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Robustness of mainstream anammox activity at bench and pilot scale

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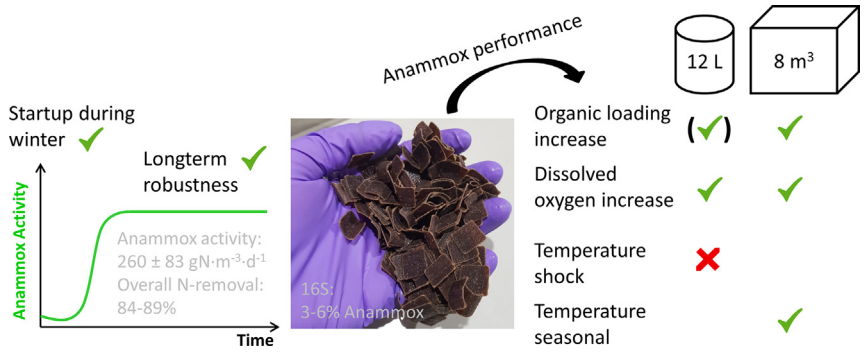
HIGHLIGHTS

- Successful startup of an 8 m^3 AMX reactor with NO_2^- amended municipal wastewater.
- AMX embedded in biofilm regain activity rapidly after exposure to dissolved oxygen.
- Organic shock loading does not impact AMX activity if nitrate is available.
- AMX community adapts to seasonal temperatures with good performance down to 13 °C.
- But, at bench scale temperature shocks strongly decrease AMX activity.

GRAPHICAL ABSTRACT



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ABSTRACT

New technologies and processes, such as mainstream anammox, aim to reduce energy requirements of wastewater treatment and improve effluent quality. However, in municipal wastewater (MWW) anammox system are often unstable due to process control disturbance, influent variability, or unwanted nitrite oxidizing bacteria (NOB). This study examines the anammox system by focusing on anammox activity and its robustness in a mainstream environment. An 8 m^3 pilot-scale sequencing batch reactor (SBR) receiving pretreated MWW (with external nitrite addition) was seeded with pre-colonized carriers. Within six months at 12–20 °C an anammox activity of 200 $gN \cdot m^{-3} \cdot d^{-1}$ was achieved. After the startup an anammox activity of 260 ± 83 $gN \cdot m^{-3} \cdot d^{-1}$ was maintained over 450 days. The robustness of the anammox activity was analyzed through three disturbance experiments. Anammox biofilm on carriers were exposed to dissolved oxygen (DO = 1.6 $mg \cdot L^{-1}$, intermittent aeration), organic loading rate (OLR, C/N increased from 2:1 to 5:1) and temperature disturbances (20 °C to 12 °C) in triplicate 12 L bench scale reactors. The anammox activity and microbial community was monitored during these disturbances. The DO and OLR disturbance experiments were replicated at pilot scale to investigate upscaling effects. Bench and pilot scale anammox activity were unaffected by the DO disturbance. Similarly, an increase in OLR did not deteriorate the bench and pilot scale anammox activity, if nitrate was available. When, at bench scale, the reactor temperature was reduced from 20 °C to 12 °C overnight, anammox activity decreased significantly, this was not the case for the slow seasonal temperature changes (12–25 °C) at pilot scale where no strong temperature dependency was detected in winter. Metagenomic analysis revealed a broad range of *Brocadiaceae* species with no single dominant anammox species. Anammox thrive under mainstream conditions and can withstand typical process disruptions.

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1. Introduction

Future wastewater treatment plants are under increasing pressure to be more climate neutral but must also improve their effluent quality. Nowadays advanced nitrogen removal requires energy intensive aeration for nitrification and, often, additional methanol dosing for denitrification. Mainstream partial nitritation and anammox (PNA, aka autotrophic deammonification (Xiang et al., 2021)) is a major step forward due to its low oxygen requirement. Furthermore, PNA requires no organic carbon for nitrogen removal (Third et al., 2001), and therefore, all carbon flows can be redirected for purposes such as biogas or bioplastics production.

Anammox processes have been employed in sidestream wastewater treatment for two decades (van Dongen et al., 2001). These sidestream treatment plants generally have performed well, but around 30% have underperformed in response to pH shocks, high C/N ratio, high influent solids, or aeration problems (Lackner et al., 2014). Furthermore, low temperatures and over-aeration can result in growth of NOB (Akaboci et al., 2018; Xu et al., 2020). Mainstream anammox systems will be confronted with similar process disturbances. For example, in a pilot scale mainstream anammox system, high solids in the effluent of the pretreatment stage (carbon removal) negatively affected the PNA performance (Hoekstra et al., 2018).

Three disturbance events which have a high likelihood to occur in a mainstream anammox system are explored in more detail.

(1) Dissolved oxygen inhibits anammox bacteria. In suspended cultures, anammox bacteria completely shut down even at oxygen concentrations below 0.2 $mgO_2 \cdot L^{-1}$ (Joss et al., 2011; Lotti et al., 2014a). In granular and biofilm anammox systems, DO inhibition concentrations will depend on the ability of other bacteria to deplete oxygen before it reaches the anammox population. In combined PNA reactors high (4 $mgO_2 \cdot L^{-1}$) and very low (0.17 $mgO_2 \cdot L^{-1}$) DO concentrations have been successfully used (Jiang et al., 2020; Laureni et al., 2019). DO can be carried over from previous aerated stages, e.g., partial nitritation or high-rate activated sludge (HRAS). Human error, equipment- (e.g., aerators) or software-failure could also introduce excess DO into an anammox reactor. Dissolved oxygen can directly affect anammox bacteria by, for example, increasing their decay coefficient or decreasing their growth rate due to recovery periods (Seuntjens et al., 2018; Wang et al., 2018b). Indirectly DO can affect anammox bacteria by promoting the growth of NOBs, which compete for nitrite. However, it is unclear to what extent these observations are relevant for the performance of a biofilm that is not dominated by anammox bacteria, as expected in a mainstream system.

(2) Increased organic loading rates can occur, e.g., due to an overloaded HRAS or other carbon capturing stages. These organics may be problematic for three main reasons. (i) They can lead to the out-competition of slow growing anammox bacteria by heterotrophs (Jenni et al., 2014). (ii) Organics can be toxic to anammox bacteria, e.g., methanol (Jin et al., 2012). (iii) The organics can induce the anammox bacteria to switch to a heterotrophic metabolism, which decreases their deammonification rate (Kartal et al., 2007a). However, it remains uncertain how a short-term increase in MWW organics will affect the anammox activity and its surrounding community.

(3) Lastly, as reported by many researchers in recent years, a decrease in temperature can induce a sharp decline in the performance of anammox reactors. This decline can significantly deviate from predications made by the Arrhenius equation (Lotti et al., 2015b). Nonetheless, high-performing anammox reactors are still possible at cold temperatures (De Cocker et al., 2018; Hendrickx et al., 2014; Kouba et al., 2018). Thus, it is worthwhile differentiating between two types of temperature change: Slow seasonal temperature changes between 12 °C and 25 °C (representative for temperate climates) and rapid but short-term temperature shifts as induced by cold rain or snowmelt. In the existing literature, contradictory results exists, concerning whether such rapid temperature shifts will permanently decrease anammox activity (Lackner et al., 2015; Laureni et al., 2016).

If an anammox system is exposed to a disturbance, two questions are important. (I): Does the anammox activity decrease in response to the disturbance? And following the disturbance: (II) is the decrease in activity temporary or permanent? In the current study, these questions are evaluated for three common disturbances: **(1)** increased DO, **(2)** increased organic loading rate, and **(3)** low temperatures. As an experimental setting, a pilot scale (8 m^3) anammox reactor, fed with nitrite-amended MWW, was started up. After the startup, biomass from this reactor was exposed to the three different disturbances, in triplicate bench scale reactors. The extent to which the disturbances influenced anammox activity and their surrounding community, and whether anammox activity rapidly recovers afterwards, was investigated. To shed light on upscaling effects, the DO and organic loading rate experiments were repeated at pilot scale. This work discusses anammox stability under mainstream conditions when nitrite is provided.

2. Materials & methods

2.1. Wastewater characteristics

All experiments were conducted with MWW from the city of Dübendorf, Switzerland. The MWW was directly pumped from the municipal sewer and mechanically pretreated (5 mm punched hole grid and grit removal), after which it entered the primary sedimentation (PS) tank (see Fig. 1). After the PS, during dry weather, the wastewater contained 492 ± 58 total COD (tCOD)·L^{-1}, 238 ± 69 mg soluble COD (sCOD)·L^{-1}, 49 ± 3 mg total nitrogen (TN)·L^{-1} and 28 ± 3 mg NH_4^+-N·L^{-1}. Wet weather wastewater characteristics are summarized in SI, Table S1.

2.2. Reactor operation

2.2.1. Pilot scale operation

R1-CARB (8 m^3) was operated as a sequencing batch reactor (SBR) with a solids retention time (SRT) of 0.5 d at temperatures >18 °C and 1 d at temperatures <18 °C. During the reaction phase a DO of 0.5–1 $mgO_2 \cdot L^{-1}$ was maintained. R2-Buf (8 m^3) was intermittently aerated at 2 $mgO_2 \cdot L^{-1}$ with a SRT of four to eight days. More information on the different SBR steps of R1-Carb and R2-Buf can be found in the supplementary information (SI, 1. Pilot scale reactor operation). These two pretreatment steps (R1-CARB + R2-Buf) aimed to reduce the C:N ratio and resulted in the following influent composition for R3-AMX: 50–60 $mg \cdot L^{-1}$ COD, 15–25 $mgNH_4^+$-N·L^{-1}, 0–1 $mgNO_2^-$-N·L^{-1} and 5–10 $mgNO_3^-$-N·L^{-1}. R3-AMX is an 8 m^3 SBR reactor with the following steps: (1) Decantation: R3-AMX was decanted from 8 m^3 to 4.5 m^3 (ca. 20 min), the carriers were retained with a sieve. (2) Filling: The reactor was filled with effluent from R2-Buf from 4.5 m^3 to 8 m^3 (60 min), i.e., 44% volume exchange. (3) Reaction phase: During the reaction phase the reactor was stirred and nitrite was continuously added (3.5 M $NaNO_2$ solution) until an ammonium concentration of <1 $mgNH_4^+$-N·L^{-1} was measured (ammonium ISE, Endress + Hauser, Switzerland) (120–1440 min). During the startup phase, nitrite flow rate was proportionally increased as anammox activity increased. This shortened the length of the reaction time and thus decreased hydraulic retention time (HRT). R3-AMX contained 167 $m^2 \cdot m^{-3}$ of the carrier material FLUOPUR® (synthetic porous fleece material, WABAG Water Technology Ltd., Switzerland) used for biofilm growth (SI, Fig. S1A). R3-AMX was

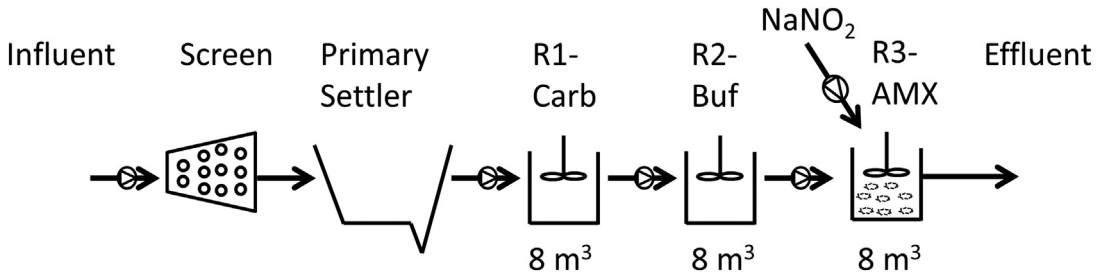

Fig. 1. Pilot scale setup. The carbon removal stage (R1-CARB) and buffer stage (R2-Buf) were suspended sludge systems. The anammox stage (R3-AMX) used carriers. Each reactor had a volume of 8 m^3 and all reactors were operated as sequencing batch reactors (SBR).

inoculated with new FLUOPUR carrier two years prior to this study. During these two years, a nitrifying community with very weak anammox activity (Fig. 2, day 0) had formed on the carrier (SI, Fig. S1B). During days 435–445 12.5% of the carriers were taken from R3-AMX to inoculate another pilot scale reactor. An overview of the whole experimental time is provided in Table 1.

2.2.2. Bench scale reactor operation

For each bench scale experiment (i.e., on day 287, 308 and 335 for DO, organics and temperature experiments, respectively) 1 kg (wet weight) of carriers were removed from R3-AMX for each replicate ($n = 3$) 12 L reactor, resulting in a specific carrier surface of 225 $m^2 \cdot m^{-3}$. The reactors were operated as SBRs, without a settling phase, since carriers were retained via a sieve. The SBR cycle involved a feeding step (6 L, 30 min), a reaction phase (length variable), a synchronization phase (i.e., idle phase until all replicates were ready to start a new cycle, length variable), and a discharge phase (6 L, 10 min). The influent for DO and temperature bench scale experiments was effluent from R2-Buf (i.e., the same influent as R3-AMX receives), and a mixture of R2-Buf effluent and PS effluent for the increased organic loading rate experiment. Additionally external ammonium was added to reach 40 ± 10 mgNH_4^+-N·L^{-1} at the start of each SBR cycle. During the reaction phase, NO_2^- (as $NaNO_2$) was continuously added until a concentration of <5 mgNH_4^+-N·L^{-1} was measured, detected by an ammonium online sensor (Endress+Hauser Ammonium Electrode ISE, 71109938). The temperature was controlled via a water heat jacket (Julabo GmbH, Germany) and set to maintain the influent MWW temperature at the start of each disturbance experiment (e.g., 25 °C, 22 °C and 20 °C for DO, organic and temperature experiments, respectively) to avoid temperature-related stress. The reactors were stirred at 130 rpm by a mechanical stirrer (Heidolph, RZR 2020, Germany). Depending on the disturbance experiment, filling and reaction steps of the SBR were altered, as described below in Section 2.3.

Before the start of a disturbance experiment, the bench scale reactors were operated for three days without applying a disturbance. On the last day, maximum anammox batch activity tests were performed (as described in Section 2.4) to find the anammox activity before any disturbance was applied. Thereafter, activity tests were performed on a daily basis.

2.3. Disturbance experiments at bench scale

Additional information for each disturbance experiment, i.e., why specific values for DO, COD and temperature were selected, is available in the supplementary information (SI, 2. Parameter selection for disturbance experiments). SBR cycle operation for R3-AMX (pilot scale) during disturbance experiments can be found in SI, Table S2.

2.3.1. Increased DO experiment

Every 9 h, fresh effluent of R2-Buf was pumped to an equilibration tank, from which the MWW was used as the influent for all bench scale reactors. The triplicate bench scale reactors were operated as described in Section 2.2.2, except for the reaction phase. For this experiment, the reaction phase had four sub steps: (i) an anoxic step where nitrite was continually added (40′), (ii) an anoxic nitrite depletion step without nitrite dosing (5′), (iii) an aerated disturbance step at 1.6 mg$O_2 \cdot L^{-1}$ (20′), and (iv) non-aerated stirring step to start DO depletion (5′). Additional information regarding these sub steps can be found in the supplementary information (SI, 5.1 Increased DO – Sub steps). These four sub steps were repeated until the reactor reached <5 mgNH_4^+-N·L^{-1}. Reactor temperature was controlled at 25 °C (maintaining current MWW temperature). This DO regime was applied for two weeks.

2.3.2. Increased organic loading experiment

Reactor filling was divided into two steps: (i) 3 L of fill volume was MWW after primary sedimentation (see SI Table S1, 200–500 mgCOD·L^{-1}) and (ii) the 3 L was effluent from R2-Buf (50–60 mgCOD·L^{-1}). Reactor temperature was set at 22 °C (maintaining current MWW temperature). The experiment had three stages, each with

Table 1
Overview of all phases and disturbance experiments that were conducted.

Phase	Experiment	Scale	Days [d]	HRT [h]	Disturbance
Phase I Start Up	–	Pilot	0–180	9–80	–
Phase II Disturbance Experiments	Increased DO	Bench Scale	290–305	24	Intermittent Aeration, at 1.6 mgDO·L^{-1}, 20 min on and 40 min off
		Pilot Scale	450–470	12	
	Increased organic loading	Bench Scale	311–331	9–12	Organic loading in influent increased by factor 3.3 by increasing influent organic concentration from 60 to 200 mgCOD·L^{-1} Operated with or without nitrate dosing
		Pilot Scale	520–547	9–12	
	Temperature	Bench Scale	338–358	6–8 (20 °C) 24–36 (12 °C)	Temperature reduced in the reactor from 20 to 12 °C
		Pilot Scale	180–650	9–12 (13–25°C)	Seasonal variability of influent temperature
Phase III Continued reactor operation	–	Pilot	548–650	9–12	–

a duration of a week. During stage I, influent COD (COD_{Inf}) was increased, as described above, and nitrate was added (20 mgNO_3^--N·L^{-1}) to the reactors at the end of the feeding phase. During stage II, the influent was switched 100% effluent of R2-Buf to recover reactor performance. During stage III, influent was switched again to 50% primary sedimentation effluent and 50% R2-Buf effluent, but nitrate was not added.

2.3.3. Decreased temperature experiment

As in Section 2.3.1, every 9 h, fresh effluent of R2-Buf was pumped to an equilibration tank, after which the MWW was used as influent for all bench scale reactors. During the first week, the temperature was set to 20 °C (maintaining current MWW temperature) and then decreased overnight (12 h) to 12 °C, where it was maintained for one week. After one week at 12 °C the reactor's temperature was increased back to 20 °C for another week.

2.4. Batch tests at bench and pilot scale

During all disturbance experiments, daily anoxic batch test were performed in situ to monitor maximum anammox activity, similarly as in Laureni et al. (2015). In brief, reactor temperature was kept the same as during normal operation. Although pH was not controlled, it always remained between 7.2 and 7.8. Before starting the batch test, 30–50 mgNH_4^+-N·L^{-1} as NH_4Cl and 10–30 mgNO_2^-·L^{-1} as $NaNO_2$ were added. Twenty minutes after the addition of ammonium and nitrite, sampling was started. Three to four samples were taken during each activity measurement and the inorganic nitrogen species were measured off-line. Sampling intervals ranged between 30 and 120 min, depending on the ammonium removal rate. Here, anammox activity is defined as the sum of the NH_4^+-N (r_{NH4}) and NO_2^--N (r_{NO2}) removal rates minus the NO_3^--N (r_{NO3}) production rate in g·m^{-3}·d^{-1}.

$$r_N = |r_{NH4} + r_{NO2}| - |r_{NO3}| \quad (1)$$

Rates for each N species (r_{NH4}, r_{NO2}, r_{NO3}) were calculated to check for anammox stoichiometry, according to Eqs. (2) and (3) (Strous et al., 1998).

$$\frac{r_{NO2}}{r_{NH4}} \approx 1.3 \quad (2)$$

$$\left|\frac{r_{NO3}}{r_{NH4}}\right| \approx 0.3 \quad (3)$$

2.5. Analytical methods

Concentration of NH_4^+, NO_2^-, and NO_3^- were measured via ion chromatography (Metrohm AG, 761 Compact IC and 881 Compact IC pro, Switzerland). All samples were centrifuged at 3000 rpm for 1 min and filtered through 0.45 μm filters (Macherey–Nagel) before analysis. COD, TN and total phosphorus (TP) were measured photometrically with test kits (Hach Lange, Düsseldorf, Germany). Total suspended solids (TSS) and volatile suspended solids (VSS) were measured according to APHA (2012) standard protocols. Total solids (TS) and volatile solids (VS) on the carriers were quantified by subtracting the total weight of uncolonized carriers from an equal number of colonized carriers, after drying (24 h) at 105 °C for total solids and incineration (2 h) at 550 °C for volatile solids. Carriers·L^{-1} of reactor volume were counted to compute the TS and VS in the reactor (SI, Table S3).

2.6. Calculations

2.6.1. Nitrogen removal efficiency

To calculate the nitrogen removal efficiency (NRE) the TN from the effluent of the primary settler ($TN_{Influent}$), TN in the effluent of R3-AMX ($TN_{Effluent}$) and the NO_2^--N added to R3-AMX ($NO2\text{-}N_{added}$) are taken into account. Similarly, to calculate NRE of R3-AMX alone, only the inorganic nitrogen is considered of the influent to R3-AMX and effluent of R3-AMX.

$$NRE = 1 - \frac{TN_{Effluent}}{(TN_{Influent} + NO2 - N_{added})} \quad (4)$$

2.6.2. Anammox performance during disturbance experiments

For the increased DO and temperature disturbance experiments, anammox activities (r_N) are normalized to the initial anammox activity before each disturbance experiment. This initial anammox activity is compared to the anammox activity achieved at the end of a disturbance, or at the end of a recovery period. For the organic loading rate disturbance experiments, the normalized r_{NH4} rates are used. If organic compounds are available under anoxic conditions, r_{NO2} and r_{NO3} could be significantly affected by denitrification, whereas r_{NH4} mainly occurs due to anammox activity (apart from ammonium assimilation, which was neglected, and dissimilatory nitrate reduction to ammonium (DNRA), which was not observed).

2.7. 16S rRNA extraction, sequencing and analysis

Samples of suspended biomass (if available) and carriers were taken at the start of the experiments and at the end of each week during the experiments. Samples were immediately frozen in liquid nitrogen and subsequently stored at −80 °C. DNA extraction was performed according to a modified protocol proposed by (Griffiths et al., 2000). In short, two carriers were cut into small squares with scissors, then lysed chemically (CTAB/$KaPO_4$), and physically, by bead beating two times for 45 s and once for 30 s. DNA was extracted with Phenol:Chlorophorm: Isoamylalcohol (25:24:1) and Chloroform:Isoamylalcohol (24:1) and resulting pellets were washed with ice-cold ethanol. The pellet was re-suspended in RNAse-free water. Sequencing was performed by Novogene (Novogene, Hong Kong) with 16S primers for V3/V4 regions. Raw sequences were analyzed within the QIIME2 framework outlined by Bolyen et al. (2019). Primer sequences were removed with the cutadapt QIIME2 plugin. After demultiplexing, read pairs were joined, low-quality reads were filtered out, and all high-quality reads were analyzed with the DEBLUR software to produce amplicon sequence variants (ASVs) based on Illumina Miseq/Hiseq error profiles. Sequences were classified against the Greengenes 16S database (version 13_8). To obtain high-resolution classification of the microbial community, one DNA sample was used for metagenomics shot-gun sequencing. This sample was sequenced on the Illumina NextSeq platform (Illumina, CA, USA) to generate 150 bp paired-end reads (350 bp mean insert size). DNA sequencing was performed at Novogene, Hong Kong. The raw sequencing reads are available on the MG-RAST server with accession number mgs709347. Kaiju (Menzel et al., 2016) was used to taxonomically assign the raw reads, using maximum exact matches of the query sequences translated to amino acids and protein database sequences. The reads were aligned against the NCBI non redundant (NR) protein sequences from all bacteria, archaea, viruses, fungi, and microscopic-sized eukaryotes, respectively.

3. Results

3.1. Startup of mainstream anammox (R3-AMX)

During Phase I (day 0 to 180), R3-AMX was started up. Fig. 2 shows the anammox activity and reactor temperature during the startup phase. Maximum anammox activity exponentially increased over the first six months. Anammox activity doubled approximately every 60 d resulting in an observed μ_{net} of 0.012 d^{-1}, as expected at mainstream conditions in winter (Lotti et al., 2014b). Importantly, anammox activity increased even as temperatures decreased from 22 °C down to 12–13 °C

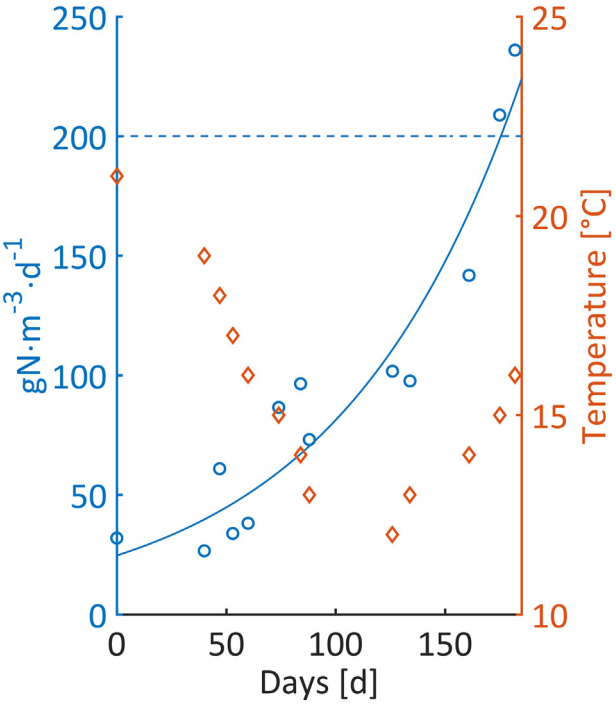

Fig. 2. Maximum anammox activity (hollow blue dots) and measured temperature in the pilot reactor (red diamonds) during the startup phase. The target activity was 200 $gN \cdot m^{-3} \cdot d^{-1}$ (dashed blue line), which was reached after 180 days. The blue line represents exponential increase with $\mu_{net} = 0.012\ d^{-1}$ from an initial activity of 25 $gN \cdot m^{-3} \cdot d^{-1}$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 2, Days 50–100). After achieving a maximum anammox activity of 200 $gN_{removal} \cdot m^{-3} \cdot d^{-1}$, on day 180, nitrogen loading was kept constant. Accordingly, from day 180 onwards, the nitrogen loading of R3-AMX was 0.8 $gN \cdot m^{-2}_{carrier} \cdot d^{-1}$, respectively 133 $gN \cdot m^{-3} \cdot d^{-1}$. The suspended biomass was very low (20–30 $mgTSS \cdot L^{-1}$) due to constant washout and low carbon inflow. Total solids on the carriers fluctuated slightly throughout the year, resulting in 1–1.5 $gTS \cdot L^{-1}$ (SI, Table S4). After R2-Buf, the MWW contained on average 20 $mgNH_4^+\text{-}N \cdot L^{-1}$ and so in R3-AMX 25–30 $mgNO_2^-\text{-}N \cdot L^{-1}$ had to be added, according to the anammox stoichiometry, to reach effluent quality <1 $mgNH_4^+\text{-}N \cdot L^{-1}$. The nitrogen removal efficiency (NRE) of the anammox reactor or over the whole treatment process corresponded to 80% and 84%–89%, respectively, under dry-weather conditions according to Eq. (4).

3.2. Disturbance experiments

Fig. 3 depicts the change of anammox activity in response to different disturbances at bench (A, B, C) and pilot scales (D and E). All activities are normalized to the initial anammox activity of each disturbance experiment.

3.2.1. Increased dissolved oxygen experiment

As presented in Fig. 3A and D (bench and pilot scale, respectively), even after two weeks of intermittent aeration, anammox activity did not decline. Anammox activity even increased at bench scale by 30%. Daily anammox activity measurements are available in the supplementary information (SI, Fig. S3). The anammox stoichiometry r_{NO2}:r_{NH4} and r_{NO3}:r_{NH4} were determined daily during activity assay and are close to theoretical values for anammox, at bench and pilot scale (SI, Figs. S4 and S5).

3.2.2. Increased organic loading rate experiment

At bench scale (Fig. 3B), if nitrate was present, the increased organic loading rate did not impact anammox activity significantly. Yet, denitrification was clearly occurring, as the r_{NO3}:r_{NH4} ratio changed from 0.3 to 0–0.1 (SI, Fig. S10C). During the recovery week, the r_{NO3}:r_{NH4} stoichiometry was again anammox dominated, i.e., at 0.3 (SI, Fig. S10C). However, in the absence of nitrate, increased organic loading rates had a negative effect on anammox activity, reducing it by 49% ± 30%. These trends are also visible in the calculated anammox contribution to overall nitrogen removal (SI, Fig. S11). The anammox activity was more strongly reduced in two of the three bench scale reactors, yielding high standard deviations (SI, Fig. S7). Furthermore, in the presence of surplus nitrate, the r_{NO2}:r_{NH4} ratio was unaffected, i.e., nitrite consumption was likely dominated by anammox activity. However, if nitrate was absent, the r_{NO2}:r_{NH4} increased sharply due to denitritation, i.e., NO_2^- reduction through heterotrophic bacteria (SI, Fig. S10A). At pilot scale (Fig. 3E), no significant changes in anammox activity were detected under all experimental conditions (SI, Fig. S8). The increase in organic loading was clearly visible in the r_{NO3}:r_{NH4} ratio, which switches from 0.3 to 0 when organic loading rate is

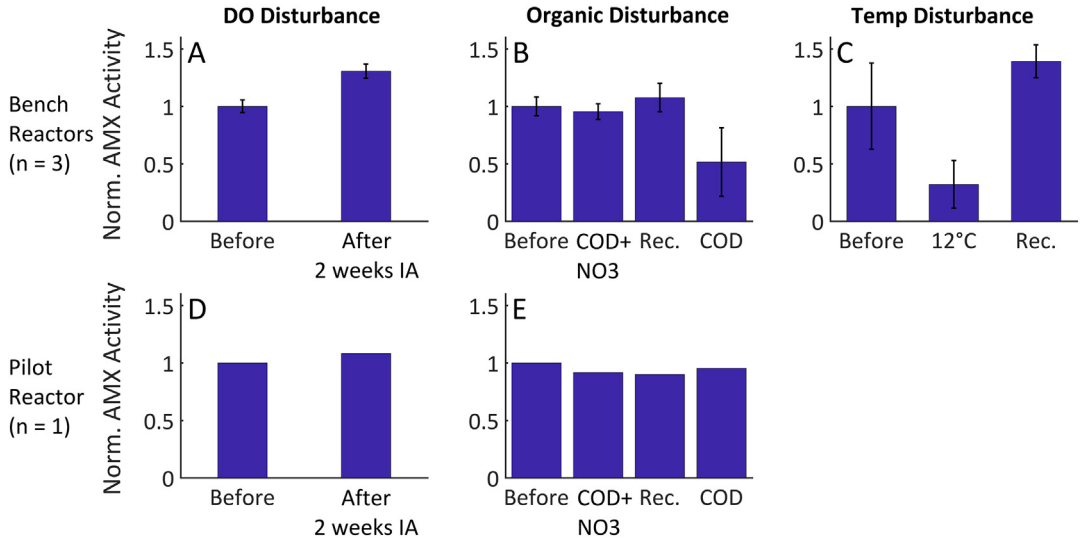

Fig. 3. Anammox activities for bench (panel **A**–**C**) and pilot (panel **D**–**E**) reactors normalized to volumetric anammox activities before the disturbance. Disturbance experiment are presented in **A**, **D** (Intermittent aeration, DO = 1.6 $mgO_2 \cdot L^{-1}$), **B**, **E** (organics increase from 60 $mgCOD \cdot L^{-1}$ to 200 $mgCOD \cdot L^{-1}$) and **C** (temperature reduction 20 °C to 12 °C). Error bars represent standard deviations ($n = 3$) in bench scale experiments. DO: dissolved oxygen; IA: intermittent aeration.

increased. Yet, the r_{NO2}:r_{NH4} ratio and anammox activity remained stable. (SI, Fig. S10B and D).

3.2.3. Temperature disturbance experiment at bench scale

In Fig. 3C, the anammox activity at bench scale at different temperatures is shown. An 8 °C temperature decrease (20 °C to 12 °C) was accompanied by a 68% ± 21% reduction in anammox activity. No increase in activity, i.e., adaptation, was observed during the weeklong operation at 12 °C (SI, Fig. S14). After temperature was reverted to 20 °C, 90% of anammox activity was immediately restored, reaching 100% after three days. One reactor had a significantly worse anammox activity in the first week (i.e., at 20 °C) than the other two reactors, yet it recovered to the same anammox activity as the other two replicate reactors in the last week (SI, Fig. S14, Reactor A4). Accordingly, a large standard deviation can be seen in the first week and more than 100% recovery in the last (Fig. 3C).

3.2.4. Seasonal temperature effects at pilot scale

To investigate temperature effects at pilot scale, anammox activity of R3-AMX was measured throughout the operational period along with ambient MWW temperature. After the initial 180 days, the nitrogen loading was not further increased but maximum anammox activity did continue to increase (Fig. 4, Days 180–345), reaching 500 $gN \cdot m^{-3} \cdot d^{-1}$ at 25 °C. From day 345 to day 425, temperature and anammox activity declined from 25 °C to 18 °C and 500 $gN \cdot m^{-3} \cdot d^{-1}$ to 310 $gN \cdot m^{-3} \cdot d^{-1}$, respectively. A sharp drop in activity (310 $gN \cdot m^{-3} \cdot d^{-1}$ to 150 $gN \cdot m^{-3} \cdot d^{-1}$) was measured prior to the start of the DO experiment at pilot scale (between day 425–448). Thereafter, activity increased again even though temperature was still decreasing (days 450–510). At the coldest MWW temperature (days 495–510, 13 °C) at pilot scale, anammox activity was 200 $gN \cdot m^{-3} \cdot d^{-1}$.

3.3. Community changes during disturbance experiments

3.3.1. Increased DO experiment

Fig. 5 presents shifts in bacterial abundance during the intermittent aeration disturbance experiment. In the top 10 most abundant phyla (Fig. 5A and B, bench and pilot scale, respectively), three aspects should be noted: First, bench and pilot scale reactor populations changed very little during the two weeks of intermittent aeration. Second, from the start of the bench scale experiment (days 290–305) to the start of the pilot scale experiment (days 450–470) the top 10 phyla had changed ranks (e.g., *Chloroflexi* changed from most abundant to fourth most abundant). Third, *Nitrospira* increased in relative abundance from 0.7% to 2.8% and from 1.8% to 7.8% in bench and pilot scale, respectively (SI, Fig. S6). Relative abundance of *Planctomycetes* remained steady around 3%.

3.3.2. Increased organic loading experiments

Fig. 6 shows the bacterial abundance changes at bench scale during the increased organic loading rate experiment. Overall, the community on the carrier (Fig. 6A) is more stable than the community in the suspended biomass (Fig. 6C). The top 10 bacterial phyla on the carrier, before organic loading rates were increased, represent the most abundant phyla during the whole experiment. The total solids on the carriers did not change significantly during the experiment (data not shown). However, in suspension, the same phyla describe 90%–95% of biomass before organic loading rates were increased but only 60% at the end (Fig. 6C). This 30% difference is related to the rapid growth of *Firmicutes* from 3% to 30% in suspension (Fig. 6D). This increase in relative abundance is linked to an absolute increase in suspended biomass from 20 to 30 to 100–200 $mgTSS \cdot L^{-1}$ (SI, Fig. S9). At pilot scale, the bacterial community is very stable on the carriers and in suspension (SI, Fig. S12).

3.3.3. Temperature disturbance experiment

During the temperature disturbance experiment at bench scale 16S sequencing revealed a consistent decrease in abundance (from 3.2% to 1.8%) of the *Planctomycetes* phylum (Fig. 7A). No major changes occurred in the top 10 phyla or on genus level (SI, Figs. S15 and S16). In the pilot scale reactor R3-AMX, the relative abundance of *Planctomycetes* was assessed at various points throughout one year (Fig. 7B). The 16S measurements are not in accordance with the measured activity, which is to say neither an increase nor a decrease in 16S correlated with anammox activity. According to the metagenomic analysis on day 200, *B. caroliniensis* (27%), unclassified *Brocadia* species (27%) and *B. sinica* (18%) were the most abundant anammox species (Fig. 7C).

4. Discussion

4.1. Anammox startup at cold temperatures

Anammox enrichment at low temperatures (10 °C), under laboratory conditions, is possible, as shown by Hendrickx et al. (2014). In that study, the installation of a membrane to withdraw effluent was crucial to retain the slow growing anammox bacteria. The study presented here, further supports the fact that anammox can be enriched at low temperatures, even with MWW. Biomass retention was achieved through biofilm formation on carriers. Anammox activity increased throughout winter at MWW temperatures of 12–15 °C (Fig. 2, days 60–170). Competitive inorganic nitrogen removal rates through

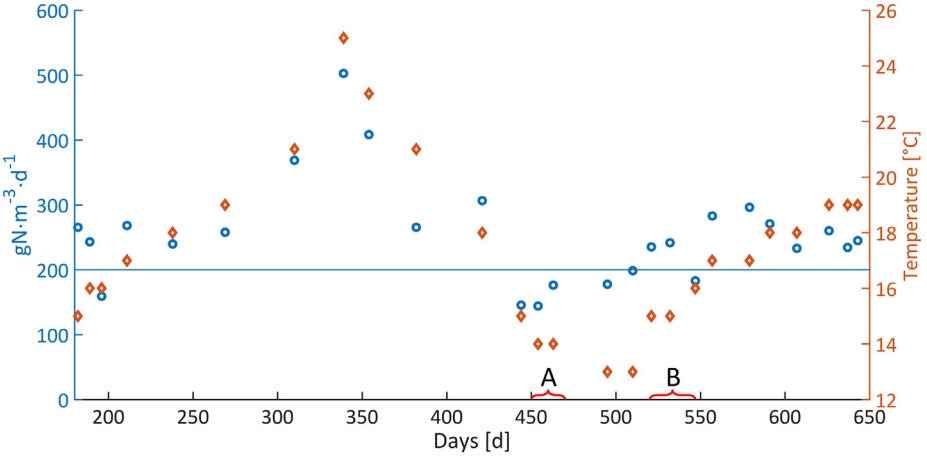

Fig. 4. Anammox activity in blue hollow dots, the blue line indicates target activity of 200 $gN \cdot m^{-3} \cdot d^{-1}$, and MWW temperature in red diamonds in the pilot scale anammox reactor (R3-AMX). Two disturbance periods in the pilot reactor are marked: **A**) DO disturbance and **B**) organic disturbance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

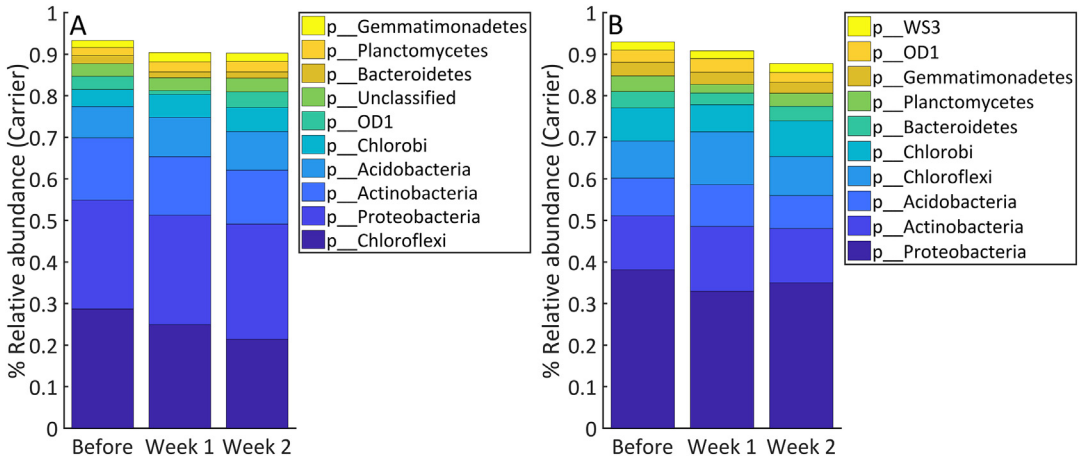

Fig. 5. A) Bench scale, **B**) pilot scale. Top 10 phyla abundance in the biofilm are shown before the experiment, as well as one week and two weeks into intermittent aeration.

anammox (200–500 $gN \cdot m^{-3} \cdot d^{-1}$), can be achieved at mainstream conditions, if nitrite supply is guaranteed. Similar rates for anammox systems have been achieved by other groups working with MWW (Hoekstra et al., 2018; Laureni et al., 2015; Lotti et al., 2014b).

4.2. Anammox activity and disturbances

4.2.1. Does increased DO lead to long recovery periods?

It has been well-established that direct exposure of anammox bacteria to oxygen has an inhibitory effect, but the data presented here show that even after repeated DO spikes performance deterioration was not observed in a mature biofilm. According to Seuntjens et al. (2018), anammox bacteria take 5–37 h to recover from oxygen spikes (0.2–2.1 $mgO_2 \cdot L^{-1}$ for 2.5–8.7 h). Additionally, they showed that the recovery from inhibition started only once micro-aerobic conditions (<0.02 $mgO_2 \cdot L^{-1}$) were reached. We found neither a dependence on micro-aerobic conditions (in the bulk phase) nor long recovery periods. The anammox bacteria immediately regained their activity even if bulk DO was still at 0.3 $mgO_2 \cdot L^{-1}$ (SI, Fig. S2). There are two key differences between their study and ours. First, our oxygen exposure periods were shorter (20 min), but they were repeated (ca. 3–10× per SBR cycle). Second, the community composition and sludge type were different. Seuntjens et al. (2018) used a floccular and anammox dominated culture (75%), whereas the mainstream community in the current study consisted of 2%–6% anammox bacteria cultured in a biofilm. This abundance is in rough agreement with the following gross abundance estimation for a mainstream application: The heterotrophic anoxic yield is 0.3 $gVSS_{Het} \cdot gCOD^{-1}$ and the anammox yield is 0.1 $gVSS_{AMX} \cdot g^{-1}NH_4^+$ (Metcalf et al., 2013; Lotti et al., 2015a). If the wastewater has a COD:N ratio of 2 (which results in a COD:NH_4^+ ratio of about 4:1, if half the ammonium is converted to nitrite) a heterotroph:anammox ratio of around 12:1 is expected, e.g., 7%–8% anammox abundance. The 2–6% relative abundance derived from 16S rRNA is likely a slight underestimate due to insufficient primer pair coverage (Orschler et al., 2019). We suspect that heterotrophs can scavenge all available DO within the biofilm and thus rapidly reverse the oxygen-induced anammox inhibition (SI, Fig. S2). Indeed, even after two weeks of regular and intense aeration, anammox activity remained stable. Thus, factors such as an increased decay coefficient in aerobic conditions, as observed by Wang et al. (2018b), may be mitigated through heterotrophs depleting oxygen. Deviation of anammox ratios r_{NO2}:r_{NH4} and r_{NO3}:r_{NH4} from theoretical values indicate increased stress levels in anammox populations (Yang et al., 2013). However, throughout the two weeks of intermittent aeration, ratios close to the theoretical values were found (SI, Figs. S4 and S5). An event that was not studied in this work was long exposure (hours) to very high oxygen concentrations (4–8 $mgO_2 \cdot L^{-1}$). Such conditions led to long recovery periods (days) for anammox bacteria (Mukarunyana et al., 2018), but, such an extreme event is unlikely to occur in conventional WWTPs. NOBs increased in abundance during the disturbance experiment (SI, Fig. S6). This demonstrates the problem of suppressing

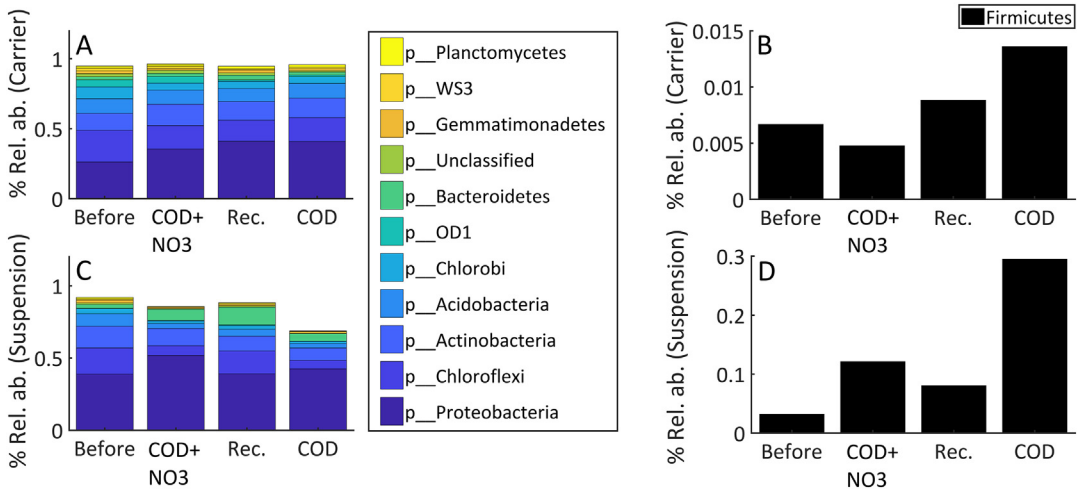

Fig. 6. Bacterial population shift during the organic loading disturbance experiment, at bench scale. Panels **A** and **C** represent top 10 bacterial phyla on the carrier (**A**) and in suspension (**C**). Panels **B** and **D** show *Firmicutes* abundance on the carriers (**B**) and in suspension (**D**).

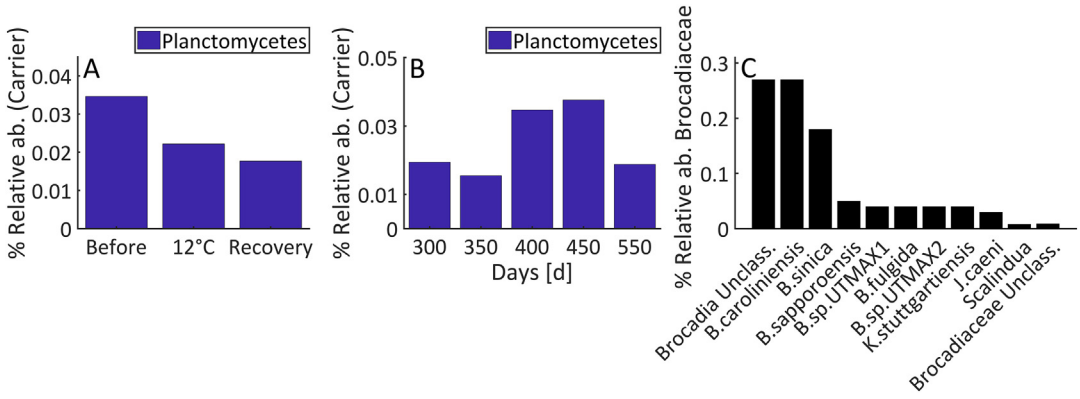

Fig. 7. *Planctomycetes* abundance at bench and pilot scale. **A**) *Planctomycetes* abundance at bench scale is shown during the temperature disturbance experiment. **B**) pilot scale *Planctomycetes* abundance over 250 days is shown. **C**) *Brocadiaceae* distribution in metagenome on day 200. The metagenome revealed a broad distribution of anammox species with none clearly dominating.

NOBs in mainstream single stage PNA systems. But in an anammox reactor this should not pose a big threat, since under normal operation no DO is available.

4.2.2. What are the dangers and advantages of organics in an anammox reactor?

Giustinianovich et al. (2016) published an extensive review on the topic of organics in anammox systems. Their review is focused on the intentional use of organics for the application of simultaneous nitrification, anammox and denitrification (SNAD). In contrast, in this study, the focus is on unexpected, high organic load into an anammox stage. Organics play diverse roles in mainstream anammox systems (Xiao et al., 2021). They can be used by the anammox bacteria themselves (Kartal et al., 2007b), or they can be used to divert nitrite and nitrate to denitrification. Organics encourage the growth of heterotrophic bacteria, which, in the long-term, could outcompete and overgrow anammox bacteria in the biofilm. For example, in a recent long term (100 days) study, anammox activity deteriorated significantly when COD/N ratios higher than 4.5 were used. The authors concluded that this was due to a shift in the microbial community as anammox were out-competed by heterotrophs and therefore displaced within the biofilm layers (Pijuan et al., 2020). However, in the short term, this study found a different outcome. Heterotrophs such as *Firmicutes* (mainly from the genus *Trichococcus*, SI, Fig. S13B) grew rapidly in suspension to take advantage of the increased organic substrate supply. But their population, as well as overall TSS, declined once influent COD was switched from 200 to 400 (C:N = 5–10) to 30–60 mgCOD·L^{-1} (C:N = 1–2) (Fig. 6). This stands in contrast with an IFAS-SBR, which had 90% NRE at a C:N ratio of 2.3, but NRE declined to 30–40% after an increase in influent C:N to 8, even though most COD was oxidized before reaching the anammox reactor (Azari et al., 2020). Furthermore, in this study nitrate seems to be preferred over nitrite for denitrification, and, hence, the anammox reaction continued to perform well in the presence of organics if nitrate for denitrification was available (SI, Fig. S10A and B). Complete depletion of nitrate was linked to (i) a strong reduction in the redox potential from, on average, −179 mV when nitrate was dosed to −277 mV when nitrate was absent (SI, Fig. S10C) and (ii) an unbalancing of the r_{NO2}:r_{NH4} ratio, which indicates denitritation (SI, Fig. S10A). Intriguingly, even though nitrite was supplied in large quantities (30 mgNO_2^--N·L^{-1}) during activity assays, anammox activity remained limited in the absence of nitrate. Thus, other factor(s) impaired anammox functionality. For example, strong anaerobic conditions might have led to the production of sulfide, which is highly toxic to anammox bacteria (Jin et al., 2012).

4.2.3. Rapid versus seasonal temperature changes

The effects of temperature change has been investigated in many different anammox systems (de Almeida Fernandes et al., 2018; De Clippeleir et al., 2013; Ma et al., 2013; Wang et al., 2018a), as it often leads to a significant performance decrease. As described by Lotti et al. (2015b), anammox activity appears to have two temperature dependencies in different ranges, i.e., between 5 and 15 °C and 15 to 35 °C. This is further supported by the recent work of Kouba et al. (2019). Many laboratory studies have either decreased the temperatures rapidly or slowly over the course of a few days to weeks, which may not reflect actual seasonal changes. In agreement with literature, a significant reduction of the anammox activity (68% ± 21%) was measured at bench scale, when exposing the anammox biofilm to a sudden temperature decrease from 20 °C to 12 °C (Fig. 3C). Full recovery was achieved within one to two days when temperature was increased (SI, Fig. S14). This is similar to the results of Laureni et al. (2016) and the moving bed biofilm reactor in Lackner et al. (2015). Interestingly, an anammox SBR with suspended sludge in the same study (Lackner et al., 2015), could not recover from cold temperatures. This suggests that biofilm systems tolerate temperature disturbances better. In the recovery week activity increased, on average, to levels exceeding those at the start of the experiment (Fig. 3C). This can be attributed to one of the replicate reactors underperforming in the first week (180–300 gN·m^{-3}·d^{-1}), yet recovering to the same activity (300–400 gN·m^{-3}·d^{-1}) as the other two replicates in the last week (SI, Fig. S14, reactor A4). The reason for this is unknown since all replicates received the same influent, shared the same inoculum, and were operated in parallel. The 16S data showed *Planctomycetes* abundance decreased during the week at 12 °C (from 3.2% to 2.2%) and further decreased in the recovery week (1.8%), even though activity was fully regained. This suggests that not all anammox species were equally active.

During steady state operation of the pilot scale anammox reactor activity fluctuated (Fig. 4, days 180–650). Initially, after the startup phase ended, maximum anammox activity kept increasing, even though nitrogen loading was constant (Fig. 4, days 180–340). The reason for this is twofold: first, the steady state anammox biomass was not yet reached, and, second, MWW temperature was increasing. The maximal activity achieved was 500 gN·m^{-3}·d^{-1} at 25 °C (Fig. 4, Day 340). A steep drop in activity (from 310 gN·m^{-3}·d^{-1} to 150 gN·m^{-3}·d^{-1}) was measured around day 440 before the intermittent aeration experiment. This can partly be attributed to the fact that 12.5% of the biomass was removed as inoculum for another anammox pilot reactor; however, this cannot fully explain the lost activity. Interestingly, when examining year-round data from the pilot scale reactor, cold temperatures do not have an outsized effect on activity, i.e., 200 gN·m^{-3}·d^{-1} is achieved even at temperatures of 13 °C. This is in stark contrast with the temperature experiment at bench scale. At bench scale, 225 m^2·m^{-3} of carrier surface was used (compared to 167 m^2·m^{-3} at pilot scale), and only 70 gN·m^{-3}·d^{-1} was achieved at 12 °C (SI, Fig. S14). If these rates are normalized to available carrier surface per volume, i.e., 1.2 gN·m^{-2}·d^{-1} and

0.31 $gN \cdot m^{-2} \cdot d^{-1}$ for pilot and bench scale respectively, the pilot scale has four times higher performance at similarly low temperatures. This is further corroborated by plotting anammox activity at pilot scale against temperature, which yields a linear relationship, although with variation (SI, Fig. S17). The metagenomic data shows a broad distribution of different anammox species, with none of them clearly dominating. We suspect that, under specific conditions, e.g., summer or winter, different anammox species have advantages and allow for more stable performance. For example, in the work of Hendrickx et al. (2014), a high rate of anammox activity was achieved at cold temperatures with *B. fulgida* as the dominant species, which was also found in the metagenomic sample of this study (Fig. 7C). Such specialism might also be responsible for the fact that *Planctomycetes* 16S abundance and activity did not increase and decrease together. If a significant subpopulation is only active under certain conditions a discrepancy between activity and abundance arises.

4.3. Reactor comparison

Bench scale reactors and pilot scale reactors are different, not only in size but also in equipment used (blowers, stirrer, and pumps) and physical parameters (reactor-walls-to-volume ratio and reactor depth). Additionally, pilot and full-scale installations are often operated for a number of years (or decades), whereas bench scale installations are often operated for weeks or months. When a system is scaled up, these differences are compounded and can lead to significantly different results, as observed in the upscaling of membrane bioreactors (Kraume et al., 2009). In this study, similar results were obtained for the DO disturbance at bench and pilot scale, but differences (i.e., anammox activity and redox potential) were observed when organic loading rate increased. We suspect that the redox potential, which was different in bench and pilot scale systems, might have played a role (SI, Fig. S10C and D). One possibility for the difference in redox potentials is the coarse bubble aeration used at pilot scale (10 s every 1200 s), which could suppress the growth of strictly anaerobic bacteria or help oxidize toxic sulfides. The response to temperature cannot be directly compared, as different time scales were used. Furthermore, temperature shocks performed in a lab are limited in their ability to mimic a rapid temperature shift in real wastewater. For example, snow melt or rainfall in a cold climate change not only temperature but also dilute wastewater. This shows the limitations of extrapolating from short lab experiments to longer processes, such as seasonal temperature changes.

4.4. Implications for mainstream anammox

From these findings, it is evident that a robust mainstream anammox system will require tradeoffs. If organics valorization is maximized, and thus no organics reach the anammox stage, then the robustness of the mainstream anammox system may be reduced. In such a system, fewer heterotrophs are available to aid in depleting the DO carried over from previous stages. Furthermore, as hypothesized by Reino et al. (2016), the heterotrophic population may even produce cryoprotective substances that increase the temperature tolerance of the system. Today, most laboratory anammox systems working with mainstream wastewater rely on a carbon-capturing pretreatment step such as HRAS, contact stabilization, micro-sieves, or precipitation (Han et al., 2016; Wett et al., 2013). Managing these carbon-capturing stages to control organic loading to the anammox stage is important, as this will allow for tailored heterotrophic growth in the anammox stage. From our results, we suggest that organics inflow can be increased as long as residual nitrate (2–5 $mgNO_3^-\text{-}N \cdot L^{-1}$) is available. Large DO peaks or organic shock loading will lead to temporary anammox inhibition or an imbalanced NO_2^-:NH_4^+ ratio; hence, to guarantee effluent quality, a polishing step is required, not only for nitrate, as reported by Gustavsson et al. (2020), but also for ammonium and nitrite. In a SBR system, one option for such a polishing step is to simply aerate the anammox stage at the end of a cycle, i.e., once there is no ammonium or no nitrite left. A small nitrifying community would grow in the same biofilm as the anammox bacteria and nitrify the remaining ammonium or nitrite. From the results of the DO experiment reported in this study, no lasting detrimental effects on the anammox population are expected due to such an aerated polishing step. Overall, this study finds that DO, organic loading, and temperature disturbances do not have a lasting impact on the slow growing anammox biomass. Thus, long recovery periods (i.e., weeks to months) for bacterial regrowth are not expected. Lastly, seasonal temperature changes may not pose a major risk to anammox activity. The anammox population will adapt or change in response to slow temperature shift and performance will not decrease drastically. However, fast temperature reduction still presents an obstacle, which may require an extensive polishing capacity.

Of course, these conclusions are only applicable if the nitrite supply, e.g., partial nitritation, is guaranteed. According to this study the anammox population can thrive in the mainstream environment; thus the challenge going forward is to find a reliable source of nitrite. Recent research has shown a variety of successful, nitrite-producing systems, e.g., via granules (Isanta et al., 2015), population segregation (Laureni et al., 2019), and denitratation (Ma et al., 2020). Further research should focus on the reliability of these systems with MWW and study performance at pilot scale.

5. Conclusion

For this study, a stable long-term pilot scale anammox reactor was operated over nearly two years. The impact of three different disturbances on mainstream anammox activity at bench and pilot scale was evaluated. The following conclusions can be drawn.

- Enough anammox biomass can be retained to support competitive volumetric removal rates in mainstream wastewater. This activity was sustained throughout the years, even during winter, which was likely due to the high diversity of anammox species on the carriers.
- Two weeks of intermittent aeration at 1.6 mg O_2/L did not lead to permanent anammox activity loss or long recovery periods. This response was consistent between bench and pilot scale.
- An increase in organic loading rates is not detrimental to anammox activity as long as residual nitrate (2–5 $mgNO_3^-\text{-}N \cdot L^{-1}$) is available.
- Short-term temperature reductions (from 20 to 12 °C) drastically decreased anammox activity, but the activity was rapidly and fully recovered once temperatures were increased again. The pilot scale system adapted well to seasonal temperatures variations sustaining the target nitrogen removal rate through anammox of 200 $gN \cdot m^{-3} \cdot d^{-1}$ down to temperatures of 13 °C (Fig. 4). After the start up, 260 ± 83 $gN \cdot m^{-3} \cdot d^{-1}$ were removed on average.
- The activity in the pilot scale systems was variable over time, but not in response to the parameters tested, i.e., DO, temperature and organic loading rates. The underlying reason for these variations is unclear and needs further investigation.

Data availability

Data used for this study is available at the Eawag Research Data Institutional Collection (ERIC) at doi:10.25678/0003ZG.

CRediT authorship contribution statement

Damian Hausherr: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Visualization. **Robert Niederdorfer:** Methodology, Resources, Writing – review & editing. **Eberhard Morgenroth:** Conceptualization, Writing – review & editing, Supervision. **Adriano Joss:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

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.scitotenv.2021.148920.

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