# Microalgae grow on source separated human urine in Nordic climate: outdoor pilot-scale cultivation

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## Abstract

Human urine contributes approximately 80% of nitrogen and 50% of phosphorous in urban wastewaters while having a volume of only 1 - 1.5 L/d per capita compared to 150 - 200 L/d per capita of wastewater generated. There is interest to study source separation of urine and search methods to recover the nutrients form the urine. In this study, the objective was to use the nutrients in source separated urine for outdoor cultivation of microalgae in Nordic climate. A freshwater green microalga *Scenedesmus acuminatus* was grown in different dilutions (1:20 and 1:15) of source separated human urine, in a semi-continuously operated outdoor raceway pond with a liquid volume of 2000 L, at hydraulic retention time of 15 d. The microalgae could remove 52% nitrogen and 38% phosphorus even at culture temperatures as low as 5 °C, while obtaining a biomass density of 0.34 g VSS/L. Harvested microalgal biomass could be used to produce methane with a yield of 285 L $CH_4$/kg volatile solids.

**Keywords:** Microalgae, nutrient recovery, raceway pond, *Scenedesmus acuminatus*, source separated human urine

## 1. Introduction

Municipal wastewaters are increasingly treated in centralized wastewater treatment units i.e. in one single treatment unit for one or a group of cities (Massoud et al., 2009). The sewers carry large volume of diluted wastewater in long pipelines involving large quantity of excavation and energy for pumping (Massoud et al., 2009; Suriyachan et al., 2012). About 80-90% of the

capital costs for centralized wastewater treatment systems are related to the collection system with potential economies of scale associated to densely populated areas (Maurer et al., 2005). On the other hand, decentralized treatment system can be defined as treatment units where the raw wastewater is collected, treated, disposed and reused close to its source (Massoud et al., 2009). Decentralized treatment systems configured with large cluster subsystems (population > 2000) was estimated to be cheaper than centralized wastewater treatment (population around 30000), with lower operation and maintenance requirement, but with higher capital cost (Jung et al., 2018).

Approximately 80% of nitrogen, 50% of phosphorous and 90% of potassium in domestic wastewater originate from human urine, depending on diet and lifestyle, use of detergents etc. (Chang et al., 2013). However, the production volume of urine is only 1 – 1.5 L per person per day (Chang et al., 2013), while the total wastewater discharge may be up to 150 - 250 L/d. In addition to large amount of N, P and K, human urine also contains trace elements like B, Cu, Zn, Mo, Fe, Co and Mn. Nitrogen and phosphorus are only partially captured in a reusable form in conventional centralized wastewater treatment systems. The development of urine sorting toilets has made it possible to separate urine for nutrient recovery instead of directing the urine to centralized wastewater treatment plants (Adamsson, 2000; Igos et al., 2017).

Several methods have been studied to capture the nutrients from source separated urine, such as struvite precipitation (Wilsenach et al., 2007), ion exchange (Lind et al., 2000), and ammonia stripping (Başakçilardan-Kabakci et al., 2007). However, nitrogen recovery is rather low in struvite precipitation along with the need for addition of magnesium, and ammonia stripping demands additional energy. Microalgae, if grown on urine, may provide an economically and environmentally sustainable way to capture nutrients from urine as nutrients are utilized by the growing microalgal cells (Feng and Wu, 2006). *Scenedesmus* species, for example, have been reported to take up high concentrations of nitrogen and phosphorus ($NH_3$-N = 273 mg $L^{-1}$, total P = 58.75 mg $L^{-1}$) (Kim et al., 2015). Choi and Lee (2015) have reported that *Scenedesmus* can grow in different media with the N/P ratio varying over a wide range of 5 to 30. .

Azov and Goldman (1982) demonstrated that the photosynthesis rates of *Scenedesmus* are reduced to 50% of their maximum rates when free ammonia reached 20 mg/L. Thus, dilution of human urine is considered necessary to reduce the toxic effect of the high ammonia concentration on the growth of microalgae. The growth of different microalgae in diluted human urine has previously been studied using cyanobacterium *Spirulina platensis* (Feng and

Wu, 2006) and microalga *Chlorella vulgaris* (Jaatinen et al., 2016), *Chlorella sorokiniana* (Tuantet et al., 2014) and *Scenedesmus acuminatus* (Adamsson, 2000). *Scenedesmus* has been reported to grow in 0.5% diluted urine supplemented with EDTA and iron up to a maximum biomass density around 133 mg/L as dry weight (Adamsson, 2000). However, there are no published studies on large scale outdoor cultivations of *Scenedesmus* or other microalgae in Nordic climate conditions with varying lengths of daylight from 23 hours to 1 hour and outside temperatures ranging from -30 to 30 °C.

Large-scale cultivation of microalgae in human urine would create a sink for nutrients. Eventually, to close the nutrient cycle, proper end use of the microalgal biomass needs to be envisaged. Use of microalgae as biofuel, nutraceutical (pharmaceutical grade nutrients), or high value chemicals is getting increasing attention (for reviews and discussion see, Kim et al., 2016; Passos et al., 2014). While majority of the biofuel production processes from microalgae requires the costly steps of drying, extraction and fuel conversion, in anaerobic digestion (AD) a natural consortium of microorganisms is able to break down the organic matter of the algal biomass into simple monomers which can then be converted into biogas (Abdelaziz et al., 2013). In addition to the produced biogas, nutrients in the substrate are retained in the AD digestate, which can be further refined as fertilizers or circulated back to algae cultivation (Kinnunen et al., 2014). Varying biochemical methane potential (BMP) between 107 and 410 L $CH_4$ $kg^{-1}$ volatile solids have been found for *Scenedesmus* dominating biomass, mainly cultivated in different synthetic media or wastewater and the BMP has been reported to vary depending on the growth media (Frigon et al., 2013; Kinnunen and Rintala, 2016; Roberts et al., 2016; Zhen et al., 2016).

City of Tampere in Finland is building a new city district in Hiedanranta that serves as an experimental living lab and promotes circular economy. In Hiedanranta, the city will proceed e.g. with the approach of decentralization, focusing on recovery of resources from wastewater generated in the area locally. In this study, the feasibility to cultivate microalga *Scenedesmus acuminatus* using source separated and hydrolyzed (stored for several months to kill the pathogens, while urea is hydrolyzed) human urine was studied in pilot scale outdoor raceway ponds in Hiedanranta. The requirements for diluting the urine for algal growth were determined both in laboratory- and pilot-scale. In addition, the biochemical methane production potential of the microalgal biomass harvested from the pilot-scale ponds was tested. To our knowledge, cultivation of microalgae in pilot-scale outdoor raceway pond to treat real human urine in Nordic climate has not been previously reported.

## 2. Materials and methods

### *2.1.Laboratory cultivation of Scenedesmus acuminatus*

*Scenedesmus acuminatus* (SAG 38.81) was obtained from the Culture Collection of Algae (SAG) at the University of Göttingen, Germany. The stock culture was maintained under continuous fluorescence illumination of 40 µmol photons/$m^2$.$s^1$ in 700 mL modified N8 medium with the following composition (per liter): 0.5055 g $KNO_3$; 0.74 g $KH_2PO_4$; 0.2598 g $Na_2HPO_4$; 0.05 g $MgSO_4$ x $7H_2O$; 0.0175 g $CaCl_2$ x $2H_2O$; 0.0115 g FeNaEDTA x $3H_2O$; 0.0032 g $ZnSO_4$ x $7H_2O$; 0.013 g $MnCl_2$ x 4H2O; 0.0183 g $CuSO_4$ x $5H_2O$ and 0.007 g $Al_2(SO_4)_3$ x $18H_2O$ at a pH of 8.5. The media was continuously purged with 5% $CO_2$ in air at a flow rates of 0.2 L/min. The initial optical density ($OD_{660}$) of the culture was 0.5. When the $OD_{660}$ reached the value of 5.5 – 5.8 (growth saturation), 5 mL of cells washed with distilled water were inoculated into 700 mL of fresh medium and the saturated culture was finally stored in the dark at 8 °C (used as inoculum in further studies).

### *2.2.Source separated urine collection and storage*

The source separated urine (later referred to as urine) was collected from the experimental cultural and seminar center in Hiedanranta (Tampere, Finland) by using urine diverting dry toilets, which were being used by the visitors of the center. The urine was collected in 1000 L plastic tanks and stored for at least six months at ambient temperature (5 - 20 °C) before use to kill the pathogens and hydrolyze the urea. The composition of the urine after storage (as average for the different samples collected over the period of operation of the ponds) was as presented in Table 1.

Table 1. Average composition (and standard deviation) of urine (five samples) after storage for at least six months at ambient temperature

| Parameter | Range |
|---|---|
| pH | 8.8 ± 0.0 |
| COD (mg/L) | 5500 ± 200 |
| $N_{tot}$ (mg/L) | 3480 ± 130 |
| $NH_4^+$-N (mg/L) | 1800 ± 750 |
| $P_{tot}$ (mg/L) | 190 ± 52 |
| Na (mg/L) | 690 ± 30 |
| K (mg/L) | 600 ± 38 |
| Mg (mg/L) | 2.3 ± 0.4 |
| Ca (mg/L) | 14 ± 5 |

COD – chemical oxygen demand,

*2.3.Screening for different dilutions of urine as growth medium*

Source separated urine (Table 1) to be used as growth medium for *S. acuminatus* was diluted with tap water to obtain the following dilutions: 0x, 2x, 3x, 4x, 5x, 10x, 15x, 20x and 25x. Here 2x dilution means 50% (v/v) urine in tap water and 25x means 4% urine in tap water. Duplicate cultures with 5 mL of *S. acuminatus* stock culture was inoculated to 100 mL of growth medium at each dilution in 250 ml Erlenmeyer flasks. The cultivations were incubated at 25 ºC at 150 rpm and under continuous illumination with white fluorescent lamps at 40 µmol/$m^2$.$s^1$ for seven days. The growth of *S. acuminatus* was followed by analyzing $OD_{660}$ every day.

*2.4.Cultivation of microalgae in raceway ponds*

Two raceway ponds (RwP), used for cultivation of microalgae on human urine, were located near to the collection site of the human urine in Hiedanranta (Tampere, Finland) inside a greenhouse (Fig. S1). The small raceway pond (SRwP; L:W:D of 3 m: 0.5 m: 0.4 m) and the big raceway pond (BRwP; L:W:D of 7 m: 1 m: 0.6 m) had liquid volumes of approximately 400 L and 2000 L, respectively. The ponds were constructed with gravel and lined with a 1.5 mm thick high-density polyethylene sheet. To maintain a constant velocity of liquid, the ponds were equipped with paddle wheels (rotation speed of 10 rpm) operated with a 0.5 HP gear wheel motor (Regal Beloit, Marathon motors, USA).

The SRwP was inoculated with 10 L of *S. acuminatus* (approximate $OD_{660}$ of 0.5), grown in modified N8 medium and was operated as batch with 20x diluted urine. After 10 days, approximately 350 L of the culture ($OD_{660}$ of 0.75) was used as inoculum for the BRwP which was filled with 20x diluted urine to reach a total volume of 2000 L. The rest of the SRwP culture (50 L) was used as inoculum for batch operation in the smaller pond, which was operated to study the maximum growth of *S. acuminatus* in 20x urine and also to act as a culture reserve. The level of medium in the SRwP was maintained constant by adding fresh water weekly (approximately 10 L) to account for the evaporation loss.

After operation in batch mode for the first 15 days, the BRwP was operated in a semi-continuous mode at a hydraulic retention time (HRT) of 15 d with 20x diluted urine for 28 days, after which the urine dilution was changed to 15x. After day 79, the BRwP was operated in batch mode up to day 148, when the pond operation was stopped due to decrease in temperature and light intensity.

The BRwP was fed two times a week by removing 500 L of the culture and replacing it with 20x (25 L of urine and 475 L of tap water) or 15x (33 L of urine and 467 L of water) diluted urine. Two samples were collected during each feeding, one before and one 15 min after the feeding. Before each feeding, 500 L of microalgal culture was collected in a drainage pit with an attached nylon filter cloth (pore size < 10 μm). The microalgal biomass was recovered from the cloth and a composite sample (500 mL) of the filtered water was collected. Harvested biomass was centrifuged for 2 min at 4000 rpm, at 20°C. Settled algal pellets were washed with deionized water, centrifuged (2 min, 4000 rpm, 20 °C) and either freeze-dried (CHRIST, Alpha 1-4 LD, Germany) for at least 17 h or stored as such at -20 °C.

*2.5.Methane production potential*

Methane production potentials of the harvested biomass was determined in triplicate batch assays using 119 mL glass serum bottles. Methane production assays were done with biomass collected from different phases of growth in BRwP namely 20x dilution phase (healthy cell growth, day 25), 15x dilution start phase (cells were dying, day 32), 15x dilution stable phase (freeze dried, day 53 and stored as such, day 79). Inoculum used in the tests was digestate from a mesophilic anaerobic digester treating municipal wastewater treatment plant sludge (Viinikanlahti, Tampere, Finland). Inoculum (30 mL) and microalgal biomass as the substrate were added into the bottles, using a $VS_{substrate}$: $VS_{inoculum}$ ratio of 1:1 (volatile solids, VS). Distilled water was added to make a total liquid volume of 60 mL, and 4 g/L $NaHCO_3$ was

added as buffer. Inoculum alone with distilled water and buffer to make a total volume of 60 mL was used as control (three replicates) and the methane volume produced in the control was subtracted from the methane volumes obtained in the bottles with the substrates. The bottles were flushed with nitrogen gas for 1 min and sealed gas tight with rubber stoppers and aluminum seals. The cultures were incubated at 35 °C without mixing.

*2.6.Analysis and calculations*

Light intensity and temperature of culture media in the ponds was measured every working day. Culture suspension samples were collected from the raceway ponds every working day and the effluent after harvesting was collected twice a week. The culture suspension samples were analyzed for OD, pH, dissolved oxygen (DO), total suspended solids (TSS), volatile suspended solids (VSS), total chemical oxygen demand (TCOD), soluble COD (SCOD), total soluble phosphorus ($P_{tot}$), total soluble nitrogen ($N_{tot}$), ammonia nitrogen ($NH_4^+$-N) and other cations namely potassium, magnesium, calcium and sodium. While TCOD is the COD of the entire sample, the SCOD was measured after filtration with 0.45 µm syringe filter.

The pH and dissolved oxygen (DO) were measured using pH electrode and DO probe (HACH Lange, HQ40D, U.S). Algal growth for all experiments was monitored by measuring optical density at 660 nm ($OD_{660}$) using UV-VIS spectrophotometer (Shimadzu UV-1700 PharmaSpec). Additional monitoring of algal growth and health was conducted using bright-field microscope (Axio-Imager, Carl Zeiss, Germany) and the samples were photographed using an AxioCam HRc CCD camera equipped with AxioVision software (Carl Zeiss, Germany). Total suspended solids (TSS) and volatile suspended solids (VSS) were analyzed according to the Standard Methods (APHA, 1998). $P_{tot}$, $N_{tot}$ and $NH_4^+$-N were determined with HACH Lange kits (LCK 350, LCK 303, and LCK 304) according to the protocols provided by the manufacturer (HACH, 2017) after filtration with 0.45 µm syringe filter. Cations in the sample were analyzed according to the ion chromatography standard SFS-EN ISO 10304–1 using an ion chromatograph (Dionex DX-120, USA) with AS40 autosampler, IonPac CS12A cation exchange column and CSRS 300 suppressor (4 mm). The eluent contained 2 mM methane sulphonic acid and the eluent flow rate was 1 mL $min^{-1}$. COD was analyzed with dichromate method according to the Finnish Standard (Standard SFS 5504). Total COD (TCOD) was analyzed from source separated urine, and soluble COD (SCOD), after filtration with 0.45 µm syringe filter, was analyzed from the effluent after collection of the algal biomass.

The harvesting efficiency was calculated using Eq. 1:

$$Efficiency = \frac{VSS_{inlet} - VSS_{outlet}}{VSS_{inlet}} * 100\% \quad \text{Eq. 1}$$

where $VSS_{inlet}$ is the VSS of the culture in the semi-continuous pond and $VSS_{outlet}$ is the VSS of the effluent after biomass harvesting.

Nutrients balance in the pond was calculated according to Eq. 2 and Eq. 3:

$$Inflow = Assimilation + Precipitation + Volatilization + Outflow \quad \text{Eq. 2}$$

$$Removal\ (\%) = \frac{Assimilation + Precipitation + Volatilization}{Inflow} \quad \text{Eq. 3}$$

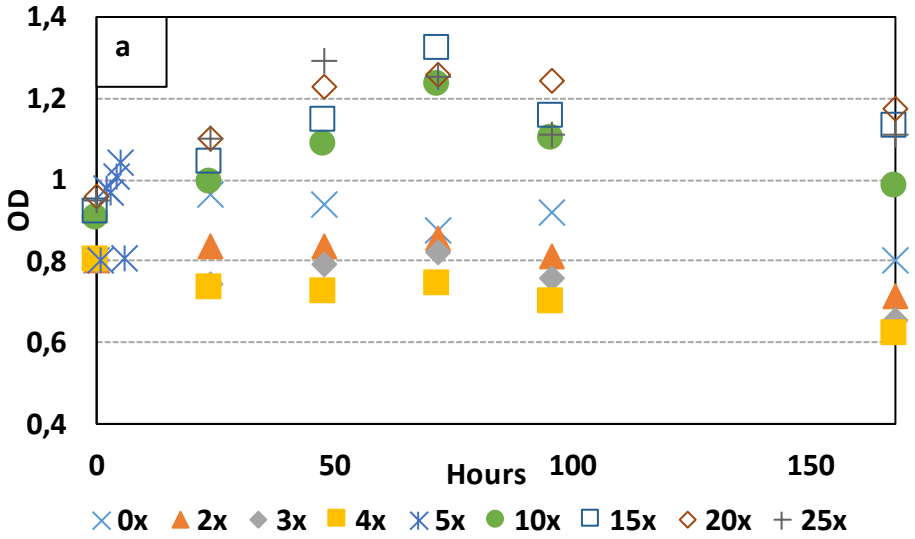
where, concentration of a specific nutrient in the inflow was measured from the fed urine samples. The pond was considered to have completely mixed conditions, so outflow sample was assumed to be representative of the pond contents. The sum of assimilation to the biomass, precipitation and volatalization as calculated from difference of inflow and outflow samples was considered as nutrient removal. Inflow and outflow concentrations were measured in laboratory from the collected samples.

The methane content and volume of the biogas, was measured with GC-FID as described in the literature (Kinnunen et al., 2015). Methane production was converted to normal conditions (NTP): temperature 273 K and pressure 1000 mbar.

## 3. Results

### *3.1.Screening of different dilutions of urine as growth medium*

Suitability of undiluted urine (0x), urine with lower dilution ratios (2x - 5x) and higher dilution ratios (10x, 15x, 20x, 25x) as a growth medium for *S. acuminatus* was studied in 7-day batch assays (Fig. 1). The first series of dilutions (0x – 5x) did not support microalgal growth. Slight increase in $OD_{660}$ up to 1 could be observed for 5x dilution on day 4, but the $OD_{660}$ soon reduced below the initial value of 0.8. The second series of dilutions (10x - 15x) showed improvement in microalgal growth for the first 3 days followed by decrease in $OD_{660}$ likely due to cell death after day 4. However, continuous growth was observed in cultures with 20x dilution where $OD_{660}$ constantly increased from 0.9 to 1.4, which was the highest $OD_{660}$ obtained throughout these batch experiments. With 25x dilution there was an initial growth with increase in $OD_{660}$ up to 1.3 after which the $OD_{660}$ decreased to 1.1. In all culture bottles with different dilutions, the starting pH was 8.7 (Fig. 1b) from which the pH continuously increased, while some decrease towards the end of the incubation (after day 4) was seen for the cultures growing on 20x and 25x diluted urine.

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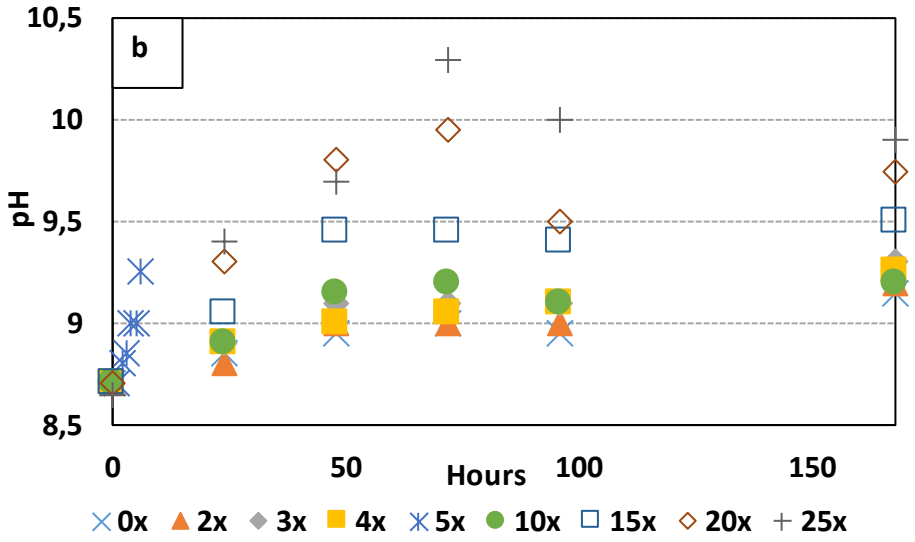


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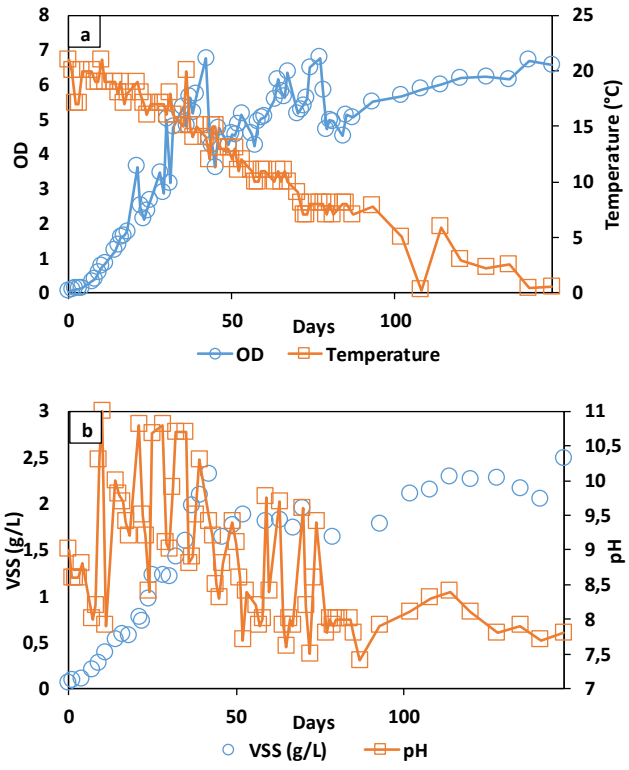
234 **Figure 1:** (a) Growth (as optical density measured at 660 nm) of *S. acuminatus* in human
235 urine at different dilutions, (b) pH during the batch experiments

236 *3.2. Raceway pond (400 L) operation in batch mode*

237 *S. acuminatus* was cultivated in batch mode in the SRwP with a working volume of 400 L
238 containing urine with 20x dilution to study the maximum possible algal growth, measured in
239 terms of $OD_{660}$ and VSS. Batch growth was conducted for almost five months spreading over
240 summer (July, days 1-14, temperature 15-20 °C), autumn (August, September and October,
241 days 15-102, temperature 10-15 °C) and onset of winter (November and December, days 103-
242 148, temperature < 5 °C). The growth of *S. acuminatus* measured as $OD_{660}$ followed a
243 continually increasing trend with sudden peaks and drops associated to increase or decrease in
244 culture solution temperature, respectively (Fig 2a). However, the microalgae recovered and

started to grow within a few days after the drops in temperature throughout the entire experimental period (Fig. 2a). The $OD_{660}$ of the microalgal culture in the pond increased to a maximum of 6.78 on day 77, and the final $OD_{660}$ value was 6.56 on day 148. Temperature in the pond was below 10 °C from day 60 onwards, while it was consistently below 5 °C from day 100 onwards. Around day 148, the outdoor temperature went subzero and the study was stopped due to freezing of the pond contents.

VSS in the pond was also monitored to determine if the biomass concentration in g per L followed similar trend as $OD_{660}$. The maximum VSS of 2.3 g/L (Fig. 2b) was obtained on day 42, when also the maximum $OD_{660}$ was recorded. After day 42, the VSS decreased suddenly and gradually started to increase again and remained around 2.11 g/L to 2.29 g/L from day 100. The pH of the ponds fluctuated widely and fast; it varied between 7.4 and 11 for the initial 60 days, after which the variation reduced and pH remained between 7.4 and 8.4 (Fig. 2b). Microscopic images from day 14 of the experiment (temperature, 19 °C) showed *S. acuminatus* growing in dense colonies of four cells (Fig. 2c), whereas Fig. 2d shows reduced density of cells on day 77 when the temperature was 8 °C.



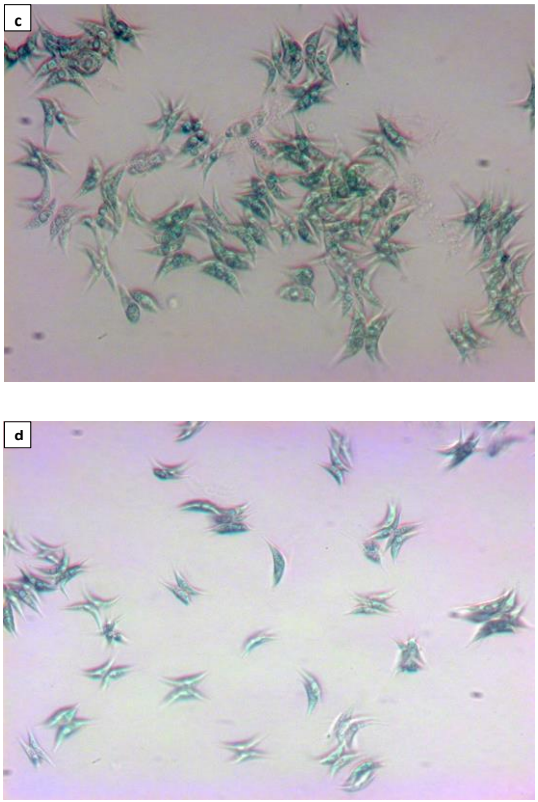



**Figure 2:** Variation of (a) optical density ($OD_{660}$) and culture temperature, (b) volatile suspended solids (VSS) and pH with time and microscopic image of *S. acuminatus* growing in the small raceway pond on (c) day 14 and (d) day 77

*3.3. Raceway pond (2000 L) operation in semi-continuous and batch mode*

*S. acuminatus* was cultivated in a semi-continuously operated raceway pond (BRwP) having a working volume of 2000 L for 148 days. The pond was fed with urine with a dilution of 20x for the first 28 days (from the middle of July to the middle of August, average temperature 17 ℃). After 28 days, the dilution of urine was reduced to 15x, the semi-continuous feeding of which was continued for 51 days (up to day 79 with a break between days 37 and 45, average temperature 11 ℃), after which batch mode operation was continued for 69 days (up to day 148, average temperature 7 ℃).

Higher dilution (20x) supported continuous microalgal growth reaching a maximum $OD_{660}$ and VSS of 1.1 g/L and 0.34 g/L, respectively, after 28 days (Fig. 3). The pH varied widely between 8.5 and 11 (Fig. 3). When the urine dilution was reduced to 15x, decrease in microalgal growth was observed with $OD_{660}$ decreasing from 0.99 to 0.68 and VSS from 0.42 g/L to 0.28 g/L in 7 days. In addition, the culture turned from healthy green to brownish colour based on visual observations. To recover from the probable shock load, the pond was fed only with tap water

for 10 days (days 37 to 45) after which feeding with 15x diluted urine was continued. Between day 49 and 79, the $OD_{660}$ and VSS of the pond culture remained at around 0.70 and 0.31 g/L, respectively. During the batch operation from day 80 onwards, the $OD_{660}$ increased slowly to a maximum of 0.97 on day 120. Though VSS of the pond contents followed a similar trend as that of $OD_{660}$ in general, it did not increase like $OD_{660}$, and was around 0.34 g/L, when the pond was operated in batch mode in the last phase of the experiment. Harvesting efficiency of the microalgal biomass was 57 ± 8% of VSS with approximately 150 g (75 g per harvesting day) of wet biomass collected every week.

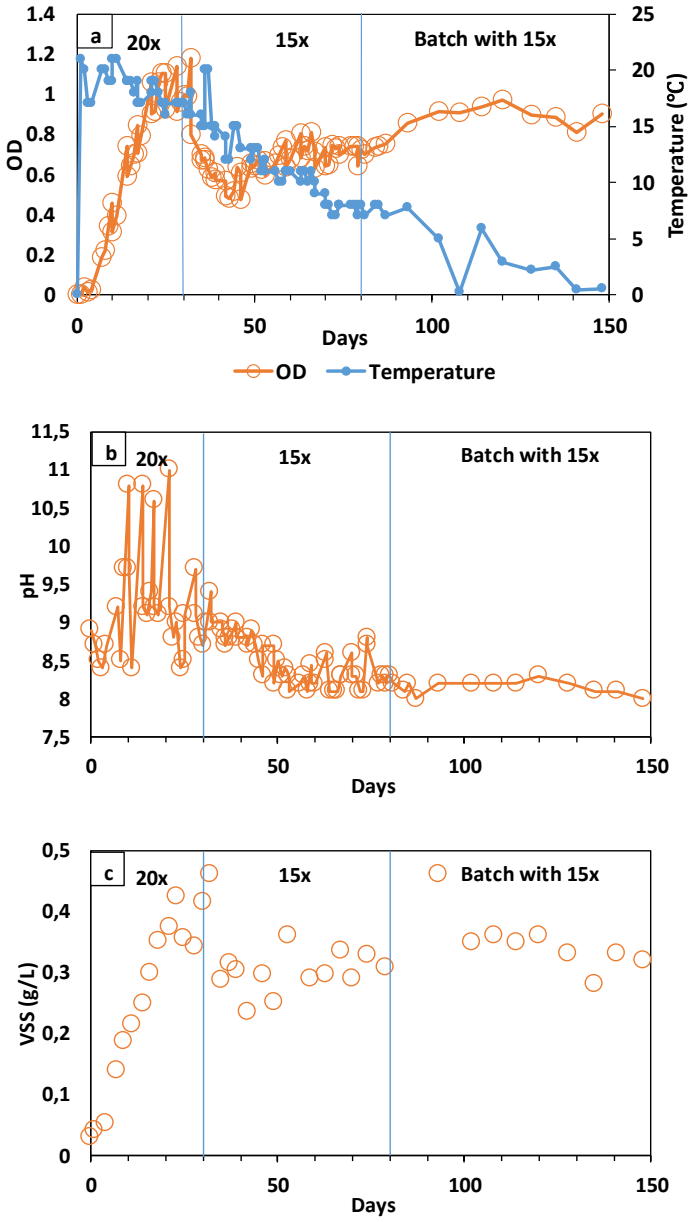



**Figure 3:** Variation of (a) temperature and optical density ($OD_{660}$), (b) pH and (c) volatile suspended solids (VSS) in the large open raceway pond operated with source segregated urine with different dilutions in semi-continuous and batch mode

The soluble phosphorus, ammonium and soluble TN concentrations in the pond were as shown in Fig. 4. When the pond was operated in semi-continuous mode with 20x diluted urine, removal of approximately 38% of phosphorus, 67% of $NH_4^+$ N and 52% of TN in a cycle of 15 days was obtained, calculated according to Eq. 2. The removal reduced to around 13%, 18% and 22% for phosphorus, $NH_4^+$ N and TN respectively, during semi-continuous operation with 15x diluted urine. Almost no removal of the nutrients was observed, while operating the pond

303 in batch mode during the last phase of the experiment. There was no measurable uptake of the
304 trace metals like Na, K, Mg and Ca during the entire period of operation of the pond.

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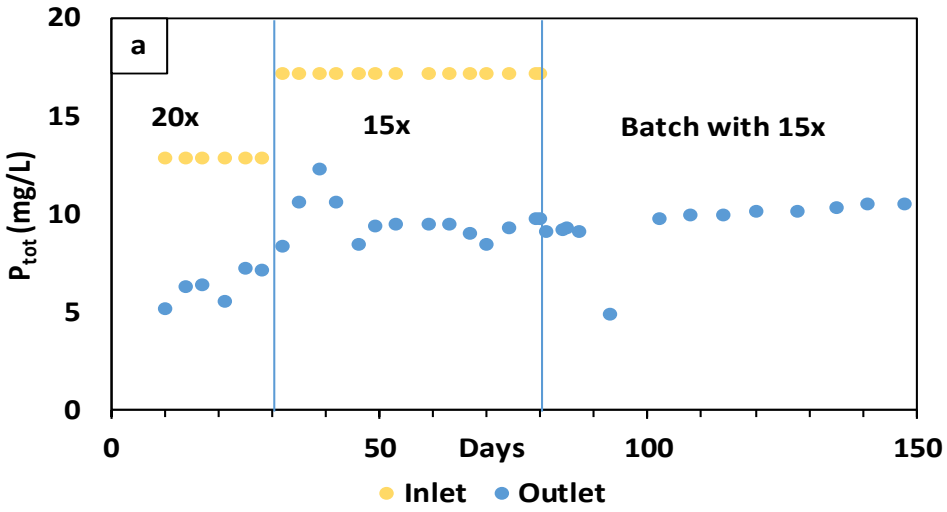


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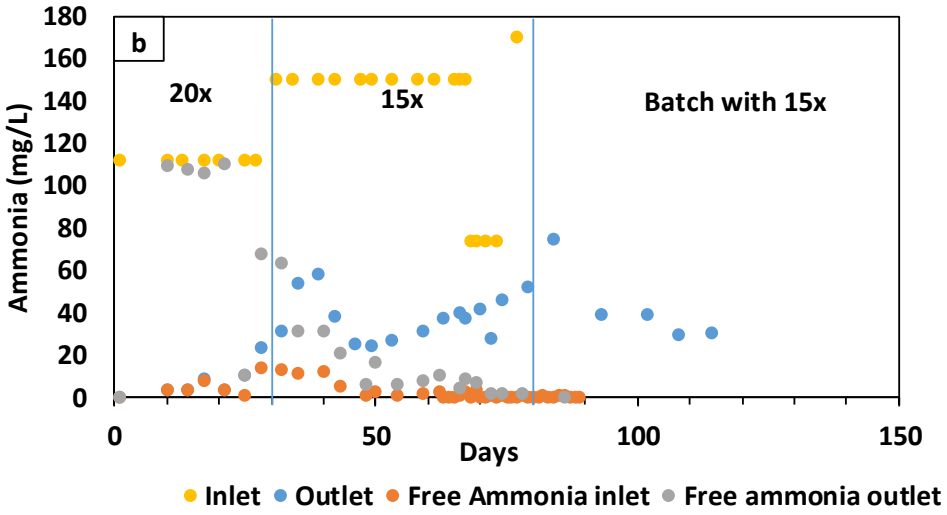


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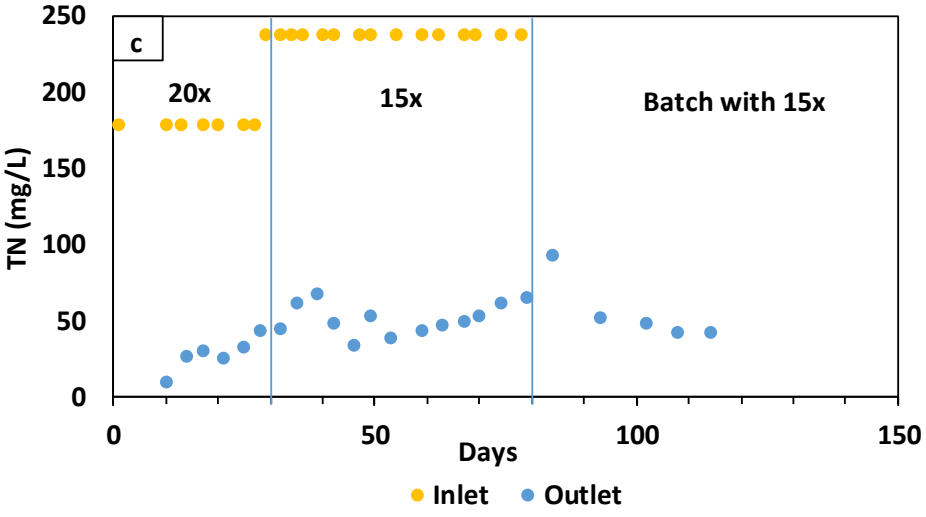


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309 **Figure 4:** Variation of soluble (a) $P_{tot}$, (b) $NH_4^+$ N, (c) TN in the large open raceway pond
310 operated with different dilutions in semi-continuous and batch mode

### *3.4.Biomethane generation potential from the harvested microalgal biomass*

Methane production assays were done on microalgal biomass collected from different phases of growth namely 20x dilution phase (healthy cell growth, day 25, freeze dried), 15x dilution start phase (cells were dying, day 32, freeze dried), and 15x dilution stable phase (freeze dried, day 53 and stored as such day 79) (Fig. 5). Methane production started immediately for two of the biomass samples, collected on 15x growth phase (freeze dried, day 53 and as such stored, day 79), and 70–90% of the total methane was formed within 10 days from the beginning of the assay. The initial methane production rate for the biomass samples collected on day 25 (20x dilution) and day 32 (15x dilution) was slower than the other two samples (Fig. 5). While the biochemical methane potential (BMP) of the harvested biomass (day 53, 15x and day 25, 20x) after freeze-drying was above 250 L/kg VS, it was less than 200 L/kg VS for the biomass without freeze-drying. For the freeze-dried biomass, maximum BMP was found during cultivation with 20x urine (285 ± 20 L/kg VS) followed by that harvested during growth phase with 15x dilution (275 ± 25 L/kg VS) and the lowest BMP (250 ± 15 L/kg VS) was observed for the time period when the algal cells were dying, during change in urine dilution (day 32).

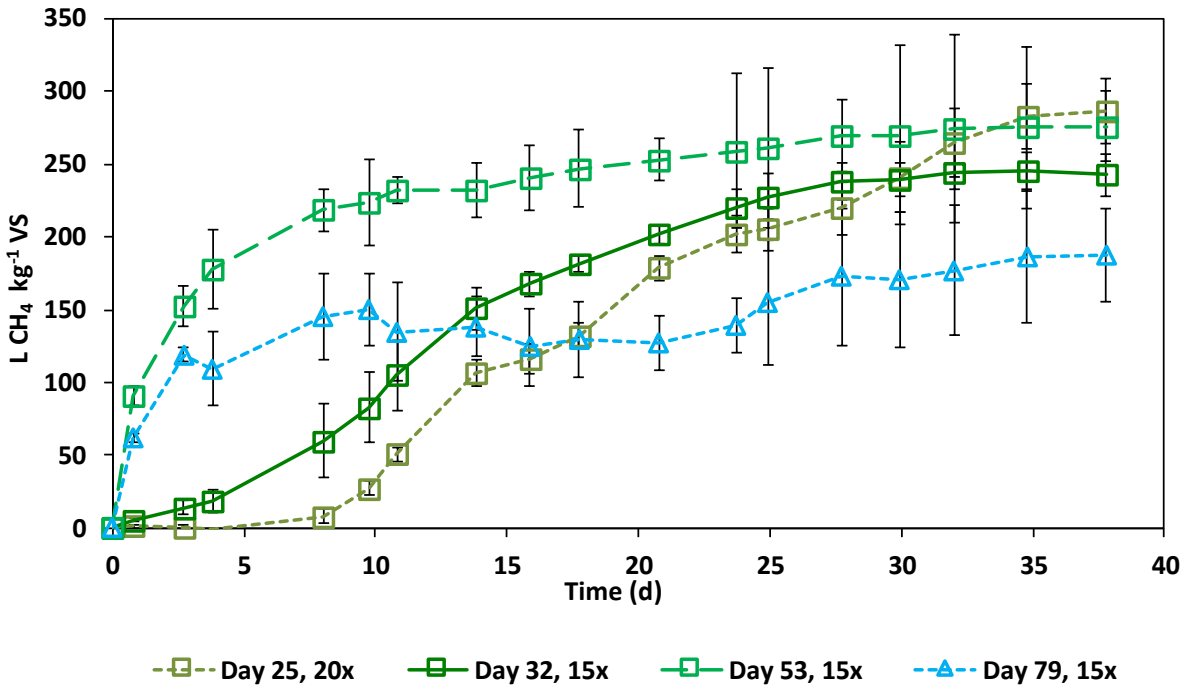


**Figure 5:** Cumulative methane production from the harvested microalgal biomass samples taken on different days of cultivation

## 4. Discussion

### *4.1. Screening urine dilutions for culturing of S. acuminatus in laboratory conditions*

*S. acuminatus* was chosen for this study because of its extensive use in wastewater treatment research for, e.g. in anaerobic digestion effluents (Tao et al., 2017b) and secondary domestic wastewater (Posadas et al., 2015), and because of its capacity to grow in high biomass concentrations of 8 – 11 g/L (Tao et al., 2017a,b). Treatment and nutrient recovery from human urine with microalgae would be most advantageous when the urine is used without dilution. Therefore, the main objective of the dilution screening experiments was to find out the lowest possible dilution of urine in which microalgae can effectively grow. The results showed that in batch experiments *Scenedesmus* could grow at 10x dilutions (initially 200 mg/L of ammonia-N) and not at lower dilutions, but proper growth without significant decrease in biomass concentration after initial growth could only be observed at 20x dilution (initially 100 mg/L of ammonia-N).

Human urine is a very concentrated nutrient solution. Among the nitrogen compounds urea is dominating in fresh urine, but when urine is stored, urea hydrolyzes to ammonia and carbon dioxide (Adamsson, 2000). Microalgae can assimilate ammonium directly into organic compounds, which could explain the rapid initial algal growth in the diluted urine. Unionized or free ammonia, which increases with increasing pH, could be toxic for algae, depending on the species, at a concentration around 50 mg/L (Azov and Goldman, 1982; Udert et al., 2006). High pH levels (pH > 10) in this study, caused by the buffering capacity of carbonate and ammonia and also due to uptake of $HCO_3^-$ ions during algal photosynthesis, can also lead to precipitation of unchelated iron, phosphate and carbonate, which then become unavailable for microalgal uptake (Kawasaki et al., 1982; Udert et al., 2006). The decreased pH at the end of incubation period was apparently due to ammonium uptake by algae, which results in $H^+$ production lowering pH. High pH can also indicate that the cultures were likely $CO_2$ limited and the decrease in pH in the end indicates reduced growth.

Also some previous studies have reported limited or no growth of microalgae on dilutions of urine lower than 20x in batch experiments. For example, limited growth of *Arthospira platensis*, with $OD_{660}$ below 0.2, was reported by Coppens et al. (2016) in hydrolyzed human urine at 5000 times dilution. When *Chlorella sorokiniana* was cultivated in 5x diluted hydrolysed urine, $OD_{750}$ increased from 0.1 to approximately 0.3 in one day but from the second day onward $OD_{750}$ did not show any increase (Tuantet et al., 2014). The same

phenomenon was observed in urine without any dilution. Tuantet et al. (2014) also tested 20x dilutions and concluded that 20x dilution showed the highest growth, as the $OD_{750}$ rose from 0.1 to 0.8 in one day without addition of trace elements.

Growth of microalgae like *Spirulina* on much higher dilutions of fresh human urine (120 times) has been reported by Chang et al. (2013). However, the fresh human urine studied by Chang et al. (2013) had higher concentrations of $N_{tot}$ (8800 mg/L) and $P_{tot}$ (792 mg/L) than the source separated stored urine used in this study ($N_{tot}$: 3480 ± 130 mg/L, $P_{tot}$: 190 ± 52 mg/L). Microalgal growth (*Chlorella vulgaris*) was reported to follow similar growth rates for all urine dilutions (1:25 to 1:100), with somewhat higher growth at a dilution of 1:100 (0.6 g/L VSS) compared to that at 1:25 dilution (0.52 g/L VSS) and 1:300 dilution (0.48 g/L VSS) (Jaatinen et al., 2016). Growth of microalgae on human urine depends a lot on the pH, temperature, light intensity and reactor configuration, as these parameters determine the availability of phosphorus,nitrogen and trace elements, and at the same time toxicity of nitrogen. Hence, it is difficult to conclude from laboratory studies conducted in controlled environment as to what would happen in real-life conditions, such as raceway ponds, necessitating pilot-scale studies.

*4.2.Cultivation of S. acuminatus in the raceway ponds*

The maximum $OD_{660}$ of 6.8 obtained in the batch raceway pond of volume 400 L fed with 20x diluted urine after 40 days of cultivation was comparable to growth in the synthetic modified N8 medium, where the maximum $OD_{660}$ was 6.4 in 8 days. The maximum OD obtained in the small raceway pond was much higher than the maximum $OD_{660}$ of 1.3 as obtained on $4^{th}$ day of cultivation in the batch bottles containing the same dilution of urine. The average light irradiation, ambient temperature, number of daily sun hours and evaporation rates decreased throughout the operational period of the pond. With the decreasing temperature (below 15 °C) and shorter days with decreasing light intensities, the microalgal growth decreased ($OD_{660}$ < 4.5) from day 43 onwards. However, the growth rate started to steadily increase after a slow growth and also probable cell death for around 20 days and $OD_{660}$ reached the value of 6.8 again on day 77 when the average temperature was just 10 °C for the last 20 days. Although optimum temperatures for microalgal growth often range from 20 to 30 °C, successful carbon and nutrient removal from piggery (Godos et al., 2009) and municipal wastewaters (Posadas et al., 2015) has been reported in raceway ponds, of respective volumes 464 L and 800 L, at average mixed liquor temperatures of 7 to 11 °C.

Increasing pH might lead to ammonia toxicity, due to increase in free ammonia concentration (Adamsson, 2000), which however, is not so pronounced at lower temperatures (Udert et al., 2006). In this study ammonia N concentration in the 15x and 20x diluted urine was around 120 and 90 mg $NH_4$-N/L, respectively, which should not be toxic to *S. acuminatus* (Tao et al., 2017a). For example, Tao et al. (2017a) observed growth of Scenedesmus in laboratory scale photobioreactors even at ammonia concentrations as high as 480 $NH_4$-N/L. Thus, hindered growth in the ponds were likely due to low temperature and shorter daylight period. However, these variations are unavoidable in the Nordic outdoor conditions.

A semi-continuous cylindrical reactor was operated by Adamsson, (2000) on 50x diluted human urine for the growth of *S. acuminatus* and a maximum biomass concentration of 287 mg/L (VSS) was reported after supplementing with iron, magnesium and EDTA. In this study, a higher biomass concentration of 424 mg VSS/L at 20x dilution and 460 mg VSS/L at 15x dilution indicates higher availability of nutrients at lower dilutions. Tuantet et al., (2014b) also observed increased growth of *Chlorella sorokiniana* in synthetic human urine in a 1 L photobioreactor with 5 times diluted synthetic urine and a light intensity of 490 mmol photons/$m^2$.s (6 g VSS/L) as compared to 20 times dilution and 990 mmol photons/$m^2$.s (2.9 g VSS/L), probably due to more availability of nutrients.

The current study is the first reporting microalgal growth in source separated human urine in open raceway ponds in Nordic climate. Growth of algae at low temperatures, lower light irradiance and shorter daylight time indicates potential of use of microalgal ponds to treat urine in Nordic areas. However, more detailed evaluation to understand the limiting factors and ultimately optimize the pond performance is necessary.

*4.3.Removal of nitrogen and phosphorus*

When the big pond was operated in semi-continuous mode with 20x diluted urine, removal of around 38% of soluble phosphorus, 67% of $NH_4^+$-N and 52% of TN was observed. The TN removal efficiencyiy was higher, while phosphorus removal was lower to the ones reported by Adamsson (2000), (23% removal of TN, 57% removal of soluble phosphorus) while cultivating *Scenedesmus* in a pilot-scale (40 L) cylindrical photobioreactor on 50x diluted urine. However, the nutrient removal in the current study is a combination of phosphate precipitation, ammonia loss to environment due to volatilization and microalgal uptake. García et al. (2000) demonstrated that the loss of ammonia to the atmosphere was the most important mechanism for nitrogen removal in algal ponds, the percentage of which increases with increasing pH. The

presence of un-ionized ammonia increases as pH rises leading to stripping off ammonia and vice versa ($NH_3 + H^+ \leftrightarrow NH_4^+$). In this study, the pH of the microalgal cultures fluctuated between 7.5 and 11 in the small pond and between 8 and 11 in the big pond. Higher pH would lead to higher loss of ammonia to the atmosphere (Chang et al., 2013). Change in temperature makes ammonia to solubilize or volatilize (increasing free ammonia concentration with increasing temperature), causing changes in pH. Fluctuations in pH reduced slowly with decreasing temperature during this study (after day 50, average temperature < 10 °C) decreasing the changes in ammonia volatilization. Future studies with continuous monitoring of pH or even pH control, temperature and ammonia concentration in different forms would give more understanding on the fate of nutrients. Again, this loss through ammonia evaporation may be the most important mechanism for N removal in this study, but this assumption requires further studies.

The concentration of soluble $P_{tot}$ decreased for the first seven days of the semi-continuous operation from 6.31 mg/L to 3.26 mg/L for 20x diluted urine and from 10.10 mg/L to 7.56 mg/L for 15x diluted urine. After seven days the concentration remained either constant or was observed to increase at some point, indicating a break in phosphate uptake by algal cells. Jaatinen et al. (2016) reported, with 1:25-diluted urine in batch cultivation, a final $P_{tot}$ concentration of 9.4 mg/L (from initial 14 mg/L) in 10 days, after which the concentration remained unchanged. Alkaline pH leads to the transformation of $H_2PO_4^-$ present in urine into $HPO_4^{2-}$ and $PO_4^{3-}$, which can precipitate with $Ca^{2+}$ and $Mg^{2+}$ in urine (Chang et al. 2013). With changing pH, there could be precipitation and dissolution of the phosphate (Chang et al., 2013). This could be the reason why in this study an increase in phosphate concentration was occasionally observed during the operation of the big pond (for example during days 35 to 49). In addition, dying algal cells can release the phosphorus and nitrogen from the cells thus increasing the phosphate in the medium (Jaatinen et al., 2016). Thus, it is difficult to quantify exact amount of phosphate uptake and detailed speciation study is required for that.

Bryant and Appah (2017) observed that *Scenedesmus* removes more phosphorus that nitrogen from diluted human urine and that removal of phosphorus increases with decreased molar ratio of nitrogen and phosphorus and also, with decreasing light intensity. The results obtained in the current study was in contrast with that reported by Bryant and Appah (2017). However, the experiments by Bryant and Appah (2017) were conducted under controlled laboratory conditions in 250 mL flasks and in this study various parameters changed simultaneously. A study by Posadas et al. (2015), also revealed lower phosphate removal (57%) than nitrogen

458 removal (79%). Hence, it is obvious that there are other factors, or probably interaction of
459 several factors that can change the nutrient uptake proportions in large scale outdoor cultivation
460 of microalgae.

461 *4.4. Methane production from harvested algal biomass*

462 One of the challenges in this study was the harvesting of microalgal biomass using simple
463 filtration. Only approximately 50% of the microalgal biomass growing in the pond could be
464 harvested with the filter cloth. Nevertheless, screening for efficient harvesting method was not
465 the main objective of this research and future studies employing efficient and continuous
466 harvesting is necessary.

467 Varying BMPs between 107 and 410 L $CH_4$ $kg^{-1}$ VS have been found for *Scenedesmus*
468 dominating biomass, mainly cultivated in different synthetic media (Frigon et al., 2013;
469 Kinnunen and Rintala, 2016; Roberts et al., 2016; Zhen et al., 2016). Roberts et al. (2016)
470 reported that the BMP of laboratory culture of *Scenedesmus* grown in artificial Jaworski's
471 medium had a much higher BMP (261 L $CH_4$ $kg^{-1}$ VS) as compared to that grown in a
472 bioreactor with synthetic fertilizers (161 L $CH_4$ $kg^{-1}$ VS) probably due to more challenging
473 growth conditions in the large-scale bioreactor. The BMP of urine grown microalgal biomass
474 in the present study (250 – 300 L $kg^{-1}$ VS) is comparable to the earlier reported values in
475 synthetic media. The differences in the methane production of algal cells harvested on different
476 days of cultivation could be explained by the different cell composition due to the difference
477 in growth conditions (Kinnunen and Rintala, 2016) like light intensity, temperature and pH.
478 The different growth conditions lead to different uptake of nutrients and likely a varying cell
479 composition. The current study was not designed to realize the impact of different parameters
480 on BMP, hence, further research is needed to understand the difference of BMP depending on
481 growth conditions and health of algal cells. The results and previous reported BMP values
482 clearly indicate that a much deeper knowledge of the factors affecting the anaerobic
483 biodegradability of microalgal biomass is desirable for understanding the heterogeneity of this
484 material as a potential renewable energy source via anaerobic digestion. However, this study
485 was sufficient to state the feasibility of using the algal cells grown on human urine for methane
486 production. Also, it could be noted here that there was a large difference between freeze-dried
487 biomass and the biomass stored as such. The freeze-drying likely acted as a pretreatment step
488 by breaking some of the microalgal cell. However, this kind of pretreatment would not be

feasible at larger scale and possibility to avoid freeze drying yet maintaining the same BMP should be explored.

### *4.5.Extrapolations towards scale up*

This study showed that microalgal cultivation is feasible in open raceway ponds on diluted human urine without any additional trace elements or $CO_2$ supplementation. City of Tampere expects around 20,000 people to start living in the old industrial area of Hiedanranta. While daily production of urine for around 20,000 people would be somewhere around 30 $m^3$/d, volume of urine to be stored for six months would be just around 5400 $m^3$. Based on the present results, 6000 $m^3$ microalgal pond with a surface area of around 2 ha would be needed to treat the urine produced from 20,000 people, when the pond is operated at an HRT of 15 d and a dilution of 15x. Final TP and TN concentrations in the pond, as achieved in this study, were around 7 mg/L and 30 mg/L, respectively. Thereby some form of post treatment is required before discharge. At this stage, use of such huge land area appears unrealistic to realize in practice. Thus, there is a need to reduce the HRT and urine dilution in order to reduce the land area requirement. It might also be interesting to try new reactor designs that would lead to more compact reactor structures and increased nutrient recovery. Research efforts are needed on efficient and automated biomass harvesting and other downstream processing of the harvested biomass. Nevertheless, based on the results of this study, microalgal pond can remove around 55 kg/d of TN and 2.3 kg/d of TP, when scaled up for 20,000 people. The algal biomass grown on urine of the entire population of the district could be used to produce around 33,000 $m^3$ of methane per year (Table 2).

Table 2: Extrapolation of the methane production potential from microalgal biomass grown in diluted human urine for nutrient recovery in a district with 20,000 inhabitants

| | |
|---|---|
| TN removed | 55 kg/d |
| TP removed | 2.3 kg/d |
| Maximum VS | 0.74 g/L |
| Methane production | 275 L/kg VS |
| For an HRT of 15 d, methane production | 27 L/d or 10,000 L/year |
| At 15x dilution, urine required | 133 L or 9 L/d for an HRT of 15 d |

## Conclusions

This study showed that diluted source separated human urine could provide enough nutrients to support microalgal growth in open raceway ponds. The study also revealed that it is possible to grow microalgae even at cultivation temperatures as low as 1 °C. *S. acuminatus* was grown on diluted urine in raceway ponds without addition of trace elements, artificial light or $CO_2$. However, in these conditions and in simple open pond systems, the urine has to be 20-times diluted to enable reasonable growth. The growth of microalgae in urine was comparable to its growth in synthetic media in laboratory reactors. *S. acuminatus* removed around 1500 kg/kg VS.d of TN, 50 kg/kg VS.d of TP from the urine and the produced biomass had a maximum methane production potential of 285 L $CH_4$/kg VS. Future studies are planned using more efficient harvesting methods, focusing on the nutrient mass balance.

## Acknowledgement

This project was funded by the city of Tampere who received funding from the European Union's Horizon 2020 research and innovation programme under Grant Agreement No. 730052. Topic: SCC-2-2016-2017: Smart Cities and Communities Nature based solutions. The

authors would like to thank Hanna Hautamäki for her assistance with the laboratory analyses and Antti Nuottajärvi for his assistance in planning and setting up the raceway ponds.

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