Nitrogen-metabolising microorganism analysis in rapid sand filters from drinking water treatment plant

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Abstract
Sand filters (SFs) are common treatment processes for nitrogen pollutant removal in drinking water treatment plants (DWTPs). However, the mechanisms on the nitrogen-cycling role of SFs are still unclear. In this study, 16S rRNA gene amplicon sequencing was used to characterise the diversity and composition of the bacterial community in SFs from DWTPs. Additionally, metagenomics approach was used to determine the functional microorganisms involved in nitrogen cycle in SFs. Our results showed that Pseudomonadota, Acidobacteria, Nitrospirae and Chloroflexi dominated in SFs. Subsequently, 85 high-quality metagenome-assembled genomes (MAGs) were retrieved from metagenome datasets of selected SFs involving nitrification, assimilatory nitrogen reduction, denitrification and anaerobic ammonia oxidation (anammox) processes. Read mapping to reference genomes of *Nitrospira* and the phylogenetic tree of the ammonia monooxygenase subunit A gene, *amoA*, suggested that *Nitrospira* is abundantly found in SFs. Furthermore, according to their genetic content, a nitrogen metabolic model in SFs was proposed using representative MAGs and pure culture isolate. Quantitative real-time polymerase chain reaction (qPCR) showed that ammonia-oxidising bacteria (AOB) and archaea (AOA), and complete ammonia oxidisers (comammox) were ubiquitous in the SFs, with the abundance of comammox being higher than that of AOA and AOB. Moreover, we identified a bacterial strain with a high NO3-N removal rate as *Pseudomonas* sp. DW-5, which could be applied in the bioremediation of micro-polluted drinking water sources. Our study provides insights into functional nitrogen-metabolising microbes in SFs of DWTPs.

Keywords Sand filters · Comammox · Metagenome-assembled genomes · Nitrogen cycling · Bioremediation · DWTP

Introduction
Conventional water treatment processes include coagulation, precipitation, filtration and disinfection, which is a very common and effective treatment method in water supply treatment. The drinking water treatment plants (DWTPs) in China that using this process are now more than 95% (Feng et al. 2012). Among these systems, the sand filters (SFs) have the advantages of small water consumption, convenient installation and easy operation, which are usually prescribed to purify water in the DWTPs (Bertelli et al. 2018; Chen et al. 2021). SFs could make an enormous contribution to transform and degrade all sorts of harmful compounds in drinking water (Association 2012, Bai et al. 2013, Terry & Summers 2018). To our knowledge, nitrogen pollution is a public enemy of all mankind due to its universal and serious (Bettez & Groffman 2013, Ming et al. 2020). All kinds of ecosystem are seriously polluted by nitrogen, which
has attracted extensive attention (Gao et al. 2020; Lin et al. 2020b; Zhang et al. 2016), including surface water, which is often used as a drinking water source (Hu et al. 2020a). A national survey in China indicating seasonal changes in ammonia levels in rivers and lakes (including the Pearl River), which are sources of drinking water (Fu et al. 2012). SFs are mainly responsible for removing ammonia from drinking water source (Lee et al. 2014), while nitrification is a simple but effective biological process to remove the ammonia in SFs (Hu et al. 2020b). To the best of our knowledge, nitrification is traditionally considered to be a two-step process, consisting of ammonia oxidation and nitrite oxidation. The priority is to convert ammonia to nitrite via archaea (AOA) or ammonia-oxidising bacteria (AOB), and then the nitrite-oxidising bacteria (NOB) is used to achieve the transition from nitrite to nitrate (Hirotugu et al. 2015). Nevertheless, our cognition about nitrification has been changed by the recently discovered complete ammonia oxidiser (comammox) (Van Kessel et al., 2015). The first pure culture of a comammox bacterium is \emph{Nitrospira inopinata}, which could oxidise ammonia to nitrate by itself. Subsequently, comammox has been discovered in certain ecological niches, such as drinking water system (Pinto et al., 2016, Wang et al. 2017). It has been reported that comammox might be more competitive than AOA and AOB under oligotrophic conditions (Kits et al. 2017). However, information on the simultaneous evaluation of AOA and AOB as well as comammox in SFs are not sufficient, and the comammox bacteria and their prospective nitrogen metabolic synergies with other microorganisms (i.e. AOA, AOB and heterotrophic counterparts) are still poorly understood (Potgieter et al. 2020).

Traditional biological denitrification occurs only under anaerobic or anoxic conditions (Joo et al. 2005). Aerobic denitrification has a higher denitrification rate than anaerobic denitrification and is easier to operate (Wu et al. 2013). Thus, aerobic denitrification has great potential for application in nitrogen-contaminated drinking water bioremediation. Aerobic denitrifiers are ubiquitously present in the natural environment. Previous studies reported that aerobic denitrifiers have been isolated from swamps (Huang et al. 2016), sediments (Wang et al. 2016), urban sewage sludge (Zhu 2008) and wastewater (Zhang et al. 2013). Compared with other environmental systems, DWTPs are oligotrophic niches. Nevertheless, they are not suitable for the growth of heterotrophic aerobic denitrifiers as they have low concentrations of carbon sources. Therefore, very few researches have concentrated on the characteristics of aerobic denitrifiers, which can remove the nitrogen from DWTPs (Huang et al. 2015). Hence, the isolation and characterisation of indigenous aerobic denitrifiers from drinking water niches are extremely meaningful.

Herein, we probed the diversity and composition of the microbial community in full-scale rapid SFs used to purify surface water by high-throughput sequencing of 16S rRNA gene amplicons. In addition, the distribution and abundance of AOA, AOB and comammox were investigated using quantitative real-time polymerase chain reaction (qPCR). Metagenomic analyses were conducted to survey the presence and function of comammox and nitrogen-cycling microorganisms in SFs. Moreover, aerobic denitrifiers were isolated, cultured and characterised. The findings of this study provide insights into nitrogen-metabolising microbes in the SFs of DWTPs in China.

Material and methods

**Sampling, DNA extraction, 16S rRNA gene PCR amplification and Illumina MiSeq sequencing**

Sand samples were collected from three SFs (SF1, SF2 and SF3) of three DWTPs in Guangzhou, South China, in December 2016. The source water of the three SFs was from the same Shunde waterway. Specifically, the source water of SF1 was from the upper reaches of the Shunde Waterway, while the source water of SF2 and SF3 was from the downstream of the Shunde Waterway. In terms of geographical location, SF1 is far from SF2 and SF3. The water treatment processes in these DWTPs were the same, including the following steps: coagulation, sedimentation, filtration and chlorination. Among which SF2 sand filter had been in operation for 10 years, whereas SF1 and SF3 for 7 years. The specific characteristics of the three SFs are shown in Supplementary Table S1. Briefly, sample SF1 consisted of 0 cm (SF1-1), 30 cm (SF1-2) and 50 cm (SF1-3) quartz sand, and which represented three replicate samples in each sand filter. Samples SF2 and SF3 followed a similar convention. In addition, triplicate influent and the corresponding effluent water samples from these three sand filters were also collected for water quality detection. Among which pH, dissolved oxygen (DO) and temperature were determined using a portable multi-parameter water quality analyzer HACH HQ40D, while ammonium nitrogen (NH$_4$-N), nitrite-nitrogen (NO$_2$-N) and nitrate-nitrogen (NO$_3$-N) were measured based on drinking water standard examination methods (GB/T 5750–2006; China). Five grams of quartz sand was used for total DNA extraction with the Fast DNA®Spin Kit for Soil (MP Biomedicals, Carlsbad, CA, USA) according to the manufacturer’s instructions. Microbial community composition was characterised via amplification the target V3–V4 regions of 16S rRNA gene using the universal primers 338F and 806R, and then sequenced on an Illumina MiSeq platform with 250PE reads. All data were processed using the Qiime2-Deblur pipeline (Prodan et al. 2020). Raw data was available from the NCBI SRA under SRR14621795-14,621,803.
Library preparation, metagenomic sequencing, assembly, gene prediction and read mapping

DNA from samples SF2 and SF3 was used for metagenomic sequencing. Briefly, DNA was sheared into 300-bp fragments using ultrasonication. The obtained DNA fragments were used for library preparation using Illumina Nextera XT DNA Library Preparation Kit. All libraries were sequenced on Illumina HiSeq 2500 platform in paired-end mode (2 × 150 cycles). Metagenomic sequencing data were trimmed as previous study (Bolger et al. 2014). The clean sequencing reads of about 30G obtained for each sample were then pooled together for assembly using MEGAHIT with the following parameters: k-min, 21; k-max, 149; and k-step, 10. In order to determine low-abundance species, the obtained clean data were mapped to their respective scaffolds, and all the reads were put together for hybrid packaging. Open reading frames (ORFs) of the contigs from each metagenomic sample were predicted using Prodigal (Hyatt et al. 2010). Identification of nitrogen cycling genes was performed using the NcycDB database (Tu et al. 2019). In addition, contigs with lengths of more than 2500 bp were extracted from the co-assembly of all samples and used to generate metagenome bins using METABAT (Kang et al. 2010). The completeness and redundancy of the metagenome bins were checked using CheckM (Parks et al. 2015). Finally, KRAKEN2 was used for taxonomic annotation (Chen & Tyler 2020). A total of 85 bins that were > 80% complete and with < 10% contamination were retained for building the phylogenetic tree using GToTree (https://doi.org/10.1101/512491) (Lee 2019). Sequences of proteins encoded by genes involved in the Kyoto Encyclopedia of Genes and Genomes (KEGG) nitrogen metabolism pathway (genes shown in Supplementary File 1 in FASTA format), including nitrification- (i.e. amoABC, hao and nrxAB), assimilatory nitrogen reduction- (i.e. nasA, nirA and nirB) and denitrification- (i.e. nirK and norBCDQ)-related genes, were obtained from the NCBI Protein database (www.ncbi.nlm.nih.gov/protein/). Protein sequences were compared to gene sequences using tblastn v 2.9.0 + searches. BLAST results were filtered for percent identity of > 30% and an e-value < 0.05. To investigate whether there are comammox in SFs, the reference genomes of three representative comammox were selected: Candidatus Nitrospira inopinata (CNI), Candidatus Nitrospira nitricicans (CNN) and Candidatus Nitrospira nitrosa (CNNS). The final assembly of the reference genomes of the three contigs was as follows: CNI, completed graph; CNNf, 36 contigs; and CNNS, 15 contigs. Then, ORFs of the non-redundant gene set alignment of SF2 and SF3 were mapped to these three reference genomes using BLASTN, with alignment parameters set as e-value = 1 − e5 and similarity > 90%. The ratio of fragment length to the total length of the ORF fragment was more than 90%. Subsequently, a circle diagram was drawn according to the abundance of the ORFs. Furthermore, to investigate whether there were comammox in SFs, comammox ammonia monoxygenase gene subunit A, amoA, was extracted from the NT library and compared using BLASTN with the comparison sets e-value = 0.00001 − max_target_seqs 1.

PCR analysis of key functional genes involved in ammonia oxidation

AOA, AOB and comammox amoA genes were amplified using genomes extracted from sand samples that mixed different depths of quartz sand together and the following PCR primers: AOA (Arch-amoAF and Arch-amoAR); AOB (AmoA-1F and AmoA-2R) (Sun et al. 2013); and comammox (amoA-189b-F and com-amoA-1-R) (Bartelme et al. 2017). PCR amplifications of AOA and AOB amoA genes were performed as previously described (Niu et al. 2019). PCR amplifications of comammox amoA genes were performed as previously described (Bartelme et al. 2017).

Quantification of AOA, AOB and comammox amoA genes

In order to investigate the abundance of AOA, AOB and comammox amoA genes in the three SFs, a triplicate of qPCR per DNA sample of quartz sand was performed. pUC57 plasmids carrying AOA, AOB and comammox amoA genes (Supplementary Table S2) were used for standard analysis. PCR amplification primers are as described in section ‘PCR analysis of key functional genes involved in ammonia oxidation’, and the PCR conditions were performed as previously described (Bartelme et al. 2017).

Enrichment and isolation of aerobic denitrifiers

Enrichment of bacteria was performed by mixing 10 g of quartz sand sample to 100 mL of heterotrophic enrichment denitrification medium (HEDM) and incubating at 30 °C and 120 rpm in the dark. The composition of the HEDM was as follows: C6H12Na2O4, 0.5 g/L; NaNO3, 0.1 g/L; K2HPO4·3H2O, 0.1 g/L; CaCl2, 0.05 g/L; MgCl2·6H2O, 0.05 g/L; and 2 mL of trace element solution (Zhu et al. 2012). The final pH of the medium was adjusted to 7.5. After incubating for 8 days, 1 mL of suspensions was inoculated into fresh medium. This process was repeated three times for 24 days. After enrichment, 1 mL of the enriched culture was diluted to 10−1–10−6 times. Subsequently, 0.2 mL of the diluted culture was retrieved and spread to HEDM agar and then incubated at 30 °C for 48 h. Single colony with different morphologies was screened and isolated.
Identification of aerobic denitrifiers

To identify bacteria, DNA of pre-cultured isolates was extracted using a bacterial DNA extraction kit (Magen, Guangzhou, China). Amplification of the 16S rRNA gene was performed using the universal bacterial primers 27f and 1492r, as described previously (Wang et al. 2021). The PCR products were sequenced by the sequencing company Liuhe Huada (Beijing, China). Phylogenetic trees were constructed as previously described (Gu et al. 2016). PCR amplification was performed as previously described (Huang et al. 2015). PCR products were sequenced by Liuhe Huada. Subsequently, the resulting sequences were compared with the National Center for Biotechnology Information (NCBI) GenBank database (www.ncbi.nlm.nih.gov/gene) using the basic local alignment search tool (BLAST) for gene determination.

Assessment of nitrogen removal performance by Pseudomonas sp. DW-5

Bacteria were pre-cultured in R2A liquid medium at 30 °C and 120 rpm for 24 h, and then 10% (v/v) was inoculated into 180 mL denitrification medium (DM) and heterotrophic nitrification medium (HNM) in a 300-mL Erlenmeyer flask. Subsequently, the inoculated cultures were incubated at 30 °C and 120 rpm for 60 h under aerobic conditions (DO was 7.0–8.0 mg/L). The composition of DM and HNM was as previously described (Lang et al. 2020). The initial concentrations of ammonium nitrogen (NH4-N) in HNM and nitrate-nitrogen (NO3-N) in DM were respectively set to a low concentration and a high concentration, 10 mg/L and 100 mg/L, and the final pH was adjusted to 7.0–7.2. During cultivation, flasks cultures were collected by three replicates every 12 h to measure optical density at 600 nm, as well as the concentrations of NH4-N, nitrite nitrogen (NO2-N) and NO3-N using ion chromatography (Tang et al. 2021; Xiao et al. 2020). In order to further evaluate nitrogen removal performance of strain DW-5 in actual water treatment, a sand filter was constructed, in which four organic glass columns with an inner diameter of 4.0 cm and a working volume of 450 mL were filled with quartz sand samples that were collected from the SF2 sand filter. Of which two columns without inoculated with strain DW-5 were defined as control group (A and B), the other two columns inoculated with 300 mL of pre-cultured strain DW-5 were used as treatment group (C and D). Additionally, sedimentation tank effluent water was also collected from the DWTP. The water quality parameters of the collected source water were as follows: 6.1 mg/L DO, 0.06 mg/L ammonia-N, 3.4 mg/L chemical oxygen demand (COD), 25.6 NTU turbidity, 7.4 pH value, 28 °C temperature. And then the source water was supplemented with 1 mg/L of ammonia-N or nitrate-N, respectively. Then, the sedimentation tank effluent water supplemented with 1 mg/L ammonia-N was pumped into columns A and C, while the sedimentation tank effluent water supplemented with 1 mg/L nitrate–N was pumped into columns B and D using peristaltic pumps. Empty bed contact time (EBCT) was set as 20 min. This process was carried out at room temperature. Both the influent water and the corresponding effluent water from A, B, C and D columns were collected for nitrogen content (NH4+ and NO3−) detection after the sand filters performed for 24 h and 48 h. Triplicate water samples were collected. Difference analysis between control group and treatment group was performed using T-test.

Results and discussion

Water quality analysis

As can be seen from Fig. 1, the pH, temperature and DO in influent and effluent water of three sand filters maintained stable, while turbidity before and after passage through sand filters decreased. After sand filtration, the final nephelometric turbidity unit (NTU) values were all below one, which could meet the requirements of Chinese standard for drinking water quality. Additionally, it could be observed that the ammonia-N in influent water was significantly removed when passed through sand filters, which suggested the occurrence of nitrification and anammox. Nitrate–N and TN reduced notably after sand filtration, which demonstrated that denitrification was ubiquitous in these three sand filters; meanwhile, we found that there was nearly no nitrite-N accumulation, which also suggested the occurrence of denitrification and anammox. Therefore, nitrification and denitrification, even anammox, were simultaneously occurred in the sand filters. Based on the findings of water quality analysis, it can be concluded that sand filters are very effective in drinking water purification, not only for turbidity removal, but also for nitrogen removal at low concentrations in water sources.

Diversity and composition of bacterial communities in SFs

As shown in Fig. 2A, at the phylum level, the main bacterial groups identified in the SFs were Pseudomonadota (21.57–48.27%), Acidobacteriota (4.70–47.83%), Nitrospirota (7.64–25.70%) and Chloroflexota (4.36–11.86%) at the phylum level. The results are in line with previous study showing that Pseudomonadota and Nitrospirota are dominant in SFs (Palomo et al. 2016). However, it can be seen that the diversity and composition of bacterial communities in SF2 and SF3 were different from that of SF1, which might be related to geographical location (Schulhof et al. 2020; Zhang et al. 2020b) and water sources. Specifically, the source water of

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SF1 was from the upper reaches of shunde Waterway, while the source water of SF2 and SF3 was from the downstream of shunde Waterway. Upon further analysis, we found that especially the relative abundance of Planctomycetota in SF2 and SF3 was much lower than that in SF1. To our knowledge, anammox bacteria belonged to Planctomycetota, so we speculate that anammox might contribute a lot for nitrogen removal in SF1. Furthermore, the microbial community in SFs is different from that in other ecological systems, such as drinking reservoirs and surrounding rock soil. It was reported that microbial composition in the drinking water reservoir was Pseudomonadota (from 20.77 to 80%), Acidobacteriota (from 5.03 to 46.34%), Cyanobacteria (from 1 to 39.89%) and Bacteroidota (from 6.54 to 22.73%) (Zhou et al. 2020). Additionally, the microbial composition in the surrounding rock soil was reported to comprise of Pseudomonadota (42.90%), Actinomycetota (23.60%), Bacteroidota (10.50%) and Gemmatimonadota (7.82%) (Jiang et al. 2020). At the genus level (Fig. 2B and Supplementary Fig. S1), the predominant taxa were Nitrospira (from 4.70 to 47.83%), norank_c_bacteriap
25 (from 0 to 12.06%), norank_f_A0839 (from 2.63 to 8.32%), unclassified_o_Burkholderiales (from 1.49 to 5.91%) and norank_f_Caldilineaceae (from 0 to 7.87%). However, most of these cases were undefined. Similarly, Nitrospira was found to be abundant in oligotrophic water environments, such as drinking water systems (Oh et al. 2017; Poghosyan et al. 2020). In addition, it has been reported that Nitrospira is the only nitrite-oxidising bacterium in SFs, with groundwater as the water source and accounts for 65% of all microorganisms (Vet et al. 2009). Interestingly, the recently discovered comammox, which completely oxidise ammonia to nitrate, belongs to the genus Nitrospira (Pinto et al. 2016). It has been reported that comammox usually exist in low ammonium loading environments and were, therefore, discovered in SFs (Fowler et al. 2017, Palomo et al., 2016). Moreover, the ammonium-oxidising bacteria (AOB), Nitrosomonas, were ubiquitously present in all three SFs and accounted for 0~6.21% of the total microbial population. In particular, the relative abundance of Nitrosomonas in SF3 was significantly higher ($P < 0.01$, Kruskal–Wallis $H$ test) than that in SF1 and SF2 (Supplementary Fig. S2). In addition, we found that Candidatus Accumulibacter was ubiquitously present in SFs, and it has been reported to be capable of using nitrate as an electron acceptor for phosphorus and nitrogen removal simultaneously under the anaerobic conditions (Zhang et al. 2018). Meanwhile, we found that some of denitrifying bacteria also occupied a certain abundance, like Flavobacterium and Comamonas (Yang et al. 2021). Taken together, there were abundant nitrogen metabolising microorganisms in sand filter, including ammonia-oxidising bacteria, nitrifying bacteria, denitrifying bacteria and anammox bacteria. Although nitrification–denitrification process is the most common process for nitrogen removal, anammox also can not be neglected. Therefore, SFs have great potential for nitrogen removal using the nitrification–denitrification and anammox process. Our results could preliminary reveal the internal mechanism of the nitrification, denitrification and anammox in the SFs.

**PCR and quantitative analysis of amoA genes in SFs**

According to the results of 16S rRNA gene amplicon sequencing, we found that abundant bacteria were involved in nitrification in SFs. To further verify the presence of ammonia-oxidising bacteria in SFs, we conducted the PCR amplification of amoA (Fig. 3). Meanwhile, qPCR of amoA gene was also used to confirm the abundance
of ammonia-oxidising microorganisms in SFs. As shown in Table 1, comparative analysis of amplified sequences showed that sequences with sizes of approximately 492 bp from samples SF1, SF2 and SF3 were 93%, 96% and 87% similar to that of uncultured bacterial amoA, respectively. In addition, sequences with sizes of approximately 635 bp from samples SF1, SF2 and SF3 were 98%, 95% and 97% similar to that of uncultured archaeal amoA, respectively. Furthermore, sequences with sizes of approximately 520 bp from samples SF1, SF2 and SF3 were 99%, 93% and 97% similar to that of uncultured comammox amoA, respectively. As shown in Fig. 4, qPCR results indicated that the abundance of AOA, AOB and comammox amoA genes in SF1 was different from that in SF2 and SF3, which might be mainly related to space and environmental factor, such as geographic location and nitrite/nitrate concentration, which has been reported in previous literatures (Bao et al. 2016; Li et al. 2022; Zhu et al. 2018). Meanwhile, the abundance of AOA, AOB and comammox amoA genes in SF2 was generally higher than that in SF1 and SF3. Interestingly, the amount of comammox amoA exceeded that of the canonical AOA and AOB amoA in SFs, which was in line with previous study that found in rapid SFs (Tatari et al. 2017). It has been reported that comammox has a strong affinity for ammonia and may be more competitive at low ammonium concentrations (Daims et al. 2015, Van Kessel et al. 2015), while ammonium concentrations in drinking water treatment plant are relatively low comparing other ecological environments. Therefore, drinking water environment with low ammonium concentration might be good for the growth of comammox. Therefore, we speculate that the reason why the abundance of comammox amoA gene in SF2 was higher than that in SF1 and SF3 might be related to the natural acclimation of SF2 microbes at a low concentration of ammonia for approximately 10 years, while SF1

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**Table 1** BLAST results of amoA genes in three sand filters (SFs)

| Gene names  | Sand filter names | Amplicon size (bp) | Nucleotide BLAST results                                      | Similarity (%) |
|------------|-------------------|-------------------|---------------------------------------------------------------|----------------|
| AOB amoA   | SF1               | 463               | Uncultured Nitrosomonadales bacterium clone PYL-FH-13 amoA gene (KJ137864) | 93             |
|            | SF2               | 465               | Uncultured beta proteobacterium clone SEP-I-B-10 amoA gene (KJ158669) | 96             |
|            | SF3               | 439               | Uncultured AOB clone N-G1-AOB-05 amoA gene (KM882239)          | 87             |
| AOA amoA   | SF1               | 609               | Uncultured AOA clone N-G3-AOA-02 amoA gene (KM881831)          | 98             |
|            | SF2               | 606               | Uncultured AOA clone J40104213_23_A_4_M13F amoA gene (KJ497144) | 95             |
|            | SF3               | 605               | Uncultured AOA clone N-G1-AOA-35 amoA gene (KM881800)          | 97             |
| Comammox amoA | SF1            | 502               | Uncultured Nitrospira sp. clone com-cladeA-OTU345 amoA gene (MT790412) | 99             |
|            | SF2               | 492               | Uncultured Nitrospira sp. clone com-cladeA-OTU310 amoA gene (MT790393.1) | 93             |
|            | SF3               | 516               | Uncultured Nitrospira sp. clone COM-Ri-LW-46 amoA gene (MG591541.1) | 99             |
and SF3 were operational for 7 years. This results indicated that comammox in SFs might play a key role in nitrogen metabolism. In addition, the amount of AOA amoA genes in SFs was generally higher than that of AOB amoA genes, which was also observed in drinking water distribution systems (Van der Wielen et al. 2009), and might be explained by the preference of AOA and AOB for low- and high-ammonia conditions, respectively (Hink et al. 2018). Taken together, our results show that AOA, AOB and comammox were ubiquitously distributed in SFs, which offer further evidence for the widespread presence of the ammonia-oxidising microorganisms in SF ecosystems. Moreover, comammox and AOA are mainly responsible for the oxidation of ammonia, and their contribution cannot be ignored in the SF niche. In addition, although the number of copies of amoA genes is lower in SF1 (Fig. 4), the efficiency of nitrogen removal in this sand filter seems similar to the others according to water quality analysis results (Fig. 1). Meanwhile, we found the relative abundance of Planctomycetota which involved in anammox in SF1 was obviously higher than that in the others (Fig. 2A). Therefore, it was not negligible that anammox might also play an important role in nitrogen removal in SF1. According to previous literatures, ammonia oxidation is the first and rate-limiting step of nitrification that is an essential part of the global nitrogen cycling process (Lin et al. 2020a), yet ammonia-oxidising bacteria, ammonia-oxidising archaea and comammox play an important role in ammonia oxidation (Beman et al. 2008). Moreover, ammonia monoxygenase subunit A (amoA) gene was a key functional gene for ammonia-oxidising microorganisms, and it was important for controlling nitrogen removal (Hoshino et al. 2001). Furthermore, amoA was often used to characterise the abundance of ammonia-oxidising archaea, archaea (Aigle et al. 2019) and comammox (Pjevac et al. 2017, Shao & Wu 2021). In order to comprehensively study nitrogen metabolism mechanisms and get more gene information on comammox and nitrification in SFs, SF2 and SF3, which presented a higher abundance of amoA than SF1, were chosen for metagenomic sequencing.

**Genome-centred metagenomics of predominantly nitrogen-metabolising species**

The phylogenomic tree was constructed based on 48 single-copy core genes.

In this study, after metagenomic binning, 85 high-quality MAGs involved in nitrogen metabolism were obtained, which mainly including members of Gammaproteobacteria (n = 9), Actinobacteria (n = 5), Deltaproteobacteria (n = 4), Planctomycetia (n = 11), Alphaproteobacteria (n = 33) and Betaproteobacteria (n = 1) (Fig. 5). The general characteristics of these 85 MAGs are shown in Supplementary Tables S3 and S4. In the 85 MAGs, the phylum Pseudomonadota (n = 67) dominated the microbial community in both SFs, followed by Planctomycetota (n = 11). Additionally, 165 rRNA gene amplicon sequencing results were found to be consistent with the metagenomic binning results, which indicates that Pseudomonadota was the predominant phylum in microbial community in SFs. A previous study showed that the Pseudomonadota phylum was correlated with NH₄-N removal and nitration efficiency (Feng et al. 2019). Moreover, Planctomycetota are also shown to play key roles in nitrogen cycles (Kallscheuer et al. 2019). Thus, the Pseudomonadota and Planctomycetota phyla might play important roles in nitrogen metabolism. Furthermore, 16 genes associated with nitrogen metabolism are shown in Fig. 5, including genes involved in nitrification (i.e. amoABC, hao and nirAB), assimilatory nitrogen reduction (i.e. nassA, nirA and nirB), denitrification (i.e. nirK and norBC-DQ) and anammox (hzo and hzA). As shown in Fig. 5, 12 MAGs included amoA. Interestingly, SF2 bin.22, identified as Pseudomonas-like MAG, simultaneously harboured the genes amoA, amoB and amoC, and SF2 bin.62, identified as Nitrosomonas-like MAG, simultaneously included the genes amoA and amoB. To our knowledge, Nitrosomonas sp. is a key ammonium-oxidising bacterium (Shitu et al. 2020) and can oxidise ammonium to nitrite (Yang et al. 2019). It has always been dominant in many natural and engineered ecosystems (Lin et al. b; Ma et al. 2020c, 2020d; Song et al. 2020; Yuan et al. 2020). Furthermore, 13 MAGs included the hydroxylamine oxidoreductase (hao) gene. For example, SF2 bin.117, identified as Bradyrhizobium-like MAG, included the hao gene. Additionally, 4 MAGs included hydrazine oxidoreductase (hzo), 36 MAGs included...
hydrazine synthetase (hznA) and interestingly nine out of 11 MAGs that identified as Planctomycetota included hznA. According to previous literatures, Planctomycetota have always been reported to contribute significantly to anammox (Speth et al. 2017; van Teeseling et al. 2016). Additionally, Planctomycetota could also assist anammox consortium settlement (Ma et al. 2020b). Furthermore, 51 MAGs included the nitrite oxidoreductase (nxrA), 28 MAGs included the
nitrite reductase (nirK) gene, 47 MAGs included the nitrite assimilation reductase (nirB) gene and 63 MAGs included the nitrate assimilation reductase (nasA) gene, the latter, accounting for 74% of total MAGs. Thus, the metagenomic binning results show that many MAGs are involved in nitrogen metabolism.

**Presence of comammox Nitrospira and NOB**

Based on amplification and qPCR results of amoA genes, we get to know comammox were ubiquitously distributed in SFs. In order to get more information on comammox and further prove the presence of comammox, ORFs of the non-redundant gene set alignment of SF2 and SF3 were mapped to these three reference genomes CNOs, CNNf and CNI. The genes from samples SF2 and SF3 matched CNNs the most, the number and the total abundance of ORFs from SF2 and SF3 matched CNNs the highest (Supplementary Table S5), followed by CNNf and CNI (Fig. 6). In addition, the gene sequences of SF2 and SF3 matched the three subunits of ammonia monoxygenase (amoA subunit A, B and C) of comammox amoA for CNI, CNNf and CNNs, respectively (Supplementary Fig. S3). Therefore, we can speculate that there might be comammox *Nitrospira* in the SFs of the DWTPs. According to high-throughput 16S rRNA gene amplicon sequencing results, we found that genus *Nitrospira* predominated in SFs (from 4.70 to 47.83%). Thus, in addition to comammox *Nitrospira* bacteria, we have also recovered the canonical nitrite-oxidising bacteria *Nitrospira* from metagenomic data, and in order to get more genomic functional information. As shown in Fig. 7, a total of five MAGs affiliated with the phylum *Nitrospirae* (completeness >75%) were reconstructed from samples SF2 and SF3 metagenomes. The five *Nitrospira*-like MAGs were named ‘*Nitrospira defluvii* SF2.bin.188’, ‘*Nitrospira defluvii* SF3.bin.113’, ‘*Nitrospira defluvii* SF2.bin.132’, ‘*Nitrospira defluvii* SF3. bin.25’ and ‘*Nitrospira defluvii* SF3.bin.52’. The genomic information of the five MAGs is shown in Supplementary Table S6. To investigate the nitrogen metabolic potential of the five MAGs, genes associated with nitrogen transformation were confirmed using *Nitrospira defluvii*. As shown in Fig. 7, all *N. defluvii*-like bins contained the nitric oxide-forming nitrite reductase genes (nirK) gene, and four out of five *N. defluvii*-like bins harbour the nitrite oxidoreductase (nirB) and nitrite assimilation reductase (nirA) genes. Notably, none of the *N. defluvii*-like bins possessed ammonia oxidation relative genes (i.e. amoA, amoB, amoC and hao), which indicates that they were representative NOB. In addition, 16S rRNA gene amplicon sequencing results (Fig. 1B) showed that *Nitrospira* spp. were the dominant bacteria in SFs, which was also observed in a previous study (Palomo et al., 2016). Therefore, the *N. defluvii*-like bins might play an important role in the nitrogen cycle of SFs.

**Isolation and identification of aerobic denitrifiers**

Based on the results of metagenomic analysis, microbes in SFs were involved in denitrification. To our knowledge, aerobic denitrification is more likely to occur than anaerobic denitrification in SF ecosystem, because of continuous water intake under the open space. In addition, in view of the fact that aerobic denitrification has a higher denitrification rate than anaerobic denitrification and is easier to operate. Thus, we considered to enrich and then isolate aerobic denitrifiers to purify nitrogen-contaminated drinking water sources in practical application. As shown in Supplementary Fig. S4, following enrichment, six colonies that could utilise NO₃⁻N under aerobic conditions were isolated and defined as DW-1, DW-2, DW-3, DW-4, DW-5 and DW-6, respectively. Strain DW-5, which had the highest removal rate of NO₃⁻N, was chosen for further study. According to the 16S rRNA gene sequences of strain DW-5, we constructed a neighbour-joining phylogenetic tree with other highly similar bacteria, showing that strain DW-5 was affiliated with the *Pseudomonas* species (Supplementary Fig. S5), and strain DW-5 had 99.64% sequence similarity with the 16S rRNA of *Pseudomonas* sp. strain HZ57. To determine the strain DW-5 was an aerobic denitrifier, we amplified the gene napA that encodes periplasmic nitrate reductase, and gained an 806-bp fragment of PCR products (Supplementary Fig. S6, Table 2).

**Assessment of nitrogen removal by *Pseudomonas* sp. DW-5**

In order to evaluate nitrogen removal performances by *Pseudomonas* sp. DW-5, two different concentrations of nitrogen were set to investigate the potential application of this strain. In particular, the nitrogen level in the oligotrophic water environment was considered. In the heterotrophic nitrification process (Fig. 8A and C), after a rapid ammonia degradation period of 24 h, strain DW-5 showed a rapid proliferation (Fig. 8A). The concentration of NO₃⁻N and NO₂⁻N and other nitrification by-products, NO₃⁻N reached the highest level, but they were still accumulated in small amounts. The possible reason is that with the continuous proliferation of bacteria, the isolate entered the stable period at 24 h, the more NH₄⁺-N was nitrified to NO₃⁻-N and NO₂⁻-N and other nitrification by-products, instead of being used for biosynthesis required by the microorganisms. Another possible reason is that the carbon source was insufficient at this time, which also affect the denitrification rate. The similar phenomenon was also observed in previous study (Lang et al. 2020). However, the N299 strain demonstrated some heterotrophic nitrification ability without NH₄⁺-N and NO₂⁻-N accumulation. Additionally, some aerobic denitrifiers even could not exhibit ammonia oxidation, such as *Pseudomonas stutzeri* C3 (Ji et al. 2015). Additionally, the NH₄⁺-N removal also mainly carried out from 0 to 24 h, with a removal rate of 86%, and the average degradation rate was 3.6 mg/L/h. When compared with other
Fig. 6 Gene circles of three reference genomes of *Candidatus Nitrospira inopinata* (CNI), *Candidatus Nitrospira nitricans* (CNNf), *Candidatus Nitrospira nitrosa* (CNNs) and open reading frames (ORFs) of the non-redundant gene set of sand filter SF2 (A) and SF3 (B) obtained in the present study. The genomes of CNI, CNNf and CNNs were selected as references presented in the third ring. The innermost ring shows the number of ORFs of SFs matched to three reference genomes using gene structure partition window calculation (log processing). The second ring shows the total abundance of ORFs of SFs matched to three reference genomes using gene structure partition window calculation (log processing).
reported strains, the ammonium removal rate by strain DW-5 was higher than *Exiguobacterium mexicanum* SND-01 (2.24 mg/L/h) (Medhi et al. 2017), *Vibrio diabolicus* SF16 (2.29 mg/L/h) (Duan et al. 2015), *Ideonella* sp. TH17 (1.4 mg/L/h) (Zhang et al. 2020b) and *Pseudomonas tolaasii* Y-11 (2.04 mg/L/h) (He et al. 2016). After 60 h, the removal rate reached 94%. Similarly, as shown in Fig. 8C, when strain DW-5 began to proliferate rapidly, the concentration of NH$_4$-N decreased rapidly. After 24 h, the removal rate reached 64%. After 60 h, the removal rate reached 89%.

Generally, these results indicate that strain DW-5 has a good heterotrophic nitrification ability. In addition, aerobic denitrification was also determined by using nitrate as the sole nitrogen source (Fig. 8B and D). Compared with the case when NH$_4$-N was the sole nitrogen source, the time taken for the strain to enter the stable growth period on NO$_3$-N as a sole carbon source was longer (36 h). Meanwhile, the growth rate of strain DW-5 was lower than that when NH$_4$-N was the sole nitrogen source. As shown in Fig. 8B, after a rapid growth period, strain DW-5 rapidly entered...
into a stable period at 36 h. During the rapid growth period, the concentration of NO\textsubscript{2}-N decreased to 14 mg/L, and the removal rate reached 86%. After 36 h, when strain DW-5 entered the stable phase, the removal rate of NO\textsubscript{2}-N was extremely low. As shown in Fig. 8D, with the continuous growth of strain DW-5, the concentration of NO\textsubscript{2}-N decreased significantly; after 36 h, the removal rate reached 76%, and the average degradation rate was 0.21 mg/L/h. Moreover, before 24 h, only a small fraction of the nitrate was converted to nitrite; the highest concentration of NO\textsubscript{2}-N reached was 0.5 mg/L, following which it disappeared quickly, which was consistent to that of Paracoccus denitrificans Z195 (Zhang et al. 2020a), but contrasted with that of Paracoccus denitrificans ISTOD1 without nitrite accumulation (Medhi et al. 2017). Similarly, columns inoculated with strain DW-5 exhibited good performance in NH\textsubscript{4}-N or NO\textsubscript{2}-N removal (Supplementary Fig. S7). At a NH\textsubscript{4}-N concentration of 1 mg/L, it could remove 92% after sand filters operated 24 h, and 99% after 48 h. In addition, when NO\textsubscript{2}-N concentration was 1 mg/L, columns inoculated with strain DW-5 could remove 88% after sand filters operated 24 h, and 93% after 48 h. NH\textsubscript{4}-N removal rates of the treatment group were significantly higher \((P<0.05, T\text{-test})\) than that of control group at 48 h. Additionally, NO\textsubscript{2}-N removal rate of the treatment group was significantly higher \((P<0.05, T\text{-test})\) than that of control group at both 24 h and 48 h. Therefore, it was observed that strain DW-5 could perform well in heterotrophic nitrification and aerobic denitrification in actual water body, and it has potential to use as bioenhanced bacteria to apply in actual water treatment for nitrogen removal.

**Genetic potential of the microbial community in SFs for nitrogen metabolism**

On the basis of metabolic reconstruction of representative MAGs that have been confirmed their role in nitrogen metabolism and the aerobic denitrifier isolated from SFs, a model of microbial nitrogen metabolism is shown in Fig. 9. Nitrogen is vital for the growth of organisms and, thus, plays an important role in aquatic ecosystems (Zhang et al. 2019). Nitrification is a critical and limiting step of nitrogen metabolism (Campos et al. 2001), due to the relatively low growth rate of the nitrifiers. Furthermore, nitrifying microorganisms are easily inhibited by various environmental factors, such as pH, temperature and toxic compounds (Farazaki & Gikas 2019). Previous studies have shown that *Nitrosomonas* and *Nitrospira* species are the major ammonia and nitrite oxidisers, respectively (Potgieter et al. 2020), which is consistent with our findings. Ammonium in the SFs was oxidised to nitrite by the typical autotrophic AOB *Nitrosomonas*-like MAG (SF2 bin.62), and the nitrite produced by SF2 bin.62 was then utilised by *Nitrospira defluvii*-like SF2 bin.188, *Nitrospira defluvii*-like SF2 bin.132, *Nitrospira defluvii*-like SF3 bin.25 and *Pseudomonas*-like MAG SF2 bin.8. Meanwhile, 16S rRNA amplicon sequencing results from our study (Fig. 1B) and those from a previous study showed that both *Nitrospira* and *Nitrosomonas* were predominant in SFs (Ma et al. 2020a). In addition, we showed that there were some comammox in the SFs that could directly oxidise nitrate to ammonium. However, denitrification mediated by microorganisms is another critical step in the nitrogen-cycling process as nitrate can be reduced by three distinct classes of nitrate-reducing systems (Moreno-Vivian et al. 1999). In our study, we observed that there were two nitrate-reducing systems (Nas and Nap) in microorganisms. For example, *Streptomyces*-like MAG (SF2 bin.167) and *Rhizobiales*-like MAG (SF3 bin.34) harboured the nasA gene, and *Pseudomonas* sp. DW-5 harboured the napA gene. Notably, in our study, approximately 74% of the total MAGs included the nasA gene in our study. Thus, nitrate assimilation reductase shares rich diversity and abundance. Subsequently, nitrite may require a reduction reaction to ammonia or nitric oxide (Li et al. 2021). Microorganisms involved in transforming nitrite to ammonia include *Bradyrhizobium*-like MAG (SF2 bin.175 and SF3 bin.90) and *N. defluvii*-like MAG (SF2 bin.188, and bin.132; SF3 bin.25). Moreover, *Nitrosomonas*-like MAG (SF2 bin.62) and *Nitrospira*-like MAG (SF2 bin.132 and SF3 bin.113) are involved in transforming nitrate to nitric oxide. Furthermore, nitric oxide was subsequently reduced to nitrous oxide by *Nitrospira*-like MAG (SF2 bin.62) and *Cupriavidus*-like MAG (SF3 bin.77). From the above model, we observe that the nitrogen-metabolising microorganisms in SFs were integrated.

Metagenomic analysis has provided a comprehensive insight into the potential nitrogen metabolism capabilities of microbial communities in drinking water biofilters. Metagenomic binning allowed us to predict the dominant nitrogen metabolism microorganisms in SFs. Read mapping to the reference genomes of comammox *Nitrospira* (Fig. 6) and the phylogenetic tree of *amoA* (Supplementary Fig. S2) showed that there were highly abundant comammox in the SFs. Furthermore, combined with pure culture techniques, we obtained the aerobic denitrifier *Pseudomonas* sp. strain DW-5, which allowed us to provide insights into the nitrogen metabolism capabilities of indigenous microorganisms in oligotrophic water environments where the nitrogen concentration is always low.

**Conclusion and prospect**

In summary, PCR amplification and quantitative analysis of *amoA* genes showed that AOA, AOB and comammox organisms were widely distributed in SFs of DWTPs, and comammox outnumbered AOA and AOB. Metagenomic analysis showed that a total of 85 high-quality MAGs were recovered from two SFs, which were found to be involved in nitrification, assimilatory nitrogen reduction, denitrification and anammox via genetic information. Meanwhile, according to further analysis, we found that comammox *Nitrospira* and NOB *Nitrospira* were also existing in the SFs. Furthermore, a strain DW-5 with high ammonia and nitrate removal ability was isolated from SFs, which was a promising
Fig. 9 Schematic overview of the representative genes and metagenome-assembled genomes (MAGs) involved in the major reactions of the nitrogen cycle in sand filters. DWTP, drinking water treatment plant.
candidate for treating trace amounts of nitrogen pollution from drinking water sources. Taken together, there are abundant nitrogen-metabolising microorganism SFs. This study improves our understanding of the nitrogen-cycling potential of SFs and provides insights into the genetic network underlying microbiologically mediated nitrogen metabolism in SFs. Additionally, this study provides a method for the targeted discovery of functional microbes in SFs. However, there is room for further improvement in our work; for example, this data is limited to three DWTPs in southern China and one season. Moreover, microbial activity and the expression of functional genes on nitrogen metabolism were not all-inclusive. Thus, the microorganisms responsible for the nitrogen cycle in SFs need to be identified in other geographical regions and different seasons to validate our findings. Comammox and canonical ammonia-oxidising microorganisms involved in the nitrification process should be further investigated, particularly focusing in the comparison of the expression of genes related to nitrification between comammox and canonical nitrifiers. In addition, the ecological characteristics of comammox and their contribution to the nitrogen cycle in DWTPs need to be studied.

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**Author contribution** Qingping Wu and Qihui Gu conceived and designed the experiments; Qihui Gu and Ming Sun performed the experiments; Qihui Gu, Ju Ma, Youxiang Zhang and Weixian Hu analysed the data; Qingping Wu, Jumei Zhang, Yu Ding, Juan Wang, Weipeng Guo and Huiqing Wu contributed reagents/materials/analysis tools; Qihui Gu wrote the paper; Moutong Chen, Liang Xue and Qihui Gu reviewed the manuscript.

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**Data availability** All data generated or analysed during this study are included in this published article.

**Declarations**

**Ethics approval and consent to participate** Not applicable.

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