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## Enrichment and adaptation yield high anammox conversion rates under low temperatures

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1        **Enrichment and adaptation yield high anammox conversion**  
2                                **rates under low temperatures**

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## **Abstract**

17 This study compared two anammox sequencing batch reactors (SBR) for one  
18 year. SBR<sub>constantT</sub> was kept at 30°C while temperature in SBR<sub>loweringT</sub> was  
19 decreased step-wise from 30°C to 20°C and 15°C followed by over 140 days at  
20 12.5°C and 10°C. High retention of anammox bacteria (AnAOB) and  
21 minimization of competition with AnAOB were key. 5-L anoxic reactors with the  
22 same inoculum were fed synthetic influent containing 25.9 mg NH<sub>4</sub><sup>+</sup>-N/L and  
23 34.1 mg NO<sub>2</sub><sup>-</sup>-N/L (no COD). Specific ammonium removal rates continuously  
24 increased in SBR<sub>constantT</sub>, reaching 785 mg NH<sub>4</sub><sup>+</sup>-N/gVSS/d, and were  
25 maintained in SBR<sub>loweringT</sub>, reaching 82.2 and 91.8 mg NH<sub>4</sub><sup>+</sup>-N/gVSS/d at 12.5  
26 and 10°C respectively. AnAOB enrichment (increasing hzsA and 16S rDNA  
27 gene concentrations) and adaptation (shift from *Ca. Brocadia* to *Ca. Kuenenia*  
28 in SBR<sub>loweringT</sub>) contributed to these high rates. Rapidly settling granules  
29 developed, with average diameters of 1.2 (SBR<sub>constantT</sub>) and 1.6 mm  
30 (SBR<sub>loweringT</sub>). Results reinforce the potential of anammox for mainstream  
31 applications.

Key words: biological nitrogen removal; shortcut nitrogen removal; cold anammox; microbial community  
structure

32

## 1. Introduction

33 Partial nitrification/anammox (PN/A) is an autotrophic shortcut nitrogen removal  
34 process in which, after partial nitrification, Anammox bacteria (AnAOB) perform  
35 anoxic oxidation of ammonium with the produced nitrite as electron acceptor.  
36 This process requires less oxygen, abandons the need for organic carbon, and  
37 yields a lower sludge production. It can thus present a more cost-efficient  
38 treatment compared to the conventional nitrification/denitrification process  
39 (Vlaeminck et al. 2012). Many advancements have been made and by the end  
40 of 2013, close to one hundred full scale side-stream anammox installations,  
41 usually treating digestate, landfill leachate or reject water, have seen the day  
42 (Lackner et al. 2014, Mulder et al. 1995, Siegrist et al. 2008, Strous et al. 1999).  
43 The next development goal is to introduce anammox in the mainstream or  
44 waterline of sewage treatment plants in order to further improve their efficiency  
45 in terms of energy consumption (and hence economics) and greenhouse gas  
46 emissions.

47

48 The main challenges for PN/A implementation on pretreated sewage, so-called  
49 mainstream PN/A, are associated with the development of robust methods to  
50 suppress nitrite oxidizing bacteria (NOB) and promote the growth and activity of  
51 AnAOB under relatively low influent nitrogen concentrations (40-80 mg  $\text{NH}_4^+$ -  
52 N/L) and non-negligible amounts of biodegradable organic carbon (which allow  
53 ordinary heterotrophic organisms, OHO, to develop and compete for nitrite  
54 through denitrification). Furthermore, and of particular interest for regions with a  
55 temperate (or cold) climate, relatively low sewage temperatures (below 15°C

56 down to 10°C, or even below) drastically decrease AnAOB growth rates and  
57 activity (Cao et al. 2017, Vlaeminck et al. 2012). Their optimal operating  
58 temperature has been reported around 35°C - 40°C (Strous et al., 1999, Isaka  
59 et al. 2008).

60

61 Several attempts have been made to understand and model the short term  
62 impact of decreasing temperature on the specific ammonia removal rate  
63 (SARR) by calculating Arrhenius temperature coefficient (also called  $\theta$ -value) or  
64 Activation Energy ( $E_a$ ). These studies reported a sharp decrease in anammox  
65 activity from 15°C and down (up to 10-fold between 30 and 10°C). Maximum  
66 reported SARR values (after conversion from  $N_2$  production to  $NH_4^+$   
67 consumption based on the theoretical stoichiometry) at 10°C did not go beyond  
68 14 mg  $NH_4^+$ -N/gVSS/d (Lotti et al. 2014c, Dosta et al. 2008). However, short-  
69 term batch tests do not include the impact of adaptation and selection on the  
70 temperature sensitivity of the biomass, and are therefore not sufficient to assess  
71 the full potential of anammox at lower temperatures.

72

73 Several reactor strategies have been tested for anammox at lower temperature,  
74 with different types of biomass (flocs, granules or carrier-supported biofilms),  
75 and types of wastewater. Adaptation could be demonstrated through a shift in  
76 optimal temperature upon quickly or gradually decreasing temperature in  
77 Moving Bed Biofilm Reactor (MBBR) (Gilbert et al. 2014, Gustavsson et al.  
78 2014) and Sequencing Batch Reactor (SBR) systems (Gilbert et al. 2015, Hu et  
79 al. 2013). The formation, enrichment and maintenance of anammox granules at

80 lower temperatures has been reported on both synthetic wastewater at 10°C  
81 (Hendrickx et al. 2014, Lotti et al. 2014a) and real wastewater at 16°C (Ma et al.  
82 2013) and 10°C (Lotti et al. 2014b). In another study where hybrid sludge (flocs  
83 + wall attached biofilm) was formed, decreasing temperature caused the SARR  
84 to drop tenfold, but the system's performance remained stable at 12°C (Laureni  
85 et al. 2015). Stable P/NA performance was also observed when decreasing the  
86 temperature to 15°C in 2 MBBRs, however a sudden drop in temperature  
87 caused a significant yet reversible drop in anammox activity (Laureni et al.  
88 2016). Interestingly, *Brocadia* was the dominant anammox genus in all of the  
89 abovementioned studies throughout the adaptation to and operation at low  
90 temperature. It should be mentioned that all those studies at low temperature  
91 were performed in the presence of oxygen and/or organic carbon and the  
92 temperature decrease was often abrupt (big steps) and/or little time (days to a  
93 couple of months) was given to renew the slow growing AnAOB. To our  
94 knowledge, no study has been done under pure anoxic autotrophic conditions  
95 with granular sludge at low temperature.

96

97 The objective of this study was to reveal the maximum potential of so-called  
98 'cold anammox' (for temperatures below 15°C), also monitoring potential  
99 change in the microbiological structure and the physical properties of granular  
100 sludge. In order to achieve an adapted and enriched anammox community, a  
101 long-term reactor experiment was designed in which the microbial competition  
102 was limited through using anoxic reactors, avoiding AOB and NOB activity, in  
103 the absence of organic carbon, avoiding OHO. To do so, an anammox

104 sequencing batch reactor (SBR) operated at high temperature (30°C) was  
105 compared to one subjected to subsequent temperature drops for almost 1 year.  
106 The impact of lowering the operational temperature from 30 over 20, 15 and  
107 12.5 to 10°C on nitrogen removal performance and factors linked to biomass  
108 enrichment/adaptation and morphology was examined.

## 109 **2 Material and Methods**

### 110 **2.1 Set-up and operation of the reactors**

111 Two anammox SBR were operated in parallel, composed of identical, airtight,  
112 mixed, and jacketed vessels (5L), equipped with pH (H 8481 HD, SI Analytics),  
113 temperature and dissolved oxygen (DO) probes (Visiferm™, Hamilton). The  
114 SBR mode is comprised of four characteristic phases: (1) filling phase (30  
115 minutes, inflow of 2.5L influent, hence a volumetric exchange ratio, VER, of  
116 50%), during which oxygen was removed from the liquid through stripping with  
117 N<sub>2</sub> gas. For the next 30 minutes, the reactors were flushed with a mixture of N<sub>2</sub>  
118 and CO<sub>2</sub> gas to ensure anoxic conditions and set the pH back to 7.5 before  
119 starting the actual (2) reaction phase with a variable duration (between 4h and  
120 11.3h). The length of the reaction phase was shortened (lengthened) to  
121 increase (decrease) the ammonium loading rate (ALR) when needed. After the  
122 reaction phase, mixing was stopped for 30 minutes during the (3) settling phase  
123 before starting the (4) discharge phase where 2.5L of supernatant was removed  
124 before starting the next cycle. Those operating conditions led to maintain in the  
125 system particles that have a settling velocity higher than 0.29 m/h.

126



127 One reactor,  $SBR_{\text{constantT}}$ , was kept at the reference temperature of 30°C  
128 throughout the entire experiment while in the second reactor,  $SBR_{\text{loweringT}}$ , a  
129 stepwise decrease from 30°C to 20°C, 15°C, 12.5°C and finally 10°C was  
130 imposed corresponding to phases I, II, III, IV and V respectively. The decrease  
131 of temperature was deliberately slow to let the time for the microbial community  
132 to adapt to new conditions. More specifically the temperature pattern was  
133 chosen according to the following philosophy:

- 134 - Step by step, each step being maintained for at least 2 months, until the  
135 Ammonium Removal Efficiency (ARE) recovered a value of 100%, and the  
136 Volumetric Ammonium Removal Rate (VARR), i.e. the anammox conversion  
137 rate, reached steady state value;
- 138 - The temperature delta was not conservative, but progressively smaller  
139 (10°C, 5°C, 2.5°C, 2.5°C), assuming that the last temperature steps would  
140 be more detrimental to the anammox bacteria considering previous  
141 literature indicating a higher activation constant at low temperature;
- 142 - The rate of temperature decrease was linear with a slope of 2°C/day,  
143 2°C/day, 1°C/day, 0.5°C/day for the last, again expecting that the last  
144 temperature step would be more critical for anammox bacteria.

145

146 Finally the decrease from 30 to 10°C took 7 months. Calculations were made in  
147 order to evaluate if this time was sufficiently long to allow the renewal of most of  
148 the biomass initially contained in the reactor considering the AnAOB biomass  
149 yield and imposed nitrogen load. These calculations are given in the discussion.

150

151 The reactors were fed with the same synthetic influent which was stored at 4°C  
152 in a 150L tank. The influent contained per liter 168.0 mg of NaNO<sub>2</sub>, 98.8 mg of  
153 NH<sub>4</sub>Cl, 165.0 mg of MgSO<sub>4</sub>•7H<sub>2</sub>O, 143.8 mg of CaCl<sub>2</sub>•2H<sub>2</sub>O, 6.3 mg of EDTA-  
154 Na<sub>2</sub>•2H<sub>2</sub>O, 775.4 mg of NaHCO<sub>3</sub>, 30.0 mg of KH<sub>2</sub>PO<sub>4</sub>, and 9.1 mg of  
155 FeSO<sub>4</sub>•7H<sub>2</sub>O. Hence, the total nitrogen content was 60 mg N/L, a typical value  
156 for municipal wastewater (Metcalf 2003), made up of ammonium and nitrite in a  
157 1/1.32 ratio correspondig to the stoichiometry found by (Strous et al. 1998): 25.9  
158 mg N-NH<sub>4</sub><sup>+</sup>/L and 34.1 mg N-NO<sub>2</sub><sup>-</sup>/L. Per liter of influent, 2 mL of a trace  
159 element solution was added, which contained per liter 430 mg of ZnSO<sub>4</sub>•7H<sub>2</sub>O,  
160 240 mg of CoCl<sub>2</sub>•6H<sub>2</sub>O, 1.21g of MnCl<sub>2</sub>•4H<sub>2</sub>O, 250 mg of CuSO<sub>4</sub>•5H<sub>2</sub>O, 183 mg  
161 of Na<sub>2</sub>MoO<sub>4</sub>•2H<sub>2</sub>O, 274.6 mg of NiSO<sub>4</sub>•7H<sub>2</sub>O, 50.1 mg of Na<sub>2</sub>SeO<sub>3</sub>•5H<sub>2</sub>O, 60.9  
162 mg of Na<sub>2</sub>WO<sub>4</sub>•2H<sub>2</sub>O, 14 mg of H<sub>3</sub>BO<sub>3</sub> and 15g of EDTA-Na<sub>2</sub>•2H<sub>2</sub>O.

## 163 **2.2 Biomass inoculum mix**

164 To provide a high microbial AnAOB diversity, four different non cold-adapted  
165 biomasses from stable, long running systems containing AnAOB and operated  
166 at rather high temperature (26-30°C) were mixed. Half of the biomass types  
167 originated from smaller lab-scale set-ups treating synthetic influent, the others  
168 originated from full or pilot scale systems treating centrate from a sewage  
169 sludge digester. Three of the systems were PN/A (aerobically operated), the  
170 fourth system was anammox (anoxic). The resulting inoculated biomass  
171 concentration was 2.3 g VSS/L.

## 172 **2.3 Anammox activity and chemical analyses**

173 The actual ARE were determined by measuring outlet concentrations of  
174 ammonium roughly every two days. Comparable information was obtained from  
175 nitrite measurements.

176

177 The systems being operated in sequencing batch mode kinetics could be  
178 followed directly during the reaction phase of the cycles. The *in situ* VARR was  
179 measured two to three times per week. Samples of mixed liquor (8mL) were  
180 taken throughout the reaction phase of one cycle. The ammonium uptake rate  
181 was calculated by linear regression (no substrate limitation for ammonium  
182 concentration higher than 5 mgN/L for which a linear decrease was observed).

183 Roughly every one or two months, mixed liquor samples were taken from the  
184 reactors to determine total (TSS) and volatile (VSS) suspended solids  
185 concentrations. For biomass sampling a protocol was carefully set up to collect  
186 representative samples. During sampling, the agitation speed was increased  
187 (from 50 to 100 rpm) for maximal homogenization of the reactor liquid (and at  
188 least one minute before sampling). Sampling and VSS measurements were  
189 done in duplicate, and the sample volume (2x50 mL) was chosen to obtain good  
190 reproducibility. For each phase, an average biomass concentration (g VSS/L)  
191 was calculated (two to three measurements) together with the average *in situ*  
192 VARR during the last two weeks (three to four measurements). From this, the *in*  
193 *situ* SARR at the end of each phase was estimated. Standard deviations were  
194 calculated for average VARR and VSS and propagated in the error bars,  
195 allowing for an objective comparison. Student tests (t-tests) were performed to

196 compare mean SARR values between reactors and between consecutive  
197 phases within the same reactor.

198

199 All water samples were centrifuged (4°C, 2 minutes at 2,591xg) and  
200 supernatant was filtered through 0.2 µm membrane filters prior to nitrogen  
201 compounds analysis. Nitrite and nitrate were quantified using  
202 spectrophotometric methods, analyses were done automatically using the  
203 SMARTCHEM200 (AMS, Italy). Ammonium concentration was measured  
204 according to the spectrophotometric Nessler method (APHA 1992). Suspended  
205 solids (TSS and VSS) were measured in duplicate according to Standard  
206 Methods 2540D and E (APHA 1992).

#### 207 **2.4 Particle size distribution of the biomass aggregates**

208 The particle size distribution (PSD) was determined after 354 days. Triplicate  
209 samples of mixed liquor were taken, and particles were visualized with a WILD  
210 M420x1.25 (optical zoom set at x6.4) stereomicroscope, operated using a  
211 NIKON DS-U2 control unit combined with NIS-elements F software (v.3.2).  
212 Photos were taken using a NIKON DS-Fi1 digital microscope camera. For each  
213 triplicate, 50 photos were taken. The three series of photos were combined to  
214 give one set of 150 photos per reactor for image analysis.

215

216 The first step of the image treatment consisted of a conversion to greyscale  
217 using IrfanView software (v.4.10). Next, using Visilog software (v.6.7), the pixel-  
218 scale values were converted by a scaling factor (which was calibrated for the  
219 magnification). Each grey-scale image underwent a binarization step after which

220 the surface of each particle was quantified and used to estimate the sphere-  
221 equivalent diameter. The used script was made so that particles touching the  
222 edge of the photo or with a diameter smaller than 15  $\mu\text{m}$  would not be detected  
223 (the detection limit corresponded to a minimal number of 10 pixels in the image  
224 analysis script). The obtained data was then treated in Excel with a visual basic  
225 macro to obtain the number, surface and volume weighted PSD along with  
226 additional information such as mean diameters.

## 227 **2.5 Microbial community analyses**

228 Triplicate samples of 1.5 mL mixed liquor were taken from each reactor, one  
229 was used for microbial community analyses, and the other two were stored as  
230 back-up. This was done on average once every two to three weeks with higher  
231 sampling density around each transition between phases. After centrifugation  
232 (20 min at 19,000xg), supernatant was removed and the remaining biomass  
233 was immediately frozen in liquid nitrogen. DNA extraction on one of these  
234 samples was performed using the FastDNA spinkit™ from MP biomedical  
235 following the provided Fastprep® protocol. The extracted DNA was stored at -  
236 20°C until further use.

237 In order to map AnAOB enrichment, qPCR was used to quantify the AnAOB  
238 based on phylogenetic primers (16S rRNA) and functional primers (hzsA). Also,  
239 to follow up the composition of the overall community, MiSeq Illumina  
240 sequencing was performed by the GenoToul Genomics and Transcriptomics  
241 facility (GeT-PlaGe, Auzeville, France). Sequencing data was processed using  
242 the pipeline FROGS, one of the tools proposed on Galaxy, an open web-based  
243 platform for genomic research.

## 244 3. Results

### 245 3.1. Reactor performance

#### 246 3.1.1. Start-up of the reactors

247 Both reactors were started with the same seeding sludge and operated  
248 identically at 30°C for 62 days to allow for adaptation to the new incubations  
249 conditions. During this period, both reactors showed a very comparable  
250 evolution both in ARE (Figure 1.b) and VARR, starting out around 48 mg NH<sub>4</sub><sup>+</sup>-  
251 N/L/d on day 19 and increasing to around 74.4 mg NH<sub>4</sub><sup>+</sup>-N/L/d on day 54  
252 (Figure 1.a). Because of this increase in volumetric activity, the duration of the  
253 reaction time was decreased twice resulting in an increase ALR: from 40.8 mg  
254 NH<sub>4</sub><sup>+</sup>-N/L/d to 47.0 mg NH<sub>4</sub><sup>+</sup>-N/L/d (on day 24) and later to 55.4 mg NH<sub>4</sub><sup>+</sup>-N/L/d  
255 (on day 31). While a biomass concentration of 2.3 g VSS/L was initially added,  
256 levels of 0.70 and 0.76 g VSS/L, for SBR<sub>constantT</sub> and SBR<sub>loweringT</sub> respectively,  
257 were retrieved on day 67. This was caused by washout of the smallest  
258 aggregates during the first two months of operation, this is illustrated by the  
259 increased loss of VSS in the effluent during phase I.

260 Figure 2 shows the average *in situ* specific ammonium removal rates (SARR) at  
261 the end of each operating phase. By the end of the phase I, in which both  
262 reactors had been operated identically, they had reached very similar *in situ*  
263 SARR (103 and 99 mg NH<sub>4</sub><sup>+</sup>-N/gVSS/d for SBR<sub>constantT</sub> and SBR<sub>loweringT</sub>  
264 respectively). On day 62, temperature in SBR<sub>loweringT</sub> was decreased to 20°C,  
265 whereas from this point on, operating conditions in SBR<sub>constantT</sub> were left  
266 unchanged for the rest of the experiment (T=30°C, ALR = 55.4 mg NH<sub>4</sub><sup>+</sup>-N/L/d).

#### 267 3.1.2. Activity evolution at constant temperature (30°C)

268 For the control reactor  $SBR_{\text{constantT}}$ , as shown in Figure 1.a, maximal volumetric  
269 activity has continuously increased upon inoculation: at the beginning of period  
270 II, it increased slightly from 79.2 mg  $\text{NH}_4^+\text{-N/L/d}$  on day 69 to 87.6 mg  $\text{NH}_4^+\text{-}$   
271  $\text{N/L/d}$  on day 103. On this day, a leak causing a slight entrance of oxygen (0.05  
272 mg  $\text{O}_2\text{/L}$ ) in the headspace was detected. After this leak was repaired, the  
273 activity increased even more rapidly to 129.8 mg  $\text{NH}_4^+\text{-N/L/d}$  in only three days.  
274 This increase in activity with time in  $SBR_{\text{constantT}}$  continued almost linearly during  
275 the overall study. Finally maximal VARR reached about six times its initial value  
276 up to 336 mg  $\text{NH}_4^+\text{/L/d}$  at the end of the experiment. At this high VARR, all of  
277 the ammonia and nitrite were consumed during the first 45 minutes of the  
278 reaction phase. The VARR was higher than the ALR, translating in a part of the  
279 reaction phase in which conditions were endogeneous. The ARE remained  
280 close to 100% during the entire running period (Figure 1.b). The average  
281 effluent concentrations were 0.6 mg  $\text{NH}_4^+\text{-N/L}$  and 0.1 mg  $\text{NO}_2^-\text{-N/L}$  over the  
282 entire operating period.

283 As VSS decreased (roughly by a factor 1.7) during the experiment, the evolution  
284 of specific activity is of particular interest: the *in situ* SARR increased by a factor  
285 7.5 between the end of phase I (103 mg  $\text{NH}_4^+\text{-N/gVSS/d}$ ) and the end of phase  
286 V (785 mg  $\text{NH}_4^+\text{-N/gVSS/d}$ ). This suggests enrichment and/or an adaptation of  
287 the anammox biomass; this point will be addressed in the discussion section.

288

### 289 3.1.3. Activity evolution at decreasing temperature (30°C to 10°C)

290 As shown in Figure 1.a, each temperature decrease step in  $SBR_{\text{loweringT}}$  resulted  
291 in a decrease of anammox activity which temporarily affected the removal

292 efficiency. This effect was most pronounced when switching from 30 to 20°C  
293 (beginning of phase II) and from 15°C to 12.5°C (beginning of phase IV),  
294 resulting in a 64.8% and 76.7% drop in activity respectively. However, partial  
295 recovery after the temperature drop from 30°C to 20°C, activity took 6 weeks,  
296 significantly more than the 3 days required for recovery after the drop from 15°  
297 to 10°C, which was probably the result of an adaptation and/or enrichment of  
298 the biomass during the 146 days between these events (see chapter 3.2). The  
299 effects of decreasing temperature from 20°C to 15°C and 12.5°C to 10°C were  
300 less significant, as activity loss was lower (36.3% and 28.4% respectively) and  
301 activity recovered within the month. Considering the activity measured at the  
302 end of each phase, maximal VARR decreased from 76.2 to 41.0 mg NH<sub>4</sub><sup>+</sup>-N/L/d  
303 for temperature decrease from 30°C to 20°C (phases I and II), but was relatively  
304 similar at the end of the next three phases (III, IV, V), reaching 24.0 mg NH<sub>4</sub><sup>+</sup>-  
305 N/L/d at the end the study. For this reason, it was possible to maintain the  
306 nitrogen load constant during the phases II, III, IV and V without impacting that  
307 much the ARE, which remained also relatively high during the study, ranging  
308 between 57-100% for SBR<sub>lowering</sub> (Figure 1.b). The average effluent  
309 concentrations were 2.7 mg NH<sub>4</sub><sup>+</sup>-N/L and 3.3 mgNO<sub>2</sub><sup>-</sup>-N/L over the entire  
310 operating period.

311

312 In SBR<sub>loweringT</sub> the VSS concentration also decreased during the experiment,  
313 from 0.76 g VSS/L to 0.24 g VSS/L. When temperature was decreased to 20°C,  
314 *in situ* SARR dropped by 38% between the ends of phase I and II. During this  
315 time, the biomass concentration decreased from 0.76 g VSS/L to 0.56 g VSS/L.



316 For the next 11 weeks, SARR remained rather stable, reaching 65.6 mg NH<sub>4</sub><sup>+</sup>-  
317 N/gVSS/d at the end of phase III while biomass concentration decreased from  
318 0.56 g VSS/L to 0.39 g VSS/L. Further decreases in temperature affected  
319 transiently the SARR but did not negatively impact the final SARR obtained  
320 after several weeks at the end of the temperature step. On the contrary SARR  
321 increased slightly to 82.2 mg NH<sub>4</sub><sup>+</sup>-N/gVSS/d at the end of phase IV and  
322 reached 91.8 mg NH<sub>4</sub><sup>+</sup>-N/gVSS/d at the end of phase V. This is the highest  
323 reported SARR at such low temperature. These results demonstrate a very  
324 successful adaptation and operation of the granular sludge anammox process  
325 at 10°C.

326 **Figure 1**

327 **Figure 2**

328 Student tests (data not shown) clearly showed that the SARR in phase II, III, IV  
329 and V were significantly different between both reactors. The tests showed no  
330 significant difference between two consecutive phases in the same reactor.

### 331 **3.2 Biomass aggregate size and microbial community analysis**

#### 332 *3.2.1 Biomass particle size distribution*

333 On day 1, both reactors contained a dark brownish and very heterogeneous  
334 hybrid sludge, made up mainly of small flocs and suspended biomass and a  
335 small amount of granules, features linked to the individual constituents of the  
336 inoculum (Table 1). From size fractionation of each biomass type (through  
337 sieving) and their respective contribution to the inoculum mix, it was estimated  
338 that that 59% of the aggregates were smaller than 315 µm (data not shown).  
339 During the first 5 months of reactor operation, the biomass evolved to a bright  
340 red, predominantly granular sludge. During the last period of operation (phase

341 V), even at 10°C, the granules demonstrated very good settling properties due  
342 to their size and density: To illustrate this, SVI was determined to be 34 mL/g  
343 and 26 mL/g for  $SBR_{constantT}$  and  $SBR_{loweringT}$  respectively on day 323.  
344 Furthermore, most of the solids were settled in less than 10 minutes during the  
345 settling phase. This corresponds to settling velocities higher than 0.88 m/h.  
346 Analysis of the particle size distribution (PSD) was performed on day 354.

347

348  $SBR_{loweringT}$  displays a broader PSD, indicating more variation in granule size  
349 compared to the narrower PSD of  $SBR_{constantT}$ , showing a rather uniform granule  
350 size. Also, the maximum observed diameter is higher at low temperature, this is  
351 also reflected in a bigger volume median diameter:  $D(0.5) = 1551\mu\text{m}$  for  
352  $SBR_{loweringT}$  vs.  $D(0.5) 1215\mu\text{m}$  for  $SBR_{constantT}$ . Some granules around 3.3-  
353 4.4mm were detected at 10°C, but not observed at 30°C. These results showed  
354 that lowering the temperature was not detrimental to the granulation process,  
355 and can even generate bigger granules. This is likely due to the lower AnAOB  
356 growth rates at low temperatures, which resulted in stronger granules with a  
357 higher density (as illustrated by their lower SVI of 26 mL/g compared to 34 mL/g  
358 at 30°C). Consequently, granules were less susceptible to breakage, explaining  
359 why larger diameters were observed at lower temperatures.

### 360 3.2.2 Evolutions in the microbial community

361 16S rRNA sequencing showed that, right after reactor start-up (day 10), AnAOB  
362 made up 64.2% and 57.7% of the total community in  $SBR_{ref}$  and  $SBR_{lowT}$   
363 respectively. By the end of phase I (day 60) the AnAOB abundances had  
364 increased to 79.2% and 80.5% respectively, enrichment was taking place. At

365 the end phase IV abundances in SBR<sub>constantT</sub> and SBR<sub>loweringT</sub> were only slightly  
366 higher, 81.2% and 87.2% respectively, indicating an AnAOB enrichment that  
367 mainly occurred in phase I. The evolution of the microbial population at genus  
368 level was monitored throughout the experiment for both reactors.

369

370 Figure 3 shows the evolution at genus level within the AnAOB community from  
371 16S rRNA sequencing data. Both reactors started with the same inoculum,  
372 which is reflected in a very similar anammox composition at day 10 with a  
373 majority of *Ca. Brocadia* (81%), complemented with minor fractions of *Ca.*  
374 *Kuenenia* (8%) and *Ca. Jettenia* (11%).

375

376 As long as both reactors were operated identically (the first 60 days), they  
377 showed a comparable evolution in their AnAOB profile: an increase in *Ca.*  
378 *Kuenenia* abundance together with a slightly lower decrease in *Ca. Brocadia*  
379 abundance. *Ca. Jettenia* abundance dropped dramatically to 1% in both  
380 reactors, and, regardless of operational temperature, could not re-establish  
381 itself dominantly within the community. The initial changes in the AnAOB  
382 community during the first 60 days are likely the result of acclimatization to the  
383 imposed reactor operating conditions. Regarding the significant loss of  
384 suspended solids observed during the first period, a selection by selective  
385 retention during settling was likely to occur. Indeed it was observed that the loss  
386 of *Ca. Brocadia* (-15%) and *Ca. Jettenia* (-10%), caused by washout of smaller  
387 aggregates, made the relative AnAOB abundance of *Ca. Kuenenia* increase  
388 (+25%).

389

390 In SBR<sub>constantT</sub> at 30°C, there was no further major shift. *Ca. Brocadia* remained  
391 dominant, and even increased its fraction during Phase II to V, at the expense  
392 of a drop in *Ca. Kuenenia* abundance. The population repartition remained  
393 roughly unchanged and *Ca. Brocadia* made up 90% of the AnAOB community  
394 on day 293 while *Ca. Kuenenia* represented less than 10%.

395

396 In contrast, when reducing the operating temperature to 20°C in SBR<sub>lowering</sub> a  
397 remarkable evolution in the AnAOB community occurred: the *Ca. Brocadia*  
398 abundance dropped quickly (from 73 to 29%) whilst the *Ca. Kuenenia*  
399 abundance increased just as quickly (from 26 to 68%). Further operation at  
400 lower temperatures (15, 12.5 and finally 10°C) did not significantly alter the  
401 dominance with the AnAOB community, which on day 293 was comprised of  
402 74% of *Ca. Kuenenia* and 26% *Ca. Brocadia*.

403

#### Figure 3

404 In parallel, the AnAOB enrichment in the biomass was assessed by qPCR at  
405 three moments in time. Results in Figure 4 indicate a considerable AnAOB  
406 enrichment occurring in both reactors, as reflected from the increase in both  
407 *hzsA* and 16S rDNA copy levels. Interestingly, in both systems most of the  
408 abundance increase occurred in the first half of the reactor operation time, by the  
409 end of Phase II, with little further enrichment over the second half. The  
410 observed trend was similar for *hzsA* and 16S rDNA copy levels expressed 'per  
411 g of biomass' or 'per ng of DNA'.

412

#### Figure 4

## 413 **4. Discussion**

### 414 **4.1 Enrichment and adaptation favoring high specific activities**

415 Our goal was to investigate the maximum potential of anammox at low  
416 temperatures ( $<15^{\circ}\text{C}$ ). After gradually decreasing from the initial temperature of  
417  $30^{\circ}\text{C}$  in  $\text{SBR}_{\text{loweringT}}$ , the activity stabilized at 82.2 and 91.8  $\text{mg NH}_4^+\text{-N/gVSS/d}$   
418 at  $12.5$  and  $10^{\circ}\text{C}$ , respectively. The *in situ* SARR in the reference reactor at  
419  $30^{\circ}\text{C}$  ( $\text{SBR}_{\text{constantT}}$ ) increased continuously, reaching up to 785  $\text{mg NH}_4^+\text{-}$   
420  $\text{N/gVSS/d}$ . Compared to literature, these specific removal rates at  $10^{\circ}\text{C}$  are  
421 considerably higher than previously reported values from reactor studies at low  
422 temperature, as illustrated in Table 1. Explanations for these differences in  
423 SARR are likely linked to differences in enrichment and/or adaptation degree,  
424 which is the consequence of a set of factors fixed in the experimental strategy.  
425 obtained a slightly higher SARR at after 131 days of operation  $12^{\circ}\text{C}$  compared  
426 to this study after 63 days at the same temperature. The final SARR obtained in  
427 this study after adaptation to  $10^{\circ}\text{C}$  was the same as the SARR obtained by Hu  
428 *et al.* at  $12^{\circ}\text{C}$  and significantly higher than the SARR obtained at  $10^{\circ}\text{C}$  without  
429 adaptation in that same study (Hu et al. 2013).

430 **Table 1**

431 Maximizing space for AnAOB in the biomass is a key factor in both enrichment  
432 and adaptation, and yielded from low competition with AnAOB and high  
433 retention of AnAOB. Feeding with an autotrophic influent in anoxic conditions  
434 indeed minimized competition with AOB, NOB and OHO, the average ratio of  
435 nitrite to ammonium removal was  $1.27\pm 0.19$  and  $1.20\pm 0.11$  for  $\text{SBR}_{\text{constantT}}$  and  
436  $\text{SBR}_{\text{loweringT}}$  respectively, which was close to the stoichiometry reported by  
437 Strous (Strous et al. 1998). Note that, the systems in other studies contained

438 COD or oxygen (Table 1). Maximizing biomass retention enables the slow(est)  
439 growing AnAOB to stay or develop in the system. In our study, this was  
440 implemented by imposing a low minimum settling velocity, keeping solids  
441 settling at > 0.3 m/h into the system. In both reactors, regardless of operational  
442 temperature, the bacteria aggregated in rapidly settling granules, which  
443 facilitated biomass retention. Nonetheless, biomass retention was not perfect,  
444 and sampling and washout renewed an estimated 70 and 100% of the produced  
445 AnAOB biomass in  $SBR_{\text{constantT}}$  and  $SBR_{\text{lowering}}$  respectively, based on the  
446 nitrogen loading and theoretical growth yield. Anoxic SRT values of 162 and  
447 164 days, for  $SBR_{\text{constantT}}$  and  $SBR_{\text{loweringT}}$  respectively, were obtained, from the  
448 estimated solids balance. In other words, operating the systems for more than  
449 two times the SRT should in theory replace more than 85% of the original  
450 biomass.

451

452 Enrichment in both systems was witnessed by the increase in relative AnAOB  
453 abundance in the biomass (qPCR data; Figure 4) in both reactors. According to  
454 the qPCR data, most of the enrichment occurred in the first half of the  
455 experiment. In  $SBR_{\text{loweringT}}$ , adaptation was observed at the level of the AnAOB  
456 community, with a major shift in dominant AnAOB genus from *Ca. Brocadia* to  
457 *Ca. Kuenenia* (16S rRNA gene sequence amplicons; Figure 3). Even though it  
458 was not studied here, physiological adaptations within a given AnAOB species  
459 could have occurred. There is for instance evidence that AnAOB are able to  
460 alter their lipid membrane composition through 'addition synthesis' as a form of  
461 homeoviscous adaptation to changes in *in situ* temperature (Rattray et al.

2010). Such type of adaptation might also explain the shift in temperature optimum for AnAOB without significant changes in the dominant genus reported by other authors (Gilbert et al. 2014, Hu et al. 2013).

In order to assess the impact of long-term enrichment and adaptation on the AnAOB performance, a parallel study was performed to test the immediate effect of exposure to low temperatures on the anammox activity, on the four types of biomass used in the inoculum mix. Based on recalculated values from this batch test, the SARR roughly doubled after 67 days of operation at 30°C (end of Phase I; Table 2). After long term operation at 30°C in SBR<sub>constantT</sub>, the SARR was 16 times higher than the activity of the inoculum at 30°C. Comparably, the final SARR at 12.5-10°C in SBR<sub>loweringT</sub> was 8-15 times higher than the recalculated inoculum rates under these respective temperatures. As a consequence the SARR ratios (low/high temperature) were similar in the short and long term tests: on short term, exposure to 12.5 and 10°C yielded respectively 20 and 13% of the SARR at 30°C, while the respective values were 17 and 12% for the long-term reactor test (see Table 2) .

Table 2

#### 4.2 Potential AnAOB genus niche differentiation

The results point towards a *Ca. Kuenenia* preference to colder temperatures (20°C and below) and a *Ca. Brocadia* preference to 30°C whereas *Ca. Jettenia* was outcompeted in both reactors. It is interesting to note that each system nonetheless maintained both genera, so the niche differentiation or competitiveness advantage was insufficiently strong to out-compete the one or the other.

487

488 In literature *Ca. Kuenenia* has been only observed as predominant in studies at  
489 relatively high temperature. A complete replacement of *Ca. Brocadia* by *Ca.*  
490 *Kuenenia* was observed in an anoxic anammox MBBR at 38°C (van der Star et  
491 al. 2008). The authors suggested that nitrite limitation was the main driver for  
492 this transition, based on the hypothesis that *Ca. Kuenenia* would be an affinity  
493 (K) strategist and *Ca. Brocadia* a growth rate (r) strategist. This hypothesis was  
494 challenged recently when the opposite switch was observed: *Ca. Brocadia*  
495 replaced *Ca. Kuenenia* as the dominant genus in the biofilm of two IFAS  
496 reactors operated between 24 and 29°C. The authors claimed that in this case,  
497 the tolerance towards higher and toxic nitrite levels affected the selection more  
498 than their affinity for low substrate levels (Zheng et al. 2016). *Ca. Kuenenia* has  
499 a higher reported tolerance towards nitrite than *Ca. Brocadia*: inhibition occurs  
500 for concentration of 180 mg/L and 70 mg/L respectively (Schmid et al. 2003).  
501 Neither of these hypotheses seems applicable to our study, since nitrite  
502 concentrations were too low to cause inhibition (maximum 17 mg N/L after  
503 feeding) yet were never limiting in any of the reactors (operated with rapid  
504 feeding in SBR mode). However, from Phase II onwards, the VARR in  
505  $SBR_{\text{constantT}}$  was higher than the VALR, meaning that the AnAOB were subjected  
506 to endogenous conditions during the final part of the cycle whereas the AnAOB  
507 in  $SBR_{\text{lowering}}$  knew no endogenous period since the VARR was stabilized  
508 around the VALR throughout the entire experiment. This presence or absence  
509 of an endogenous period may have also played a role in the niche  
510 differentiation between *Ca. Brocadia* to *Ca. Kuenenia*.



511

512 This is the first time a switch from *Ca. Brocadia* to *Ca. Kueneria* is observed at  
513 low temperature. Almost every other low temperature study, reported that *Ca.*  
514 *Brocadia* was and remained the dominant genus throughout the experiment,  
515 suggesting that this genus had a competitive advantage over other anammox  
516 genera at low temperature (Gilbert et al. 2015, Hendrickx et al. 2014, Hendrickx  
517 et al. 2012, Hu et al. 2013, Laureni et al. 2016, Laureni et al. 2015, Lotti et al.  
518 2014a, [Sánchez Guillén et al., 2016](#)). Note that during these studies there was  
519 always a presence of oxygen and/or organic carbon. [In the study by Sánchez](#)  
520 [Guillén and colleagues the maximum DO was 0.2%, but even at such low](#)  
521 [concentration AnAOB activity can be impacted \(observed in this study when a](#)  
522 [leak was detected on day 69\). It is interesting to note that in the before](#)  
523 [mentioned study, average granules size decreased throughout the reactor](#)  
524 [operation. The fact that granule size increased in this study was probably due to](#)  
525 [the higher NLR and lower mixing intensity \(the SBR reactor in the other study](#)  
526 [was fitted with baffles which most likely increased the shear force on the](#)  
527 [granules\).](#) Only one study reported on *Ca. Jettenia* as most dominant AnAOB  
528 genus in a PN/A Expanded Granular Sludge Bed (EGSB) reactor operated  
529 under low DO conditions (0.8 mg/L) to treating COD containing effluent of an  
530 upflow anaerobic fixed bed (UAFB) executing anaerobic digestion (AD) (Gao et  
531 al. 2015). It is possible that the absence of inhibition (in the case of oxygen) and  
532 of competition/partnerships with other bacteria (AOB, NOB and OHO) played a  
533 role in the niche differentiation between *Ca. Brocadia* and *Ca. Kueneria*  
534 observed in this study at low temperature. Available data on activation energies

535 ( $E_a$ ) do not allow to differentiate in temperature sensitivity between the two  
536 discussed genera. Two studies working with *Ca. Kueneenia* reported  $E_a$ -values  
537 of 63 kJ/mol between 10°C-40°C (Dosta et al. 2008) and 93 kJ/mol between  
538 6°C and 22°C (Isaka et al. 2008), these values fall within the same range as the  
539 ones obtained for *Ca. Brocadia* (Hendrickx et al. 2014, Lotti et al. 2014c).

540

541 Further research should focus on understanding the influence key drivers such  
542 as high/low affinity for substrate, sensitivity to low temperature and/or to  
543 inhibitors etc. have on the niche differentiation between different AnAOB genera  
544 and if/how this affects process kinetics/stoichiometry.

#### 545 **4.3 Towards implementation of partial nitrification/anammox**

546 The high activities obtained at low temperatures in this study reinforces the  
547 potential of anammox for mainstream application. A key strategy to obtain  
548 enrichment and adaptation was to eliminate competition and maximize retention  
549 while applying a step-wise decrease in temperature. **Process design and  
550 operation will define what activity level can be reached and should therefore be  
551 aimed at maximizing the abundance of adapted AnAOB in the biomass.**

552

553 In the end, the high maximal potential AnAOB activities under low temperatures  
554 provide a basis for to conceive new PN/A design solutions and associated  
555 operational strategies. If 50% of the activity at 10°C could be maintained in a  
556 full-scale process with a solids content of 3 g VSS/L, it would work at 135 g  
557  $\text{NH}_4^+\text{-N/m}^3/\text{d}$  which corresponds to a hydraulic retention time of about 8 hours,  
558 this is a conventional value for activated sludge treating systems domestic

559 sewage. Given the nitrite feeding and anoxic conditions in this study, a two-  
560 stage PN/A approach seems to be suitable to go as close as possible to the full  
561 potential of an enriched/adapted community, as it avoids the competition with  
562 nitrifiers, aerobic heterotrophs and with part of the anoxic heterotrophs.  
563 Selective AnAOB retention and enrichment can also be implemented in a **one-**  
564 **stage approach**, by selectively increasing the SRT of AnAOB compared to the  
565 SRT of the competitors, like in IFAS PN/A systems (Veuliet et al. 2014) or  
566 through the use of a selective screen (Han et al. 2016). Future work should  
567 verify whether equally high potential rates can be obtained when making a  
568 faster and seasonally realistic transition from higher to lower temperatures. Five  
569 year temperature gradient data from a WWTP in Germany showed a  
570 temperature decrease from 20°C (summer) to 10°C (winter) at a rate of 2°C per  
571 month. During the winter temperature could acutely drop down to 7°C for a  
572 short period of time (weeks) (Gilbert et al. 2014). In addition, more research  
573 should be dedicated to determine any influence of low temperatures on the  
574 growth yield. One study in a PN/A SBR estimated that the growth yield lowered  
575 with decreasing temperature (Hu et al. 2013) while in another anammox  
576 granular MBBR/SBR study no such impact was observed (Hendrickx et al.  
577 2014).

## 578 **5. Conclusions**

579 **Minimal competition and high AnAOB retention resulted in unprecedented**  
580 **removal rates of 82 and 92 mg NH<sub>4</sub><sup>+</sup>-N/g VSS/d at 12.5 and 10°C respectively.**  
581 **AnAOB enrichment (indicated by increasing hzsA and 16S rRNA gene**  
582 **concentrations) and adaptation at genus level (indicated by a shift from Ca.**

583 *Brocadia* to *Ca. Kueneenia* in SBR<sub>loweringT</sub>) contributed to this performance. Well  
584 settling granules were formed and maintained at both 30°C and 10°C, indicating  
585 that lowering temperature is not detrimental to granulation and can even  
586 increase granule size. These results provide new insights that reinforce the  
587 potential of cold anammox applications for mainstream N-removal.

588

589 E-supplementary data of this work can be found in online version of the paper

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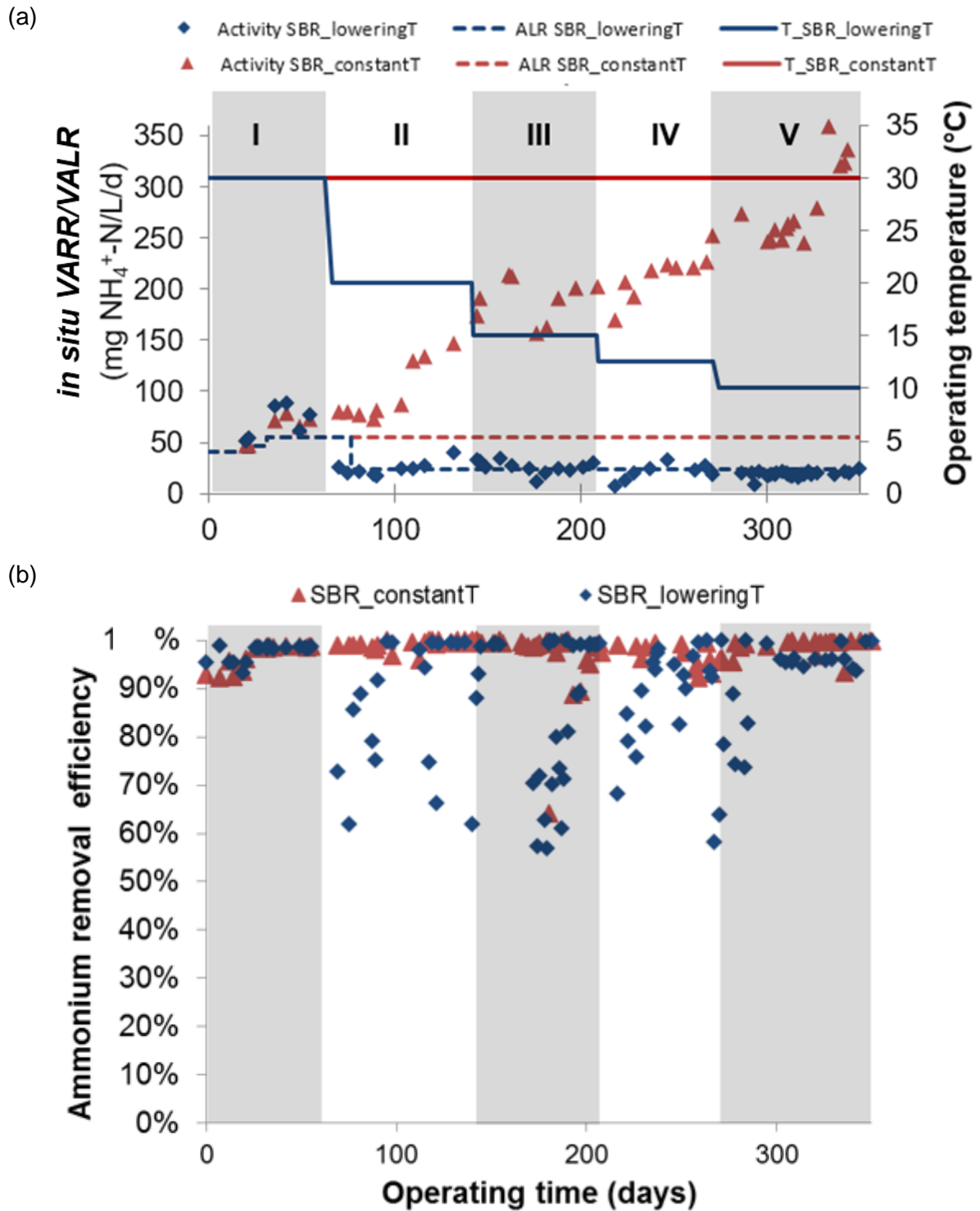


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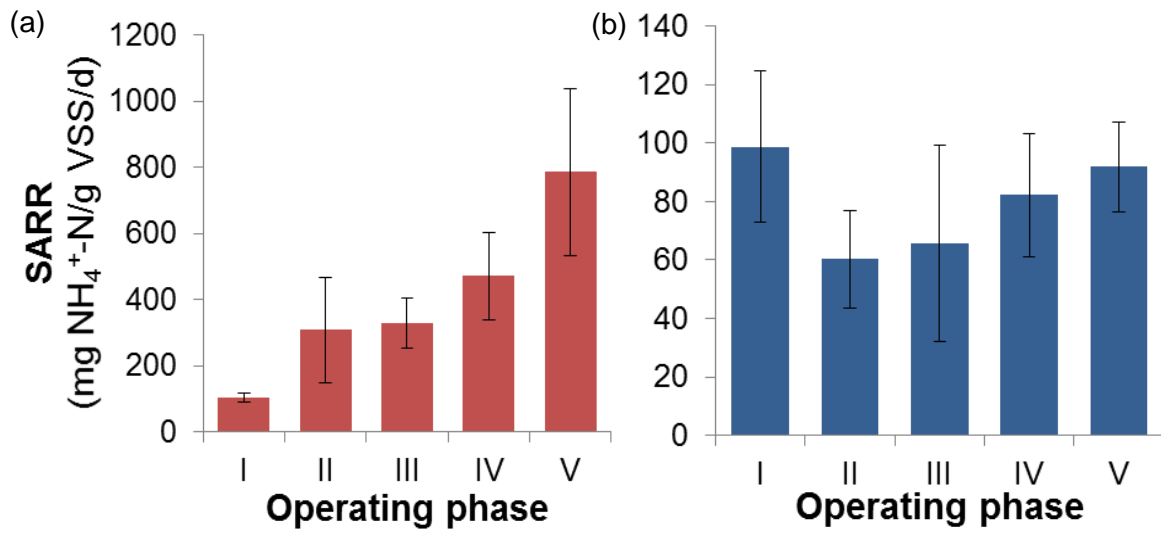
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708 two pilot-scale one-stage anammox reactors. Bioresource Technology 211,  
709 273-279.



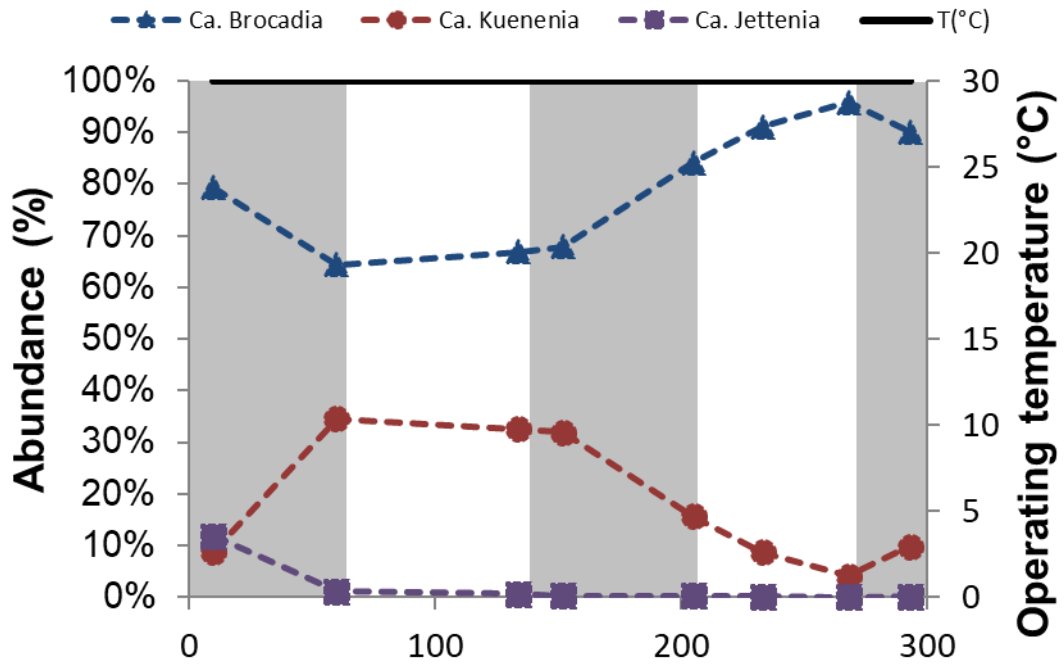
710 **Figure 1** – Evolution of the operating temperature, *in situ* volumetric ammonium removal rate and  
 711 volumetric ammonium loading rate (a) and ammonium removal efficiency (b) in SBR<sub>constantT</sub> (red) and  
 712 SBR<sub>loweringT</sub> (blue).  
 713



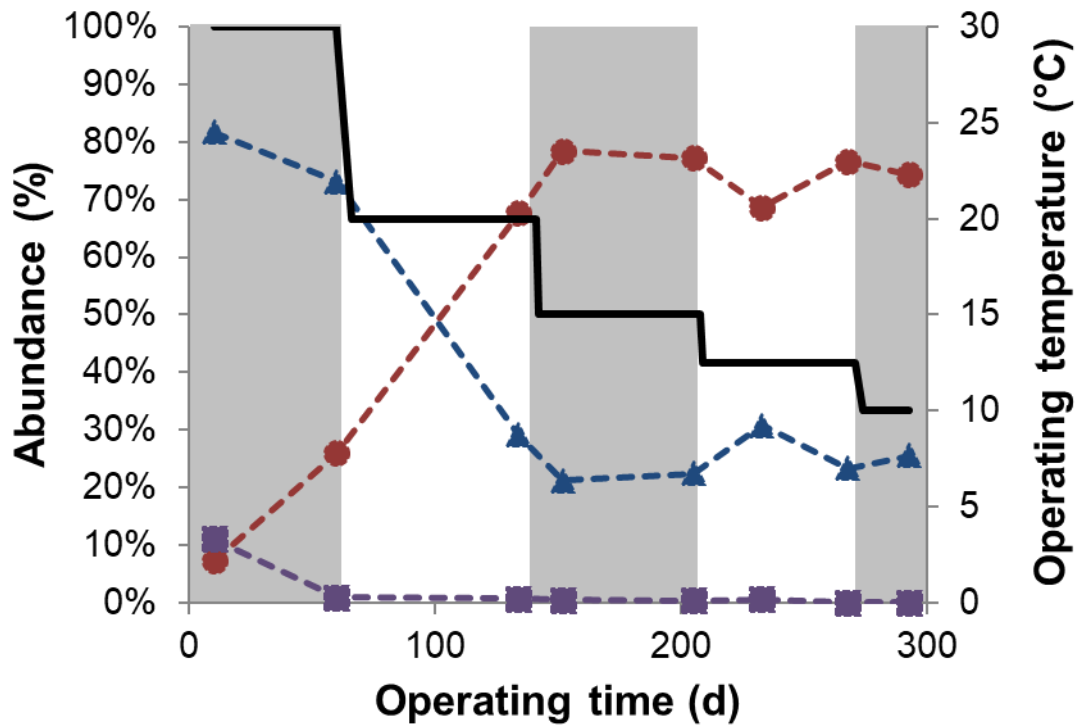
714 **Figure 2** – Average *in situ* specific ammonium removal rates (SARR) at the end of each operating phase  
 715 for SBR<sub>constantT</sub> (a) and SBR<sub>loweringT</sub> (b). The corresponding days at the end of each phase are 62 (I), 141  
 716 (II), 208 (III), 271 (IV) and 349 (V). Error bars represent standard deviations (n≥3)

717

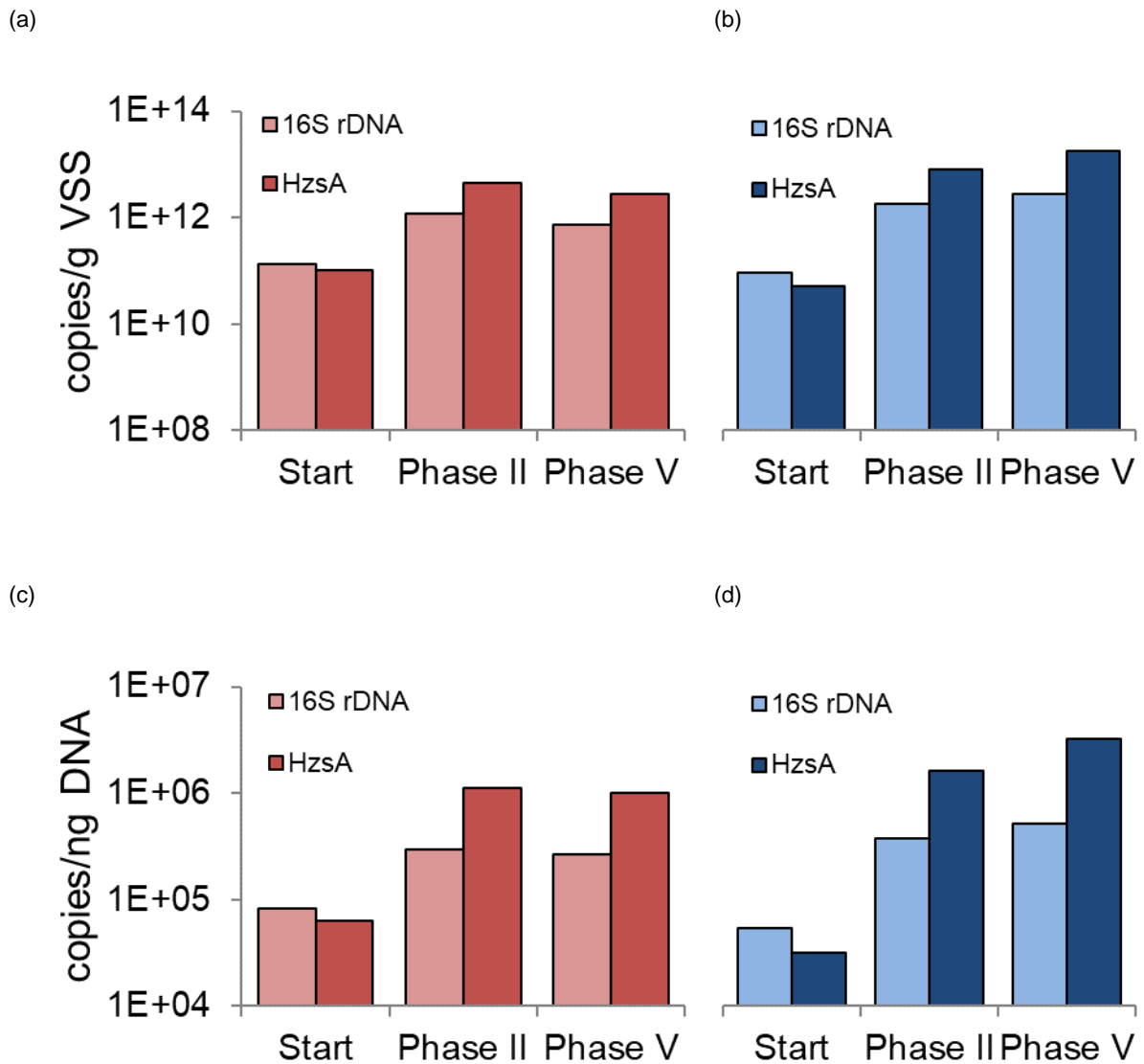
(a)



(b)



718 **Figure 3** – Evolutions within the AnAOB community for SBR<sub>constantT</sub> (a) and SBR<sub>loweringT</sub> (b): relative  
719 abundances of *Ca. Brocadia*, *Ca. Kuenenia* and *Ca. Jettienia* genera, along with the operating  
720 temperature.



721 **Figure 4** - Evolution of overall AnAOB abundance as determined by qPCR analyses based on AnAOB  
 722 16S rDNA (lighter bars) and the HzsA functional gene (darker bars). Top row shows the data expressed  
 723 per g of VSS for SBRconstantT (a) and SBRLoweringT (b). Bottom rows show the same data expressed  
 724 per ng of DNA for SBRconstantT (c) and SBRLoweringT (d). The corresponding sampling times for Start,  
 725 Phase II and Phase V are 10, 134 and 293 days, respectively.

726 **Table 1** - Specific ammonium removal rates (SARR, expressed in mg NH<sub>4</sub><sup>+</sup>-N/g VSS/d) and selected  
 727 influencing factors for anammox based processes at low temperatures (<15°C) and in different reactor  
 728 configurations. Moving Bed Biofilm Reactor (MBRR), Sequencing Batch Reactor (SBR), Partial  
 729 Nitritation/Anammox (PN/A), Chemical oxygen demand (COD)

Process configuration; Process type	Substrates for competitors	T <sub>low</sub> (°C)	Period lowering T (d)	Period at T <sub>low</sub> (d)	SARR	Reference
Granular MBBR/SBR; Anammox	COD	10	-	722	13-19	Hendrickx <i>et al.</i> 2014
SBR; PN/A	O <sub>2</sub>	12	10 (25-12°C)	131	93	Hu <i>et al.</i> 2013
Granular upflow fluidized bed; Anammox	COD	10	127 (20-10°C)	151	35	Lotti <i>et al.</i> 2014b
Granular SBR; Anammox	O <sub>2</sub>	13.2	523 (30.5-13.2°C)	75	15.1	Sánchez Guillén <i>et al.</i> , 2016
Granular SBR; Anammox	-	12.5	208 (30-12.5°C)	63	82	This study
		10	257 (30-10°C)	78	92	

730

731 **Table 2** – Comparison of the short vs. long term effect of temperature decrease on SARR. Between  
 732 brackets the Phase number from the long-term reactor experiment is given. For the short term test,  
 733 biomass was directly exposed to lower temperatures and the data from individual biomass tests were used  
 734 to calculate a weighted average, based on the different biomass contributions in the inoculum of the  
 735 reactor test.

	Short term experiment	Long term experiment	
	Inoculum mix	SBR <sub>constantT</sub>	SBR <sub>loweringT</sub>
SARR 30°C (mg NH <sub>4</sub> <sup>+</sup> -N/gVSS/d)	49.2	104 (I); 471 (IV); 785 (V)	98.7 (I)
SARR 12.5°C (mg NH <sub>4</sub> <sup>+</sup> -N/gVSS/d)	9.99	-	82.2 (IV)
SARR 10°C (mg NH <sub>4</sub> <sup>+</sup> -N/gVSS/d)	6.30	-	91.8 (V)
SARR ratio 12.5°C/30°C (%)	20	-	17*
SARR ratio 10°C/30°C (%)	13	-	12**

736 \* ratio SBR<sub>loweringT</sub>/SBR<sub>constantT</sub> for Phase IV

737 \*\* ratio SBR<sub>loweringT</sub>/SBR<sub>constantT</sub> for Phase V

**Electronic Annex**

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