Immobilized Microalgae for Nutrient Recovery from Source Separated Human Urine

Inaugural – Dissertation

zur

Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität zu Köln

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Summary

Shortages in supply of nutrients and freshwater for a growing human population are critical global issues. Traditional centralized sewage treatment can prevent eutrophication and provide sanitation, but is neither efficient nor sustainable in terms of water and resources. Source separation of household wastes, combined with decentralized resource recovery, presents a novel approach to solve these issues. Urine contains within 1 % of household waste water up to 80 % of the nitrogen (N) and 50 % of the phosphorus (P). Since microalgae are efficient at nutrient uptake, growing these organisms in urine might be a promising technology to concomitantly clean urine and produce valuable biomass containing the major plant nutrients. While state-of-the-art suspension systems for algal cultivation have mayor shortcomings in their application, immobilized cultivation on Porous Substrate Photobioreactors (PSBRs) might be a feasible alternative. The aim of this study was to develop a robust process for nutrient recovery from minimally diluted human urine using microalgae on PSBRs. The green alga *Desmodesmus abundans* strain CCAC 3496 was chosen for its good growth, after screening 96 algal strains derived from urine-specific isolations and culture collections. Treatment of urine, 1:1 diluted with tap water and without addition of nutrients, was performed at a light intensity of 600 µmol photons m^{-2} s^{-1} with 2.5 % CO_2 and at pH 6.5. A growth rate of 7.2 g dry weight m^{-2} day^{-1} and removal efficiencies for N and P of 13.1 % and 94.1 %, respectively, were determined. Pre-treatment of urine with activated carbon was found to eliminate possible detrimental effects of pharmaceuticals. These results provide a basis for further development of the technology at pilot-scale. If found to be safe in terms human and environmental health, the biomass produced from three persons could provide the P for annual production of 31 kg wheat grain and 16 kg soybean, covering the caloric demand in food for almost one month of the year for such a household. In combination with other technologies, PSBRs could thus be applied in a decentralized resource recovery system, contributing to locally close the link between sanitation and food production.

Zusammenfassung

Die Verknappung von Nährstoffen und Frischwasser für eine wachsende menschliche Bevölkerung sind globale Probleme von großer Tragweite. Traditionelle zentralisierte Abwasserreinigungssysteme können zwar Eutrophierung verhindern und Siedlungshygiene erreichen, sind aber in Bezug auf Wasserverbrauch und Ressourcen weder effizient noch nachhaltig. Die getrennte Sammlung von Haushaltsabwässern an ihrer Quelle, kombiniert mit dezentraler Rückgewinnung von Ressourcen, ist ein neuartiger Ansatz um diese Probleme zu lösen. Urin beinhaltet in 1 % des Gesamtabwassers eines Haushalts bis zu 80 % des Stickstoffs (N) und 50 % des Phosphors (P). Da Mikroalgen Nährstoffe effizient aufnehmen können, ermöglicht die Kultivierung dieser Organismen auf Urin dessen gleichzeitige Reinigung sowie Gewinnung von Biomasse, welche die hauptsächlichen Pflanzennährstoffe enthält. Während die Suspensionskultur von Mikroalgen nach dem Stand der Technik erhebliche Defizite in ihrer Anwendbarkeit hat, könnte die immobilisierte Kultur auf Porous Substrate Photobioreaktoren (PSBRs) eine mögliche Alternative sein. Ziel dieser Arbeit war die Entwicklung eines robusten Prozesses zur Nährstoffrückgewinnung aus minimal verdünntem Urin mit PSBR-immobilisierten Mikroalgen. Die Grünalge *Desmodesmus abundans* Stamm CCAC 3496 wurde unter 96 Stämmen, aus spezifischen Isolationen und Kultursammlungen, aufgrund seines guten Wachstums ausgewählt. Die Behandlung von Urin, 1:1 verdünnt mit Leitungswasser und ohne Zusatz von Nährstoffen, fand bei einer Lichtintensität von 600 µmol Photonen m^{-2} s^{-1}, bei 2.5 % CO_2 und pH 6.5 statt. Eine Wachstumsrate von 7.2 g Trockenmasse m^{-2} Tag^{-1} und Aufnahmen von 13.1 % und 94.1 % von N und P wurden erreicht. Die Behandlung von Urin mit Aktivkohle konnte mögliche hemmende Effekte von Pharmazeutika verhindern. Diese Ergebnisse bilden eine Basis für die weitere Entwicklung der Technologie im Pilot-Maßstab. Falls Risiken für die Gesundheit von Mensch und Umwelt ausgeschlossen werden können, könnte die Algenbiomasse eines 3-Personen-Haushalts genutzt werden, um jährlich 31 kg Weizen und 16 kg Soja zu produzieren, was den kalorischen Nahrungsbedarf für ca. einen Monat des Jahres decken würde. In Kombination mit anderen Technologien könnten PSBRs in einem dezentralen System zur Ressourcenrückgewinnung angewendet werden und dazu beitragen, die Verbindung zwischen den Nährstoffen in Abfall und der Lebensmittelproduktion lokal herzustellen.

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1 Introduction

1.1. The global nutrient and water crises

The availability of fresh water and nutrients to supply basic human needs can be considered to be among the mayor future challenges facing humanity as a whole. Of the 9 billion people estimated to inhabit the earth in 2050, 2-7 billion will expectedly be facing water shortages (Cordell et al. 2011; UNESCO 2006). In order to produce food for this rapidly growing human population, industrial agriculture is in demand of large quantities of mineral fertilizer. The principal nutrients in plant fertilizer are nitrogen (N) and phosphorus (P), both vital elements needed by all organisms. Nitrogen for fertilizer is commercially produced using the Haber-Bosch process, fixing atmospheric di-nitrogen into a reduced, bio-available form: Ammonia (Haber 1910). Global synthesis of ammonia amounted to ~ 100 Tg per year in 2008 and is projected to rise in parallel with the growing population (Erisman et al. 2008). The energy demand of this process is at least 32 MJ kg^{-1} N, making up for approximately 1-2 % of the total global energy consumption (Erisman et al. 2008). In contrast, phosphorus is a finite resource which is commercially mined from mineral rock deposits. The mayor reserves are spatially confined to 5 countries: Morocco, China, USA, Jordan and South Africa (Jewell & Kimball 2015). Mining of phosphate rock for fertilizer amounts to ~ 20 Tg per year (Cordell et al. 2011) and different projections predict the depletion of land-based reserves in 50-250 years (Cordell et al. 2009; Isherwood 2000; Smil 2000). Although the exact timing is difficult to foresee and further P reserves might be tapped into by dredge mining of seamounts in the future (Jewell & Kimball 2015), the irreplaceable and finite nature of this element is obvious. In the words of biochemist and famous science writer Isaac Asimov:

"Life can multiply until all the Phosphorus has gone and then there is an inexorable halt which nothing can prevent." (Asimov 1974)

Besides the energetic, economic and societal concerns linked to fertilizer production, its extensive use also has mayor environmental implications. Over-application, inefficient conversion and run-off from fields into aquatic ecosystems leads to eutrophication and is considered the main cause of hypoxic "dead zones", found in more than 400 marine systems globally (Diaz & Rosenberg 2008). Toxic cyanobacteria blooms and the associated loss of freshwater supply are also mainly linked to the input of nutrients from agriculture (Michalak et al. 2013). Apart from run-off, N is also lost from animal- and plant-producing agricultural systems in form of the greenhouse gas nitrous oxide (N_2O) (Mosier et al. 1998). For P it is assumed that only about 16 % of the extracted resource is being consumed as human food, while the rest is lost by erosion or in animal waste (Chen & Graedel 2016; Rittmann et al. 2011). Besides an urgent need to optimize agricultural practices towards higher nutrient efficiency, the nutrients that do end up in human consumption present a valuable source for recovery and reuse. Indeed, the P present in human urine and feces could account for up to 22 % of global P-demand if it were recovered as fertilizer (Glibert et al. 2006).

The treatment of human excreta thus presents a valuable opportunity to close the link between sanitation and food, however currently used technology is not designed for this purpose (Verstraete et al. 2009; Ashley et al. 2011). Centralized municipal wastewater treatment plants (WWTPs), as employed in most industrialized countries, have the main purposes of preventing environmental degradation by removing pollutants and protecting public health by providing sanitation (Hammer & Hammer 2012). A central technology in such operations is the activated sludge process, in which organic compounds are microbially converted, mainly into gaseous carbon dioxide (CO_2). Due to its need for aeration, this process makes up between 45-75 % of the total energy demand in a WWTP and is the mayor cost factor in its operation (Henze et al. 2008). Nitrogen is conventionally removed by a sequence of nitrification and denitrification steps, microbial processes under aerobic and anaerobic conditions, respectively. The desired outcome is the conversion of fixed nitrogen into gaseous di-nitrogen (N_2), however N_2O is also produced and may account for up to 3.4 % of total N converted (Wang et al. 2016). Phosphorus can be efficiently removed from municipal waste water either chemically, by precipitation with iron or aluminium or biologically, by phosphate accumulating organisms (PAO) (de-Bashan & Bashan 2004).

In both cases the P ends up in sludge, together with contaminants such as heavy metals and pharmaceutical residues, significantly reducing its reuse potential (Smil 2000). In practice, the sludge from such treatments is often burned (Hammer & Hammer 2012) and thus more than 10.5 million tons of P are not recovered for reuse annually (Liu et al. 2008). Another seemingly wasteful aspect in the centralized system of municipal WWT is using large amounts of (drinking-) water to transport (flush) relatively small amounts of liquid and solid waste. The installation of piping for sewage disposal is the mayor cost in implementation of municipal WWTPs, while the dilution of waste streams impedes the potential to recover resources (Dockhorn 2016). Pipes for sewage disposal are considered an inappropriate technology in regions which are water stressed (Fry et al. 2008). Diffuse emissions of CH_4 and H_2S from sewers are further reasons to question the sustainability of such systems (Verstraete et al. 2009). It can be concluded that centralized WWT is an end-of-pipe technology which is effective at removing pollutants from water in the forms of gas and sludge, but largely disregards the potential of resource recovery and water savings.

In developing countries, where most of the global population increase is expected, water and nutrient scarcity as well as lack of sanitation are concerns of an even greater dimension. Open defecation is still a widespread practice in large parts of Africa and Asia, where 2.6 billion people have no access to improved sanitation facilities (WHO & UNICEF 2012). The lack of processing of human waste brings about serious health risks (epidemics, malnutrition) as well as environmental degradation, endangering the availability of potable water (Corvalan et al. 2005). At the same time, rising mineral fertilizer prices are increasing the economic pressure on farmers who are relying on their acquisition (Agoramoorthy 2008; Hossain & Singh 2000). Taken in concert, these arguments are calling for a paradigm shift in the way in which human waste is viewed: Not only as a source of pollutants, but at the same time as a mine of valuable resources to be recovered. In order to fulfill future needs of human nutrition and health and to conserve global ecosystems, the unidirectional flow of nutrients and water should be changed into to a system of locally closed loops. Both in industrialized and developing countries, there is an urgent need for solutions that combine adequate sanitation with a valorization of the nutrients from human waste.

1.2. Source separation and resource recovery

A relatively new idea in applied research on municipal wastewater treatment is source separation combined with decentralized sanitation and resource recovery. The concept is being investigated under various labels such as DESAR (decentralized sanitation and reuse; (Kujawa-Roeleveld & Zeeman 2006), ECOSAN (ecological sanitation; (Langergraber & Muellegger 2005)) or NoMix (Larsen & Lienert 2007). While different combinations of technologies for collection and treatment are being proposed, the unifying principle is to separately collect waste streams and to treat them on-site, with the aims of maximal resource recovery and water savings. Pilot operations for > 100 inhabitants or workers are currently being operated at the head-offices of the German technical co-operation GTZ, Kullön residential area (Sweden), Sneek residential area (the Netherlands), Erdos Eco-Town (China), the EAWAG research institute (Switzerland) and other places worldwide (Lienert & Larsen 2010; Kvarnström et al. 2006; Zeeman & Kujawa-Roeleveld 2011; Zhou et al. 2010). Sweden is the pioneer in No-mix technology, with over 135,000 urine-diverting toilets installed before 2006 (Lienert & Larsen 2010). Figure 1 schematically shows the flow of the main liquid and solid waste streams in a model household:

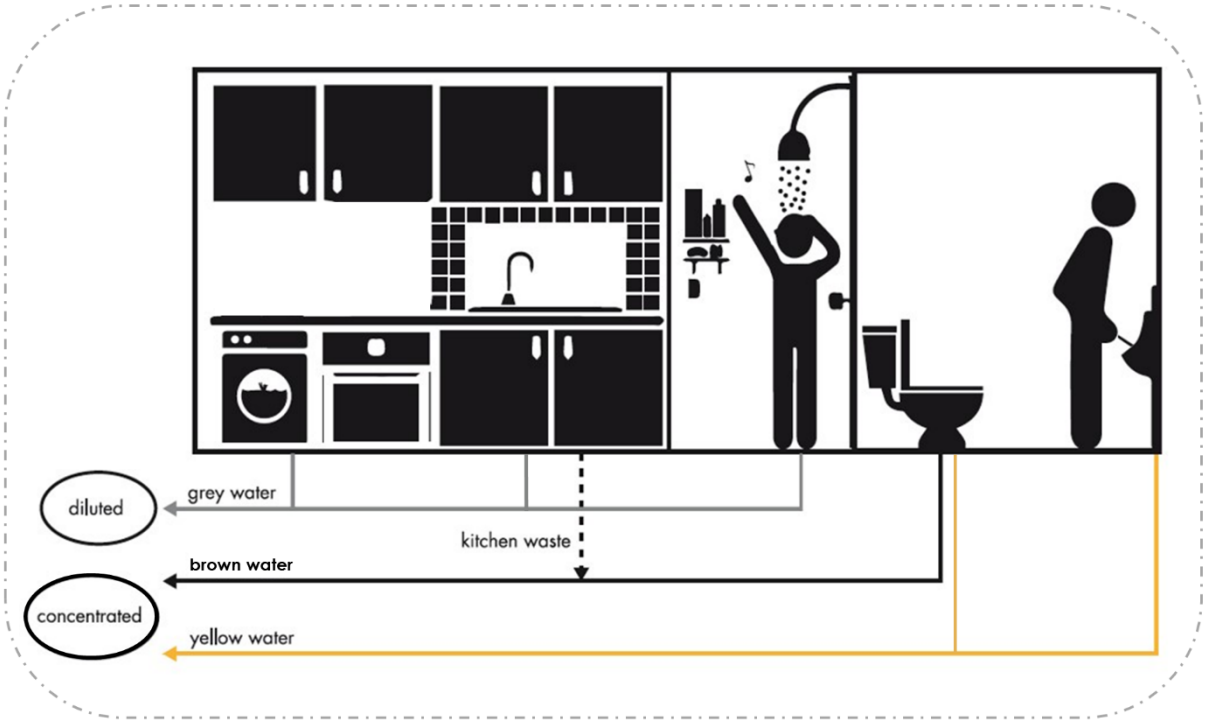

Figure 1: Schematic drawing showing source separation of waste streams in a model household. Image courtesy of Alex Graf Illustration with modifications by the author.

Grey water, originating from showering, dishwashing and laundry makes up the largest volume of household waste waters (~80 %) (Li et al. 2009). The concentration of pollutants is relatively low and it can be upgraded to safe irrigation- or flush-water by simple processes, such as settling and sand filtration (Finley et al. 2009) or treatment in a rotating biological contractor (Friedler et al. 2005). Toilet wastes can be collected by urinals and No-mix toilets, a technology which is generally viewed positively by potential users (Lienert & Larsen 2006; Pahl-Wostl et al. 2003). The type of toilet defines the degree of dilution, with water saving options such as vacuum- and low flush toilets preferable (Johansson 2000). Brown water, comprised of feces, paper and flush water, contains most of the organic compounds in household waste (50-70%) in a concentrated form (Kujawa-Roeleveld & Zeeman 2006). It can be combined with kitchen- and other organic waste and treated by anaerobic digestion, a microbial conversion producing biogas. The gas, mainly consisting of CO_2 and methane (CH_4), can be used for cooking as well as heating of houses and of the treatment systems itself, making anaerobic digestion a core technology in most decentralized sanitation systems (Wendland et al. 2007; Zeeman & Kujawa-Roeleveld 2011).

Urine, which together with flush water is collectively termed yellow water, contains within less than 1 % of the total volume of household waste water up to 80 % of the N and 40-50 % of the P, as urea and ortho-phosphate, respectively (Wilsenach & van Loosdrecht 2006). These high concentrations make it an attractive stream for the recovery of nutrients. Indeed, urine can be directly applied to agricultural crops, a practice that has historically been widely used e.g. in China ~5000 years ago (Ashley et al. 2011) and is recently being tested at larger scales, for example in Sweden (Johansson 2000). Although results from growth experiments with various crops have been positive (Akpan-Idiok et al. 2012; Pradhan et al. 2007), several problems are associated with the direct application of urine: Runoff to the environment in case of rain, volatilization of N depending on application technique and climate (Rodhe et al. 2004), and large volumes of liquid that need to be stored, since constant application would oversupply the fields (Winker et al. 2009). Storage of urine under non sterile conditions leads to the bacterial hydrolysis of urea to ammonium (NH_4^+) / ammonia (NH_3), according to the following formula (from Udert et al. 2003):

$$NH_2(CO)NH_2 + 2H_2O \rightarrow NH_3 + NH_4^+ + HCO^-$$

The reaction increases the pH of the solution, shifting the equilibrium between NH_4^+ and NH_3 towards the uncharged ammonia (pKa 9.3) (Warner 1942). The high pH can lead to volatilization of ammonium to the gas phase as well as precipitation of phosphate-containing minerals (e.g. struvite ($NH_4MgPO_4 \cdot 6H_2O$) or calcium-phosphate ($Ca(H_2PO_4)_2$), making urine an unstable liquid in storage (Maurer et al. 2006). Furthermore, the application of urine to soils can lead to increases in salinity, threatening their long term fertility (Mnkeni et al. 2008; Jönsson 2004).

A technique to temporally uncouple the application of fertilizer from urine excretion and to solve the issue of salinity increase makes use of the bacterial hydrolysis of urea by intentional precipitation of struvite (Magnesium ammonium phosphate; MAP), a mineral composed of the three elements in an equimolar ratio (Ronteltap et al. 2007; Ban & Dave 2004). The process can be realized with the simple means of a stirred tank reactor and addition of a magnesium source, and has been demonstrated at pilot-scale in various studies (Adnan et al. 2003; Lind et al. 2000; Etter et al. 2011). Struvite has been used in greenhouse trials for various crops and proved to be a good source of phosphorous (Gell et al. 2011; Antonini et al. 2012). Despite the advanced status of this technology, its mayor shortcoming is the limited nitrogen recovery potential, which is dictated by the 1:1 molar ratio of N and P in struvite. Since atomic N:P ratios can range between 30:1 – 50:1 in human urine (Putnam 1971), struvite precipitation leaves the largest fraction of nitrogen unused. For the same reason it cannot suffice as a complete fertilizer for plants. Indeed, pot trials on wheat (*Triticum aestivum*) fertilized with struvite showed that plants were nitrogen limited (Ganrot et al. 2007).

1.3.Microalgae for nutrient recovery

Microalgae are very efficient at taking up mineral nutrients, using sunlight as their sole source of energy. The concept of phototrophic municipal waste water treatment employing these organisms has first been proposed almost 60 years ago with High Rate Algal Ponds (HRAPs) for concomitant nutrient recovery and oxygenation (Oswald et al. 1957). The number of studies in the field has since risen rapidly, especially due to the recent efforts of producing biofuels from algae (e.g. Pittman et al. 2011). Although

practical experience is still scarce, microalgal biomass grown on wastewater might realistically be used as an organic slow release fertilizer, due to its elemental composition being similar to that of plants (Kebede-Westhead et al. 2004; Mulbry et al. 2005). Besides a suitable content of N and P, algal biomass also contains plant growth-promoting microelements (e.g. Fe, Mn, Zn, Cu), which may increase its usefulness as fertilizer (Shaaban 2001). Alternatively, high value compounds (e.g. pigments) could be extracted from biomass, enhancing the economic feasibility of this approach (Cai et al. 2013). Using microalgae to recover nutrients from source separated urine thus presents an added-value alternative to struvite treatment.

To-date, there are ten published studies dealing with microalgal nutrient recovery from human urine, all of which employed suspension systems for cultivation: Adamsson (2000) used *Scenedesmus acuminatus* to provide a feed for *Daphnia magna* in combination with the culturing of tomatoes. In this study, urine was applied at a 1:100 dilution, with the addition of Fe and Mg. Feng & Wu (2006), Yang, Li, et al. (2008) and Yang et al. (2008) as well as Chang et al. (2013) cultivated *Spirulina (Arthrospira) platensis* in 1:140 dilutions of synthetic human urine, with the addition of micronutrients and vitamins, placing their studies in the context of life-support systems for space travel. Zhang et al. (2014) encountered problems of mineral precipitation when using urine at moderate dilutions (between 1:1 and 1:5) and circumvented the problem by dosing small amounts of urine as N and P source to a conventional growth medium for *Chlorella sorokiniana*. Using the same algal strain, Tuantet et al. were the first to establish stable growth on minimally diluted (1:1) and undiluted human urine, in both microtiter plate experiments (Tuantet, Janssen, et al. 2014) and continuously operated flat plate reactors (Tuantet, Temmink, et al. 2014). In their studies, the addition of trace elements as well as Mg and P was considered necessary, mainly due to loss of the latter two elements by uncontrolled precipitation (Tuantet, Temmink, et al. 2014). Jaatinen et al. (2016) used dilutions from 1:25 up to 1:300 at low light intensities to cultivate *Chlorella vulgaris* and found that under these conditions, bacteria were dominating their cultures. Finally, Coppens et al. (2016) cultivated *Arthrospira platensis* on urine which was pre-stabilized by a biological nitrification step.

These studies provide a framework for the further development of urine treatment with microalgae towards a realistic application. In this context the use of low dilution factors without the addition of chemicals should be imperative, in order to maximize the water- and resource-efficiency of the process. Human urine contains compounds of interest for algal growth in the following order of concentration: Urea, Cl, Na, K, S, P, Ca and Mg (Putnam 1971). Trace elements such as Fe, Cu and Zn are also usually present, with their concentration depending on individual habits (Rodríguez R. & Díaz R. 1995). Fresh urine contains urea as the main source of nitrogen, which is known to be used by a variety of microalgae (Antia et al. 1991; Neilson & Larsson 1980). The uncharged free ammonia, formed by hydrolysis of urea and a shift of the NH_3/NH_4^+ equilibrium at high pH, can diffuse freely across cell membranes and is toxic to most organisms, including microalgae (Azov & Goldman 1982). Furthermore, the precipitation of phosphate containing minerals, typical for hydrolyzed urine (Udert et al. 2006), can deprive algae of phosphorus for their growth. Thus, in order to prevent the formation of toxic NH_3 and P-precipitates, using fresh urine to grow an alga that can metabolize urea while exhibiting tight pH-control appear to be of utmost importance. The selection of an algal strain that is adapted to growth on urine might furthermore circumvent the need for addition of nutrients.

Despite the relative success of the abovementioned studies, the employed systems of suspension based cultivation have mayor shortcomings when practically applied: The most common large-scale algal cultivation system is the raceway pond or HRAP, as it is usually called in wastewater treatment. Due to the shallow light penetration, HRAPs show low productivities and resulting low biomass concentrations, leading to high energy consumption and costs for separating biomass and water (harvesting) (Hoffmann 1998). Furthermore, controlling the strain of algae which dominates in these open systems is difficult and contamination with grazing organisms frequently lead to culture crashes and operational downtime (Cai et al. 2013). Closed photobioreactors (e.g. tubular or flat plate PBRs) can be operated much more efficiently, due to short light paths and controlled process conditions (Ugwu et al. 2008). However, their investment costs are relatively high and the problem of harvesting algae from the suspension still poses a significant economic hurdle for their application in wastewater treatment (Acién et al. 2012). Submerged biofilm systems solve some of these issues by growing a productive and dense biomass that

is simple to harvest (Kesaano & Sims 2014). Nevertheless, washout of cells is commonly observed in such systems (Boelee et al. 2011; Posadas et al. 2013), leading to problems especially in regions were the concentration of suspended particles in discharge water is controlled (Mallick 2002).

A relatively new approach to the technical cultivation of algal biofilms are Twin Layer - porous substrate bioreactors (TL-PSBRs), where water and biomass are effectively separated. The method was invented at the University of Cologne and initially used for small scale cultivation of algae in biosensors and culture collections (Melkonian & Podola 2004; Nowack et al. 2005; Podola & Melkonian 2003). At full scale, these immobilized systems might overcome many of the shortcomings of conventional algal cultivation, since they combine high productivities with simple construction and operation (Podola et al. 2016; Schultze et al. 2015). Figure 2 shows a schematic representation of the PSBR technology at different levels of magnification:

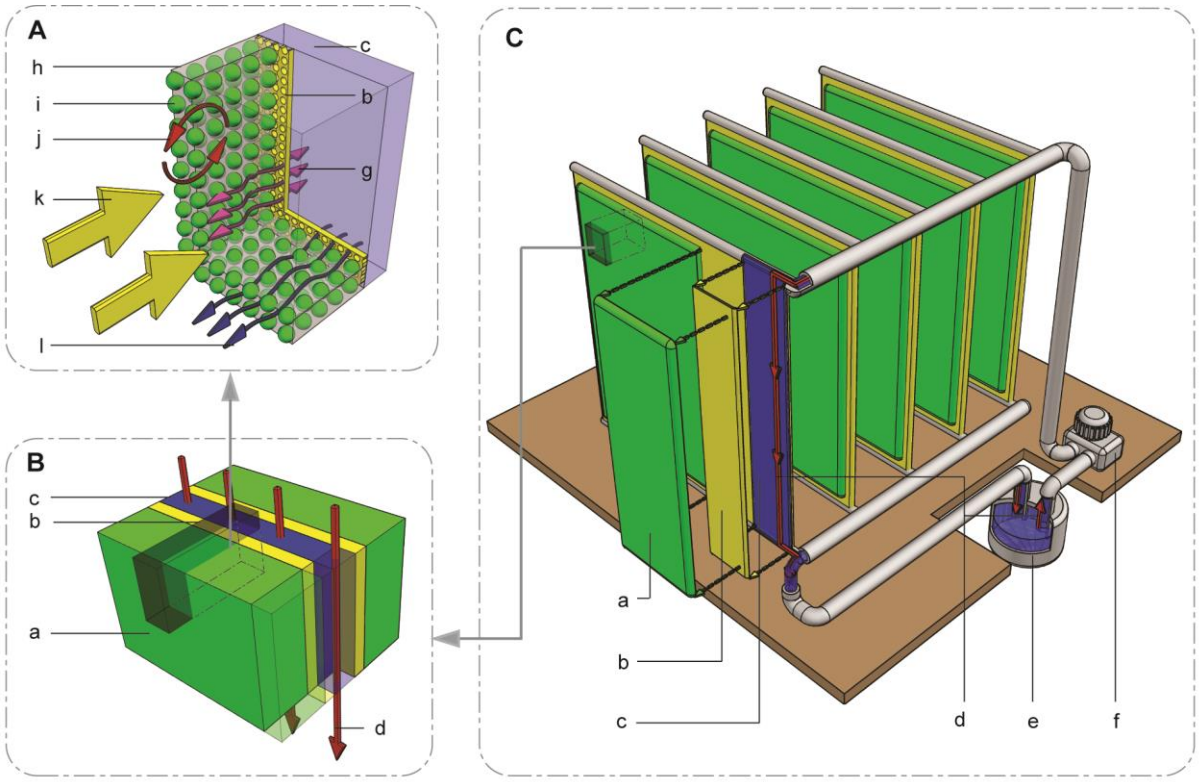

Figure 2: Schematic representation of a Twin Layer Porous Substrate Biofilm Photobioreactor (PSBR). (A) PSBR-immobilized cells and physical processes. (B) Arrangement of layers in a vertical PSBR. (C) Large-scale vertical PSBR with multiple sheets. a: algal biofilm, b: microporous membrane, c: culture medium, d: direction of medium flow, e: medium reservoir, f: medium pump, g: diffusion of solutes, h: extracellular matrix, i: algal cells, j: gas exchange, k: irradiance, l: evaporation. Image from (Podola et al. 2016), reprinted with permission from Cell Press.

Cells are immobilized on a sheet-like porous membrane impermeable to the cells due to its pore size, but permeable for liquids, in order to supply dissolved nutrients (substrate layer; Fig. 2, b). The liquid medium (wastewater) is applied to a glass fiber or grid by dripping and is transported through this layer by gravity (source layer; Fig. 2, c). The low energy demand for water circulation as well as the low water content of biomass and associated ease of harvesting and processing of biomass can make these systems economically highly attractive (Podola et al. 2016), which is especially important for wastewater applications. The spread of contamination is usually minimal, since invasion from the medium is prevented by means of the substrate layer while organisms coming from the air are immobilized along with the algal biomass. Furthermore, the low shear forces to which cells are exposed enable the cultivation of a diverse variety of microalgal species (Nowack et al. 2005; Benstein et al. 2014; Naumann et al. 2013; Kiperstok et al. 2016). The system has been employed for nutrient recovery from various municipal waste waters, both at laboratory-scale (Shi et al. 2007) and prototype-scale (Shi et al. 2014), as well as for anaerobically digested black water (pers. obs., unpublished data), but so far not for the treatment of source separated human urine.

1.4. Outline of this study

The aim of this study was to establish a robust process in which PSBR-immobilized microalgae would recover nutrients from minimally diluted, unamended human urine. To achieve this, three distinct steps were taken:

(1) Isolation of algae from the environment and screening of strains from culture collections

Algae which are able to grow on human urine were enriched from the environment and established as clonal cultures. Furthermore, strains derived from other wastewater sources or showing potential of utilizing urea were selected. In a comprehensive screening several promising strains were chosen for further experiments.

(2) Investigation of the nitrogen metabolism of selected strains with focus on urea

Selected axenic strains were tested for their ability to grow on various nitrogen sources and their metabolism of urea was investigated in liquid cultures, to draw conclusions on the use of this molecule.

Using the information from steps (1) and (2), one strain was selected to:

(3) Optimize process parameters for stable growth and nutrient recovery

The selected strain was grown under semi-realistic laboratory conditions on a Twin Layer PSBR system. Process parameters as well as necessary pre-treatments of urine were optimized to ensure stable operation of the treatment. The potential for recovery of nitrogen and phosphorus was determined.

2 Materials and Methods

2.1. Human urine

Four batches of human urine were used in the course of this study. Batch A and B were collected in 2014 from 1 and 3 male members of the Melkonian laboratory, respectively. Batches C and D were collected during the 2015 and 2016 editions of the animal physiology practical course at University of Cologne, respectively, from student volunteers of both genders. The details of the collections are summarized in table 1.

Table 1: Urine batches used in the various experiments, their volumes, number of donors and experiments they were used in.

Urine batch	Number of donors	Volume (L)	Experiment (chapter #)
A	1	3	Isolation (3.2)
B	3	6	Suspension Screening (3.3)
C	53	25	Prevention of precipitation (3.5) Growth on TL-PSBR (3.6)
D	57	40	Growth on TL-PSBR (3.6) Nutrient recovery (3.7)

Collections lasted 3-5 days. During the time of collection, urine was stored in 10 and 25 l plastic containers at 4 °C. Once the collection of one batch was complete, the whole volume was mixed and stored frozen in plastic bottles (0.5 – 1 l) at -20 °C until use. To formulate a growth medium for microalgae, urine was diluted with different kinds of water. Waters used were: Ultrapure water (MQ), Tap water from Cologne (TapCGN), and tap water from Bonn (TapBN). The elemental composition of these waters was analyzed by inductively coupled plasma –mass spectrometry (ICP-MS) at the MS-platform at University of Cologne using an ICP-MS 7700 (Agilent, Santa Clara, USA). Electrical conductivity and pH of urine as well as mixtures of urine and water were measured regularly.

2.2. Nutrient determination

Concentrations of the mayor forms of nitrogen and phosphorus were measured routinely for new batches of urine as well as during some experiments. All nutrient measurements are based on spectrophotometric analyses, carried out using an infinite PRO 200 multiplate reader (Tecan, Männedorf, Switzerland) in 96-well microtiter plate format. Calibration curves were measured whenever a new batch of color reagent was used.

2.2.1. Urea

Urea was determined using a method modified from Orsonneau et al. (1992). The principle was quantification of the color change of a pH indicator upon enzymatic hydrolysis of urea. In short, 300 µl of color reagent containing 2.5 mM indicator grade σ-cresolphtalein complexone (Alfa Aesar, Karlsruhe, Germany) were placed in the microtiter plate, mixed with 6 µl of sample and the absorbance at 575 nm was measured as background value. After this, 30 µl of enzyme solution (200 kUnits l^{-1} of urease from jack bean (SERVA electrophoresis GmbH, Heidelberg, Germany) in 150 mM NaCl) were added and the mixture was incubated in the dark at 37 °C for 10 min. Absorbance at 575 nm was measured again and the difference between absorbances was compared to a calibration curve (0-500 mM urea) to give the sample concentration of urea. Since this method was newly established in the laboratory, tests were conducted to determine if matrix-associated effects would jeopardize the accuracy of the method when employed in urine. In a standard addition procedure, known concentrations of urea were added to a sample of urine and compared with a calibration curve. The concentration of 64.5 mM in the urine sample could be back-calculated with >95% accuracy at added urea concentrations between 0-500 mM. Thus, the method was found to be suitable, the results are presented in appendix I.

2.2.2. Ammonia / Ammonium

Concentrations of ammonia and ammonium were determined using the indophenol method based on Verdouw et al. (1978), modified to suit the microtiter plate format. Briefly, 200 µl of reagent 1 (5 % sodium salicylate) and 10 µl of sample were mixed with 50 µl of reagent 2 (0.1 M NaOH solution with 5 % sodium citrate, 0.8 % sodium dichloroisocyanurate and 0.3 % sodium nitroprusside). The color

reaction was performed at room temperature in the dark for 60 min, after which the absorbance at 660 nm was measured. Calibration curves were prepared with standards of 1-8 mM NH_4^+.

2.2.3. Nitrate / Nitrite

Nitrate and nitrite (NO_x) concentrations were determined using a method according to Miranda et al. (2001). Calibration curves were prepared using standards of 0.1-3 mM NO_3^-.

2.2.4. Phosphate

Ortho-phosphate (PO_4^-) concentrations were determined according to Murphy & Riley (1962) with modifications to suit the microtiter plate format. Briefly, 2.5 µl of sample was diluted in 200 µl MQ water and mixed with 40 µl of the color reagent in the well. Incubation was performed in the dark at 40 °C for 30 min after which the absorbance at 882 nm was measured. Calibration curves were prepared using standards of 0.1-2 mM PO_4^-.

2.2.5. Total nitrogen and phosphorus

Total nitrogen and total phosphorus concentrations in digests of liquid samples were measured as NO_3^- and PO_4^-, respectively. To oxidize all forms of nitrogen and phosphorus present, a persulfate digestion, based on the method of Cabrera & Beare (1993), was performed. The digestion solution contained 5 % $K_2S_2O_8$, 1.68 % NaOH and 3 % H_3BO_3. The sample was added in a ratio of 1:1 and the digestion performed at 95 °C for 3h. Measurements of nitrate and phosphate in the digestate were performed as described above.

2.2.6. Identification of precipitates

Precipitation of phosphorus-containing minerals (e.g. struvite ($NH_4MgPO_4 \cdot 6H_2O$) or calcium-phosphate ($Ca(H_2PO_4)_2$) is a well-known phenomenon in urine, especially when the pH rises due to hydrolysis of urea under non sterile conditions (Udert et al. 2006). Since precipitated nutrients are not available for algal growth, experiments to control the pH were conducted to test methods of counteracting precipitation. 500 ml of urine, 1:1 diluted with TapBN, were placed 1l glass bottles which were left under non sterile conditions at 23 °C, as in later Twin Layer experiments. To one set of bottles, constant sparging of air with 2.5 % CO_2 at 1 l min^{-1} was applied. As a control, another set of bottles was sparged

with just air at the same flow rate. Experiments were carried out in triplicate. The pH, as well as concentrations of ammonium and urea were measured, to monitor the effect of hydrolysis. The formed precipitates were analyzed after 14 days, with a method based on the works of Doyle et al. (2003) and Hen Sabbag et al. (2016). The precipitate was washed 3-fold by centrifugation and replacement of supernatant with MQ water. Precipitates were then dissolved by addition of 1 M HCL. Cations (Mg^{+}, Ca^{+}) were bound by addition of 0.25 mM ETDA and the pH was brought back to neutrality by addition of 1 M NaOH. No more precipitates were visible at this point and the solution was used for measurement of ammonium and phosphate, as described above.

2.3. Algal strains

To find algal strains optimally suited for growth on human urine, two different strategies were employed: (A) Isolation of new strains from the environment on human urine and (B) Testing of established strains, mainly isolated from wastewater sources or with other indications to be suitable for growth on urine such as literature on the use of urea as nitrogen source.

2.3.1. Isolation

For the isolation of algae from the environment, open plastic containers filled with 250 ml of urine of batch A, diluted with MQ at 1:1, 1:5 and 1:10 dilutions were placed on the premises of the "Versuchsgärtnerei" at University of Cologne, sheltered from rain. This setup is from hereon called "urine trap". The urine traps were left in the open for 6-8 weeks in April and May of 2015, until algal growth was visible. The establishment of clonal cultures was attempted both directly from the raw samples and from enrichment cultures. The latter were derived by placing a subsample in artificial growth media and incubating it at 23 °C and ~100 µmol photons m^{-2} s^{-1} light intensity using a 14:10 h light:dark cycle. The media used were Waris-H (McFadden & Melkonian 1986) and BG-11 (Stanier et al. 1971) as modified by Naumann et al. (2013). In several cases Waris-H+Si was used, which additionally contained 0.5 mM Na_2Si_3. Isolation of single cells was performed using the microcapillary technique, as described by Pringsheim (1946), with the aid of an inverted microscope. At this level, cultivation was performed in 24-well microtiter plates and 50 ml Erlenmeyer flasks. Once unialgal cultures were established, they were attempted to be made axenic by spraying onto an agar-dish and

subsequent picking of a single colony, as described by Surek & Melkonian (2004). For this purpose cultures were washed several times by gentle centrifugation (300 g) and re-suspension in fresh culture medium, to discard most bacterial contaminants. The dilute algal suspension was then placed in a sterile reagent tube with a glass capillary held in place by cotton. A stream of filter-sterilized air was guided to the opening of the capillary, producing a fine mist that was sprayed onto the petri dish, which contained the respective growth medium solidified by 1 % agar. After several weeks, plates were inspected visually and bacteria-free colonies that were derived from a single algal cell were transferred into liquid medium with a sterile toothpick.

2.3.2. Identification of isolates

Identification of algal strains isolated in this study was based mainly on morphological characteristics. The guidelines used can be found in Linne von Berg et al. (2012). As a further tool for identification and taxonomic placement, the information found on algaebase.org (Guiry & Guiry 2016) was used.

To obtain unambiguous identities and allow for strain comparisons with the literature, isolates of interest for further experiments were also identified by sequencing of the nuclear encoded small subunit rRNA operon (SSU rRNA) and subsequent database comparison. DNA was isolated using the CTAB method (Stewart & Via 1993), with the modification of adding acid washed glass beads for more efficient cell disruption, and stored at -20 °C until further use. Fragments of interest were amplified via Polymerase Chain Reaction (PCR) (Saiki et al. 1988). PCR reagents of type "Dreamtaq" (Fermentas, Waltham, USA) were used throughout. DNAse-free water used was "molecular grade water" (Applichem, Omaha, USA). The composition of one reaction is presented in table 2.

Table 2: List of PCR components

Component	Volume
Reaction buffer	2.5 µl
dNTP mix (0.25 mM)	2.5 µl
Forward primer	0.25 µl
Reverse primer	0.25 µl
Polymerase	0.125 µl
Matrix-DNA	0.5 µl
Molecular grade water	25 µl

Primary amplification was performed with EAF3 forward and ITS055 reverse primers, while in the secondary partially nested amplification, BR was used as reverse primer while EAF3 remained the forward primer (Marin et al. 2003). Reactions were performed in a thermocycler as follows: 3 min denaturation at 95 °C, followed by 30 cycles of 0:45 min denaturation (95 °C), 1 min annealing (55 °C) and 3 min extension (72 °C). Final extension was performed for 5 min at 62 °C, followed by a continuous hold at 10 °C. As a control for the success of amplification, 3 µl of PCR product mixed with 3 µl of "orange loading dye" (Fermentas, Waltham, USA) were run on a 1 % agarose gel in TAE buffer (2 mM Tris, 1 mM Na-acetate, 5 µM Na_2-EDTA). The voltage was 100 V and separation continued for 15-30 min. 1.5 µl of "1 Kb DNA ladder" (Fermentas, Waltham, USA) served as reference concerning the amount and length of PCR product. To purify the product, 100 µl of isopropanol were added and the DNA left to precipitate overnight at -20 °C. After centrifugation, the resulting pellet was washed 3 times with 80 % ice-cold ethanol. Finally the pellet was air-died and re-suspended in 50 µl of molecular grade water. Sequencing was performed commercially in "prepaid plate kits for PCR product" (Eurofins Genomics, Ebersberg, Germany). Obtained sequences were aligned and corrected manually with AlignIR software V 2.0.48 (LI-COR Biosciences, Bad Homburg, Germany). Next relatives were found in the public databases by BLAST-algorithm search (Altschul et al. 1990) on the NCBI website (http://blast.ncbi.nlm.nih.gov; accessed 06.07.2015 and 20.07.2016).

2.3.3. Stock cultures

Stock cultures were kept in 50 ml Erlenmeyer flasks at 16 °C and ~30 µmol photons m^{-2} s^{-1} light intensity under a 14:10 h light:dark cycle using Waris-H medium. Cultures were transferred regularly, approximately every 6 weeks. Cultures were transferred ~1 week before using them as inoculum for an experiment, to ensure logarithmic growth. The following table lists the 96 different microalgal strains that were used in the course of this work:

Table 3 A: Microalgal strains used in this study, their growth media and sites of origin. CCAC: Culture Collection of algae at university of Cologne (letter B stands for presence of bacteria); CCAP: Culture Collection of Algae and Protozoa; BI: Brazil Isolate provided by Alice Kiperstok; U: Urine isolates from this study; VZ: isolates provided by Veronica Zilz (AG Melkonian). WWTP – waste water treatment plant.

Identifier	Species	Medium	Origin
BI-11	*Chlamydomonas sp.*	Waris-H	municipal WWTP, settlement
BI-16	*Desmodesmus sp.*	Waris-H	municipal WWTP, settlement
BI-19	*Chlorella sp.*	Waris-H	municipal WWTP, settlement
CCAP 211/8K	*Chlorella sorokiniana*	BBM	freshwater
U 10.1	*Chlamydomonas sp.*	BG 11	urine enrichment
U 10.10	*Chlorella sp.*	BG 11	urine enrichment
U 10.2	*Cocconeis*	BG 11	urine enrichment
U 10.3	*Stichococcus sp.*	Waris-H+Si	urine enrichment
U 10.3	*Stichococcus sp.*	Waris-H+Si	urine enrichment
U 10.4	*Synechococcus sp.*	BG 11	urine enrichment
U 10.5	*Navicula sp.*	Waris-H+Si	urine enrichment
U 10.6	*Heterococcus sp. (?)*	Waris-H+Si	urine enrichment
U 10.7	*Chlorella sp.*	Waris-H+Si	urine enrichment
U 10.8	*Chlorella sp.*	Waris-H+Si	urine enrichment
U 10.9	*Trebouxia sp. (?)*	BG 11	urine enrichment
U 2.1	*Chlorella sp.*	Waris-H+Si	urine enrichment
U 2.2	*Synechocystis sp.*	Waris-H+Si	urine enrichment
U 2.3	*Phormidium sp.*	BG 11	urine enrichment
U 2.4	*Desmodesmus sp.*	Waris-H+Si	urine enrichment
U 5.1	*unidentified coccal*	BG 11	urine enrichment
U 5.2	*Sphaerocystis / Coenochloris*	Waris-H+Si	urine enrichment
U 5.3	*Chlamydomonas sp.*	Waris-H+Si	urine enrichment
U 5.4	*Chlorella sp.*	Waris-H+Si	urine enrichment
U 5.5	*Chlamydomonas sp.*	Waris-H+Si	urine enrichment
U 5.6	*Chlamydomonas sp.*	Waris-H+Si	urine enrichment
U 5.7	*unidentified coccal*	Waris-H+Si	urine enrichment
U 5.8	*Scenedesmus sp.*	BG 11	urine enrichment
U 5.9	*Chlorella sp.*	BG 11	urine enrichment

Table 3 B (continued): Microalgal strains used in this study, their growth media and sites of origin. CCAC: Culture Collection of algae at university of Cologne (letter B stands for presence of bacteria); CCAP: Culture Collection of Algae and Protozoa; BI: Brazil Isolate provided by Alice Kiperstok; U: Urine isolates from this study; VZ: isolates provided by Veronica Zilz (AG Melkonian). WWTP – waste water treatment plant.

Identifier	Species	Medium	Origin
VZ 147	*Acutodesmus sp.*	Waris-H+Si	municipal WWTP, inflow
VZ 148	*Acutodesmus sp.*	BG 11	municipal WWTP, inflow
VZ 150	*Acutodesmus sp.*	Waris-H+Si	municipal WWTP, inflow
VZ 165	*Chlamydomonas sp.*	Waris-H+Si	municipal WWTP, inflow
VZ 185	*Chlorella sp.*	Waris-H+Si	municipal WWTP, inflow
VZ 199	*Dictyosphaerium sp.*	Waris-H+Si	municipal WWTP, inflow
VZ 200	*Dictyosphaerium sp.*	Waris-H+Si	municipal WWTP, inflow
VZ 205	*Haematococcus sp.*	BG 11	municipal WWTP, inflow
VZ 213	*Kirchneriella sp.*	Waris-H+Si	municipal WWTP, inflow
VZ 223	*Klebsormidium sp.*	BG 11	municipal WWTP, inflow
VZ 228	*Monoraphidium cf. arcuatum*	Waris-H+Si	municipal WWTP, inflow
VZ 237	*Monoraphidium cf. litorale*	BG 11	municipal WWTP, inflow
VZ 246	*Monoraphidium cf. litorale*	Waris-H+Si	municipal WWTP, inflow
VZ 252	*Mougeotia sp.*	Waris-H+Si	municipal WWTP, settlement
VZ 276	*Oocystis sp.*	Waris-H+Si	municipal WWTP, inflow
VZ 278	*Oocystis sp.*	Waris-H+Si	municipal WWTP, inflow
VZ 279	*Oocystis sp.*	Waris-H+Si	municipal WWTP, inflow
VZ 280	*Oocystis sp.*	Waris-H+Si	municipal WWTP, inflow
VZ 281	*Oscillatoria sp.*	BG 11	municipal WWTP, settlement
VZ 289	*Phormidium sp.*	Waris-H+Si	municipal WWTP, inflow
VZ 304	*Stauroneis ?*	Waris-H+Si	municipal WWTP, inflow
VZ 305	*Stauroneis ?*	Waris-H+Si	municipal WWTP, inflow
VZ 309	*Stichococcus bacillaris*	Waris-H+Si	municipal WWTP, inflow
VZ 340	*Uronema sp.*	Waris-H+Si	municipal WWTP, inflow
VZ 344	*Uronema sp.*	BBM	municipal WWTP, inflow
VZ 351	*Acutodesmus cf. acuminatus*	BG 11	municipal WWTP, inflow
VZ 355	*Acutodesmus cf. dimorphus*	Waris-H+Si	municipal WWTP, inflow
VZ 356	*Acutodesmus cf. dimorphus*	Waris-H+Si	municipal WWTP, inflow
VZ 392	*Chlorella sp.*	SFM	municipal WWTP, inflow
VZ 393	*Chlorella sp.*	BG 11	municipal WWTP, inflow
VZ 437	*Klebsormidium cf. dissectum*	Waris-H+Si	municipal WWTP, inflow
VZ 462	*Phormidium sp.*	Waris-H+Si	municipal WWTP, inflow
VZ 462	*Phormidium sp.*	BG 11	municipal WWTP, settlement
VZ 466	*Pseudoanabaena sp.*	BG 11	municipal WWTP, settlement
VZ 472	*Rhoicosphenia sp.*	BG 11	municipal WWTP, settlement
VZ 473	*Sphaerobotrys cf. fluviatilis*	Waris-H+Si	municipal WWTP, inflow
VZ 475	*Sphaerobotrys cf. fluviatilis*	Waris-H+Si	municipal WWTP, inflow
CCAC 1964 B	*Stichococcus bacillaris*	BBM	drainage metal waste deposit
CCAC 3524 B	*Desmodesmus maximus*	Waris-H	freshwater
CCAC 3552 B	*Scenedesmus quadricauda*	Waris-H	freshwater
CCAC 3521	*Kirchneriella sp.*	Waris-H	freshwater
CCAC 3526 B	*Kirchneriella sp.*	Waris-H	freshwater
CCAC 3525 B	*Kirchneriella sp.*	Waris-H	freshwater
CCAC 2143 B	*Chlamydomonas sp.*	Waris-H	freshwater
CCAC 2844 B	*Synechococcus sp.*	Waris-H	freshwater
CCAC 3271 B	*Chlorella vulgaris*	BBM	industrial WWTP (automotive)
CCAC 0041	*Chlamydomonas terricola*	BBM	squeeze sample sewage field
CCAC 3496	*Desmodesmus abundans*	Waris-H	industrial WWTP (automotive)
CCAC 3315 B	*Euglena sp.*	Waris-H	freshwater
CCAC 0126	*Halochlorella rubescens*	BBM	marine
CCAC 3520 B	*Kirchneriella sp.*	Waris-H	freshwater

2.4. Experimental cultivation

2.4.1. Screening

The initial screening for growth on urine and determination of an appropriate dilution factor was performed in 24 well microtiter plates with 1 ml cultures, using absorbance at 750 nm as measure of growth in undiluted, 1:1 diluted and 1:5 diluted urine. Most cultures used in the screening were not axenic. In preliminary trials, both the absorbance at 680 nm (chlorophyll absorption maximum) and 750 nm (general turbidity without pigment interference) were tested and compared (see appendix II). The two measures showed a strong linear relationship (r^2>0.9724), thus 750 nm was chosen to prevent interference from differences in chlorophyll content due to the physiological state of cells, as other authors have done before (e.g. Griffiths et al. 2011). To prevent excessive bacterial growth, urine was sterile filtered through 0.2 µm filter cartridges into autoclaved flasks in a laminar-flow cabinet before using it as culture medium. Inoculation was performed from log-phase cultures, which were harvested and washed by centrifugation (800 *g*, 7 min) and three times replacement of the medium with sterile urine of the appropriate concentration. Cultures were inoculated at a density yielding an absorbance at 750 nm of 0.1 and then grown at 23 °C and 80 µmol photons m^{-2} s^{-1} with 14:10 h light:dark cycle on a LED-table for 6 days. Cultures were set up in triplicates. Cultures grown in Bold's Basal Medium (BBM; Bischoff & Bold 1963), as modified by CCAP, Oban, UK (www.ccap.ac.uk), were used as control.

A comprehensive screening of 96 algal strains was subsequently performed in 96-well microtiter plates on 1:1 diluted urine. Cultures had a volume of 200 µl. To prevent excessive evaporation of water from the cultures, the outer wells of the microtiter plate were filled with water only, causing a moist atmosphere in the plate. Absorbance at 750 nm was measured on days 1, 4 and 5. Two reference strains (CCAC 0126 and U 5.5) as well as a negative control (non-inoculated urine) were used in each plate experiment. Besides these changes, experiments were conducted under the same conditions as stated above.

2.4.2. Nitrogen metabolism

To determine the capabilities of using different nitrogen sources, experiments under axenic conditions were performed with seven axenic strains, using Bolds Basal Medium (BBM) and modifications thereof. In the modifications nitrate was exchanged for either urea (BBM_{urea}) or ammonium (BBM_{NH4}) at the same final nitrogen content. Cultures were grown in Erlenmeyer flasks or on agar plates (with addition of 1 % agar to the medium). Before the start of an experiment, axenicity of stock cultures was verified by sub-culturing in Bacterial Standard Medium (BSM), as prepared by CCAC, Cologne, Germany (www.ccac.uni-koeln.de). A drop of culture was placed in BSM, which was diluted 1:1, 1:10 and 1:100 with Waris-H medium in reagent test tubes or 24 well microtiter plates. These were incubated for a total of 7 days at the growth conditions of the algal culture as well as at 37 °C in the dark. As negative control sterile culture medium was used, while a culture known to be contaminated by bacteria served as positive control. Bacterial contamination could be seen as turbidity in the medium.

Erlenmeyer flask

Cultures in BBM_{urea} medium were set up in four replicates. After washing cells three times in sterile medium, cultures were inoculated at 2 x 10^5 cells ml^{-1} and incubated at 23 °C and a light intensity of 100 µmol photons m^{-2} s^{-1} on a LED-table for 23 days. Negative controls consisted of un-inoculated media. Samples to determine cell numbers and dissolved nutrients were taken at regular intervals. Cells were counted using a hemocytometer and nutrients were measured as described above. A liquid sample to determine dry biomass and pH was taken at day 9 and at the end of the experiment. For biomass determination, 10 ml of culture were filtered onto a polycarbonate filter of known weight. The filter was dried at 105 °C for at least 3 h and left to cool down in a desiccator until a stable weight was reached. Addition of 10 µl of a urease solution of 220 U ml^{-1} (Serva Electrophoresis, Heidelberg, Germany), dissolved in 0.15 M NaCl, was performed at day 9 to those cultures which showed no growth until that time point (i.e. were unable to use urea as N-source).

Agar Plate

Cultures were set up in four replicates. Media used were BBM, BBM_{urea} and BBM_{NH4}. As negative control, an agar medium without nitrogen source was used. Before inoculation, cells were harvested and washed three times in sterile medium, after which the suspension was diluted to contain 100 cells in 50 µl of medium. This drop was placed on the agar dish and spread on the surface with a sterile glass reagent tube. Plates were incubated at 23 °C and 50 µmol photons m^{-2} s^{-1} for 4 weeks. After this time, algal colonies were counted and their diameter measured, using a binocular with camera and the image analysis software Image J version 1.51d (Schneider et al. 2012). The average total colony area per plate was used as a means of assessing algal growth in this experiment.

2.4.3. Cultivation on Twin Layer-PSBR

Laboratory scale Twin Layer experiments were conducted as described by Schultze et al. (2015), based on Shi et al. (2007). A dry weight inoculation density of 2.5 g m^{-2} on polycarbonate filter disks was used in experiments for determination of growth, while a fully inoculated nylon membrane was used for the nutrient recovery experiment, as described in chapter 2.4.3.3. All Twin Layer experiments were conducted at temperature-controlled conditions, at 21 °C. Medium was exchanged after 4 days, if not mentioned otherwise. In order to work under more realistic conditions, dilutions of urine and refilling of evaporated water was performed with either MQ water, tap water from Bonn (TapBN) or tap water from Cologne (TapCGN) in this experimental section. In some of the experiments, the pH was kept constant by means on-demand CO_2 addition in the medium. This was realized by means of a PH-803 pH-controller (Analytical Instruments, Colombo, Sri Lanka), connected to a solenoid valve type 356 3/2NC G1/8 (ASCO-Numatics, Michigan, USA) which regulated a flow of 2.5 % CO_2 when the set pH value was exceeded. The full experimental setup, as it was used in this experimental part, is shown in figure 3.

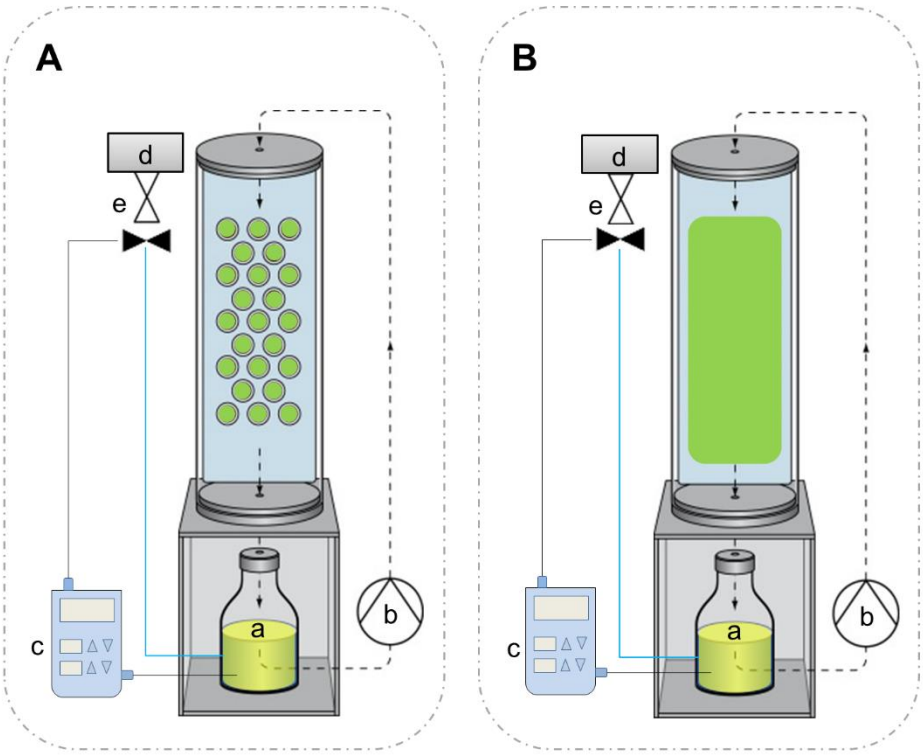

Figure 3: Schematic representation of the experimental setup in Twin Layer experiments with pH control. (A) Setup with inoculated polycarbonate disks for growth rate determination. (B) Setup with inoculated nylon membrane for nutrient recovery. a: medium reservoir, b: peristaltic pump, c: pH-meter, d: CO_2-source, e: solenoid valve. Black dashed lines: medium flow, black unbroken lines: electrical connection, blue lines: gas tubing. Graphic courtesy of Björn Podola and modified by the author.

2.4.3.1. Screening

An initial screening, to test the capability of strains to grow immobilized with urine as medium and to select the strain for further optimization, was performed with 9 algal strains. The conditions were: 1:1 diluted urine (Urine batch C : TapBN); constant 2.5 % CO_2 supplied to the medium and atmosphere; 600 µmol photons m^{-2} s^{-1}.

2.4.3.2. Optimization

A series of experiments was conducted to optimize growth of the selected strain. The variables that were tested and respective experimental conditions are summarized in table 4.

Table 4: Summary of experimental parameters for optimization of growth of strain CCAC 3496 on Twin Layer. Resp. water stands for use of the same type of water as used for dilution/evaporation refill.

Experimental variable	Urine batch	Diluent water	Evaporation refill	Light intensity (µmol photons m^{-2} s^{-1})	pH control	Pre-treatment/ comment
Light intensity	C	TapBN	MQ	400, 600, 800, 1000	constant CO_2	
pH value	C	TapBN	TapBN	600	6, 6.5, 7, 7.5	
Diluent water	D	MQ, TapBN or CGN, Undil.	resp. water	600	6.5	
Evaporation refill	D	TapBN or CGN	resp. water or MQ	600	6.5	& regrowth experiments
Pre-Treatment	D	TapBN	MQ	600	6.5	activated carbon, sterile filtration

When using urine of batch D, an inhibitory effect on growth was detected, together with a bleaching of biomass. To investigate the cause of this, several experiments were performed:

- Freshly inoculated filters were grown on the same batch of urine which had previously caused inhibition, to check whether a limitation was present in the medium.
- Biomass (approx. 1 cm² surface area) from partially bleached filters was scraped off and used as inoculum for a culture in Waris-H medium, to check whether the effect was reversible. These cultures were prepared in 50 ml Erlenmeyer flasks and illuminated at 100 µmol m^{-2} s^{-1}. Cells were counted regularly with a hemocytometer.
- A growth experiment on Twin Layer was conducted with urine that had been treated with activated carbon as well as sterile filtered, to check if a dissolved molecule (e.g. pharmaceutical compound) or a bacterial component caused the inhibition and if the negative effect could be alleviated.

Activated carbon treatment was performed in the following way: 10 g of activated carbon type "Carbopal MB4" (Donau Carbon, Frankfurt, Germany) per 1 l of 1:1 TapBN diluted urine were suspended in the liquid and mixed thoroughly on a magnetic stirrer for 10 min at room temperature. The

particles were then filtered out by a series of decantations over coffee filters of type “Aromata No 4” (Lidl, Neckarsulm, Germany). Sterile filtration was performed as described previously.

2.4.3.3. Nutrient recovery

To determine the removal and uptake of nitrogen and phosphorus from activated carbon-treated urine, a Twin Layer experiment with a fully inoculated surface was performed. A nitrocellulose membrane of type “Zeta-Probe” (BIO RAD, Hercules, USA) with 0.45 µm pore size was used as substrate layer and a surface of 300 cm² (dimensions 10 cm x 30 cm) was inoculated by means of gentle filtration with a stencil to reach a biomass density of 2.5 g m^{-2}. The source layer was applied to the wet substrate layer (glass fiber) and secured by plastic paper-clips. The experiment was conducted at the previously defined optimal growth conditions. Concentrations of N and P were measured daily and the experiment was conducted until no more reduction in phosphorus concentration was detectable. The medium was not changed during this period. Algal biomass was then completely scraped off from the surface and washed in MQ water by repeated centrifugation and exchange of the liquid. Part of this suspension was brought onto polycarbonate filters for dry weight determination. After weighing, the biomass was grinded with a mortar and pestle and ~5 mg were weighed into tin cups for analysis of nitrogen content with a Flash 2000 elemental analyzer (Thermo Scientific, Waltham, USA). Phosphorus content of the ground biomass was determined by acid digestion according to Hu & Barker (1999) and subsequent measurement of dissolved orthophosphate, as described above.

2.5. Statistics

All statistical analyses were performed with GraphPad Prism software for Windows, version 6.01 (GraphPad Software, La Jolla, California, USA). Comparison of replicate measurements were analyzed by one-way ANOVA with multiple comparison and Turkey’s post-hoc test. Rates were calculated as linear regressions over a certain time period. Comparison of rates was performed by analysis of covariance (ANCOVA) with multiple comparison.

3 Results

3.1. Analysis of urine batches

To evaluate the variability of urine used in this study, each batch was analyzed for its nitrogen and phosphorus content, conductivity and pH value before any experiments were carried out with it. Table 5 summarizes the results of these measurements.

Table 5: Chemical characteristics of the different urine batches used in this study.

Urine batch	[total N] (g/L)	[Urea-N] (g/L)	[NH_4-N] (g/L)	[total P] (g/L)	[PO_4-P] (g/L)	Atomic N:P ratio	Conductivity (mS/cm)	pH
A	5.76	5.70	0.06	0.29	0.28	43	15.75	6.74
B	4.50	4.41	0.09	0.29	0.28	34	11.85	6.83
C	3.70	3.52	0.15	0.21	0.21	38	6.51	6.97
D	2.50	2.38	0.09	0.17	0.17	31	8.83	7.02

The variability in composition of urine of different batches was quite large. Batch A showed the highest content in total N, with 5.76 g L^{-1} while batch D had the lowest total N content of 2.5 g L^{-1}. Nitrogen was present mainly (>95%) as urea in all cases, while ammonium made up only a minor fraction. The variability in total P content was similarly high, with 0.29 and 0.17 g L^{-1} as the maximum and minimum values in batch A and D, respectively. Phosphate was the mayor form of phosphorus present in all batches. The atomic ratio of N:P ranged between 31:1 and 43:1. Conductivity ranged between 6.51 and 15.75 mS cm^{-1}. The pH of urine was similar in all batches, with minor variations between 6.74 and 7.02.

3.2. Diversity of isolates

From the urine traps, 23 unialgal cultures were established. Table 6 gives an overview of the strains, their identities based on morphological characteristics and the conditions of their isolation.

Table 6: Isolated strains, identification based on morphology and conditions of isolation. Isolations were performed with urine of batch A diluted with MQ water at the indicated levels. Secondary enrichments were done by placing a subsample into the respective growth medium and isolating from there after ~ 1-3 weeks. Cultures were kept in the indicated medium.

Strain	Class	Species (morphology)	Dilution	Secondary enrichment	Medium
U 2.1	Trebouxiophyceae	*Chlorella sp.*	1:1	x	Waris-H + Si
U 2.2	Cyanobacteria	*Synechocystis sp.*	1:1	x	Waris-H + Si
U 2.3	Cyanobacteria	*Phormidium sp.*	1:1		BG-11
U 2.4	Chlorophycae	*Chlamydomonas sp.*	1:1		Waris-H + Si
U 5.1	Chlorophycae	*unidentified coccal*	1:5	x	BG-11
U 5.2	Chlorophycae	*Sphaerocystis sp.*	1:5	x	Waris-H + Si
U 5.3	Chlorophycae	*Chlamydomonas sp.*	1:5	x	Waris-H + Si
U 5.4	Trebouxiophyceae	*Chlorella sp.*	1:5	x	Waris-H + Si
U 5.5	Chlorophycae	*Chlamydomonas sp.*	1:5	x	Waris-H + Si
U 5.6	Chlorophycae	*Chlamydomonas sp.*	1:5	x	Waris-H + Si
U 5.7	Chlorophycae	*unidentified coccal*	1:5	x	Waris-H + Si
U 5.8	Chlorophycae	*Scenedesmus sp.*	1:5		BG-11
U 5.9	Trebouxiophyceae	*Chlorella sp.*	1:5	x	BG-11
U 10.1	Chlorophycae	*Chlamydomonas sp.*	1:10	x	BG-11
U 10.2	Bacillariophyceae	*Cocconeis sp.*	1:10		BG-11
U 10.3	Trebouxiophyceae	*Stichococcus sp.*	1:10	x	Waris-H + Si
U 10.4	Cyanobacteria	*Synechococcus sp.*	1:10	x	BG-11
U 10.5	Bacillariophyceae	*Navicula sp.*	1:10	x	Waris-H + Si
U 10.6	Xanthophyceae	*Heterococcus sp. (?)*	1:10	x	Waris-H + Si
U 10.7	Trebouxiophyceae	*Chlorella sp.*	1:10	x	Waris-H + Si
U 10.8	Trebouxiophyceae	*Chlorella sp.*	1:10	x	Waris-H + Si
U 10.9	Trebouxiophyceae	*Trebouxia sp. (?)*	1:10	x	BG-11
U 10.10	Trebouxiophyceae	*Chlorella sp.*	1:10	x	BG-11

Of the 23 isolates, 10 were recovered from the 1:10 dilution, 9 from the 1:5 dilution and 4 from the 1:1 dilution. Of all isolates, 1 was of the class xanthophyceae, 2 bacillariophyceae, 3 cyanobacteria, 8 trebouxiophyceae and 9 chlorophyceae. The most abundant species were *Chlorella ssp.* and *Chlamydomonas ssp.*. About 17 % of the isolates were derived directly from the urine samples, while the others were isolated from secondary enrichments.

Several of the isolates, and other strains with which the main experiments were carried out, were also identified by molecular methods (SSU rRNA sequencing). The full sequences are given in appendix III. Table 7 gives the results of the closest relatives found when comparing the obtained sequences with the public database. Only those sequences originating from validly published research or from public culture collections were included in the list.

Table 7: Molecular identification of selected strains. BI: Brazil Isolate; CCAC: Culture Collection of Algae at University of Cologne; U: Urine isolate; CCAP: Culture Collection of Algae and Protozoa. Nucleotide blast queries were made on NCBI-website (http://blast.ncbi.nlm.nih.gov/Blast.cgi; accessed: 15.07.2016).

Sequenced strain		**Comparison**		**Closest relative**		
ID	**Length (bp)**	**Coverage (%)**	**Identity (%)**	**Species**	**Accession #**	**Publication/ Culture collection**
BI-11	1687	99	99	*Chlamydomonas zebra*	U70792.1	(Buchheim et al. 1997)
CCAC 3496	895	97	99	*Desmodesmus abundans*	KR904903.1	(Barbano et al. 2015)
		97	99	*Scenedesmus abundans*	X73995.1	(Kessler et al. 1997)
U 2.1	1694	100	99	*Chlorella sorokiniana*	KU948990.1	(Li, Q. and Chen 2016)
U 2.4	1206	96	99	*Desmodesmus bicellularis*	KP726231.1	(Kaplan-Levy et al. 2016)
U 5.5	1698	100	99	*Chlamydomonas moewusii*	FR865598.1	CCAP 11/5D
U 10.10	1695	100	99	*Chlorella luteoviridis*	FR865678.1	CCAP 211/5B

Molecular identifications were identical with those based on morphology to the genus level in all cases. More resolved identifications were obtained by finding close relatives, with high sequence identities, in all cases. Strain CCAC 3496 yielded 2 close relatives which were validly published and had the same values for sequence coverage as well as sequence identity. *Scenedesmus abundans*, as used by (Kessler et al. 1997) can be viewed as a synonym for *Desmodesmus abundans* (Guiry & Guiry 2016). In a previously published physiological test, this strain was found to produce secondary carotenoids, possessed a functional nitrate reductase and did not rely on thiamine or vitamin B_{12} for its growth (Kessler et al. 1997).

A large degree of morphological variability was detected in suspension cultures of strain CCAC 3496 (Fig. 4), although this has not been validated statistically: When growing strain CCAC 3496 in Waris-H culture medium cells were elongated oval shaped and ornamented with spines, mainly arranged in groups of four in one row (Fig. 4 B). Single cells and groups of two cells were also observed. When

growing the organism in 1:1 diluted urine cells were slightly enlarged and round to oval shaped (Fig 4 A). Some were arranged in groups of four or two cells while many were also present as single cells. Spines were absent in cells from this medium.

Figure 4: Photographs of colonies of *Desmodesmus abundans* CCAC 3496 grown in different media in suspension culture. A: Urine (1:1 diluted with TapBN), B: Waris-H. Images were taken after 3 days in the medium. Images are magnified 1000x and were taken with differential interference contrast.

3.3. Screening for growth on human urine

3.3.1. Choice of dilution

The initial screening to determine an appropriate dilution factor of urine with 6 selected strains was performed using the absorbance at 750 nm as measure of algal growth. The results are represented in figure 5.

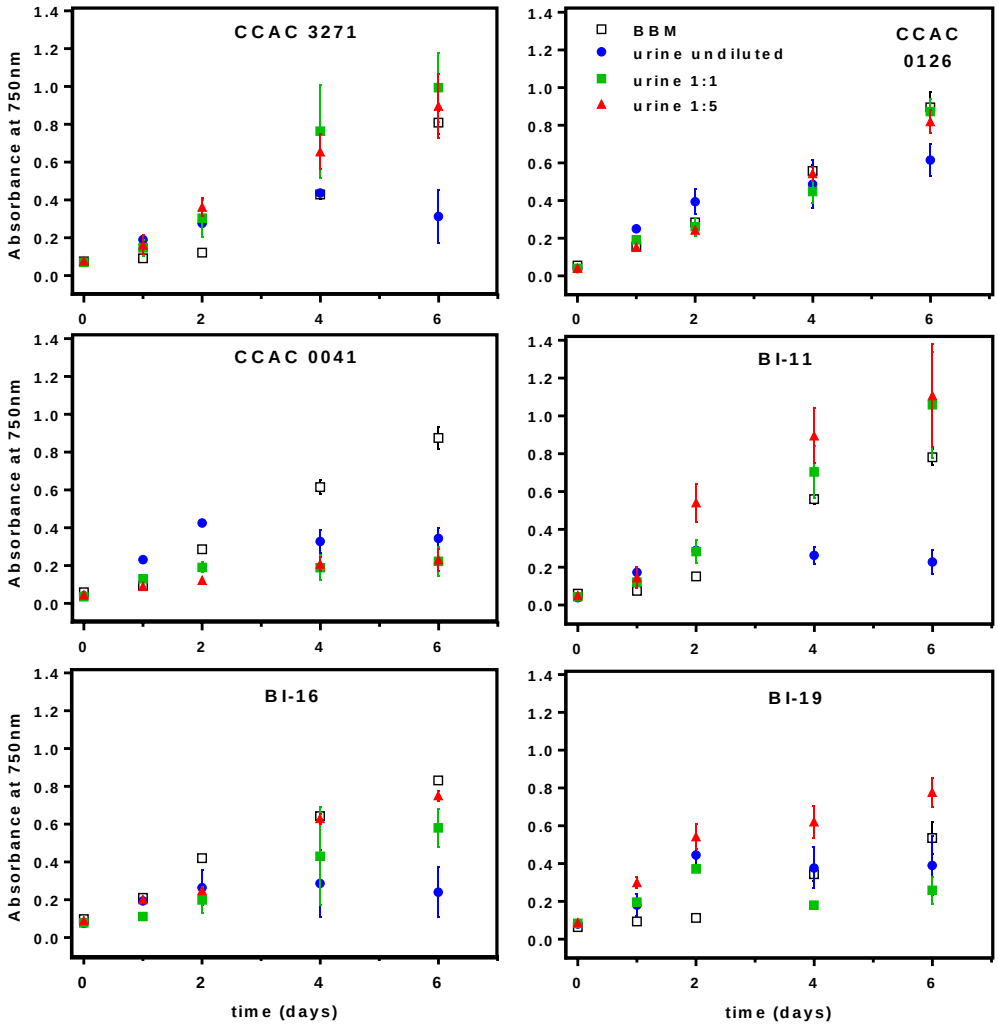

Figure 5: Summary of the dilution screening. Absorbance at 750 nm was used as growth measure of algal strains in urine of different dilutions (diluted with MQ) in suspension cultures in 24-well microtiter plates. Captions denote the algal strain. BBM medium was used as control. Values represent the mean +/- SD (n=3).

The assessment of growth performance is based on ANOVA comparison of the final absorbance values on day 6. Strain BI-11 showed no significant difference between 1:1 dilution and either 1:5 dilution or BBM growth medium. Undiluted urine alone showed a significantly lower absorbance than all the other treatments ($p<0.001$). In strain BI-16, BBM medium yielded the highest growth, which was however statistically not different to growth in 1:5 diluted urine ($p>0.1$). The 1:1 dilution resulted in an intermediate growth, which was statistically not different from the value reached in the 1:5 dilution. Undiluted urine yielded the lowest growth in this strain, clearly different from all other treatments ($p<0.01$). In strains CCAC 3271, BI-11 and BI-19 there was a lag period of 2 days observed in BBM

medium. In strain BI-19 there was also a decrease in growth between days 2 and 4. Due to this irregular behavior, this strain was excluded from further analysis. In strain CCAC 0126 undiluted urine yielded the lowest final absorbance value, which was significantly lower than all others (at least $p<0.05$). The other treatments were statistically not different from one another. In strain CCAC 0041 growth in BBM was significantly higher than in all other treatments ($p<0.0001$). The dilutions of urine resulted in a similar final measure of growth. For strain CCAC 3271 there was a clear decrease in absorbance in undiluted urine between days 4 and 6, which resulted in the lowest final value, significantly lower from the other treatments (at least $p<0.05$). The other dilutions as well as BBM medium resulted in similar final absorbance values.

Taken in concert, it can be seen that for most of the strains tested, undiluted urine yielded the lowest final absorbance at day 6. The differences between the 1:1 and the 1:5 dilutions were in most cases not significant. Therefore, a dilution of 1:1 of urine with water was chosen for all following experiments.

3.3.2. Choice of algal strains

To narrow down the selection of strains to be used for physiological characterization and experiments on Twin Layer, a screening of 96 strains for short term growth on urine was performed. Figures 5 A-C show the results of the comparative screening in 96-well microtiter plate cultures carried out in 6 separate experiments, with absorbance at 750 nm used as measure of growth.

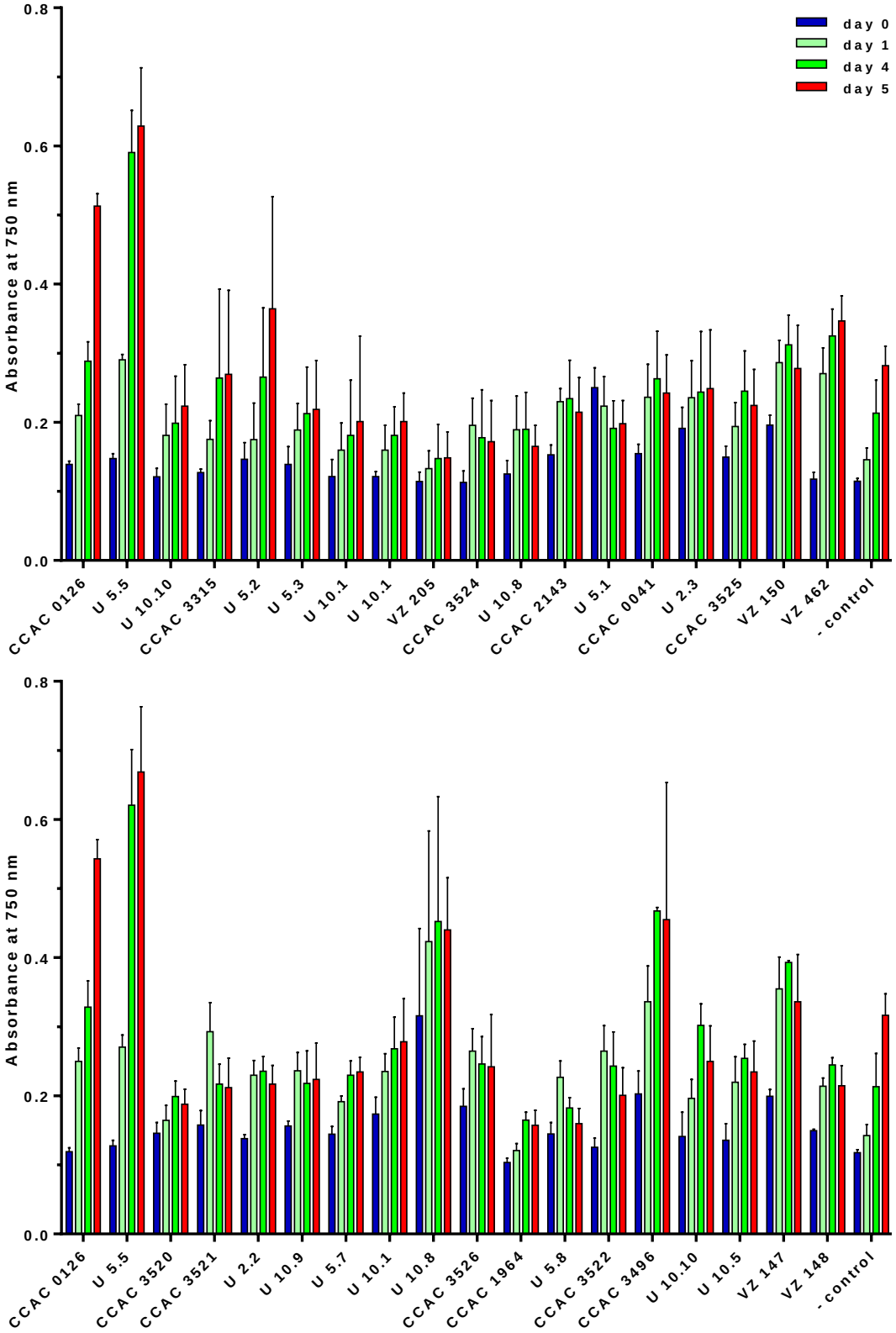

Figure 6 A: Strain screening. Absorbance at 750 nm was used as growth measure in 1:1 diluted urine (with MQ) in suspension cultures in 96-well microtiter plates. Non-inoculated urine was used as negative control. Values represent the mean +/- SD (n=3).

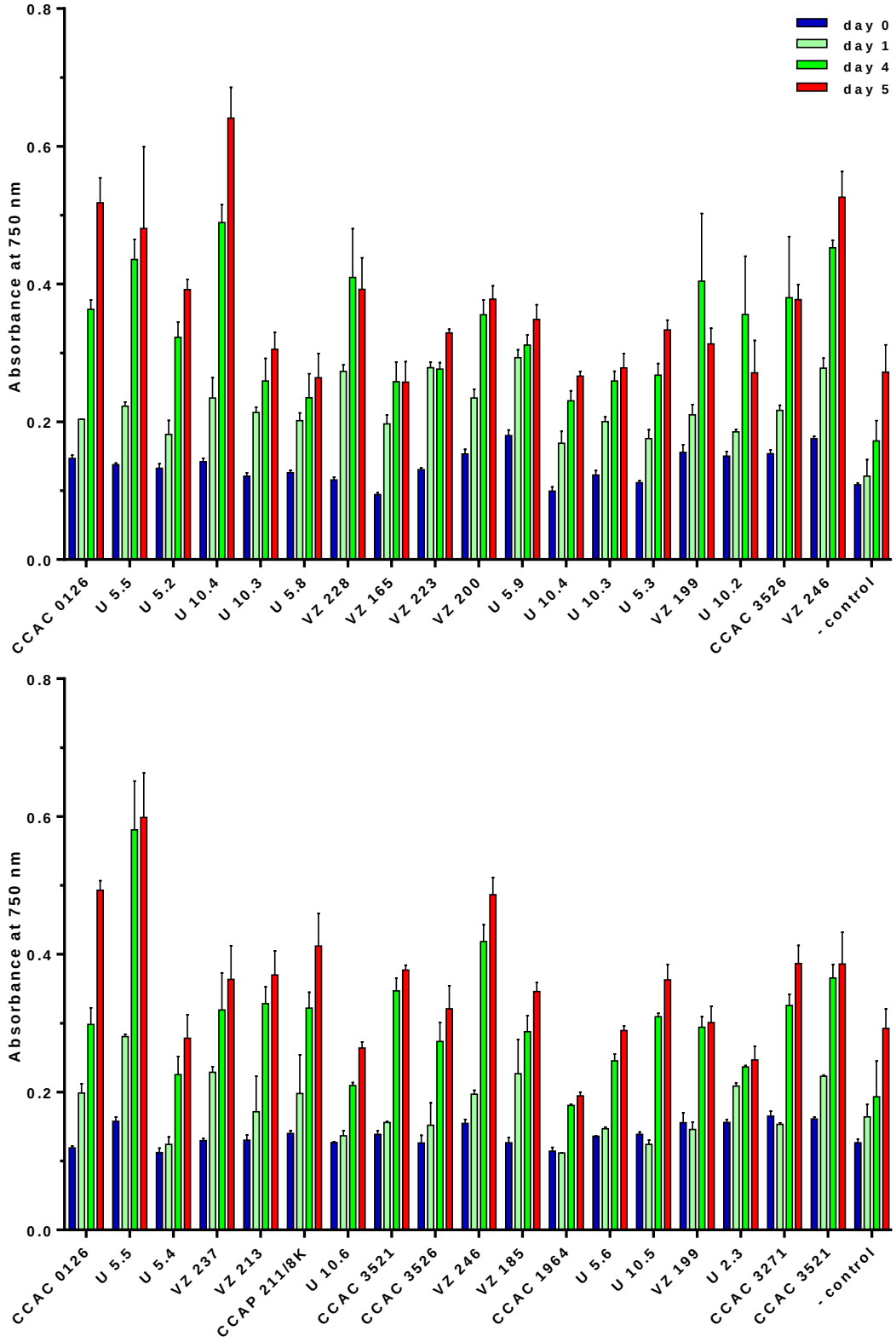

Figure 6 B (continued): Strain screening. Absorbance at 750 nm was used as growth measure in 1:1 diluted urine (with MQ) in suspension cultures in 96-well microtiter plates. Non-inoculated urine was used as negative control. Values represent the mean +/- SD (n=3).

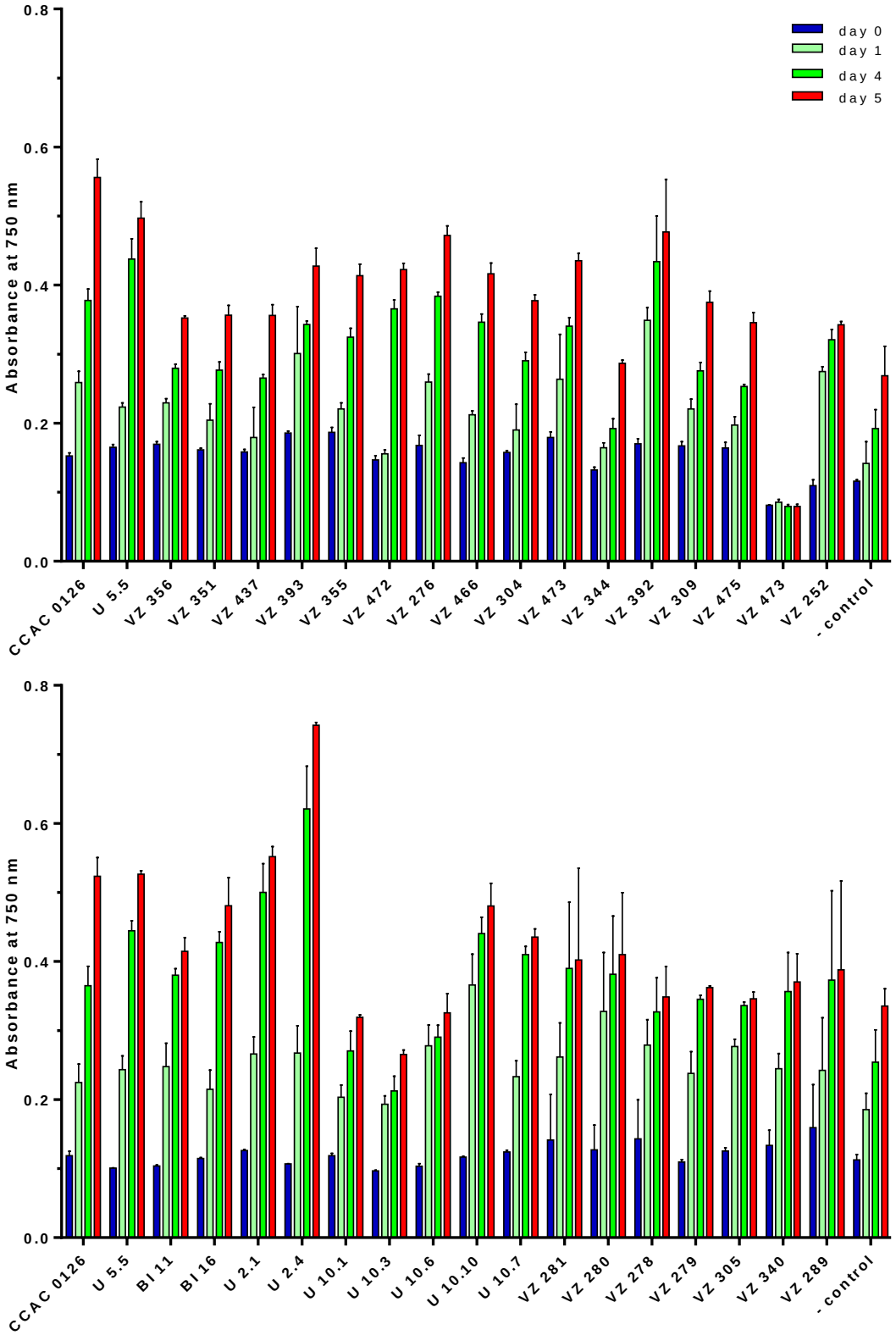

Figure 6 C (continued): Strain screening. Absorbance at 750 nm was used as growth measure in 1:1 diluted urine (with MQ) in suspension cultures in 96-well microtiter plates. Non-inoculated urine was used as negative control. Values represent the mean +/- SD (n=3).

In all but 8 strains, a statistically relevant increase in absorbance at 750 nm was observable in the timeframe of the experiment ($p<0.01$). The largest increase was in most cases found in the first day of the experiment. Some strains increased in absorbance throughout the experimental period, while in others there was no more increase visible between days 4 and 5. The positive controls (U 5.5 and CCAC 0126) showed similar behavior in all cases and yielded final absorbances on day 5 between 0.480-0.628 and 0.493-0.556, respectively. The single values for each of the two were statistically not different from each other ($p<0.005$). The negative control, which was un-inoculated urine and principally represented bacterial growth and other forms of turbidity increase (e.g. precipitation of minerals) increased linearly in all cases and resulted in absorbances at day 5 between 0.268 and 0.335. When this background absorbance at day 5 of each experimental run was subtracted from the respective values of the strains tested, 9 remained which showed an actual increase in absorbance associated with algal growth. These are shown in figure 7. Those strains represent the ones which were used in further experiments, screening their growth on Twin Layer (section 3.6).

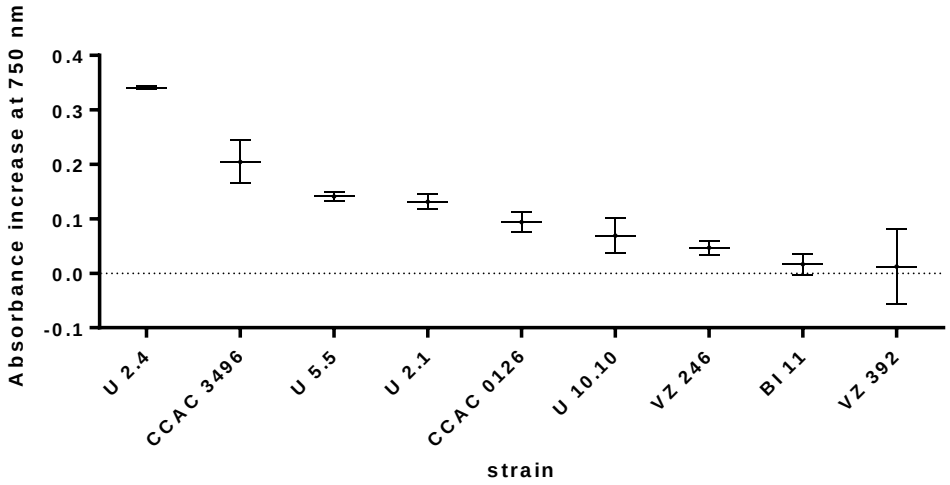

Figure 7: Summary of strain screening. Increase in absorbance at 750 nm was used as growth measure in 1:1 diluted urine (diluted with MQ) in suspension cultures in 96-well microtiter plates. The absorbance of negative controls (bacterial background) was subtracted and only positive values are shown. Values represent the mean +/- SD (n=3).

The largest increase in absorbance in the 5 days of growth period was observed for strain U 2.4, followed by strain CCAC 3496. The final increase of strains U 5.5 and U 2.1 were statistically not different from each other and other strains showed increases of less than 0.1 absorbance units.

3.4. Nitrogen metabolism of axenic strains

3.4.1. Urea utilization

In the next step several strains, from which axenic cultures were present, were used to investigate their nitrogen metabolism with emphasis on the use of urea as nitrogen source. Figure 8 shows the results of cell counts (C) as well as urea (B) and ammonium (A) measurements in suspension cultures of 3 selected strains using a modified BBM medium which contained urea as sole nitrogen source.

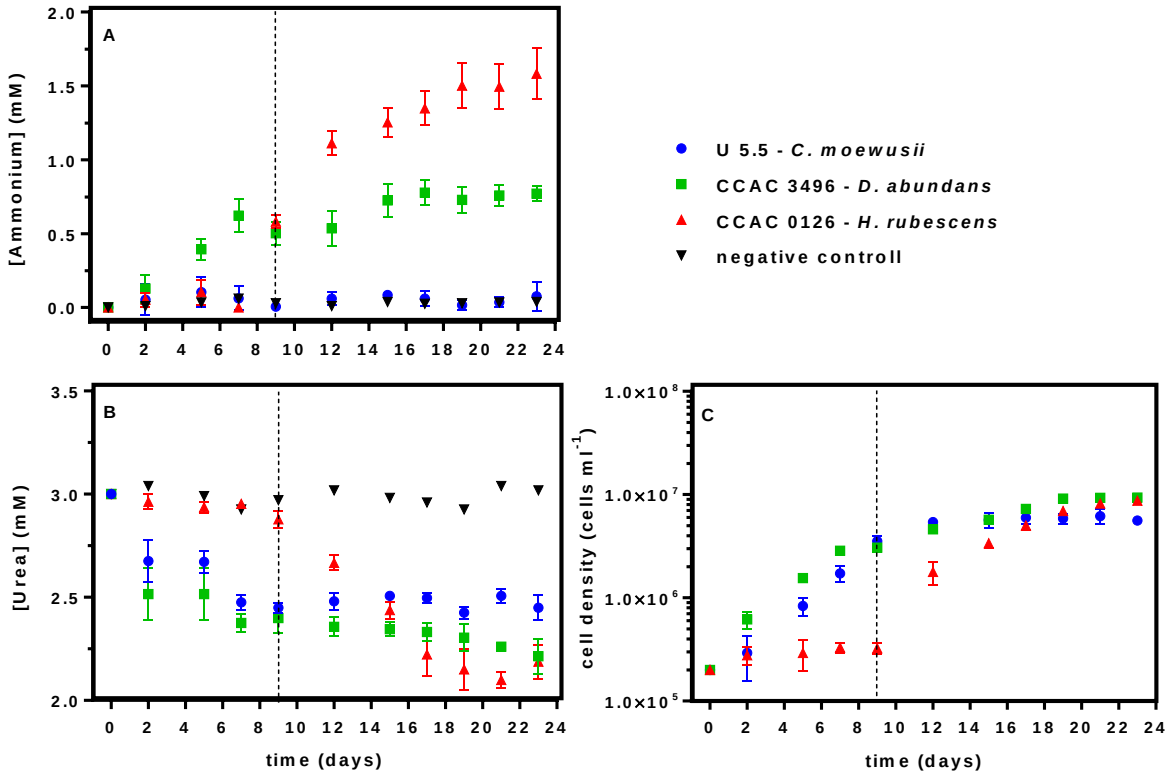

Figure 8: Cell growth and nitrogen species development in axenic suspension cultures using modified BBM medium with urea as only N-source. A: Ammonium concentration; B: Urea concentration; C: Cell numbers. Dashed line indicates the time of urease addition to *H. rubescens* CCAC 0126 culture. Values represent the mean +/- SD (n=4).

While cultures of U 5.5 and CCAC 3496 seemed to grow exponentially for the first 9 days, CCAC 0126 showed no significant growth in this period. On day 9, urease enzyme was added to the cultures of this strain, which immediately alleviated the limitation in growth. At the end of the experiment (day 21) all 3 strains showed similar final cell densities, which were statistically not different from one another. Urea concentrations in U 5.5 and CCAC 3496 decreased in parallel with the increase in cell numbers during

the first 9 days, after which there were only minor changes detectable. In CCAC 0126 the concentration of urea was constant until day 9, as in the negative control. Upon the addition of urease, urea concentrations in the cultures of this strain decreased rapidly. Ammonium concentrations in cultures of strain U 5.5 were almost not detectable throughout the experimental period, while in cultures of CCAC 3496 there was an immediate release of ammonium, which occurred for as long as cells were growing. In the cultures of strain CCAC 0126, ammonium release was observed only after addition of the enzyme. Figure 9 shows the results of dry biomass and pH development in the same experiment.

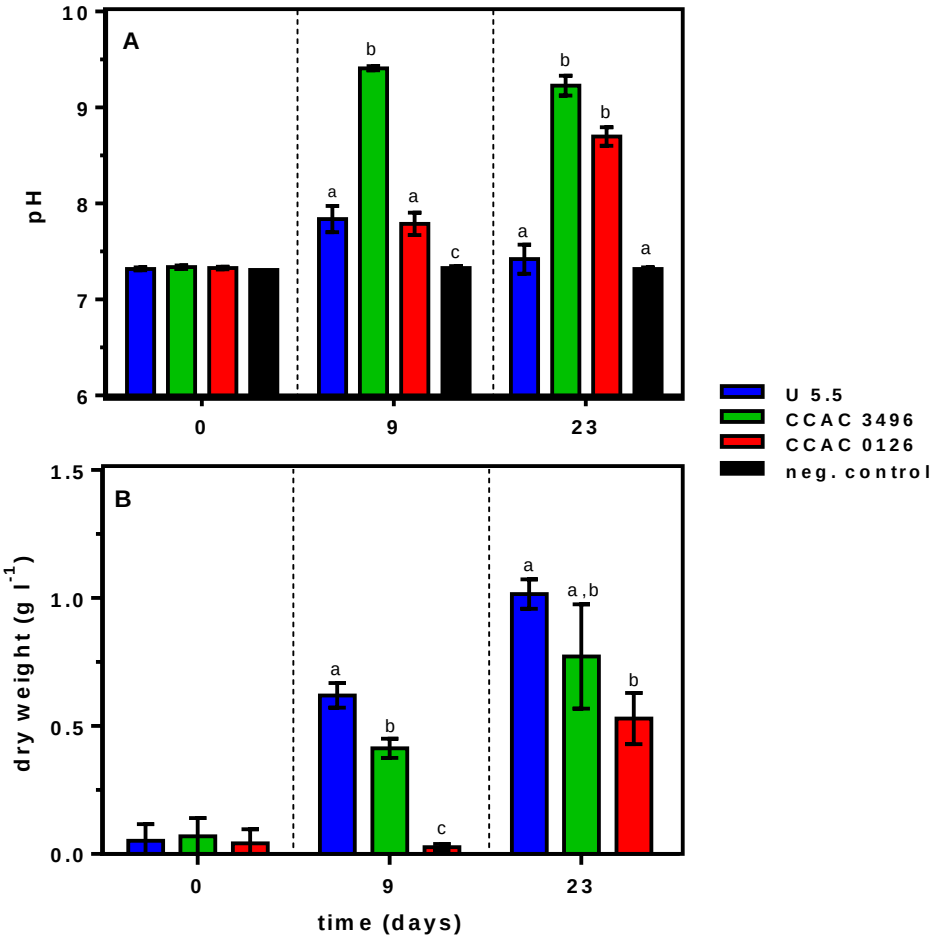

Figure 9: Biomass and pH development in axenic suspension cultures using modified BBM medium with urea as only N-source. A: pH; B: dry biomass. Dashed lines separate the days of sampling. Small letters above bars represent significant differences according to one way ANOVA with Turkey's post-hoc test (at least $p<0.05$). Values represent the mean +/- SD (n=4).

In strain CCAC 3496 there was a strong increase in pH value between day 0 and 9, from 7.4 up to 9.45, after which pH stayed stable. CCAC 0126 showed only a minor increase in pH until urease enzyme was added at day 9. From this time on pH increased from 7.7 up to 8.8 on day 23. Strain U 5.5 showed only

a minor increase in pH, which stayed rather stable from day 9 onwards. Biomass increased throughout the experiment for strains CCAC 3496 and U 5.5, while it stagnated for CCAC 0126, until the addition of urease enzyme on day 9, after which this strain also showed an increase in biomass dry weight.

3.4.2. Various nitrogen sources

To further elucidate the nitrogen use capabilities of selected strains, a growth experiment was performed on agar plates, using BBM medium with different nitrogen sources. The results of this are shown in figure 10.

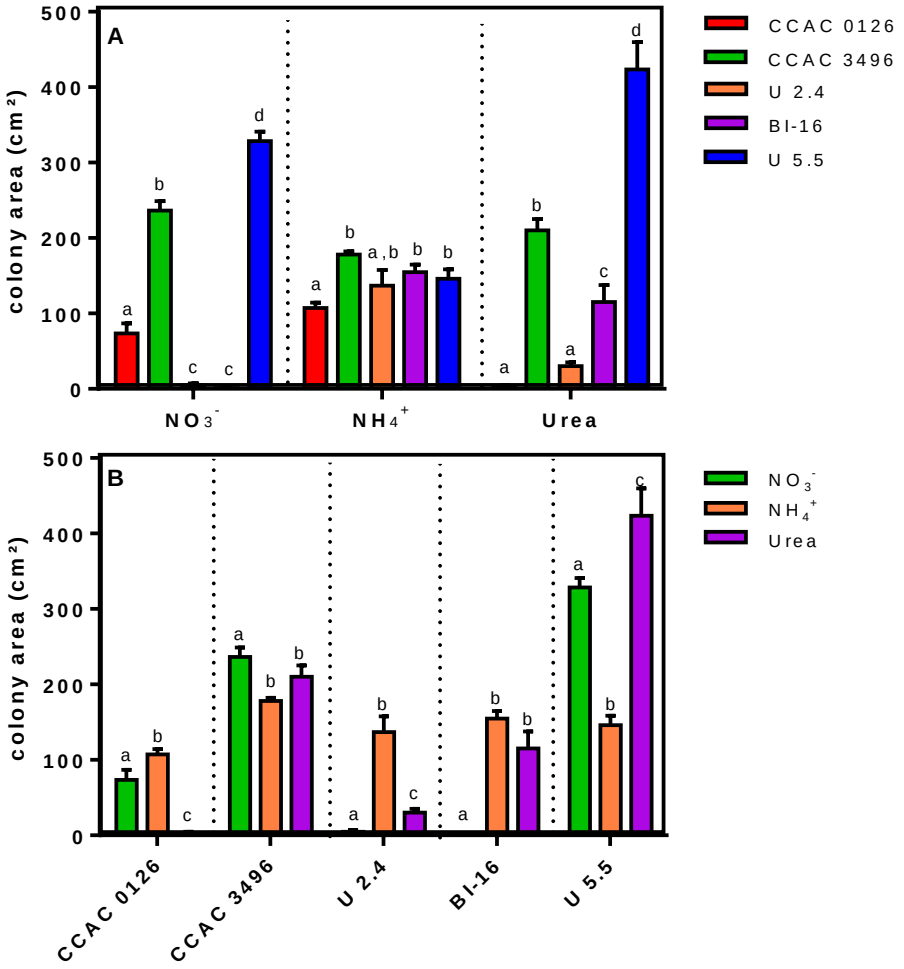

Figure 10: Colony growth of axenic cultures on agar plates with modified BBM containing different nitrogen sources. Colony area is calculated by the mean number of colonies per plate times the mean diameter of colonies. The same data is plotted twice for ease of comparison: A: comparison by nitrogen source; B: comparison by strain. Small letters above bars represent significant differences according to one way ANOVA with Turkey's post-hoc test (at least $p<0.05$).Values represent the mean +/- SD (n=4).

Ammonium served as a nitrogen source for all the five strains tested. Strains BI-16 and U 2.4 were unable to grow on nitrate. CCAC 0126 was unable to grow on urea and U 2.4 showed only low growth

on this N-source. Strain U 5.5 showed the highest growth on nitrate and urea and strain CCAC 3496 grew similarly well on all three nitrogen sources.

3.5. Water analysis and prevention of precipitation

3.5.1. Water analysis

In order to perform them under more realistic conditions, tap water was used to prepare the dilutions of urine for Twin Layer experiments. Initial experiments with TapCGN water indicated that there was a large potential for the formation of precipitates when used for dilutions of urine. To make a more informed choice on the source of water to be used, an ICP-MS analysis of mayor solutes in TapBN and TapCGN waters was performed. The main elements of interest for algal cultivation are presented in Figure 11.

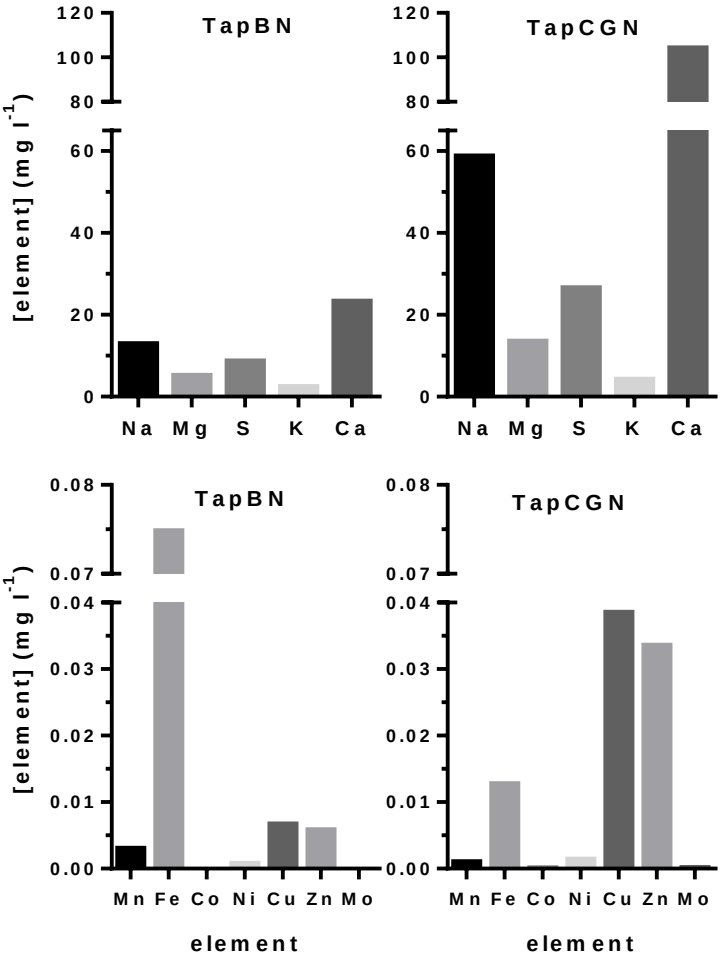

Figure 11: Concentrations of elements relevant for algal growth in tap waters from Bonn and Cologne. Top graphs show macro-elements, bottom graphs show micro elements. Refer to the difference in scale. Values represent a single measurement.

The most striking differences are the elevated concentrations of calcium and sodium in TapCGN, which are about 5x and 6x higher, respectively, than in TapBN. Values of sulfur and magnesium are also elevated in TapCGN, showing about 3x the concentration of TapBN. Among the micro-elements, TapBN showed elevated concentrations of iron, approximately 6x higher than in TapCGN, while TapCGN had elevated concentrations of copper and zinc, about 8x and 7x higher, respectively, than in TapBN. Electrical conductivity in TapCGN and TapBN was 0.531 and 0.340 mS cm^{-1}, respectively. Due to the lower content in elements that might form precipitates (Ca and Mg) and the elevated concentration in iron, which might promote algal growth, TapBN was chosen as the standard water for dilution in most experiments.

3.5.2. Prevention of precipitation

An experiment to assess the means of preventing the precipitation of phosphorus-containing minerals in a setting similar to a growth experiment on Twin Layer-PSBR was performed. Urine diluted 1:1 with TapBN was placed under non-sterile conditions and aerated with either normal air or air amended with 2.5 % CO_2. After 14 days, there was visible precipitation in the air-treated urine, while none was visible in the CO_2-treatment. Table 8 shows the results of the analysis of the precipitates.

Table 8: Prevention of precipitation by pH control with constant addition of 2.5 % CO_2. Values were determined in 1:1 diluted urine (TapBN) after 14 days of development under non-sterile conditions at 23°C. Starting pH was 6.7. Concentrations of ammonium and phosphate were measured from dissolved precipitate. n.d. stands for not detectable. Values represent the mean +/- SD (n=3).

Variable	CO_2 controlled pH	no pH control
Final pH	7.02 +/- 0.01	9.1 +/- 0.25
Precipitate (g l^{-1})	11.01 +/- 1.32	157.90 +/- 11.35
[Ammonium] (mM)	0.49 +/- 0.02	7.09 +/- 0.24
[Phosphate] (mM)	n.d.	7.11 +/- 0.11
Ratio NH_4:PO_4	n.d.	**1.00** +/- 0.12

In the air-treatment, pH rose from 6.7 to 9.1 at the end of the experiment, while in the CO_2-treatment pH only went up to a value of 7.1. There was about 14x more precipitate (by weight) present in the air-treatment. Analysis of NH_4^+ and PO_4^- showed that the precipitate contained the two molecules in an almost equimolar ratio.

3.6. Growth on Twin Layer-PSBR

3.6.1. Screening of strains on Twin Layer

To assess their potential for immobilized growth and treatment of urine, an experiment on Twin Layer with the 9 best growing strains (as found in the microtiter plate screening (Fig. 6)) was performed. Figure 12 shows the results of this screening.

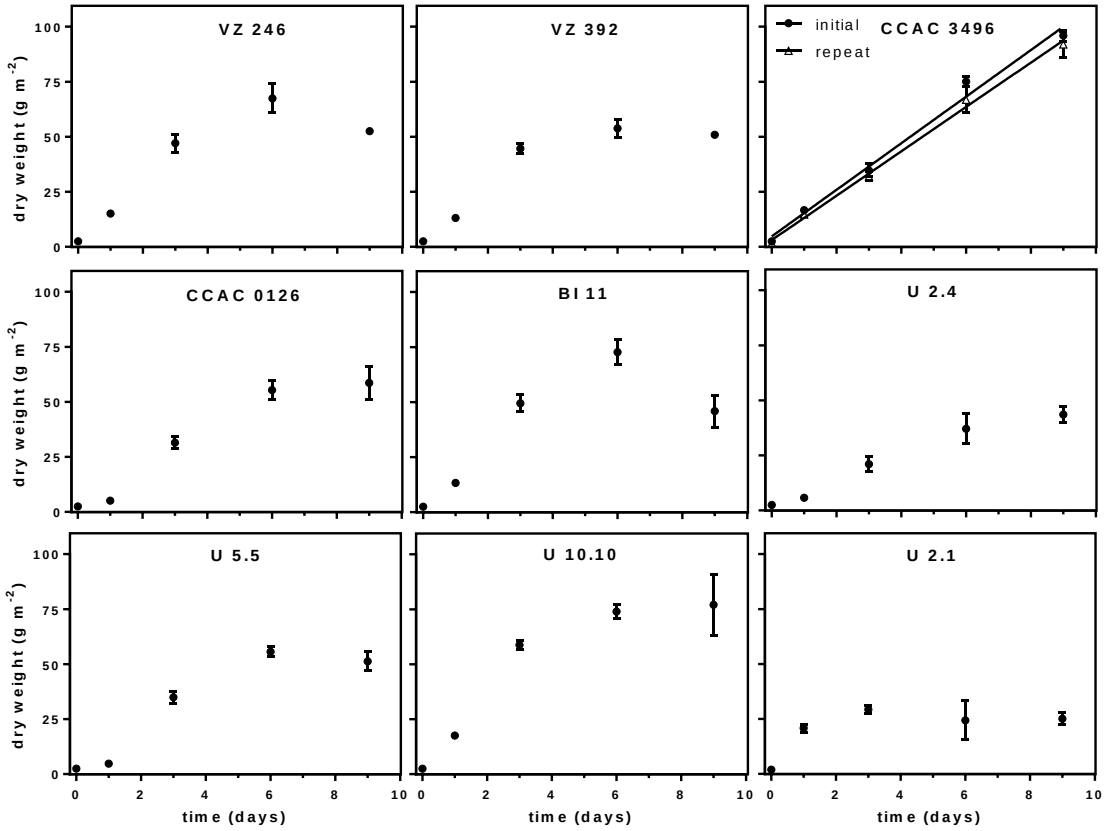

Figure 12: Immobilized growth of different algal strains on 1:1 (TapBN) diluted urine of batch C. Conditions were the following: 600 µmol photons m^{-2} s^{-1} light intensity; 2.5 % CO_2 constantly supplied to the medium vessel and the atmosphere. The medium was changed every 4 days. Evaporation was refilled daily with MQ water. The experiment with CCAC 3496 was repeated (triangles). Lines represent linear regressions. Values represent the mean +/- SD (n=3).

Most of the tested strains grew in a linear fashion for the first 6 days, except for U 2.1, which showed a decrease in biomass already from day 3 onwards. Between days 6 and 9 all strains except for CCAC 3496 showed a decline or stagnation of biomass. Only strain CCAC 3496 showed linear growth throughout the experimental period of nine days. To validate this result, the experiment with this strain was repeated. The slopes (growth rates) of the two experiments conducted with *Desmodesmus abundans* CCAC 3496 were compared by analysis of covariance (ANCOVA) and were found to be statistically

not different (P=0.2906). The pooled growth rate can be expressed as 10.33 +/- 0.354 g m^{-2} day^{-1}. This strain was selected for further experiments.

3.6.2. Optimizing growth of CCAC 3496

As a next step, it was attempted to optimize growth performance of CCAC 3496 by changing several parameters, in order to maximize the nutrient recovery potential of this strain.

3.6.2.1. Light intensity

An experiment was conducted to find the optimal light intensity for this strain. The results are presented in figure 13.

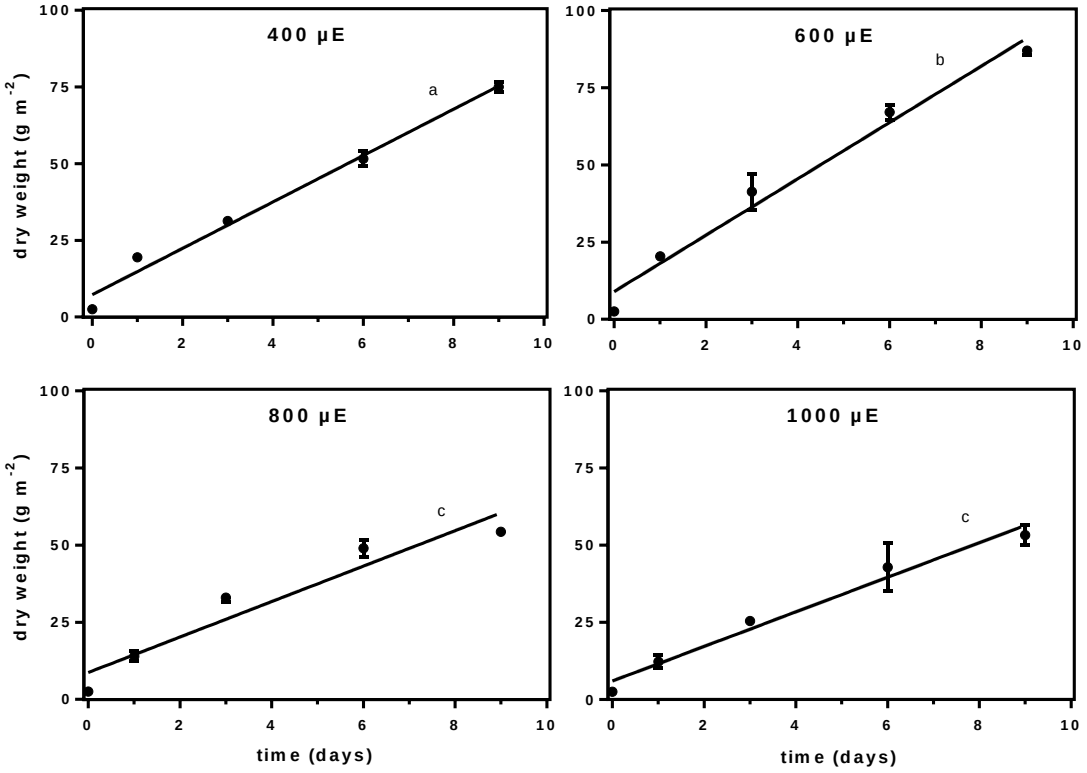

Figure 13: Immobilized growth of *Desmodesmus abundans* CCAC 3496 on 1:1 (TapBN) diluted urine of batch C at different light intensities. Conditions were the following: 400-1000 µmol photons m^{-2} s^{-1} light intensity; 2.5 % CO_2 constantly supplied to the medium vessel and the atmosphere. The medium was changed every 4 days. Evaporation was refilled daily with TapBN water. Lines represent linear regressions and different letters indicate significant differences between them according to ANCOVA and Turkey's post-hoc test ($p<0.0001$). Values represent the mean +/- SD (n=3).

All treatments displayed linear increase in dry weight throughout the experimental period of 9 days. The growth rates of the 400 and 600 µmol photons m^{-2} s^{-1} treatments were significantly different from one

another (P=0.0045; F=9.66616). Likewise, the growth rate of the 600 µmol photons m^{-2} s^{-1} treatment differed significantly from the 800 and 1000 µmol photons m^{-2} s^{-1} treatments (P<0.0001) according ANCOVA analysis. The highest growth rate reached in this comparison of light intensities was 9.130 +/- 0.4223 g m^{-2} day^{-1} at 600 µmol photons m^{-2} s^{-1}. Thus, further experiments were conducted at this light intensity.

3.6.2.2. pH-value

For the following experiment, the pH control system of on-demand CO_2 addition to the medium (Fig.3) was implemented to identify the optimal pH for growth of strain CCAC 3496. The results are shown in figure 14.

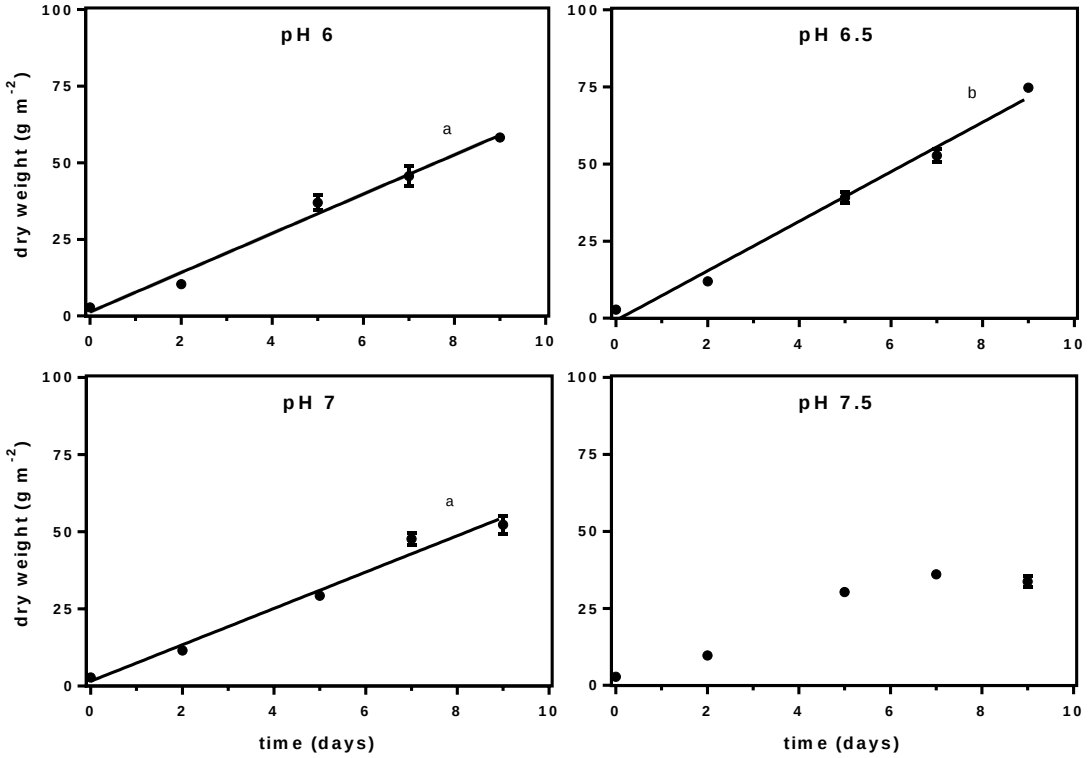

Figure 14: Immobilized growth of *Desmodesmus abundans* CCAC 3496 on 1:1 (TapBN) diluted urine of batch C at different set pH values, regulated by CO_2 addition. Conditions were the following: 600 µmol photons m^{-2} s^{-1} light intensity; pH 6-7.5; 2.5 % CO_2 constantly supplied to the atmosphere. The medium was changed every 4 days. Evaporation was refilled daily with TapBN water. Lines represent linear regressions and different letters indicate significant differences between them according to ANCOVA and Turkey's post-hoc test (p<0.00015). Values represent the mean +/- SD (n=3).

Dry weight increased linearly during the 9 days of experiment for the treatments of pH 6, 6.5 and 7. At pH 7.5, growth decreased between days 5 and 7 and stagnated towards the end of the cultivation period.

The highest growth rate reached in this comparison of pH values was 8.033 +/- 0.2638 g m^{-2} day^{-1} at pH 6.5. This value was significantly different from all other pH treatments (P<0.00015) according to ANCOVA analysis and the highest among the treatments. Therefore, 6.5 was chosen as the pH set-point for all further experiments.

3.6.2.3. Source of diluent water

As the next step, an experiment was conducted to test the effect of using different waters for the dilution of urine, as well as for replacing the evaporated water. The results are shown in figure 15.

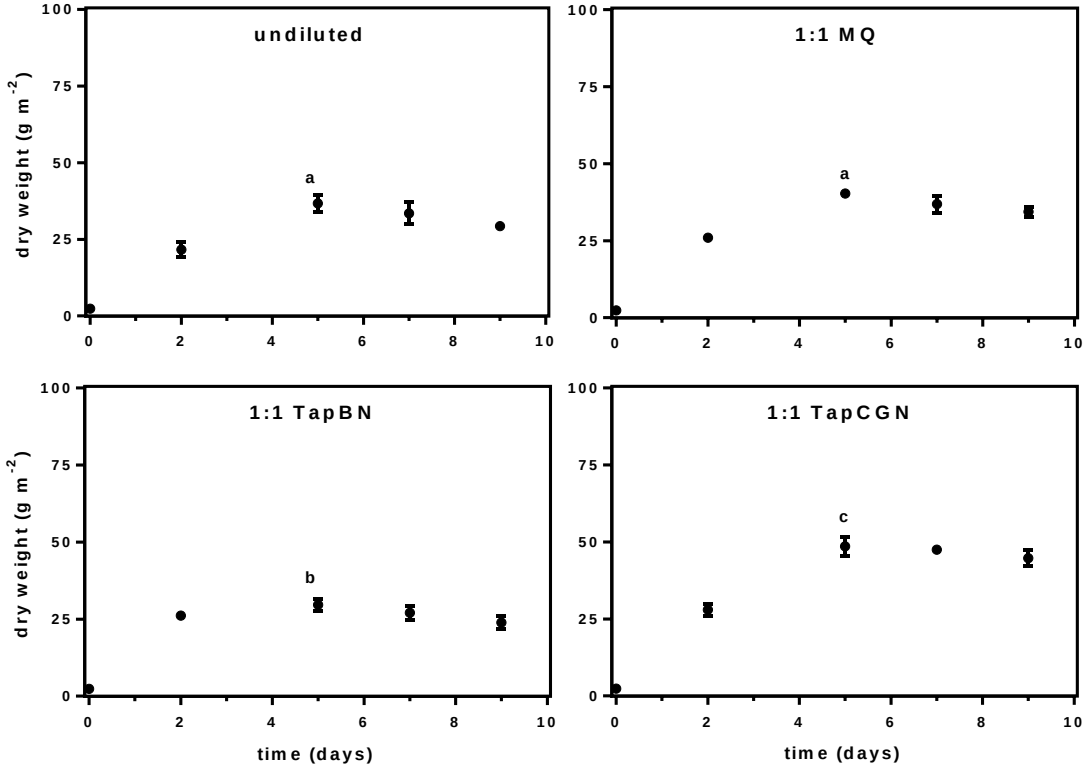

Figure 15: Immobilized growth of *Desmodesmus abundans* CCAC 3496 on urine of batch D diluted with water from different sources. Conditions were the following: 600 µmol photons m^{-2} s^{-1} light intensity; pH regulated at 6.5; 2.5 % CO_2 constantly supplied to the atmosphere. The medium was changed every 4 days. Evaporation was refilled daily with the respective water. Different letters indicate significant differences in the biomass on day 5, according to one-way ANOVA with Turkey's post-hoc test (p<0.005). Values represent the mean +/- SD (n=3).

This experiment yielded unexpected results. Biomass increase was linear in all treatments, except TapBN, for the first 5 days, after which biomass growth stagnated and even slightly decreased in all treatments. In TapBN biomass growth stagnated already after 2 days. The cease in biomass growth was concurrent with a gradual bleaching of biomass observed in all treatments. Due to the non-linear growth

in this experiment, a calculation of growth rates was not possible. Instead the maximal biomass reached on day 5 was compared. The treatment with 1:1 dilution with TapCGN water yielded the highest biomass of 48.56 +/- 3.18 g m^{-2}. This value was statistically different from the maxima in all other treatments according to one-way ANOVA with Turkey's post-hoc test ($p<0.001$).

To exclude a negative effect of replacing the evaporated water with tap water, which might have caused the observed inhibition, a new experiment was conducted in which evaporation was replaced with either the respective tap water or MQ water. The results are shown in figure 16. Since an inhibition of growth (concurrent with bleaching of biomass) was again occurring in all treatments after 6 days, freshly inoculated biomass was applied on new filters and brought to the glass fibers. It was grown with the same bottle of medium for another 9 days, to investigate if a limiting factor in this batch of urine was the cause of inhibition.

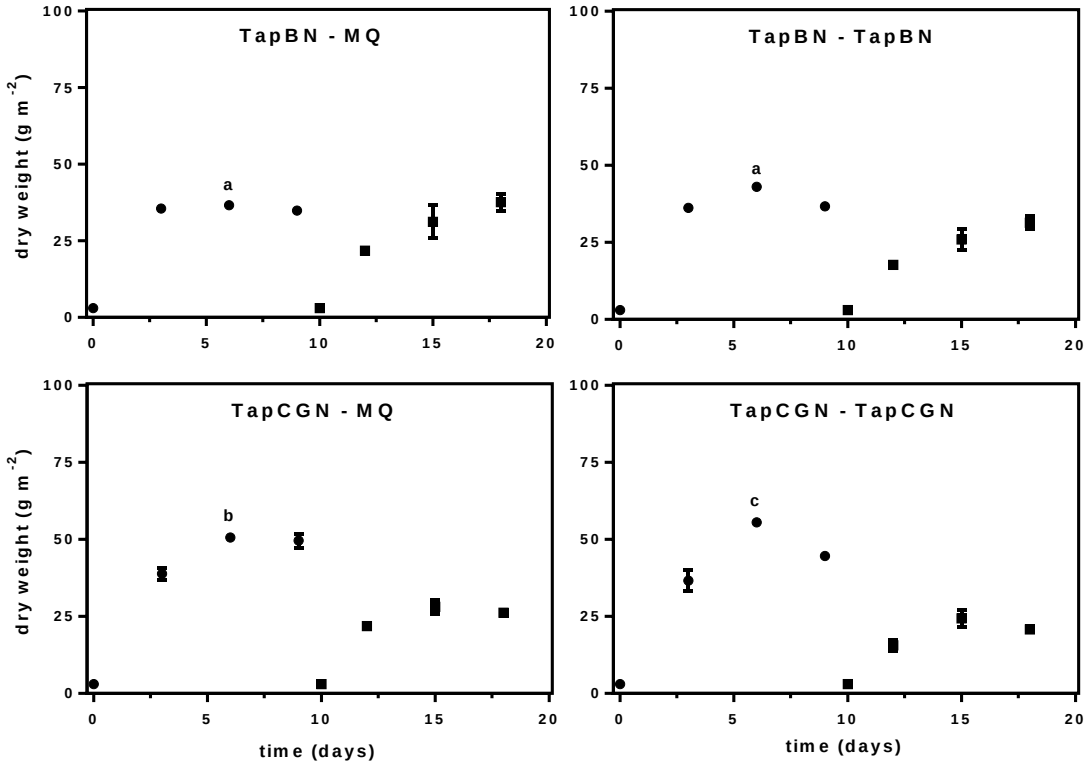

Figure 16: Immobilized growth of *Desmodesmus abundans* CCAC 3496 on urine of batch D diluted and evaporation replaced with water from different sources. The captions denote the diluent water and the refill water before and after the dash, respectively. Conditions were the following: 600 µmol photons m^{-2} s^{-1} light intensity; pH regulated at 6.5; 2.5 % CO_2 constantly supplied to the atmosphere. Circles represent the initial growth, squares represent the growth of newly inoculated filters. The medium was changed every 4 days until day 8, after which it was no longer changed. Different letters indicate significant differences in the biomass on day 5, according to one-way ANOVA with Turkey's post-hoc test ($p<0.005$). Values represent the mean +/- SD (n=3).

Biomass growth showed a similar pattern of stagnation and/or decrease as previously observed. In the first growth cycle the TapCGN – TapCGN treatment showed the highest biomass with 55.51 +/- 0.25 g m^{-2}. This value was statistically different from all other treatments according to one-way ANOVA with Turkey's post-hoc test ($p<0.05$). In the regrowth experiment, the treatment TapBN – MQ yielded the highest biomass at day 18 with a value of 37.58 +/- 2.78 g m^{-2}, which was statistically different from all other treatments according to one-way ANOVA with Turkey's post-hoc test ($p<0.001$). In all cases a regrowth was visible, however at a decreased rate compared to the initial growth and with a similar pattern of inhibition after 6 days.

To investigate whether the inhibition was permanent, a portion of bleached biomass was taken from the filters of the previous experiment and inoculated into Waris-H growth medium in suspension cultures.

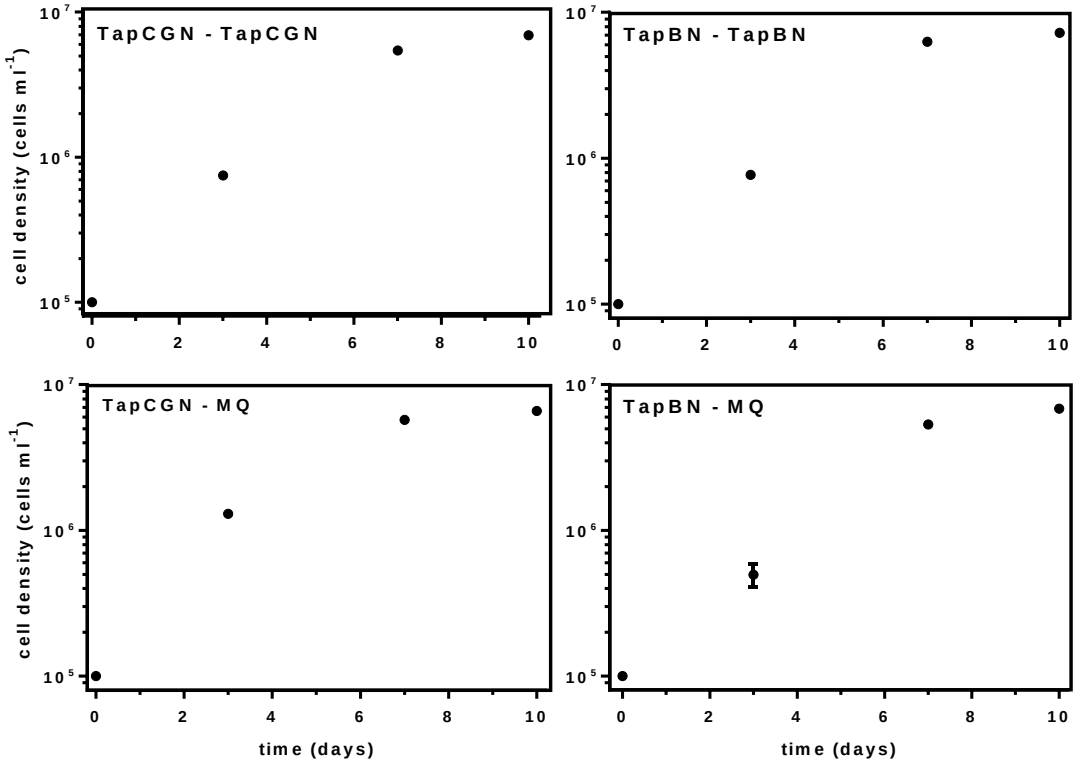

Figure 17: Cell growth of *Desmodesmus abundans* CCAC 3496 (taken from a partially bleached filter which had been cultivated on urine batch D) in Waris-H medium. The captions denote the treatment from which the inoculum was taken. Conditions were the following: 100 µmol photons m^{-2} s^{-1} light intensity, 20 ml medium in 50 ml Erlenmeyer flasks. Values represent the mean +/- SD (n=3).

Figure 17 shows that in all cases a logarithmic growth was observed for 7 days, after which cultures ceased to grow. A final cell density of 6.59 +/- 0.21 x 10^6 cells ml^{-1} was statistically not different between the different treatments, according one-way ANOVA with Turkey's post-hoc test ($p<0.001$).

3.6.2.4. Filtration and activated carbon treatment

The inhibitory substance, or microorganism, was attempted to be excluded from the urine by sterile filtration and/or activated carbon treatment. Before a growth experiment was conducted, potential effects of these treatments on the nutrient content of urine were determined. Sterile filtration did not have an effect on nutrient concentrations, while activated carbon treatment resulted in 17.19 % and 10.11 % removal of total nitrogen and total phosphorus, respectively. Since significant quantities of total N (2.07 g l^{-1}) and total P (0.15 g l^{-1}) were still present, a growth experiment on Twin Layer was performed. The results are shown in figure 18.

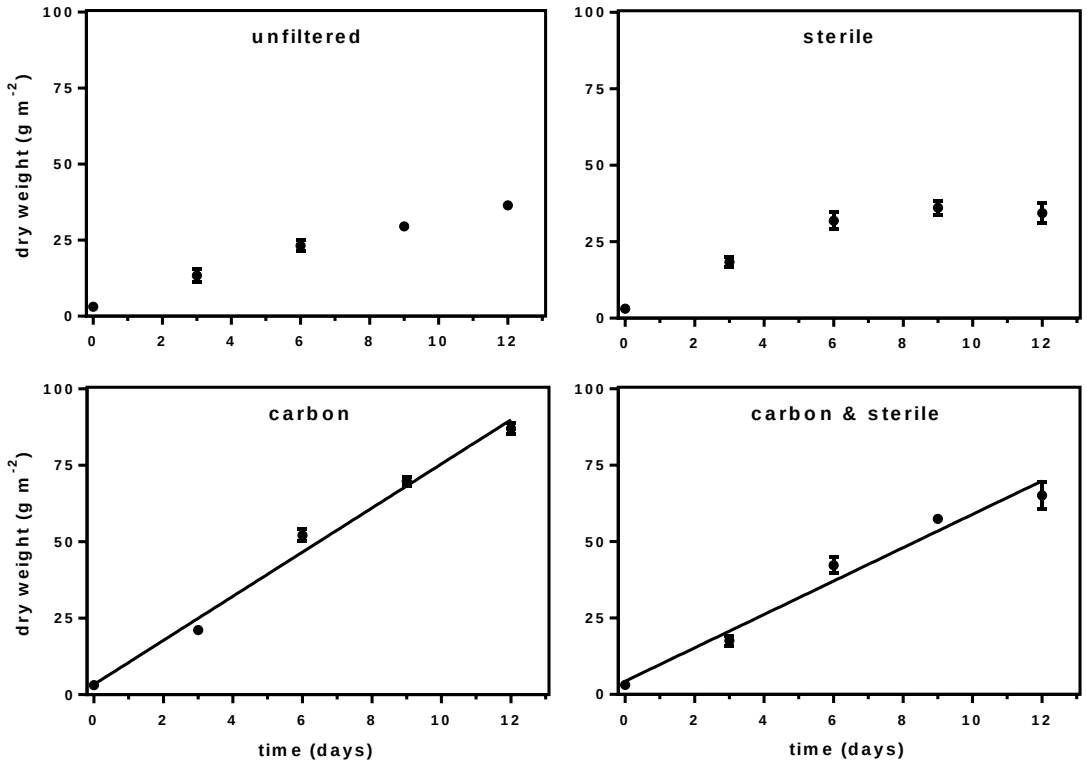

Figure 18: Immobilized growth of *Desmodesmus abundans* CCAC 3496 on urine of batch D diluted with TapBN water and treated in different ways. The captions denote method of filtration and treatment used. Conditions were the following: 600 µmol photons m^{-2} s^{-1} light intensity; pH regulated at 6.5; 2.5 % CO_2 constantly supplied to the atmosphere. The medium was changed every 4 days. Lines represent linear regressions. Values represent the mean +/- SD (n=3).

It is apparent that the activated carbon treatment yielded the highest growth rate of this comparison (7.216 +/- 0.2319 g m^{-2} day^{-1}), which was also statistically different from the growth rate of the combined treatment with activated carbon and sterile filtration, according ANCOVA analysis with Turkey's post-hoc test ($p<0.0001$). Furthermore, activated carbon treatment alleviated the bleaching effect on biomass that was otherwise observed with urine of batch D (Fig. 19). Interestingly there seemed to be an enhanced bleaching of biomass in the treatments with sterile filtration (B compared to A).

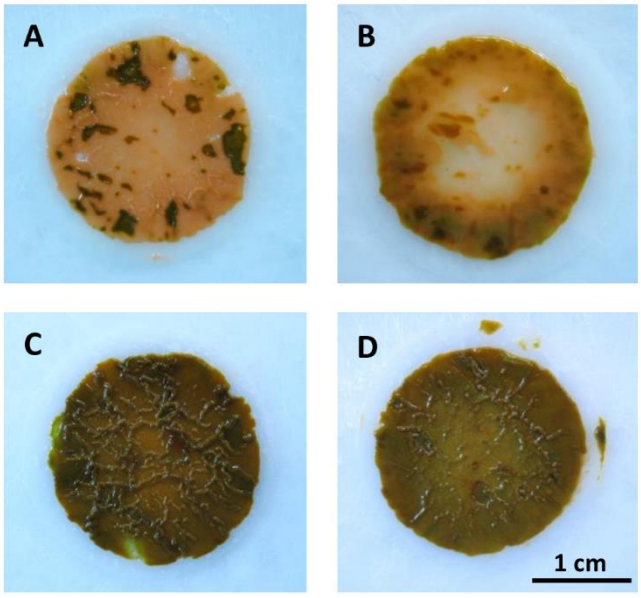

Figure 19: Photographs of filters with immobilized *Desmodesmus abundans* CCAC 3496 on day 9 grown on urine of batch D diluted with TapBN water and filtered in different ways. A: unfiltered; B: sterile filtered; C: carbon-treated; D: carbon-treated and sterile filtered.

Due to the alleviation of the inhibitory effect, activated carbon treatment was selected as pre-treatment for the following experiment, determining nutrient recovery from urine of batch D.

3.7. Removal and recovery of nutrients

To assess the potential of recovering nitrogen and phosphorus with immobilized strain CCAC 3496, an experiment was performed in which a surface (~300 cm^2) was inoculated and the urine was recirculated and not exchanged during the experimental period. Nitrogen and phosphorus concentrations were measured daily and the experiment was conducted until no more significant removal was observable. Figure 20 shows the results of this experiment.

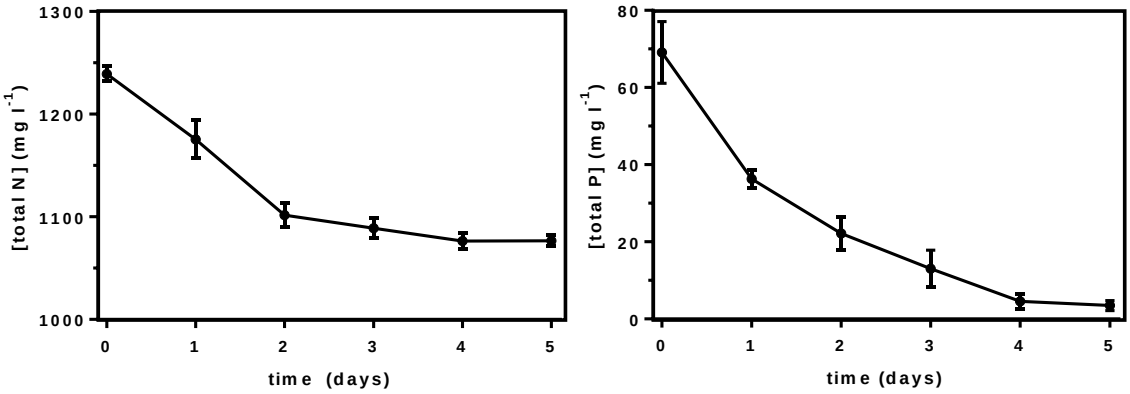

Figure 20: Nutrient concentrations in urine of batch D, diluted with TapBN water and treated with activated carbon, using immobilized *Desmodesmus abundans* CCAC 3496. Conditions were the following: 600 µmol photons m^{-2} s^{-1} light intensity; pH regulated at 6.5; 2.5 % CO_2 constantly supplied to the atmosphere. Values represent the mean +/- SD (n=4).

Total nitrogen was reduced most rapidly during the first two days, when the rate of removal was 68.75 mg N l^{-1} day^{-1}. After this, removal of nitrogen slowed down and stagnated between days 4 and 5. All remaining nitrogen in solution was present as ammonium (total-N = ammonium-N) at the end of the experiment. Total phosphorus showed the highest rate of removal in the first day of treatment, which resulted in a removal rate of 32.79 mg P l^{-1} day^{-1}. After this, removal gradually decreased until it stagnated between days 4 and 5. During the first day, a molar ratio of uptake of ~5:1 (N:P) was calculated. The final biomass on the reactor surface was 36.19 +/- 2.945 g m^{-2}, which can be converted into an overall growth rate of 7.238 g m^{-2} day^{-1}, assuming linear growth. The nitrogen and phosphorus content of the biomass at the end of the experiment was found to be 5.36 % and 2.1 %, respectively. Thus it can be calculated, that the portion of the removed elements, that was recovered in the form of biomass (recovery efficiency) was 87.1 and 87.5 % for N and P, respectively (Tab. 9).

Table 9: Nutrient removal and recovery by immobilized *Desmodesmus abundans* CCAC 3496. Recovery efficiency is the percentage of nutrients removed from the medium that is found in biomass. Removal efficiency is the percentage of total nutrient that was removed in the treatment. Values represent the mean +/- SD (n=4).

Parameter	**Nitrogen**	**Phosphorus**
removed from medium (mg)	81.25 +/- 8.6	32.81 +/- 3.9
recovered in biomass (mg)	71.87 +/- 2.26	26.8 +/- 1.4
recovery efficiency (%)	**87.1**	**87.5**
initial amount (mg)	619.6 +/- 15.74	34.55 +/- 3.56
left in medium (mg)	538.34 +/- 11.32	1.74 +/- 1.21
removal efficiency (%)	**13.1**	**94.1**

The removal of N was calculated to be 13.1 %, hence there were still significant quantities of nitrogen present after the 5 days of treatment. The efficiency of removal for P was 94.1 %, hence almost all phosphate from urine was removed at the end of the treatment.

4 Discussion

4.1. Microalgal strains

Previous studies that have dealt with the treatment of human urine with microalgae largely relied on using established strains from culture collections. Species that have been commonly used are *Chlorella sorokiniana* (Tuantet, Janssen, et al. 2014; Zhang et al. 2014; de Wilt et al. 2016), *Chlorella vulgaris* (Jaatinen et al. 2016), *Scenedesmus acuminatus* (Adamsson 2000) and *Spirulina platensis* (*Arthrospira sp.*) (Feng & Wu 2006; Yang, Liu, et al. 2008; Chang et al. 2013; Coppens et al. 2016). The approach of focusing on the optimization of process parameters and bioreactors sets aside the untapped potential that lies in natural microalgal diversity. To fill this gap in knowledge and to make use of specialist strains, a bioprospecting approach of isolating algae adapted to growing in urine together with a comprehensive screening of established strains was chosen as the starting point in this thesis.

4.1.1. Diversity of isolates

The majority of strains in this study were isolated from the higher dilutions of urine (Tab. 6), indicating that 1:1 diluted urine is a harsh environment for algae from the environment, possibly due to high pH and resulting high concentrations of NH_3 after hydrolysis of urea. Another point that speaks for this conclusion is that most isolates (83%) were isolated from secondary enrichment cultures. This means that the initial selection of algae occurred in the urine traps, while fastidious growth of isolatable populations occurred mainly in the enrichment media into which they were placed afterwards. Strains from secondary enrichments might simply have survived in urine (e.g. in the form of resting stages) rather than thriving on it. Most of the isolated strains belonged to three mayor phylogenetic groups, chlorophyta (green algae), cyanobacteria and bacillariophyta (diatoms) (Tab. 6). The green algae are considered to be the most tolerant to high ammonium concentrations among unicellular algae (Collos & Harrison 2014), which were most certainly encountered in the urine traps. Some cyanobacteria can also tolerate high ammonium concentrations (Collos & Harrison 2014) and occur in extreme environments

in general (Rothschild & Mancinelli 2001). Diatoms are also known to be relatively tolerant to high ammonium concentrations, ranking behind the chlorophyta and cyanobacteria in the review of Collos & Harrison (2014). Some diatom species (e.g. *Nitzschia, Navicula*) are known to occur in environments with high nutrient inputs and the composition of diatom communities in nature is commonly used as an indicator for the eutrophication status of a water body (e.g. Kelly & Whitton 1995).

Other studies in which algae were isolated from wastewater environments report a similar diversity as presented here. Zilz (2013) studied the diversity of microalgae in several municipal wastewater treatment plants in western Germany and established a large collection of strains, some of which were used in the screening of this study (designated VZ). Considering only those algae which were present in the inflows of the treatment plants, before the steps of nitrification and phosphorus removal, her samples were dominated by chlorophytes, followed by bacillariophytes and cyanobacteria. Dominant green algal species were *Chlorella ssp, Chlamydomonas ssp* and various scenedesmaceae, which correlates well with the species isolated from the urine traps (Tab. 6). A very similar pattern of dominant green algae, followed by diatoms and cyanobacteria was found by Chinnasamy et al. (2010), who isolated microalgal strains from carpet mill effluents, where ammonia was the mayor form of nitrogen. Thus, it can be concluded that the diversity of isolated strains largely represented those species which are typically present in wastewater environments.

One isolate, strain U 10.6, was identified as the xanthophyte *Heterococcus* sp., a group of organism which is not usually found in wastewater environments. It must be pointed out that this species is difficult to identify based on morphology alone, since it can undergo variations in appearance depending on environmental conditions and life-cycle stage (Darling et al. 1987). Therefore, it might have been wrongly identified. The same might be true for the isolate U 10.9, which was identified as *Trebouxia* sp.. Curiously, both these species are known to occur as photobionts in lichens (Darling et al. 1987; Kroken & Taylor 2000). In these symbiotic systems, urea is considered to play a role in carbon and nitrogen nutrition of both the fungus and the alga (Vicente et al. 1984), while the enzyme urease has in some cases been attributed to the algal partner (Millanes et al. 2004). Thus, the algae isolated might

indeed belong to these species and could occur in the urine traps in their free living form as early colonizers, since they might possess the enzymatic machinery necessary for hydrolysis of urea.

Isolating algae by providing a niche habitat naturally provides only a snapshot of the true diversity of strains capable of growing there. The full diversity was most likely not captured, since only one attempt of isolation was made in one specific season (spring), due to a limited amount of enrichment media and limited experience of the isolator with the manual techniques. Therefore, the diversity of algae is most likely biased towards fast growing and easy-to-isolate strains. However, this is in line with the overall aim of finding strains suitable for treatment of urine, where ease of cultivation and speed of growth are the main criteria. A more specific isolation strategy, closer to the treatment situation, might have been setting up a non-inoculated open Twin Layer system supplied with fresh urine, replaced at regular intervals and kept at a constant pH. Such a system would have provided a growth surface for environmental algae which are optimally adapted for growing in an immobilized way under the treatment conditions, and might have yielded even more suitable isolates.

4.1.2. Choice of organisms

During the suspension culture screening in 1:1 diluted urine, several strains which had been isolated from urine traps did not grow well (e.g. strains U 10.8, U 10.1, U 5.1 and U 5.8; Fig. 6 A, B and C). Those strains had however been derived from higher dilutions of urine, while two strains isolated from 1:1 urine traps ranked among the highest in this screening (U 2.1 and U 2.4; Fig. 7). This further highlights the fact that the dilution of urine is a critical factor for the growth of some strains.

Chlamydomonas moewusii strain U 5.5, which had performed among the best three strains in the initial screening in suspension culture (Fig. 7), did not show good growth when immobilized on Twin Layer PSBR (Fig. 12). This deviation might be explained by the morphology of the organism, since some flagellate algae have previously been observed to show only poor growth when immobilized (B. Podola, pers. comm.). Surprisingly, *Desmodesmus bicellularis* strain U 2.4 as well as *Chlorella sorokiniana* strain U 2.1, which showed among the highest growths in the initial screening (Fig. 6 C), also grew only poorly when immobilized on Twin Layer PSBR (Fig. 12). The reason for this divergence is not clear,

but it indicates that using an immobilized system for screening (e.g. the "Phycomat" (Nowack et al. 2005)) might have resulted in a more suitable selection of strains for further experiments. *C. sorokiniana* (CCAP 211/8K), which has previously been chosen for studies of nutrient recovery from urine (Tuantet, Temmink, et al. 2014; Zhang et al. 2014), ranked on place 24 of the 96 strains tested in the suspension screening (Fig. 6 B). This further points to the potential of using the biological approach to improve the selection of organisms most suited for the treatment of urine.

Desmodesmus abundans (CCAC 3496) was chosen as the algal strain for further work because it displayed, as the only tested organism, linear growth for up to 9 days when immobilized on Twin Layer with 1:1 diluted urine as medium (Fig. 12). The organism also grew rapidly in suspension culture, further simplifying the preparation of inocula for Twin Layer cultures. This strain originated from the wastewater of a paint-finishing system of an automotive company, speaking for a very robust organisms tolerant to heavy inorganic pollution. Members of the genus *Desmodesmus* are indeed known for their cosmopolitan distribution in freshwater habitats and are commonly found at sites of high nutrient input (Sheath & Vis 2013). Morphological polymorphism, as observed in this study (Fig. 4), is a well-described phenomenon in the genera *Scenedesmus* and *Desmodesmus* (Trainor 1966; Lürling 2003) and in some cases functional conclusions have been drawn. The disappearance of spines, as was observed in cultures of *D. abundans* CCAC 3496 when grown in urine, has previously been attributed to adverse growth conditions such as high salt concentration (conductivity), nutritional deficiencies or presence of inhibiting substances (Marčenko 1969). All of these conditions might have been encountered when growing in urine. A decreased spine formation upon exposure to high conductivities has also been observed for the desmid *Staurodesmus omearii* (Miebach 2014). In that species, it might be interpreted as a mechanism to avoid low nutrient concentrations in the natural situation, since cells with spines (mainly composed of cell-wall material) become heavier, sinking to the bottom of a water body were nutrient concentrations are generally higher than in the open water (M. Melkonian, pers. comm.). The slight enlargement of *D. abundans* cells in the present work might further be due to the inability to divide, which could be interpreted as a deficiency in some critical nutrient when growing in urine.

4.2.Nitrogen metabolism

To elucidate their physiological capacities and to aid in the selection of a suitable organism for treatment of urine, several axenic strains were tested for their use of different nitrogen sources.

4.2.1. Differences in nitrogen metabolism

As can be seen in figure 10, all tested strains were able to utilize ammonium as sole nitrogen source and grew similarly well on this N-source on agar plates. This is in line with the textbook concept that this most reduced mineral form of nitrogen is in many cases the preferred nitrogen source for microalgae, as well as for most other photolithotrophs (Raven et al. 1992; Giordano & Raven 2014). Indeed, it has been shown in *C. vulgaris*, that the expression of nitrate reductase is repressed in the presence of ammonium, leading to a preferred uptake of ammonium (Morris & Syrett 1963). Energetically ammonium is preferable over nitrate, since its use does not require any further reduction before incorporation into amino acids (reviewed by Cai et al. 2013). Nitrate served as a good N-source for strains U 5.5 and CCAC 3496, while in strain CCAC 0126 it resulted in growth similar to that on ammonium (Fig. 10). The fact that the *Desmodesmus* strains BI-16 and U 2.4 did not grow on nitrate (Fig. 10 A) is unusual, since nitrate is generally considered a good nitrogen source for most microalgae (Healey 1973). However, these strains also only showed intermediate/low growth on the other N-sources in this experiment (Fig. 10 B). Thus it might be the case that these strains are generally not suited for growth on agar plates under the given conditions. To substantiate this claim, one would have to perform a test in suspension culture, providing nitrate as sole N-source. The highest growth on urea as N-source was achieved by strains U 5.5 and CCAC 3496, while CCAC 0126 was seemingly unable to use this N-source. These 3 strains were chosen for a further elucidation of their urea use capabilities in suspension cultures (Figs. 8 & 9).

Halochlorella rubescens CCAC 0126 showed no growth (cells and dry weight) with urea as sole N-source, until an external dose of urease enzyme was added on day 9. From then onwards cell numbers increased and ammonium release was detected in parallel with a decrease in urea concentration. After the addition of enzyme there was also an increase in pH, typical for the hydrolysis of urea. Taken in

concert, these results point to the conclusion that the organism does not possess a functional enzyme capable of splitting urea.

Chlamydomonas moewusii U 5.5 grew exponentially with urea as sole N-source for the first 9 days (Fig. 8). In this timeframe concentrations of urea decreased steadily, while ammonium concentrations remained close to the detection limit throughout the experimental period. Since there was also no significant rise in pH (Fig. 9), the external hydrolysis of urea and presence of ammonium in the medium can be excluded. These results can be seen as evidence that *C. moewusii* strain U 5.5 possesses all the transport and enzymatic components necessary to take up urea and intracellularly hydrolyze it. As with many other soluble nutrients, uptake of urea can occur via high- and low-affinity uptake systems, their regulation depending on the concentration gradient between the environment and the cell. In the model chlorophyte *Chlamydomonas reinhardtii*, a distant relative to *C. moewusii*, the active transporter DUR3 has been identified as an important uptake system for urea (Kirk & Kirk 1978). The gene family coding for this transmembrane protein complex has later been identified in a number of other eukaryotic algae (Solomon et al. 2010). This high affinity transporter uses the energy from a sodium gradient across the cell membrane; it belongs to the sodium:solute symporter family also present in higher plants and fungi (Wang et al. 2008). Activity of this system is considered to be light-dependent and is down-regulated by the presence of ammonium in cultures as well as in natural phytoplankton populations (Rees & Syrett 1979; Lomas et al. 2002). Transport of urea into the algal cell can also occur via a low-affinity passive transport system, the mayor intrinsic proteins (MIPs) or aquaporins (reviewed by Giordano & Raven 2014). Little is known about regulation of urea transporting MIPs in algae, however it can be deduced from higher plants that these channels are especially important at high external urea concentrations, as would be the case under agricultural fertilization (Kojima et al. 2006) or in urine. The type of urea splitting enzyme present in *C. moewusii* is an open question. However *C. reinhardtii* is known to possess the ATP dependent UALase enzyme instead of urease (Solomon et al. 2010). The following formula shows the overall reaction catalyzed by UALase *in vivo* (from Syrett & Leftley 1976):

$$CO(NH_2)_2 + ATP + H_2O \rightarrow CO_2 + 2NH_3 + ADP + P_i$$

The ATP dependency is a puzzling feature, since there seems to be no thermodynamic requirement in the breakdown of urea by the urease enzyme. Syrett & Leftley (1976) have speculated that the reason might be a tight metabolic control of UALase, requiring the input of energy. It has been shown in *C. reinhardtii*, that UALase synthesis is indeed substrate-controlled (Hodson et al. 1975), while evidence of urease activity in *Phaeodactylum tricornutum* speaks for a constitutive mode of expression (Leftley & Syrett 1973). Another distinguishing feature are the differences in Michaelis-Menten constants between the two enzymes. Comparing values from various organisms, urease can have 150-500 fold higher K_m than UALase (Syrett & Leftley 1976; Reithel 1971), speaking for an advantage of UALase-possessing microalgae at low environmental concentrations of urea.

Desmodesmus abundans CCAC 3496 showed growth on urea as sole N-source, both in cell numbers and dry weight (Figs. 8 & 9). In the logarithmic growth phase, from day 0 until day 9, the pH in the culture rose substantially from 7.3 to 9.45. In the same timeframe concentrations of urea dropped, while an increase in the concentration of ammonium was detected. This experiment clearly shows that the strain possesses the enzymatic machinery to hydrolyze urea and thus to make use of the compound as sole N-source. Non-enzymatic hydrolysis can be excluded, since the half-life time of urea is 3.6 years at 38 °C (Udert et al. 2003). Indeed, a related organism "*Scenedesmus sp.*" has previously been found to utilize urea, showing growth similar to that on nitrate (Ren et al. 2013). However, the fact that relatively high concentrations of ammonium were found in the medium is puzzling and can lead to two separate conclusions: (a) The alga secretes excess ammonium after intracellular hydrolysis, because the nitrogen in the molecule is in excess of the physiological need. This mechanism was proposed by Price & Harrison (1988), who concluded that urea serves as both carbon and nitrogen source in the diatom *Thalassiosira pseudonana* and that carbonate and/or ammonium are excreted from the cell in case the it does not require one or the other. Little & Mah (1970) also observed ammonium production in photoheterotrophic urea-grown cultures of *Chlorella ellipsoidea*, however only in the stationary phase. These authors suggested that ammonia might be excreted if growth is carbon limited. (b) Another explanation for the occurrence of ammonium in the medium might be the presence of hydrolytic enzymes on the cell surface or their release into the environment, which would then supply the alga with

ammonium as exogenous nitrogen source. This would be analogous to the secretion of phosphatases, which is a well-studied mechanism by which algae and other microorganism can mobilize organic P-sources in their environment (Patni et al. 1977). For ureases, enzyme secretion is known from the intestinal pathogen *Helicobacter pylori*, which uses the hydrolysis to locally raise the pH and protect itself in the extremely acidic conditions of the stomach (Marshall et al. 1990). Closer to the phototrophic realm, several species of lichen have been shown to secrete urea-splitting enzymes in the presence of external urea (e.g. Perez-Urria et al. 1989). In one study of the lichen *Evernia prunastri*, it has been shown that ureolytic activity was mainly associated with the photobiont, while the fungal partner did not harbor the enzyme (Legaz & Vicente 1981). These authors later showed that the enzyme was located within the photobiont's cell wall (Millanes et al. 2004). While from the experimental data in this thesis the location of hydrolysis in CCAC 3496 cannot be concluded, it is nevertheless clear that this organism possesses the enzymatic capacity to utilize urea as nitrogen source. Whether the alga contains the urease enzyme or the ATP-dependent UALase enzyme cannot clearly be deduced. One hint that the enzyme in this organism might not be under tight metabolic control, typical of urease rather than UALase (Solomon et al. 2010), is that when the culture started entering senescence, a further decrease in urea concentration was observed (Fig. 8). This might be explained by ATP-independent activity of enzymes from lysed cells. A similar situation can be found in soils, where a mayor part of urease activity is attributed to free urease from lysed bacterial cells or excreted by plants (Hasan 2000).

To further elucidate the mechanisms involved, one should study the enzyme activity of cell free culture medium in which the organism was grown and compare the urease activity in different compartments of the cell. Furthermore, uptake characteristics of urea and urea-derived N could be investigated in short term incubations with stable isotope-labelled urea, measuring the different intra- and extracellular pools of nitrogen. Presence of UALase enzyme activity could be proven by means of demonstrating nickel-independent hydrolysis of urea and tolerance to inhibition by hydroxyurea, as has been done for *Chlorella vulgaris* and *Scenedesmus obliquus* (Syrett & Al-Houty 1984). For a certain determination which enzyme is involved, the presence or absence of the urease-encoding gene sequence could be tested (Baker et al. 2009).

4.2.2. Implications for treatment of urine

Fresh urine contains urea as the mayor nitrogen source. While urine from healthy individuals is generally considered sterile, this paradigm has recently been challenged (Wolfe & Brubaker 2015) and minor loads of bacteria are usually carried from the skin or through fecal cross contamination and thus enter collected urine (Winker et al. 2009). Furthermore, when kept under non-sterile conditions at moderate temperatures, such as in the open Twin Layer system, bacteria are inevitably introduced and cause the hydrolysis of urea to ammonium (Table 8 and Udert et al. (2006)). Thus, hydrolysis of urine in an open system treating urine is inevitable. Strain CCAC 0126, for example, would be able to grow only under these conditions, relying on hydrolytic bacteria to release ammonium from urea. While it was shown that CCAC 0126 could grow relatively well on Twin Layer with urine for 6 days (Fig. 12), such a dependency would decrease the robustness and reproducibility of the process. Growth would depend on the presence of bacteria, which could vary due to external factors (e.g. loading of the urine and of air). Furthermore, the inability to metabolize urea might lead to a lag-period in algal growth before bacterial hydrolysis takes place. Therefore, in order to achieve reliable growth on urine, a strain should be used which has the ability to utilize urea as a source of nitrogen in an axenic situation as well as grow well on Twin Layer, as is the case for *Desmodesmus abundans* strain CCAC 3496.

4.3. Operation of Twin Layer PSBRs

4.3.1. Operational parameters

In order to maximize the capacity of nutrient recovery from urine, several parameters (namely light, pH-value, type of diluent water and pretreatment of urine) were adjusted before employing a laboratory-scale treatment system. Optimization was performed using the filter-disk system (Fig 3 A) to accurately determine growth rates, while treatment was performed on a fully inoculated membrane (Fig. 3 B) to follow the uptake of nutrients. It has to be noted here, that even though parameters were attempted to be optimized for maximal growth, the highest growth rate was reached in the initial screening of strain CCAC 3496 (compare Figs. 12-14). Determined maximal growth rates varied between 7.24 and 10.43 g m^{-2} day^{-1}. This apparent fluctuation in growth performance might, for example, be attributed to the presence or absence of particular bacterial populations during the different experiments, which might

have affected the chemical parameters of the medium (e.g. by increased or decreased rates of hydrolysis). In the case of the nutrient uptake experiment (chapter 3.7) the decreased growth performance was most likely due to the difference in composition of this batch of urine (Tab. 5; batch D) as well as the activated carbon pre-treatment, which removed some of the nutrients.

4.3.1.1. Light and CO_2

Light intensities between 400 and 1000 µmol photons m^{-2} s^{-1} were tested for their effect on growth of *D. abundans* CCAC 3496. This range was chosen on the basis of previous experimental work with PSBR-systems (Schultze et al. 2015; Li et al. 2015) and because this range is close to daytime averages of incident radiation in the tropics, as well as in the temperate regions during summer (Norsker et al. 2011). It has to be noted, that in the case of higher incident solar radiation, the light reaching the growth surface on Twin Layer sheets could simply be reduced by hanging sheets more closely to each other (light dilution principle; see Fig. 2 C). Optimal growth was found at 600 µmol photons m^{-2} s^{-1}, while higher and lower light intensities resulted in decreased growth rates (Fig. 13). In an algal biofilm, light arriving at the surface inevitably decreases with depth into the biofilm, until, depending on biofilm thickness, there might be no more light at all. On the surface there is usually a zone of light inhibition of photosynthesis, after which there is a zone of optimal growth, followed by an increasing impact of light limitation (Li et al. 2015). The overall growth of the biofilm is therefore determined by the relative impact of these three zones. The effect of applying CO_2 to the gas phase was not investigated in detail. However, from previous experience with Twin Layer type PSBRs, it is known that even at relatively low light intensities of 200 µmol photons m^{-2} s^{-1}, the addition of 3 % CO_2 can increase algal growth rates by up to 50 % (Schultze et al. 2015). Therefore, in the present study, the use of supplementary CO_2 was considered necessary to obtain optimal algal growth.

4.3.1.2. Water and pH

In order to design a more realistic treatment system, which does not rely on costly water purification, tap water was used to prepare the 1:1 dilutions of urine used in this part of the study. The use of undiluted urine was tested in one instance only, using the urine of batch D, which allowed only poor algal growth generally (Fig. 15). Thus, it cannot be said with certainty that the use of undiluted urine is not possible.

However, the 1:1 dilution was chosen to accommodate the nature of vacuum low-flush source separating toilets for collection, which would yield a similar dilution factor in a realistic situation (Johansson 2000). The direct comparison of different waters used for dilution of urine was also carried out using the urine of batch D, which did not allow sustained algal growth in any of the cases (Fig. 15). Thus, drawing a direct conclusion as to which water is more suitable in a realistic situation is difficult to do. However, the elemental composition of the tap waters yielded very different results (Fig. 11). Most notably, TapBN was found to contain relatively high amounts of iron, an element of mayor importance for phototrophic growth due e.g. to its presence in cytochromes and ferredoxin (Healey 1973). Iron has indeed been found to be a limiting factor for algal growth on urine in a previous study (Tuantet, Janssen, et al. 2014). In TapCGN, high concentrations of calcium were detected. Calcium is known to form precipitates with phosphate at pH values above 9 (Udert et al. 2006), as are often found in non-buffered algal growth systems. In preliminary suspension culture experiments using urine diluted with TapCGN under non pH-controlled conditions, crystalline precipitates were indeed observed (data not shown). When using adequate pH control, precipitation of minerals could be effectively prevented (Tab. 12), making the choice of dilution water less critical. Using different waters to refill the evaporated water, which is inevitably occurring in a cultivation system with a large exposed surface, seemed to make a slight difference for algal growth (Fig. 16). The use of tap waters (TapBN or TapCGN) for refilling water lost by evaporation yielded in both cases significantly higher biomass densities at day 6 than when MQ water was used. This might speak for the addition of trace elements through tap waters, however this result is ambiguous since urine of batch D was used, which resulted in poor growth generally.

The control of pH below a value of 7 was found to be a critical factor in the studied system, both to prevent precipitation and to allow optimal algal growth. Figure 14 shows that a pH of 6.5 yielded optimal algal growth, while a pH of 7 resulted in reduced growth rate. At pH of 7.5 growth rate decreased further and linear growth ceased after 7 days. The most likely explanation is that at this pH, the fraction of ammonia/ammonium which was present as NH_3 became high enough to have a toxic effect on the cells. Indeed, modelling of ammonia/ammonium speciation in hydrolyzed urine at pH 7.5 and room temperature results in up to 10 % of the molecule being present as NH_3 (Siegrist et al. 2013). In the case

of urine of batch D, this would result in a theoretical [NH_3] of 264 mM, which is far higher than the previously determined limits of tolerance of 1.2 mM and 10 mM for a *Scenedesmus obliquus* and a *Chlorella vulgaris*, respectively (Azov & Goldman 1982; Konig et al. 1987). This would also explain the increasingly negative effect of high pH over time (Fig. 14), since towards the end of the experiment urea should have been completely hydrolyzed, resulting in increased concentrations of NH_3. At a pH below 7, the speciation of ammonium ions and free ammonia is completely shifted towards NH_4^+ (Siegrist et al. 2013), making 6.5 a suitable pH value for this treatment system. The use of fresh (or stored frozen) urine under controlled pH conditions prevented uncontrolled hydrolysis and the resulting formation of struvite prior to - as well as during - the treatment. The occurrence of struvite when pH was not controlled was shown by measuring the equimolar presence of N and P in precipitates which formed from urine without adding CO_2, while the same was prevented when pH was controlled by addition of CO_2 (Tab. 8).

4.3.1.3. Inhibition and activated carbon

The most prominent challenge that occurred in the experimental work in this thesis was the unexpected inhibition of growth that took place when using untreated urine of batch D (Figs. 15, 16 & 18). With this batch, biomass development was normal for the first 4-5 days, after which it severely slowed down and in some cases decreased towards the end of an experiment. The inhibition of growth was paralleled by a progressive bleaching of biomass (Fig. 19 A & B), which speaks for a degradation of chlorophyll in the algal cells. The inhibition was not due to a limiting nutrient in the medium (e.g. a trace element which was present at lower concentration than in other batches), since newly inoculated biomass exhibited a similar pattern of growth followed by inhibition when growing on the same bottle of urine (Fig. 16). Inhibition caused by batch D urine was reversible, since almost completely bleached biomass taken from the filters grew back normally when inoculated into Waris-H medium in suspension culture (Fig. 17). It was possible to alleviate the inhibitory effect by treating the urine of batch D with activated carbon, but not by sterile filtration (Fig. 18). Thus, it can be concluded that this batch of urine contained a dissolved compound or element, which had an inhibitory effect on algal growth.

The most plausible explanation appears to be the presence of an inhibitory pharmaceutical compound, or residue thereof, in this batch collected from a large group of 57 people (Tab. 5). Urine is known be the mayor excretion route of pharmaceutical compounds leaving the human body (Bester et al. 2008; Jjemba 2006). Among the most commonly used pharmaceuticals are non-steroidal anti-inflammatory drugs (NSAIDs; e.g. ibuprofen and diclofenac) and antibiotics. The mode of action of toxicity of NSAIDs to microalgae has been proposed to be non-specific and mediated by damage to cell membranes due to the lipophilic nature of the molecules (Escher et al. 2005). NSAIDs have recently been tested for their effect on axenic laboratory cultures of eukaryotic algae (Bácsi et al. 2016). In this study, the growth of *Desmodesmus communis*, a relative to *D. abundans* CCAC 3496, was inhibited by various NSAIDs at concentrations of 100 mg l^{-1}. Interestingly the inhibition by ibuprofen showed its effect after 4-6 days of incubation, the same timeframe as observed in this study. This is by no means a proof for the action of this compound, but together with the potentially widespread use of this drug among the donors of urine could indicate that such a drug might have been the causative agent of inhibition. Cleuvers (2004) performed inhibition experiments with *D. subspicatus* and found, that while acute inhibitory effects of the single NSAIDs were relatively low, the combination of four different compounds increased the inhibition up to 75 fold. In another recent study, several NSAIDs did not inhibit growth of *Chlorella sorokiniana* at concentrations of 100-300 µg l^{-1} (de Wilt et al. 2016). In both recent studies of NSAIDs effect on algal growth, only minor uptake or adsorption of the compounds to algal biomass was reported (de Wilt et al. 2016; Bácsi et al. 2016), which is in line with the observation that placement of affected cells in fresh media resulted in immediate regrowth of the cultures (Fig. 17). A contrasting result was observed by Escapa et al. (2016), who showed that biomass was increased in cultures of three green algal species to which the NSAID diclofenac had been added. The authors attributed this to the use of the compound as an organic carbon source and argued that an algal post-treatment might enhance the otherwise unsatisfactory removal of such compounds in municipal WWTPs. However, their study used a relatively low concentration of 25 mg l^{-1}. Clearly, inhibition by any toxic compound is concentration dependent and concentrations of pharmaceuticals in source separated urine can be expected to be much higher than what is commonly found in municipal waste waters.

Antibiotics are another type of pharmaceutical that might have inhibited algal growth in this study. Although typically used to suppress the growth of prokaryotic organisms, they are also known to be able to exhibit toxic effects on eukaryotic microalgae (Provasoli et al. 1951). Although the purpose of these early studies was to eliminate bacterial contaminants from algal cultures, it was found that depending on the concentration and mode of application, there might be harmful effects on algal photosynthesis (Hunter & McVeigh 1961; Melkonian & Weber 1975). There are various kinds of antibiotics and their mode of actions vary widely. Most of the studies concerning microalgae have been conducted in the framework of aquaculture larval hatcheries (Eguchi et al. 2004; Lützhøft et al. 1999) or related to the sensitivity of indicator species in the environment (Campa-Córdova et al. 2006; Lai et al. 2009; Zhang et al. 2013). For example, the broad spectrum antibiotic chloramphenicol acted inhibitory both at chronic and acute exposure on growth of *Scenedesmus obliquus* (Zhang et al. 2013).

Although the exact nature of the inhibitory compound is unknown, it is clear that the treatment with activated carbon was able to alleviate the inhibitory effect of urine batch D on growth (Fig. 18) as well as the bleaching effect on biomass (Fig. 19 B & C). The achieved growth rate was somewhat lower than those measured before (7.22 vs. 10.33 g m^{-2} day^{-1}), which might have been due to the presence of residual amounts of the inhibitory substance, the difference in composition of this batch of urine (Tab. 9), or the decrease in P-content of the urine due to the activated carbon treatment (chapter 3.7). Activated carbon (AC) is known as an effective adsorbent for a variety of organic and inorganic molecules from liquids, mainly due to its large surface area, its internal porosity structure and presence of surface-binding groups (Yin et al. 2007). Thermally treated AC, as the one used in this study, is especially suited for binding of organic molecules and is often used in small scale waste water treatment operations for the removal of pharmaceuticals (Donau Carbon, personal comm). Indeed, activated carbon treatment has been proven to remove pharmaceutical contaminants (e.g. Antibiotics, beta-blockers and NSAIDs) from human urine (Özel 2012) and has been proposed as a viable option for reducing the risk of the enrichment of these substances in decentralized sanitation and resource reuse systems (Udert et al. 2015). In the case of the algal treatment studied here, it appears that AC can be used as a "safety net" to ensure that algal growth is not inhibited, even though the drug intake of urine donors is not controlled.

4.3.2. Efficiency of treatment

Of the N and P that was removed from the medium, 87.5 and 87.1 % were recovered as algal biomass, respectively (Tab. 9), speaking for high recovery efficiencies and only minor fractions of nutrients which were "lost" from the system. This lost fraction might be present in the form of bacterial and algal biomass which had formed in the tubes and media reservoirs of the systems. In terms of removal efficiency, phosphorus was removed almost completely (94.1 %), while nitrogen was only partially removed (13.1 %). It has to be mentioned that these experiments were carried out on urine of batch D, which had the lowest N-content of all urine batches (Tab. 5). Had another batch of urine been used for the uptake experiments, then the percentage of recovered N would likely be even lower. The following paragraphs will elaborate on the shortcoming of nitrogen recovery this and will attempt to explain this finding:

The phosphorus content found in strain CCAC 3496 after 5 days of urine treatment was 2.1 % (w/w), while the nitrogen content was 5.36 % (w/w), resulting in an atomic N:P ratio of 5.8:1. This is a low ratio, when compared with the canonical work of Redfield (1958), who determined a statistical mean N:P ratio of 15:1 in phytoplankton-dominated surface water samples across the global oceans. However, the universal applicability of this ratio to algal biomass has to be questioned (Geider & Roche 2002). For example, in a freshwater ecosystem a mean ratio of 12:1 has been determined in one study of 24 algal strains (Ahlgren et al. 1992). Phosphorus is generally considered the limiting element for photosynthesis in aquatic systems, and lower N:P ratios can be expected in nutrient-replete systems (Geider & Roche 2002). Indeed, the P content of algal cells is known to show considerable plasticity, ranging between 0.5-33 % (w/w) depending on environmental conditions and physiological status of the cells (Healey 1973). Thus, generalizations on what a "normal" N:P ratio for algal cells is, are difficult to make. Nevertheless, a concentration of 2.1 % can be considered high for algal cells with a balanced nutrient status (Healey 1973). There are two distinct explanations possible as to why the observed biomass contained elevated amounts of phosphorus:

Luxury uptake of phosphorus is a well-known process, which describes the uptake and storage of P beyond the physiological requirements of the organisms for replication, usually taking place when P is present in excess (Azad & Borchardt 1970; Eixler et al. 2006; Borchardt & Azad 1968; Kuenzler &

Ketchum 1962). In this case, P is stored in the form of polyphosphate in vacuoles, which can be remobilized and provide phosphorus for phases of limitation. This phenomenon is known for a variety of microalgae (reviewed by Cembella et al. 1984). Through this mechanism, cells can uncouple growth from the external concentration of a limiting nutrient, as described in the cell-quota model (Droop 1977). In the present study, the initial N:P ratio of urine (batch D) was 31:1, well above the critical N:P ratios between 5:1 and 15:1 considered to favor luxury uptake (Ptacnik et al. 2010). Therefore, it seems likely that a limitation by another element came into play, by which the uptake of phosphorus was slowed down after 1 day, and which resulted in incomplete recovery of P (Fig. 20). The limiting element might have been iron or another critical trace metal, such as boron, copper, manganese or zinc, all of which have been suggested to limit algal growth in urine previously (Tuantet, Janssen, et al. 2014). Other elements that might have limited growth are vitamins, such as thiamine or cobalamine, which are known to be critical for the growth of wide range of microalgae (reviewed by Croft et al. 2006). A simple test to answer this would be to try to grow the axenic strain in a medium which is devoid of vitamins. The low N:P ratio in the final biomass might be explained by an initial luxury uptake of P into the cells, which was followed by the limitation of a critical growth factor, making the use of internal phosphate reserves for further cell multiplication impossible. To evaluate luxury uptake, the presence or absence of polyphosphate granules in the cells could be evaluated by cytochemical staining and microscopic observation (Ebel et al. 1958). To shed further light on what the limiting factor was, a stepwise addition of trace elements and vitamins to stationary cultures in urine could be performed. The critical element should alleviate growth inhibition.

Precipitation of phosphate-minerals in the biofilm is another possible reason for the high P content observed in the produced biomass. Extracellular P adsorption has previously been described for natural assemblages of the colonial marine cyanobacterium *Trichodesmium* (Sañudo-Wilhelmy et al. 2004), where it was hypothesized to serve as an external pool of P storage. In a biotechnological situation, crystallization of calcium-phosphate in the vicinity of cells of *Chlorella emersonii*, cultivated at high densities in a membrane bioreactor, has recently been observed (Xu et al. 2014; Tang & Hu 2016). Especially figure 4 in Tang & Hu (2016) is of interest here, since it shows crystal formation next to algal

cell. The authors do not state this conclusion, but it seems likely that precipitates were formed due to high pH conditions (> pH 9), which are generally favorable for the precipitation of phosphate minerals. A similar conclusion was drawn by Craggs et al. (1996), who determined chemical precipitation on their algal turf scrubber to be a mayor mechanism for P-removal from wastewater. The same might have occurred in the PSBR system employed in this study, although pH in the medium was tightly controlled. In a comparable system with PSBR immobilized *Halochlorella rubescens*, it was shown that *in situ* pH in the depth of the biofilm can vary vastly from the pH in the medium and may reach values up to 9, even when CO_2 is applied to the atmosphere at the same concentration as in this study (Li et al. 2015). Such a localized peak in pH might have been present in the biofilm grown on urine and could have caused the precipitation of, for example, calcium-phosphate. This would explain the high P content (and low N:P ratio) in the biomass that was observed in this study. Struvite as precipitate is in this case unlikely, since it would not affect the N:P ratio of harvested biomass. To validate this hypothesis, microscopic observations of harvested biomass as well as separate measurements of intracellular and extracellular P should be employed. If found to be indeed the case, this phenomenon could be described as a practical way of recovering more phosphorus in solid form than would be possible in the biomass alone. Especially in a PSBR system, this would mean an enhanced extraction of P from the liquid phase into a harvestable, dry product. However the effect on fertilizer quality would need to be examined, since P-containing mineral precipitates might not possess the slow-release properties which are attributed to algal biomass.

4.3.3. Comparison to published studies

Since most other studies on using algae to treat urine have been conducted with other strains under dilute conditions in suspension-based systems, direct comparisons of growth rates and nutrient uptake are difficult to make. However, when the biomass productivity achieved in this study is related to the volume of urine used, a productivity of 14.5 g l^{-1} day^{-1} can be calculated for a growth surface of 0.3 m^2 (chapter 3.7). This compares favorably with the 9.3 g l^{-1} day^{-1} which were reported for a continuously operated flat plate photobioreactor treating urine augmented with trace elements, in the only published study using urine at the same dilution factor (Tuantet, Temmink, et al. 2014). When the authors additionally

enriched urine with Mg and P and shortened the light path of the reactor, productivity increased to 14.8 g l^{-1} day^{-1}. In the same study, nutrient removal was achieved at 87 % and 76 % for N and P, respectively. The N removal is much higher than the 13.1 % obtained in this study, which can be explained by the addition of P, optimizing the N:P ratio. P removal was achieved at 94.1 % in the present study and the difference might also be due to the addition of P by Tuantet, Temmink, et al. (2014), combined with an enrichment of P in the biofilm biomass, as elaborated above. It has to be stated that the operation in continuous culture mode can compromise on performance as opposed to batch mode, as it was employed in this study. Furthermore, since other operational parameters such as light intensity and temperature also differed between studies, these comparisons are inherently only approximations.

Comparing the system in this study to other biofilm systems, used for the treatment of municipal wastewaters, has the same inherent inaccuracies. However, an attempt will be made in order to situate the results among other algal systems for nutrient recovery. Based on biomass growth and phosphorus content, an overall P-removal rate of 0.152 g m^{-2} day^{-1} can be calculated for this study (chapter 3.7). This is somewhat lower than the rate found in a study of an algal turf scrubber treating municipal wastewater by Craggs et al. (1996), who reported 0.73 g P m^{-2} day^{-1}, but similar to the P removal rate of 0.13 g m^{-2} day^{-1}, reported for another algal biofilm system treating municipal wastewater (Boelee et al. 2011). The same study reported a nitrogen removal rate of 1 g N m^{-2} day^{-1}, which is more than twice the N removal rate determined in this study (0.413 g N m^{-2} day^{-1}; chapter 3.7). The rates measured in this study thus fall into similar ranges as other algal biofilm systems for nutrient recovery. However, the practical advantages of the Twin Layer PSBR in terms of reproducible culture stability and lack of outflow of biomass might enhance the applicability of such a system.

4.4 Implications for application

In the following section, treatment of urine with a Twin Layer PSBR system will be considered at a realistic scale and recommendations will be made on how to optimize and apply such a treatment in a decentralized sanitation and resource recovery concept in combination with additional technologies.

4.4.1 Optimization and complementation

It has been concluded that under the given conditions, algae were most likely limited by a critical element other than N or P. The fact that in the treatment situation, a growth rate of 7.238 g m^{-2} day^{-1} (chapter 3.7) was determined while when growing *Desmodesmus abundans* CCAC 3496 on polycarbonate filters, growth rates of up to 10.33 +/- 0.354 g m^{-2} day^{-1} were measured (Fig. 12), is another point speaking for this conclusion. The lowered growth rate was most likely due to, besides differences in the composition of the used urine batches, the larger amount of biomass grown on the same volume of urine and thus a relatively lower availability of growth-limiting components. This effect might be reduced by using a larger volume of urine to be treated by the same amount of biomass. However, such a strategy would only shift the limitation to a later stage, when algae will nonetheless cease to grow without having taken up all nutrients. Furthermore, the fact that nitrogen was not completely taken up is most likely due to the high N:P ratio in urine. An interesting option to explore might be the use of certain cyanobacteria, storing excess nitrogen in the form of cyanophycin, and might thus be able to take up relatively more nitrogen than most eukaryotic algae (Lawry & Simon 1982). For this, a dedicated screening to find cyanobacteria suitable for growth on urine would be necessary. As mentioned before, other authors have argued for the complementation of nutrient deficiency of urine with additional phosphate and trace elements, in order to sustain optimal growth of algae. However, when producing a relatively low-value and potentially contaminated product such as algal biomass grown on excreta, the addition of "clean chemicals" does not seem like a rational choice. If maximal algal production is the target, the missing elements should rather be sourced from other waste streams, which could be blended in at the right concentration to obtain an optimal growth medium. In a decentralized sanitation system with source separation, food waste digestate, animal manure digestate or digested black water could be valuable sources of additional phosphorus and trace elements (Bjornsson et al. 2013). When working with digestates, it is however critical to have an efficiently running anaerobic process, in order to achieve low organic carbon loading in the wastewater. If this is not the case, bacterial growth can be uncontrolled and might compromise on algal biomass production (pers. obs., unpublished data).

In case an amendment with missing nutrients is not feasible, but full removal of nitrogen from urine is desired, the algal treatment should be followed up by a secondary treatment step. This could be, for example, physical adsorption and desorption to zeolites, minerals which bind positively charged ions and can thus be used for ammonium recovery from liquids (Beler-Baykal et al. 2004; Ban & Dave 2004), or volatilization and re-suspension of ammonium via a microbial fuel cell (Kuntke et al. 2012). A low energy requiring option for the removal of ammonium via conversion into atmospheric di-nitrogen gas could be the completely autotrophic nitrogen removal via nitrate process (CANON) or another variation of anaerobic ammonium oxidation (ANNAMOX) (Ahn 2006). The first is a proven technology with relatively large need for handling (rinsing, reactivation etc.) of minerals, while the latter two are partially still experimental technologies, which require very tight process control. The choice of technology to be employed for achieving full nitrogen removal thus strongly depends on the framework situation in which it is to be used. A large portion of pollutants, such as pharmaceuticals, should already be removed during the active carbon treatment, which seems to be needed to ensure the stability of the algal process. Final polishing of the effluent, i.e. removal of trace amounts of nutrients and other pollutants, could be performed in a sand- or reed bed filtration (Ellis 1987; Green & Upton 1994), after which the purified water might be used for irrigation of plants, toilet flushing or could be discharged.

4.4.2 Scaling and operation

Scaling of the model system will be based on achieving maximal P-recovery as determined experimentally in this study (95% of initial concentration). Optimal conditions of light intensity and duration of illumination, pH control and dilution of urine are assumed. Urine is considered fresh and non-hydrolyzed, which might be realized in the form of a small cooled buffer tank (cooled by heat exchangers or located underground). The design is proposed for a 3 person household, with an average daily urine excretion of 1.4 l person^{-1} (Tortora & Derrickson 2006b). Averages from all collections in this study are used for concentrations of nutrients in urine (Tab. 5).

Long term nutrient uptake rates are can be realistically calculated using the biomass growth rate and the determined content of the nutrient of interest. In this way fluctuations in uptake rate, e.g. due to luxury uptake mechanisms are levelled out (Shi et al. 2007; Shi et al. 2014). Therefore, rates calculated in this

way can be assumed to behave linearly over the time for which linear growth has been observed (12 days; Fig. 18). For P-uptake, a rate of 0.15 g m^{-2} day^{-1} can thus be estimated. A treatment system for the daily volume of urine produced by 3 people would then require a growth surface of 6.5 m^2. Leaving room for increased loading of urine at peak times of use (guests), a system of 9 m^2 growth surface, based on a single Twin Layer module of 1.5 m height and 3 m width (inoculated on both sides) is proposed. The system could be located on the flat roof of a building, covered with a greenhouse or similar shelter, minimizing the use of extra space while maximizing available illumination. Treatment would take place in a sequencing-batch mode, recirculating a given volume of urine until the target P concentration is reached.

Harvesting and restart of this system would be necessary only when the growth rate decreases dramatically due to the thickness of the biofilm. No long term data is available for *Desmodesmus abundans* CCAC 3496, however linear growth at 600 µmol photons m^{-2} s^{-1} can be assumed for at least 12 days from the experimental data. In a study with *Halochlorella rubescens* CCAC 0126, decrease in growth rate at low light occurred only after >40 days (Schultze et al. 2015), presumably because of dark respiration in the depth of the biofilm (Li et al. 2015). For an optimization of harvesting cycles (balancing the decrease in growth rate over time with the effort of harvesting), one option could also be to enlarge the algal growth surface. However detailed studies with the specific strain and conditions are needed to substantiate this. The feasibility of different modes of harvesting and regrowth on Twin Layer PSBRs is still an open field of research and development, thus the optimal strategy cannot be presented here. However some degree of automation seems necessary to make the operation of a wastewater treatment module feasible and safe to handle. Harvesting of biomass might be realized by means of a soft scraper or by application of a stream of air. Regrowth might be possible from remaining biomass on the surface or by re-applying part of the harvested biomass by spray-nozzles. Whether or not regrowth of biomass at comparable rates is achievable needs to be determined for this system. Problems that might be associated with the prolonged treatment of wastewater in a system, such as bacterial growth in the tubing and on surfaces and associated material degradation, will have to be evaluated in detail. Especially the durability of the substrate layer to be employed has to be weighed carefully with the price associated with the material.

4.4.3 Economic considerations

Light is clearly the mayor variable when thinking about the application of an algal wastewater treatment system. The previously considered scenario treats light as an unlimited resource, however this is not the case in most parts of the world. Thus, to uncouple nutrient recovery with algae from the availability of sunlight, and to make such a system globally applicable, artificial light would have to be used. Utilizing the modelling approach of Blanken et al. (2013), based on using LED lighting for the cultivation of microalgae and assuming an average energy price of 0.21 € kWh^{-1} for household consumers in the EU (Eurostat 2016), illumination of the immobilized cultivation system would cost approximately 522.50 € per year. The price of energy consumption might be reduced to 347 € per year in the future, if advances in LED efficiency are being made as projected (Pimputkar et al. 2009; Blanken et al. 2013). The cost for illumination might be further reduced substantially if lamps are only used in half of the year (winter) or only during certain hours of the day (morning and evening), when natural light intensities so not suffice for optimal growth. The cost for activated carbon treatment can be estimated at 36.80 € per year, assuming one-time application of 10 g powdered activated carbon l^{-1} urine, as performed in this study, at a bulk price of 2.4 € kg^{-1}, as stated by the distributor (Donau Carbon, Frankfurt, Germany). The application of activated carbon could presumably be optimized with lower quantities and partial reuse, which would further lower the cost of this treatment. For a comparison, the annual cost of wastewater disposal was about 96 € per person in 2010 on a European average (Rehberg 2010), resulting in an annual price of 288 € for the 3 person household considered. Thus at this point, even if the proposed treatment system would replace public wastewater disposal completely, it would economically not be feasible to employ artificial light for microalgal growth in this urine treatment system. It also has to be mentioned that these calculations do not take into account the cost of the lamps nor of the carbon filtration unit or the Twin Layer PSBR itself. However it has to be noted that with increasingly stringent legislation on the discharge limits of nutrients and pharmaceuticals in the EU and other places, higher prices can be assumed in the future (Christodoulou & Stamatelatou 2016). In such a new framework, the economic feasibility of artificial light should be revisited in detail.

For the sake of this discussion it is assumed that applicability of the system is limited to areas with ample sunshine during the period of use. In the temperate regions (e.g. in southern or central Europe) a feasible example might be holiday regions, where the highest demand for wastewater treatment occurs during summer. In such a location, source separation of urine and consequent nutrient recovery with microalgae could decrease the pressure on centralized wastewater operations, which are in some cases not adapted to the high seasonal loads (Goronszy et al. 1995; Larsen & Gujer 1996). Another application in these latitudes could be houses in rural settings, where a connection to a sewage system is not possible and recovery of nutrients is desirable (e.g. for local food production). To maximize on the outreach effect of such a system, modern eco-villages and research centers would be also be suitable locations for operation and show-casing, even if an economic need does not exist.

In the equatorial, subtropical and tropical regions of the world, an algal nutrient recovery system could operate year-round and might be a simple decentralized technology to valorize the nutrients present in human urine. Implementing it would decrease environmental pollution through untreated runoff, while at the same time providing an economic benefit in the form of fertilizer, decreasing the dependency on externally-sourced mineral fertilizers (Damodar Reddy et al. 1999). The system could be applied in any rural place which does not have existing sewage treatment (Kvarnström et al. 2006) as well as in urban developments, which are switching to the use of source separation technologies, as is for example under research in southern China (Medilanski et al. 2007).

4.4.4. Integration into a decentralized concept

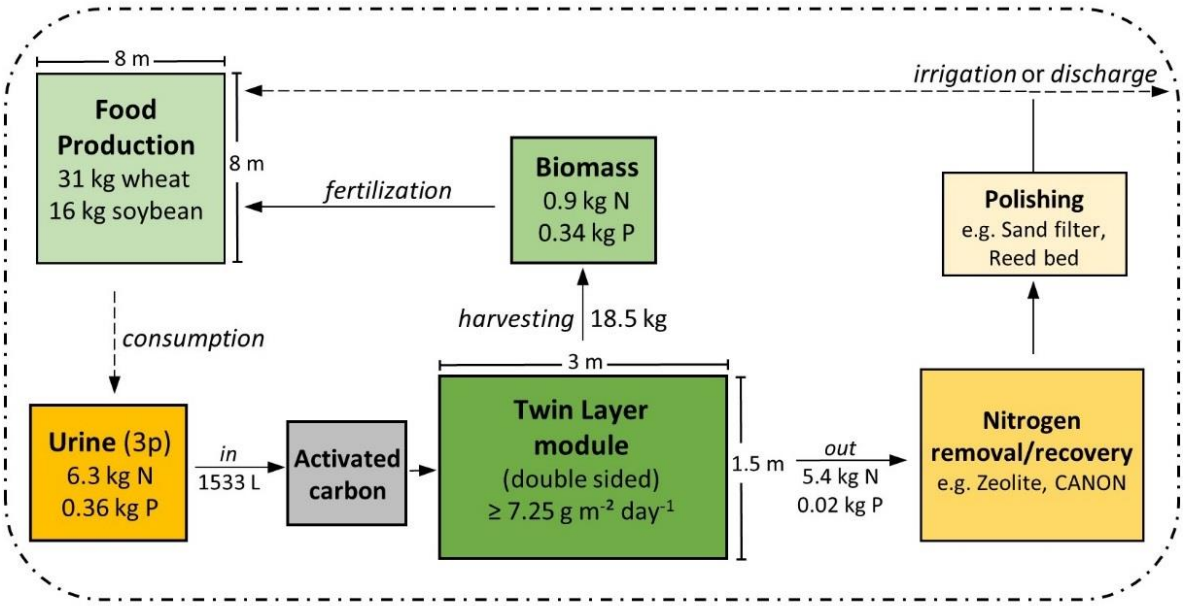

Figure 21: Flow diagram of nutrients and materials in a proposed decentralized treatment and resource recovery system of source separated urine. Values are based on the estimates for a household of three people over one year. See text for further explanation.

Figure 21 shows a schematic representation of a treatment system based on abovementioned criteria and the considerations explained below:

Assuming year-round operation, a Twin Layer PSBR running on the urine of a three person household could produce ~18.5 kg dry algal biomass per year, containing 348 g of phosphorus and 991 g of nitrogen, which could be utilized as fertilizer for growing crops. The yield that can be achieved on these nutrients is a rough estimate, due to the limited experience with algal biomass as fertilizer, large variations in the properties of soils and crops, effects of agricultural methods and other factors such as local climate conditions (Syers et al. 2008). Nevertheless, an estimation will be calculated here, based on available data for a wheat-soybean cropping system in India: In a crop rotation, wheat (*Triticum aestivum*) and soybean (*Glycine max*) are cultivated over an annual cycle on the same plot of land. In semi-arid subtropical climates, soybean is cultivated during the monsoon season as a rain fed crop, while wheat is cultivated in the dry season with additional irrigation (Aulakh et al. 2003; Damodar Reddy et al. 1999). This type of cultivation might replace large parts of the dominant rice-wheat double crop system in south and east Asia, due to changes in dietary demands (Khoury et al. 2014) as well as lower

water consumption and less potential for nitrogen losses (Aulakh et al. 2003). Considering phosphorus as the limiting resource on a nutrient poor Indian soil, an optimal dosage/yield relation has been determined for an application rate of 2.62 g P m^{-1} a^{-1} in this type of cropping system (Aulakh et al. 2003). Assuming a short-term P-availability from algal biomass of 50 % in soil (based on Mulbry et al. 2005), an agricultural plot of approximately 66.5 m² could be optimally supplied with the phosphorus recovered from the urine of 3 people. Using the pooled averages of 8 years from two research sites, it can be estimated that about 31 kg of wheat grain and 16 kg of soybean could be produced per year on this land (Damodar Reddy et al. 1999; Aulakh et al. 2003). Assuming an average intake of 2000 kcal $person^{-1}$ day^{-1} (Tortora & Derrickson 2006a) and using the caloric values of 4490 kcal kg^{-1} and 3370 kcal kg^{-1} for dry soybean and wheat, respectively (USDA 2016), the food produced could supply ~8.1 % of the annual dietary need for energy of a three person household. This means that the energetic demand of almost one month of the year could be covered by the nutrients derived from urine. Furthermore, due to the slow release properties of algal biomass, soils would become enriched with additional N, P as well as other plant essential nutrients in the long term.

The decentralized sanitation and resource recovery system could further be upgraded with the integration of an anaerobic digester, processing black water and/or kitchen waste to produce biogas, a mixture of CH_4 and CO_2 (e.g. Wendland et al. 2007). While the methane can be used as energy source e.g. for cooking or conversion into electricity in a co-generation unit, the CO_2 might be diverted into the algal module, to ensure optimal growth. With a smart engineering of gas-flow and an algal strain tolerant to high CO_2 and CH_4 concentrations, the PSBR module might thus serve as an upgrading step for biogas, removing CO_2 and increasing its combustion efficiency. Furthermore, the effluent of anaerobic digestion is typically rich in inorganic nutrients and trace elements available for algal growth (Vasconcelos Fernandes et al. 2015). Depending on the exact inputs and conditions of digestion, this stream might be used to complement the nutrient balance of urine, as proposed in chapter 4.4.1. To-date, practical knowledge on the application of algal biomass as fertilizer in agriculture is scarce, but a number of other uses could also be of interest. Anaerobic digestion for biogas production might be an option if the biomass is found to be contaminated and thus unsuitable as fertilizer (Santos-Ballardo et al. 2016).

Potential uses of higher value could be, depending on the strain of algae cultivated, the extraction of pigments, fatty acids or the production of chemical building blocks in a biorefinery approach (Milledge 2011; Olguín 2003).

The safety of application of any new system for the recycling of nutrients from urine has to be duly considered, especially with regard to new transmission routes for pathogens as well as potential enrichment of organic pollutants and heavy metals (Winker et al. 2009). In such a small scale setting, the input of pharmaceuticals and pathogens into the treatment system could be minimized or avoided, if proper practices are adhered to by the users. Nevertheless fecal cross-contamination and input by sick users could introduce human pathogens into the algal biomass. There are some indications that drying of biomass and subsequent application to soil might inactivate most pathogens originating from urine (e.g. due to UV-stress and desiccation), however much more research is necessary to substantiate this (Winker et al. 2009; Mulbry et al. 2005). The pre-algal activated carbon treatment proposed in this study provides some means of lowering the concentration of pharmaceuticals and other potentially harmful substances. This is a relatively well established process (Snyder et al. 2007), however regular controls for hazards in biological and chemical forms have to be included in such a system, where closed loops can potentially lead to the enrichment of toxic substances and organisms. Before application can be considered, the absolute safety for human and environmental health and wellbeing has to be ensured by rigorous long term testing at full scale and under realistic conditions.

5 Conclusion and Recommendations

In summary, it can be concluded that a robust treatment for the recovery of phosphorus and partial recovery of nitrogen from un-amended and minimally tap-water-diluted human urine has been realized by using a selected microalgal strain under controlled process conditions at laboratory scale. Such a system might be applied to recover P and partially recover N from source separated urine in a household. Should the stability and safety of the process be validated at pilot-scale, it could be integrated with other technologies in a decentralized sanitation and resource recovery framework, providing substantial amounts of biomass that could serve as a slow-release fertilizer in food production, partially closing local nutrient cycles and decreasing pressure on freshwater reserves.

For future studies with a similar purpose and for further development of the technology, the following recommendations are proposed:

- When using algae for the treatment of wastewater, it is recommended to test a large number of diverse strains before deciding on a strain to use. When attempting to isolate algae for this purpose, researchers should use conditions that are as close as possible to the intended treatment situation, to select specialist strains most suitable for the application at hand.
- When dealing with concentrated human waste, precautions must be taken to mitigate negative effects of unknown substances (e.g. pharmaceuticals). Activated carbon can be a simple and effective means of preventing inhibition of growth and eliminating chemical contaminants.
- The type of water and the applied process conditions (e.g. tap-water and pH control) have to be carefully chosen and tuned to each other in order to allow stable operation and to prevent chemical precipitation. This is the same for all types of mass cultivation systems of algae.
- The usefulness and safety of applying algal biomass grown on urine as plant fertilizer should be studied in field trials with a large variety of crops, using various agricultural practices under various climatic conditions.

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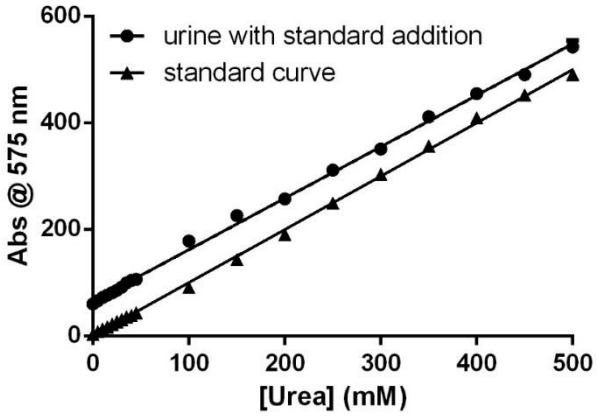
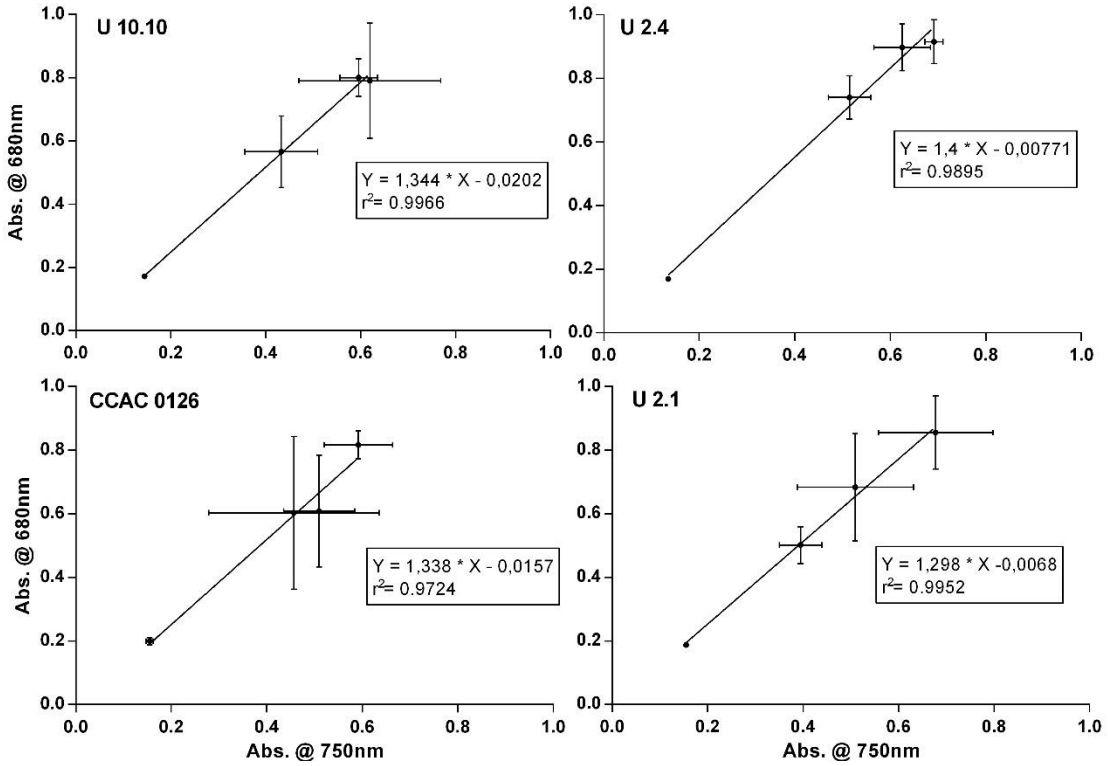
Appendix I - Standard additions to verify the urea measurement method



Appendix II - Comparison of 680 and 750 nm as measures of algal growth



Appendix III - SSU rRNA sequences of selected strains in FASTA format

>BI11

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CTACTCGGATAACCGTAGTAATTCTAGAGCTAATACGTGCGCACAACCCGACTTCTGGAAGGGTCGTATTTATTAGATAAAAG
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TGATCAAGAACGAAAGTTGGGGGCTCGAAGACGATTAGATACCGTCGTAGTCTCAACCATAAACGATGCCGACTAGGGATTG
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>CCAC3496

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>U2_1

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>U2_4

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