



Coupling anammox and heterotrophic denitrification activity at mainstream conditions in a single reactor unit

Xènia Juan-Díaz, Lluc Olmo, Julio Pérez^{*}, Julián Carrera

GENOCOV Research Group, Department of Chemical, Biological and Environmental Engineering, School of Engineering, Universitat Autònoma de Barcelona, Ed. Q-Campus UAB, Spain

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ABSTRACT

Mainstream partial nitrification/anammox (PN/AMX) has attracted large attention in the last decade. Two-stage configurations have been pointed out as an appealing technology for the application of the PN/AMX process at mainstream conditions. However, the process requires of an efficiency improvement by designing a new technology to remove the nitrate produced by anammox bacteria. A new reactor configuration was developed by coupling anammox to heterotrophic denitrification in a single reactor unit while avoiding competition between both processes. The addition of acetate as an external C-source allowed the removal of nitrate by heterotrophic microorganisms without hampering the anammox process by maintaining high nitrogen removal rates ($0.16 \pm 0.03 \text{ g N L}^{-1} \text{ d}^{-1}$) and nitrogen removal efficiencies ($91 \pm 8 \%$) (on average from acetate addition periods). Further, a proper organic load management showed to be effective to face the drawbacks derived from previous mainstream treatment stages (i.e. undesired nitrate production), without compromising effluent COD concentrations. By withdrawing samples along the sludge bed, nitrogen compounds and COD concentrations were determined at different reactor heights. This set of experimental data demonstrated that the combination of anammox and heterotrophic denitrification in a single reactor unit was possible as anammox activity dominated within bottom sludge bed sections, while heterotrophic denitrification occurred within middle and upper sludge sections. Microbial diversity results of 16S rRNA gene-targeted sequencing analyses confirmed that anammox and heterotrophic denitrifiers communities occupied two differentiated sludge bed sections along the reactor being dominated by *Candidatus Brocadia* (30 %) and *Thauera* (33 to 46 %), respectively. When heterotrophic denitrification occurred, the average N_2O emissions with COD addition was ca. 50 % lower than that in periods without COD addition.

1. Introduction

Anammox bacteria oxidize ammonium by using nitrite as electron acceptor to produce nitrogen gas [1]. The implementation of the anammox process at mainstream conditions offers the possibility to move from energy consuming to neutral (or even positive) wastewater treatment plants (WWTPs) [2]. Compared to conventional nitrification/denitrification, nitrogen removal via the anammox process allows to reduce oxygen consumption by a 60 % and does not require any organic C-source [3]. The treatment of municipal sewage via anammox-based processes allows the uncoupling of organic matter (i.e. chemical oxygen demand, COD) and nitrogen removal. A widely accepted configuration for the implementation of the anammox process at mainstream conditions is the A/B process [4–6]. The incoming organic matter will be

captured within the A-stage via a high-rate activated sludge (HRAS) reactor in which a short SRT is imposed to enhance the removal of COD through adsorption and to enhance higher yields of biogas production from the digestion of the obtained sludge [2,3,7]. The remaining nitrogen is autotrophically removed in the B-stage by the partial nitrification/anammox (PN/AMX) process. To date, the PN/AMX process stability has been reported by using one-stage and two-stage configurations at temperatures as low as 11 °C with biofilms developed on plastic carriers [8,9] and by using granular sludge [10,11], respectively. However, the obtained nitrogen removal rates (NRR) of one-stage configurations were not competitive compared to conventional treatments. This is mainly due to the fact that this type of biofilm reactors with ammonium oxidizing bacteria (AOB) and anammox growing on inner surfaces of plastic carriers are strongly rate limited by the external mass

^{*} Corresponding author.

E-mail address: julio.perez@uab.es (J. Pérez).

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transfer rate [12]. For this reason, the use of granular sludge reactors for two-stage PN/AMX configurations might be an appealing alternative to grant process stability and allow for high nitrogen loading rates [10,11,13–15]. The maximum nitrogen removal efficiency of the PN/AMX process is limited by the nitrate produced by anammox bacteria (up to 10 % of the influent nitrogen). The increasing stringent discharge limitations requires of further increase in the process efficiency by designing a process that includes the removal of the nitrate produced by anammox bacteria [16]. The conventional process upgrade would be to enhance heterotrophic denitrification by adding an external C-source (e. g., acetate). An efficient design of this last treatment unit might also contribute to mitigate the negative effects of (already identified) process challenges such as the instability of the previous PN reactor during short periods of time (for instance, nitrate production at the lowest temperatures in winter [17]); potential nitrate production in the A-stage during warm seasons [18,19]; non-ideal nitrite to ammonium feeding ratios to the anammox reactor [13]; or the daily nitrogen loads oscillations of the main water line [15].

An appealing alternative would be to add the external C-source into the anammox reactor. The combination of anammox and heterotrophic denitrification in a single reactor unit would allow to enhance the robustness of the PN/AMX process at mainstream conditions. Nevertheless, heterotrophic denitrifying bacteria might deteriorate the anammox process, as they present higher growth rates and could outcompete anammox bacteria utilizing nitrite as electron acceptor [20]. Also, high COD/N ratios and long-term exposure to organic substrates might result into a loss of anammox activity [21,22].

An ad hoc reactor design has been developed to convert an Up-flow Anammox Sludge Bed (UAnSB) reactor into a reactor unit with minimal nitrate production (i.e. CANDLE, Coupling ANammox and Denitrification in a single unit). This reactor therefore would be integrated in the treatment of the main waterline as depicted in Fig. 1. The proposed configuration enabled the use of anammox and heterotrophic denitrifying bacteria in a single unit by preventing competition for nitrite between both groups of bacteria. The CANDLE reactor was operated for 200 days at temperatures ranging from 20 to 14 °C by treating a real mainstream wastewater. Substrate distribution along the sludge bed sections was investigated to determine the distribution of the anammox and heterotrophic denitrifying activities in the reactor. Nitrous oxide (N₂O) emissions were monitored, whereas process reactor performance was complemented by 16S rRNA gene-targeted sequencing analyses for the characterization of the microbial community.

2. Materials and methods

2.1. Reactor operation and configuration

An UAnSB reactor with an effective working volume of 13 L, excluding the gas–liquid–solid (GLS) separator phase (12 L), was converted into a CANDLE reactor for the combination of the anammox and heterotrophic denitrifying activity in a single reaction unit. The inner diameter of the column was 120 mm and the total-reactor-height to column-diameter ratio was 12:1. The CANDLE reactor was previously operated as an UAnSB reactor for 350 days at temperatures ranging from 20 to 10 °C treating a real mainstream wastewater by maintaining a rather constant NRR ($0.10 \pm 0.01 \text{ g N L}^{-1} \text{ d}^{-1}$) [13]. The hydraulic residence time (HRT) was calculated by considering the liquid fraction within the sludge bed. The liquid fraction volume corresponded to the voidage of the sludge bed (i.e., the fraction of the sludge bed volume that is occupied by the voids) and was experimentally determined. The average HRT was $1.0 \pm 0.2 \text{ h}$. 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. 2). The main difference (compared to the UAnSB reactor) is that the CANDLE reactor presents two inlets with the corresponding diffusers located (i) in the bottom section to feed a real mainstream wastewater and (ii) at 0.16 m from the reactor bottom (i.e. at the height of sampling S3) to feed an external C-source (i.e. sodium acetate) (Fig. 2). 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. CANDLE reactor operation was divided in four periods.

- **Period I.** To evaluate reactor performance without the addition of an external C-source, i.e. only mainstream wastewater addition at 20 °C. This period will be used as base line in terms of reactor performance for the assessment of the effects of adding an external C-source. During this period disturbances were applied in the inflow with regards to: (i) the nitrite to ammonium ratio compared to the theoretically desired for the anammox process; (ii) the inflow nitrate concentrations, mimicking a low performance of the previous treatment stages (e.g., nitrification during carbon removal in the A-stage and nitrate produced in the PN reactor at low temperatures).
- **Period II.** Reactor performance was evaluated by adding acetate as an external C-source at 20 °C. Additionally, the same disturbances described in period I were applied. Further, N₂O emissions were

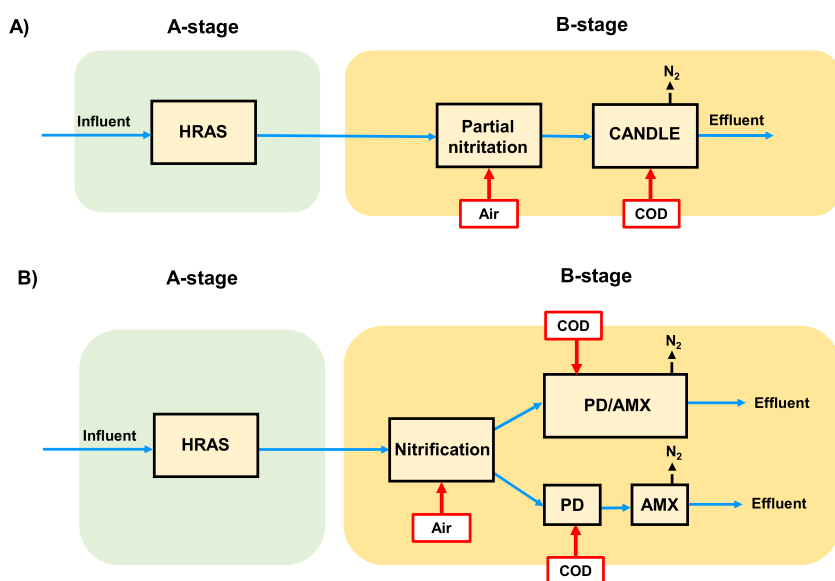


Fig. 1. Two potential treatment schemes for the integration of anammox in the main waterline of urban WWTPs. The treatment scheme is based on the A/B configuration, where the A-stage is in charge of the COD removal and the B-stage is in charge of the N removal. The A-stage is a high rate activated sludge (HRAS) reactor to enhance both the COD removal through adsorption and the energy recovery from the excess sludge produced. The B-stage removes the nitrogen with anammox, through two different approaches: A) Two-stage PN/AMX process, splitting PN and anammox in two reactor units, being the second one the focus of research in the present study and named Coupling ANammox and Denitrification in a single unit (CANDLE). B) The partial denitrification/anammox (PD/A) process (with either one or two stages) fed with the effluent of a nitrification reactor.

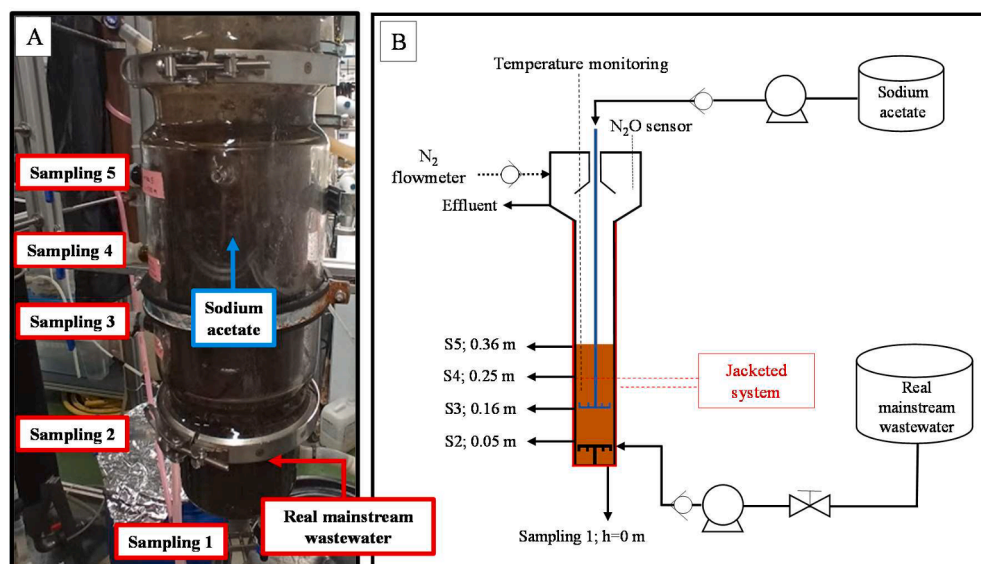


Fig. 2. (A) Picture of the lab-scale CANDLE reactor (sludge bed section). (B) Schematic diagram of the reactor set-up with the corresponding peripheral instrumentation, feeding and sampling points. The fraction of the CANDLE reactor vessel highlighted in solid orange corresponds to the sludge bed. Sampling 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).

assessed under optimal and limited COD conditions for heterotrophic denitrification.

- **Period III.** To re-evaluate reactor performance with only mainstream wastewater addition at 20 °C. This experimental period is designed to analyse the potential negative effects of the external COD addition on the anammox process after stopping the previous acetate addition (i.e. period II). Additionally, it was also used to assess the N₂O emissions without external COD addition, for enabling comparison with periods in which the external organic source was fed to the reactor.
- **Period IV.** Reactor performance was assessed when adding acetate whereas a temperature decrease from 20 to 14 °C was applied, to explore the influence of temperature on both process (anammox and heterotrophic denitrification) taking place in a single reaction unit. N₂O emissions were also evaluated at 17 °C under over-dimensioned COD conditions.

2.2. Wastewater characteristics

The CANDLE reactor was fed with real mainstream wastewater coming from the effluent of an urban WWTP located in an industrial area of Catalonia, NE Spain, therefore the wastewater contained almost no biodegradable COD. As an effluent coming from a PN reactor at mainstream conditions was not available at the time of this study, nitrite or ammonium were added according to the needs of anammox stoichiometry [1]. Therefore, this study is not aiming to validate the performance of the PN reactor. Ammonium and nitrite were added as (NH₄)₂SO₄ and NaNO₂, respectively to maintain a ratio between the nitrite and ammonium concentrations of 1.2 ± 0.2 . Further characterization of the real mainstream wastewater after the ammonium and nitrite addition is provided in Supporting Information (Table A.1). The real mainstream wastewater was stored in a tank within lab-facilities. The storing tank containing the influent was flushed with N₂ every time the tank was refilled (3–5 weeks) to keep DO concentration in the range of 0.2 to 2 mg O₂ L⁻¹ and maintained at 20 ± 1 °C. The DO concentration measured in the influent did not negatively affect the performance of the anoxic processes occurring in the reactor (i.e., anammox and heterotrophic denitrification). These potential negative effects could be ruled out because the performance of the reactor was not altered when the DO concentration in the tank, where the inflow wastewater was stored, changed from 0.2 to 2 mg O₂ L⁻¹. The stored wastewater experienced some degree of nitrification, which allowed to simulate a potential nitrate production of the previous mainstream

treatment stages. Consequently, variable nitrate concentrations were measured in the inflow real mainstream wastewater throughout reactor operation, as well as variations in the feeding ammonium/nitrite concentration ratio, diverging from the theoretical one desired for the anammox process (see Supporting Information Figs. A.3 and A.2, respectively).

2.3. Profile concentrations along the sludge bed

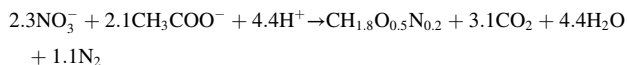
Ammonium, nitrite, nitrate, COD and acetate concentrations were measured along the different sampling points of the CANDLE reactor at steady state conditions to assess the distribution and contribution of anammox and of heterotrophic denitrification activities. Further, acetate concentrations were measured along the sludge bed on day 132. Distribution of sampling points is depicted in Fig. 2.

2.4. Heterotrophic denitrifying ex-situ batch activity tests

Ex-situ batch tests were used to assess heterotrophic denitrifying activity before and during acetate addition on days 43 (period I) and 98 (period II), respectively. Tests were carried out in duplicates using septum-closed bottles of 125 mL with 2.5 ± 0.3 g VS L⁻¹ and 0.3 ± 0.1 g VS L⁻¹ for tests of days 43 and 98, respectively. For tests of day 43, biomass from sampling S3 was employed as it corresponded to the sludge bed section where acetate was added. For tests of day 98, the employed biomass corresponded to the sludge located within the GLS separator phase. The distinct sample point explains the difference of biomass concentrations among the performed tests. The biomass was washed and re-suspended in a medium containing the corresponding nitrite and/or nitrate and C-source (i.e. acetate) concentrations together with the macro and microelements to avoid nutrient limitation [23].

All septum bottles were flushed with N₂ to maintain anoxic conditions. Tests were conducted at 180 rpm and at 20 °C. Two sets of experiments were performed for each sampling period (days 43 and 98): (1) the consumption of nitrite or nitrate in the presence of acetate as an easily biodegradable C-source and (2) the consumption of nitrite and nitrate in the presence of acetate to evaluate if the biomass presented a preference for any of these two electron acceptors. Sampling time depended on the consumption rate of substrates in each experiment. Substrate consumption rates were calculated by linear regression of nitrite or nitrate concentrations of three to six bulk liquid-phase grab samples. Specific heterotrophic denitrifying activities (mg N g VS⁻¹ d⁻¹) were calculated by dividing the substrate consumption rates (mg N L⁻¹ d⁻¹)

¹) by the biomass concentration (g VS L⁻¹) of each performed test. The employed COD/N ratio (4.2 ± 0.4 g COD g⁻¹N-NO₃⁻) was slightly higher than the theoretical stoichiometric relationship for the biological heterotrophic denitrification process using acetate as a C-source (3.9 g COD g⁻¹N-NO₃⁻) (Eq. (1), [24]).



Further information regarding COD, nitrite and nitrate concentrations for each performed *ex-situ* batch test can be found in [Supplementary Information \(Table A.2\)](#).

2.5. Analytical methods

Liquid samples from influent and effluent of the CANDLE reactor were analysed 4 to 5 times per week. Samples were filtered (0.22 µm) before analysis. Nitrite (influent) and nitrate (influent and effluent) concentrations were analysed off-line with ionic chromatography using ICS-2000 Integrated Reagent-Free IC system (DIONEX Corporation, USA). For accurate results, effluent nitrite concentrations were measured by using colorimetric Hach Lange kits (LCK342, Hach Lange, Germany). Ammonium concentrations (influent and effluent) were analysed off-line by means of a gas selective electrode (GSE) (AMTAX sc, Hach Lange, Germany). Ammonium and nitrite samples from profile concentrations and from *ex-situ* batch activity tests were determined by colorimetric Hach Lange kits (LCK303 and LCK342, respectively, Hach Lange, Germany). Total and soluble COD concentrations were analysed by using colorimetric Hach Lange kits (LCK314 Hach Lange, Germany). Acetate concentrations were measured by gas chromatography (7820A, Agilent Technologies, USA). Total solids (TS) and volatile solids (VS) concentration were analysed according to Standard Methods [25]. Biomass characteristics were monitored using a stereomicroscope. Average particle size was measured by a laser diffraction analysis system (Malvern Mastersizer Series 2600, Malvern instruments Ltd., UK). N₂O off-gas concentrations were analysed within the GLS phase of the CANDLE reactor with a Clark-type N₂O microsensor calibrated according to manufacturer's instructions (UNISENSE, Denmark).

2.6. Calculations

Nitrogen loading rates (NLR), NRR and organic loading rates (OLR) were calculated by using the effective CANDLE reactor volume of 13 L, excluding the GLS separator phase. NLR was calculated considering the ammonium, nitrite and nitrate concentrations of the real mainstream wastewater. Likewise, NRR was calculated considering the removed ammonium, nitrite and nitrate concentrations. The OLR was calculated considering the necessary acetate to reduce (i) the nitrate produced by the anammox process and (ii) the inflow nitrate according to the stoichiometric relation of 3.9 g COD g⁻¹N-NO₃⁻ (see Eq. 1) considering a ratio of 0.78 g COD g⁻¹ sodium acetate. The variations of the acetate flow rate to meet the required OLR were manually and periodically implemented. As a result, the requirements just described were not always fully achieved due to the fluctuating inflow nitrate concentrations throughout periods II and IV. In case of a full-scale application, this could be easily managed by a proper automatic control strategy. Nitrogen removal efficiency (NRE) was calculated based on Eq. (2):

$$\text{NRE} = \frac{(\text{N-NH}_4^+ + \text{N-NO}_2^- + \text{N-NO}_3^-)_{\text{inf}} - (\text{N-NH}_4^+ + \text{N-NO}_2^- + \text{N-NO}_3^-)_{\text{eff}}}{(\text{N-NH}_4^+ + \text{N-NO}_2^- + \text{N-NO}_3^-)_{\text{inf}}} \quad (2)$$

where N-NH₄⁺, N-NO₂⁻ and N-NO₃⁻ are the ammonium, nitrite and nitrate concentrations in the influent (inf) and in the effluent (eff), respectively.

The emission factor (EF) for N₂O was calculated based on the N₂O

emission rates (Eq. (3)) to the relative nitrogen removed by the anammox process or by both the anammox and heterotrophic denitrifying processes during a defined period.

$$r_{\text{N}_2\text{O}} = Q_{\text{N}_2} \cdot C_{\text{N}_2\text{O}} \quad (3)$$

where C_{N₂O} corresponds to the measured N₂O off-gas concentrations (mg N-N₂O L⁻¹) and Q_{N₂} corresponds to the nitrogen gas flow (13 L d⁻¹) added within the GLS separator phase (see Fig. 2) and controlled by a means of flowmeter (Bronkhorst, The Netherlands).

Nitrogen gas production by the anammox and heterotrophic denitrification processes were not considered in Q_{N₂} as they were 7-to-10 times lower than the external nitrogen gas supply.

2.7. Microbiological quantification and characterization

Microbiological community composition was identified by using next-generation sequencing analysis. Two biomass samplings were carried out: (i) before acetate addition (day 42) from samplings S1 and S3 and (ii) during acetate addition (day 97) from sampling points S1, S3, S4 and from the GLS separator phase. DNA was extracted by using the Soil DNA Isolation Plus Kit™ (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 1.9 ± 0.1 was used as quality cut-off and a minimum of 17 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 [Supporting Information \(A1.3\)](#).

3. Results and discussion

3.1. Combining anammox and heterotrophic denitrifying activity in a single reactor unit: Process performance

The CANDLE reactor configuration was proposed to couple anammox and heterotrophic denitrification processes in a single reactor unit. To prevent the competition among anammox and heterotrophic denitrifying bacteria, an external C-source was fed (i.e. acetate) to the middle sludge bed section (i.e. at the height of sampling S3), whereas the real mainstream wastewater was fed to the bottom sludge section (see Fig. 2). The obtained results showed that acetate addition (period II and IV, respectively) allowed the removal of the nitrate produced by anammox bacteria, without hampering the anammox process (Fig. 3, Table 1). This, in turn, significantly improved effluent quality while maintaining rather high and constant NRR (0.16 ± 0.03 g N L⁻¹ d⁻¹) and high NRE (91 ± 8 %) (on average from acetate addition periods, Fig. 3). Reactor performance was successfully maintained when temperature decreased from 20 to 14 °C (Fig. 3). In case of increasing inflow nitrate concentrations (i.e. eventual nitrate production in previous treatment stages), the OLR was adjusted to maintain a good effluent quality, without hampering the autotrophic nitrogen removal by anammox bacteria (Fig. 3). Even when acetate was added over the stoichiometric requirements (day 93, from days 111 to 114, day 132, from days 172 to 182 and 184 to 192), COD effluent concentrations remained in the range of COD inflow concentrations (on average for acetate addition periods 40 ± 5 and 47 ± 10 mg COD L⁻¹ for influent and effluent, respectively), showing that mainly the non-biodegradable COD entering the reactor was the only organic source discharged, so that the external acetate was either directly used for heterotrophic denitrifiers or may have been stored as polymers by the cells (Fig. 4 and Table A.3, [Supporting Information](#)). Furthermore, despite acetate was added as an external C-

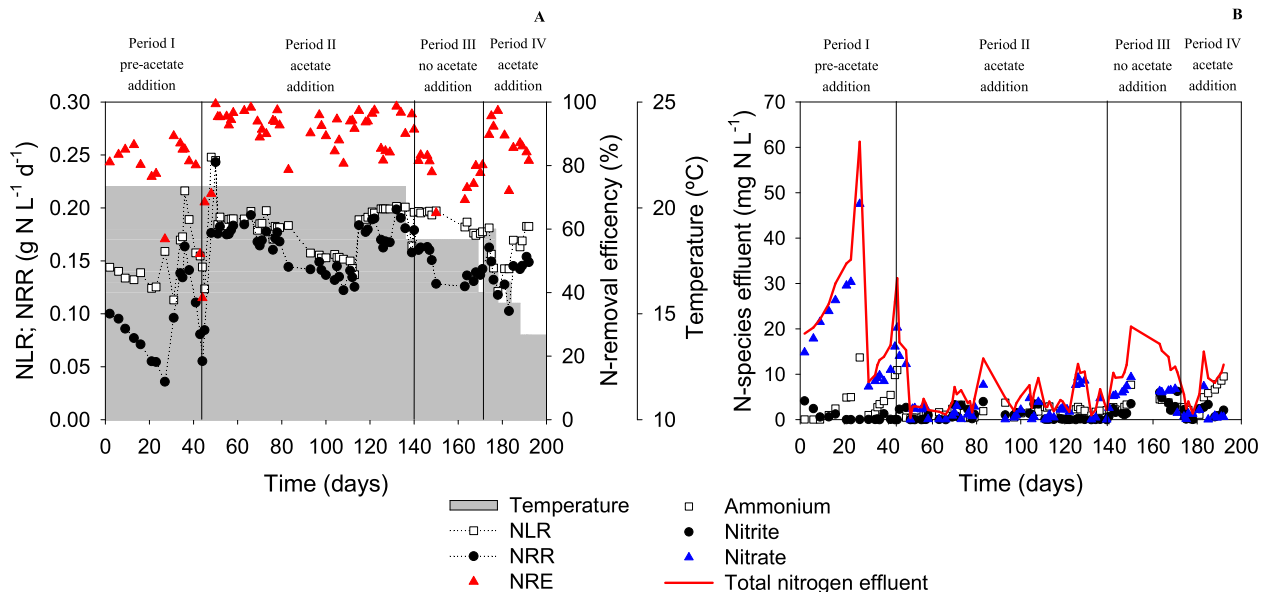


Fig. 3. Long-term operation of the CANDLE reactor at mainstream conditions. (A) Nitrogen loading and removal rates (NLR and NRR, respectively) and nitrogen removal efficiency (NRE). (B) Effluent concentrations of ammonium, nitrite and nitrate. Total nitrogen concentrations in the effluent refer to the sum of ammonium, nitrite and nitrate.

Table 1

Operational parameters for the different periods without acetate addition (I and III) and during acetate addition (II and IV). NLR: Nitrogen Loading Rate; NRR: Nitrogen Removal Rate; NRE: Nitrogen Removal Efficiency.

Period	Time lapse (days)	NLR ($\text{g N L}^{-1} \text{d}^{-1}$)	NRR ($\text{g N L}^{-1} \text{d}^{-1}$)	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/ammonium consumed	Nitrate produced/ammonium consumed	Nitrite/ammonium influent
Period I pre-acetate addition	43	0.15 ± 0.03	0.10 ± 0.03	83 ± 4	18 ± 11	4 ± 4	1 ± 2	17 ± 9	1.3 ± 0.1	0.24 ± 0.04	1.2 ± 0.3
Period II acetate addition	97	0.18 ± 0.02	0.17 ± 0.03	92 ± 7	5 ± 4	2 ± 2	1 ± 2	3 ± 4	1.2 ± 0.2	–	1.2 ± 0.2
Period III no-acetate addition	31	0.19 ± 0.01	0.15 ± 0.02	79 ± 8	13 ± 4	4 ± 2	3 ± 2	7 ± 2	1.3 ± 0.1	0.24 ± 0.10	1.3 ± 0.1
Period IV acetate addition	21	0.16 ± 0.02	0.14 ± 0.01	88 ± 8	8 ± 5	5 ± 4	2 ± 2	2 ± 2	1.4 ± 0.2	–	1.2 ± 0.2

source to enhance heterotrophic denitrification, this did not affect effluent COD concentrations which remained under $125 \text{ mg COD L}^{-1}$ throughout the operation (see Fig. 4), below the discharging limit according to the European legislation.

Reactor operation period was divided into four different phases (Table 1). The high total nitrogen concentrations in the effluent of period I (i.e. without acetate addition) was caused by the high incoming nitrate concentrations and by the oscillating nitrite to ammonium feeding ratios (1.2 ± 0.3) (see Figs. A.3 and A.2, respectively in Supporting Information). As soon as heterotrophic denitrifying activity was promoted by adding acetate (period II), higher NRE ($92 \pm 7\%$) and NRR ($0.17 \pm 0.03 \text{ g N L}^{-1} \text{d}^{-1}$) and a better effluent quality ($5 \pm 4 \text{ mg N L}^{-1}$) were achieved by maintaining high NLR ($0.18 \pm 0.02 \text{ g N L}^{-1} \text{d}^{-1}$) (Table 1).

From day 140 to 171 (period III), reactor performance was reassessed to rule out the possible negative effects of the previous external C-source addition on the anammox process (i.e. only mainstream wastewater feeding) by maintaining a similar NLR than that of previous periods ($0.19 \pm 0.01 \text{ g N L}^{-1} \text{d}^{-1}$). During the first 10 days after stopping acetate

addition, from days 140 to 149, the nitrate production to ammonium consumption ratio was lower than the stoichiometrically proposed for the anammox process (i.e. 0.17 ± 0.05), still detecting a small degree of heterotrophic denitrifying activity. However, reactor performance decreased compared to that of period II, resulting into lower NRE ($79 \pm 8\%$) and NRR ($0.15 \pm 0.02 \text{ g N L}^{-1} \text{d}^{-1}$) (Table 1). The lower reactor performance was attributed to the lack of an external C-source, highly reducing the heterotrophic denitrifying activity and, thus, into high effluent nitrate concentrations ($7 \pm 2 \text{ mg N L}^{-1}$, Table 1). Also, the non-adjusted nitrite to ammonium feeding ratio (Fig. A.2, Supporting Information) of this period resulted into an excess of ammonium in the effluent ($4 \pm 2 \text{ mg N L}^{-1}$, Table 1) and, thus, worsened effluent quality.

During period IV, the performance of the CANDLE reactor at low temperatures was assessed. For this purpose, reactor temperature was decreased from 20 to 14°C while acetate addition was reactivated by maintaining the NLR in the range of previous periods ($0.16 \pm 0.02 \text{ g N L}^{-1} \text{d}^{-1}$, Table 1). As the OLR was mainly over-dimensioned during period IV, nitrate concentrations remained very low in the effluent (Table 1). Simultaneously, high effluent ammonium concentrations

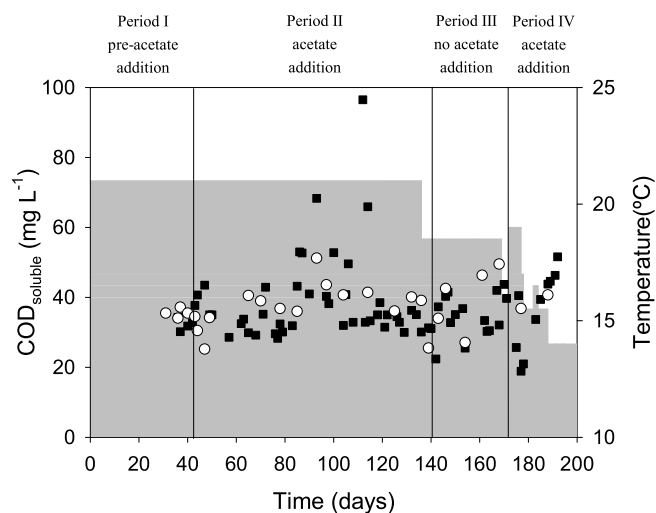


Fig. 4. Soluble chemical oxygen demand (COD) concentrations of the influent real mainstream wastewater (white dots) and of the effluent (black squares) during CANDLE reactor operation. The external C-source (i.e. acetate) is considered within effluent concentrations.

were detected ($5 \pm 4 \text{ mg N L}^{-1}$, Table 1) caused by a misbalanced nitrite to ammonium feeding ratio to the anammox process (1.2 ± 0.1 , see Fig. A.2 in Supporting Information for information of the nitrite to ammonium feeding ratio). Overall, a better effluent quality and NRE were achieved compared to periods without acetate addition (Table 1). Nevertheless, the $\text{N-NO}_2^-/\text{N-NH}_4^+$ influent ratio needs to be better controlled to avoid excessive discharging nitrogen compounds, whereas the COD load should be properly managed to completely reduce nitrate concentrations. Despite the temperature decrease, reactor performance of period IV was only slightly lower compared to the previous acetate addition period at 20°C (period II), as NRR and NRE only decreased from $0.17 \pm 0.03 \text{ g N L}^{-1} \text{ d}^{-1}$ and $92 \pm 7 \%$ (period II) to $0.14 \pm 0.01 \text{ g N L}^{-1} \text{ d}^{-1}$ and $88 \pm 8 \%$ (period IV) (see Table 1). The capability of an UAnSB reactor of dampening a temperature drop at mainstream conditions was previously demonstrated to be a result of the so-called reactor overcapacity [13]. This is the capacity of the middle and upper part of the sludge bed to counterbalance the activity decrease of the bottom sludge bed section when temperature drops. According to this, the CANDLE reactor also seemed to show overcapacity to damp temperature drops. Thus, the main reason of the lower NRR and NRE in period IV can be attributed to the non-adjusted nitrite to ammonium feeding ratio, rather than to the effects of temperature decrease.

The coexistence of anammox and heterotrophic denitrifying activities in a single reactor was demonstrated to be a promising technology to overcome the associated drawbacks of the two-stage autotrophic biological nitrogen removal process at mainstream conditions; that is, potential nitrate production or a misbalanced nitrite to ammonium ratio in the effluent of the PN reactor. Further studies should be performed to evaluate the CANDLE reactor performance under fluctuating nitrogen and organic loading rates as well as under different organic carbon sources at pilot scale.

3.2. Location of anammox and heterotrophic denitrifiers along the bed prevents competition of nitrite

Substrate concentration measurements (nitrogen compounds and COD) were performed along the different sampling points of the sludge bed (see Fig. 2) for three different operational days: during acetate supply (period II, on days 125 and 132, when acetate addition was under and over-dimensioned, respectively) and without acetate addition (day 168, period III). Additionally, biomass characteristics were examined using a stereomicroscope and particle size was measured for different

withdrawn samples of the sludge bed.

Anammox activity was detected within bottom sludge sections, as observed by the reduction of ammonium and nitrite compounds from S1 to S3 sludge bed sections in both conditions, with and without acetate addition (Fig. 5A). The biomass located within this sludge section presented a brown-to-reddish colour, with a dense granule structure (see Fig. A.5, Supporting Information).

3.3. Effect of heterotrophic denitrifying activity on N_2O emissions

Nitrate concentrations increased along the bottom sludge bed sections, according to the nitrate produced by anammox bacteria (Fig. 5A). When acetate was supplied, nitrate concentrations decreased after reaching the sludge bed section corresponding to the acetate feeding point (i.e. sampling S3) (Fig. 5A). The highest COD concentrations corresponded to the acetate bed section addition and decreased along the upper sludge bed sections, in agreement with the reduction of nitrate concentrations by heterotrophic denitrifiers (Fig. 5B). The biomass located beyond the acetate feeding point presented fewer compact aggregates with a brownish colour and fluffy structure (see Fig. A.5, Supporting Information), although the sludge presented similar average diameter sizes with those of the bottom bed sections (Fig. A.4, Supporting Information).

The obtained results indicate that heterotrophic denitrifying activity was located within middle and upper sludge sections, while anammox activity was relegated to the bottom sludge section. The reason was attributed to the suitable distribution of acetate addition beyond the middle sludge section of the CANDLE reactor. This was proved when measuring acetate concentrations along the sludge bed sections, as they were only detected within middle sludge sections, while no acetate diffusion was observed to the bottom sludge bed sections, ensuring the performance of the anammox process (Fig. 5B). Further, the lower granule size and fluffy structure of heterotrophic denitrifying bacteria could have also favored heterotrophic denitrifying community to remain within the middle and upper sludge bed section.

When acetate was added above the stoichiometric requirements (day 132), the COD effluent concentration remained in the range of the COD inflow concentration, whereas a residual nitrate concentration was detected (ca. 0.1 mg N L^{-1}) (Fig. 5A). Additionally, mass balances indicated that COD consumption was higher than the stoichiometrically required for nitrate reduction by using acetate as an organic source (Fig. 5B). Acetate can be intracellularly accumulated as polyhydroxybutyrate (PHB) [26–28]. This is consistent with results from period III, as heterotrophic denitrification still occurred after stopping acetate addition (days 140 to 149, see section 3.1). This suggests that heterotrophic denitrifying bacteria could have stored organic compounds when acetate addition was over-dimensioned and that they were capable of further consume them under starvation conditions of an external organic feeding. However, the effect of heterotrophic denitrifiers of consuming COD from lysis or endogenous material under organic starvation conditions cannot be neglected. Further studies should be performed to evaluate if acetate was accumulated as PHB and how this affected heterotrophic denitrifiers when they were exposed to substrate limiting conditions.

Different studies reported that anammox activity became deteriorated when influent with high COD concentrations or high COD/N ratios was fed in different reactor configurations [21,29]. However, the results of this study not only provided evidence that anammox activity remained unaffected after the continuous external C-source supply, but that the coexistence of anammox and heterotrophic denitrification processes was possible regardless the COD/N ratio and the incoming acetate concentrations thanks to the employed reactor configuration (i.e., two different feeding points along the sludge bed) which avoided acetate diffusion to the bottom sludge bed section, mainly colonized by anammox.

N_2O off-gas concentrations were monitored within different

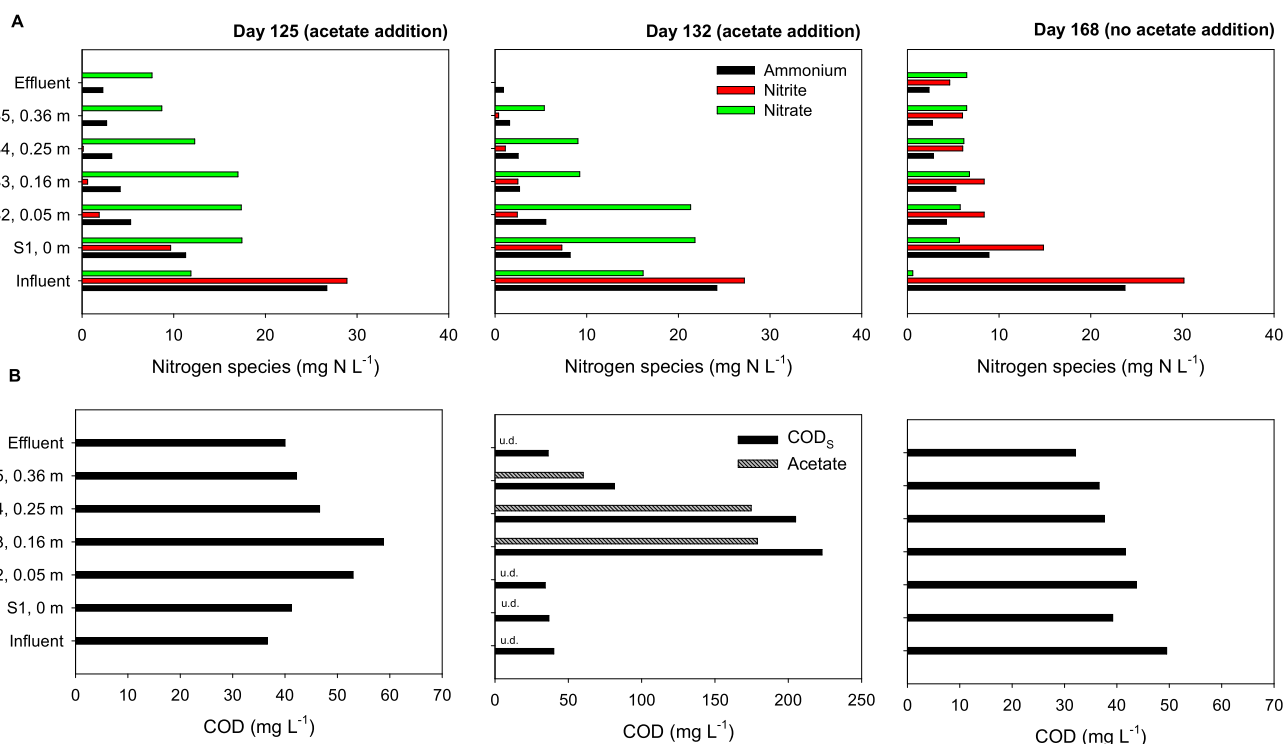


Fig. 5. Ammonium, nitrite, nitrate concentrations (A) and soluble chemical oxygen demand (COD_s) and acetate (measured as COD) concentrations (B) corresponding to different sludge bed heights sections 0 m (S1), 0.05 m (S2), 0.16 m (S3), 0.25 m (S4) and 0.36 m (S5) of the CANDLE reactor during (period II, days 125 and 132, when acetate addition was under and over-dimensioned, respectively) and without acetate (period III, day 168) addition. *u.d., under detection limit. (For interpretation of the references to colour, the reader is referred to the web version of this article).

operational periods of the CANDLE reactor with and without acetate addition at 20 °C (period II and III, respectively) and with acetate addition at 17 °C (period IV) (Fig. 6). The measured N₂O emission factor (EF) (0.002 %, on average from all available measurements) was maintained low during the different operational periods, with and without acetate addition. This is consistent for a reactor where the main biological activity is the anammox process since previous studies indicated that anammox lacks of genes producing N₂O [30]. As anammox do not produce N₂O, the measured N₂O emissions may account on other microbial pathways. They can be attributed to incomplete heterotrophic denitrification activity [31] due to the limited organic matter contained in the mainstream wastewater or because of the imposed external C-source limiting conditions. They can also be related to the AOB of the incoming wastewater transported to the CANDLE reactor and located at the bottom of the sludge bed “i.e. just in the entry”, where they can benefit from the residual dissolved oxygen (DO) of the inflow wastewater. Actually, N₂O production by AOB is enhanced under low oxygen concentrations or anoxic environments, prevailing conditions within anammox reactors [32].

The measured average N₂O off-gas concentrations of the CANDLE

reactor were lower when acetate addition occurred (Fig. 6). This agrees with the fact that heterotrophic denitrifiers can act as an N₂O sink when COD is not limiting denitrification [33]. From days 125 to 130 (period II), acetate addition was deliberately under-dimensioned, resulting into a peak of N₂O off-gas concentrations (Fig. 6). This confirmed that the amount of N₂O that the denitrifiers can consume is smaller under COD limiting conditions [31]. To demonstrate the positive effects of COD for mitigation of N₂O emissions, acetate addition was completely stopped on day 140 (period III, see Fig. 3). This resulted in a gradual increase of the N₂O off-gas concentrations (Fig. 6). However, this slow and progressive increase was not expected. During the first days after stopping acetate addition (days 140 to 149), nitrate reduction was still active (i.e. the nitrate production to ammonium consumption ratio was 0.17 ± 0.05 , see Fig A.1, Supporting Information). A plausible possibility of the gradual N₂O off-gas concentrations increase is related to the capability of heterotrophic denitrifying bacteria to reduce a fraction of the nitrate produced by the anammox process by using the stored organic compounds during the previous acetate addition period (i.e. exposed to organic limiting conditions). When the nitrate produced to the ammonium consumption ratio agreed with the stoichiometric produced by the

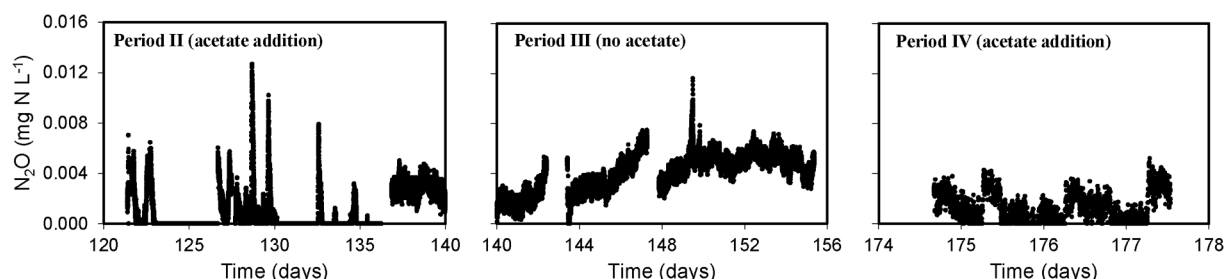


Fig. 6. N₂O off-gas concentrations during period II (acetate addition) and period III (no acetate) at 20 °C and during period IV (acetate addition) at 17 °C.

anammox process (0.29 ± 0.03 ; Fig. A.1), i.e. heterotrophic denitrifying activity was severely limited by the absence of organic matter, N_2O off-gas concentrations increased and became rather stable at a maximum value (Fig. 6). Finally, when acetate addition was re-established and over-dimensioned (period IV, from days 175 to 177), N_2O emissions decreased to minimal values, confirming that heterotrophic denitrifiers acted as an N_2O sink (Fig. 6).

3.4. Ex-situ assessment of heterotrophic denitrifying activity

Ex-situ heterotrophic denitrifying batch tests aimed to assess heterotrophic denitrifying activity of biomass located above the external C-source feeding before (day 43, period I) and during (day 98, period II) acetate addition to the CANDLE reactor by using nitrite and/or nitrate as different electron acceptors. Before acetate addition (period I), nitrite and nitrate consumption rates were in the same range (Fig. 7). When both electron acceptors were added together, they were simultaneously reduced although nitrate consumption rates were higher (see Table A.2 in Supporting Information). When assessing heterotrophic denitrifying activity after a period of acetate supply to the CANDLE reactor (i.e. day 98), nitrite and nitrate consumption rates clearly increased compared to previous tests (Fig. 7). Nitrate consumption rates were ca. four times higher than those of nitrite, indicating a preference of the denitrifying biomass towards nitrate as electron acceptor (see Table A.2 in Supporting Information). This was confirmed when both electron acceptors were added together, as nitrate was firstly reduced. Under this situation, nitrite consumption started right after nitrate depletion (ca. 22 h after starting batch activity tests). Nitrite consumption rates were ca. 5 times higher than the achieved when only nitrite was used as electron acceptor (Fig. 7). This might be attributed as nitrite reduction occurred during the exponential growth phase of heterotrophic denitrifying bacteria, which present doubling times of ca. 9 h at 20 °C [34]. The ex-situ batch tests performed during the period of acetate addition showed that heterotrophic denitrifiers preferred to use nitrate over nitrite as electron acceptor, although in the absence of nitrate, nitrite reduction was also possible.

3.5. Impact of acetate addition on microbial community

The total number of sequences for each library after quality analysis

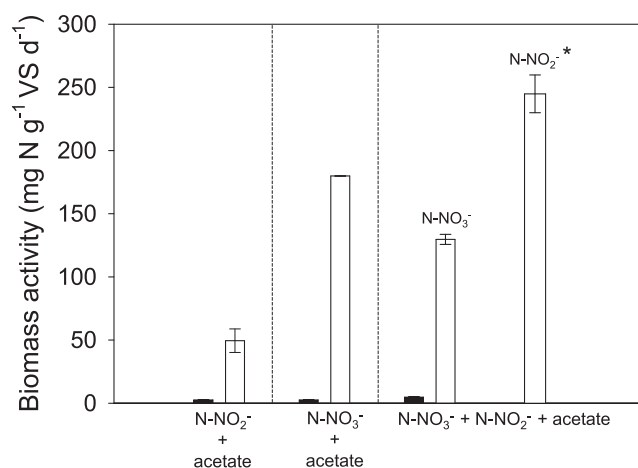


Fig. 7. Specific heterotrophic denitrifying activities from ex-situ batch tests before (black) and during acetate addition (white) on days 43 and 98, respectively. Activities before acetate addition (in black) have been plotted at a smaller Y axis scale in the Supporting Information (Fig. A.6). The applied COD/ $N-NO_x$ ratios and nitrate and nitrite concentrations of each ex-situ batch test can be found in Supplementary Information (Table A.2). *Corresponds to the nitrite consumption rate after nitrate depletion within the ex-situ batch tests where both electron acceptors were added.

and removal of low-quality sequences can be found in Supplementary Information (Fig. A.9). All libraries presented an average length of 290 bps per sequence.

During period I (before acetate addition), the microbial community presented a high abundance of *Candidatus Brocadia* in both S1 (28 %) and S3 (12 %) (Fig. 8). Also, different abundances of heterotrophic denitrifying bacteria were detected. Chloroflexi, Acidobacteria and Chlorobi (specifically *Ignavibacterium* genus) phylum were observed with abundances ranging from 8 to 21 % (Fig. 8). These phyla represent a large fraction of the microbial diversity within anammox reactors without the addition of any external C-source [35–37]. Under these conditions, heterotrophic denitrifying bacteria can degrade the extracellular polymeric substances (EPS) produced by anammox bacteria to use it as an organic carbon source while encoding nitrite and/or nitrate respiration [37,38]. Overall, no significant differences were detected on the microbial community within the bottom and middle sections of the sludge bed (see similitude results from Jaccard Index, Table A.4 in Supporting Information). Further, anammox bacteria were present in both sludge bed sections in a significant amount.

After ca. 2 months of acetate addition, microbial composition changed within the middle sludge section S3, while it remained almost unaffected within the sludge bed section S1 (see Table A.4 in Supporting Information). *Candidatus Brocadia* still presented a high abundance within the bottom sludge bed section (30 %), while its abundance became significantly reduced within upper and middle sludge sections (<3 %) (Fig. 8 and Fig. A.7 in Supporting Information). However, the small anammox fraction of S3 was still dominated by the genus *Candidatus Brocadia*, according to the obtained results from the specific primer (Fig. A.8 in Supporting Information). Within middle and upper sludge layers, the microbial composition was strongly dominated by the genus *Thauera* (33 to 46 %), resulting into a decrease of the previously detected heterotrophic denitrifiers before acetate supply (Fig. 8 and Fig. A.7 in Supporting Information). *Thauera* was reported to provide nitrite for anammox bacteria through reducing nitrate to nitrite in denitrification/anammox processes [38–41]. Also, a high abundance of *Thauera* was found in a CANON reactor [42] and when coupling anammox and denitrification in a single reactor fed with acetate as a C-source [39]. *Thauera* prefers to use nitrate over nitrite as electron acceptor [39]. Nevertheless, nitrite can be also reduced by *Thauera* denitrifiers after nitrate depletion if a sufficient amount of organic matter is still available [39]. These results are in accordance with the ex-situ batch activity tests performed during acetate addition (i.e. day 98) when both electron acceptors were added, as nitrite reduction occurred right after nitrate consumption.

Microbial diversity results confirmed that anammox activity dominated the bottom sludge bed section of the CANDLE reactor. Results from profile concentrations showed that nitrate reduction took place beyond the acetate feeding point (i.e. sampling S3), in accordance with the developed heterotrophic denitrifying community (i.e. *Thauera*). The obtained microbial community data confirmed that the development of a significant denitrifying community in the middle and upper sections of the CANDLE reactor did not hinder the stability of the anammox community located within the bottom sludge bed section.

3.6. Implication of the findings

This research proposed a new reactor configuration to couple anammox and heterotrophic denitrifying processes in a single reactor unit by treating a real mainstream wastewater (containing almost no biodegradable COD and amended with ammonium and nitrite) at temperatures from 20 to 14 °C. A high and stable nitrogen removal performance was obtained, even under extra incoming nitrate concentrations (i.e., when simulating a poor performance of previous mainstream stages). Full-scale implementation of anammox in the main waterline is known to currently present as main bottleneck the production of nitrite (i.e., circumventing the production of nitrate by nitrite

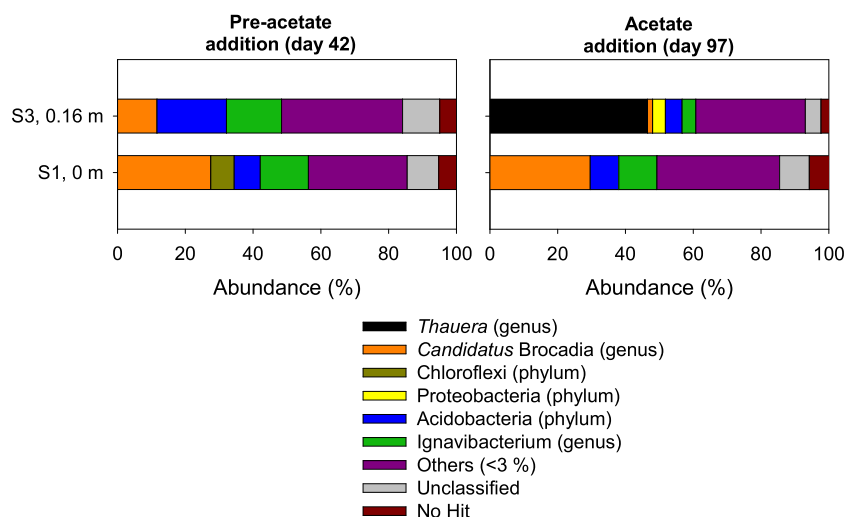


Fig. 8. Microbial diversity using the 515F-806R primer for libraries before and after ca. two months of acetate addition (day 42 and 97, respectively) at different CANDLE reactor heights: 0 m (S1) and 0.16 m (S3). Relative abundance was calculated by only considering those microorganisms in which the 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).

oxidizing bacteria (NOB)). Despite previous reports have highlighted the stability of the partial nitrification in granular sludge reactors (when the PN/AMX is split in two different units, as in Fig. 1A) [10,11], this was never demonstrated feasible with real wastewater with large reactor volumes. If this technology would demonstrate its feasibility, still there is the risk of potential episodes of nitrate production, particularly during the colder days in the winter season. The CANDLE reactor offers a new approach for mainstream anammox as it allowed to overcome the associated drawbacks of previous mainstream stages (e.g., NOB proliferation at low temperatures within the PN step) as demonstrated by the low total nitrogen effluent concentrations ($\leq 8 \text{ mg N L}^{-1}$; see Table 1). A better reactor performance could have been obtained by optimizing the inflow nitrite to ammonium ratio for the anammox process and by constantly adapting the organic load to the nitrate concentrations. For future studies, a proper automatic control strategy of the incoming ammonium load should be desirable (see [15]). The automatic control of the COD addition is a complex problem because requires of online measurements of the nitrogen load to the CANDLE reactor, and the control strategy and reactor sensors to be used require for further research.

The use of acetate as an organic source was proposed in this work. Acetate is preferentially used for nitrate reductase rather than nitrite reductase within the electron transfer chain [38], and thus, beneficial to reduce the produced nitrate by anammox bacteria. Further, acetate has been reported to be mostly converted to PHB as a storage compound rather than being used for biosynthesis [28,38]. This offers an advantage for mainstream anammox as it allows to buffer the instability of previous mainstream stages by reducing the extra incoming nitrate (e.g., PN destabilization at low temperatures). However, the use of acetate requires for higher economical costs compared to other organic sources [43]. Future studies should focus on investigating the feasibility of using other organic sources such as glycerol or ethanol, among others [41,44]. The direct use of the sewage stream as an organic source is not recommended; the incoming nitrogen from the sewage (i.e., ammonia) could not be converted as the reactor configuration of this study was designed to add the C-source beyond the anammox sludge bed, to avoid negative effects on anammox performance.

Recently, different studies proposed the application of a partial denitrification/anammox (PD/AMX) process to the main water line to overcome the associated drawbacks of achieving a stable nitrite production within PN/AMX systems at low ammonium strength and low temperature conditions. Promising results in terms of attaining high

nitrogen removal rates for one-stage PD/AMX systems at 27°C [39] and at temperatures ranging from 30 to 20°C [45] were reported.

For a qualitative comparison of the PN/AMX process proposed in this study with the PD/AMX configuration, two possible main water lines of an urban WWTP are defined in Fig. 1. Considering the main objective of both configurations is to enable maximum energy recovery as biogas, both processes (PD/AMX and PN/AMX) should be integrated in the mainstream with a previous organic matter recovery step, such a HRAS system, to redirect the organic matter to anaerobic digestion. After this first step, both configurations will receive an ammonia nitrogen inflow with a low C/N ratio. The main advantage of PD/AMX over PN/AMX systems is the removal of the nitrification step, which requires a strict control of NOB proliferation. Despite this, a proper control strategy to obtain a stable denitrification (i.e. by controlling the adequate nitrate to ammonium ratio) is needed for PD/AMX systems. Further, the PD/AMX stability at temperatures below 20°C have not been yet proved, which is probably linked to the complexity of maintaining a high anammox activity at low temperatures due to the low anammox abundance populations detected within the employed SBR configurations [41,45,46]. In comparison to PD/AMX systems, the PN/AMX process coupled to full heterotrophic denitrification (i.e. the so-called CANDLE configuration proposed in this study) requires for a 20 to 30 % lower oxygen consumption as partial nitrification instead of nitrification is performed [39]. In addition, a higher amount of organic dosage is required for PD/AMX than for PN/AMX configurations; for the PD/AMX process, the nitrate obtained from the nitrification step (50 % of the incoming nitrogen) is denitrified to nitrite, whereas only the produced nitrate by anammox bacteria needs to be denitrified in PN/AMX systems (i.e. 10 % of the incoming nitrogen).

4. Conclusions

- The ad hoc reactor design for Coupling ANammox and Denitrification in a single unit (i.e. the CANDLE reactor) allowed the addition of an external organic source for the removal of nitrate produced by anammox bacteria without compromising the stability of the anammox process.
- The coexistence of anammox and heterotrophic denitrifying bacteria in a CANDLE reactor was possible as anammox activity dominated within bottom sludge bed sections, while heterotrophic denitrification occurred within middle and upper sludge bed sections.

- N₂O emissions were lower during the addition of an external carbon source, indicating that heterotrophic denitrifiers acted as an N₂O sink.
- Microbial diversity results confirmed that both populations (anammox and heterotrophic denitrifiers) occupied differentiated niche compartments in a CANDLE reactor.
- Anammox population was dominated by the genus *Candidatus Brocadia* while the *Thauera* genus dominated among heterotrophic denitrifiers.
- The CANDLE reactor has been shown to be a feasible technology to enhance the robustness of the two-stage mainstream partial nitrification/anammox process, alleviating the negative effects from the previous treatment stages.

Declaration of Competing Interest

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cej.2021.134087>.

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