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Continuous cultivation of microalgae yields high nutrient recovery from nitrified urine with limited supplementation

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ABSTRACT

Microalgae can play a key role in the bioeconomy, particularly in combination with the valorisation of waste streams as cultivation media. Urine is an example of a widely available nutrient-rich waste stream, and alkaline stabilisation and subsequent full nitrification in a bioreactor yields a stable nitrate-rich solution. In this study, such nitrified urine served as a culture medium for the edible microalga *Limnospira indica*. In batch cultivation, nitrified urine without additional supplements yielded a lower biomass concentration, nutrient uptake and protein content compared to modified Zarrouk medium, as standard medium. To enhance the nitrogen uptake efficiency and biomass production, nitrified urine was supplemented with potentially limiting elements. Limited amounts of phosphorus (36 mg L^{-1}), magnesium (7.9 mg L^{-1}), calcium (12.2 mg L^{-1}), iron (2.0 mg L^{-1}) and EDTA (88.5 mg Na_2-EDTA.$2H_2O$ L^{-1}) rendered the nitrified urine matrix as effective as modified Zarrouk medium in terms of biomass production (OD_{750} of 1.2), nutrient uptake (130 mg N L^{-1}) and protein yield (47%) in batch culture. Urine precipitates formed by alkalinisation could in principle supply enough phosphorus, calcium and magnesium, requiring only external addition of iron, EDTA and inorganic carbon. Subsequently, the suitability of supplemented nitrified urine as a culture medium was confirmed in continuous *Limnospira* cultivation in a CSTR photobioreactor. This qualifies nitrified urine as a valuable and sustainable microalgae growth medium, thereby creating novel nutrient loops on Earth and in Space, i.e., in regenerative life support systems for human deep-space missions.

KEYWORDS: urine source separation; resource recovery; MELiSSA; *Spirulina*; *Arthrospira;* cyanobacteria

1. INTRODUCTION

The transition from a linear economy to a sustainable circular bioeconomy will be key in the future to face the growing demand for food and other bio-based products while reducing our dependence on natural resources (Antranikian and Streit, 2022; Verstraete et al., 2016). Closing and/or shortening the nutrient cycles by recovery from waste streams could reduce the need for energy intensive ammonia production using the conventional Haber-Bosch process and mining of non-renewable phosphorus and potassium (Spiller et al., 2022). Urine is one of the targeted waste streams, as it presents the major nutrient source in domestic wastewater and has good plant fertilization properties (i.e., contains all nutrients and trace elements required for plant growth) (Bonvin et al., 2015; Randall and Naidoo, 2018). Urine recycling is also of key interest for the purpose of nutrient and water recovery in regenerative life support systems (RLSS) for deep-space exploration (Clauwaert et al., 2017).

Besides the use as an alternative fertiliser in agriculture, urinary nutrients can be valorised directly or indirectly for microbial biotechnology, e.g. for microbial protein (MP) applications (Christiaens et al., 2017; Coppens et al., 2016). In recent years, MP, i.e., the protein-rich biomass of cultivated microorganisms including for instance bacteria and microalgae, has gained a revived interest as a more sustainable alternative to plant and animal protein (Pikaar et al., 2017). In contrast to plants and animals, microorganisms can be highly efficient in their nutrient conversions and can be cultured in fully controlled bioreactors at high volumetric biomass production rates, enabling near full nutrient recycling from a recovered stream with a minimal land or space usage (Clauwaert et al., 2017; Fabregas et al., 1986). Depending on the production process and the final quality, in the food production chain, MP may be used as a food ingredient for humans, as a feed ingredient for livestock production, or as an organic fertiliser for plant nutrition (Chang et al., 2013; Pikaar et al., 2018; Spanoghe et al., 2020). Furthermore, microbial biomass has been proposed as a source for biofuel (biodiesel and bioethanol), bioplastics, biochemicals, cosmetics and

pharmaceuticals (Antranikian and Streit, 2022; Fernández et al., 2021; Hamed, 2016; Mapstone et al., 2022). Microalgae are an interesting source of MP and have been cultivated and used as food or feed supplements/ingredients for many years (Muys et al., 2019; Richmond, 2004; Sui and Vlaeminck, 2020). As photoautotrophic organisms, they use light as an energy source and inorganic carbon (CO_2) as carbon source to produce organic compounds via photosynthesis (Hamed, 2016). Microalgae cultivation is particularly relevant in RLSS, such as MELiSSA, the Micro-Ecological Life Support System Alternative under development by the European Space Agency (ESA) (Fahrion et al., 2021; Lasseur et al., 2010). Besides being a proteinaceous food source for the astronauts, microalgae can be used for cabin atmosphere revitalisation as they produce O_2 and consume CO_2 and as a source for biopolymers (Fahrion et al., 2021; Matula and Nabity, 2019; Yang et al., 2019). One of the commercially most widely-produced microalgae is known as “Spirulina” (Spolaore et al., 2006), which refers to cyanobacteria belonging to the genera *Arthrospira* or *Limnospira* as recently proposed by Nowicka-Krawczyk et al. (2019). These filamentous microalgae are rich in protein (46-71% protein on a dry weight basis), vitamins, minerals (e.g. iron), essential amino acids and fatty acids, photosynthetic pigments (chlorophylls and carotenoids) and antioxidants (Hamed, 2016; Muys et al., 2019).

Several studies reported successful batch cultivation of microalgae, including *Arthrospira/Limnospira,* on urine (Supporting Information (SI), Table S1), but reports on continuous reactors producing microalgae on urine are scarce. Most of the studies using fresh or stored urine (with urea or TAN (total ammonia nitrogen, i.e., sum of NH_3-N and NH_4^+-N) as nitrogen source) as culture medium for *Arthrospira/Limnospira* reported that the biomass was yellow-green and had a lower protein content compared to the dark green biomass obtained on standard medium such as (modified) Zarrouk medium containing nitrate as N source (Chang et al., 2013; Feng and Wu, 2006; Feng et al., 2007). Feng et al. (Feng et al., 2007; Feng et al., 2008) and Coppens et al. (2016) pre-treated urine in a

nitrification bioreactor (converting the urea/TAN into nitrate), and obtained a reduced lag phase and higher cell concentration on nitrified urine compared to fresh or stored urine. Although nitrified urine yielded biomass with a similar ash, protein, lipid and chlorophyll content as on (modified) Zarrouk medium, a 33% lower dry weight was obtained in batch culture experiments by Coppens et al. (2016), which could possibly be linked to specific (micro)nutrient limitations. Tuantet et al. (2014b), for instance, identified phosphorus and magnesium as the limiting elements for continuous *Chlorella sorokiniana* cultivation in urine (with urea/TAN as N source and supplemented with Fe-EDTA and trace elements). Supplementing urine with phosphorus and magnesium increased the biomass productivity and nitrogen removal efficiency (Tuantet et al., 2014b).

As a high resource usage efficiency is of utmost importance to circularize agricultural nutrient management on Earth or to reduce the need for resupply in RLSS, the goal of this study was to optimise growth of and nitrogen uptake by *Limnospira indica,* the selected microalga for MELiSSA, on real human urine. Urine was first stabilised by alkalinisation and biological treatment. Alkalinisation (OH^- addition to pH>12) was applied immediately after collection to prevent enzymatic urea hydrolysis , which would result in nutrient losses and malodour during storage (De Paepe et al., 2020). Subsequent treatment in a nitrification bioreactor converted instable urea and/or ammoniacal nitrogen into nitrate (nitrification) and removed most of the biodegradable organics (COD (chemical oxygen demand) mainly oxidized to CO_2), thereby suppressing spontaneous uncontrolled microbial growth in urine. To improve the biomass production and nitrogen uptake efficiency, the nitrified urine was supplemented with, amongst others, phosphate, calcium, magnesium, iron and trace elements. With the aim of identifying the limiting compound(s) in the nitrified urine, different mixtures were evaluated in batch culture experiments by monitoring the optical density (OD), dry weight, nutrient uptake and biomass composition. Finally, to evaluate the optimised

nitrified urine solution for continuous cultivation of *Limnospira*, a photobioreactor was operated for more than 6 hydraulic retention times (HRT).

2. MATERIALS AND METHODS

2.1. Urine pre-treatment through alkalinisation and biostabilisation

Nitrified urine (Table 1) was collected from a bioreactor equipped with electrochemical pH control, described in De Paepe et al. (2021). Briefly, immediately after collection in a nonwater urinal, the urine was diluted with water (33% urine - 67% H_2O) to simulate dilutions that would be obtained with flush water and the pH was increased to 12 by means of an electrochemical cell to prevent urea hydrolysis during storage. Alkalinisation removed on average ~90% of the calcium and magnesium, 78% of phosphate, 58% of sulfate and 64% of chloride by precipitation and/or electromigration (De Paepe et al., 2021). The alkalinised urine was fed into a moving-bed biofilm reactor (MBBR) for nitrification and COD removal. Hydroxide production at the cathode of a dynamically controlled electrochemical cell compensated for the acidification caused by nitrification, enabling full nitrification. The effluent from the reactor was sent through the middle compartment of the electrochemical cell to capture all nitrate that migrated over the anion exchange membrane separating the cathodic and middle compartment. By redirecting the nitrified urine through the middle compartment, the urine was acidified (pH 1.3) due to proton migration from the anode to the middle compartment. KOH was added to neutralise the nitrified urine prior to medium preparation. Phosphate diffusion from the anolyte to the middle compartment increased the phosphate concentration with a factor ~4.5. For more details about the urine pre-treatment, we refer to the original publication (De Paepe et al., 2021).

TABLE 1

2.2. Batch culture experiments

2.2.1 Optimisation of a nitrified urine-based culture medium

Batch culture experiments were performed to optimise the medium composition for the growth of *Limnospira indica*, with the aim to maximise the nitrogen uptake efficiency on nitrified urine. As benchmark medium, modified Zarrouk medium was used, a synthetic alkaline saline culture medium first described by Zarrouk (1966) and modified by Cogne et al. (2003). Since the nitrogen uptake is limited by the biomass concentration, which is in turn limited by the light availability in the culture flask, the maximum nitrate uptake in the given setup was determined in a preliminary batch experiment with modified Zarrouk medium (Cogne et al., 2003). Based on the nitrate uptake in this experiment, the initial nitrate concentration was set at ~165 mg N L^{-1} for the following experiments (except NU 4x).

2.2.2 Batch culture set-up

The batch culture experiments were conducted in 1L Erlenmeyer flasks with a vented cap (0.22 µm vent filter) under axenic conditions. The culture medium was composed of three or four solutions (i.e., i) a urine solution, ii) a phosphate and inorganic carbon solution, iii) a (micro)nutrient solution, iv) a trace elements solution) (Table 2), which were autoclaved separately to avoid precipitation, and aseptically added to the Erlenmeyer flasks (total volume of 490 mL) in a laminar flow cabinet. $NaHCO_3$ (10.5 g L^{-1}) and Na_2CO_3 (7.6 g L^{-1}) were used in all experiments as a carbon source (2.4 g C L^{-1}). Each culture medium was tested in triplicate (except for ZM N_{low} (4 replicates), NU all (5 replicates) and NU all-Ca^{2+} (6 replicates)). The flasks were inoculated with 5-10 mL of *Limnospira indica* PCC8005 culture originating from an axenic 83 L pilot photobioreactor operated on modified Zarrouk medium (OD_{750} of 2-3) or from the previous batch culture experiment (OD_{750} of ~1.2) (resulting initial OD_{750} of ~0.05), and incubated for two weeks at 35°C. The incubator (F-4 refrigerated incubator, Ibercex, Spain) was equipped with fluorescent lamps (average light intensity of 40 µmol m^{-2} s^{-1}) and magnetic stirrers. Sampling was performed every 2-3 days in a laminar

flow cabinet with sterile pipettes. After sampling, the flasks were randomised in the incubator to minimise positional effects on growth. The OD_{750}, pH and electrical conductivity (EC) were measured immediately after sampling. Samples were filtered (Philic PTFE 0.2 µm, Millex-LG, Merck Millipore, Germany) prior to analyses. The axenicity was verified by plating immediately after inoculation and at the end of the experiment. The biomass was harvested at the end of the experiment to determine total suspended solids (TSS) and the biomass composition (after lyophilization).

TABLE 2

2.3. Continuous cultivation in a photobioreactor

Continuous cultivation of *Limnospira indica* on supplemented nitrified urine was evaluated in a bench scale photobioreactor (SI Figure S1, F1 Laboratory bioreactor, Bionet, Spain). The reactor had a total volume of 5 L, inner diameter of 13 cm and was equipped with a pH probe (EasyFerm Plus, Hamilton, Switzerland), dissolved oxyen (DO) probe (VisiFerm, Hamilton, Switzerland), temperature sensor and Rushton impeller. The temperature was controlled at 36°C with a double-wall water jacket and the pH was controlled at 9.5 by means of base (2M NaOH) and acid (2M H_2SO_4) addition. Antifoam (Y-30, Sigma Aldrich, United States) was added manually during sampling. An air flow of 1.01 L min^{-1}, enriched in CO_2 (1%, i.e., 0.01 L min^{-1}) was supplied to the reactor, providing inorganic carbon for the microalgae. The reactor content was gently stirred at 80 rpm. Continuous illumination was provided by a light module consisting of 6 racks, each with 30 LEDs (LUXEON SunPlus 20 Line Cool White, Lumileds, USA), surrounding the bioreactor. The light intensity was increased in a stepwise manner during the batch cultivation from 31 µmol m^{-2} s^{-1} (6.7 W m^{-2}) to 923 µmol m^{-2} s^{-1} (198 W m^{-2}) (SI Table S2). After autoclaving, the reactor was inoculated with 200 mL of *Limnospira* culture with an OD_{750} of ~2, resulting in an initial OD_{750} of ~0.2 (active volume of 2 L). After 6-7 days of operation in batch mode (no influent and effluent

flow), the active volume was decreased to 1.5 L and autoclaved fresh medium was fed at a flow rate of 0.5 L d^{-1} (dilution rate of 0.33 d^{-1}). Effluent left the reactor via overpressure and was collected in a sterile bottle with venting filter. The first reactor run was performed with modified Zarrouk Medium (2.5 g $NaNO_3$ L^{-1}, without $NaHCO_3$ and Na_2CO_3). Subsequently, the reactor was emptied, cleaned and autoclaved prior to the reactor run on nitrified urine (29% corresponding to ~500 mg N L^{-1}) supplemented with K_2HPO_4 (53 mg P L^{-1}), $MgSO_4.7H_2O$ (7.89 mg Mg^{2+} L^{-1}), $CaCl_2.2H_2O$ (12.19 mg Ca^{2+} L^{-1}), $FeSO_4.7H_2O$ (2.01 mg Fe^{2+} L^{-1}), Na_2-EDTA.$2H_2O$ (88.5 mg L^{-1}), $MnCl_2.4H_2O$ (0.064 mg Mn^{2+} L^{-1}), $ZnSO_4.7H_2O$ (0.025 mg Zn^{2+} L^{-1}) and $CuSO_4.5H_2O$ (0.0076 mg Cu^{2+} L^{-1}). Both experiments lasted for 3 weeks (6 HRT). Samples were taken five times a week and analysed for OD_{750}, NO_3^-, TIC, pH and EC. Axenicity was verified once a week by means of plating. TSS/VSS and the phosphate concentration were determined once a week. At the end of the experiment, the biomass was harvested and lyophilised prior to protein and biomass analyses.

2.4. Analytical methods

The OD was measured at 750 nm with a spectrophotometer (DR6000, Hach, Germany). Anion chromatography (Dionex ICS2000, equipped with AS18 anion-exchange column and ASRS-ULTRA II suppressor, Thermo Fisher Scientific, USA) was used to determine the nitrate concentration. The phosphate concentration was determined with phosphate Hach tube test kits (LCK 350, Hach, Germany) and TIC was measured on a TIC/TOC/TN analyser (Multi N/C, model 2100S/1, Analytikjena, Germany). EC was measured by means of a conductivity meter (Cond 8 Basic, XS Instruments, Italy) and pH measurements were performed with a pH meter (pH 50+ DHS, XS Instruments, Italy). Axenicity was checked by plating (in duplicates) on PDA plates (potato dextrose agar, 30°C, 13 days) for fungi, PCA plates (plates count agar, 37°C, 5 days) for aerobic bacteria and plates with modified Zarrouk medium supplemented with Tryptone Soy Agar (35°C, 13 days) for aerobic bacteria able to grow on modified Zarrouk medium. Total suspended solids (TSS) and volatile

suspended solids (VSS) were determined by filtering the culture medium over borosilicate glass microfiber filters (GMFC-52047 (1.2 µm), Scharlab, Spain) and subsequent drying at 105°C (Universal oven UF75, Memmert, Germany) and 550°C for at least 2 hours. The biomass was centrifuged (Avanti^{TM} J-20 centrifuge, Beckman Coulter, USA), washed, frozen at -80°C for at least 24 hours and lyophilised (VirTis Sentry Lyophilizer, Scientific Products, USA) prior to protein and biomass analyses. Proteins were measured using the Lowry method (with BSA standard). The C, H, N and S content of the biomass was determined by combustion of the biomass samples at 1200°C and subsequent quantification by gas chromatography (Flash 2000, quantification limit of 0.1%, Thermo Fisher Scientific, USA). The P content of the biomass and Ca^{2+}, Mg^{2+}, Fe^{2+}, Mn^{2+}, Zn^{2+} and Cu^{2+} content of the medium was determined by inductively coupled plasma mass spectrometry (ICP-MS, Model 7900, Agilent, USA). Prior to operation, photon flux densities were measured on 24 spots distributed homogeneously over the inner wall of the photobioreactor using a Li-Cor 190R Quantum sensor. The reported light intensity was averaged over the reactor surface.

2.5 Statistical analysis

To assess the primary hypothesis that nitrified urine supports *Limnospira* growth equally well as modified Zarrouk control medium, univariate statistical hypothesis testing was performed. Different endpoints of *Limnospira* growth (Max OD_{750}; TSS; total N, P and TIC uptake; N, P and TIC conversion efficiency; biomass protein content; C, H, N, S and P content of the biomass) were compared on the Zarrouk control medium (ZM N_{low}) versus different formulations of nitrified urine (NU 1x, NU 4x and NU all). As a secondary aim, specific growth limiting micronutrients were identified. To this end, the statistical significance of supplementing nitrified urine with P, Ca^{2+}, Mg^{2+}, Fe^{2+} and trace elements was assessed for each endpoint.

ANOVA was used for normally distributed, homoscedastic data with residuals falling on a straight line in QQ-plots and Shapiro-Wilk and Bartlett p-values >0.05. Welch's ANOVA was performed when the assumption of equal variances was violated. Non-parametric Kruskal-Wallis rank sum tests were applied if departures from normality were established.

If a significant (Welch's) ANOVA p-value was obtained, (Welch's) pairwise t tests were carried out. Likewise significant non-parametric effects were further explored with pairwise Wilcoxon Rank Sum Tests. Keeping the small sample size and research hypotheses in mind, groups were only compared to the reference (ZM N_{low} or NU all) to preserve statistical power after correction for multiple testing with Benjamini-Hochberg post hoc tests.

3. RESULTS

3.1 Optimising urine dilution is essential to maximise N uptake efficiencies

Both on Earth and in Space, an efficient use of recovered resources is essential. Before optimising the nitrified urine based culture medium, the optimal urine dilution to maximize the N uptake efficiency was determined, as N is the second most important macronutrient (after C) required for the synthesis of macromolecules including proteins, peptides, enzymes, chlorophyll and nucleic acids (DNA/RNA) (Salama et al., 2017). N uptake levels in *Limnospira* cultivation depend on the biomass concentration. As microalgae require light as an energy source, growth stagnates in dense cultures because of a reduced light availability due to self-shading.

In the batch culture set-up, the maximum N uptake was determined in a preliminary batch experiment with modified Zarrouk medium. The maximum OD_{750} (1.2) was reached after two weeks (SI Figure S2) (final TSS of ~1.35 g L^{-1}). Afterwards, the OD_{750} decreased, and the colour of the culture changed from dark green to yellowish green, indicating decay. The nitrate concentration in the culture medium slowly decreased and stabilised when the maximum OD_{750} was reached (SI Figure S2). This removed 110-120 mg N L^{-1}

(corresponding to 22-27% of the initial N concentration) from the culture medium. Based on these results, it was decided to lower the initial nitrate concentration to ~165 mg N L^{-1} for the following experiments, providing ~50 mg N L^{-1} in excess. This enables higher N uptake efficiencies, while avoiding N limitation, which can result in an increased lipid and a reduced protein content (Feng and Wu, 2006). To obtain this concentration of 165 mg N L^{-1}, the nitrified (3 times diluted) urine (containing ~1800 mg N L^{-1}, Table 1) was diluted more than 10 times, corresponding to a more than 30 times dilution of the fresh urine before stabilization.

In the photobioreactor, a higher N uptake could be obtained because of a higher light availability, enabling a higher biomass concentration (Section 3.5). N supply was therefore increased to 500 mg N L^{-1}, corresponding to 29% diluted nitrified urine. Nonetheless, even with a more optimised short light-path reactor system, biomass concentrations are limited to several grams per liter, corresponding to a N uptake below 1 g N L^{-1} as demonstrated by Tuantet et al. (Tuantet et al., 2014b; Tuantet et al., 2019). Since undiluted urine can contain up to 9 g N L^{-1} (Udert et al., 2006), urine dilution is essential to achieve high N uptake efficiencies. This was also concluded by Tuantet et al. (2019) who found that extremely low dilution rates would be required to accomplish complete N removal by *Chlorella sorokiniana* grown on undiluted urine in a short light-path photobioreactor. In practice, the nitrified urine could be diluted by redirecting a part of the N-depleted effluent back to the reactor after biomass separation (e.g., by filtration or settling), as was also suggested by Tuantet et al. (2019).

3.2. Nitrified urine sustains *Limnospira* biomass production, but growth stagnates earlier than on modified Zarrouk medium

Limnospira batch cultivation on nitrified urine was benchmarked against cultivation on N-reduced modified Zarrouk medium (ZM N_{low}, i.e., modified ZM with a lower sodium nitrate

concentration). Similar as in the preliminary experiment with modified Zarrouk medium (Section 3.1), a maximum OD_{750} of 1.23 ± 0.05 and N uptake of 130 ± 4 mg N L^{-1} was reached after two weeks on ZM N_{low} (Figure 1A, Table 3). Because of the lower initial N concentration, a higher N uptake efficiency could be obtained (~71% instead of ~25%). Due to CO_2 uptake by *Limnospira*, TIC decreased by 25% (but was still >1.5 g L^{-1}) and the pH increased from ~9.8 to ~11.5 (SI Figures S5-S6).

FIGURE 1 (color) / TABLE 3

The increase in OD_{750} and decrease in nitrate concentration in the flasks with nitrified urine (NU 1x) followed the same trend as ZM N_{low} during the first 7-10 days, but then stagnated (Figure 1A). The maximum OD_{750} amounted to 0.95 ± 0.01 and only 64 mg N L^{-1} or 36% (compared to 71% on ZM N_{low}) of the nitrate was removed from the medium. This was also reflected in the lower TSS concentration (1.5 mg TSS L^{-1}) and protein content of the biomass (only 21%) (Table 3). Furthermore, the P uptake on NU 1x was significantly ($p=0.026$) lower compared to ZM N_{low}. The nitrified urine only contained 12 mg P L^{-1}, compared to ~100 mg P L^{-1} in ZM N_{low}, but, in principle, this should be sufficient since the P uptake on ZM N_{low} was ~10 mg P L^{-1}. Yet, only 2 mg P L^{-1} was removed from the NU 1x medium. The biomass contained significantly less nitrogen (4%, $p= 0.000114$) and phosphorus (0.2%, $p= 0.00744$) compared to the biomass grown on ZM N_{low} (9% N and 0.7% P). The growth profile on NU 1x was similar to the one obtained by Coppens et al. (2016) using nitrified urine (20%, containing 1.3 g N L^{-1}) supplemented with trace elements. Also in the latter study with *Arthrospira platensis*, nitrified urine yielded a similar growth rate but a 33% lower biomass concentration compared to modified Zarrouk medium.

These results clearly demonstrate that some compounds limited growth on NU 1x. Next to P, NU 1x also contains less Ca^{2+}, Mg^{2+} and other micronutrients compared to ZM N_{low} (SI Table S3-S4). It was hypothesised that a lack of one or more of these elements is limiting

the growth of *Limnospira* on nitrified urine. To confirm this hypothesis, the dilution of the nitrified urine was lowered to increase the concentrations of P and micronutrients in the culture medium. The N uptake (132 ± 40 mg N L^{-1}), P uptake (7.8 ± 0.7 mg P L^{-1}) and protein content (46%) were higher on four times more concentrated nitrified urine (NU 4x) compared to NU 1x and approaching ZM N_{low} (p-values of 0.905, 0.472 and 0.503 respectively). Also the biomass content was very similar to the biomass grown on ZM N_{low} (Table 3, Table S5). P was probably no longer the limiting nutrient, since the N:P uptake ratio decreased from 32:1 (NU 1x) to 16:1 (NU 4x), which is close to the ratio obtained on ZM N_{low} (14:1). The OD_{750} and TSS, however, were significantly lower (Figure 1A, p-values of 0.0283 and 0.00914 respectively), indicating that there was still a limitation. Moreover, due to the higher N concentration in the growth medium, only 17% of the nitrate was consumed. Further reducing the dilution is not a good strategy to increase the supply of growth limiting compounds, as it would further decrease the N uptake efficiency and could even result in nitrate inhibition. Coppens et al. (2016) reported inhibition of *Arthrospira platensis* (same strain as used in this study) at nitrate concentrations above 1 g N L^{-1}.

3.3. Supplemented nitrified urine yielded the same biomass production and N uptake as modified Zarrouk medium

As an alternative to the use of less diluted urine, nitrified urine was supplemented with chemicals to increase the concentration of P, Ca^{2+}, Mg^{2+} and/or other micronutrients. In a first stage, all compounds in ZM, except for $NaNO_3$, NaCl and K_2SO_4, were added to the nitrified urine in the same concentration as in ZM N_{low}, except for K_2HPO_4 (concentration was reduced to 0.2 g K_2HPO_4 L^{-1} (36 mg P L^{-1}) to maximize the P conversion efficiency since only ~10% of the P was consumed in ZM N_{low}). The increase in OD_{750} and decrease in nitrate concentration in the flasks with the supplemented nitrified urine (NU all) coincided with the curves of ZM N_{low} (Figure 1B, p-values of 0.832 and 0.905 respectively). Also the biomass and protein content (p-values>0.05, Table S5), P uptake (p=0.472) and TIC removal

(p=0.730) were very similar to ZM N_{low} (Table 3). These results demonstrate that *Limnospira* growth on nitrified urine without supplements was limited by a deficiency of one or more of the supplied elements (i.e., P, Ca^{2+}, Mg^{2+}, Fe^{2+}, EDTA and trace elements).

3.4 Identifying the limiting compound(s) for *Limnospira* growth in nitrified urine

Supplementing nitrified urine with P and micronutrients yielded the same biomass production as N-reduced modified Zarrouk medium. In a next phase, one or more elements were removed from the medium in order to determine which supplement(s) is/are essential to ensure optimal growth on nitrified urine.

Precipitation as a consequence of alkalinisation removed a significant fraction of the phosphorus (~78%), calcium (~90%) and magnesium (~90%) in urine (De Paepe et al., 2021). In a first experiment, it was decided to add these compounds to the nitrified urine given their important roles. P is an essential macronutrient in microalgal metabolism, given its presence in nucleotides, energy molecules, lipids and polysaccharides (Nagarajan et al., 2020). The low P uptake on nitrified urine without supplements (NU 1x) already pointed out that the P concentration in the nitrified urine was insufficient to sustain high biomass concentrations and N uptake efficiencies. Mg^{2+} is pivotal for photosynthesis as the central element of the chlorophyll molecule (Encarnação et al., 2012), and is furthermore involved in the aggregation of ribosomes in functional units, in the formation of catalase and enzyme catalysis. It is for instance an important enzyme cofactor catalyzing reactions of RuBisCo (CO_2 fixing enzyme complex) (Encarnação et al., 2012; Kirkby and Mengel, 1976). Ca^{2+} is important to form rigid gels in microalgae cell walls by crosslinking pectin polymers (Hanifzadeh et al., 2018). Two different P concentrations (+8 mg P L^{-1} and +36 mg P L^{-1}) were tested, whereas Ca^{2+} and Mg^{2+} were added in the same concentration as ZM N_{low}. In NU+Ca^{2+}/Mg^{2+}/P_{low}/TE, only 8 mg P L^{-1} (0.0453 g K_2HPO_4 L^{-1}) was supplemented to the medium to maximize the P conversion efficiency and thus minimize the P addition to the

medium. Yet, in the flasks with this low P concentration , the maximum OD_{750} (0.86 ± 0.09, p=0.0710), nitrate uptake (93 ± 24 mg N L^{-1}, p=0,00109) and TIC removal efficiency (17.3 ± 1.5 %, p=0.000169) were lower compared to the nitrified urine with all supplements (NU all) (Figure 1C, Table 3). Higher OD_{750} values and TIC removal efficiencies were reached in the flasks with the same P concentration (+36 mg P L^{-1}) as NU all (NU+Ca^{2+}/Mg^{2+}/P), but the growth was still not as optimal as for NU all (Table 3, Table S5), indicating that a deficiency in at least one of the other elements (EDTA, Fe^{2+} or trace elements) was still limiting *Limnospira* growth.

In a next set of experiments, one element at a time was removed from the culture medium NU all. Removing Ca^{2+} from the culture medium (NU all-Ca^{2+}) resulted in a very poor growth in five out of six replicates. The OD_{750} did not exceed 0.25 and less than 50 mg N L^{-1} was removed from the medium (Figure 1D, Table 3), which is even lower compared to the unsupplemented nitrified urine. This can possibly be attributed to the addition of EDTA in NU all-Ca^{2+}. While EDTA was added with the aim to increase Ca^{2+} bioavailability by avoiding precipitation, an adverse effect on *Limnospira* growth was observed, likely due to the formation of a strong EDTA-Ca^{2+} complex which made the Ca^{2+} in nitrified urine less available (EDTA first binds with Ca^{2+} and then with Mg^{2+} (Muralikrishna and Manickam, 2017)). Surprisingly, one of the six flasks performed well (OD_{750} of 1.06 and N uptake of 110 mg N L^{-1}) (SI Figures S3-S4).

Removing Mg^{2+} from the nitrified urine with all supplements (NU all-Mg^{2+}) yielded a similar growth rate and final OD_{750} (p=0.107, Figure 1D), but a significantly lower N (p=0,000834) and P uptake ($p=2.80*10^{-8}$) and protein content (p=0.00208) compared to NU all. This translated in a low N and P content of the biomass (Table 3). Also the colour of the culture changed to yellowish green instead of dark green (SI Figure S6), which could be related to the fact that Mg^{2+} is a key compound of the chlorophyll pigment.

Likewise, Fe^{2+} amendment proved to be important to obtain a high N uptake and N content in the biomass. The N uptake on NU all-Fe^{2+} was about 15% lower compared to NU all ($p=0.0724$). The increase in OD_{750} ($p=0.786$) and P uptake ($p=0.677$), on the other hand, were similar to NU all (Figure 1D, Table 3). Iron is an important micronutrient given its ability to function as electron donor/acceptor and plays a role in many metabolic functions, including electron transport in the Calvin cycle, respiratory electron transport, synthesis of DNA and chlorophyll, detoxification/degradation of reactive oxygen species and nitrite reduction (Encarnação et al., 2012; Ermis et al., 2020). The latter could possibly explain the lower N uptake on NU all-Fe^{2+} as nitrite reduction is essential for nitrate assimilation in microalgae. In contrast to ammonium which can be directly incorporated into amino acids, nitrate has to be converted to ammonium via nitrite (Tao et al., 2020). The conversion of nitrite into ammonium is catalysed by ferrodoxins, i.e., clusters of iron and sulfur (Tao et al., 2020). Iron deficiency could thus affect the nitrate uptake. Moreover, iron is one of the main contributors determining the nutritional value of *Spirulina/Arthrospira* (Delrue et al., 2017).

The medium without trace elements (i.e., Zn^{2+}, Cu^{2+} and Mn^{2+}) performed equally good as NU all (Figure 1D, Table 3, Table S5). Trace elements are only required in small amounts. Hence, the concentrations present in urine might have been sufficient to ensure optimal growth or the inoculum (<2% of culture medium) may have contained enough trace elements to support microalgae growth. The nitrified urine contained less trace elements compared to ZM N_{low}, except for zinc whose concentration was 4-5 higher compared to ZM N_{low} (SI Table S3-4). A deficiency in trace elements, however, might still appear during long-term continuous culture or after subculturing. The Mn^{2+} and Cu^{2+} uptake was substantially lower in NU all-TE compared to ZM N_{low} and NU all (SI Table S4).

EDTA could not be left out of the medium as absence resulted in precipitation and the formation of a pink coloured nutrient solution during autoclaving (presumably due to iron oxidation). EDTA is used as a chelate/complexing agent in the culture medium, enhancing

the solubility of metal ions (Fe^{2+}, Mg^{2+} and Ca^{2+}), thereby increasing their availability to microalgae (Ren et al., 2014). Too high levels of EDTA, on the other hand, are known to decrease the bioavailability since the metal ions are strongly chelated to EDTA and thus no longer available (Gerringa et al., 2000; Ren et al., 2014).

3.5. Long-term continuous *Limnospira* cultivation in a photobioreactor confirmed the application potential of supplemented nitrified urine as a culture medium

Given the promising results in batch culture, supplemented nitrified urine was evaluated for continuous (steady-state) *Limnospira* cultivation in a photobioreactor. Due to the higher light availability, a higher biomass concentration and N uptake could be achieved in the photobioreactor. Hence, modified Zarrouk medium and more concentrated nitrified urine (29% corresponding to ~500 mg N L^{-1} instead of 9.2%) were used as culture media. This resulted in a higher N uptake, requiring a proportional increase in supplemented P compared to the batch culture experiments (0.3 g L^{-1} K_2HPO_4 instead of 0.2 g L^{-1}). $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, $FeSO_4.7H_2O$, EDTA.$2H_2O$ and trace elements ($MnCl_2.4H_2O$, $ZnSO_4.7H_2O$ and $CuSO_4.5H_2O$) were supplemented in the same concentration as in modified ZM.

After inoculation, the photobioreactor was started in batch mode on modified Zarrouk medium at an initial OD_{750} of 0.2. The light intensity was stepwise increased from 31 to 923 µmol m^{-2} s^{-1} (SI Table S2) to prevent photoinhibition in the early stages of the batch culture. After 6 days, an OD_{750} of 2.7 was reached and ~68% (320 mg N L^{-1}) of the N had been removed from the culture medium. Subsequently, the reactor was operated in continuous mode with an HRT and SRT of 3 days. The OD_{750} and effluent N concentration remained stable at 2.7 ± 0.2 and 178 ± 19 mg N L^{-1}, respectively (Figure 2). The volumetric biomass production amounted to 1 g VSS L^{-1} d^{-1}. On average 331 mg N L^{-1} and 25 mg P L^{-1} were assimilated, corresponding to volumetric uptake rates of 110 mg N L^{-1} d^{-1} and 8.3 mg P L^{-1} d^{-1} and uptake ratios of 0.11 g N g^{-1} VSS and 0.009 g P g^{-1} VSS.

FIGURE 2 (color)

In a second experiment, the reactor was operated on supplemented nitrified urine. After 7 days of batch culture, an OD_{750} of 2.8 was reached and ~62% (314 mg N L^{-1}) of the N had been removed from the culture medium. After shifting to continuous mode, the OD_{750} further increased to 3.9 and then dropped and stabilised at 2.9, whereas the cell dry weight (CDW) remained stable at 3.9 g TSS L^{-1} throughout the experiment. Some changes in cell morphology probably affected the scattering properties of the cell, resulting in fluctuations in OD_{750} and OD_{750}/CDW ratio. The N concentration remained stable at 166 ± 28 mg N L^{-1} (Figure 2). The volumetric biomass production amounted to 1.1 g VSS L^{-1} d^{-1}. On average 331 mg N L^{-1} was assimilated, corresponding to a volumetric uptake rate of 110 mg N L^{-1} d^{-1} and an N uptake ratio of 0.11 g N g^{-1} VSS. Supplemented nitrified urine yielded biomass with a similar CHNSP content as modified ZM (48.4%C, 7.0% H, 10.7%N, 0.6% S and 1.0% P in biomass cultivated on modified ZM and 47.3% C, 6.9%H, 9.9%N, 0.6%S and 1.1% P in biomass cultivated on supplemented nitrified urine).

With an HRT of 3 days, about 30% of the N was not consumed by the microalgae. Light was the limiting growth factor since the microalgae were grown at their optimal conditions (temperature of 36 °C and pH of 9.5) and the effluent was still rich in nutrients (~180 mg N L^{-1} and ~50 mg P L^{-1}) and inorganic carbon (0.8-2 g C L^{-1}). Increasing the HRT would probably have resulted in a higher biomass concentration and nutrient removal. At long HRT, dense microalgae cultures develop, favouring nutrient removal (as nutrients are assimilated in the biomass). However, due to increased self-shading in such dense cultures, dark zones can occur in the reactor, resulting in a decreased biomass productivity (due to biomass loss through cellular maintenance or endogenous respiration) and photosynthetic efficiency (Tuantet et al., 2019). Alternatively, increasing the urine dilution factor could improve the N removal while still maintaining a low HRT and high productivity rate (Tuantet et al., 2019).

4. DISCUSSION

This study aimed to evaluate and optimise nitrified urine as a culture medium for *Limnospira indica*. Urine is a very rich medium, containing all macro- and micronutrients required for microalgae growth, but not necessarily in the right proportions. Pre-treatment (alkalinisation and biological treatment) moreover reduced the concentration of some essential elements. As a result, nitrified urine yielded a lower biomass concentration compared to modified Zarrouk medium.

P was clearly limiting the growth on nitrified urine. The N:P ratio in fresh urine (~12 g N:g P (Udert et al., 2006)) corresponds rather well to the N:P uptake ratio on modified Zarrouk medium (i.e., ~14). However, precipitation caused by alkalinisation or during cultivation reduced the P concentration in urine with 78%, resulting in a poor P uptake and a high N:P uptake ratio (32 compared to 14 on ZM N_{low}). In contrast, P addition resulted in a N:P uptake ratio close to 14 in the batch culture experiments (Table 3), except for NU all-Mg^{2+} (N:P of 43), NU+Ca^{2+}/Mg^{2+}/P (N:P of 7.4) and NU all-Ca^{2+} (N:P of 7.1). A low N:P uptake ratio can be caused by luxury P uptake (i.e., P accumulation as polyphosphate granules) or by P precipitation (Tuantet et al., 2014b). The former is unlikely since luxury P uptake is induced by high phosphate levels and all culture media contained less P compared to ZM N_{low}. The P uptake ratio was moreover in line with the other experiments (5.6 mg P g^{-1} TSS (NU all-Ca^{2+}) and 8.3 mg P g^{-1} TSS (NU+Ca^{2+}/Mg^{2+}/P)). Precipitation might occur during batch cultivation, triggered by the increase in culture pH (up to 11.5) (Chang et al., 2013). In NU 1x and NU 4x, precipitation of P was however limited by the low Ca^{2+} and Mg^{2+} concentrations (most of the Ca^{2+} and Mg^{2+} had been removed by the pH increase to 12 in the alkalinisation step). In the other experiments with supplemented Ca^{2+}, Mg^{2+} and/or Fe^{2+}, EDTA addition limited precipitation of P. Only in NU+Ca^{2+}/Mg^{2+}/P in the absence of EDTA, precipitation might have occurred. The N:P ratio in the obtained biomass (7.1) corresponded however to the N:P uptake ratio (7.4) (Table 3), indicating that precipitation was limited.

Besides the N:P ratio, the N:Mg^{2+} ratio is important. In ZM N_{low} and NU all, about ~3 mg Mg^{2+} g^{-1} TSS was removed from the medium, which is in line with the Mg^{2+} uptake reported in literature (around 2 mg Mg^{2+} g^{-1} *Arthrospira* biomass (dry weight)) (Campanella et al., 1998; Cogne et al., 2003; Johnson, 1986). Considering that about 10% of the dry matter consists of N, a N:Mg^{2+} ratio of 60 is required in the medium. Interestingly, both the N:P and N:Mg^{2+} ratio in fresh urine (before alkalinization) approaches the uptake ratios on modified Zarrouk medium (12 g N:g P and 60 g N:g Mg^{2+}) (Udert et al., 2006). If the precipitates formed during alkalinisation could be captured, they could be used as a source of P, Ca^{2+} and Mg^{2+} to restore the optimal N:P, N:Ca^{2+} and N:Mg^{2+} ratio in urine (Figure 3). Alternatively, struvite could be a cheap and effective source of P and Mg^{2+} for microalgae, as demonstrated by Muys (2019).

FIGURE 3 (color)

Besides addition of P, Ca^{2+} and Mg^{2+}, supplementing nitrified urine with EDTA and Fe^{2+} proved to be key in obtaining a high biomass concentration and maximising the N uptake efficiency. The Fe^{2+} concentration in nitrified urine was much lower than the Fe^{2+} uptake in the experiments with ZM or Fe^{2+} supplementation (SI Table S4). Further research in continuous culture or with multiple subcultures (to deplete trace elements in the original culture medium) is necessary to investigate whether addition of trace elements (i.e., Zn^{2+}, Mn^{2+} and Cu^{2+}) to nitrified urine is required. On the other hand, urine contains a broader range of trace elements than modified Zarrouk medium including molybdenum, cobalt and vitamins which may benefit *Limnospira* growth (Feng et al., 2008).

The above findings are in line with several studies reporting a microalgae growth stimulation by the addition of P, Mg^{2+}, Fe^{2+}, EDTA and/or trace elements to synthetic or real urine. However, unlike the current work, none of these studies systematically evaluated the growth enhancing impact of each of these elements separately. Adamsson (2000) found that

addition of Mg^{2+}, Fe^{2+} and EDTA improved the growth of *Scenedesmus acuminatus* in hydrolysed urine. Tuantet et al. (2014a) showed that enriching urine with Fe^{2+}, Mg^{2+} and trace elements improved the growth of *Chlorella sorokiniana* in batch culture whereas the biomass production and nitrogen removal in continuous culture on synthetic and hydrolysed urine was enhanced by supplementing P and Mg^{2+} (Tuantet et al., 2014b). Coppens et al. (2016) added trace elements to nitrified urine, but obtained a 33% lower biomass yield compared to ZM, indicating that other micronutrients were limiting growth. On the other hand, Chang et al. (2013) obtained >95% of N and P removal on real, fresh urine without additions, but used a high dilution factor (1.25% of urine, N:P ratio of 11), which probably prevented P precipitation, and only evaluated the growth of *Arthrospira platensis* over 10 days.

Finally, the three major compounds in modified Zarrouk medium (i.e., $NaNO_3$, NaCl and K_2SO_4) did not need to be amended to the nitrified urine, since the observed growth on nitrified urine supplemented with P, Ca^{2+}, Mg^{2+}, Fe^{2+}, EDTA and trace elements was equivalent to the growth on modified Zarrouk medium. This qualifies nitrified urine as a valuable and sustainable alternative microalgae growth medium. Since urine can contain micropollutants and pathogens, an additional treatment step (e.g., activated carbon adsorption, UV or ozone treatment) is recommended before microalgae cultivation. With recycling of the urine precipitates formed by alkalinisation, external addition of Fe^{2+} and EDTA is likely sufficient to obtain maximum N uptake. Carbon dioxide (in the atmosphere or in flue gases) can be used as inorganic carbon source, rendering CO_2 sequestration (Cheah et al., 2015). Further research is required to investigate whether addition of trace elements is required and to evaluate the nutritional quality of the biomass.

4. CONCLUSIONS

- Nitrified human urine can be used as a culture medium for *Limnospira indica*. The biomass production and N uptake were promoted by addition of P and micronutrients.
- Nitrified urine supplemented with phosphorus, magnesium, calcium, iron, EDTA and trace elements was as effective as modified Zarrouk medium. Urine precipitates harvested in the alkalinisation step could potentially supply enough P and magnesium, as the N:P and N:Mg^{2+} ratio in fresh urine corresponds to the typical uptake ratios.
- Continuous *Limnospira* cultivation on supplemented nitrified urine was, for the first time, evaluated and yielded similar results as modified Zarrouk medium.

SUPPLEMENTARY INFORMATION

Supplementary data of this work can be found at x.

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COMPETING INTEREST

The authors have no competing interests to declare.

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FIGURE/TABLE CAPTIONS

Table 1. **Composition of the nitrified urine used in this study (before addition of KOH and supplements).** The urine was diluted (33% urine - 67% demineralised water) prior to alkalinisation and biostabilisation.

Table 2. **Overview of culture media in the batch culture experiments based on modified Zarrouk medium (ZM) and pre-alkalinised nitrified urine (NU).** ZM: modified Zarrouk medium from Cogne et al. (2003), ZM N_{low}: N-reduced modified Zarrouk Medium with a lower $NaNO_3$ concentration, NU 1x: nitrified urine with the same N concentration as ZM N_{low}, NU 4x: nitrified urine, four times more concentrated than NU 1x, NU all: nitrified urine (1x) with all supplements, NU + Ca^{2+}/Mg^{2+}/TE/P_{low}: nitrified urine (1x) supplemented with Ca^{2+}, Mg^{2+}, P (low concentration) and trace elements, NU + Ca^{2+}/Mg^{2+}/P: nitrified urine (1x) supplemented with Ca^{2+}, Mg^{2+} and P, NU all – x: nitrified urine (1x) with all supplements except x. (TE: trace elements.) Each culture medium was tested in three or more replicates.

Table 3. **Biomass production, N, P and TIC consumption and biomass content**. The linear growth rate equals the slope of the linear range of the OD curve. The maximum linear growth rate is determined by the light availability in the given setup. AV: average, SD: standard deviation. The labels of the different conditions are explained in the legend of Table 2. p-values can be found in SI, Table S5.

Figure 1. **Batch culture experiments: evolution of optical density (OD_{750}, measured at 750 nm) and nitrate concentration. A: ZM N_{low}, NU 1x, NU 4x, B: ZM N_{low}, NU 1x, NU all, C: NU all, NU + Ca^{2+}/Mg^{2+}/P_{low}/TE, NU + Ca^{2+}/Mg^{2+}/P, D: NU all, NU all – Ca^{2+}, NU all – Mg^{2+}, NU all – Fe^{2+}, NU all – TE.** The vertical error bars represent the standard deviation. Horizontal error bars indicate slight variations in sampling time across replicates. The nitrate concentration in NU 4x is displayed on the right y-axis.

Figure 2. **Continuous culture of *Limnospira indica* on modified Zarrouk medium (left) and supplemented nitrified urine (right): evolution of optical density (OD_{750}) and nitrate concentration.** The black dashed line indicates the shift from batch to continuous culture (HRT of 3 days). The blue dashed lines represent the nitrate concentration in the influent.

Figure 3. **Optimised system design to maximise the nutrient potential of urine and minimise the external input.**

TABLES AND FIGURES

Table 1

Nitrified urine (33%)		
NO_3^--N	[mg N L^{-1}]	1788
NH_4^+-N	[mg N L^{-1}]	10
TN	[mg N L^{-1}]	1840
COD	[mg L^{-1}]	188
PO_4^{3-}	[mg P L^{-1}]	93
K^+	[mg L^{-1}]	1210
Na^+	[mg L^{-1}]	1066
Cl^-	[mg L^{-1}]	767
SO_4^{2-}	[mg L^{-1}]	271
Ca^{2+}	[mg L^{-1}]	13
Mg^{2+}	[mg L^{-1}]	2.2
EC	[mS cm^{-1}]	42

Table 2

			ZM	ZM N_{low}	NU 1x	NU 4x	NU all	NU + Ca^{2+}/ Mg^{2+}/ P_{low}/TE	NU + Ca^{2+}/ Mg^{2+}/P	NU all – Ca^{2+}	NU all – Mg^{2+}	NU all – Fe^{2+}	NU all – TE
Dilution and neutralisation													
Urine %		[%]	0	0	3.1	12.3	3.1	3.1	3.1	3.1	3.1	3.1	3.1
H_2O		[mL L^{-1}]	1000	1000	908	632	908	908	908	908	908	908	908
Nitrified urine		[mL L^{-1}]			92	368	92	92	92	92	92	92	92
KOH		[g L^{-1}]			1	3.7	1	1	1	1	1	1	1
Phosphate and inorganic carbon													
P	(K_2HPO_4)	[mg P L^{-1}]	89	89			36	8	36	36	36	36	36
HCO_3^-	($NaHCO_3$)	[g C L^{-1}]	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
CO_3^{2-}	(Na_2CO_3)	[g C L^{-1}]	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
Other nutrients													
N	($NaNO_3$)	[g N L^{-1}]	0.412	0.165									
K^+	(K_2SO_4)	[g K^+ L^{-1}]	0.224	0.224									
Na^+	(NaCl)	[g Na^+ L^{-1}]	0.393	0.393									
Mg^{2+}	($MgSO_4.7H_2O$)	[mg Mg^{2+} L^{-1}]	7.89	7.89			7.89	7.89	7.89	7.89		7.89	7.89
Ca^{2+}	($CaCl_2.2H_2O$)	[mg Ca^{2+} L^{-1}]	12.19	12.19			12.19	12.19	12.19		12.19	12.19	12.19
Fe^{2+}	($FeSO_4.7H_2O$)	[mg Fe^{2+} L^{-1}]	2.01	2.01			2.01			2.01	2.01		2.01
Na_2-EDTA.$2H_2O$		[mg L^{-1}]	88.5	88.5			88.5	88.5		88.5	88.5	88.5	88.5
Trace elements													
Mn^{2+}	($MnCl_2.4H_2O$)	[µg Mn^{2+} L^{-1}]	64	64			64	64		64	64	64	
Zn^{2+}	($ZnSO_4.7H_2O$)	[µg Zn^{2+} L^{-1}]	25	25			25	25		25	25	25	
Cu^{2+}	($CuSO_4.5H_2O$)	[µg Cu^{2+} L^{-1}]	7.6	7.6			7.6	7.6		7.6	7.6	7.6	

Table 3

		ZM N_{low}	NU 1x	NU 4x	NU all	NU + Ca^{2+}/Mg^{2+}/ P_{low}/ TE	NU + Ca^{2+}/ Mg^{2+}/P	NU all – Ca^{2+}	NU all – Mg^{2+}	NU all – Fe^{2+}	Nu all – TE
Biomass production											
TSS	[g L^{-1}]	1.73 ± 0.13	1.52 ± 0.16	1.36 ± 0.18	1.49 ± 0.12	1.50 ± 0.21	1.45 ± 0.15	0.74 ± 0.39	1.34 ± 0.03	1.48 ± 0.12	1.52 ± 0.19
Max OD750		1.23 ± 0.05	0.95 ± 0.01	0.94 ± 0.27	1.21 ± 0.09	0.86 ± 0.09	0.95 ± 0.03	0.43 ± 0.55	1.18 ± 0.10	1.21 ± 0.09	1.21 ± 0.16
Linear growth rate	[d^{-1}]	0.01 ± 0.01	0.09 ± 0.00	0.06 ± 0.02	0.09 ± 0.01	0.09 ± 0.00	0.08 ± 0.01	0.02 ± 0.05	0.10 ± 0.00	0.09 ± 0.01	0.10 ± 0.02
N consumption [mg NO_3-N L^{-1}]											
Initial N concentration		183 ± 1	180 ± 2	792 ± 7	176 ± 13	188 ± 4	161 ± 2	204 ± 16	216 ± 3	208 ± 13	192 ± 1
Total N uptake		130 ± 4	64 ± 2	132 ± 40	131 ± 1	93 ± 24	89 ± 5	30 ± 43	91 ± 14	113 ± 15	146 ± 2
N conversion efficiency	[%]	71.0 ± 1.9	35.6 ± 1.6	16.7 ± 5.1	74.4 ± 5.8	49.5 ± 11.5	55.0 ± 3.4	33.2 ± 24.6	42.0 ± 7.0	54.5 ± 10.7	76.1 ± 1.1
Biomass protein content [a]	[%]	51.9 ± 9.4	21.3 ± 3.9	46.5 ± 6.8	48.0 ± 8.9	30.0 ± 4.7	41.1 ± 9.3	57.9 ± 3.8	24.8 ± 3.0	43.5 ± 3.7	
P consumption [mg PO_4-P L^{-1}]											
Initial P concentration		97.2 ± 1.5	11.7 ± 0.1	39.9 ± 0.1	49.4 ± 0.2	18.4 ± 0.9	49.6 ± 0.0	51.2 ± 1.6	51.9 ± 0.6	52.1 ± 0.5	32.6 ± 0.7
Total P uptake		9.2 ± 2.3	2.0 ± 0.1	7.8 ± 0.7	10.2 ± 1.0	7.3 ± 0.8	12.0 ± 0.4	4.2 ± 4.9	2.1 ± 0.6	9.9 ± 0.8	10.7 ± 0.9
P conversion efficiency	[%]	9.4 ± 2.3	17.1 ± 1.0	19.6 ± 1.8	20.8 ± 2.0	39.5 ± 2.5	24.2 ± 0.8	16.7 ± 5.8	4.1 ± 1.2	18.9 ± 1.5	33.0 ± 2.4
N:P uptake ratio	[g/g]	14.2	32.0	16.9	12.7	12.8	7.4	7.3	42.6	11.4	13.6
TIC consumption [g C L^{-1}]											
Initial TIC concentration		2.27 ± 0.05	2.20 ± 0.04	2.26 ± 0.07	2.27 ± 0.09	2.26 ± 0.01	2.34 ± 0.02	2.20 ± 0.07	2.14 ± 0.03	2.19 ± 0.05	2.16 ± 0.02
Total TIC uptake		0.56 ± 0.05	0.35 ± 0.06	0.54 ± 0.05	0.55 ± 0.06	0.39 ± 0.03	0.52 ± 0.03	0.36 ± 0.12	0.39 ± 0.04	0.39 ± 0.05	0.48 ± 0.03

TIC conversion efficiency	[%]	25 ± 2		16 ± 2		24 ± 2		25 ± 2		17 ± 2		22 ± 1		16 ± 6		18 ± 2		18 ± 2		22 ± 2	
Biomass composition																							
Carbon	[%]	43.06	± 1.11	39.35	± 0.93	43.29	± 2.22	44.62	± 2.66	32.13 ± 2.48		40.65 ± 2.19		44.38	± 1.67	45.26	± 0.76	42.23	± 1.66	43.28	± 1.18
Hydrogen	[%]	6.62 ± 0.22		6.10 ± 0.09		6.20	± 0.32	6.39	± 0.36	5.03 ± 0.62		5.98 ± 0.28		6.44	± 0.11	6.67	± 0.06	6.16	± 0.29	6.35	± 0.08
Sulphur	[%]	0.55 ± 0.07		0.23 ± 0.02		0.54	± 0.08	0.57	± 0.01	0.27 ± 0.07		0.32 ± 0.06		0.52	± 0.02	0.36	± 0.04	0.38	± 0.05	0.51	± 0.03
Nitrogen	[%]	8.92 ± 0.93		4.18 ± 0.03		8.92	± 0.88	9.7 ± 0.63		5.57 ± 0.93		6.42 ± 0.74		9.40	± 0.51	6.43	± 0.60	6.90	± 0.96	8.70	± 0.34
Phosphorus	[%]	0.66 ± 0.03		0.18 ± 0.01		0.53	± 0.10	0.56	± 0.25	0.63 ± 0.04		0.90 ± 0.14		1.40	± 0.53	0.22	± 0.03	0.89	± 0.05	0.70	± 0.02
N/P ratio biomass		13.5		23.2		16.8		17.3		8.8		7.1		6.7		29.2		7.8		12.4	

[a] Protein concentration divided by biomass concentration (5 mg lyophilised biomass dissolved in 10 mL of MilliQ water)

Figure 1

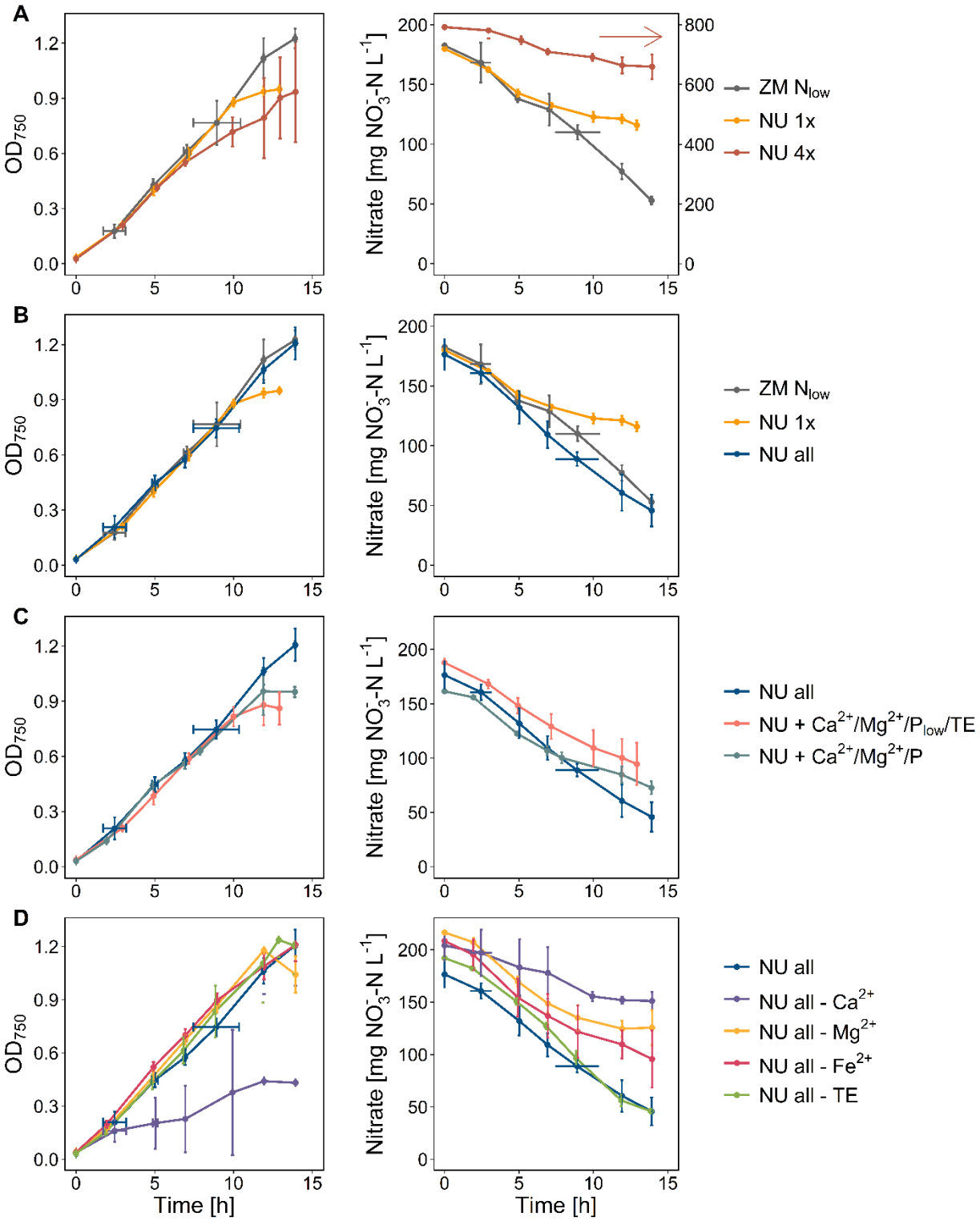

A
B
C
D
OD750
Nitrate [mg NO3-N L-1]
Time [h]
ZM Nlow
NU 1x
NU 4x
NU all
NU + Ca2+/Mg2+/Plow/TE
NU + Ca2+/Mg2+/P
NU all - Ca2+
NU all - Mg2+
NU all - Fe2+
NU all - TE

Figure 2

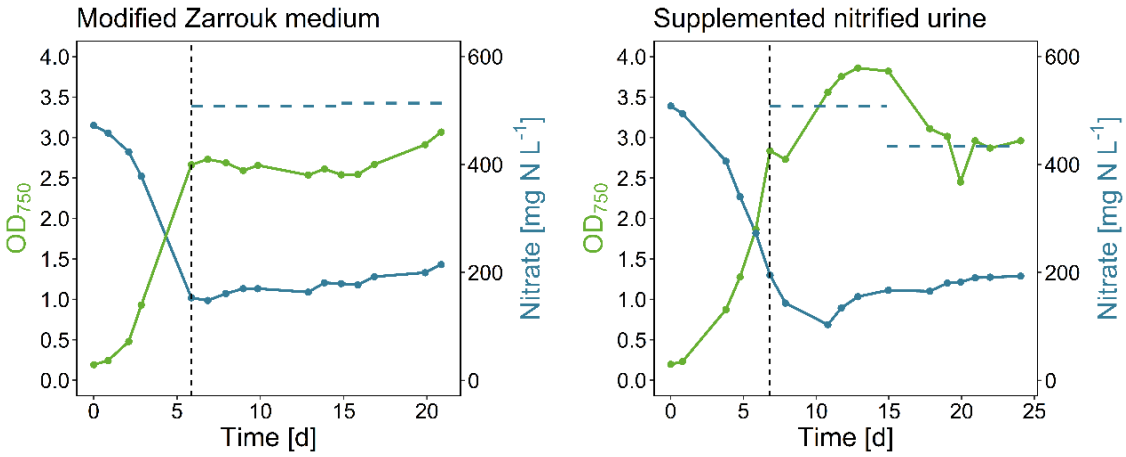
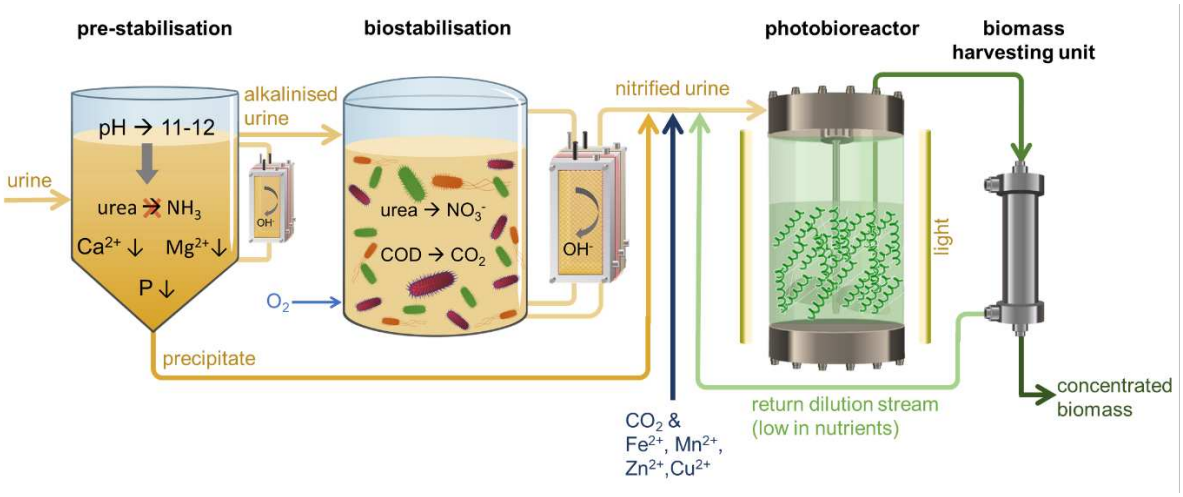

Modified Zarrouk medium
Supplemented nitrified urine
OD750
Nitrate [mg N L-1]
Time [d]
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
0
200
400
600
0
5
10
15
20
25

Figure 3


pre-stabilisation
biostabilisation
photobioreactor
biomass harvesting unit
urine
pH → 11-12
urea → NH3
Ca2+ ↓ Mg2+ ↓
P ↓
alkalinised urine
OH-
O2
urea → NO3-
COD → CO2
OH-
nitrified urine
light
precipitate
CO2 & Fe2+, Mn2+, Zn2+, Cu2+
return dilution stream (low in nutrients)
concentrated biomass