Water Research 229 (2023) 119475

Contents lists available at ScienceDirect

Water Research

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

C-N-S synergy in a pilot-scale mainstream anammox fluidized-bed membrane bioreactor for treating chemically enhanced primary treatment saline sewage

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ARTICLE INFO

Keywords:
Saline mainstream anammox
Fluidized membrane bioreactor
Kuenenia
Synergetic C-N-S loop
Saline sewage

ABSTRACT

Seawater for toilet flushing conserves the scarce freshwater resources in Hong Kong and similar densely-populated coastal cities. Saline sewage treatment using energy-efficient anammox-based processes appears to be beneficial, with notable potential for the future. However, the feasibility of this process remains uncharted, especially from its start-up to its steady-state cooperation. In this study, a pilot-scale mainstream anammox process was succeeded in a granular activated carbon fluidized-bed membrane bioreactor (FMBR) with only inoculating saline anaerobic digestion sludge. The FMBR, operating at the dissolved oxygen (DO) of 0.04-0.16 mg/L, achieved comparable nitrogen removal rates of 50.1-78.3 g N/m^3/d while treating real chemically enhanced primary treatment saline sewage at 3.2 m^3/d. Excluding *Scalindua*, 16S rRNA gene amplicon sequencing revealed a high abundance of common-freshwater-observed *Kuenenia* from 0.2% (day 259) to 1.3% (day 332) in biofilms. *Nitrosomonas,* responsible for ammonium oxidation, dominated in biofilms and accounted for 1.8%-9.2%. Nitrite oxidizing bacteria incursion happened unexpectedly, due to high oxygen exposure/supply from temporary on-site technical difficulties; however, NOB suppression was achieved by controlling DO at <0.16 mg/L, leading the FMBR's stability over the 343-day operation. Sulfate-reducing bacteria and sulfur-dependent denitrifiers propagated with high abundance, representing 14.0±10.6% and 6.0±2.0% in suspensions and 7.2±1.8% and 15.9±2.3% in biofilms, respectively. Further, metagenomic sequencing analysis indicated the C-N-S synergy of nitritation, anammox, sulfate reduction, and mixotrophic denitrification in the FMBR. Importantly, this research found that such a novel C-N-S synergy was made by the scavenges of hydrogen sulfide by *Gammaproteobacteria sp.* (B01 and B19) and *Thioalkalispiraceae sp.* (B03 and B04), species having ascendancy subsisting in the presence of oxygen owing to their aptitude of detoxifying reactive oxygen species. Knowledge gleaned from this study, as well as a complete set of pilot experimental data, could serve as a strong technical base for the larger-scale application of this process.

1. Introduction

The scarcity of clean, fresh water has become an unrelenting, undeniably serious global problem, due to the rapidly increasing world population. This insufficiency highlights the pressing need for alternative water resources globally. Since more than half the world's population resides in coastal areas (Merkens et al. 2016), the utilization of seawater for toilet flushing has been adopted in many places to alleviate the freshwater resources shortage (Li et al. 2005, Mackey et al. 2019, Sun et al. 2009). Hong Kong has used seawater for toilet flushing

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https://doi.org/10.1016/j.watres.2022.119475
Received 30 September 2022; Received in revised form 24 November 2022; Accepted 6 December 2022
Available online 8 December 2022

(SWTF) for over 60 years (Li et al. 2005), thereby reducing freshwater consumption by over 22% while serving up to 80% of the city's population (Leung et al. 2012). However, the introduction of SWTF produces sewage salinity ranging from 1.0 to 1.7% and approximately 2 million m^3/day of saline sewage (Yang et al. 2015). This requires a new process to supplement the nitrification/denitrification (N/DN) approach, which is commonly applied for freshwater municipal wastewater treatment.

Currently, anammox is being applied for treating ammonium-rich wastewater; this is known as the sidestrea anammox (Lackner et al. 2014). Mainstream anammox has been successfully tested at the laboratory-, bench-, and pilot-scale in many locations (Hoekstra et al. 2019, Laureni et al. 2016, Lotti et al. 2015, Lotti et al. 2014). Full-scale anammox is an encouraging process in temperate and tropical areas, such as South America, Singapore, and Hong Kong. For example, a full-scale mainstream anammox application was first achieved at the Changi Water Reclamation Plant in Singapore, contributing to about 38% of total nitrogen removal (Cao et al. 2016). However, its mainstream saline anammox has not yet been sufficiently studied. Saline mainstream anammox inherits two restrictions: 1) nitrite oxidizing bacteria (NOB) induced process instability; and 2) saline anammox bioaugmentation.

Firstly, NOB incursion could provoke the process deterioration by competing with anammox bacteria for nitrite. Recent studies have proven that NOB suppression is feasible in hybrid floc-biofilm mainstream anammox processes in long-term operation, despite its consistent presence (Laureni et al. 2016, Laureni et al. 2019, Malovanyy et al. 2015). This feasibility was explained by proof of concept: NOB in biofilms is suppressed by limited DOs and selective NOB washout from flocs is achievable via a decrease in DO and/or an increase in floc discharge (Laureni et al. 2019). For this process, a low DO supply was suggested (e.g., 0.15-0.18 mgO_2/L (Laureni et al. 2016), 0.17±0.04 mgO_2/L (Laureni et al. 2019), below 0.3 mgO_2/L (Gilbert et al. 2015), and 0.1-0.16 mgO_2/L (full-scale) (Cao et al. 2016)).

Secondly, excellent biomass retention with fouling-mitigated-efficient membrane bioreactor (Shen et al. 2012) has been proven to be an ideal tool to grow anammox bacteria (van der Star et al. 2008). Granular activated carbon (GAC) fluidized membrane bioreactor (FMBR) has been successfully developed to treat diluted wastewater for methane generation (Shin et al. 2016, Shin et al. 2014). In these applications, membrane fouling was effectively controlled over long-term operations (i.e., 485 days (Shin et al. 2014) and 2 years (Shin et al. 2016)). The introduction of GAC served as biomass carrier and membrane fouling mitigator via scouring force. In this regard, the FMBR could be nominated as a suitable approach to implementing the mainstream anammox processes.

Thirdly, the detection of *Scalindua* in saline sewage treatment facilities (Wu et al. 2019) demonstrated the feasibility of saline mainstream anammox. Evidence also stemmed from the ubiquity of *Scalindua, Brocadia*, and *Kuenenia* in saline ecosystems (Sonthiphand et al. 2014, Zheng et al. 2016), providing analogous niches for the mainstream treatment (low ammonium and varied seasonal temperatures). The toxic concern of hydrogen sulfide (derived from sulfate reduction) on nitrogen-metabolizing microorganisms is likely to be exempted; this result was reported in a study of sulfate reduction, autotrophic denitrification, and nitrification integrated process (known as SANI) with saline sewage (Wang et al. 2009). Taking the extant research into consideration, the saline mainstream anammox seemed feasible in the GAC-FMBR.

In this study, mainstream anammox was implemented in a pilot-scale GAC-FMBR to treat chemically enhanced primary treatment (CEPT) saline sewage. Such sewage has a lower C/N ratio (e.g., 2.1-4.7), suitable for mainstream anammox applications. The pilot-scale GAC FMBR was initially seeded with saline anaerobic digestion sludge to start, and its long-term performance under variable nitrogen loads and temperatures was monitored. In addition, microbial community analyses were carried out using 16S rRNA gene amplicon and metagenomic sequencing. The overarching objectives of this study were to: 1) evaluate the feasibility of the saline mainstream anammox; and 2) disclose symbiotic patterns of functional microbial consortia, especially for S metabolism linking with C and N. By finding a spatially-ordered oxygen gradient (i.e., 0.08±0.03 mg/L) of the C-N-S synergic microbial consortia, the outcome of this study should aid the significant broadening of anammox technology to the treatment of saline sewage.

2. Materials and methods

2.1. Pilot plant description and operation strategies

A pilot-scale FMBR (Fig. 1), installed at the Stonecutters Island Sewage Treatment Works (SCISTW) in Hong Kong, was used to treat the effluent of the CEPT process. SCISTW, the fourth largest WWTP in the world, is currently serving up to 80% of the local sewage treatment with 1.7 million m^3 saline sewage generated daily. The characteristics of CEPT sewage were pH 7.0±0.2, alkalinity 240.2±81.3 mg/L, salinity 1.1 ±0.1%, ammonium 21.5±5.1 mgN/L, BOD_5 80.4±19.6 mg/L, COD 142.3±35.5 mg/L, and TN 26.8±6.7 mgN/L. The rectangular-shaped stainless steel (STS 304) FMBR had an effective working volume of 1.06 m^3 (0.85 m by 0.51 m in area with 2.84 m depth). A settler (1.25 m by 0.91 m in area with 0.7 m depth) containing a submerged weir (0.85 m by 0.51 m with 1.5 cm V-notch height), with a total liquid volume of 0.49 m^3, was connected on the reactor top to prevent GAC overflow into the recycle line. Five membrane modules – each consisting of 2.36 m long hollow fiber polyvinylidene fluoride (PVDF) membranes with the pore size of 0.1 μm (provided by KOLON Membrane, Korea) – were installed in the FMBR. The total surface area of the membranes was 55 m^2. The FMBR was introduced with 332.3 kg of granular activated carbon (GAC) (Calgon F300, USA), occupying approximately 56% of the reactor working volume when settled. The recycle flow rate was set to 30 m^3/h, responding to an up-flow velocity of 1.15 m/min, which was sufficient to expand the GAC to 100% of the reactor working volume; this enabled effective scouring of all membrane surfaces. A tubular diffuser was installed at the reactor bottom to implement efficient aeration.

The pilot-scale FMBR was automated using an integrated programmable logic controller (PLC) unit (Fig. S1). Operational pH and temperature were monitored by the submerged sensors installed on the reactor top. Sodium carbonate solution was added automatically when pH was below 7.2. Temperature was not controlled and varied according to the actual temperature of the SCISTW sewage (14.5-34.5 °C, Table 1). The dissolved oxygen (DO) was automatically controlled using a sub-PLC unit (Burkert GmbH & Co. KG, Ingelfingen, Germany) throughout the trial, and its set value was 0.1 mg/L. The DO was measured by taking the mean of readings obtained from five DO sensors which were vertically installed on the reactor wall. Aeration was implemented with an air-compressor. However, the aeration was stopped while the FMBR was operating under the anoxic condition temporarily; this occurred when incidents happened onsite (i.e., power supply circuit, the PLC crash, water level sensor malfunction, sewage supply shortage due to power outrage, breakage and/or damage of influent pipeline, and influent sewage pump malfunction, as documented in Table S1). The transmembrane pressure was recorded with a pressure gauge installed in the effluent line. Calibrations of the DO sensor and pH sensor were conducted monthly. The temperature sensor was calibrated when starting up the system; it functioned stably during the entire operation. A 2.0 mm mesh sieve was installed on the top of the influent storage tank to sort sundries (e.g., leaves, foam rubber, plastic, and hair). The sieve was cleaned every week and replaced every month due to saline sewage corrosion.

In this study, the pilot-scale FMBR was started with inoculating 500 L of digested sludge obtained from a full-scale anaerobic digester operated at Tai Po Sewage Treatment Works in Hong Kong. It contained 5.7 g/L total suspended solids (TSS) and 4.3 g/L volatile suspended solids (VSS).

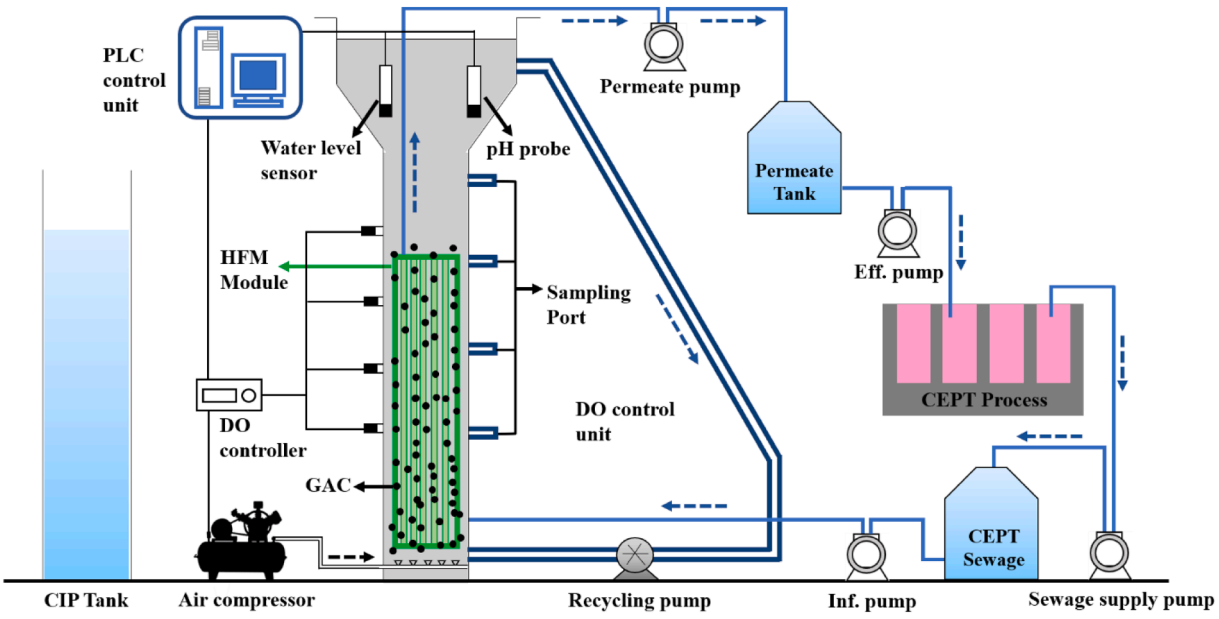

Fig. 1. The schematic diagram of pilot-scale FMBR treating CEPT sewage.

Table 1
Summary of the experimental setup and performance of the pilot-scale FMBR

Operation mode	Period	Temp.	HRT	NRR	NRE	CODRR	CODRE	Effluent concentration (mg/L)				
	Day	°C	hr	g N/m^3/d	%	g COD/m^3/d	%	NH_4^+-N	TN	BOD_5	COD	TSS
Intermittent	1-33	23.2-27.5	N.A.	N.A.	N.A.	N.A.	N.A.	42.5±23.4	–	3.6±2.9	14.3±3.2	–
	34-82	15.4-26.1	N.A.	N.A.	N.A.	N.A.	N.A.	23.0±22.8	–	1.0±0.2	13.2±1.1	35.7±4.9
	83-146	14.5-24.0	N.A.	N.A.	N.A.	N.A.	N.A.	11.7±14.6	–	1.3±0.6	4.9±2.0	27.7±0.3
Continuous	147-161	20.5-26.3	12	5.9±5.3	11.3±10.5	–	–	24.4±4.7	26.2±4.6	–	–	12.4±0.3
	162-180	20.7-28.0	30	12.8±1.4	62.7±3.4	74.0±0.0	91.7±2.6	2.4±1.8	9.5±0.5	6.0±0.5	8.3±1.5	22.7±0.4
	181-278	24.8-34.5	15	27.3±7.0	65.6±13.7	275.0±96.0	93.8±3.4	4.7±3.9	9.2±4.2	3.4±3.6	10.9±7.0	17.6±7.3
	279-320	27.9-33.2	10	48.4±29.7	69.4±9.9	274.0±38.9	95.0±3.9	0.5±0.9	7.6±1.3	1.5±0.8	5.7±4.1	19.7±0.4
	321-343	28.1-31.1	8	60.6±13.3	71.1±8.0	388.6±39.1	94.7±2.4	1.2±1.7	8.1±2.1	1.8±0.6	7.4±4.2	18.7±0.5

HRT, hydraulic retention time; NRR, nitrogen removal rate; NRE, nitrogen removal efficiency; CODRR, chemical oxygen demand removal rate; CODRE, chemical oxygen demand removal efficiency; TN, total nitrogen; BOD_5, 5-days biochemical oxygen demand; COD, chemical oxygen demand; TSS, total suspended solid; N.A., not available.

The reactor was initially operated in an intermittent mode for the first stage, lasting 146 days. During the first 33 days, tap water was used to replenish the liquid loss via evaporation and sampling. Ammonium chloride of 70 g/d (equivalent to 10.0 mgN/L) was fed on days 10-33. From day 34 onwards, the FMBR was fed with the saline CEPT sewage. On days 34-82, 0.2-0.4 m^3/d sewage and 115 g/d ammonium chloride (equivalent to 16.5 mgN/L) were supplemented. From days 83-146, supplementation of ammonium chloride was suspended, and the sewage load was increased from 0.5 to 1.2 m^3/d. This suggests that the FMBR began to operate under mainstream conditions from this point onward. In addition, ammonium chloride dosing of 140 g/d (equivalent to 20.0 mgN/L) was supplemented on days 98-107 when the sewage supply was not available (due to influent sewage pump failure). From day 147 onwards, the FMBR was changed to a continuous flow mode with a starting HRT of 12 hr. From day 162, the HRT was prolonged to 30 hr, since elevated ammonium remained in the effluent (i.e., 24.4±4.7 mgN/L). Furthermore, the HRT was halved on day 178, and then progressively reduced to 10 hr on day 278 and 8 hr on day 321, respectively. During the continuous flow operation period, ammonium chloride (i.e., approximately 30 mgN/L ammonium) was added when sewage supply was not available, due to the incidents listed in Table S1.

2.2. Analytical methods

The determination of nitrogen compounds (TN, NH_4^+-N, NO_2^--N, and NO_3^--N), TSS, VSS, alkalinity, salinity, chemical oxygen demand (COD), and 5-day biochemical oxygen demand (BOD_5) were carried out according to the Standard Methods (Andrew et al. 2005). The calculations of ammonium removal efficiency (ARE), nitrogen removal efficiency (NRE), nitrogen loading rate (NLR), nitrogen removal rate (NRR), COD loading rate (CODLR), COD removal rate (CODRR), and COD removal efficiency (CODRE) were provided in our previous study (Huang and Lee 2021).

2.3. DNA extraction, 16S rRNA gene amplicon and metagenomic sequencing

Biomass samples (S, suspension sludge in bulk; B, biofilm attached grew on GAC) collected on days 216 (S1 and B1), 259 (S2 and B2), 308 (S3 and B3), and 332 (S4 and B4) were used for microbial community analysis. DNA extraction for suspension and biofilm samples was performed according to Yang et al. (2019). Total genomic DNA was extracted using the PowerSoil DNA extraction kit (MoBio Laboratories, Carlsbad, CA, USA) according to manufacturer's protocol. Qualification and quantification of extracted DNA was carried out using Nanodrop 2000c UV-vis Spectrophotometer (Thermo Scientific, Beverly, MA, USA). Bacterial 16S rRNA gene hypervariable V4 region was amplified with barcoded primers 515F (5'-GTG CCA GCM GCC GCG GTA A -3') and 806R (5'- GGA CTA CHV GGG TWT CTA AT -3'). 16S rRNA gene amplicon sequencing was performed on Illumina Mi-Seq platform

(PE250) at BGI (Wuhan, China), and over 50,000 raw reads were generated for each DNA sample. S4 and B4 were adopted for metagenomic sequencing performed on Illumina Hi-Seq 4000 platform at BGI (Wuhan, China). Approximately 30 Gbp of metagenomic sequence data were generated for each DNA sample. The raw 16S rRNA gene amplicon and metagenomic sequence data have been deposited in NCBI under the BioProject PRJNA544020. Sequences of the C-N-S metabolizing microorganisms are accessible with MW136667-MW136692.

2.4. 16S rRNA gene amplicon and metagenomic sequencing analyses

The analysis of 16S rRNA gene amplicon sequences was described in our previous study (Huang and Lee 2021). Alpha diversity statistics were calculated with Mothur. The weighted UniFrac principal coordinate analysis (PCoA) was employed to evaluate the beta diversity. The PCoA plot was carried out with the 'vegan' package and visualized via ggplot2. The phylogenetic tree of observed microorganisms and reported relatives was constructed with MEGA7. Microbial symbiotic patterns were investigated with a co-occurrence network. The linear discriminant analysis effect size (LEfSe) was applied to explore the hub microbes of the microbial community in suspension sludge and biofilms. Tax4Fun and FAPROTAX were utilized to exploit the functional profiles of the microbial community.

The analysis of metagenomic sequences was conducted according to Bolger et al. (2014). Khmer scripts were used for digital normalization to remove redundant sequences. The trimmed clean paired-end reads were further de novo assembled using SPAdes Genome Assembler (ver. 3.9.0) with the default kmer settings of 19, 33, 47, 61, 75 (Nurk et al. 2013). The quality of assembled contigs was assessed with Quast (Gurevich et al. 2013). Based on research by Wu et al. (2014), MaxBin was applied to divide cluster contigs into individual draft genomes. The quality of recovered genome bins was evaluated with CheckM (Donovan H. Parks et al. 2015). The abundance of genome bins was calculated as per the previous study (Langmead and Salzberg 2012). Gene annotation was performed with Prokka (ver. 1.11) (Seemann 2014). Genome-wide average nucleotide identity (ANI) and average amino acid identity (AAI) were used to differentiate the draft genomes. Taxa classification followed the AAI cutoff of 40%, 55%, and 85% for the family, genus, and species, respectively (Rodriguez-R and Konstantinidis 2014). The genome functions characterization was determined with Local Blastp and KEGG BlastKOALA, while BlastKOALA was further utilized to reconstruct functional pathways (Kanehisa et al. 2016).

3. Results

3.1. Reactor performance

The pilot-scale FMBR operated at an intermittent flow mode from day 1 to 146 and a continuous flow mode from day 147 to 343 (Table 1). Initially, ammonium chloride was supplemented to foster the growth of AOB and anammox bacteria. On days 10-82, the effluent ammonium concentration fluctuated in the range of 27.5±24.1 mgN/L whilst 10.0-16.5 mgN/L of ammonium chloride was supplemented. The effluent nitrite concentration was first kept below 5 mgN/L on days 10-27. Thereafter, it was accumulated at the elevated levels of 21.2-105.7 mgN/L on days 30-82, corresponding to over 94.1% of nitrite accumulation efficiency. On days 83-146, the reactor was fed with an incremental sewage flow from 0.5 to 1.2 m^3/d. The nitrite-dominated effluent demonstrated a diminishing trend from 79.8 to 1.5 mgN/L. Effluent ammonium remained at the levels of 9.3±11.3 mgN/L. Nitrate was detected at 1.6±1.5 mgN/L and 0.4±0.4 mgN/L on days 10-82 and 83-146, respectively. At the intermittent operation phase, partial ammonium oxidation and high-efficient nitrite accumulation (i.e., 90.2%-100%) were achieved concurrently. Subsequently, the FMBR operated with the continuous feeding at varied HRTs (Table 1). Initially, the reactor was overrated and started with an HRT of 12 h, leading to poor conversion of ammonium, with the high remaining of over 19.2 mg NH_4^+-N/L. Afterwards, the HRT was prolonged to 30 h and, on average, the reactor achieved 88.3% ammonium conversion. When an HRT of 15 h was implemented, the system obtained an average NRE of 65.6% with the effluent ammonium below 5.0 mgN/L, except for the incidental periods. For instance, from day 220 to 234, effluent ammonium ranging 6.9-12.3 mgN/L was detected due to insufficient oxygen supply (i.e., malfunction of air-compressor). During the last forty days at the HRT of 15 h, the FMBR reached a quasi-steady-state and achieved an average NRE of 69.8% with a mean effluent TN of 8.1 mgN/L. Further, the FMBR was operated at the HRT of 10 h and 8 h successively, achieving average NREs of 69.4% and 71.1%, respectively. The pilot plant eventually obtained NRRs ranging 50.1-78.3 (60.6±13.3) g N/m^3/d with the effluent TN below 10 mgN/L at the HRT of 8 h. This was comparable with the levels of the reported mainstream anammox treating freshwater wastewaters (e.g., 47.0-83.2 g N/m^3/d) (Ji et al. 2020, Kao et al. 2022, Laureni et al. 2016, Laureni et al. 2019, Malovanyy et al. 2015).

The operating DO was controlled at <0.16 mg/L consistently, which led nitrate, the dominant nitrogen species in the effluent, to remain at below 4.5 mgN/L on days 147-270; this finding suggested a robust suppression of NOB theretofore. On day 278, the FMBR system encountered a water-level sensor failure; as a consequence, a certain amount of biomass (including GAC and suspended sludge) sloped over the reactor. Unsurprisingly, the exposure of biomass to air rendered rising nitrate in the effluent on days 279-343, i.e., 6.8±2.2 mgN/L; however, the FMBR system retained NREs of 70.0±8.9% in this time, suggesting the resilience of the FMBR system during a short shock.

Furthermore, the FMBR established relatively steady NREs of 66.8 ±11.4% over the 180 days' continuous operation (days 162-343) despite confronting sundry incidents (Table S1). This echoed the materiality of insuring low DOs during the operation of mainstream anammox processes. As for COD, the FMBR obtained effluent COD of 2.1-7.1 mg/L at intermittent flow operation period and COD removals of >90% at the continuous flow operation phase (Table 1). In brief, the pilot plant obtained the effluent quality of BOD_5 below 10 mg/L, COD below 20 mg/L, TSS below 30 mg/L, ammonium below 5 mgN/L, and TN below 10 mgN/L while treating the CEPT sewage at a capacity of 3.2 m^3/d.

3.2. Microbiomes: statistical results of 16S and meta-genomics

3.2.1. Microbial population dynamics based on 16S rRNA gene

Table S2 contains the summary of 16S rRNA gene amplicon sequencing analysis. Good's coverage >95.7% was obtained for all samples, suggesting a satisfactory sequencing depth (Claesson et al. 2010). The microbial consortia associated with the GAC was enriched, as inferred by the increasing diversity and richness indices (Table S2). As for the taxonomic classification, at Phylum level, Bacteroidetes and Proteobacteria dominated all samples and in total represented 69.1%, 77.4%, 60.0%, 56.2%, 70.9%, 93.4%, 90.6%, 77.0%, and 76.3% in Seed, S1-S4, and B1-B4, respectively. At Genus level, *Nitrosomonas* was detected as the corresponding AOB and dominated in biofilm ranging between 1.8%-9.2% (Fig. 2A). *Nitrospira*, a typical NOB, was detected at below 0.04% in all suspension and biofilm samples collected at the HRTs of 30 h and 15 h; this finding was in line with the reactor performance, since nitrate was mostly detected at below 1 mgN/L. Later, *Nitrospira* proliferated to 0.8% and 0.9% in relation to S3 and B3 (day 308), and further rose to 1.2% and 1.3% in S4 and B4 (day 332), respectively. This *Nitrospira* incursion was ascribed to refilling overflowed sludge that was exposed to air for several hours on day 278, as noted above. As for the denitrifying populations, *Arcobacter* and *Pseudomonas*, which accounted for 21.5% and 17.3% in Seed, respectively, were eliminated along with the operation. Instead, typical denitrifiers (i.e., *Hyphomicrobium* and *Paracoccus*) proliferated and dominated in biofilms, representing 1.5% and 0.7% on average, respectively (Fig. 2A). Meanwhile, microbe affiliated with *Thalassospira*, a known facultative denitrifier (Liu et al. 2007), was significantly enriched from below 0.05% in Seed to 6.1% in S3,

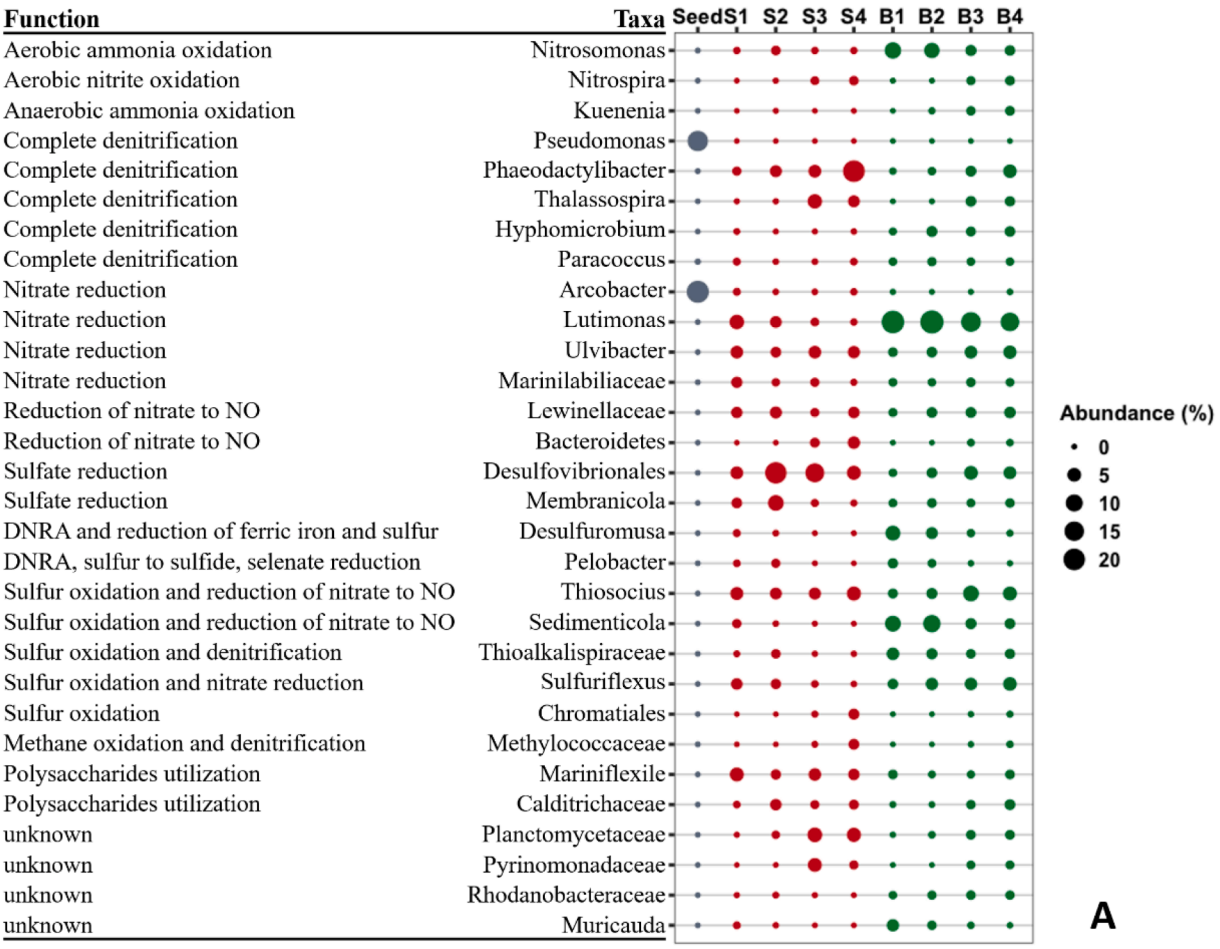

Fig. 2. A, Bubble plot indicates the relative abundances of dominant taxa in the pilot-scale FMBR. B, Phylogenetic tree of observed functional microorganisms based on 16S rRNA gene.

3.2% in S4, 2.1% in B3, and 1.7% in B4, respectively. Remarkably, microorganism affiliated with *Phaeodactylibacter* (Fig. 2B), which performs aerobic denitrification (Lei et al. 2015), was enriched from 1.0% to 20.5% in bulk sludge, and from 0.2% to 4.7% in biofilms. *Lutimonas* and *Ulvibacter,* which are capable of reducing nitrate to nitrite aerobically (Kim et al. 2014, Nedashkovskaya et al. 2004), were detected with high abundances and, in total, accounted for 10.1%, 4.9%, 4.5%, and 3.7% in S1-S4, and 24.0%, 26.2%, 19.5%, and 17.6% in B1-B4, respectively. Furthermore, microbes (OTU19, 22, 39, and 48) sharing the best hit with *Lewinella xylanilytica*, a bacterium capable of reducing nitrate to nitric oxide aerobically (Sung et al. 2015), occupied 3.8±2.1% in suspension sludge and 2.0±0.9% in biofilms, in total. These aerobic and facultative anaerobic denitrifiers either require Na^+ for growth (*Thalassospira, Lutimonas*, and *Ulvibacter*) or prefer to live in the presence of NaCl (*Phaeodactylibacter* and *Lewinella*) with the optimal value of 2.0% (w/v). The propagation of halophilic and facultative denitrifying populations coincides with the operation conditions of the FMBR. In this study, *Candidatus* Kuenenia sp. (freshwater anammox bacteria) rather than *Scalindua* (marine anammox bacteria) proliferated in the FMBR and performed anaerobic ammonium oxidation. *Kuenenia* was detected in biofilms only and accounted for 0.2% in B2, 1.1% in B3, and 1.3% in B4, respectively. Notably, *Kuenenia* was enriched while NOB invaded and persisted in the system (when B3 and B4 were sampled), indicating that *Kuenenia* could contend for a survival niche with NOB at the saline mainstream conditions.

In addition, diverse sulfur cycling populations proliferated and occupied high abundances. *Membranicola*, performing sulfate reduction, initially established high abundances of 2.1% in S1 and 8.0% in S2 and thereafter reduced to 0.5% in S3 and 0.2% in S4, while keeping relatively stable proportions in biofilms (1.0±0.3%). *Thiohalophilus* coupling sulfur oxidation and denitrification was enriched in biofilms, representing 2.3±1.2%. *Sulfuriflexus, Sedimenticola*, and *Thiosocius*, which are capable of sulfur oxidation and reduction of nitrate and nitrite, were detected in high abundances. *Sulfuriflexus* and *Sedimenticola* dominated in biofilms and occupied average 5.9% and 3.5%, respectively. *Thiosocius* represented 4.3%, 3.1%, 3.3%, and 5.8% in S1-S4, and 1.4%, 2.3%, 8.1%, and 5.4% in B1-B4, respectively. Correspondingly, OTU6 and OTU17 shared 97.2% similarity of the partial 16S rRNA sequence with *Thiosocius teredinicola*, a facultative denitrifying SOB (Altamia et al. 2019). In particular, OTU2 and OTU3 with their respective closest relatives of *Desulfovibrio indonesiensis* and *Desulfovibrio salexigens*, also known as sulfate reducing bacteria (SRB) (Feio et al. 1998), accounted for an average of 10.9% in suspension sludge and 3.0% in biofilms, in total. Together, these microbes actuated the sulfur turnover in the saline mainstream anammox process.

The microbial co-occurrence network analysis implied the C-N-S symbiosis in positive patterns (Fig. S2). Exploitations of microbial functional profiles also supported the finding that nitritation, anammox, denitrification, sulfate respiration, and oxidation of reduced sulfur compounds synchronized in the FMBR (Fig. S3). The LEfSe analysis further identified the SRB (*unclassified Desulfovibrionales*) and sulfur-dependent autotrophic denitrifiers (*Thiohalophilus, Sedimenticola, Sulfuriflexus*, and *Thiosocius*) (LDA >3.7) as biomarkers (Fig. S4); this suggested their pivotal roles in constituting the C-N-S network,

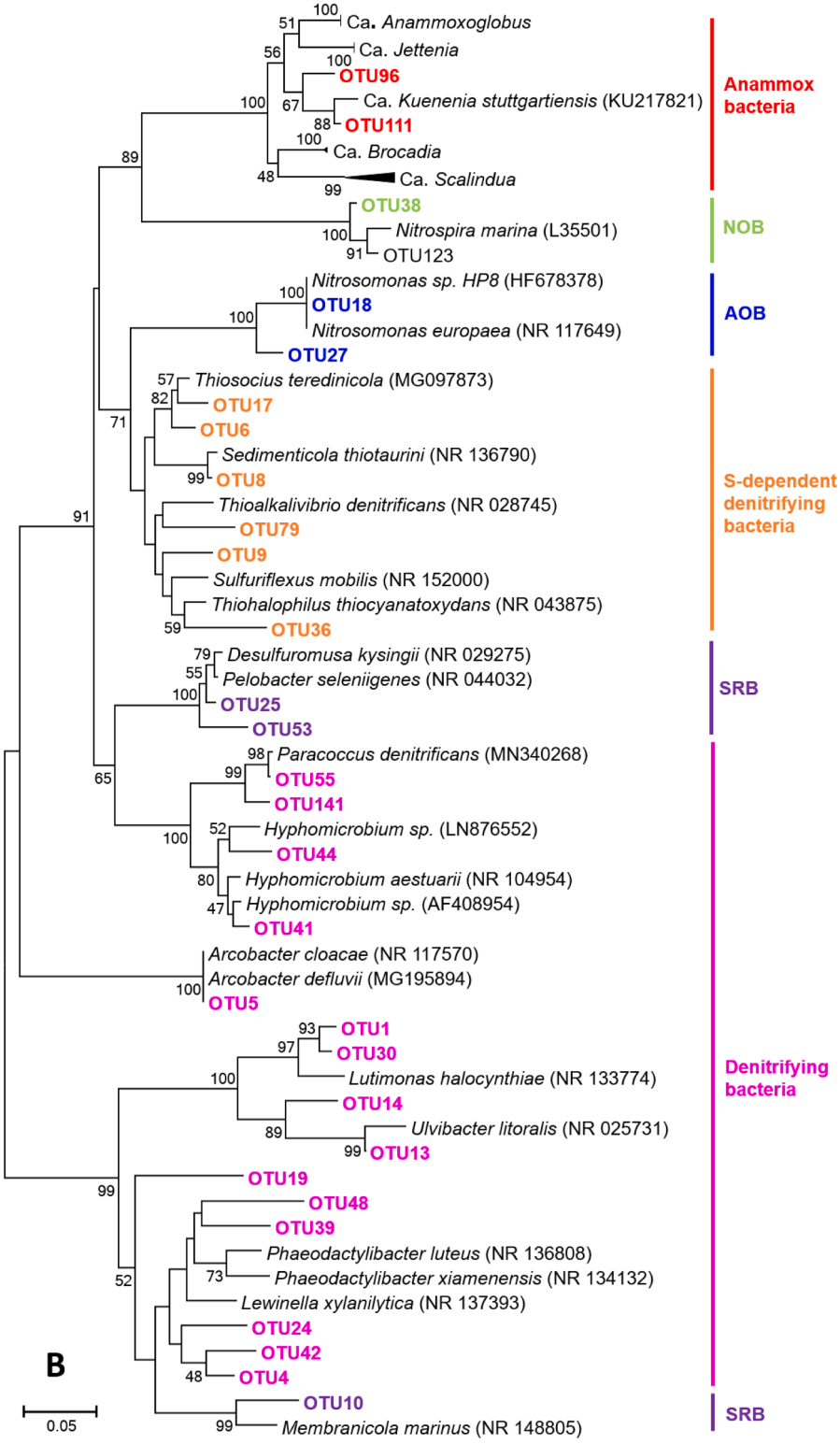

Fig. 2. (*continued*).

accomplishing the co-removal of nitrogen and COD from the saline sewage.

3.2.2. *Metagenomic insights into the metabolic versatilities of the microbiome*

To explore the symbiotic metabolic pathways, metagenomic analysis was performed with S4 and B4 collected on day 332. In total, 29 and 32 high-quality bins (>85% genome completeness and <10% contamination) were assembled from S4 and B4, respectively (See details of the genome quality in Table S3). 23 out of the 29 bins and 25 out of the 32 bins, accounting for 32.3% and 26.6%, respectively, were annotated with nitrogen and/or sulfur metabolizing properties. For B4, 14, 12, and 9 out of the 32 bins were annotated with the functions of dissimilatory nitrate reduction to ammonium (DNRA), denitrification, and nitrate

reduction to nitrite, respectively; these occupied 15.4%, 16.9%, and 9.3% in sequence. In addition, 13, 4, and 4 out of the 32 bins were annotated with the function of assimilatory sulfate reduction, dissimilatory sulfate reduction, and thiosulfate oxidation, amounting for 11.5%, 9.2%, and 9.2%, respectively. In particular, Bin B01 (occupying 6.8%) shows the best hit with *Sedimenticola thiotaurini* (AAI, 53.9%), which was a known SOB capable of coupling oxidation of thiosulfate, tetrathionate, elemental sulfur, and sulfide with reduction of nitrate, nitrite, and selenate (Flood et al. 2015); this bin harbored a full set of genes encoding proteins responding to DNRA, thiosulfate oxidation, denitrification, and dissimilatory sulfate reduction (Table S4). Bin B19 accounted for 1.0% shares the ANI of 84.2% with and all the N-S metabolizing genes of B01; its closest relative was identified to be *Sedimenticola thiotaurini* (AAI, 53.41%). Bin B03 and B04, representing 3.3% and 1.9% in sequence, showed the best hit to *Sulfurivermis fontis* (known for coupling nitrate reduction and oxidation of reduced sulfur) (Kojima et al. 2017) with the AAI score of 47.8% and 50.2%, respectively. Both bins possessed the full set of genes encoding the proteins carrying out assimilatory sulfate reduction (Table S4), not sulfur oxidation. In addition, Bin B01, B03, B04, and B19 were temporarily designated as *Gammaproteobacteria sp.* (B01), *Thioalkalispiraceae sp.* (B03), *Thioalkalispiraceae sp.* (B04), and *Gammaproteobacteria sp.* (B19), respectively.

For S4, 15 (26.1%) and 14 (10.2%) out of the 29 bins were annotated with nitrogen and sulfur metabolizing functions, respectively (Table S3). Among them, 7, 5, and 8 bins were annotated with the capabilities of DNRA, denitrification, and nitrate-to-nitrite reduction, respectively. In total, these bins successively accounted for 4.6%, 3.2%, and 21.9%. Specially, Bin S02, occupying 10.7% and showing the best hit to *Chitinophaga pinensis* (AAI, 44.5%), was annotated with the function of nitrate-to-nitrite reduction (Table S4). Bin S04 (occupying 6.7%) was also annotated with the aptitude of nitrate-to-nitrite reduction (Table S4); its closest relative was identified to be *Geoalkalibacter subterraneus* (AAI, 41.7%), a species gaining energy from the reduction of ferric iron, nitrate, and elemental sulfur with various electron donors (e.g., alcohols, organic acids, and hydrogen) (Greene et al. 2009). Bin S02 and S04 were designated as *Chitinophagaceae sp.* (S02) and *Desulfuromonadales sp.* (S04), respectively. Besides, the 14 bins identified as S-metabolizing microbes are restricted to assimilatory sulfate reduction. High-quality genomes of sulfur-facilitated autotrophic denitrifying bacteria were assembled only for B4. Further, the key bioprocesses and symbiotic metabolic pathways of the representative microbes actuating the C-N-S cycle in biofilms were constructed (Fig. 3) (See Table S4 for the respective coding DNA sequences predicted to involve these key pathways). In brief, microorganisms participating in denitrification, DNRA, nitrate reduction, and sulfate reduction resided in both suspension sludge and biofilms with the dominance on GAC. This was in line with the 16S rRNA gene-based sequencing result that shows denitrifying bacteria and SRB dominated on GAC (Fig. 2). Unfortunately, high-quality genome bins of *Nitrosomonas* sp. and *Ca.* Kuenenia sp. were not obtained. This may be due to the limitations of assembly and binning methods (Donovan H. Parks et al. 2015). Even so, the metagenomics approach revealed that a synergetic C-N-S loop formulated and responded in the FMBR with CEPT saline sewage.

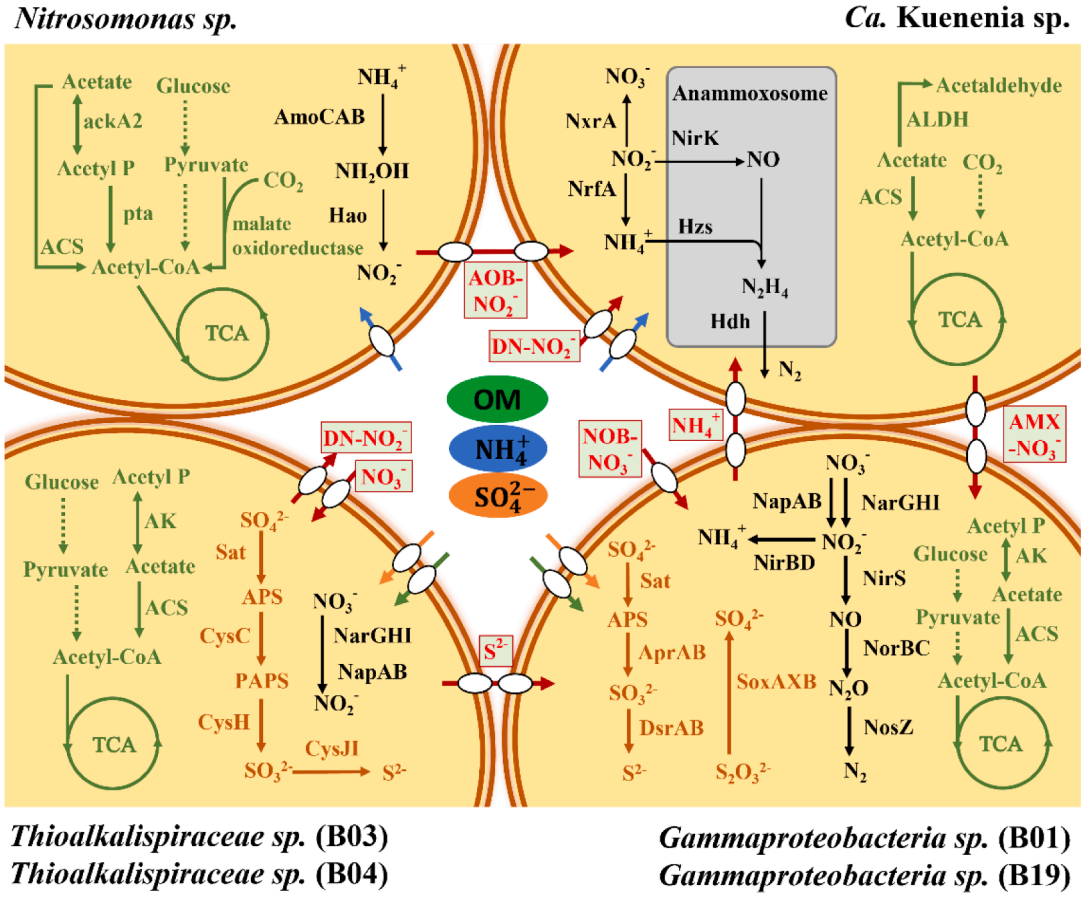

Fig. 3. Key metabolic pathways of the microbial C-N-S loop in biofilms revealed by metagenomics approach. OM, organic matters; AOB, aerobic ammonium oxidizing bacteria; NOB, aerobic nitrite oxidizing bacteria; AMX, anaerobic ammonium oxidizing bacteria; DN, denitrifying bacteria.

4. Discussion

4.1. Factors determining the success of pilot-scale saline mainstream anammox from conventional sludge source

The present study successfully started a pilot-scale mainstream anammox process from a conventional anaerobic digestion sludge with the GAC-fluidized membrane bioreactor; this result subverts the stereotype that the mature anammox seed is necessary to initiate a mainstream anammox scale-up. The utilization of GAC and membrane facilitated the fast start-up by improving sludge retention and stability of the FMBR operation. Firstly, the hollow fiber membrane (0.1 μm) enabled complete retention of the biomass through microfiltration, which is crucial for the cultivation of slow-growth anammox bacteria (van der Star et al. 2008). Secondly, the application of GAC yielded multiple benefits: 1) enforcing membrane fouling prevention with the aid of GAC scouring (Kim et al. 2011); 2) expediting the formation of biofilms, which helped retain denser biomass and provided an optimal ecological niche of a spatially-ordered oxygen gradient, enabling the symbiosis of aerobic and anaerobic functional microbiota; and 3) probably created a microenvironment with concentrated ammonium and nitrite via adsorption. The biofilm-based biomass averaged 1.6 times (while HRT was 10 hr) and 1.8 times (while HRT was 8 hr) the biomass of the suspension sludge (Table S5). This finding was in accordance with peer studies that the media-based (high-density polyethylene K series carriers and PE synthetic porous fleece sheet) biofilms dominated the biomass and were crucial to, as well as effective in, the maintenance of sufficient AOB and anammox bacteria during the long-term operation of mainstream anammox processes (Lackner et al. 2014, Laureni et al. 2016).

Results also demonstrated that limited oxygen supply was crucial to the robust performance of the saline mainstream anammox-based community. In particular, NOB repression and the cultivation of anammox bacteria and AOB were the determinant for the stability of partial nitritation and anammox (PN/A) processes, regardless of the reactor's configuration (Lackner et al. 2014). Given that the ammonium and operating temperature stayed at low values under mainstream conditions, it was not feasible to adopt free ammonium (FA) to inhibit the NOB. In this study, the pilot-scale FMBR was persistently operated at the DO of <0.16 mg/L, which facilitated the progressive gemination of the anammox bacteria; this process delivered stable nitrogen removal although it encountered unpredicted incidents in pilot trials (Table S1). More notably, the steady proliferation of *Kuenenia* on biofilms occurred while the *Nitrospira* erupted and persisted in the system (days 279-343). This finding conflicts with the previous assumption that overgrowth of NOB traditionally depressed the growth of anammox bacteria. Therefore, the research team for this study proposes two potential explanations for our impactful findings.

Firstly, although NOB subsisted in the FMBR, its activity might be halted due to the oxygen deficiency. This was mutually validated by Laureni et al. (2016), who found that stable repression of NOB was achieved during a 400-day operation by controlling DO as low as 0.18 mg/L. This explanation is supported by a recent study concluding that NOB inactivation – through a short-term anaerobic/anoxic operation – is effective in suppressing the regrowth of NOB in the long-term operation of bench- and large-scale (8 m^3) mainstream PN/A processes (Hausherr et al. 2022).

Secondly, bacteria capable of DNRA and nitrate-to-nitrite reduction inhabited both suspension sludge and biofilms and redirected nitrate produced by the NOB to ammonium and nitrite, respectively; this situation might offer a better survival niche for anammox bacteria. In particular, *Chitinophagaceae sp.* (Bin S02, 10.7%) and *Desulfuromonadales sp.* (Bin S04, 6.7%) were observed in the suspension sludge; their presence likely served the conversion of nitrate-to-nitrite given that NOB dominated in an oxygen-accessible niche. This further implied a simultaneous nitrogen removal path through partial denitrification and anammox (Deng et al. 2021, Ji et al. 2020, Kao et al. 2022). Additionally, the NO-forming bacteria – in total accounting for 12.8% in S4 and 10.8% in B4 (Fig. 2A) – potentially supported the metabolism of anammox bacteria given its versatility of NO-dependent anaerobic ammonium oxidation (Hu et al., 2019). In summary, this study supports and intensifies our understanding that it is feasible to suppress NOB; further, NOB washout would not be required towards stable mainstream anammox processes, even if unexpected germination occurred (Haushherr et al. 2022, Laureni et al. 2016).

4.2. Metagenomic insights into the microbe-driven C-N-S loop in the GAC-biofilms

Due to the unique features of sulfate in the saline sewage (i.e., approximately 0.5 g/L) (Yang et al. 2015), compared with the freshwater, S-metabolizing bacteria concatenating the C-N-S loop were identified (Fig. 3). Hydrogen sulfide produced from SRB remained a concern due to its potential threat to other C-N-S functionalizing microorganisms. In this study of biofilms, diverse bacteria dominated by *Thioalkalispiraceae sp.* (B03) and *Thioalkalispiraceae sp.* (B04) performed the sulfate reduction, occupying 20.7%. Meanwhile, dominant bacteria capable of respiring nitrate with reduced sulfur compounds as the electron donor, i.e., *Gammaproteobacteria sp.* (B01) and *Gammaproteobacteria sp.* (B19), were identified. In total, these bacteria (consisting of 7.8% of the B4's microbial community) not only contributed to the TN removal through denitrification, but also scavenged hydrogen sulfide. This property endowed these bacteria with competitive advantages over others in the presence of hydrogen sulfide; this quality also protected other microbes from the toxicity of hydrogen sulfide. Significantly, the bacteria functioning DNRA occupied 15.4% of the B4's populations (when only high-quality genomes were accounted therein). This unique function not only pointed to the versatility and superiority of these bacteria in harnessing nitrogen source at substrate scarcity, especially for CEPT sewage, but also potentially led to improved TN removal whilst the ammonium was retaken by the anammox bacterium. Thus far, two scenarios in support of DNRA have been proposed: 1) low C concentration with CEPT saline sewage triggers DNRA (Friedl et al. 2018, Li et al. 2020); and 2) low redox potential by low DO supply (0.5 mg/L) favors the NO_3^- partitioning via DNRA rather denitrification (Friedl et al. 2018). Therefore, the operation conditions likely fostered the bacteria with DNRA function. Collectively, these novel bacteria – along with *Nitrosomonas* and *Kuenenia* – established the C-N-S loop in the FMBR system and responded to the removal of nitrogen, COD, and properly sulfate from the saline sewage.

Another compelling finding was that *Gammaproteobacteria sp.* (B01) and *Gammaproteobacteria sp.* (B19) have bi-directional sulfur metabolism, i.e., reducing sulfate to sulfide through assimilatory and/or dissimilatory paths and oxidizing sulfide to sulfate via the SOX system (Table S4). Moreover, this study documented that *Gammaproteobacteria sp.* (B01) and *Gammaproteobacteria sp.* (B19) possessed the detoxification pathways of reactive oxygen species (ROS): 1) ROS reduction to hydrogen peroxide and oxygen by superoxide dismutase (SOD), and 2) hydrogen peroxide reduction to water and oxygen via catalase peroxidase (CAT) and peroxiredoxin (PRDX) (Table S6). More interestingly, these bacteria might have led an alternative ROS detoxification trick by integrating SOD and Cytochrome c551 peroxidase (Ccp) (Table S6). However, it is worth noting that the Ccp expressed only in the presence of hydrogen peroxide and the absence of oxygen (Khademian and Imlay 2017); this further complicated the oxygen tradeoff of these two bacteria. In addition, a full set of proteins involving the ROS detoxification were also annotated in B03, B04, S02, and S04 (Table S6), indicating their ascendancy subsisting in the presence of limited oxygen. Thus, three routes were hereby proposed for the oxidation of reduced sulfur by coupling: 1) respiration of nitrate or nitrite; 2) DNRA; and 3) respiration of oxygen. Collectively, this indicates that the role of N-S co-metabolizing microorganisms (i.e., B01, B03, B04, and B19) in

regulating the ecological C-N-S flux might be underrated. Thus, the evolution of these N-S co-metabolizing bacteria associated with oxygen deserves extensive future research.

5. Conclusions and implications

This study investigated the feasibility of the GAC FMBR configuration with CEPT saline sewage. Operating the GAC FMBR at a limited DO supply (i.e., 0.08±0.03 mg/L) was determined to be the vital factor leading to stable performance. The GAC, aggregated rich functional bacteria and higher nitrogen strength in microenvironment, insured robust removals of nitrogen and COD. The integrated nitritation, anammox, sulfate reduction, and mixotrophic denitrification scenario manifested in the saline sewage treatment. Exploitation of the synergetic microbial symbiosis patterns rendered insights into the scavenging of hydrogen sulfide by *Gammaproteobacteria sp.* (B01 and B19) and *Thioalkalispiraceae sp.* (B03 and B04) associating with nitrogen transforming anammox-based microbes. In conclusion, this work significantly informs our understanding of ecological interactions feeding with saline sewage. In addition, our findings provide direction for future vital research.

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.

Data Availability

The data that has been used is confidential.

Acknowledgements

The authors wish to acknowledge the Innovation and Technology Fund (ITS 188/15FP) and Theme-based Fund (T21-711/16-R) for providing financial support.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2022.119475.

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