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Effective dampening of temperature effects in an anammox reactor treating real mainstream wastewater

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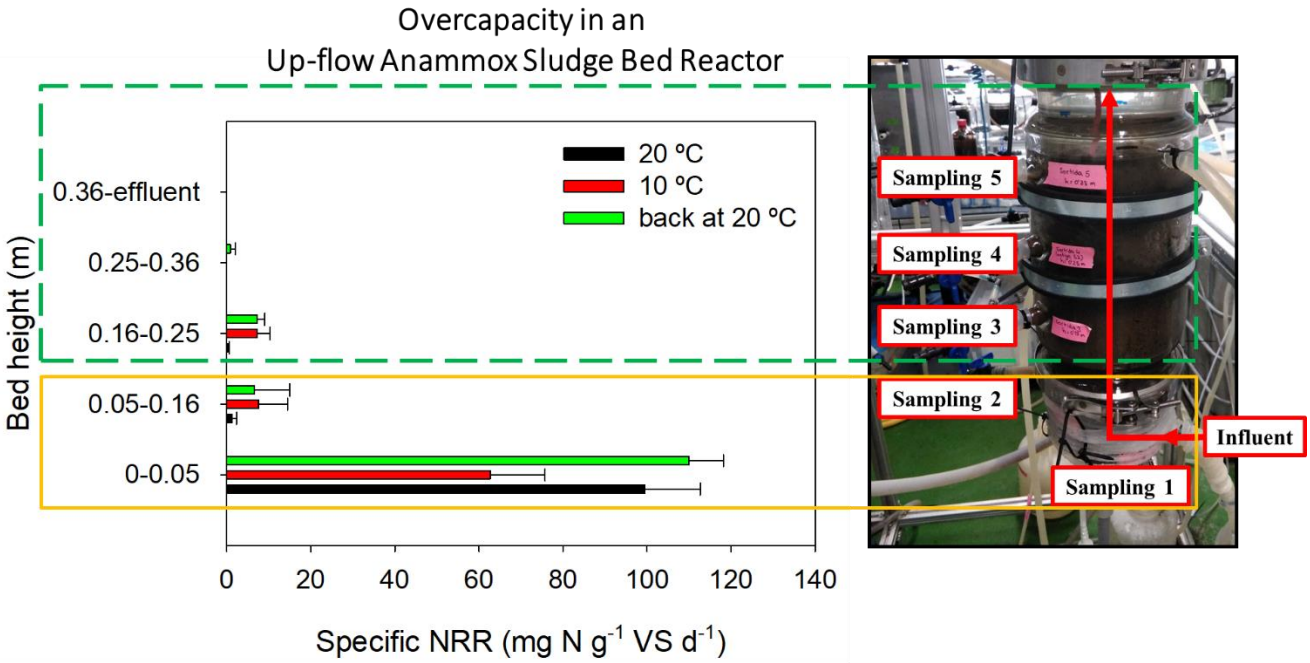
	Taavo Tenno, PhD Tartu University taavo.tenno@ut.ee Taavo Tenno has recently published different research papers related to anammox technologies for the treatment of urban wastewaters.
	Robert Kleerebezem, PhD TU Delft: Technische Universiteit Delft r.kleerebezem@tudelft.nl Robert Kleerebezem has a wide experience on microbial ecosystems and its modelling. He has published a lot of works related to partial nitritation and anammox systems at both lab and pilot-scales conditions.
	Jose Luis Campos, PhD Universidad Adolfo Ibáñez: Universidad Adolfo Ibanez jluis.campos@uai.cl Jose Luis Campos has published a lot of works within the field of partial nitritation and anammox for its application in sidestream and industrial wastewaters. Further, he also

	investigates on aerobic granular sludge and its applications.
Opposed Reviewers:	
Response to Reviewers:	

Highlights

- The up-flow anammox sludge bed reactor is able to treat real mainstream wastewater
- Up-flow anammox sludge bed allows dampening the effect of 10°C temperature drop
- Heterogeneous substrate distribution granted assistance of intra-bed overcapacity
- Intra-bed reactor overcapacity allows constant loading rates over the seasons
- Despite influent COD, anammox conversion dominates over heterotrophic even at 10°C

Graphical abstract



Effective dampening of temperature effects in an anammox reactor treating real mainstream wastewater

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Abstract

The aim of the present study was to evaluate the capability of an up-flow anammox sludge bed reactor (13 L) of dampening a 10 °C temperature drop at mainstream conditions while operating at constant nitrogen loading rates (0.11 ± 0.01 g N L^{-1} d^{-1}). The up-flow anammox sludge bed reactor was fed with an aerobically pre-treated mainstream wastewater. The reactor temperature was controlled as to mimic a (realistic) summer-to-winter transition from 20 to 10 ºC plus a three months period at 10 ºC. During the 350 days of operation, the average nitrogen removal rate remained high (0.10 ± 0.01 g N L^{-1} d^{-1}), indicating that the 10 °C drop did not affect the overall efficiency of the reactor. This can be explained as anammox activity differed among the different sludge bed sections. At 20ºC, anammox activity was mainly located at the bottom of the sludge bed. At 10ºC, the anammox activity decrease of the bottom sludge bed was compensated by an activity increase within the upper sludge bed sections. The contribution of heterotrophic denitrifying activity to the total nitrogen removal rate was assessed to be 3-to-5 times lower than that of anammox, even at 10 °C. Microbial community of 16S rRNA gene-targeted sequencing analyses resulted in the identification of an uncharacterized Planctomycetes in high numbers. This study demonstrated that the low temperatures should not be an obstacle for the feasibility of anammox bacteria in the main water line of an urban wastewater treatment plant.

Keywords

Mainstream anammox, urban wastewater, overcapacity, substrate concentration profiles.

1. Introduction

The implementation of the anaerobic ammonium oxidation (anammox) process at mainstream conditions for nitrogen removal is an attractive alternative to achieve energy neutral (or even energy producing) urban wastewater treatment plants (Kartal et al., 2010). The anammox process has been successfully used to treat side-stream wastewaters (the so called sludge liquor) (Lackner et al., 2014), but it still faces several challenges as to be applied in the main water line of urban wastewater treatment plants (WWTPs). The bottlenecks at mainstream conditions are related to (1) the low temperatures, (2) the low nitrogen concentrations and (3) the presence of organic matter which hinder anammox performance (Kartal et al., 2010; Pijuan et al., 2020). Furthermore, the main challenges are focused on the need to operate at nitrogen removal rates (NRRs) similar to conventional WWTPs (Metcalf & Eddy, 2003) and, at the same time, meeting (or even improving) the strict effluent requirements (<10 mg N L^{-1}) according the European Council Directive 91/271/ECC.

Previous studies pointed out the possibility to implement the anammox process with the so-called one-stage configuration (with partial nitritation and anammox in the same reactor), as it is the most widespread approach for side-stream treatment (Lackner et al., 2014). Some one-stage configurations studies reported high NRRs in a membrane biological bioreactor (MBBR) and in rotating biological contactor (RBC) treating synthetic wastewater but nitrite accumulated and nitrate was produced by nitrite-oxidizing bacteria (NOB) when temperature decreased (De Clippeleir et al., 2013; Gilbert et al., 2015). Interestingly, other studies achieved a good effluent quality working at NRRs close to those reported for conventional WWTPs in one-stage configurations treating an aerobically pre-treated mainstream wastewater in MBBR at 16 and 15 ºC

(Laureni et al., 2019, 2015). However, they experienced an anammox activity suppression at 11 ºC (Laureni et al., 2015) or needed to daily filter and centrifuge the effluent to reintroduce the solids into the reactor to improve floc retention (Laureni et al., 2019), which could not be implemented at full-scale. Despite one-stage systems gained a lot of interest due to the successful attained results at sidestream conditions and the lower investment costs, two-stage systems have been recognized as a good alternative to avoid the associated problems in terms of nitrate production (i.e. NOB proliferation) and of anammox deterioration (Gonzalez-Martinez et al., 2016; Hendrickx et al., 2012; Pérez et al., 2015). In fact, stable partial nitration with an effective NOB repression has been reported (Isanta et al., 2015b; Ma et al., 2011; Poot et al., 2016; Reino et al., 2018, 2016; Soler-Jofra et al., 2019). The advantage when splitting the nitrogen removal into two granular sludge reactors is that stratification of nitrifier guilds is feasible (Poot et al., 2016; Soler-Jofra et al., 2019), with ammonia-oxidizing bacteria (AOB) occupying the external shell of the granules whereas NOB are relegated to deeper layers where oxygen is not available. Additionally, the organic matter not totally converted in the pre-treatment could be also consumed before reaching the anaerobic ammonia oxidation reactor. In the subsequent anammox reactor, then, NOB cannot compete for nitrite with anammox because of the anoxic conditions imposed. Recently, further confirmation of the convenience of using the two-stage nitrogen removal approach has been obtained from shotgun metagenomics. Results obtained indicated that the two stage approach led to the substantial anammox enrichment, making mainstream anammox viable (Annavajhala et al., 2018).

Using the two-stage approach, few studies reported a long-stable anammox operation treating real wastewater at mainstream conditions. Some studies reported high NRRs but

with reactor set-up that required high recirculation flow rates (Lotti et al., 2014), which prevent scaling-up that technology. Another study reported a three-fold anammox activity decrease when temperature was decreased from 20 to 15 ºC treating a municipal wastewater (Laureni et al., 2015). In addition, high NRRs in an up-flow anammox sludge bed (UAnSB) reactor were reported at 11 ºC suggesting that the high concentrations of biomass in this type of reactor helped to face temperature decrease (Reino et al., 2018). However, NRRs decreased when changing from synthetic to an amended pre-treated mainstream wastewater which resulted into accumulation of substrates in the effluent. In all previous studies, it should be stressed that when the effect of low temperatures was studied, nitrogen loading rates (NLR) were decreased as to avoid the accumulation of substrates (ammonium and nitrite) due to the decrease of anammox activity. Thus, according to the state-of-the-art, one of the main challenges in this technology is still to demonstrate that an anammox reactor can effectively dampen the temperature effects in the mainstream line of an urban WWTP under roughly a constant NLR throughout the year. This proof of concept should be carried out by: (i) using real mainstream wastewater, (ii) employing a reactor easy to scale-up, (iii) attaining and maintaining a constant NLR higher than those usually reported for urban WWTPs and (iv) performing a realistic temperature decrease throughout a long-term experiment. Additionally, providing the reason why this technology can achieve these objectives would be a clear step forward in its development.

The UAnSB reactor was operated for almost a year as to mimic a (realistic) temperature change from 20 ºC to 10 ºC (with a period of ca. three months at 10 ºC), by working at a constant loading rate treating a real mainstream wastewater throughout the operation. An in-depth characterization of the reactor operation by evaluating substrate

distribution along the sludge bed reactor was used to assess the variation of anammox activity contribution. The contribution of heterotrophic denitrification activity to the overall reactor performance was also assessed. Additionally, microbiological population analyses assisted the characterization of the microbial community in the sludge bed at 20 and 10 ºC.

2. Materials and methods

2.1. Reactor configuration and operation

A lab-scale UAnSB reactor with an effective working volume of 13 L, excluding the gas-liquid-solid separator phase (12 L), was used for the implementation of the anammox process. The inner diameter of the column was 120 mm and the total-reactor-height to column-diameter ratio was 12:1. The UAnSB reactor of this study was different than the one used in Reino et al. (2018) since the total volume was higher (25 L vs 2 L) and the tube diameter to particle diameter ratio (D/d) was larger (166 vs 70). The change in the reactor design was planned as to minimize potential wall effects, which could lead to a flow maldistribution. The reactor had five different sampling points at heights of 0 m (S1), 0.05 m (S2), 0.16 m (S3), 0.25 m (S4) and 0.36 m (S5) (Fig. 1). The pH of the reactor bulk liquid was not controlled but measured off-line. Temperature was maintained by means of a cooling system connected to the reactor jacket. Reactor temperature was continuously monitored and recorded on-line using a Pt1000 sensor (Axiomatic, S.L, Spain) located in the core of the sludge bed. Different operational periods can be distinguished as a function of the reactor temperature (Table 1).

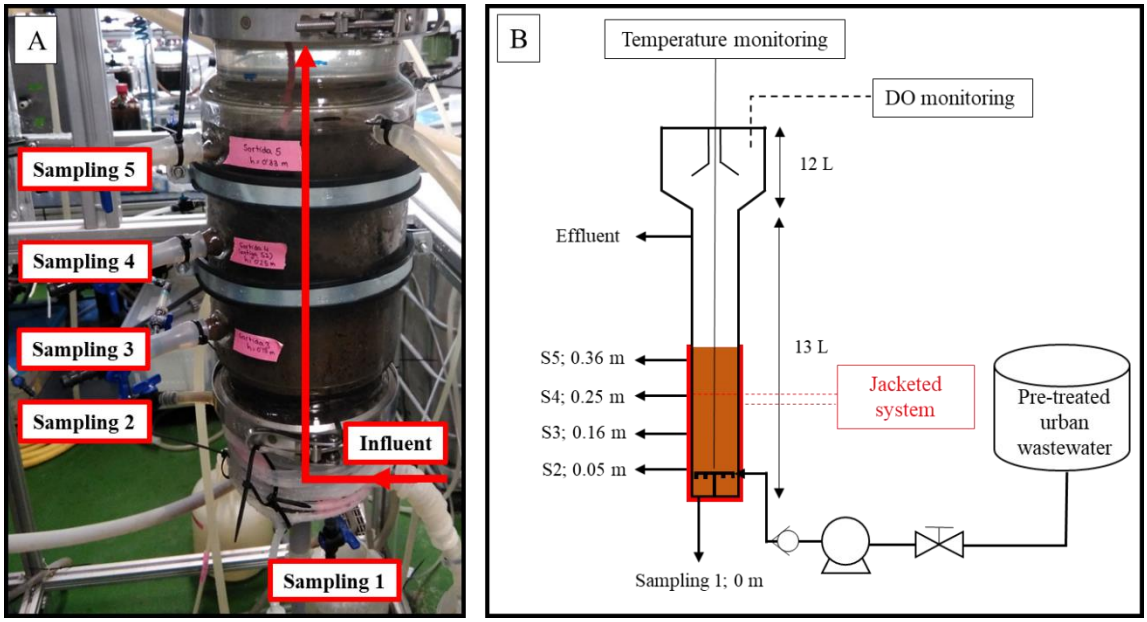

Fig 1. (A) Picture of the lab-scale UAnSB reactor (sludge bed section). **(B)** Schematic diagram of the reactor set-up with the corresponding peripheral instrumentation and sampling points. The fraction of the UAnSB reactor vessel highlighted in solid orange corresponds to the sludge bed. The effective working volume is of 13 L and the gas-liquid-solid separator is of 12 L. Sampling points are denoted as S1, S2, S3, S4 and S5 and the height from the reactor base is provided for each one of them. (For interpretation of the references to colour, the reader is referred to the web version of this article).

2.2. Long-term reactor operation and wastewater characteristics

The UAnSB reactor was inoculated with granular biomass from an anammox SBR operated at 33 ± 1 ºC with synthetic wastewater (Isanta et al., 2015a). The inoculum was enriched in *Candidatus* Brocadia fulgida (65 %) estimated by 16S rRNA gene-targeted sequencing analyses. The inoculum presented a maximum specific anammox activity of 0.14 ± 0.01 g N g VS^{-1} h^{-1}. The anammox granules had an average size of 868 ± 20 µm and the biomass concentration was 4.9 ± 0.3 g VS L^{-1}. After inoculation, the UAnSB reactor was operated for ca. one year at 20 ± 1 ºC (data not shown). The objective of this previous experimental period was to find the best operational strategy to obtain high and stable loading rates together with a good effluent quality without the need of using a recirculation flow-rate. At the same time, it was important to ensure the maintenance of granular sludge throughout the start-up period by controlling the up-flow velocities. The mainstream wastewater used in this study came from an urban WWTP located in an industrial area of Catalonia, NE Spain. This wastewater was a mixture of effluents coming from: (i) a high-rate activated sludge reactor (after primary sedimentation) working at sludge retention times (SRTs) of 1-2 days and (ii) a partial nitritation pilot-scale reactor treating reject water from the dewatering process of the digested sludge. Both effluents were mixed in the appropriate proportion to obtain a pre-treated mainstream wastewater suitable to be added into an anammox reactor. The wastewater was stored in a tank at 20 ± 1ºC, which was refilled every 4 to 5 weeks. The tank was flushed with N_2 every time it was refilled to keep DO concentration in the range of 0.3 to 1.3 mg O_2 L^{-1}. Further information about the characteristics of the mainstream wastewater as well as influent concentrations can be found in Supplementary Information (see Table A.1 and Fig. A.4, respectively).

2.3. Calculations within the UAnSB reactor

NLRs and NRRs were calculated by using the effective UAnSB reactor volume of 13 L, excluding the gas-liquid-solid separator. NRR was calculated as the removal of ammonium and nitrite without considering the nitrate produced in the anammox reaction. However, nitrate production was considered for the quantification of the nitrogen removal efficiency (NRE) to clearly indicate what are the needs for a post-treatment, in case of an eventual full-scale implementation of this technology.

2.4. Concentration of nitrogen compounds and anammox activity at different bed sections of the UAnSB reactor

Ammonium, nitrite and nitrate concentrations were measured along the different sampling points of the UAnSB reactor at steady state conditions to evaluate nitrogen compounds concentrations distribution on the sludge bed. Volumetric and specific NRRs corresponding to the different sludge bed sections delimited by the sampling points were calculated using the measured nitrogen compounds concentrations. Distribution of sampling points and the related calculations can be found in Fig. 1 and in Supplementary Information (A1.3), respectively.

2.5. Heterotrophic denitrification *ex-situ* batch activity test

Ex-situ batch tests were used to assess heterotrophic activity within the sludge bed following the procedures detailed in van Loosdrecht et al. (2016). Tests were carried out in duplicates using septum-closed bottles of 125 mL with 2.0 ± 0.3 g VS L^{-1} from biomass of sampling S3. Biomass from sampling S3 was chosen to carry out the experiment since, because of the plug-flow reactor configuration, it was likely that the reduction of the produced nitrate by the anammox process was taking place within middle and upper sludge bed sections (see section 3.2 in Discussion). All bottles and substrates were flushed

with N_2 to maintain anoxic conditions. Tests were conducted at 180 rpm and at 20 ºC. Three sets of experiments were performed: (1) the consumption of nitrite or nitrate without the presence of any external C-source to evaluate heterotrophic denitrification by organic matter from biomass decay, (2) the consumption of nitrite and/or nitrate in the presence of acetate as an easily biodegradable carbon source under non-limiting chemical oxygen demand (COD) concentrations and (3) the consumption of nitrite and/or nitrate in the presence of acetate as an easily biodegradable carbon source under limiting COD concentrations. Sampling time depended on the consumption rate of substrates in each experiment. Substrate consumption velocities were calculated by linear regression of the nitrite or nitrate concentrations of three to six bulk liquid-phase grab samples. The employed COD/N ratios and the detailed calculations be found in Supplementary Information (Table A1.2 and section A1.4, respectively).

2.6. Analytical methods

Liquid samples from influent and effluent of the UAnSB reactor were analysed 3 to 4 times per week. Samples were filtered (0.22 μm) before analysis. Nitrite and nitrate concentrations were analysed off-line with ionic chromatography using ICS-2000 Integrated Reagent-Free IC system (DIONEX Corporation, USA). Ammonium concentrations were analysed off-line by means of a gas selective electrode (GSE) (AMTAX sc, Hach Lange, Germany). For concentration profiles and for *ex-situ* batch activity tests, concentrations of ammonium and nitrite were determined by colorimetric Hach Lange kits (LCK303 and LCK342, respectively, Hach Lange, Germany). Soluble COD was analysed by using kits ranging from 0 to 1500 mg COD L^{-1} (Scharlab, Spain). Total solids (TS) and volatile solids (VS) concentration were analysed according to Standard Methods (APHA, 2005). Average biomass particle size was measured by a laser

diffraction analysis system (Malvern Mastersizer Series 2600, Malvern instruments Ltd., UK).

2.7. Microbiological quantification

Microbiological compositions were identified by using next-generation sequencing analysis. DNA was extracted from three different sampling points (S1, S3 and S5) by using the Soil DNA Isolation Plus KitTM (Norgen Biotek Corp, Canada) following the manufacturer protocol. The quantity and quality of the extracted DNA was measured by using the NanoDrop 1000 Spectrophotometer (Thermo Fischer Scientific, USA). A 260/280 nm ratio of 1.8 ± 0.1 was used as quality cut-off and a minimum of 20 ng μL^{-1} of extracted DNA was guaranteed to perform sequencing. Paired-end sequencing of the extracted DNA was performed on an Illumina MiSeq platform by the Research and Testing Laboratory (Lubbock, Texas, USA). Bacterial 16S rRNA variable regions V2-V4 were targeted using the primer pair 515F-806R for general bacteria and the specific primer pair 368F-820R for the anammox population. Additional information of the bioinformatics protocol can be found in Supplementary Information (A1.5).

3. Results and discussion

3.1. Performance of the UAnSB reactor during a summer-to-winter transition period by treating a real mainstream wastewater

3.1.1. UAnSB reactor operation

The UAnSB reactor was operated by maintaining a constant NLR (0.11 ± 0.01 g N L^{-1} d^{-1}) for 350 days (Table 1). Reactor temperature was controlled to mimic a realistic summer-to-winter transition period as reported to happen in urban WWTPs operated in temperate climates decreasing from 20 to 10 ºC in ca. 3 months (slightly more pronounced than that described in Gilbert et al. (2014)). In addition, the reactor operation was

maintained at 10 ºC for c.a. 3 months to evaluate long-term effects of such low temperature in the anammox process. Overall, in the 350 days period the reactor performance was high; in average, the NRR was 0.10 ± 0.01 g N L^{-1} d^{-1} and an NRE of 83 ± 8% (see the overall performance in "all periods" row, Table 1 and Fig. 2). These results indicate that the UAnSB reactor was able to perform at rather constant loading rates by maintaining high NRRs during a realistic summer-to-winter temperature decrease (Fig. 2). Besides the capacity of the UAnSB reactor to withstand the temperature change as well as the cold temperatures, it is important to highlight that the average NRR achieved (0.10 ± 0.01 g N L^{-1} d^{-1}) was ca. two times higher than that reported for conventional urban WWTPs (Metcalf & Eddy, 2003).

In addition to the overall achievements already highlighted, a more detailed analysis of the experimental data is here also discussed. The reactor operation period was divided into several phases (Table 1). For temperatures between 20 and 12°C (periods I and II), the removal of nitrogen at high rates was achieved as reflected by the high NREs of 87 ± 4 %. During period III (10°C), an unintended increase in loading rates resulted in a slight decrease in NREs efficiencies up to 71 ± 6 %. The lack of data between days 182 and 224 corresponds to technical problems in the analysis of nitrogen species. In period IV, temperature was increased back to 20 ºC to proof the stability of the anammox process after long exposure to cold temperatures. During period IV, the UAnSB was operated at similar NLR to those of period III attaining NREs of 78 ± 8 % (Table 1). Changes in influent concentrations occurred throughout the operation due to variability of the real wastewater, which resulted into variations of the nitrite to ammonium feeding ratios. Interestingly, the decrease in NREs during period III and IV were related with the high nitrite to ammonium ratio fed into the reactor which led to an excess of nitrite

concentrations in the influent (Table 1), worsening the performance of the process (see Effluent quality section for further details).

3.1.2. Effluent quality

Ammonium, nitrite, nitrate and total nitrogen concentrations in the effluent throughout the study were plotted in Fig. 2. The total average effluent nitrogen concentration was slightly higher (11 ± 4 mg N L^{-1}) than legal discharge limits according to EU legislation (i.e. 10 mg N L^{-1}) (Table 1). The UAnSB reactor was almost able to provide a suitable effluent for an urban WWTP according to the European legislation. Nevertheless, further strategies should be implemented to guarantee a good effluent quality. One option might consist of optimizing the previous partial nitritation stage to provide a suitable nitrite to ammonium ratio for the subsequent anammox process (Guo et al., 2020; Pérez et al., 2015). In fact, the results of this study pointed out that a non-suitable nitrite to ammonium feeding ratio had a direct effect on the effluent quality of the UAnSB reactor. This was especially important on periods III and IV, where rather high total nitrogen concentrations were detected in the effluent (15 ± 4 mg N L^{-1} and 11 ± 4 mg N L^{-1}, respectively) especially in the form of nitrite (7 ± 4 mg N L^{-1} and 5 ± 4 mg N L^{-1}, respectively) (Table 1). Further data concerning the nitrite to ammonium feeding ratios can be found in Fig. A.2 of Supplementary Information. In addition, a post-treatment by adding an external C-source to enhance heterotrophic denitrification via nitrate could also be considered according to the overall effluent nitrate concentrations of 5 ± 3 mg N L^{-1} (Table 1) (Li et al., 2020). Interestingly, another reason for the high nitrogen concentrations in the effluent could be related with preferential ways or to

external mass transfer limitations, although with the present analysis this remains speculative.

Table 1. Operational parameters for the different periods I, II, III, IV and V. NLR: Nitrogen Loading Rate; NRR: Nitrogen Removal Rate; NRE: Nitrogen Removal Efficiency. *Temperature is presented in accordance of the temperature range from 20 to 10 ºC for the whole operation.

Period	Time lapse (days)	Temperature (ºC)	NLR (g L^{-1} d^{-1})	NRR (g L^{-1} d^{-1})	NRE (%)	Total effluent nitrogen (mg N L^{-1})	Ammonium effluent (mg N L^{-1})	Nitrite effluent (mg N L^{-1})	Nitrate effluent (mg N L^{-1})	Nitrite consumed/ ammonium	Nitrate produced/ ammonium consumed	Nitrite/ Ammonium influent	Up-flow velocity (m h^{-1})
all periods	350	15 ± 5*	0.11 ± 0.01	0.10 ± 0.01	83 ± 8	11 ± 4	3 ± 3	3 ± 3	5 ± 3	1.4 ± 0.2	0.19 ± 0.05	1.4 ± 0.2	0.08 ± 0.02
I (0-81)	81	20	0.10 ± 0.02	0.10 ± 0.02	87 ± 3	10 ± 3	2 ± 2	2 ± 2	6 ± 2	1.4 ± 0.1	0.20 ± 0.03	1.3 ± 0.2	0.07 ± 0.01
II (81-155)	74	19 to 12	0.09 ± 0.01	0.09 ± 0.01	86 ± 5	9 ± 2	4 ± 2	1 ± 1	7 ± 2	1.3 ± 0.1	0.23 ± 0.04	1.3 ± 0.1	0.07 ± 0.02
III (155-260)	105	10	0.11 ± 0.01	0.09 ± 0.01	71 ± 6	15 ± 4	4 ± 2	7 ± 4	4 ± 2	1.5 ± 0.2	0.20 ± 0.05	1.4 ± 0.2	0.10 ± 0.01
IV (255-350)	90	20	0.11 ± 0.02	0.09 ± 0.02	78 ± 8	11 ± 4	3 ± 2	5 ± 4	3 ± 2	1.4 ± 0.2	0.10 ± 0.01	1.4 ± 0.2	0.10 ± 0.01

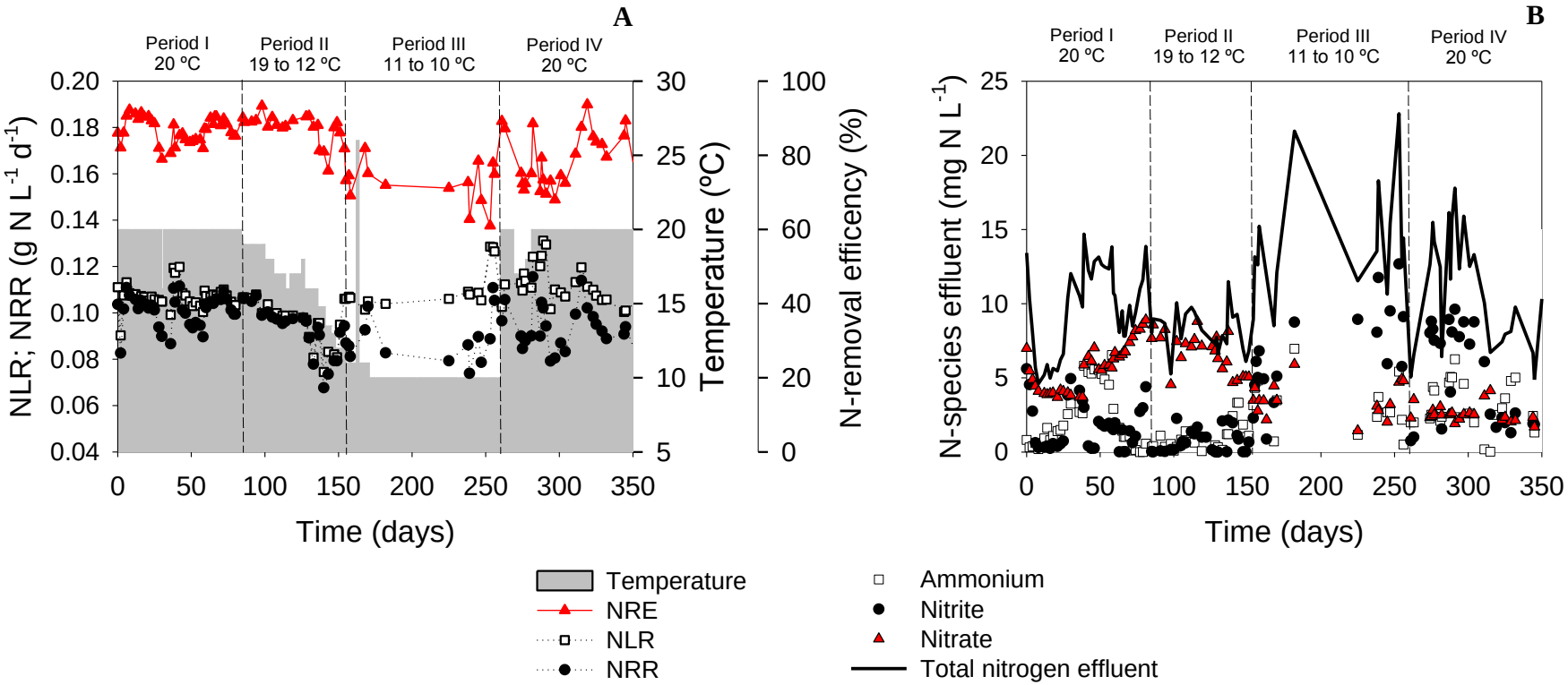

Fig. 2. Long-term operation of the UAnSB reactor at mainstream conditions at different temperatures. **(A)** Nitrogen loading and removal rates (NLR and NRR, respectively) and nitrogen removal efficiencies (NRE). **(B)** Effluent concentrations of ammonium, nitrite and nitrate. Total nitrogen concentrations in the effluent refer to the sum of ammonium, nitrite and nitrate.

3.1.3. Solids, granule size and up-flow velocity

Throughout the reactor operation period, the average biomass concentration was 14 ± 5 g VS L^{-1} with a VS/TS ratio of 0.4 ± 0.1. Mean average size of the granules was maintained stable throughout the operation within the different sampling points (see Fig. A.5 in Supplementary Information) despite working at low up-flow velocities (0.08 ± 0.02 m h^{-1}) and at temperatures as low as 10 ºC (Table 1). It should be emphasized that the up-flow velocities of periods III and IV were slightly higher than those of period I and II (Table 1). Maintaining roughly constant loading rates throughout the operation was challenging since variations in influent concentrations had to be correspondingly compensated by manipulating the influent flow rate (Fig. A.4, Supplementary Information). Thus, this resulted into slight variations of the applied up-flow velocities.

The obtained results are in contrast to those reported by Reino and Carrera (2017), who suggested to operate at up-flow velocities higher than 0.4 m h-1 in an UAnSB reactor to maintain granulation. Interestingly, the applied up-flow velocities within the UAnSB reactor were significantly lower than those conventionally applied to up-flow anaerobic sludge bed (UASB) reactors used in anaerobic digestion (0.7 m h^{-1}) (Metcalf & Eddy, 2003). The reasons for the stability of the granule structure (in average 717 ± 43 µm, see Fig. A.5 in Supplementary Information) were probably linked to the reactor design used. The rather high H/D ratio (12:1) selected could have been favorable to preserve granule morphology and, thus, avoid biomass loss. The obtained results are in accordance with different studies that reported granule stability at low temperatures within an UAnSB reactors (He et al., 2018; Ma et al., 2013; Wang et al., 2020), while granule deterioration occurred in an SBR-anammox reactor (Sánchez-Guillén et al., 2016). This is a significant fact since our results indicate that anammox granulation can be maintained without the

need of applying high up-flow velocities, which can only be achieved by applying high recirculation flow rates, and thus, resulting into unattainable operation costs at mainstream conditions.

3.2. Heterogeneous distribution of the anammox activity in the sludge bed as the key point to face low temperatures at mainstream conditions

The anammox activity within the different sludge bed sections was calculated using the nitrogen compounds concentrations measured along the sampling points of the UAnSB reactor. These activities were quantified as the specific NRR achieved in each sludge bed section and they were calculated under steady state conditions at periods I (20ºC), III (10ºC) and IV (back to 20ºC) (Figure 3).

At 20 ºC (period I), the specific NRR associated to the S1-S2 sludge bed section (100 ± 14 mg N g^{-1} VS L^{-1}) (see Figure 1 for a detailed situation of this section in the UAnSB) was ca. 50 times higher than those specific NRRs determined for upper sludge bed sections (< 4 mg N g^{-1} VS L^{-1}) (Fig. 3). This fact is correlated with the almost complete absence of nitrite from section S4 onwards, resulting into substrate limitation for anammox biomass of upper sludge bed sections (Fig. 3). Thus, there was a significant amount of biomass not contributing to the overall anammox activity at high temperatures.

At 10 ºC (period III), the specific NRR from sludge bed section S1-S2 showed a significant decrease (63 ± 14 mg N g^{-1} VS L^{-1}) from that obtained at 20 ºC, resulting into a higher substrate availability for upper sludge bed sections (Fig. 3). The increase of substrate availability resulted into an activation of the starved anammox biomass of middle and upper sludge bed sections. Consequently, almost a 10-fold increase in the specific NRR was detected within sludge bed sections S2 onwards (ranging from 4 to 14

mg N g^{-1} VS L^{-1}) (Fig. 3). Therefore, the biomass activity decrease of the bottom sludge bed section (from 20 to 10 ºC) was counterbalanced by the activity increase of middle and upper sludge bed sections at 10 ºC. That means that the sludge bed of the UAnSB reactor presented an overcapacity that allowed to dampen a 10 ºC temperature drop while operating at high and constant nitrogen loading rates.

When temperature was increased back to 20 ºC (period IV), the specific NRR within S1-S2 sludge bed section increased up to 110 ± 9 mg N g^{-1} VS L^{-1} (Fig. 3), in the range of those of period I, confirming that activity within the bottom sludge bed was fully recovered after a long-period exposure to low temperatures. However, middle and upper sludge sections (from S2 to S4) did not decrease their specific NRR but maintained in the range to those values obtained at 10 ºC (Fig. 3). This might be related to the fact that the applied NLRs of period IV were slightly higher than the ones applied in period I (Table 1). This was translated into lower NREs and, thus, into higher residual ammonium and nitrite concentrations to middle and upper sections of the anammox sludge (Fig. 3) Further, the nitrite to ammonium stoichiometry ratios within top sludge sections of period IV were more favourable for the anammox reaction than the ones of period I (i.e. where nitrite was limiting), and this resulted into a higher specific activity within upper sludge bed sections.

The significant amount of biomass concentration (14 ± 5 g VS L^{-1}) of the sludge bed and the low up-flow velocities applied in the UAnSB reactor resulted into a heterogeneous substrate distribution along the sludge bed; that is, into a plug-flow substrate hydrodynamic pattern. Different authors suggested that this hydrodynamic pattern could limit the practical application of an UAnSB reactor because it could lead to have a pull of biomass exposed to substrate limitation, and thus, to a lower reactor performance (Ma

et al., 2017; Strous et al., 1998). However, the overall NRR of the UAnSB did not decrease throughout the operation but maintained rather constant even after a long-term exposure to low temperatures (Fig. 1), in contrast of what should have been expected from the temperature dependence of the anammox reaction rate (Hendrickx et al., 2012; Lotti et al., 2015; Sobotka et al., 2016). Therefore, the heterogenous substrate concentrations gradients within the anammox granular sludge bed granted the assistance of a sludge bed overcapacity that has been crucial to dampen a 10 ºC drop by working at high and constant loading rates.

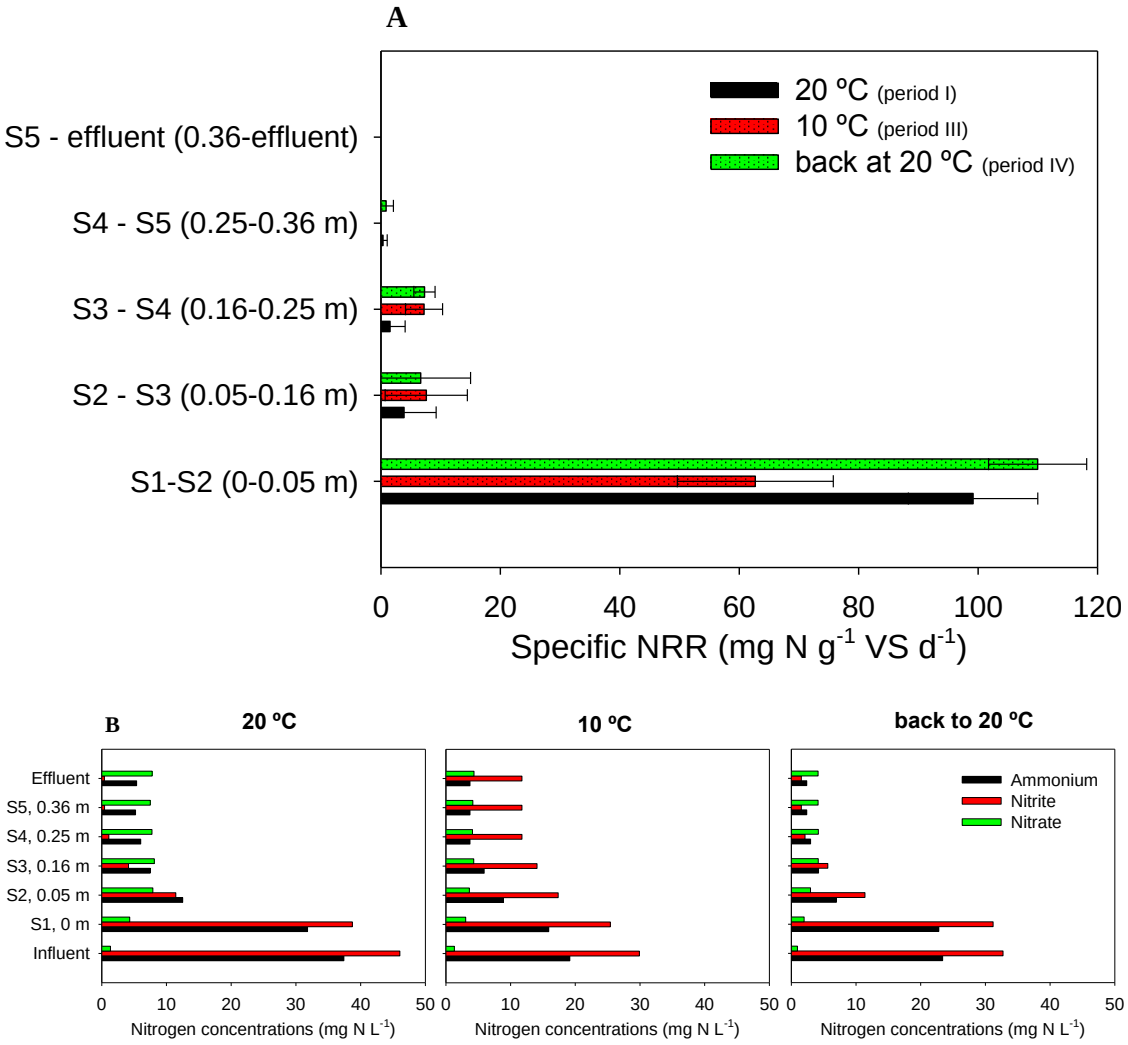

Fig. 3. (A) Average specific nitrogen removal rates (NRRs) and **(B)** nitrogen compounds concentrations corresponding to different sludge bed heights sections of the UAnSB reactor delimited by the sampling points (see Fig. 1) at different operation periods and temperatures. Nitrogen concentrations at 20 ºC, 10 ºC and back at 20 ºC correspond to days 42, 239 and 282, respectively. (For interpretation of the references to colour, the reader is referred to the web version of this article).

3.3. Heterotrophic denitrifying activity within the UAnSB reactor

The mainstream wastewater treated in this study contained an organic matter concentration of 60 ± 14 mg COD L^{-1} and a COD/N ratio of 1.0 ± 0.1 mg COD mg^{-1} N. Throughout the operational period, a COD removal efficiency of 60 ± 16 % was achieved in the UAnSB reactor. This result is in accordance with nitrogen mass balances of the UAnSB reactor (see. Fig. A.3 in Supplementary Information), which indicated a contribution of heterotrophic denitrification (5 ± 5 %) in the total nitrogen removal. Likewise, throughout the operational period, the produced nitrate to consumed ammonium ratio (Table 1) was lower than the proposed by Strous et al. (1998), indicating that part of the produced nitrate by anammox was being heterotrophically denitrified.

To characterize the heterotrophic activity of the sludge bed, *ex-situ* heterotrophic batch activity tests were performed using nitrite and/or nitrate at different COD/N ratios with sludge from sampling S3 (see Table A.2 in Supplementary Information). The heterotrophic denitrifying activities from decay products (i.e. no without addition of an external C-source) were hardly detected while the activities achieved with the addition of acetate as external C-source ranged from 3 to 13 mg N g^{-1} VS d^{-1} depending on the electron acceptor (nitrite or nitrate) and on the employed COD/N ratio (Fig. 4). At low COD/N ratios, the heterotrophic activities achieved with nitrite, nitrate or a mix of both electron acceptors were almost the same (Fig. 4), indicating that heterotrophic bacteria did not present a preference for any of these two electron acceptors under limiting COD conditions. However, at high COD/N ratios, the heterotrophic activity achieved with nitrate was almost twice that achieved with nitrite (Fig. 4), indicating that heterotrophic bacteria seem to prefer nitrate under non-limiting COD conditions. This fact is interesting as it could enhance effluent quality by reducing part of the produced nitrate without

competing with anammox for nitrite. Heterotrophic bacteria could outcompete anammox bacteria due to their higher growth rates (Güven et al., 2005; Lackner et al., 2008; Leal et al., 2016; Molinuevo et al., 2009; Pijuan et al., 2020). Further, the presence of several organic compounds present in real wastewaters may hinder anammox activity (Güven et al., 2005; Molinuevo et al., 2009). However, in this study, the detected heterotrophic denitrifying activities were five to three times lower than the overall NRRs measured within the UAnSB reactor regardless the reactor temperature (i.e., at 20 or 10 ºC) (Fig. 4), even under non-limiting COD concentrations, which are supposed to enhance heterotrophic denitrifying activity (van Loosdrecht et al., 2016). Therefore, anammox activity dominated the overall nitrogen conversion while heterotrophic denitrifying activity slightly contributed to improve effluent quality by mainly decreasing the nitrate concentrations produced by the anammox process.

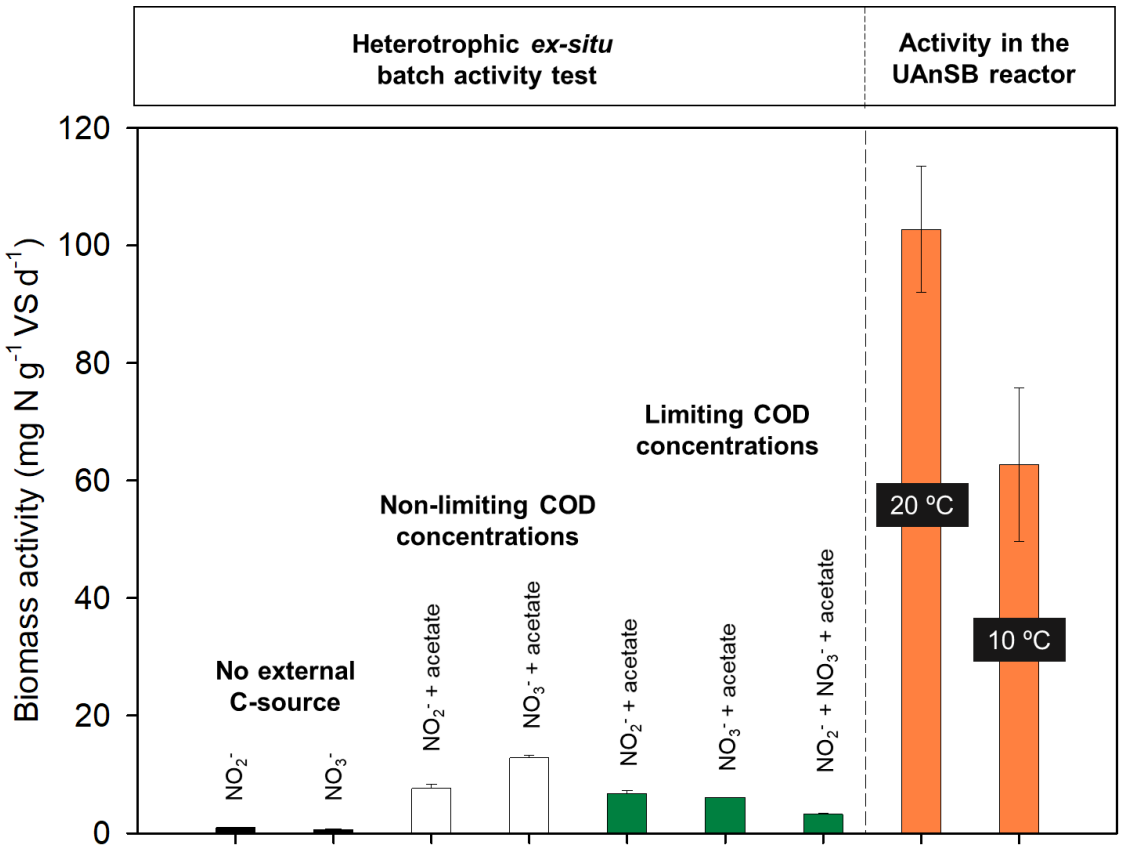

Fig. 4. Specific heterotrophic denitrifying activities at different COD/N ratios from *ex-situ* batch tests conducted at 20 ºC employing biomass from sampling S3. Average specific nitrogen removal rates (NRRs) within the UAnSB reactor at 20 and 10 ºC are also depicted. The applied COD/N ratios and the operational days of each batch test can be found in Supplementary Information (Table A.2).

3.4. Microbiological characterization

The microbial community composition was studied by 16S rRNA gene-targeted sequencing analyses when UAnSB reactor reached steady state conditions. Six different libraries of reactor sampling points S1, S3 and S5 (see Figure 1 for a detailed situation of these sampling points) at operational days 17 (20 ºC) and 203 (10 ºC) were constructed. The total number of sequences for each library after quality analysis and removal of low-quality sequences can be found in Supplementary Information (see. Fig. A.7). All libraries presented an average length of 290 bps per sequence.

The microbial community for both temperatures and for the three sampling points was dominated by species inside the Planctomycetes phylum, ranging from 12 to 36 %. The unclassified Planctomycetes sequence was run against BLAST and matched with an uncultured Planctomycetes (MG099764) found in a CANON bioreactor acclimated from high to low temperatures (Gonzalez-Martinez et al., 2016). The genus *Candidatus* Brocadia was also identified at both temperatures and at different reactor sampling points with an abundance from 5 to 19 %. Accordingly, the results of the specific primer used to identify the different species among the anammox population pointed out that the major abundance corresponded to the *Candidatus* Brocadia genus, while an increase of *Candidatus* Kuenenia was observed at low temperatures (Fig. 5). These results agree with the experimental observations indicating that the anammox process dominates the nitrogen conversions in the reactor.

In addition, different abundances of heterotrophic denitrifying bacteria as *Ignavibacterium* (Chlorobi phylum) (8 to 14 %) and of Caldilineales order (4 to 5 %) were detected at 20 ºC at all the different sampling points. Also, a small abundance of other Chloroflexi (3 to 5 %) was detected at 20 ºC. Interestingly, the decrease of

temperature seemed to decrease the percentage of heterotrophic denitrifying bacteria whereas the percentage of anammox bacteria increased. This is in agreement with He et al. (2018) who found a higher abundance of Planctomycetes after decreasing temperature from 33 to 13 ºC in an UASB reactor, pointing out that anammox microbes had been adapted to cold temperatures.

The obtained sequencing analyses are in accordance with other studies where a community of heterotrophic denitrifying bacteria were detected within anammox reactors. In fact, it seems that Chlorobi bacteria may degrade and catabolize extracellular peptides bound in the EPS matrix of anammox bacteria while respiring the nitrate produced by anammox bacteria (Lawson et al., 2017). Similarly, a high abundance of *Ignavibacterium* was found after analysing different one- and two-stage partial nitritation/anammox (PN/AMX) reactor configurations at main and side-stream conditions (Annavajhala et al., 2018). Likewise, it was found that the second highest abundance in a full-scale partial-nitritation-anammox reactor corresponded to Chlorobi bacteria, indicating that these organisms could cooperate with Brocadia *sp* in anammox wastewater treatment systems (Speth et al., 2016). The coexistence of uncultured Chloroflexi bacteria was also found in anammox reactors fed with synthetic medium (i.e. no external organic matter), pointing out that these bacteria degrade and use cellular components produced by anammox bacteria while reducing nitrite and/or nitrate (Kindaichi et al., 2012). As suggested by literature, the hydrolysis of extracellular compounds bound in the extracellular polysaccharides (EPS) of anammox bacteria by Chlorobi and Chloroflexi bacteria could provide short-chain volatile fatty acids (VFAs) and alcohols which would support other microbial communities as Proteobacteria (Bhattacharjee et al., 2017). Those findings would support the obtained results found in

this study since a significant abundance of Proteobacteria (7 to 10 %) and more specifically of *Denitratisoma* genus (7 to 8 %) was detected at 20 ºC, while its abundance decreased at 10 ºC. *Denitratisoma* is able to grow using different fatty acids using nitrate as electron acceptor (Fahrbach et al., 2006). As mentioned, different heterotrophic denitrifying bacteria can grow from decay products or hydrolysis of EPS which might be enhanced by the residual COD concentration from the pre-treated mainstream wastewater. As suggested by previous reports, the metabolic contribution of denitrifying bacteria in terms of the nitrogen cycle is expected to be from nitrate. These would be in accordance with the operational results of the UAnSB reactor where a COD consumption together with a lower yield of nitrate produced to ammonium consumed was observed (Table 1), indicating that nitrate reduction was taking place.

Despite the UAnSB reactor presented high diversity according to biological diversity indices (see. Fig. A.6 in Supplementary Information), high NREs were attained throughout the operation. Similarly, a two times higher anammox bacteria abundance was found in a suspended than in an attached anammox reactor although both reactors presented similar NREs, suggesting that engineering reactors do not need to be highly enriched in anammox bacteria to achieve high removal efficiencies, endorsing the feasibility of mainstream anammox at full scale conditions (Bhattacharjee et al., 2017). Additionally, biological diversity indices as well as microbial communities did not present significant differences among the different sludge bed sections. This agrees with the reported UAnSB sludge bed reactor overcapacity, which indicated that anammox activity persisted within middle and upper sludge bed sections despite a long-term exposure to starvation conditions.

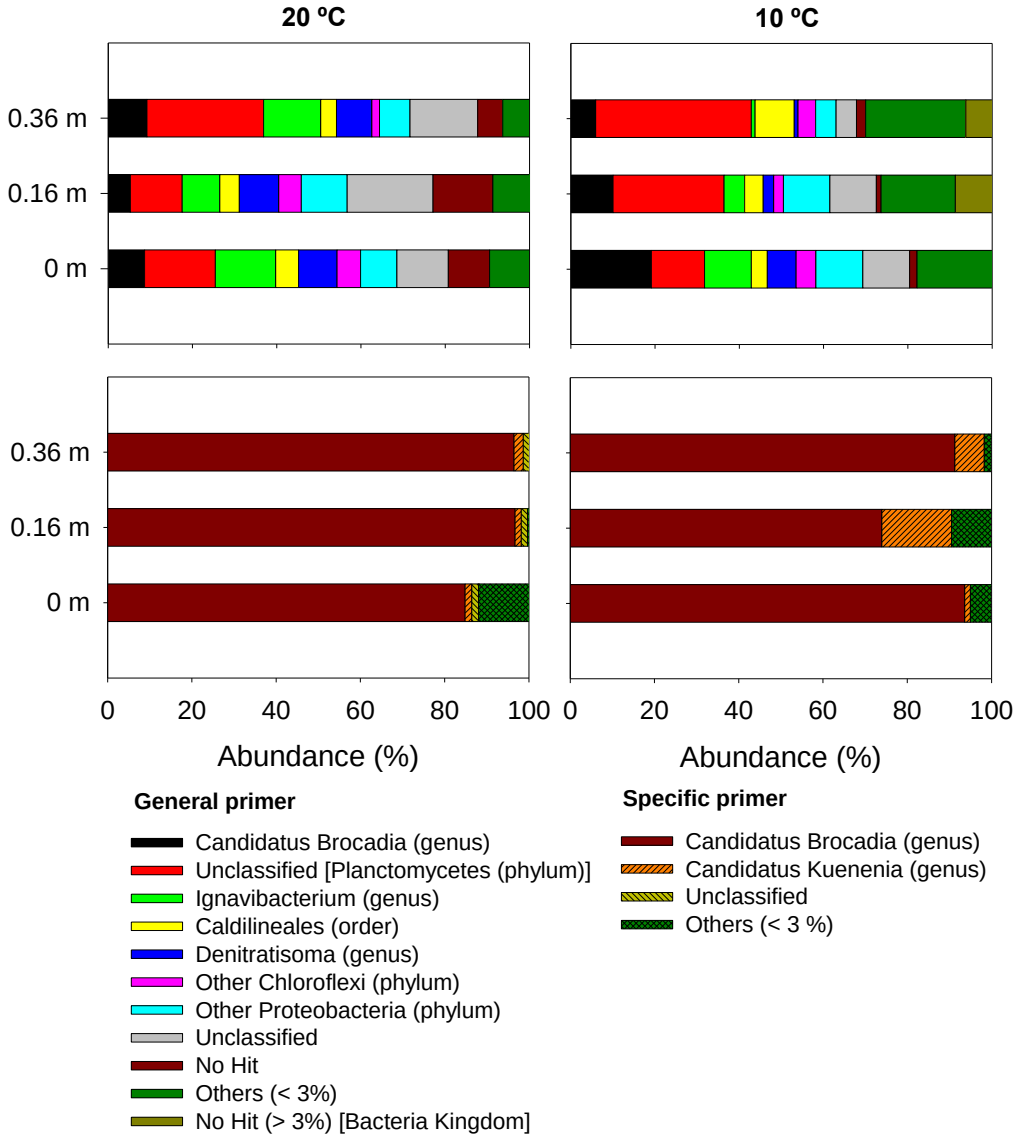

Fig. 5. Microbial diversity using the 515F-806R primer (top) and the specific primer 368F-826R (bottom) of libraries at 20 ºC (day 17) and at 10 ºC (day 203) within the different UAnSB reactor heights 0 m (S1), 0.05 m (S3) and 0.36 m (S5). Relative abundance was calculated only considering those microorganisms in which the number of 16S copies was higher than 3 % of the total copies. (For interpretation of the references to colour, the reader is referred to the web version of this article).

4. Conclusions

- An UAnSB reactor treating mainstream wastewater fed at nearly constant loading rates (0.11 ± 0.01 g N L^{-1} d^{-1}) fully damped the effects of a 10ºC temperature drop, by roughly maintaining stable removal rates (ca. 0.10 ± 0.01 g N L^{-1} d^{-1}).
- The anammox activity differed along the UAnSB reactor, being concentrated at the bottom sludge bed section, yielding heterogeneous substrate profiles along the sludge bed.
- The biomass not contributing to the overall activity at high temperatures (i.e., the so-called reactor overcapacity) assisted to maintain high nitrogen removal rates at low temperatures (ca. 10ºC, three months).
- Due to the presence of COD in the mainstream wastewater, heterotrophic denitrification activity was detected in the sludge bed. However, anammox activity dominated the overall nitrogen conversion, even at low temperatures.

Acknowledgments

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Appendix A.

Supplementary Information of this article can be found in Appendix A.

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Appendix A. Supplementary Information

Effective dampening of temperature effects in an anammox reactor operated on real mainstream wastewater

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A1. Materials and methods

A1.1. Wastewater characteristics

Table A.1. Characterization of the real mainstream wastewater of the UAnSB reactor. This wastewater was a mixture of effluents coming from: (i) a high-rate activated sludge reactor (after primary sedimentation) working at sludge retention times (SRTs) of 1-2 days and (ii) a partial nitritation pilot-scale reactor treating reject water from the dewatering process of the digested sludge. Both effluents were mixed in the appropriate proportion to obtain a pre-treated mainstream wastewater suitable to be added into an anammox reactor. DO is the dissolved oxygen concentration. COD is the soluble chemical oxygen demand. COD/N corresponds to the ratio of soluble chemical oxygen demand to total nitrogen of the influent.

Parameter	Value	Units
[N-NH_4^+]	26 ± 8	mg N L^{-1}
[N-NO_2^-]	36 ± 9	mg N L^{-1}
[N-NO_3^-]	2 ± 2	mg N L^{-1}
pH	8.1 ± 0.3	-
DO	0.3 to 1.3	mg O_2 L^{-1}
COD	63 ± 15	mg COD L^{-1}
COD/N	1.0 ± 0.2	mg COD mg^{-1} N

A1.2. Heterotrophic denitrifying *ex-situ* batch activity test

Table A.2. Substrates and COD/N ratios used for the *ex-situ* heterotrophic denitrifying batch activity tests performed using acetate as C-source with sludge from sampling S3 of the UAnSB reactor. COD/N ratios were calculated following van Loosdrecht et al. (2016) (see section A1.4).

Performed test	Day of operation	Nitrite (mg N L^{-1})	Nitrate (mg N L^{-1})	COD (mg COD L^{-1})	COD/N
Only Nitrite	320	24 ± 1	-	-	-
Only Nitrate	320	-	23 ± 1	-	-
Nitrite + Acetate (non-limiting COD)	343	49 ± 1	-	873 ± 37	18 ± 1
Nitrite + Acetate (limiting COD)	end of operation	35 ± 1	-	158 ± 37	4.5 ± 1.0
Nitrate + Acetate (non-limiting COD)	343	-	37 ± 1	879 ± 1	24 ± 1
Nitrate + Acetate (limiting COD)	end of operation	-	37 ± 1	160 ± 1	4.3 ± 1.0
Nitrate + Nitrite + Acetate (limiting COD)	end of operation	24 ± 1	25 ± 1	172 ± 63	3.6 ± 1.3

A1.3. Calculation of volumetric and specific nitrogen removal rates

Volumetric section nitrogen removal rate (NRR, g N L^{-1} d^{-1}) and specific section nitrogen removal rate (NRR, g N g^{-1} VS d^{-1}) were calculated for each bed section (Eq. A.1 and Eq. A.2).

$$\text{Volumetric NRR } (h_{i+1} - h_i) = \frac{C(h_{i+1}) - C(h_i) \cdot Q}{\text{section volume } (h_{i+1} - h_i)} \qquad \text{(Eq. A. 1)}$$

$$\text{Specific NRR } (h_{i+1} - h_i) = \frac{C(h_{i+1}) - C(h_i) \cdot Q}{\text{section volume } (h_{i+1} - h_i)} \cdot \frac{1}{y} \qquad \text{(Eq. A. 2)}$$

where $C(h_i)$ and $C(h_{i+1})$ are the two concentrations at sampling points i and $i+1$, respectively in g L^{-1}, Q corresponds to the influent flow rate in L d^{-1} and y corresponds to the biomass concentration being 14 ± 5 g VS L^{-1} along the whole sludge bed. Finally, section volume, in L, is calculated as follows (Eq. A.3),

$$\text{section volume } (h_{i+1} - h_i) = \pi \cdot r^2 \cdot (h_{i+1} - h_i) \cdot 1000 \qquad \text{(Eq. A. 3)}$$

being h_i and h_{i+1} the heights at the initial sampling i and $i+1$ in m and r corresponds to the inner radius of the UAnSB reactor in m.

A1.4. Calculations of the COD/N ratios of heterotrophic denitrification *ex-situ* batch activity test

The calculations of the employed COD/N ratios for the heterotrophic denitrification *ex-situ* batch activity tests followed the methodology detailed in van Loosdrecht et al. (2016). To determine the maximum heterotrophic denitrification specific consumption rates (i.e. non-limiting COD concentrations) via nitrate or nitrite at least twice of the stoichiometric COD/N relationship of Eq. A.4 and Eq. A. 5 was respectively considered. To determine heterotrophic denitrifying activities under limiting COD concentrations via nitrate and/or

nitrite, approximately half of the COD/N ratios of Eq. A.4 and Eq. A. 5 were applied, respectively.

$$\frac{\mathrm{COD}}{\mathrm{N}} = \frac{2.86}{1 - \mathrm{Y_{OHO}}} \qquad (\mathrm{Eq.\,A.\,4})$$

$$\frac{\mathrm{COD}}{\mathrm{N}} = \frac{1.71}{1 - \mathrm{Y_{OHO}}} \qquad (\mathrm{Eq.\,A.\,5})$$

where $\mathrm{Y_{OHO}}$ is the heterotrophic anoxic growth yield for acetate 0.66 g COD g^{-1} COD (Ficara and Canziani, 2007).

A1.5. Biodiversity analysis and phylogenetic classification

Bioinformatics data was performed by the Research and Testing Laboratory. For denoising performance, sequence reads were merged together using the PEAR Illumina paired-end merger (Zhang et al., 2014) and sorted by length from longest to shortest. Then, prefix dereplication and clustering at a 4 % divergence was performed using the USEARCH (Edgar, 2010) clustering algorithm. Chimera checking was performed on the selected OTUs using the UCHIME chimera detection software executed in de novo move. The different clusters were classified into Operational Taxonomic Units (OTUs) using the UPARSE OUT selection algorithm (Edgar, 2013). Each OTUs was then identified using the USEARCH global alignment algorithm or the RDP Classifier against a data base of high-quality sequences derived from the NCBI database. For each OTU, the top six matches from the high-quality database were kept and confidence values were assigned to each taxonomic level by taking the number of taxonomic matches that agreed with the best match at that level and dividing that by the number of high-quality sequences matches that were found. Each OTU was then assigned taxonomic information using the lowest common taxonomic level whose confidence value was above 51%. OTUs that received no matches against the high-quality sequences were identified as “No Hit”. After

resolving the number of sequences per OTU, the percentage of each organism was individually calculated for each sample. Relative abundances of reads were calculated by taxonomic level for each library. Values represent the percentage of reads of sequences obtained at each taxonomic identity (according to the degree that of similarity described above) within the total set of readings from the library. When the taxonomy of an OTU was not assigned by using the protocol mentioned above (resulted as either in "Unclassified", "Unknown" or "No Hit"), an attempt to identify it was made by using the Basic Local Alignment Search Tool (BLAST) from the U.S. National Library of Medicine freely available at https://blast.ncbi.nlm.nih.gov/Blast.cgi. To assign a taxonomic classification a quality control cut-off higher than 97 % for the identity and the query cover values together with an E-value equal to zero were set.

Biological diversity indices (Shannon, Chao1 and Evenness index) were obtained for all libraries at 97 % of similitude to confirm enough expression of bacterial diversity by high-throughput sequencing methods. Rarefaction curves were also performed at 95, 97 and 99 % of similitude to test proper expression of bacterial assembly. Calculation of the biological diversity index and rarefaction curves were performed by using the on-line free available RDPipeline software. The Shannon, the Chao1 and the Evenness index estimated at 97 % of similarity for all samples showed an equally species richness and diversity among the different libraries. These results indicate no differences between the reactor operation temperatures (20 and 10 ºC) as well as in the different reactor heights. Rarefaction curves indicated a good coverage of diversity.

A2. Results

A2.1. Nitrite to ammonium and nitrate to ammonium yields.

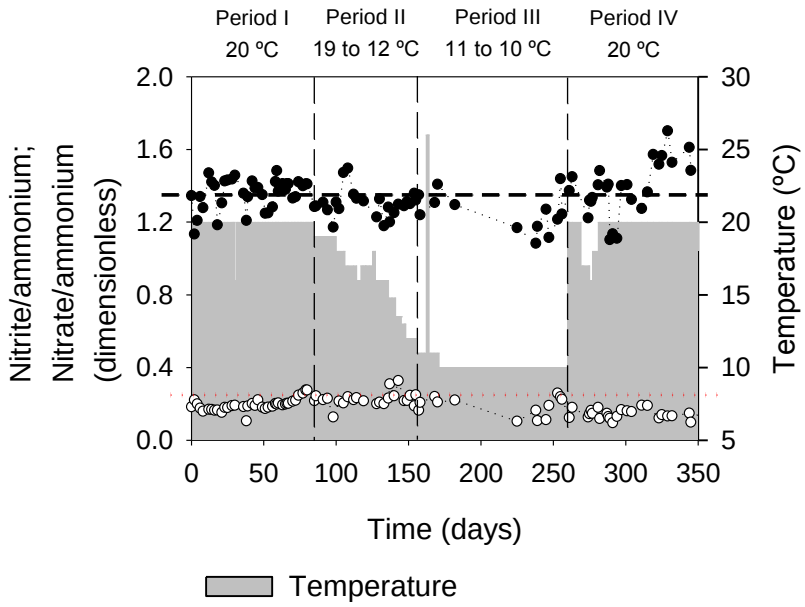

Fig A.1. Nitrite to ammonium consumed (black dots) and nitrate produced to ammonium consumed (white dots) yields throughout the UAnSB reactor operation treating a real mainstream wastewater at each temperature period. Dashed and botted lines correspond to the stoichiometric ratio of nitrite to ammonium consumed and to the produced nitrate to ammonium consumed ratio, respectively according to Strous et al. (1998) stoichiometry.

A2.2. Nitrite to ammonium feeding ratio

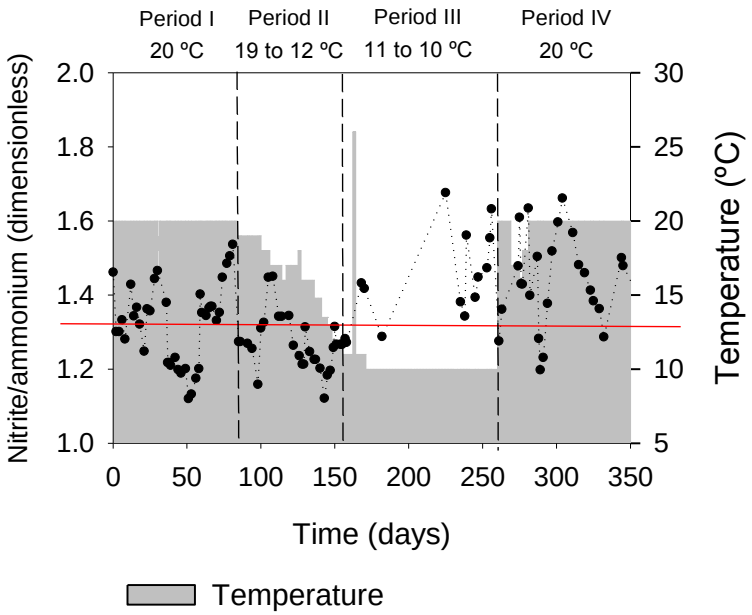

Fig A.2. Nitrite to ammonium feeding ratio throughout the UAnSB reactor operation treating a real mainstream wastewater at each temperature period. Red line corresponds to the stoichiometrically ratio of anammox bacteria according to Strous et al. (1998) stoichiometry.

A2.3. Nitrogen balance fulfilment

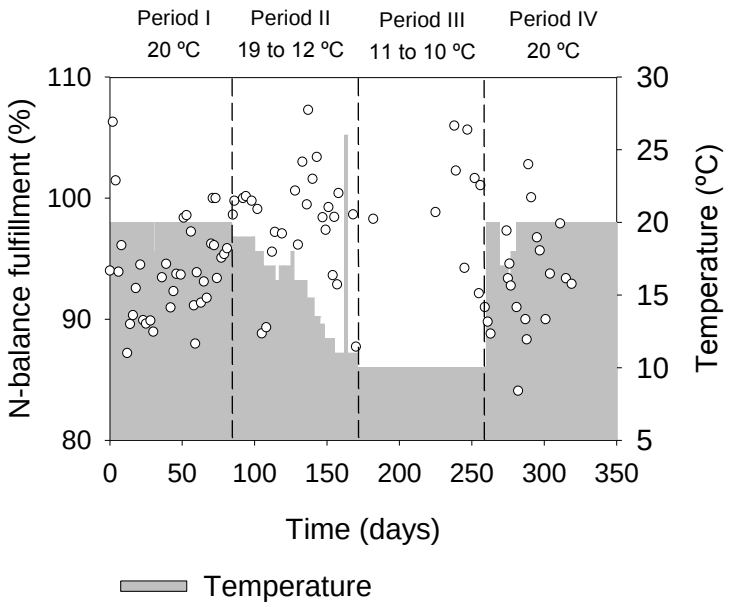

Fig. A.3. Fulfillment of the nitrogen balance of the UAnSB reactor operation treating a real mainstream wastewater at each temperature period.

A2.4. Total nitrogen concentrations in the influent and in the effluent

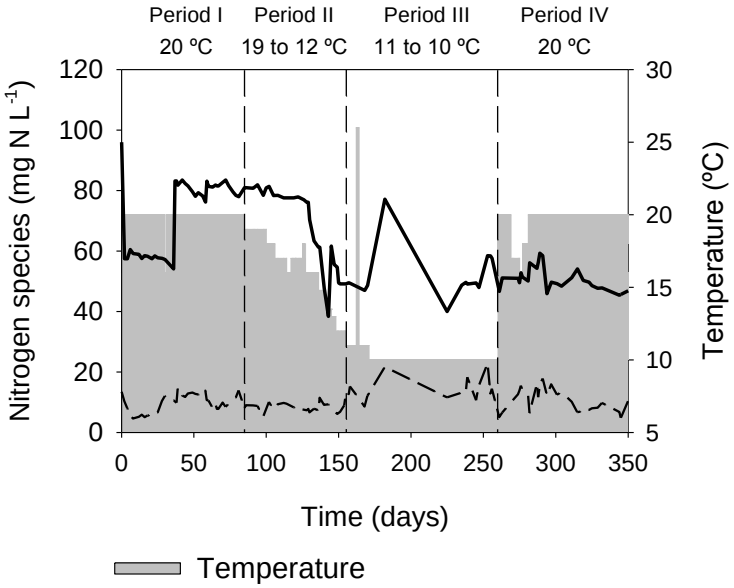

Fig. A.4. Total nitrogen concentrations in the influent as the sum of ammonium and nitrite (solid line) and in the effluent (dashed line) as the sum of ammonium, nitrite and nitrate throughout the UAnSB reactor operation treating a real mainstream wastewater at each temperature period.

A2.5. Granule size

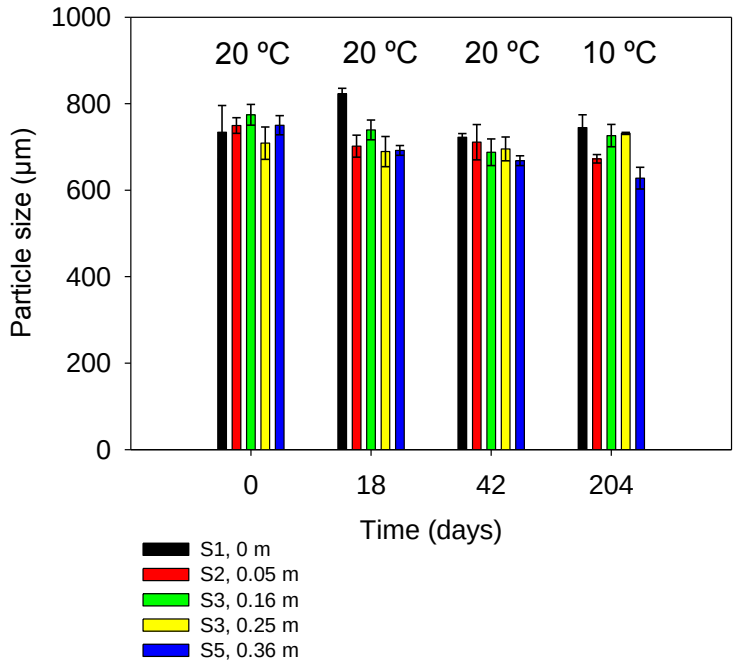

Fig. A.5. Particle size at different reactors sampling points (S1, S2, S3, S4 and S5) of the UAnSB reactor at 20 and 10 ºC.

A2.6 Microbiological quantification

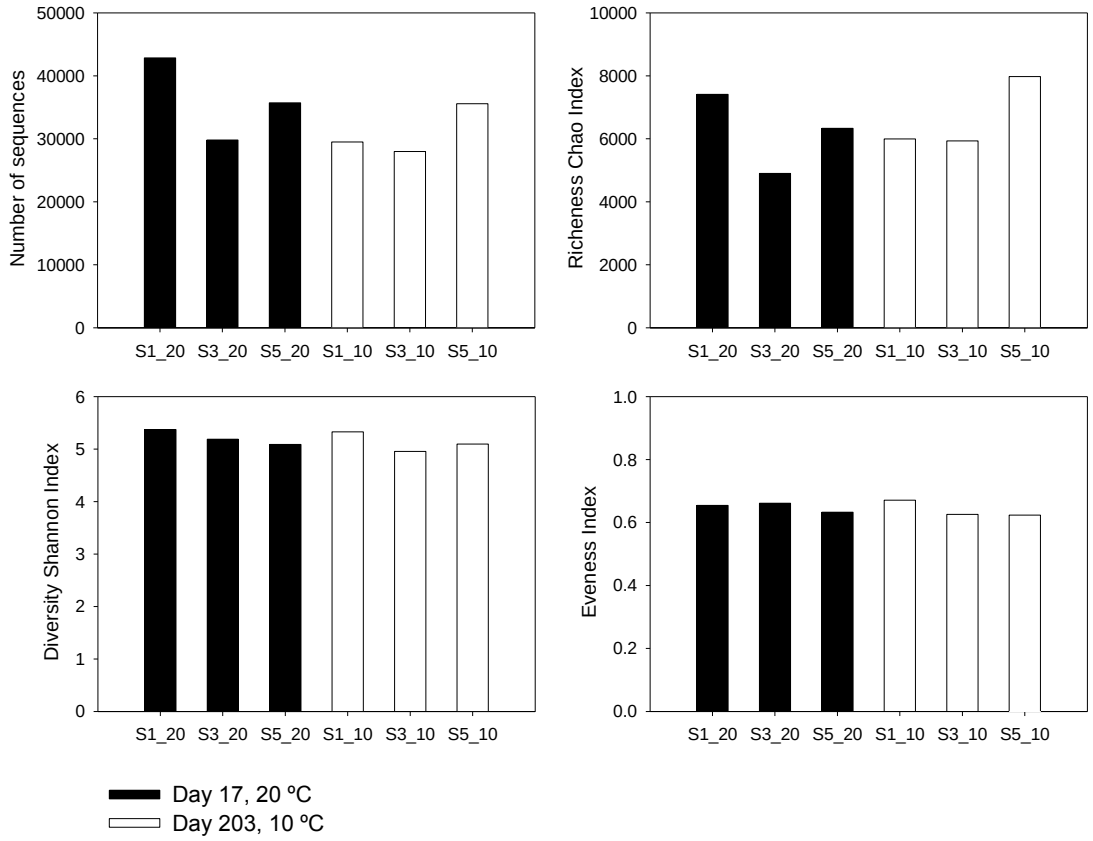

Fig. A.6. Number of sequences and biological diversity indices (Chao, Shannon and Evenness) for the libraries of day 17 (20 ºC) (black columns) and of day 203 (10 ºC) (white columns) for sampling points S1, S3 and S5.

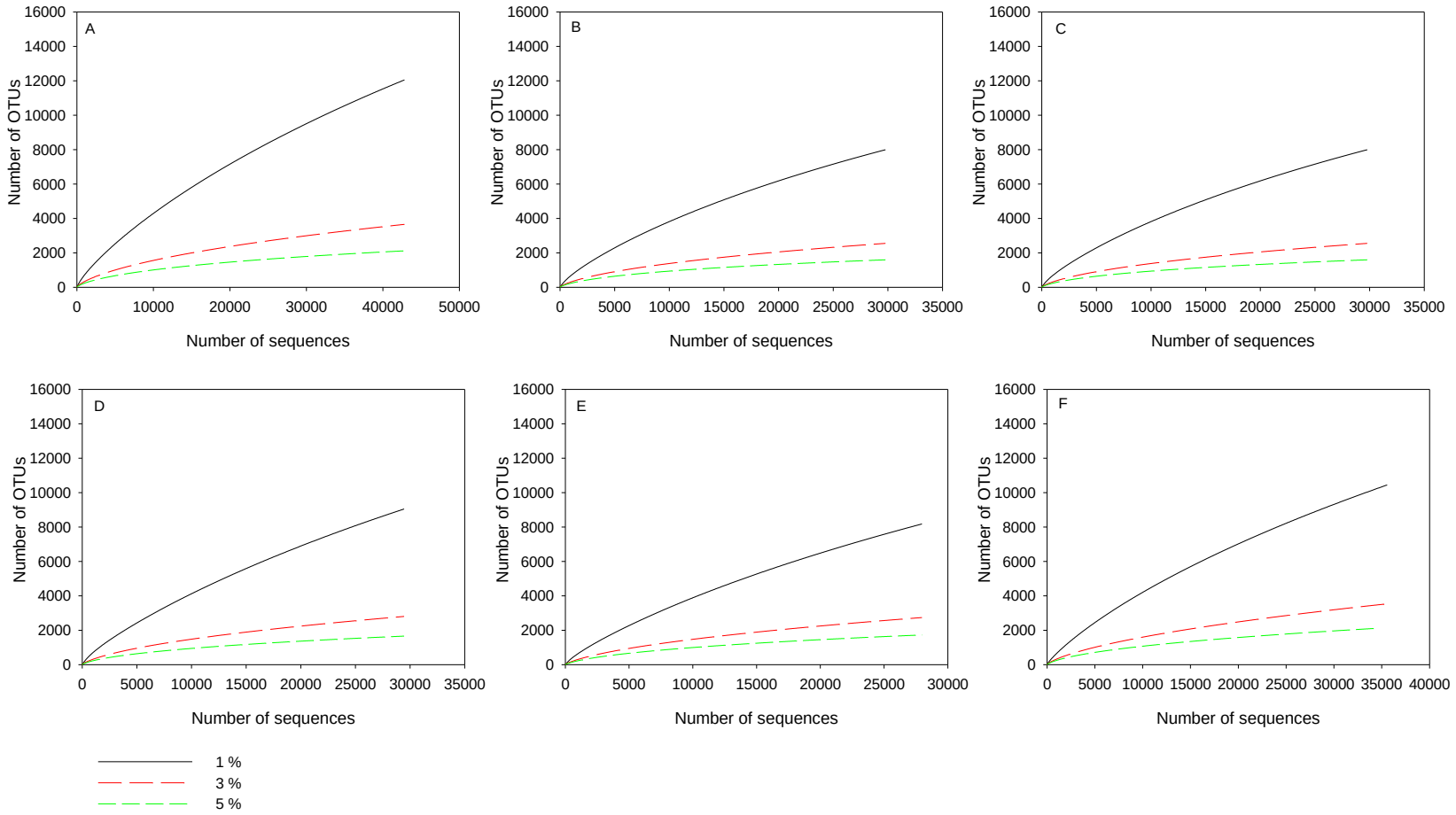

Fig. A.7. Rarefaction curves for the libraries of day 17 at 20 ºC for sampling points S1 (A), S3 (B) and S5 (C) and of day 203 at 10 ºC for sampling points S1 (D), S3 (E) and S5 (F). OTUs were defined at 1 %, 3 % and 5 % distances, respectively.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: