Fast DNA-analyses for surveillance of microbial communities in full-scale deammonification tanks

Potential for control and troubleshooting

Andersen, Martin Hjorth; Thomsen, Lisette; Stokholm-Bjerregaard, Mikkel; Eriksen, Søren; Hansen, Susan Hove; Albertsen, Mads; Nielsen, Per Halkjær

Published in:
Water Research

DOI (link to publication from Publisher):
10.1016/j.watres.2023.119919

Publication date:
2023

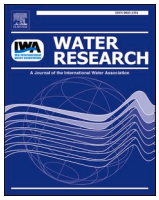
Document Version
Publisher's PDF, also known as Version of record

Water Research 236 (2023) 119919

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres



Fast DNA-analyses for surveillance of microbial communities in full-scale deammonification tanks: Potential for control and troubleshooting

Martin Hjorth Andersen [a], Lisette Thomsen [a], Mikkel Stokholm-Bjerregaard [b], Søren Eriksen [c], Susan Hove Hansen [a], Mads Albertsen [a], Per Halkjær Nielsen [a,*]

[a] Center for Microbial Communities, Department of Chemistry and Bioscience, Aalborg University, Aalborg, Denmark
[b] Krüger A/S, Aalborg, Denmark
[c] VandCenter Syd A/S, Odense, Denmark

ARTICLE INFO

Keywords:
Deammonification
Nanopore DNA sequencing
Anammox
Process surveillance and control

ABSTRACT

The partial nitritation/anammox process is a popular process for sidestream nitrogen removal, but the process is sensitive to disturbances and requires extensive surveillance and monitoring for optimal performance. We followed two parallel sidestream full-scale deammonification reactors treating digester centrate for a year with high time-resolution of both online sensor data and microbial community as measured by Nanopore DNA sequencing. DNA surveillance revealed system disturbances and allowed for detection of process and equipment upsets, and it facilitated remediating operational actions. Surveillance of anammox bacteria (*Ca.* Brocadia) revealed unexpected variations, and the composition and dynamics of the flanking community indicated causes for occasional process disturbances with poor nitrogen removal. Monitoring the ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) could potentially allow reactor operation with increased dissolved oxygen (DO), yielding higher ammonia conversion while keeping NOB in control. The use of fast and frequent DNA sequencing (sampling 3–5 times a week, analysed once per week) was an important supplement, and in many cases superior, to the online sensor data for process surveillance, understanding and control.

1. Introduction

Efficient nitrogen (N) removal is crucial for water resource recovery facilities (WRRFs) to adhere to sustainable development goal six, concerning clean water and sanitation (UN General Assembly, 2015). Nitrogen is most commonly removed by nitrification-denitrification in the activated sludge process, but the application of the anaerobic ammonium oxidation (anammox) is becoming increasingly popular. It usually takes place as a sidestream process, although there are also reports about inclusion of anammox as a mainstream process, primarily in warmer climates (Cao et al., 2018). In the sidestream process, centrate from anaerobic digestion with high ammonia concentrations is treated by partial nitritation and anammox (PN/A), also called de-ammonification (Jetten et al., 1997). Compared to conventional denitrification, it has three advantages: reduction in oxygen demand, no carbon demand for denitrification, and significant reduction in sludge production (Daigger, 2014; Jetten et al., 1997; Wett, 2007).

Removal of nitrogen by the sidestream process in PN/A reactors rely on the presence of anaerobic anammox bacteria (AnAOB) as well as ammonia oxidising bacteria (AOB), the latter to ensure oxidation of ammonium to nitrite, the key substrate for anammox bacteria (Chen et al., 2023; Jetten et al., 1997; Zhang and Okabe, 2020). However, nitrite oxidising bacteria (NOB) may also be present converting nitrite to nitrate. This is highly unwanted as it removes the substrate from the process and thereby starves the anammox bacteria. Nitrite is not only an important substrate, but also an inhibitor for the anammox process in high concentrations, with reported inhibition in the range from 30 to 350 mg N/L (Dapena-Mora et al., 2007; Fux et al., 2004; Strous et al., 1999). However, inhibition depends on the duration of high nitrite concentrations and is reversible by lowering the nitrite concentration (Bettazzi et al., 2010; Lotti et al., 2012).

The optimal condition for retaining AnAOB and AOB, but no NOB in one reactor is obtained by growing the communities in granules and keeping the oxygen level low to ensure an anaerobic inner niche for the AnAOB and enough oxygen to keep AOB active at the granules surface (Manonmani and Joseph, 2018; Zhang et al., 2008). The NOB are

* Corresponding author.
E-mail address: phn@bio.aau.dk (P.H. Nielsen).

https://doi.org/10.1016/j.watres.2023.119919
Received 7 August 2022; Received in revised form 17 March 2023; Accepted 26 March 2023
Available online 28 March 2023

typically controlled either by keeping the oxygen concentrations very low or by intermittent aeration (Ma et al., 2015). In full-scale reactors the performance is typically surveyed and controlled by pH (DEMON® (Wett, 2006)), NH_4^+ (Joss et al., 2009), a ratio between NO_2^-/NH_4^+ concentration or, in the case of ANITAMox™, by a ratio of influent/effluent ammonium concentrations and the nitrate production in the system (Lackner et al., 2014). These parameters are used to control the daily feeding, aeration cycles and dissolved oxygen (DO). Identifying problems building up over longer periods is not always straightforward. Issues affecting reactor performance are often related to the influent feed, where a high or varying concentration of organic matter, often analysed as total suspended solids, as well as inhibitory substances, can lead to a decrease in reactor performance (Lackner et al., 2014; Li et al., 2020; Qian et al., 2023).

The development of new sequencing technologies, where the Nanopore MinION is of particular interest due to its size and low price, makes it possible to analyse microbial communities from day to day and use this information for surveillance and for control by changing operational conditions (Pomerantz et al., 2018). Traditionally, the turn-around time for obtaining reliable community data by DNA sequencing is weeks, thus not providing community analyses in time for control purposes. However, with the development of the MinION technology this problem is now solved (Ciuffreda et al., 2021), and reliable community analysis can take place within a few hours on-site at the WRRF. In addition, the ecosystem-specific reference databases MiDAS 3 and MiDAS 4 allow unique and robust species-level identification, producing high-resolution insight into the dynamics of microbial communities (Dueholm et al., 2022; Nierychlo et al., 2020), allowing for cross study comparisons.

The overall aim of this study was to monitor the process stability in two full-scale deammonification reactors, based on physicochemical and microbiological data, and investigate whether the microbiological data would give additional value for surveillance and control. The specific objectives were to: 1) evaluate the use of AOB and NOB abundances dynamics for process monitoring, 2) observe the stability and dynamics of AnAOB, 3) investigate the composition and dynamics of the flanking community, and 4) to evaluate whether changes in any of these communities could be used as alarms to the operator for potential control measures.

2. Materials and methods

2.1. Reactor details, online sensors and operation

The study included two full-scale PN/A reactors with DEMON® configurations located at Ejby Mølle WRRF in Odense, Denmark and they have been in operation for 5 years. The reactors are each 320 m^3 and designed to treat 197 kg N/day each (0.62 $kg_N\ m^{-3}d^{-1}$). They were operated in parallel, treating ammonium-rich centrate from four mesophilic anaerobic digesters at the WRRF. The reactors were fitted with hydrocyclones to retain granular sludge (Table 1).

The timeline for operation in this study was divided into an introductory phase with normal operation and collection of only operational data and online sensor surveillance of chemical parameters (day −155–0), and three experimental phases with additional biomass sampling and DNA analyses: Phase I (day 0–57) to establish a baseline at normal conditions (DO setpoint of 0.3 mg/L); phase II (day 58–180) with an increased DO set point of 1.0 mg/L to increase the activity of AOB combined with close surveillance of NOB to observe potential growth, and phase III (day 181–224) with a lowered DO setpoint (0.3 mg/L) to re-establish baseline. Due to fouling of the membranes in the aeration system during phase II, this phase was divided into two phases: Phase IIa (day 58–88) with membrane fouling and Phase IIb (day 89–180), where the DO levels could again reach 1.0 mg/L. For the experimental phases, only reactor R1 changed DO level. The other reactor, R2, had continuously normal conditions with a DO setpoint of 0.3 mg/L. Mean operational values for the two reactors are available in Table 2 (reactor 1) and Table S1 (reactor 2).

Table 1
Design and operation of the two deammonification reactors, R1 and R2 at Ejby Mølle WRRF. The two reactors are identical, operated in parallel and values are per reactor.

Volume (each reactor)	320 m^3
Loading capacity (each reactor)	197 kg NH_4^+-N d^{-1}
Granule selection	Hydrocyclone
Online sensors (IQ system, Xylem Analytics, Germany)	O_2 (FDO 700 IQ), NH_4^+ (AmmoLyt plus 700 IQ), NO_2^- (NitraVis 700 IQ NI), NO_3^- (NitraVis 700 IQ NI), SS (Visolid 700 IQ), pH (SensoLyt 700 IQ), Temperature
Influent concentration (mean ±SD and range):	
NH_4^+ (mg NH_4^+-N L^{-1})	922±105, range: 196–5304
Total COD (mg O_2 L^{-1})	922±105, range: 196–5304
SS (mg L^{-1})	200–500, occasionally higher peaks
Total P (mg P L^{-1})	No data
Oxygenation (through membrane systems)	Intermittent aeration, 10–40 min up to setpoint (0.3 or 1.0 mg/L). Also controlled by nitrite level and nitrite removal rate

2.2. Biomass sampling

Due to the granularity of the biomass, it was investigated whether sampling variance was larger than the variance introduced in a sequencing workflow by drawing 10 biomass samples with 5 min. intervals and analysing each sample in duplicates. Only minor sample heterogeneity was observed, and the variance introduced in the applied sequencing workflow was even smaller (Fig. S2). Some temporal variation was observed, as a 25-minute break between samples 5 and 6 was visible in the resulting data. Sampling of biomass was performed by taking five subsamples from the sampling port (top of reactor), pooling them and homogenising them using a T18 Ultra Turrax mixer (IKA®) for 2 min at 10,000 rpm. The homogenised biomass was aliquoted into cryotubes and stored at −18 °C until DNA extraction. Sampling occurred 3 times a week for the first 5 weeks, and 4–5 times a week from day 36. All samples were homogenised, aliquoted and frozen on-site within hours, whereas downstream sample preparation and sequencing was carried out once a week, if possible. Due to covid-19 restrictions samples were often delayed and processed together in larger batches.

2.3. Chemical analyses

Laboratory analyses of chemical parameters were performed routinely to evaluate sensor accuracy. SS concentrations were determined by filtering the sludge through a glass fibre filter GA-55 (ADVANTEC®) followed by drying at 105 °C for two hours. NH_4^+ concentrations were analysed using an Ammonium 50 (NANOCOLOR®, Macherey-Nagel, Germany) kit according to the manufacturers' protocol.

2.4. DNA extraction

DNA was extracted using a custom workflow, based on the studies by Albertsen et al. (2015), here developed for on-site extraction with minimal equipment. 500 μL biomass, 480 μL phosphate-buffered saline (PBS) (MP Biomedicals, USA) and 120 μL MT buffer (MP Biomedicals, USA) were added to a Lysing Matrix E tube (MP Biomedicals, USA). Bead-beating was performed using a SuperFastPrep-1™ (MP Biomedicals, USA) for 2 × 30 s, with the tube on ice for 2 min between turns. The lysed samples were centrifuged at 2000 *x* g for 60 s, and the supernatant was transferred to 250 μL CD2 buffer (QIAGEN, USA) in a new tube, which was then inverted 10 times by hand and subsequently incubated on ice for 5 min. After incubation, the sample was centrifuged

at 2000 *x* g for 2 min, 750 μL of the supernatant was transferred to a 1.5 mL tube for final DNA purification using a custom CleanNGS beads solution (CleanNA, Netherlands) in a sample/bead ratio of 1:1. The custom bead solution was made by diluting CleanNGS beads 1:10 with a 20% w/w PEG-8000 and 2.5 M NaCl solution. The tubes were inverted by hand for 5 min followed by two washing steps with 80% ethanol and subsequently eluted in 63 μL nuclease-free water. DNA concentrations were measured using Qubit™ dsDNA BR Assay Kit and a Qubit® 4 Fluorometer (Thermo Fisher Scientific, USA).

2.5. Sequencing library preparation and DNA sequencing

Sequencing libraries were prepared by creating 16S rRNA gene V4 amplicons ([515F] GTGCCAGCMGCCGCGGTAA and [806R] GGACTACHVGGGTWTCTAAT (Caporaso et al., 2011)), and subsequently barcoding with Nanopore-compatible custom adapters. The amplicons were prepared in duplicate reactions using (per reaction) 25 μL PCRBIO 2X Ultra Mix (PCR Biosystems, UK), 20 μL tailed primer mix in a 1 μM concentration to obtain a final 400 nM concentration, 1 μL nuclease-free water and 4 μL template DNA in a 5 ng/μL concentration. The PCR program for the amplicon PCR was 95 °C for 2 min, 30 cycles of 95 °C for 15 s, 55 °C for 15 s, 72 °C for 50 s and a final elongation at 72 °C for 5 min. The duplicate amplicon libraries were pooled and purified using CleanNGS beads (CleanNA, Netherlands) in a sample/bead ratio of 5/4. Sequencing libraries were prepared from the amplicons, multiplexed and adapted for Nanopore sequencing based on a custom ligation protocol based on the Sequencing Kit (SQK-LSK109) as described by Uri-Carreño et al. (2023). The libraries were loaded onto a MinION 106 v.9.4.1 flow cell in a MinION Mk1C sequencer (Oxford Nanopore Technologies, UK) according to the manufacturer's protocol. Live base-calling was enabled using Guppy 3.6.0 (Oxford Nanopore Technologies, UK) with the fast model. Fastq files are available at http://www.ncbi.nlm.nih.gov/bioproject/789450

2.6. Bioinformatic pipeline

The base-called fastq files were processed using a snakemake workflow for trimming, demultiplexing, mapping to MiDAS 3.7 database and quality filtering. The workflow is available at https://github.com/martinhjorth/onlineDNA-workflow and also described by Uri-Carreño et al. (2023). Nanopore sequencing can give species-level identification (Ciuffreda et al., 2021). The error rate is now around 1% (previously much higher) and by using the high-quality 16S rRNA gene reference database for WWTP systems (MiDAS 3 or 4), species-level classification could be obtained of most ASVs. The SILVA or Greengenes reference databases would not give the same resolution (Dueholm et al., 2020, 2022). Illumina and MinION sequencing gave very similar community structure at genus and species level in the DEMON reactor (see later, 3.2).

2.7. Data analysis

Operational and sequencing data were analysed and visualised in the R environment (v. 3.6.3 (R Core Team, 2020)) using RStudio (Rstudio Team, 2020) with the ampvis2 package (v. 2.6.1 (Andersen et al., 2018)) and the tidyverse (v. 1.3.0 (Wickham et al., 2019)). Rmarkdown files can be obtained through https://github.com/martinhjorth/Publications/tree/master/Andersen2022_deammonification.

3. Results and discussion

3.1. Reactor performance

The two full-scale sidestream PN/A reactors treating digester centrate were identical in design and operation and have been running for 5 years, generally showing, according to the operator, stable operation (Table 2). We found the two reactors to be very similar in operation during the period surveyed (Fig. 1 and Fig. S1). The period from day −155 to day 57 was surveyed to establish a baseline, prior to process perturbations in phase II with elevated DO. We observed variations in the performance, where NO_2^- increased prior to phase I and dropped suddenly in both reactors around day 35. NH_4^+ removal efficiency fluctuated but declined from ~92.5% at its peak to ~82.5% at the end of phase I (Fig. 2B).

Increasing DO in phase II did not result in an increase in nitrate concentration as we had expected, but rather a drop in both reactors during phase IIb. The increased DO did, however, cause an increase in NH_4^+ removal efficiency once the DO concentration of 1 mg/L was successfully maintained in phase IIb. The removal efficiency peaked around day 140 and started to decline towards 80%. The same trends were observed in the control reactor (R2), but without a peak at day 140, indicating that the higher DO concentration had an effect in reactor 1 (Fig. 1 and Fig. S1). Overall, the increased DO in reactor 1 appeared to increase the NH_4^+ removal capacity at a slightly higher level than in the control reactor.

The daily operation of the reactors was based on information from the chemical analyses, sensor data and the operator's experience in running the reactors. Although the operator was working on a timescale with higher resolution, some simple alarms could perhaps have been implemented and triggered based on the sensor data reported after day ≈ 150, which could have warned the operator about potential operational problems (Fig. 2A and 2B). The decline in NO_3^- during phase IIb indicated that either a washout/suppression of NOB had occurred, or that the reactors performed heterotrophic denitrification. Alarms could thus have been triggered in reactor 1 around day 35–45 (NO_2^- drop), day 70–90 (NH_4^+ increase), day 100–125 (NO_2^- decrease) and day 150–160 (NH_4^+ removal efficiency decrease). Increasing the AnAOB performance may be solved by a lower feeding and a higher biomass retention (Lackner et al., 2014), resulting in a lower load and better accumulation of the AnAOB biomass.

3.2. Sampling strategy and analysis of community structure

Prior to biomass sampling for DNA analyses at day 0, we conducted experiments to determine the optimal sampling strategy as the reactor hydraulics and different granules sizes may cause some sample heterogeneity. Based on the results (Fig. S2), we decided to perform all biomass sampling from the reactors in replicates of 5 to minimise intra-sample heterogeneity. The biomass replicates were then pooled and homogenised in a blender prior to aliquoting biomass in cryotubes. Biomass was sampled 3–5 days a week to obtain a high resolution of the time-series to reveal possible strong temporal variations.

The community composition was analysed on Nanopore MinION and validated with Illumina MiSeq platform, using the 16S rRNA gene V4 region to enable comparison between platforms. We developed a workflow where amplicons were barcoded with Nanopore-compatible Illumina adapters, thereby enabling sequencing on both platforms. The V4 primer set was selected because the commonly used V1–V3 primers do not target the AnAOB very well (Mazzoli et al., 2020). The two platforms gave very similar results at genus level (Fig. S3), while abundances varied slightly on species level. *Ca.* Brocadia species were especially affected, where abundances in the same libraries varied up to 4.1%-point between the two platforms. The results could indicate that *Ca.* Brocadia species are so similar in the V4 region that species-level cannot readily be resolved by low-accuracy reads and should instead be classified by the full length of the 16S rRNA gene. It has previously been shown that sequencing of the entire 16S rRNA gene can provide strain-level resolution (Dueholm et al., 2020; Johnson et al., 2019), but we chose the V4 region for comparison with the Illumina platform.

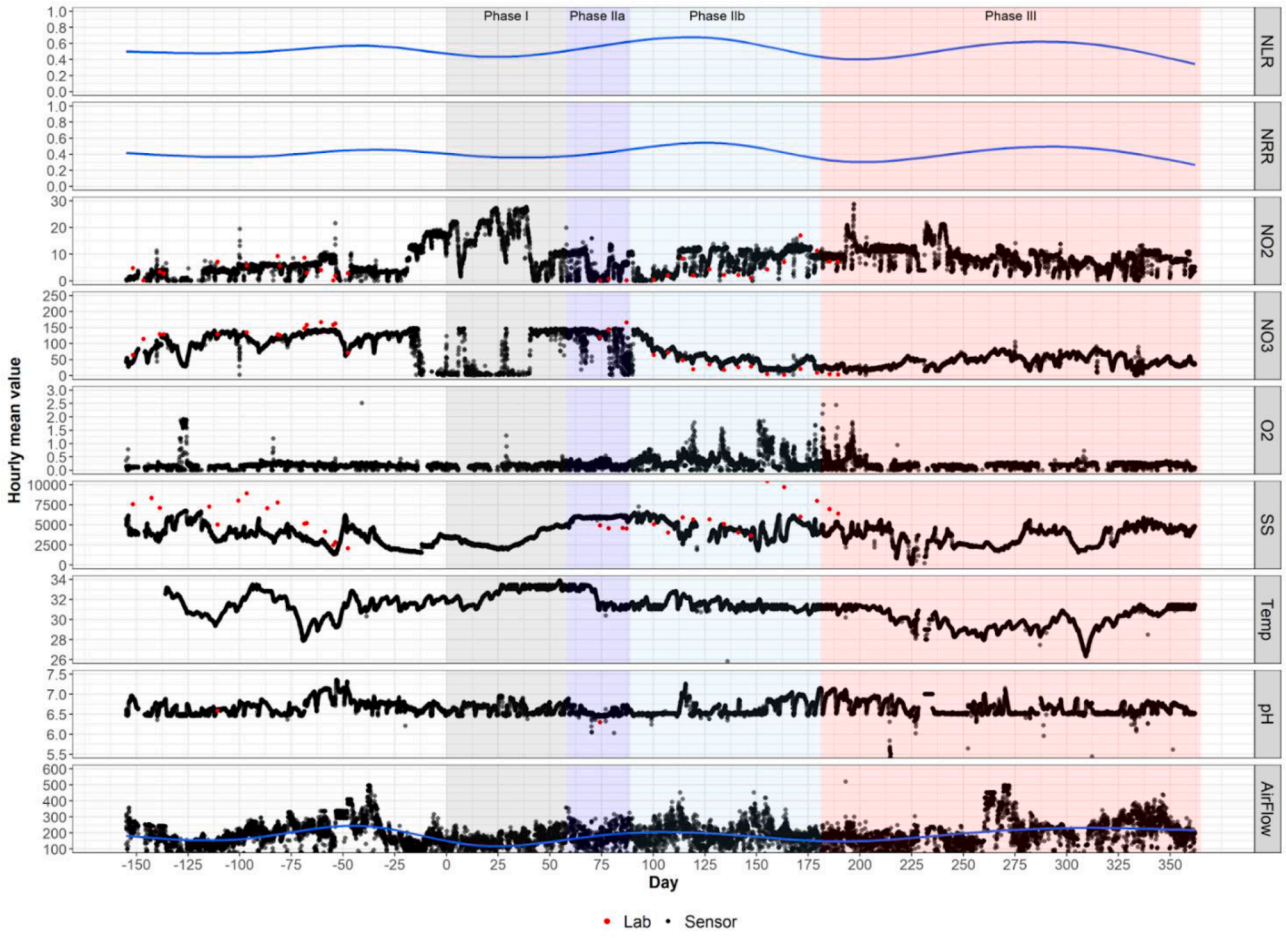


Fig. 1. Variations in nitrogen loading rate, removal rate, and nutrient concentrations. Timeline of reactor 1 online sensors and lab analyses for NO_2^-, NO_3^- (mg N/L), O_2 (mg O_2/L), SS (mg SS/L), temperature (Temp., °C), pH and airflow (m^3/h). A regression line (in blue) has been added to airflow, as well as to the calculated parameters nitrogen loading rate (NLR, kg N/m^3*d) and nitrogen removal rate (NRR, kg N/m^3*d), where points are omitted. Colours indicate phases. Phase I: DO setpoint of 0.3 mg/L, phase II: DO setpoint of 1.0 mg/L, and Phase III: DO setpoint of 0.3 mg/L.

3.3. General microbial community structure

While online sensor data was shown from day −155, biomass sampling and community analysis for the baseline commenced at day 0. The community analysis, conducted weekly with the developed MinION workflow, showed that the microbial composition in the two reactors was almost identical and only minor differences were observed in the relative read abundances for some taxa throughout the three phases. During phase I, the communities were fairly stable and dominated by AnAOB besides approx. 20 genera in relative read abundances above 1% (Fig. 3 for reactor 1, Fig. S4 for reactor 2). Only one anammox genus was observed, *Ca.* Brocadia, and it varied in relative abundances of 11–24% (Fig. 2C). Three different *Ca.* Brocadia species were identified, with *Ca.* B. caroliniensis as most abundant, *Ca.* B. sapporoensis less abundant, and with few *Ca.* B. sinica (Fig. 2D). *Ca.* Brocadia has previously been reported as the dominant AnAOB in full-scale de-ammonification plants, occasionally with *Ca.* Jettenia or *Ca.* Kuenenia in high abundance as well (Annavajhala et al., 2018; Gonzalez-Martinez et al., 2015; Zhang and Okabe, 2020; Zhao et al., 2023).

The genus *Nitrosomonas* was the only known AOB detected in the reactors and was the fourth-most abundant genus overall (1.4–5.8% in phase I). A high diversity of *Nitrosomonas* species was detected, with *Nitrosomonas* midas_s_5118 and *Nitrosomonas* midas_s_639 as most abundant and *N. europaea* and *Nitrosomonas* midas_s_6235 in lower abundance (Fig. 2E).

Among the known NOB, we only detected genus *Nitrospira*, and it reached a relative read abundance of 0.4–1.3%. We detected five different species, but only *N. defluvii* was abundant. Species from *Nitrosomonas* and *Nitrospira* are commonly found as the dominant nitrifiers in full-scale sidestream PN/A reactors (Al-Hazmi et al., 2019; Annavajhala et al., 2018; Gonzalez-Martinez et al., 2015), but as these studies did not have species resolution, it remains unknown whether the species are identical.

The flanking community consisted of many genera, severalwithout any known, or only poorly described function. Most notable was genus OLB8 (Bacteroidetes phylum) with one dominant species (midas_s_3279) in abundances up to 11% of the total community (Fig. 3). It may participate in the denitrification but does not have a full pathway (Kondrotaite et al., 2022). Other abundant genera were the genus *Denitratisoma,* previously described as an AnAOB symbiontic denitrifier (Fahrbach et al., 2006; Qian et al., 2023) and a Chloroflexi genus (midas_g_731).

Interestingly, the methanogenic genera *Methanomethylovorans* and *Methanothrix* were also present in high abundance (Fig. 3). *Methanothrix* is commonly found in high abundance in Danish mesophilic ADs, while *Methanomethylovorans* is less common, but has been observed in high abundance in the digesters at the treatment plant (Jiang et al., 2020). These methanogens, along with other genera commonly present in mesophilic digesters (*Thermovirga* and *Thermomonas*), indicated that biomass from the anaerobic digesters was transferred to the deammonification reactors.

The overall community structure remained very similar in both

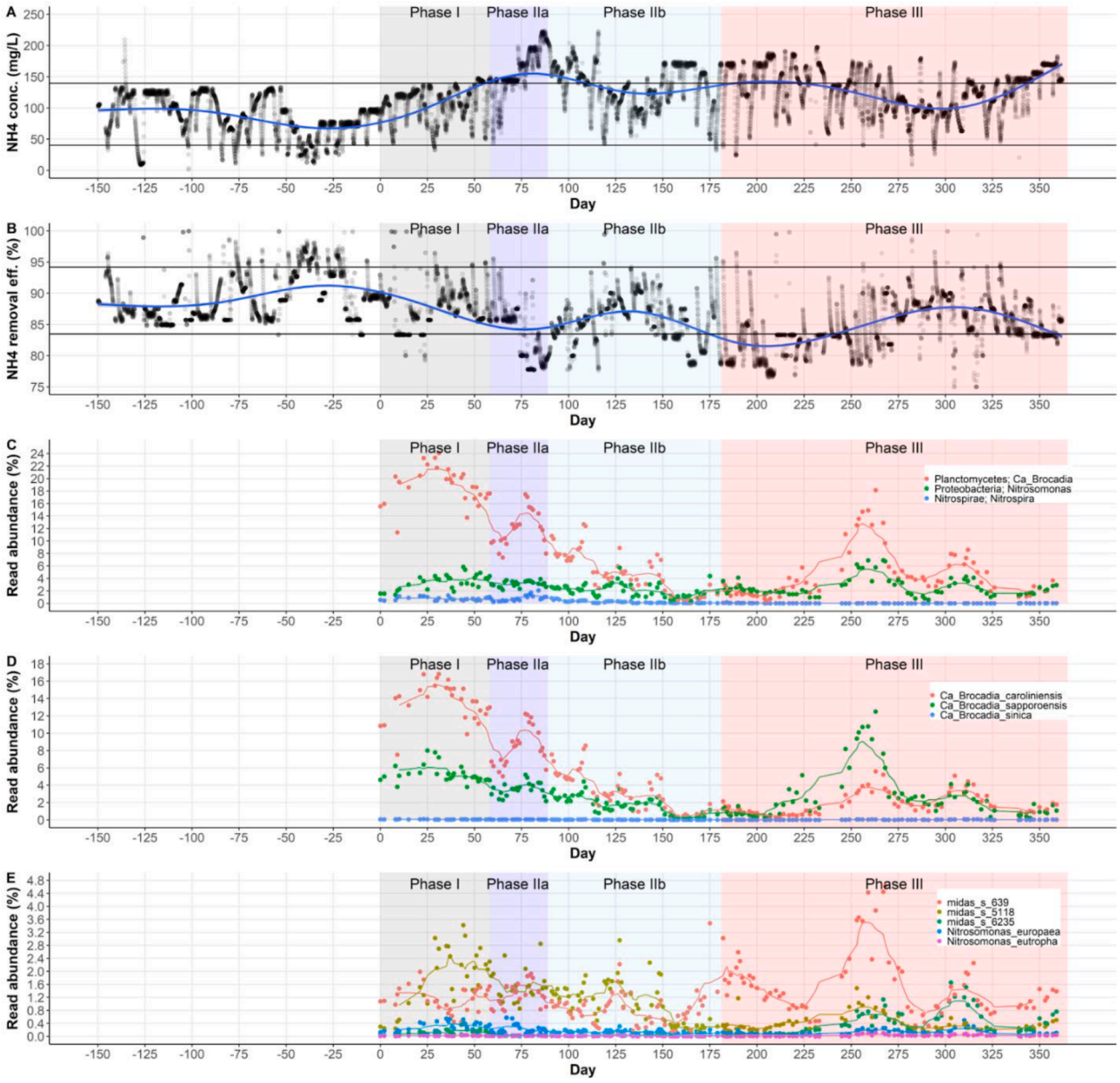


Fig. 2. Development of ammonium concentrations, removal efficiencies and nitrifier abundances in reactor 1 through phase I-III. Threshold levels are shown in horizontal black lines, calculated from baseline mean ± 1.5*standard deviation. A) Ammonium concentrations with an added regression line in blue. B) Ammonium removal efficiency in percentage with an added regression line in blue. C) Nitrifier relative read abundance and 10-sample moving average (14 days). D) Relative read abundance for *Ca.* Brocadia species and 10-sample moving average (14 days). E) Relative read abundance for *Nitrosomonas* species and 10-sample moving average (14 days).

reactors also during phase IIa-b and III (Fig. S5), and only the abundances of several genera/species changed as described below.

3.4. Changes in abundances of AOB, NOB and AnAOB

The AOB (*Nitrosomonas*) and NOB (*Nitrospira*) abundances were relatively constant during phase I in both reactors (Fig. 2C and Fig. S6). The AnAOB (*Ca.* Brocadia) relative read abundance varied between 12% and 24% and was in a downward trend at the end of phase I. We followed the community structure by weekly analyses, consisting of separate samples at a resolution of 2 (day 0–25) to 4–5 (day 29–198) times per week to ensure high time-resolution.

In phase I, the abundance of *Nitrosomonas* was rather stable (2–5%) and there was a constant low level of *Nitrospira* (0.5–1.5%). By increasing the oxygen level in phase IIa and IIb in R1, we tried to increase the nitritation while observing for any unwanted increase of NOB (Fig. 2C). The increased oxygenation did not clearly increase the abundance of AOB. The NOB remained unexpectedly at low abundances (1–2%) through phase II although a small increase took place around day 80. Normal operation (phase III) was re-established on day 181. In phase III both AOB and NOB dropped further to very low levels (0.59–6.91% and 0.00–0.18% relative read abundance, respectively).

The abundance of *Ca.* Brocadia declined dramatically during phase IIa-IIb. It dropped from 18% to only 1–2% in relative abundance with some temporal variations. This low level remained throughout most of phase III, where *Ca.* Brocadia started to increase in abundance again. Interestingly, the dominant *Ca.* Brocadia at the end of phase III was *Ca.* B. sapporoensis, whereas *Ca.* B. caroliniensis had been dominant before the abundance drop. The disappearance of *Ca.* Brocadia was possibly a result of the increased and unstable SS levels in the influent, as it would cause some heterotrophic activity and reduce substrates available for the anammox process. The decline in *Ca.* Brocadia was in agreement

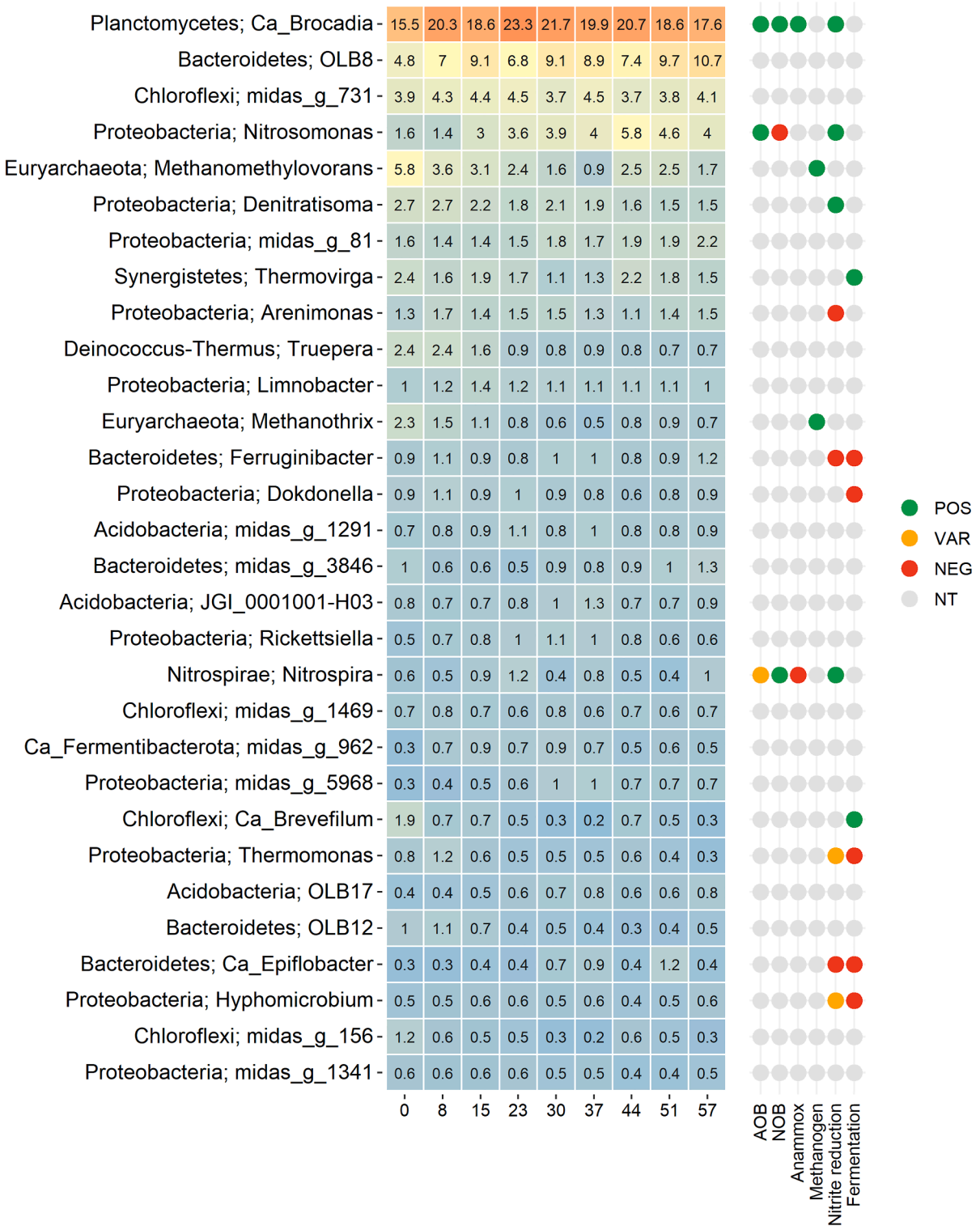


Fig. 3. The 30 most abundant genera in reactor 1 during phase I. Only samples with ~7 day intervals are shown. Selected known functions are indicated as "POS" for positive, "VAR" for variable, "NEG" for negative and "NT" for not tested. Functional information was downloaded from the MiDAS field guide (midasfieldguide.org).

with the declining NH_4^+ removal efficiency and increasing NH_4^+ levels in the sensor data (Fig. 2A and 2B). The trends were the same in both reactors, suggesting that the increased DO did not strongly affect the abundance of *Ca.* Brocadia.

The changes in the nitrifying community in both reactors prompted us to look more into the species-level changes. For the NOB community, the same single species (*Nitrospira defluvii*) dominated throughout all phases without changes (Fig. 2C, Fig. S6 for reactor 2), but for AOB, interesting changes were observed in both reactors (Fig. 2E, Fig. S6 for reactor 2). Two *Nitrosomonas* species were dominating, midas_s_5118 and midas_s_639. The former dominated in both reactors throughout phase I, although midas_s_639 was dominant at the start of phase I. There were some differences in abundance developments for the two species through the three phases, all together indicating that growth

conditions changed over time and were different in the two reactors (Fig. 2E, Fig. S6 for reactor 2).

3.5. Dynamics of flanking community explain deteriorated reactor performance

The microbial community changed in both reactors during phase IIb and into phase III where both AnAOB and AOB decreased and almost disappeared, resulting in a deteriorated N-removal. *Ca.* Brocadia was the most abundant genus throughout phase I, but other species became more abundant in phase II and III (Fig. 4A and B). Some of these were also abundant in phase I and have previously been found in full-scale sidestream PN/A, such as *Dokdonella* and *Denitratisoma* (Gonzalez-Martinez et al., 2015). Interestingly, several genera usually present in the digester also increased, such as the three methanogens, *Methanothrix, Methanomethylovorans* and *Methanolinea,* besides the fermenting genus *Thermovirga*. The increase of these genera commonly present in the anaerobic digesters indicated that large amounts of biomass from the digesters were added to both reactors due to insufficient removal of SS in the feedline. Upstream SS was removed using centrifuges with the operator aiming for <500 mg SS/L.

The increased level of other heterotrophic and potential denitrifiers in phase II and III, such as *Denitratisoma* and genera belonging to Chloroflexi, and the decline of AOB and particularly NOB also indicated that the organic load to the two reactors had increased. Similar observations are described in other recent studies when the organic load increases (Chen et al., 2022; Qian et al., 2023). The removal of ammonium seemed to have changed from a well-functioning sidestream PN/A to partly heterotrophic removal by denitrification, primarily via nitrite, as NOB was almost absent. The very similar community changes in the two reactors also supported that this was the main cause for the deteriorated community composition.

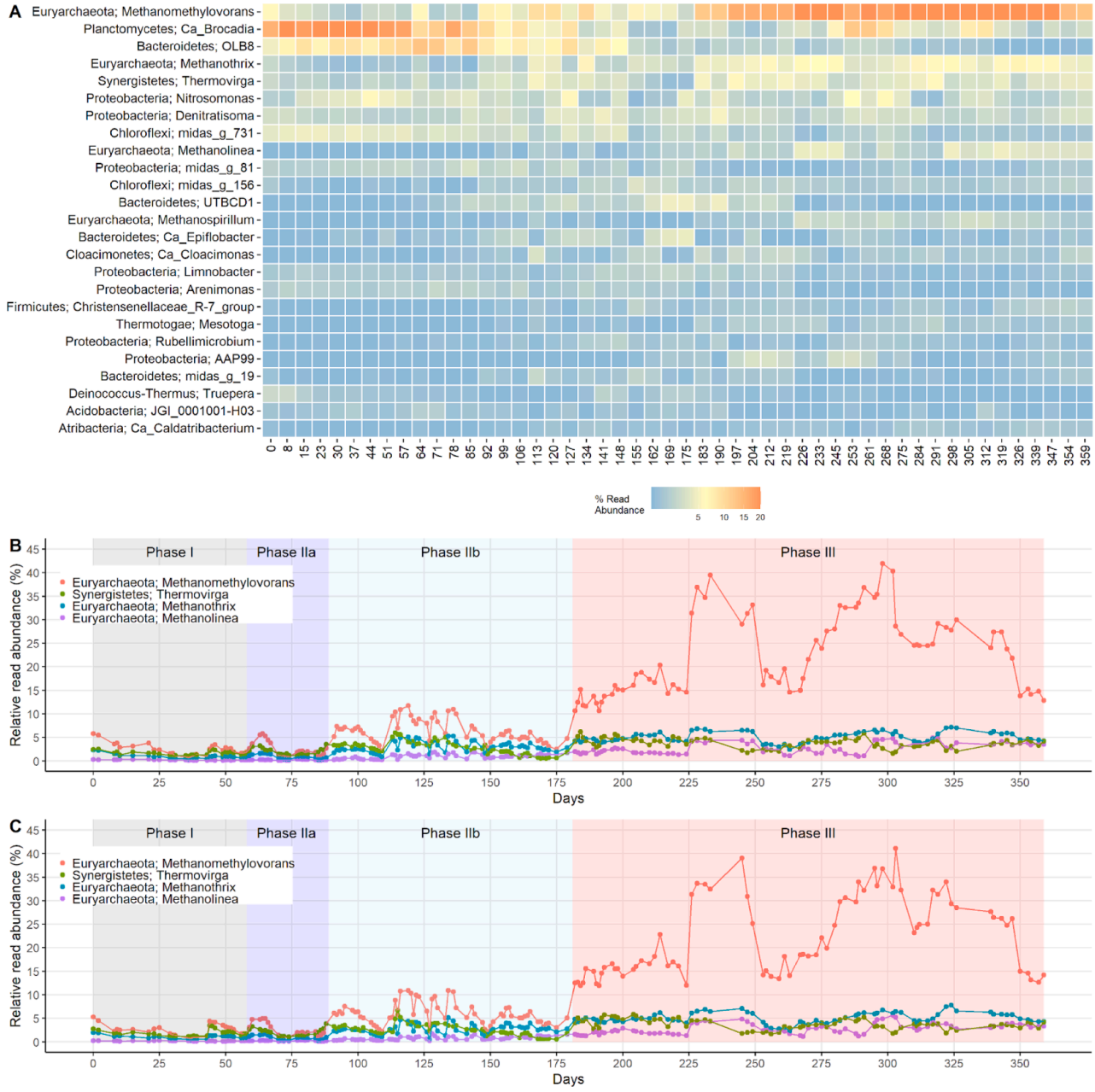


Fig. 4. A) Overview of the 25 most abundant genera in reactor 1 through all phases. Only samples with ~7-day intervals are shown. B + C) Anaerobic digester genera and their relative read abundances in reactor 1 (B) and reactor 2 (C).

Table 2
Mean operational values and standard deviations for selected parameters in reactor 1. DO is dissolved oxygen, HRT is hydraulic retention time. HRT values are median values. See Table S1 for reactor 2 values.

Phase	Time [d]	DO [mg O_2 L^{-1}]	HRT [h]	NH_4^+-load [kg NH_4^+ $m^{-3}d^{-1}$]	Temperature [°C]	pH
Normal operation	−155 - −1	0.19±0.21	42.7	0.42±0.18	31.3 ± 1.21	6.68±0.20
I	0–57	0.17±0.08	51.2	0.40±0.18	32.6 ± 0.77	6.63±0.15
IIa	58–88	0.19±0.1	40.0	0.48±0.19	32.2 ± 0.95	6.55±0.15
IIb	89–180	0.37±0.3	38.2	0.51±0.22	31.4 ± 0.48	6.62±0.26
III	181–224	0.3 ± 0.31	42.7	0.41±0.14	30.8 ± 0.86	6.76±0.27

3.6. Fast community analyses support online sensors for surveillance and establishing of alarms for control measures

The study showed that the fast surveillance of the entire community composition and structure created important added value to the general online monitoring. Microbial surveillance revealed system disturbances, allowed for detection of process and equipment upsets, and it could facilitate remediating operational actions. Based on the microbial community data, the operator was able to track the cause of disturbance back to instability in the feed SS. The problems were solved by minimising the incoming SS and removing more suspended biomass from the reactors while increasing the retention of heavy granules in the reactor through the hydrocyclones. We did not use the microbial information for direct control in this study as we wanted to see how the normal control measures were reflected in the microbial data and use this information to evaluate whether changes in the microbial communities could be applied as potential alarms to select for appropriate control measures.

The community analysis provided important information about the identity and diversity of the important functional groups. The identity of the microbes is important as knowledge about their function can be transferred from one study to another. This is possible because we have used the MiDAS reference database with species-level taxonomy that can be compared across studies. It is, however, important to further study the microbiology of full-scale PN/A reactors. Few studies exist on full-scale communities and many members are likely yet to be identified (Speth et al., 2016; Zhang and Okabe, 2020).

A number of issues should be considered in relation to applying routine DNA analysis for control purposes. These include sample frequency, alarms based on the entire community profile or specific species, and whether an alarm should be based on abundance change over time (rate) or based on abundance thresholds. Temporal changes, in our case based on analyses of 2–5 samples a week, in the microbial composition may form the basis for establishing alarms about potentially critical operational issues. These can, together with the normal online measurements, provide inputs to the control strategies. The most obvious measure, changes in the NOB/AOB ratio, was not possible to evaluate in this study due to the very low NOB abundances. We expect that an NOB/AOB ratio could be useful for surveillance of the NOB and controlling oxygenation, so DNA surveillance can allow a tighter control with smaller safety margin in DO setpoint.

The change in the AnAOB abundance may also be converted to an alarm. The drop in *Ca.* Brocadia abundance from day 40–65 was not directly detected in the online sensor data, although perhaps indicated by the drop in NO_2^- around day 40 along with an increase in NO_3^-, (which could indicate higher NOB activity and lower AnAOB activity), but an alarm could have been triggered based on DNA analysis. The subsequent *Ca.* Brocadia decline between day 80 and 100 was seen in the sensor data as an NH_4^+ increase in the same period (topping at ~225 mg N/L around day 90), which could have alerted the operator as the concentrations were above normal baseline values (~10–150 during day −155–57). Here DNA analysis would have given the operator added information about the state of the AnAOB community. Also, after the NH_4^+peak around day 90, the NO_3^-concentration dropped to all-time lows after day 110. Low NOB abundances and thus low NO_3^-concentrations are of course desirable, but the rate of decline could have alerted the operator of abnormal operation. DNA analysis would clarify what had appeared, thus providing valuable insight about the potential cause. *Ca.* Brocadia remained in relatively stable abundance (2–8%) between day 120 and 150, after which it dropped below 2%. This drop was also visible in the sensor data, as the NH_4^+ removal efficiency decreased between day 150 and 160. In the same period, an SS measurement from the laboratory showed elevated SS concentration from the digesters, as indicated from the analyses of the anaerobic community, e.g. the methanogens. In this case the analysis of the flanking community gave important information, revealing the heavy load of SS from the digesters, and it revealed the increase in denitrifiers, explaining the fairly good nitrate removal despite the lack of AnAOB. It could be considered whether an alarm could be defined based on the relative abundance of key species originating from the digesters.

The approach demonstrated here for surveillance and potential control can be applied to all systems related to wastewater treatment, including DEMON reactors. However, as shown here, it may be difficult to interpret the microbial data and use them for active control. Therefore, we recommend running analyses weekly or biweekly for one to two years to gain experience with the plant (all plants are different) to learn what is normal community structure, which variations can be expected, how does the community react to operational changes etc. In this way, the operators build up experiences that later can be used for control. Furthermore, the community analyses could also be related to the physical structure of the granules and the spatial distribution of AOB, NOB, AnAOB and coexisting heterotrophic bacteria and suspended solid adhering onto the granular surface for optimized surveillance.

The costs are decreasing as the technology develops. Amplicon sequencing provides a near-complete community overview in contrast to qPCR and is not much more costly. With time, when the genome databases are complete, metagenomics will be the choice, but presently we recommend amplicon sequencing. Our cost price to MinION amplicon sequencing and chemicals is 30–40 € per sample (+ working hours) when running 10–20 samples at a time. We collect samples from several plants and run them weekly or biweekly at a central laboratory with the equipment and expertise necessary.

4. Conclusion

- Two full-scale sidestream de-ammonification (PN/A) reactors treating digester centrates were followed for one year by online sensors for the surveillance and control of operation. The overall operation showed good N-removal efficiencies of 85-95%, although it dropped to 80% in a period.
- The microbial communities were analysed with a resolution of 3-5 samples/week by Nanopore MinION DNA sequencing. The nitrifiers consisted of one genus of AOB (*Nitrosomonas*) and one NOB (*Nitrospira*), and the anammox (AnAOB) of *Ca.* Brocadia.
- Experiments with elevated levels of DO in one reactor did not stimulate the growth of NOB as expected. An increase would have enabled monitoring of an NOB/AOB ratio, which is a potential tool for surveillance and control of aeration and operation of the reactors.
- *Ca.* Brocadia dominated the reactors during stable operation but declined substantially during a period, causing poor N-removal. The

decrease in species-abundance could be detected before it affected the overall reactor performance.
- Detailed analyses of the dynamics of nitrifiers and the flanking community revealed possible causes for the poor reactor performance and enabled the operator to make changes to the operation.
- Temporal changes of several species had the potential to act as an alarm to the operator for control purposes.
- Overall, the ability to obtain high-resolution DNA sequencing results weekly was an important supplement to the online sensor data for process surveillance, understanding, and control. We recommend fast DNA sequencing as an important tool for surveillance of the PN/A process. Building up experiences over one to two years may be needed before use in control.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Per H Nielsen reports financial support was provided by Innovation Fund Denmark (OnlineDNA, grant 6155-00003B). MA and PHN are co-owners of DNASense ApS, which specialises in microbial community analyses. MS-B is employed by Krüger A/S, part of Veolia. MHA, SE and LT declare no competing financial interests.

Data availability

All links to data are available in the M&M

Acknowledgments

The project was funded by the Innovation Fund Denmark (OnlineDNA, grant 6155-00003B).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2023.119919.

References

Albertsen, M., Karst, S.M., Ziegler, A.S., Kirkegaard, R.H., Nielsen, P.H., 2015. Back to basics – the influence of DNA extraction and primer choice on phylogenetic analysis in activated sludge communities. PLoS One 10, e0132783. https://doi.org/10.1371/journal.pone.0132783.

Al-Hazmi, H., Grubba, D., Majtacz, J., Kowal, P., Makinia, J., 2019. Evaluation of partial nitritation/anammox (PN/A) process performance and microorganisms community composition under different C/N ratio. Water (Basel) 11, 2270.

Andersen, K.S.S., Kirkegaard, R.H., Karst, S.M., Albertsen, M., 2018. ampvis2: an R package to analyse and visualise 16S rRNA amplicon data. bioRxiv. https://doi.org/10.1101/299537.

Annavajhala, M.K., Kapoor, V., Santo-Domingo, J., Chandran, K., 2018. Structural and functional interrogation of selected biological nitrogen removal systems in the united states, denmark, and singapore using shotgun metagenomics. Front. Microbiol. 9, 2544.

Bettazzi, E., Caffaz, S., Vannini, C., Lubello, C., 2010. Nitrite inhibition and intermediates effects on Anammox bacteria: a batch-scale experimental study. Process Biochem. 45, 573–580.

Cao, Y., Kwok, B.H., van Loosdrecht, M.C.M., Daigger, G., Png, H.Y., Long, W.Y., Eng, O. K., 2018. The influence of dissolved oxygen on partial nitritation/anammox performance and microbial community of the 200,000 m^3/d activated sludge process at the Changi water reclamation plant (2011 to 2016). Water Sci. Technol. 78, 634–643.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., Fierer, N., Knight, R., 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc. Natl. Acad. Sci. U.S.A. 108 (Suppl 1), 4516–4522.

Chen, H., Liu, K., Yang, E., Chen, J., Gu, Y., Wu, S., Yang, M., Wang, H., Wang, D., Li, H., 2023. A critical review on microbial ecology in the novel biological nitrogen removal process: dynamic balance of complex functional microbes for nitrogen removal. Sci. Total Environ. 857, 159462.

Chen, J., Hai, Y., Zhang, W., Zhou, X., 2022. Insights into deterioration and reactivation of a mainstream anammox biofilm reactor response to C/N ratio. J. Environ. Manag. 320, 115780.

Ciuffreda, L., Rodríguez-Pérez, H., Carlos Flores, C., 2021. Nanopore sequencing and its application to the study of microbial communities. Comput. Struct. Biotechnol. J. 19, 1497–1511. https://doi.org/10.1016/j.csbj.2021.02.020.

Daigger, G.T., 2014. Oxygen and carbon requirements for biological nitrogen removal processes accomplishing nitrification, nitritation, and anammox. Water Environ. Res. 86, 204–209.

Dapena-Mora, A., Fernández, I., Campos, J.L., Mosquera-Corral, A., Méndez, R., Jetten, M.S.M., 2007. Evaluation of activity and inhibition effects on Anammox process by batch tests based on the nitrogen gas production. Enzyme Microb. Technol. 40, 859–865.

Dueholm, M.S., Andersen, K.S., McIlroy, S.J., Kristensen, J.M., Yashiro, E., Karst, S.M., Albertsen, M., Nielsen, P.H., 2020. Generation of comprehensive ecosystem-specific reference databases with species-level resolution by high-throughput full-length 16s rrna gene sequencing and automated taxonomy assignment (AutoTax). MBio 11. https://doi.org/10.1128/mBio.01557-20.

Dueholm, M.K.D., Nierychlo, M., Andersen, K.S., Rudkjøbing, V., Knutsson, S., Consortium, MiDASG., Albertsen, M., Nielsen, P.H., 2022. MiDAS 4: a global catalogue of full-length 16S rRNA gene sequences and taxonomy for studies of bacterial communities in wastewater treatment plants. Nat. Commun. 13, 1908.

Fahrbach, M., Kuever, J., Meinke, R., Kämpfer, P., Hollender, J., 2006. Denitratisoma oestradiolicum gen. nov., sp. nov., a 17beta-oestradiol-degrading, denitrifying betaproteobacterium. Int. J. Syst. Evol. Microbiol. 56, 1547–1552.

Fux, C., Huang, D., Monti, A., Siegrist, H., 2004. Difficulties in maintaining long-term partial nitritation of ammonium-rich sludge digester liquids in a moving-bed biofilm reactor (MBBR). Water Sci. Technol. 49, 53–60.

Gonzalez-Martinez, A., Osorio, F., Morillo, J.A., Rodriguez-Sanchez, A., Gonzalez-Lopez, J., Abbas, B.A., van Loosdrecht, M.C.M., 2015. Comparison of bacterial diversity in full scale anammox bioreactors operated under different conditions. Biotechnol. Prog. 31, 1464–1472.

Jetten, M.S.M., Horn, S.J., van Loosdrecht, M.C.M., 1997. Towards a more sustainable municipal wastewater treatment system. Water Sci. Technol. 35, 171–180.

Jiang, C., McIlroy, S.J., Qi, R., Petriglieri, F., Yashiro, E., Kondrotaite, Z., Nielsen, P.H., 2020. Identification of microorganisms responsible for foam formation in mesophilic anaerobic digesters treating surplus activated sludge. Water Res. 191, 116779.

Johnson, J.S., Spakowicz, D.J., Hong, B.-Y., Petersen, L.M., Demkowicz, P., Chen, L., Leopold, S.R., Hanson, B.M., Agresta, H.O., Gerstein, M., Sodergren, E., Weinstock, G.M., 2019. Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. Nat. Commun. 10, 5029.

Joss, A., Salzgeber, D., Eugster, J., König, R., Rottermann, K., Burger, S., Fabijan, P., Leumann, S., Mohn, J., Siegrist, H., 2009. Full-scale nitrogen removal from digester liquid with partial nitritation and anammox in one SBR. Environ. Sci. Technol. 43, 5301–5306.

Kondrotaite, Z., Valk, L.C., Petriglieri, F., Singleton, C., Nierychlo, M., Dueholm, M.K.D., Nielsen, P.H., 2022. Diversity and ecophysiology of the genus OLB8 and other abundant uncultured Saprospiraceae genera in global wastewater treatment systems. Front. Microbiol. 13, 917553.

Lackner, S., Gilbert, E.M., Vlaeminck, S.E., Joss, A., Horn, H., van Loosdrecht, M.C.M., 2014. Full-scale partial nitritation/anammox experiences–an application survey. Water Res. 55, 292–303.

Li, W., Zhuang, J.-l., Zhou, Y.-y., Meng, F.-g., Kang, D., Zheng, P., Shapleigh, J.P., Liu, Y.-d., 2020. Metagenomics reveals microbial community differences lead to differential nitrate reduction in anammox reactors with differing nitrogen loading rates. Water Res. 169, 115279.

Lotti, T., van der Star, W.R.L., Kleerebezem, R., Lubello, C., van Loosdrecht, M.C.M., 2012. The effect of nitrite inhibition on the anammox process. Water Res. 46, 2559–2569.

Ma, B., Bao, P., Wei, Y., Zhu, G., Yuan, Z., Peng, Y., 2015. Suppressing nitrite-oxidizing bacteria growth to achieve nitrogen removal from domestic wastewater via anammox using intermittent aeration with low dissolved oxygen. Sci. Rep. 5, 13048.

Manonmani, U., Joseph, K., 2018. Granulation of anammox microorganisms for autotrophic nitrogen removal from wastewater. Environ. Chem. Lett. 16, 881–901.

Mazzoli, L., Munz, G., Lotti, T., Ramazzotti, M., 2020. A novel universal primer pair for prokaryotes with improved performances for anammox containing communities. Sci. Rep. 10, 15648.

Nierychlo, M., Andersen, K.S., Xu, Y., Green, N., Jiang, C., Albertsen, M., Dueholm, M.S., Nielsen, P.H., 2020. MiDAS 3: an ecosystem-specific reference database, taxonomy and knowledge platform for activated sludge and anaerobic digesters reveals species-level microbiome composition of activated sludge. Water Res. 182, 115955.

Pomerantz, A., Peñafiel, N., Arteaga, A., Bustamante, L., Pichardo, F., Coloma, L.A., Barrio-Amorós, C.L., Salazar-Valenzuela, D., Prost, S., 2018. Real-time DNA barcoding in a rainforest using nanopore sequencing: opportunities for rapid biodiversity assessments and local capacity building. Gigascience 7, 1–14.

Qian, F., Liu, Y., Mo, C., Zhang, H., Yan, F., Gao, J., Zhuang, J., 2023. Response of partial nitritation/anammox granules to biodegradable organic input in bacterial community and functions. J. Water Process. Eng. 51, 103442.

R Core Team, 2020. R: A language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

RStudio Team, 2020. RStudio: Integrated Development for R. RStudio, PBC.

Speth, D.R., In 't Zandt, M.H., Guerrero-Cruz, S., Dutilh, B.E., Jetten, M.S.M., 2016. Genome-based microbial ecology of anammox granules in a full-scale wastewater treatment system. Nat. Commun. 7, 11172.

Strous, M., Kuenen, J.G., Jetten, M.S., 1999. Key physiology of anaerobic ammonium oxidation. Appl. Environ. Microbiol. 65, 3248–3250.

UN General Assembly, 2015. Transforming our world: the 2030 agenda for sustainable development.
Uri-Carreño, N., Nielsen, P.H., Gernaey, K.V., Wang, Q., Nielsen, U.G., Nierychlo, M., Hansen, S.H., Thomsen, L., Flores-Alsina, X., 2023. The effects of low oxidation-reduction potential on the performance of full-scale hybrid membrane-aerated biofilm reactors. Chem. Eng. J. 451, 138917.
Wett, B., 2007. Development and implementation of a robust deammonification process. Water Sci. Technol. 56, 81–88.
Wett, B., 2006. Solved upscaling problems for implementing deammonification of rejection water. Water Sci. Technol. 53, 121–128.
Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L., François, R., Grolemund, G., Hayes, A., Henry, L., Hester, J., Kuhn, M., Pedersen, T., Miller, E., Bache, S., Müller, K., Ooms, J., Robinson, D., Seidel, D., Spinu, V., Takahashi, K., Vaughan, D., Wilke, C., Woo, K., Yutani, H., 2019. Welcome to the tidyverse. J. Open Source Softw. 4, 1686.
Zhang, L., Okabe, S., 2020. Ecological niche differentiation among anammox bacteria. Water Res. 171, 115468.
Zhang, L., Zheng, P., Tang, C.-J., Jin, R.-C., 2008. Anaerobic ammonium oxidation for treatment of ammonium-rich wastewaters. J. Zhejiang Univ. Sci. B 9, 416–426.
Zhao, Y., Li, J., Liu, Q., Qi, Z., Li, X., Zhang, Q., Sui, J., Wang, C., Peng, Y., 2023. Fast start-up and stable operation of mainstream anammox without inoculation in an A^2/O process treating low COD/N real municipal wastewater. Water Res. 231, 119598.