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1 **Nitrification and microalgae cultivation for two-stage biological nutrient valorization from**
2 **source separated urine**

3

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18 **Abstract**

19 Urine contains the majority of nutrients in urban wastewaters and is an ideal nutrient recovery
20 target. In this study, stabilization of real undiluted urine through nitrification and subsequent
21 microalgae cultivation were explored as strategy for biological nutrient recovery. A nitrifying
22 inoculum screening revealed a commercial aquaculture inoculum to have the highest
23 halotolerance. This inoculum was compared with municipal activated sludge for the start-up of
24 two nitrification membrane bioreactors. Complete nitrification of undiluted urine was achieved in
25 both systems at a conductivity of 75 mS cm^{-1} and loading rate above 450 mg N L^{-1} d^{-1}. The
26 halotolerant inoculum shortened the start-up time with 54%. Nitrite oxidizers showed faster salt
27 adaptation and *Nitrobacter* spp. became the dominant nitrite oxidizers. Nitrified urine as growth
28 medium for *Arthrospira platensis* demonstrated superior growth compared to untreated urine and
29 resulted in a high protein content of 62%. This two-stage strategy is therefore a promising
30 approach for biological nutrient recovery.

31

32 **Keywords:**

33 Spirulina, single cell protein, nitrogen recovery, salt adaptation, source separation

34 **1 Introduction**

35 In the light of a growing global population, rising resource scarcity and environmental awareness,
36 the transition towards a sustainable food production system has become increasingly important
37 (Sutton et al., 2013). The implementation of advanced nutrient recycling technologies is thereby
38 essential to reduce nutrient losses and improve nutrient use efficiencies. At present, advanced
39 wastewater treatment facilities focus on resource recovery mainly through the energetic
40 valorization of organic waste compounds as biogas to improve energy autonomy and
41 sustainability. The implementation of recovery technologies for nitrogen (N) and phosphorus (P)
42 can nonetheless generate profits similar to carbon recovery by decreasing the dependency of
43 inorganic fertilizers and creating additional revenues for waste processing facilities (Verstraete &
44 Vlaeminck, 2011).

45 Nutrient recovery can be energetically feasible for concentrated waste streams (Maurer et al.,
46 2003). In this context, source separated urine is an ideal target stream as it contains the majority
47 of nutrients present in urban wastewaters. Urine contributes for 70% of the nitrogen (N), 40% of
48 the phosphorus (P), and 60% of the potassium (K) load in domestic wastewater, while it only
49 accounts for 1% of the volume (Zeeman et al., 2008). Source separated urine therefore allows for
50 the efficient recovery of nutrients, while it offers additional benefits to existing centralized
51 wastewater treatment plants due to the decrease of nutrient loads (Wilsenach & Van Loosdrecht,
52 2004).

53 However, source separated urine is highly unstable, as microbial activity during transportation
54 and storage hydrolyzes the urea present to ammonia, inducing a rise in pH. In this manner
55 nitrogen can volatilize as free ammonia, causing nitrogen losses and environmental and health
56 concerns. The high pH also induces the uncontrolled precipitation of salts such as struvite and

57 calcium phosphate, which result in the loss of phosphorus and can obstruct pipelines and other
58 equipment (Udert, 2002). Furthermore, the presence of high free ammonia (FA) concentrations in
59 untreated urine limits the possibilities of biological nutrient recovery through microalgae
60 cultivation. Although previous studies demonstrated the potential of human urine as a microalgal
61 growth medium, this required urine dilutions from 20 to more than 120 times to prevent ammonia
62 toxicity and obtain satisfactory growth (Adamsson, 2000; Feng et al., 2007; Tuantet et al., 2014;
63 Yang et al., 2008). The stabilization of urine prior to storage is therefore a prerequisite to ensure
64 appropriate processing.

65 Biological urine stabilization through nitrification shows to be a promising approach, as it allows
66 to produce a chemically stable solution in which all nutrients are preserved (Feng et al., 2008b;
67 Udert et al., 2015; Udert & Wachter, 2012). The nitrified urine can subsequently be valorized as a
68 fertilizer solution or further processed for inorganic fertilizer production and water recovery
69 (Udert et al., 2015; Udert & Wachter, 2012). Alternatively, nitrified diluted urine has been
70 applied as a feedstock for the production of high value bioproducts through the cultivation of the
71 cyanobacterium *Arthrospira platensis* (Feng et al., 2007; Feng et al., 2008b). While these studies
72 demonstrated better growth on nitrified urine than on untreated urine, the urine solution still
73 required a 10-fold dilution to ensure stable nitrification. The high salinity limits the activity of
74 unadapted ammonia oxidizing bacteria (AOB), archaea (AOA) and nitrite oxidizing bacteria
75 (NOB), which results in the accumulation of ammonium and/or nitrite (Bassin et al., 2012). This
76 further inhibits the nitrification process, as both ammonia oxidation and nitrite oxidation are
77 susceptible to free ammonia and nitrous acid (FNA) inhibition. As a result, the continuous
78 operation of urine nitrification reactors is highly sensitive to instabilities (Feng et al., 2007; Udert

79 et al., 2015). A suitable nitrifying inoculum and adaptation strategy are therefore indispensable to
80 achieve adequate reactor performance.

81 In this study, the stabilization of source separated urine through nitrification and subsequent
82 cultivation of microalgae are explored as a strategy for biological nutrient valorization. In a first
83 stage, the effect of salinity on ammonium and nitrite oxidizing organisms was assessed through a
84 screening of nitrifying inocula, originating from a spectrum of wastewaters with different
85 nitrogen and salinity levels. Afterwards, the best performing inoculum from the screening was
86 compared with activated sludge of a sewage treatment plant for the start-up of a urine nitrification
87 membrane bioreactor (MBR). The nitrifying communities were closely monitored through batch
88 activity tests and molecular analyses, in order to elucidate the salt adaptation process and shifts in
89 the microbial population. Additionally, the valorization of the nitrified solution as a growth
90 medium for the cyanobacteruim *A. platensis* was evaluated. This species was chosen as a first
91 exploration step for microalgae cultivation on high-concentration nitrified urine, given its high
92 commercial interest (Spolaore et al., 2006) and comparison potential with earlier tests with this
93 organism on a urinary matrix (Chang et al., 2013; Feng et al., 2007; Feng et al., 2008a; Filali et
94 al., 1997; Yang et al., 2008). The influence of salinity, nitrogen source and nitrogen concentration
95 on the growth of *A. platensis* were first evaluated, after which the influence of the urine matrix
96 was further examined. Finally the growth and biomass composition of *A. platensis* grown on
97 nitrified urine was compared to standard growth medium.

98 **2 Materials and methods**

99 **2.1 Screening of nitrifying inocula**

100 Twelve nitrifying inocula were selected for the screening of maximum nitrification rates in order
101 to select an optimal inoculum for the urine nitrification reactor. Nitrifying inocula were collected
102 from a wide spectrum of wastewater treatment installations, characterized by different salinities
103 and nitrogen loading rates (Table A1). The nitrifying inocula were stored for maximum seven
104 days in the dark at 4°C until experiments were started. Electrical conductivity (EC), as an
105 indication for ionic strength and osmotic pressure, was measured in the supernatant of the
106 collected samples. Reactivation of the nitrifying inocula was performed prior to the nitrification
107 activity tests. The nitrifying inocula were reactivated in 2L Erlenmeyer flasks in a medium with
108 a final concentration of 50 mg NH_4^+-N L^{-1}, 50 mg NO_2^--N L^{-1}, 8.3 g KH_2PO_4 L^{-1}, 14.5 g
109 K_2HPO_4 L^{-1} and 2.2 g $NaHCO_3$ L^{-1}. The pH was corrected to 7 with HCl (1M) or NaOH (1M).
110 Erlenmeyers were incubated in the dark at 20 °C and continuously shaken at 120 rpm using an
111 orbital shaker (New Brunswick, The Netherlands) until the substrates were depleted. Dissolved
112 oxygen (DO), pH and ammonium and nitrite concentrations were monitored daily. Afterwards,
113 the biomass was washed two times with a phosphate buffer solution on the original EC of the
114 nitrifying inocula. Afterwards the biomass was washed with a buffer solution at the desired EC
115 for the activity test. The biomass was separated through centrifugation for 10 minutes at 3000
116 rpm (Beckman Coulter, USA). Buffer solutions contained 4.15 g KH_2PO_4 L^{-1}, 7.25 g K_2HPO_4 L^{-1}
117 and 1.1 g $NaHCO_3$ L^{-1}. The pH was corrected to 7 by addition of HCl (1M) or NaOH (1M). The
118 buffer solution was adjusted to the desired EC with sodium chloride (NaCl, Fig. A1). Batch
119 ammonia oxidation (nitritation) and nitrite oxidation (nitratation) activity tests were conducted
120 separately and in quadruplicate. The activity tests were initiated by adding 5 mL of substrate
121 stock solution of NH_4Cl or $NaNO_2$ to obtain a final concentration of 50 mg NH_4^+-N L^{-1} and 50

122 mg NO_2^--L^{-1}, respectively. Erlenmeyer flasks were incubated in the dark at 20°C and shaken
123 continuously at 120 rpm using an orbital shaker (New Brunswick, The Netherlands). Similar to
124 the nitrification activity test, the urease activity of the biomass was determined in a batch activity
125 test through the addition of 50 mg urea-N L^{-1} whilst inhibiting ammonia oxidation with 1000 mg
126 allylthiourea (ATU) L^{-1}. Samples were filtered (0.45 μm), stored at 4°C and analysed within two
127 days. Dissolved oxygen (DO) and pH levels were monitored during sampling.

128 **2.2 Nitrification reactor set-up and operation**

129 Two submerged membrane bioreactors (MBR) with a working volume of 8L were used for the
130 continuous nitrification experiments (Fig. 1). The flat sheet polyvinylidene fluoride (PVDF)
131 membranes (Kubota, Japan) had a pore size of 0.4 μm and a surface of 0.08 m². Reactor aeration
132 and mixing was achieved using an air pump (KNF, Germany). Dissolved oxygen (DO) levels
133 were maintained above 6 mg L^{-1}. Process operation was identical for the two reactors. A pH
134 controller (Prominent GmbH, Germany) controlled the pH between 6.9 and 7.1 through the
135 addition of 0.1M NaOH and HCl. Each reactor was inoculated with a different nitrifying
136 inoculum, at a biomass concentration that corresponds to an ammonia oxidation rate of 125 mg N
137 L^{-1} d^{-1}. The reactors were initially fed with synthetic hydrolysed urine which contained 5.21 g
138 $Na_2SO_4.10H_2O$ L^{-1}, 2.42 g $NaH_2PO_4.H_2O$ L^{-1}, 3.60 g NaCl L^{-1}, 4.20 g KCl L^{-1}, 9.60 g
139 $C_2H_3O_2NH_4$ L^{-1}, 9.22 g NH_4Cl L^{-1}, 6.89 g NaOH L^{-1} and 21.40 g NH_4CO_3 L^{-1} and was
140 supplemented with trace elements (Kuai & Verstraete, 1998). At the start, a solution of 10%
141 synthetic hydrolysed urine was obtained through dilution with demineralized water. The nitrogen
142 loading rate of the reactors was increased by decreasing the influent dilution. Once the reactors
143 were operated with undiluted urine, the loading rate was corrected by adjusting the influent flow
144 rate. Ammonium and nitrite concentrations were measured twice per day to ensure no

145 accumulation occurred. The reactors were run in semi-continuous feeding mode. No biomass was
146 wasted from the reactors, except for analysis of total suspended solids (TSS) and volatile suspend
147 solids (VSS). After the start-up period with synthetic urine, one reactor was fed with non-
148 hydrolysed real urine. Real urine was collected from healthy male volunteers which were not
149 taking antibiotics or other medication. After collection, the fresh urine from different individuals
150 was pooled and frozen in batches sized to feed the reactor for two days.

151 **2.3 Cultivation of *Arthrospira platensis***

152 **2.3.1 Influence of salinity, nitrogen source concentration on the growth of *A. platensis***

153 An axenic culture of *A. platensis* was provided by SCK-CEN (Belgium). Batch cultivation tests
154 were performed to assess the influence of salinity, the nitrogen source and concentration on the
155 growth of *A. platensis*. The influence of the nitrogen source was determined through the use of
156 ammonium, nitrate or urea as the respective sole nitrogen sources in modified Zarrouk medium
157 (Zarrouk, 1966). For nitrate, concentrations of 410, 1000, 2000 and 5300 mg N L^{-1} were tested,
158 while for ammonium and urea, 100, 410, 1000 and 2000 mg N L^{-1} were evaluated. For every
159 nitrogen species and concentration the growth of *A. platensis* was analyzed at 20, 30, 45 and 60
160 mS cm^{-1}, as amended through NaCl addition.

161 Growth tests were conducted under axenic conditions in 96 well plates with a working volume of
162 300 µL. Tests were performed in quintuplicate. Plates were sealed with a gas permeable
163 membrane (Thermo Scientific, USA) to prevent water evaporation, after which they were
164 incubated at 28°C and continuously shaken at 700 rpm using an orbital microplate shaker
165 (Thermo Scientific, USA). Continuous illumination was provided at the surface of the 96 well
166 plates by means of Grolux T5 24W fluorescent growth lamps (Osram Sylvania, USA) at a light
167 intensity of 200 µmol photons m^{-2} s^{-1}. Biomass growth was followed up by measuring the optical

168 density (OD) at 680 nm (Tecan Infinity 200 PRO NanoQuant; Tecan, Switzerland). Each well

169 was inoculated with 10 µL of the *A. platensis* culture in order to obtain an initial OD of 0.1.

170 2.3.2 Influence of the urine matrix on the growth of *A. platensis*

171 Non-hydrolyzed, hydrolyzed and nitrified real urine were used to investigate the influence of the

172 urine matrix on the growth of *A. platensis*. For each medium both synthetic and real urine were

173 tested, supplemented with trace elements (Kuai & Verstraete, 1998). Synthetic non-hydrolyzed

174 urine was prepared according to Brooks and Keevil (1997). For each urine type, growth of *A.*

175 *platensis* was determined on undiluted urine and urine diluted to a final concentration of 3 g N L^{-}

176 ^{1}, 2 g N L^{-1}, 1 g N L^{-1}, 0.5 g N L^{-1} and 0.1 g N L^{-1}. Additionally, the synthetic and real hydrolyzed

177 urine were stripped from ammonia by adjusting the pH to 12 through the addition of 10 M NaOH

178 and aerating the urine solution until all ammonia was removed. Afterwards, a dilution series of

179 100%, 50%, 33%, 20% and 10% was prepared. The pH was adjusted to 8.2 with 5M HCl and the

180 solutions were spiked to a final ammonium and phosphate concentration of 100 mg N L^{-1} and mg

181 50 P L^{-1}, respectively. All solutions were filter sterilized (0.22 µm) prior to the growth

182 experiments. Growth tests were conducted under axenic conditions in 96 well plates as described

183 above.

184 2.3.3 Cultivation of *A. platensis* on nitrified urine

185 Growth experiments were performed in 0.8 L batch tests. *A. platensis* in exponential growth was

186 inoculated at an OD_{680} of 0.1 in 20% nitrified urine and standard Zarrouk medium. Cultures were

187 aerated with 0.22 µm filter sterilized 0.7 L_{air} L_{medium}^{-1} and continuously shaken at 120 rpm using

188 an orbital shaker (New Brunswick, USA). The cultivation temperature was 28°C and the pH was

189 maintained between 8.2–8.5 throughout the experiment using 1M HCl. Continuous illumination

190 was provided from the top at a light intensity of 160 µmol photons m^{-2} s^{-1} near the surface of the

191 medium. Tests were performed during 10 days and nitrate, nitrite, ammonium, and phosphate

192 concentrations in the medium were measured daily. Growth was monitored daily by measuring

193 TSS and OD_{680}, and yielded following linear relationships: $OD_{680} = 0.01 + 2.92 \times TSS$ in Zarrouk

194 medium ($R^2 = 0.998$), and $OD_{680} = -0.02 + 2.19 \times TSS$ in nitrified urine ($R^2 = 0.993$). All batch

195 tests were performed axenically and in quadruplicate. Bacterial contamination of the cultures was

196 checked throughout the experiments using phase contrast microscopy (Axioskop 2, Carl Zeiss

197 AG, Germany).

198 **2.4 Analytical techniques**

199 DO and pH levels were measured with an HQ40d DO meter (Hach Lange, Germany) and a

200 Dulcotest pH-electrode PHEP 112 SE (Prominent GmbH, Germany), respectively. Nitrate, nitrite

201 and phosphate were analyzed using anion chromatography (Metrohm 930 Compact IC,

202 Switzerland). EC was measured with a C833 Multi-channel analyzer (Consort, Belgium),

203 equipped with a Pt1000probe (Metrohm, Switzerland). Ammonium (Berthelot reaction) was

204 determined according to Bucur et al. (2006). TSS, VSS, total Kjeldahl nitrogen (TKN), and total

205 phosphorus (TP; molybdene–vanadate method) were determined according to standard methods

206 (Greenberg et al., 1992). Chemical Oxygen Demand (COD) was determined with Nanocolor

207 COD kits (Machery-Nagel, USA). The levels of chlorophyll and carotenoids of *A. platensis*

208 biomass were determined according to Lichtenthaler (1987).

209 **2.5 Molecular analysis**

210 Real-Time PCR was applied to analyze the nitrifying communities in the MBRs. Total DNA was

211 extracted according to Vilchez-Vargas et al. (2013). A SYBR Green assay (Power SyBr Green,

212 Life Technologies, USA) was used to quantify the 16S rRNA of *Nitrospira* spp. and *Nitrobacter*

213 spp. and the functional amoA gene for AOB and AOA (Table A2). Plasmid DNAs carrying

AOB, AOA functional amoA gene and *Nitrobacter* and *Nitrospira* 16SrRNA gene, respectively, were used as standards for qPCR.

2.6 Statistical analysis

For the nitrifying inoculum screening, the statistical significance ($p<0.05$) between activities at original and normal salinity was tested using a Shapiro-Wilk test. A one-way ANOVA test was performed and combined with an all pairwise multiple comparison Bonferroni test ($p<0.05$) to evaluate the growth of *A. platensis* under the different growth conditions (Prism 5.0, Graphpad Software, USA). A non-parametric Wilcoxon signed-rank test was used if the hypothesis of normality was rejected. A non-parametric non-paired Mann-Whitney test was performed to compare the composition of *A. platensis* biomass grown on nitrified urine and standard Zarrouk medium.

225 **3 Results and discussion**

226 **3.1 Screening of nitrifying inocula**

227 A halotolerant nitrifying inoculum is indispensable in order to facilitate a rapid start-up of urine
228 nitrifying bioreactors (Cui et al., 2014; Feng et al., 2008b). A screening was therefore performed
229 of a spectrum of nitrifying inocula, characterized by different nitrogen loading rates and
230 salinities, to assess their potential for urine nitrification. The resilience of nitrification to salt
231 stress was analyzed for inocula from wastewater treatment units treating salt and fresh water
232 aquaculture effluents, landfill leachate, pig manure and domestic and industrial wastewater
233 (**Error! Reference source not found.**).

234 Nitrification activities at the inoculum's original salinity were in line with the specific nitrogen
235 loading rates of the corresponding reactors, although the standardized test conditions (pH 7, 20°C
236 and synthetic medium) diverged from the original reactor conditions (Fig. 2, Table A1). The
237 commercial nitrifying inoculum showed the highest specific ammonia oxidation rate (143 mg N
238 g^{-1} VSS d^{-1}; Fig. 2a) and nitrite oxidation rate (555 mg N g^{-1} VSS d^{-1}; Fig. 2b). The relatively
239 higher nitrite oxidation activity is explained by the presence of both ammonium and nitrite in the
240 breeding reactor's influent (Avecom, personal communication). The municipal activated sludge
241 of the AB process for domestic wastewater treatment had an ammonia oxidation rate of 59 mg N
242 g^{-1} VSS d^{-1} and a nitrite oxidation rate of 86 mg N g^{-1} VSS d^{-1}. The high specific oxidation rates
243 of the commercial inoculum are explained by the synthetic autotrophic influent, which allows for
244 a high specific enrichment of nitrifiers in the microbial community (Courtens et al., 2014).
245 Domestic wastewater, landfill leachate and manure treatment units are characterized by both high
246 nitrogen and organic loading rates. This stimulates the growth of heterotrophs and hence

247 decreases the share of the nitrifying populations in the total microbial community (Wagner &
248 Loy, 2002; Whang et al., 2009; Ye et al., 2011). As a result, the specific nitrification rates of the
249 different inocula remain difficult to compare. Also, the low nitrification activity obtained from
250 the aquaculture and aquarium biofilters are attributed to the low nitrogen concentrations and
251 nitrogen loading rates which characterize these systems (Bagchi et al., 2014).

252 A direct correlation between the relative nitrification inhibition and osmotic shock is observed
253 when comparing the nitrification activity of the different nitrifying inocula at their original EC
254 and at an EC of 45 mS cm^{-1} (20 g NaCl L^{-1}; Fig. 2). The inocula originating from fresh water
255 sources were severely inhibited at an EC of 45 mS cm^{-1} as they had to cope with a thirteen-fold
256 increase in EC and associated change in osmotic pressure. The activated sludge of the municipal
257 wastewater treatment plant was subjected to a salt shock of 40 mS cm^{-1}, which resulted in a
258 decrease of the ammonia and nitrite oxidation by 98% and 97%, respectively (Fig. 2a-b). On the
259 other hand, for the commercial nitrifying inoculum a salt shock of 24 mS cm^{-1} resulted in
260 decreases of ammonia oxidation and nitrite oxidation with 33% and 23%, respectively (Fig. 2a-
261 b). This high salt-stress resilience of the inoculum compared to previous findings, indicates that
262 the inoculum is halotolerant (Hunik et al., 1993; Hunik et al., 1992; Moussa et al., 2006).

263 Furthermore, for the majority of inocula tested a higher inhibition of ammonia oxidation activity
264 was obtained compared to nitrite oxidation. This indicates a higher sensitivity of the ammonia
265 oxidizing organisms towards short-term salt stress. This confirms previous findings in which the
266 ammonia oxidation activity of non-adapted nitrifying sludge was more affected to salt stress than
267 nitrite oxidation (Bassin et al., 2012; Hunik et al., 1993; Hunik et al., 1992; Moussa et al., 2006;
268 Sudarno et al., 2011).

269 **3.2 Urine nitrification reactor**

270 **3.2.1 Nitrification reactor performance**

271 The adaptation of the nitrifying community towards the high salinity and nitrogen loading rates
272 which characterize an undiluted urine nitrification system was further assessed in a continuous
273 reactor configuration. One reactor was inoculated with activated sludge from a sewage treatment
274 plant, which showed to have low salt-stress resilience. The performance of this system was
275 compared with an identical reactor inoculated with the salt-stress resilient commercial nitrifying
276 inoculum. Synthetic hydrolyzed urine was used to investigate the treatment of stored source
277 separated urine.

278 The MBR inoculated with activated sludge from the sewage treatment plant was started at an
279 initial EC of 5 mS cm^{-1} and a volumetric loading rate of 70 mg N L^{-1} d^{-1}. A loading rate of 300
280 mg N L^{-1} d^{-1} was obtained after 6 days of operation by increasing the influent concentration from
281 10% to 40% urine (Fig. 3b). The long HRT and increasing urine concentration resulted in a
282 gradual rise in salinity of on average 0.56 mS cm^{-1} d^{-1} throughout the period of reactor operation.
283 When the EC in the reactor reached 15 mS cm^{-1} on day 7, severe foaming and ammonium and
284 nitrite accumulation indicated inhibition of the nitrifying biomass due to salt stress. This salt
285 inhibition was confirmed in parallel batch activity tests (Fig. A2). Decreased nitrogen loading
286 rates were maintained in the reactor in order to prevent ammonium and nitrite accumulation until
287 the target volumetric loading rate of 450 mg N L^{-1} d^{-1} was reached on day 43 and the reactor was
288 fed with undiluted urine. This volumetric loading rate was attained until day 58, when the EC in
289 the reactor gradually increased to 63 mS cm^{-1}. Ammonium accumulation (between day 58 and
290 84) indicated inhibition of ammonia oxidizers, while no nitrite accumulation occurred in the
291 reactor. Unstable reactor operation was attained until the system returned to the target loading

292 rate of 450 mg N L^{-1} d^{-1} on day 94. The reactor was finally operated at a volumetric loading rate
293 of 466±24 mg N L^{-1} d^{-1}, which corresponds to a specific loading rate of 72±4 mg N g^{-1} VSS d^{-1},
294 for a period of twice the hydraulic retention time (HRT = 11 days) to demonstrate stable reactor
295 performance. Complete nitrification of the undiluted urine at an EC of 74±0.5 mS cm^{-1} was
296 thereby obtained with a nitrification efficiency of above 95%.

297 The reactor inoculated with the commercial nitrifying inoculum was started at its original EC of
298 20 mS cm^{-1}. The volumetric loading rate was increased from 60 mg N L^{-1} d^{-1} at day 1 to 450 mg
299 N L^{-1} d^{-1} at day 12 by gradually increasing the influent concentration from 10% to 100% synthetic
300 hydrolyzed urine. This resulted in a salinity increase of 0.76 mS cm^{-1} d^{-1}. A stable volumetric and
301 specific loading rate of 478±34 mg N L^{-1} d^{-1} and 60±5 mg N g^{-1} VSS d^{-1} was achieved from day
302 43 on and was maintained for a period of twice the HRT with a nitrification efficiency of above
303 95%. Furthermore, a COD removal efficiency of 96% was achieved. Following a technical
304 malfunctioning of the pH controller, the loading rate of the reactor was afterwards lowered and
305 stabilized at 250 mg N L^{-1} d^{-1} to maintain stable reactor performance. The utilization of the salt-
306 adapted commercial nitrifying inoculum thus shortened the start-up time of the urine nitrification
307 system with about a factor 2, compared to the municipal activated sludge. The reactor also
308 performs well compared to previous salt adaptation studies. Bassin et al. (2012) obtained a
309 salinity increase of 20 g NaCl L^{-1} (44 mS cm^{-1}) at a lower conductivity increase rate (0.40 mS cm^{-}
310 ^{1} d^{-1}) and nitrogen loading rate (48 mg N g^{-1} VSS d^{-1}). Moussa et al. (2006) obtained a higher
311 final salinity of 66 g NaCl L^{-1} (144 mS cm^{-1}) and conductivity increase rate (1.03 mS cm^{-1} d^{-1}),
312 but obtained over 95% nitrification inhibition at a loading rate of 60 mg N g^{-1} VSS d^{-1}.

313 In order to assess the influence of real urine on the reactor performance, the reactor with the
314 fastest start-up, i.e. the one inoculated with the commercial nitrifying inoculum, was afterwards

315 fed with fresh, undiluted real urine. Prior to switching to the real non-hydrolyzed influent, the
316 urease activity of the biomass in the reactor was determined in a batch activity test. This revealed
317 a ureolytic activity of 202±23 mg urea-N g^{-1} VSS d^{-1}, which was 7 times higher than the target
318 specific nitrogen loading rate of the reactor and indicates urea hydrolysis would not limit the
319 nitrification process. While the synthetic hydrolyzed urine had an ammonia concentration of 8 g
320 N L^{-1} and a COD:N ratio of 1, the real urine had an organic nitrogen concentration of 6.3 g N L^{-1}
321 and COD:N ratio of 1.25. The reactor was operated in a stable manner at a volumetric and
322 specific loading rate of 225±33 mg N L^{-1} d^{-1} and 39±4 mg N g^{-1} VSS d^{-1} for a period of 51 days
323 (2.4 times the HRT), with a nitrification efficiency above 95% and a 94% COD removal
324 efficiency. The use of non-hydrolyzed real urine therefore had no effect on the reactor
325 performance.

326 The performance of the urine nitrification reactors in this study demonstrate a rapid start-up and
327 stable reactor operation at high salinities and nitrogen loading rates. While previous urine
328 nitrification studies sometimes achieved similar loading rates, those studies were performed using
329 diluted urine solutions (10-30%; Chen (2009); Feng et al. (2008b); Sun et al. (2012)) or with
330 stored urine with low N concentrations (< 2.4 g N L^{-1}; Udert et al. (2015); Udert and Wachter
331 (2012)). This indicates that those systems were operated at lower salinities compared to our
332 study. Our study therefore demonstrates that complete nitrification of undiluted source-separated
333 urine can be achieved.

334 **3.2.2 Salt tolerance shifts in nitrification activity**

335 The adaptation of the nitrifying sludge towards the increasing salinity in the reactors was
336 monitored with parallel batch activity tests. For each time point, the conductivity with the highest
337 activity was indicated as the optimal conductivity and the activity at other conductivities was

338 depicted relative to this. The municipal activated sludge was adapted to an EC of 5 mS cm^{-1} in
339 the sewage treatment plant and showed low salt-stress resilience (Fig. 2). The increase in salinity
340 in the urine nitrification MBR initially caused a decrease of the specific ammonia and nitrite
341 oxidation activity of the biomass by 41% and 58% (day 21), respectively, compared to the
342 inoculum. Afterwards, the nitrifying community gradually adapted to the elevated salinity. At the
343 end of the reactor operation (day 124), the optimal conductivity for aerobic ammonia oxidation
344 shifted to 15 mS cm^{-1}, while maximal nitrite oxidation occurred at 30 mS cm^{-1}. At the reactor
345 conductivity (75 mS cm^{-1}) ammonia oxidation performed at 41% of the maximal activity, while
346 nitrite oxidation exhibited a relative activity of 58%. This indicates that only partial adaptation to
347 the elevated salinity took place. The stable reactor performance at 75 mS cm^{-1} is therefore
348 attributed to the increase in specific activity of the biomass through an enrichment of the nitrifiers
349 in the microbial community (Fig. 3d, right panel).

350 The commercial nitrifying inoculum initially had an optimal EC 23 mS cm^{-1}. After 145 days the
351 optimum for ammonia and nitrite oxidation had shifted to 30 mS cm^{-1}. At an EC of 75 mS cm^{-1},
352 the batch tests demonstrated a relative activity of 57% and 76% for ammonia and nitrite
353 oxidation, respectively. Furthermore, at 5 mS cm^{-1}, ammonia and nitrite oxidation preserved a
354 relative activity of 87% and 86%, respectively. The good performance over the broad range of
355 salinities shows that the nitrifying community is more robust towards salinity change, which is an
356 indication of halotolerance.

357 The monitoring of the nitrification activity in both reactors demonstrated a clear shift of the
358 optimal salinity for nitrification. Salt inhibition nevertheless still occurred, as the nitrifying
359 communities only partially adapted to the elevated salinity. Furthermore, the nitrite oxidizing
360 community in both reactors showed to have adapted better to the salinity increase than the

361 ammonia oxidizing community. This confirms the outcome from the preliminary inoculum
362 screening and is in agreement with previous findings (Bassin et al., 2012; Moussa et al., 2006;
363 Sudarno et al., 2011).

364 When comparing the nitrification performance of both reactor systems, the commercial nitrifying
365 inoculum shortened the start-up time by half and its nitrifying community adapted better to the
366 high salinity and showed superior performance over the broad range of salinities. This confirms
367 the importance of an appropriate nitrifying inoculum to enhance the reactor performance when
368 treating saline wastewaters. The elevated costs attributed to the utilization of the commercial
369 inoculum could be compensated by the economic benefits offered by a shorter start-up time and
370 stable reactor operation during salt shocks (Kuhn et al., 2010).

371 **3.2.3 Evolution of the nitrifying community members**

372 Next to the physiological salt adaptation process of the nitrifying community, also the dynamics
373 of its main contributor groups were monitored. The municipal activated sludge inoculum
374 contained 10^9 copies g^{-1} VSS of AOB, while AOA copies were below the detection limit (Fig.
375 3d). For NOB, *Nitrospira* spp. (10^{10} copies g^{-1} VSS) dominated over *Nitrobacter* spp. (10^9 copies
376 g^{-1} VSS) in the inoculum. At the end of the reactor operation, a clear enrichment of AOB in the
377 total community was obtained (10^{11} copies g^{-1} VSS) compared to the inoculum. Interestingly, a
378 shift in the NOB community was observed, as the community was now dominated by *Nitrobacter*
379 spp. (10^{10} copies g^{-1} VSS) and the abundance of *Nitrospira* spp. (10^9 copies g^{-1} VSS) decreased.
380 This could be explained by the somewhat elevated nitrite (max. 10 mg N L^{-1}) and oxygen
381 concentrations, which allowed this r-strategist NOB to outcompete *Nitrospira* spp. (Schramm et
382 al., 2000).

383 For the commercial nitrifying inoculum, the high specific ammonia and nitrite oxidation rates
384 were supported by a high abundance of nitrifiers in the microbial community. The ammonia
385 oxidizing community was dominated by AOB (10^{11} copies g^{-1} VSS). However, in contrast to the
386 municipal activated sludge, also a high abundance of AOA (10^{9} copies g^{-1} VSS) was present. The
387 nitrite oxidizing community was dominated by *Nitrobacter* spp. (10^{12} copies g^{-1} VSS) but also a
388 high abundance of *Nitrospira* spp. (10^{11} copies g^{-1} VSS) was observed. At the end of the
389 operation phase with synthetic urine, the ammonia oxidizing community remained unchanged, as
390 AOB and AOA were present in the same order of magnitude as in the inoculum. When the
391 reactor was operated on real non-hydrolyzed urine, the abundance of AOA (10^{7} copies g^{-1} VSS)
392 in the ammonia oxidizing community decreased with two log units. For NOB, the ratio of
393 *Nitrobacter* spp. (10^{11} copies g^{-1} VSS) to *Nitrospira* spp. (10^{9} copies g^{-1} VSS) was unaffected,
394 making *Nitrobacter* spp. the dominant NOB in both nitrification systems.

395 While the nitrification activity tests showed a shift of the optimal salinity, the microbial
396 adaptation strategy which enables this remains complex. Several end-member species of the
397 *Nitrosomonas*, *Nitrospira*, and *Nitrobacter*-genera are classified as halotolerant or moderately
398 halophilic (Koops & Pommerening-Röser, 2001). More in-depth knowledge up to species and
399 gene expression level are therefore required to acquire further insights in whether population
400 shifts and/or salt acclimation is the driving factor towards salt adaptation.

401 **3.3 Valorization of nitrified urine through *A. platensis* cultivation**

402 Source separated urine contains key components for plant cultivation such as the macronutrients
403 N, P and K and micronutrients Fe, B, Cu and Zn. Through nitrification, a chemically stable
404 solution was obtained with a residual COD concentration of 340 mg L^{-1} and a nitrate and
405 phosphate concentration of 5300 mg N L^{-1} and 340 mg P L^{-1}, respectively. The high salinity and

406 nutrient concentrations which characterize the undiluted urine can however constrain the growth.
407 In order to accurately assess the potential of nitrified urine as a microalgal growth medium, the
408 influence of salinity, nitrogen source and nitrogen concentration on the growth of *A. platensis*
409 were first evaluated, after which the influence of the urine matrix was further examined.

410 **3.2.1 Influence of salinity, nitrogen source and concentration on growth of *A. platensis***

411 *A. platensis* showed good tolerance towards high salinities in the synthetic growing medium (Fig.
412 A3). Up to a conductivity of 45 mS cm^{-1} (21 g NaCl L^{-1}) there was no significant effect on
413 microalgal growth compared to the standard growth medium (20 mS cm^{-1}; 10 g NaCl L^{-1}). At 60
414 mS cm^{-1} (27 g NaCl L^{-1}), salt stress resulted into a prolonged lag phase but still comparable
415 growth rates were obtained. *A. platensis* was also able to grow on nitrate, ammonium and urea as
416 the sole nitrogen source. Nitrate is the conventional nitrogen source for *A. platensis* cultivation
417 and resulted in the best growth under the tested conditions. Compared to ammonium and urea, no
418 lag phase and higher cell concentrations were obtained, thereby confirming previous findings
419 (Costa et al., 2001). When evaluating the effect of the nitrate concentration on microalgal growth,
420 there was no significant effect on growth up to 1000 mg N L^{-1}. Microalgal growth was severely
421 inhibited at higher concentrations, thereby confirming previous findings which showed inhibition
422 of *A. platensis* at nitrate concentrations higher than 1400 mg N L^{-1} (Filali et al., 1997). *A.*
423 *platensis* was able to withstand high ammonia concentrations. There was no significant difference
424 in growth between 100 and 410 mg N L^{-1} and growth was moderately inhibited at 1000 mg N L^{-1}
425 (Fig. A8-11). Growth on urea was characterized by a prolonged lag phase and by lower maximal
426 cell densities. No significant effect of the nitrogen concentration was thereby observed (Fig. A12-
427 15). These preliminary growth tests indicate that the high salinity inherent to source separated
428 urine would not inhibit the growth of *A. platensis*. Furthermore, the superior growth observed

429 with nitrate as the nitrogen source suggests that pretreatment of urine through nitrification is a
430 suitable process to promote microalgal growth.

431 **3.2.2 Influence of the urine matrix on microalgal growth**

432 In order to further evaluate the optimal use of urine as a growth medium, the influence of the
433 urine matrix on *A. platensis* growth was examined. When *A. platensis* was inoculated in real non-
434 hydrolyzed urine only limited growth occurred at the highest dilution (2%; Fig. 4). This is in
435 agreement with Chang et al. (2013), which observed optimal growth of *A. platensis* at 120-fold
436 diluted urine. In the case of real hydrolyzed urine, no growth was observed, despite the addition
437 of trace elements (Fig. 4). To exclude ammonia toxicity, the hydrolyzed urine was stripped and
438 subsequently spiked with ammonium. Only at 50% and 33% dilution significant growth of *A.*
439 *platensis* occurred in the stripped real urine. This is possible explained by the dark color of the
440 undiluted stored urine that limits light penetration. Compared to the untreated urine solutions,
441 nitrified real urine yielded the best growth and highest cell densities (Fig. 4). The best result was
442 achieved for a 20% nitrified urine solution (1 g N L^{-1}), as no significant difference in growth was
443 observed between the nitrified urine and the standard Zarrouk medium, although a lower final
444 cell density was obtained. The optimal growth on nitrified urine compared to untreated urine
445 demonstrates the additional growth benefits obtained through the nitrification pretreatment. The
446 high nitrate concentrations of undiluted nitrified urine completely inhibited *A. platensis* growth,
447 which confirms previous results (Fig. 4). However, nitrate inhibition would be prevented when *A.*
448 *platensis* is cultivated in a (semi-) continuous reactor operation, therefore potentially allowing
449 biomass production on undiluted nitrified urine.

450 ### 3.3.3 Nutrient uptake and biomass composition of *A. platensis* cultivated on nitrified urine

451 The cultivation of *A. platensis* on nitrified urine as a pathway for nutrient recovery was further
452 examined by analyzing the nutrient uptake and biomass composition in an additional batch
453 cultivation experiment. When comparing the cultivation of *A. platensis* on 20% nitrified urine
454 and standard Zarrouk medium, similar growth rates can be observed despite a 33% higher
455 biomass productivity for the standard medium (Fig. 5). The biomass composition of *A. platensis*
456 was compared for the two treatments in the stationary phase at the end of the experiment. No
457 significant difference in the nitrogen, phosphorus, protein and carotenoid content was observed
458 (Table 1). Considering the specific total nitrogen-to-protein conversion factor of 6.25 for *A.*
459 *platensis*, a 62% protein content was obtained for the urine-grown biomass. The high protein
460 content and growth rate confirm the excellent properties of nitrified urine as a growth medium for
461 *A. platensis*. The biomass produced can subsequently be valorized as a high-value fertilizer or
462 can function as a protein supplement in fodder (Crab et al., 2007; Mulbry et al., 2007). While
463 heavy metal concentrations are generally low in source separated urine, the presence of
464 pathogens and unwanted micropollutants such as pharmaceuticals or hormones might require
465 additional processing of the nitrified urine prior to *A. platensis* production through the
466 implementation of a chemical oxidation or activated carbon unit (Udert et al., 2015).

467 **4 Conclusions**

468 This study demonstrated complete nitrification of undiluted urine at high salinities and nitrogen
469 loading rates. A halotolerant inoculum enhanced reactor performance and reduced the reactor
470 start-up time. The nitrite oxidizing community showed faster adaptation and in both systems
471 *Nitrobacter* spp. became the dominant nitrite oxidizers. The high reactor salinity suggests that
472 this stabilization strategy can be expanded to other saline wastewaters. *A. platensis* cultivation on
473 nitrified urine resulted in high growth rates and a high biomass protein content. Biological
474 stabilization of source-separated urine through nitrification and subsequent *A. platensis*
475 cultivation is therefore an interesting strategy for nutrient recovery.

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600

601 **6 Table and Figure Captions**

602 **Table 1.** Biomass composition expressed per dry weight of *A. platensis* cultivated on nitrified
603 urine and standard Zarrouk medium

604 **Fig. 1.** Schematic overview of the urine nitrification membrane bioreactor set-up

605 **Fig. 2.** Overview of specific ammonium (a) and nitrite (b) oxidation rates, along with relative
606 inhibition

607 **Fig. 3.** Operation and performance characteristics of the MBR inoculated with the commercial
608 nitrifying inoculum (left) and the conventional municipal activated sludge (right). (A) Reactor
609 effluent characteristics. (B) Volumetric nitrogen loading rates (C) Specific nitrogen loading rates
610 and sludge content. (D) Abundance of nitrifying community groups as determined by qPCR

611 **Fig. 4.** Growth of *A. platensis* on different dilutions of male non-hydrolyzed, hydrolyzed,
612 stripped and nitrified urine

613 **Fig. 5.** Growth and nutrient uptake of *A. platensis* cultivated in Zarrouk medium (open symbols)
614 and in 20% nitrified urine (filled symbols)

615 ## 7 Tables and figures

616 **Table 1.** Biomass composition expressed per dry weight of *A. platensis* cultivated on nitrified

617 urine and standard Zarrouk medium[1]

Composition	Zarrouk medium	20% nitrified urine
N (%)	10.7±0.4	10.0±0.2
P (%)	8.5±0.4	9.1±0.4
Protein (%)	66.8±2.4	62.4±1.1
Total chlorophyll (%)	1.78±0.10[a]	1.35±0.1[b]
Total carotenoids (%)	0.31±0.04	0.26±0.03
Ash (%)	6.87±0.17[a]	7.21±0.14[b]

[1] Mean values and standard deviations are displayed (n =4). Sampling was performed at t=10 days and t=9 days for Zarrouk medium and nitrified urine, respectively. A Mann-Whitney test was performed to assess differences between treatments. Parameters not sharing superscripts are significantly different from each other (p=0.05).

618

Figure 1
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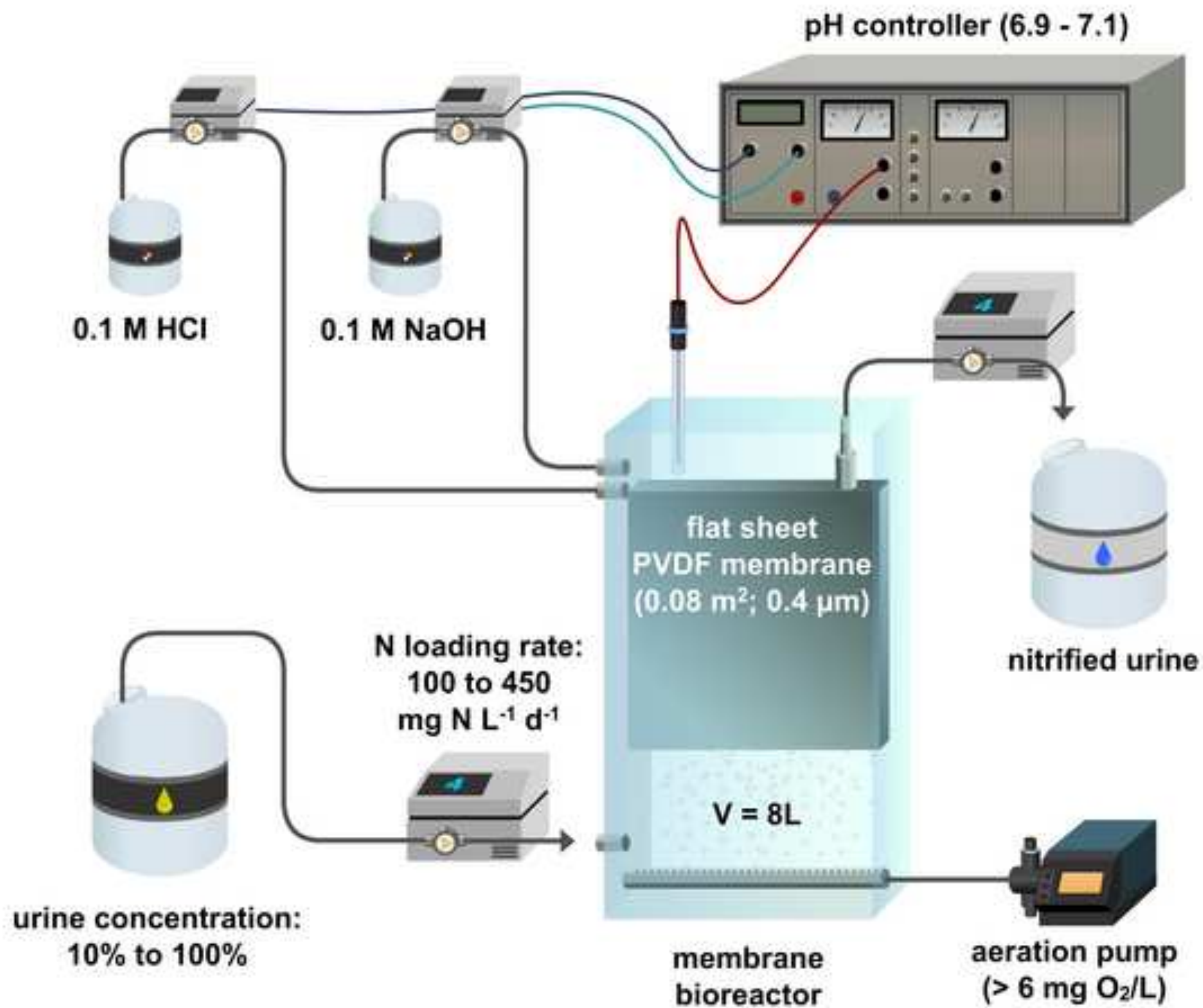

Figure 2

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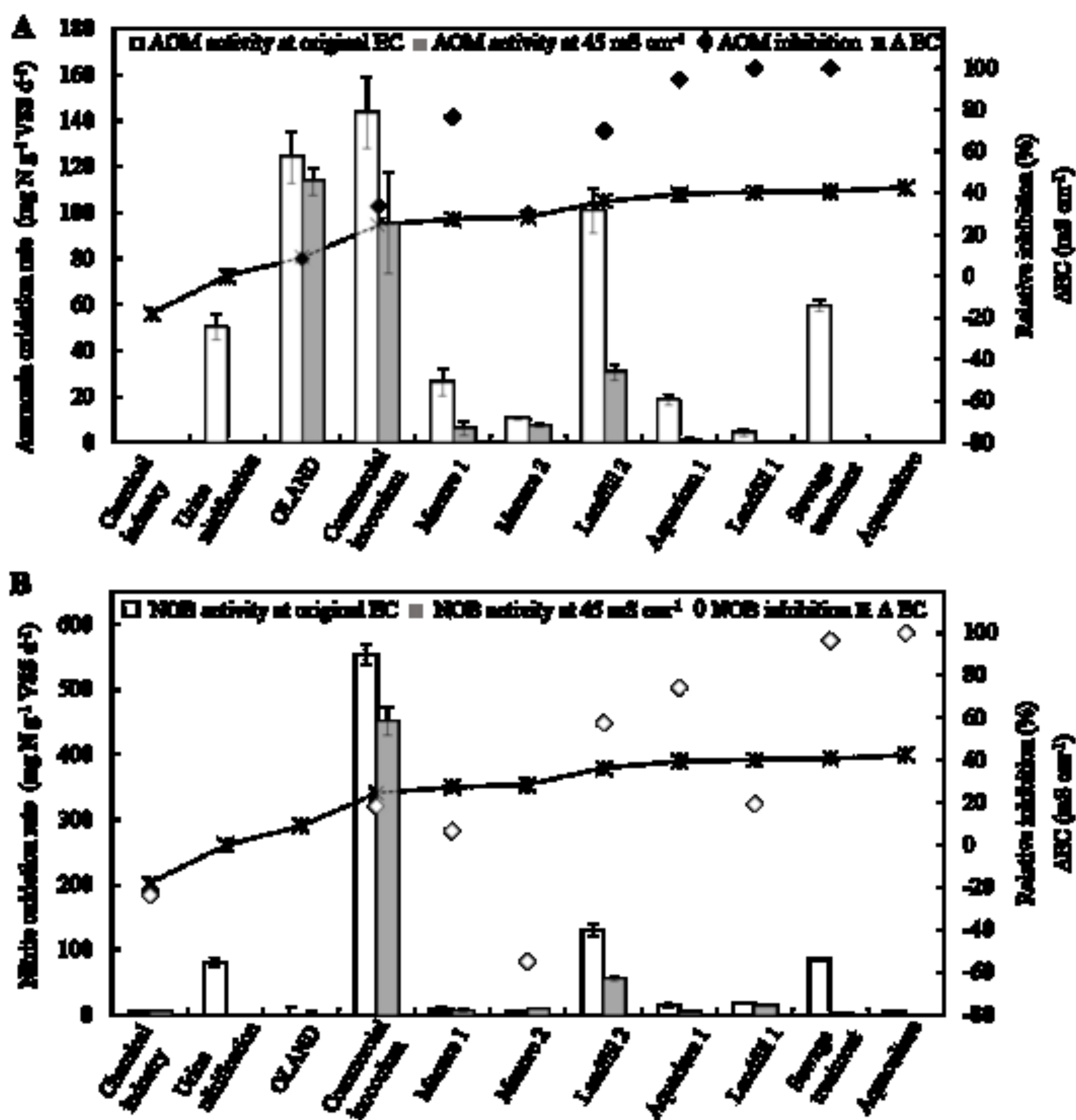

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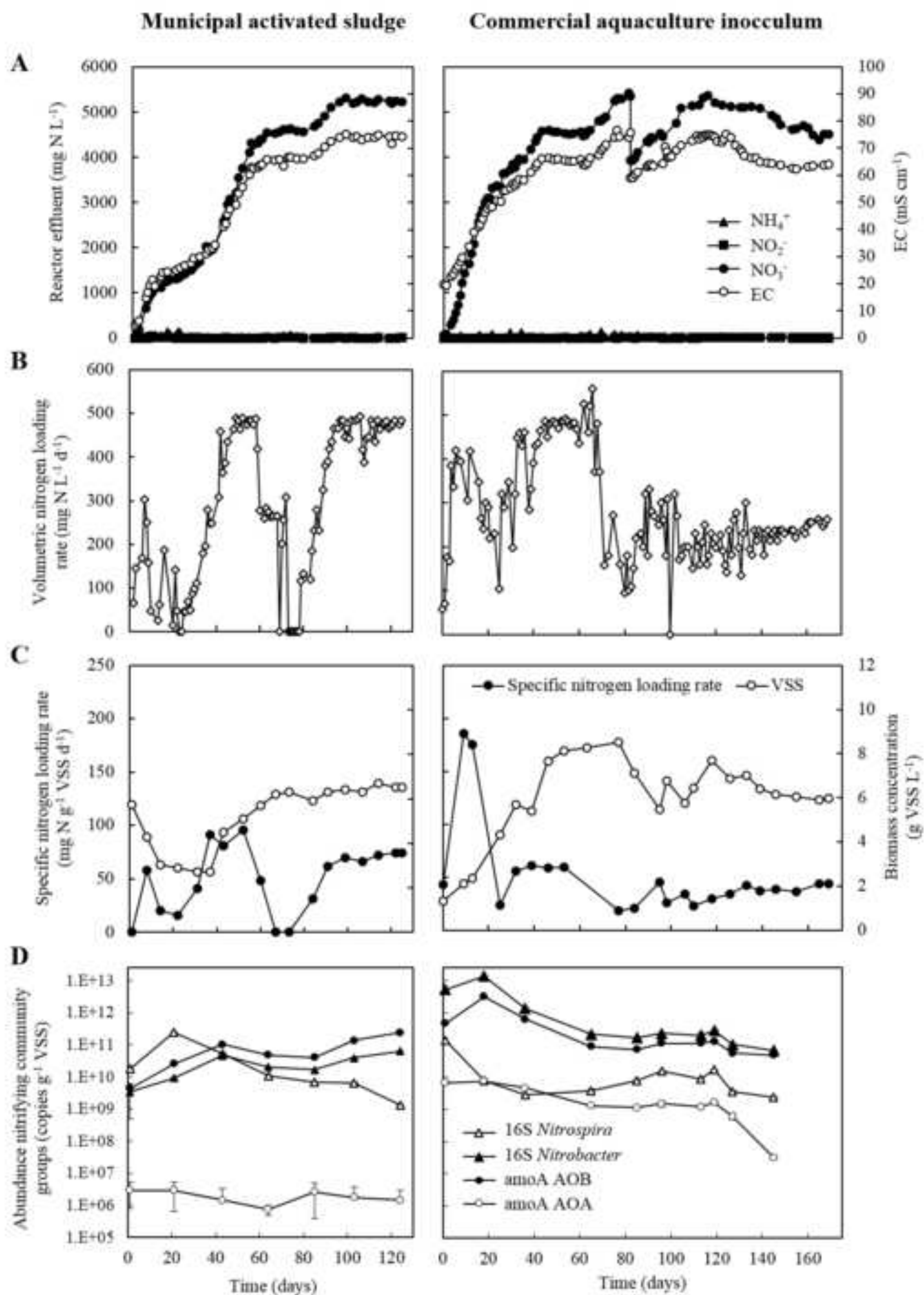

Figure 4
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Non-hydrolyzed urine

100% urine: 6.3 g N/L
48% urine: 3 g N/L
32% urine: 2 g N/L
16% urine: 1 g N/L
8% urine: 0.5 g N/L
2% urine: 0.1 g N/L

OD680

Hydrolyzed urine

100% urine
50% urine
33% urine
20% urine
10% urine

Stripped urine

100% urine: 5.7 g N/L
55% urine: 3 g N/L
35% urine: 2 g N/L
20% urine: 1 g N/L
10% urine: 0.5 g N/L
2% urine: 0.1 g N/L

OD680

Time (hours)

Nitrified urine

Standard Zarrouk medium: 0.4 g N/L
100% urine: 5.3 g N/L
60% urine: 3 g N/L
40% urine: 2 g N/L
20% urine: 1 g N/L
10% urine: 0.5 g N/L
2% urine: 0.1 g N/L

Time (hours)

Figure 5
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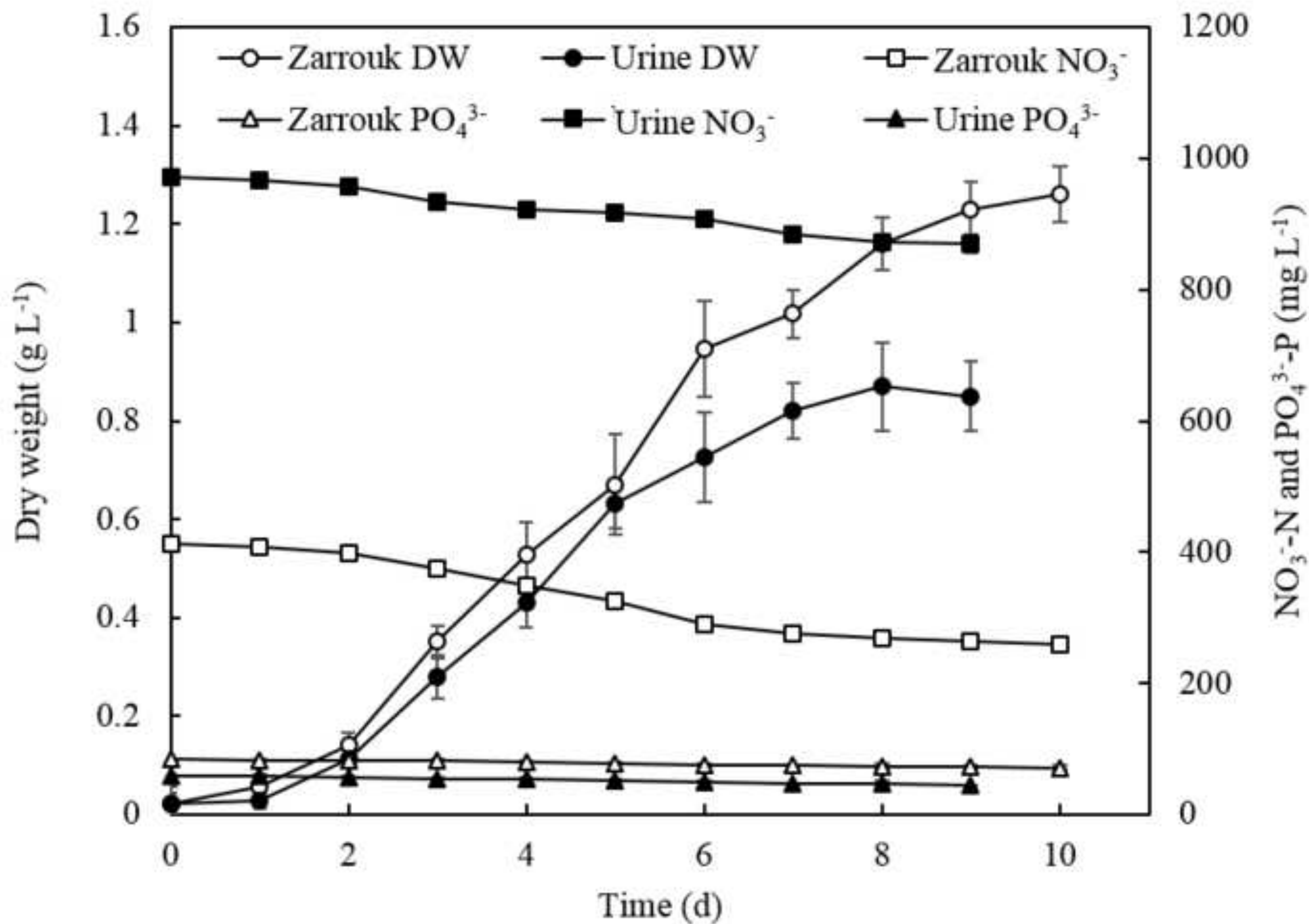

Electronic Annex
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