Nitrogen-metabolising Microorganism Analysis in Rapid Sand Filters From Drinking Water Treatment Plant by Culturing and Metagenomics

Qihui Gu

GDIM: Guangdong Institute of Microbiology

Jun Ma

Shaanxi University of Science and Technology

Jumei Zhang

Guangdong Institute of Microbiology

Weipeng Guo

Guangdong Institute of Microbiology

Huiqing Wu

Guangdong Institute of Microbiology

Ming Sun

Guangdong Institute of Microbiology

Juan Wang

South China Agricultural University

Xianhu Wei

Guangdong Institute of Microbiology

Youxiong Zhang

Guangdong Institute of Microbiology

Montong Chen

Guangdong Institute of Microbiology

Liang Xue

Guangdong Institute of Microbiology

Yu Ding

Jinan University

Qingping Wu (✉ wuqp203@163.com)

Guangdong Institute of Microbiology

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Abstract

Sand filter (SFs) are common treatment processes for nitrogen pollutants 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 Proteobacteria, 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, and denitrification 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 isolates. Quantitative real-time polymerase chain reaction (PCR) 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., 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.

1. Introduction

Conventional water treatment process is a very common and effective treatment method in water supply treatment, and the drinking water treatment plants (DWTPs) in China that using this process is 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 universality and seriousness (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. 2020, 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-oxidizing bacteria (AOB), and then the nitrite-oxidizing bacteria (NOB) is used to achieve the transition from nitrite to nitrate (Hirosugu et al. 2015). Nevertheless, our cognition about nitrification has

been changed by the recently discovered complete ammonia oxidizer (comammox)(Van Kessel et al. 2015). The first pure culture of a comammox bacterium is *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. 2016b, 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 is 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 abundance and distribution of AOA, AOB and comammox were investigated using quantitative real-time polymerase chain reaction (PCR). 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-metabolizing microbes in the SFs of DWTPs in China.

2. Material And Methods

2.1 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. All of the DWTPs used surface water as their source water. The water treatment processes in these DWTPs were the same, including the following steps: coagulation, sedimentation, filtration, and chlorine disinfection. 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 were 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. Samples SF2 and SF3 followed a similar convention. In addition, influent water samples and the corresponding effluent water samples from these three sand filters were also periodically 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 ($\text{NH}_4\text{-N}$), nitrite-nitrogen ($\text{NO}_2\text{-N}$) and nitrate-nitrogen ($\text{NO}_3\text{-N}$) were measured based on drinking water standard examination methods (GB/T 5750-2006; China). Total DNA was extracted from these sand samples using the Fast DNA® Spin Kit for Soil (MP Biomedicals, Carlsbad, CA, USA). Microbial community composition was characterized 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 SRR14621795-14621803.

2.2 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 Scaffigs, 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 2,500 bp were extracted from the co-assembly of all samples and used to generate metagenome bins using METABAT (Kang et al. 2019). 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 phylogenomic 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 *nxrAB*), 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 nitrificans* (CNNf), 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 = 1×10^{-5} 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 monooxygenase gene subunit A, *amoA*, was extracted from the NT library and compared using BLASTN with the comparison conditions set as e-value = 0.00001 - max_target_seqs 1.

2.3 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 (pmoA-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).

2.4 Quantification of AOA, AOB and comammox *amoA* genes

In order to investigate the abundance of AOA, AOB, and comammox *amoA* genes, quantitative real-time PCR was conducted. 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 2.3, and the PCR conditions were performed as previously described(Bartelme et al. 2017).

2.5 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: $C_4H_4Na_2O_4$, 0.5 g/L; $NaNO_3$, 0.1 g/L; $K_2HPO_4 \cdot 3H_2O$, 0.1 g/L; $CaCl_2$, 0.05 g/L; $MgCl_2 \cdot 6H_2O$, 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 were 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 were screened and isolated.

2.6 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). The *napA* gene was amplified using the primers, NAP1 and NAP2 (Kong et al. 2006). PCR amplification was performed as previously described (Huang et al. 2015). PCR products were sequenced by Liuhe Huada.

2.7 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 ($\text{NH}_4\text{-N}$) in HNM and nitrate-nitrogen ($\text{NO}_3\text{-N}$) in DM were respectively set to two different concentrations, 10 mg/L and 100 mg/L, and the final pH was adjusted to 7.0-7.2. During cultivation, flask cultures were sampled every 12 h to measure optical density at 600 nm, as well as the concentrations of $\text{NH}_4\text{-N}$, nitrite nitrogen ($\text{NO}_2\text{-N}$), and $\text{NO}_3\text{-N}$ 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 (NH_4^+ and NO_3^-) detection after the sand filters performed for 24 h and 48 h. Triplicate water samples were collected.

3. Results And Discussion

2.1. 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 changed a lot. 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 that the occurrence of nitrification. 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. Therefore, the sand filters in these DWTPs could be responsible for nitrogen removal.

2.2. Diversity and composition of bacterial communities in SFs

As shown in Fig. 2A, the main bacterial groups identified in the SFs were Proteobacteria (21.57–48.27%), Acidobacteria (4.70–47.83%), Nitrospirae (7.64–25.70%), and Chloroflexi (4.36–11.86%) at the phylum level. The results are in line with previous study showing that Proteobacteria and Nitrospirae are dominant in SFs(Palomo et al. 2016b). However, 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 Proteobacteria (from 20.77–80%), Acidobacteria (from 5.03–46.34%), Cyanobacteria (from 1–39.89%), and Bacteroidetes(from 6.54–22.73%)(Zhou et al. 2020). Additionally, the microbial composition in the surrounding rock soil was reported to comprise of Proteobacteria (42.90%), Actinobacteria (23.60%), Bacteroidetes (10.50%), and Gemmatimonadetes (7.82%)(Jiang et al. 2020). At the genus level (Fig. 2B and Supplementary Fig.S1), the predominant taxa were *Nitrospira* (24.33%), *norank_c_bacteriap* 25 (4.72%), *norank_f_A0839* (4.69%), *unclassified_o_Burkholderiales* (3.05%), and *norank_f_Caldilineaceae* (2.82%). 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. 2016a). 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. 2016a). Moreover, the ammonium-oxidizing bacteria (AOB) *Nitrosomonas*, were ubiquitously present in all three SFs and accounted for 1.81% of the microbial population. In particular, the relative abundance of *Nitrosomonas* in SF3 was significantly higher than that in SF1 and SF2. Taken together, our results suggest that SFs have great potential for nitrogen transformation.

2.3. PCR analysis of *amoA*

To study the ammonia-oxidising microorganisms in SFs, *amoA* of AOA, AOB, and comammox was amplified (Fig. 3). The sequence comparison 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(Table 1). In addition, sequences with sizes of approximately 635bp 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 99%

similar to that of uncultured comammox *amoA*, respectively. Therefore, our results clearly show that AOA, AOB, and comammox were ubiquitously present in SFs, which indicates that SFs have a great potential to remove ammonia.

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 <i>amoA</i>	SF1	463	Uncultured Nitrosomonadales bacterium clone PYL-FH-13 <i>amoA</i> gene (KJ137864)	93
	SF2	465	Uncultured beta proteobacterium clone SEP-I-B-10 <i>amoA</i> gene (KJ158669)	96
	SF3	439	Uncultured AOB clone N-G1-AOB-05 <i>amoA</i> gene (KM882239)	87
AOA <i>amoA</i>	SF1	609	Uncultured AOA clone N-G3-AOA-02 <i>amoA</i> gene (KM881831)	98
	SF2	606	Uncultured AOA clone J40104213_23_A_4_M13F <i>amoA</i> gene (KJ497144)	95
	SF3	605	Uncultured AOA clone N-G1-AOA-35 <i>amoA</i> gene (KM881800)	97
Comammox <i>amoA</i>	SF1	502	Uncultured Nitrospira sp. clone com-cladeA-OTU345 <i>amoA</i> gene (MT790412)	99
	SF2	492	Uncultured Nitrospira sp. clone com-cladeA-OTU310 <i>amoA</i> gene (MT790393.1)	93
	SF3	516	Uncultured Nitrospira sp. clone COM-Ri-LW-46 <i>amoA</i> gene (MG591541.1)	99

2.4. Quantitative analysis of *amoA* genes in SFs

As shown in Fig. 4, the abundance of AOA, AOB, and comammox *amoA* genes in SF2 was 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 (Tatari et al. 2017). This results indicated that comammox in SFs might play a key role in nitrogen metabolism. This might be related to the natural acclimation of SF2 microbes at a low concentration of ammonia for approximately 10 years, while SF1 and SF3 were operational for 7 years. 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 (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). Our results show that comammox and AOA are mainly responsible for the oxidation of ammonia, and their contribution cannot be ignored in the SF

niche. In order to comprehensively study nitrogen metabolism mechanisms in SFs, SF2 and SF3, which presented a higher abundance of *amoA* than SF1, were chosen for metagenomic sequencing.

2.5. 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 S5 and S6. In the 85 MAGs, the phylum Proteobacteria (n = 67) dominated the microbial community in both SFs, followed by Planctomycetes (n = 11). Additionally, 16S rRNA gene amplicon sequencing results were found to be consistent with the metagenomic binning results, which indicates that Proteobacteria was the predominant phylum in microbial community in SFs. A previous study showed that the Proteobacteria phylum was correlated with $\text{NH}_4\text{-N}$ removal and nitrification efficiency (Feng et al. 2019). Moreover, planctomycetes are also shown to play key roles in nitrogen cycles (Kallscheuer et al. 2019). Thus, the Proteobacteria and Planctomycetes phyla might play important roles in nitrogen metabolism. Furthermore, 14 genes associated with nitrogen metabolism are shown in Fig. 4, including genes involved in nitrification (i.e., *amoABC*, *hao*, and *nxrAB*), assimilatory nitrogen reduction (i.e., *nasA*, *nirA*, and *nirB*), and denitrification (i.e., *nirK* and *norBCDQ*). As shown in Fig. 4, 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*. *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. 2020, Ma et al. 2020b, Ma et al. 2020c, Song et al. 2020, Yuan et al. 2020). Furthermore, 13 MAGs that included the hydroxylamine oxidoreductase (*hao*) gene, and SF2 bin.117, identified as *Bradyrhizobium*-like MAG, included the *hao* gene. Additionally, 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.

2.6. Presence of comammox *Nitrospira*

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 S3), followed by CNNf and CNI (Fig. 6). In addition, the gene sequences of SF2 and SF3 matched the three subunits of ammonia monooxygenase (*amoA* subunit A, B, and C) of comammox *amoA* for CNI, CNNf, and CNNs, respectively (Supplementary Fig.S2). Therefore, we can speculate that there might be comammox *Nitrospira* in the SFs of the DWTPs.

2.7. Presence of NOB

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 S4. 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 harboured the nitrite oxidoreductase (*nxrB*) 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. 2016a). Therefore, the *N. defluvii*-like bins might play an important role in the nitrogen cycle of SFs.

2.8. Isolation and identification of aerobic denitrifiers

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 (Supplementary Fig.S3). 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.S4), 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.S5, Table 2).

Table 2
BLAST results of the *napA* sequences in the strain DW-5

Primer	Amplicon size(bp)	Nucleotide blast results	Similarity (%)
<i>napA</i>	806	<i>Pseudomonas mendocina</i> strain LerA1 <i>napA</i> gene (MK217475)	89.79

2.9. 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 degradation period of 24 h, strain DW-5 showed a rapid proliferation

(Fig. 8A). After approximately 36 h, strain DW-5 got to the stable phase; meanwhile, the concentration of $\text{NO}_3\text{-N}$ reached the highest level, and the $\text{NH}_4\text{-N}$ removal mainly carried out from 0 to 36 h, with a removal rate of 76%. Additionally, only a very small amount of $\text{NO}_3\text{-N}$ and $\text{NO}_2\text{-N}$ accumulated in this process. This phenomenon indicates that strain DW-5 might have heterotrophic nitrification ability. Similarly, as shown in Fig. 8C, when strain DW-5 began to proliferate rapidly, the concentration of $\text{NH}_4\text{-N}$ decreased rapidly. After 24 h, the removal rate reached 86%, and the average degradation rate was 3.5 mg/L/h. After 60 h, the removal rate reached 94%. This indicates 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 $\text{NH}_4\text{-N}$ was the sole nitrogen source, the time taken for the strain to enter the stable growth period on $\text{NO}_3\text{-N}$ as a sole carbon source was longer (24 h and 36 h, respectively). Meanwhile, the growth rate of strain DW-5 was lower than that when $\text{NH}_4^+\text{-N}$ was the sole nitrogen source. Thus, there was a positive correlation between removal rate and growth rate. As shown in Fig. 8B, with the continuous growth of strain DW-5, the concentration of $\text{NO}_3\text{-N}$ 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 $\text{NO}_2\text{-N}$ reached was 0.5 mg/L, following which it disappeared quickly. As shown in Fig. 8D, 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 $\text{NO}_3\text{-N}$ decreased to 14 mg/L, and the removal rate reached 86%. After 36 h, when strain DW-5 entered the decline phase, the removal rate of $\text{NO}_3\text{-N}$ was extremely low. Similarly, columns inoculated with strain DW-5 exhibited good performance in $\text{NH}_4\text{-N}$ or $\text{NO}_3\text{-N}$ removal (Supplementary Fig.S6). At a $\text{NH}_4\text{-N}$ concentration of 1 mg/L, could remove 92 % after sand filters operated 24 h, and 99 % after 48 h. In addition, when $\text{NO}_3\text{-N}$ concentration was 1mg/L, columns inoculated with strain DW-5 could remove 88% after sand filters operated 24 h, and 93 % after 48 h. Removal rates of the treatment group were significantly higher than that of control group. 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.

2.10. Genetic potential of the microbial community in SFs for nitrogen metabolism

On the basis of metabolic reconstruction of MAGs 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(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 were 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 defluvi*-like SF2 bin.132, *Nitrospira defluvi*-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. *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.defluvi*-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 nitrite to nitric oxide. Furthermore, nitric oxide was subsequently reduced to nitrous oxide by *Nitrosomonas*-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 have 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.

4. Conclusion

In summary, 85 high-quality MAGs were discovered in two SFs of DWTP in China. All of these genes were found to be involved in nitrogen metabolism, including nitrification (i.e., *amoABC*, *hao*, and *nxrAB*), assimilatory nitrogen reduction (i.e. *nasA*, *nirA*, and *nirB*), and denitrification (i.e. *nirK* and *norBCDQ*), via genetic information. Surveyed metagenomes showed that comammox *Nitrospira* existed in the SFs, and fluorogenic quantitative PCR further demonstrated that the comammox organisms were widely distributed and outnumbered AOA and AOB in SFs of DWTPs. However, 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. Furthermore, the strong adaptability of strain DW-5 to an oligotrophic environment makes it a promising candidate for treating trace amounts of nitrogen pollution from natural regions; thus, in this work, we identified more indigenous functional microorganisms, particularly nitrogen removal microbes, which could be recovered from SFs because of its high ammonia-nitrogen removal effect in practical production. This study improves our understanding of the nitrogen-cycling potential of SFs and provides insights into the genetic network underlying microbially-mediated nitrogen metabolism in SFs. Additionally, this study provides a method for the targeted discovery of functional microbes in SFs.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare no competing interests.

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Authors' contributions

Qingping Wu and Qihui Gu conceived and designed the experiments; Qihui Gu and Ming Sun performed the experiments; Qihui Gu, Ju Ma, Youxiong Zhang, and Weixian Hu analyzed the data; Qingping Wu, Jumei Zhang, Yu Ding, Juan Wang, Weipeng Guo, 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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References

Association AWW (2012): Water treatment plant design. McGraw Hill Professional

Bai Y, Liu R, Liang J, Qu J (2013): Integrated Metagenomic and Physiochemical Analyses to Evaluate the Potential Role of Microbes in the Sand Filter of a Drinking Water Treatment System. Plos One 8, e61011

Bartelme RP, McLellan SL, Newton RJ (2017): Freshwater Recirculating Aquaculture System Operations Drive Biofilter Bacterial Community Shifts around a Stable Nitrifying Consortium of Ammonia-Oxidizing Archaea and Comammox Nitrospira. Frontiers In Microbiology 8

Bertelli C, Courtois S, Rosikiewicz M, Piriou P, Aeby S, Robert S, Loret J-F, Greub G (2018): Reduced Chlorine in Drinking Water Distribution Systems Impacts Bacterial Biodiversity in Biofilms. Frontiers In Microbiology 9

Bettez ND, Groffman PM (2013): Nitrogen Deposition in and near an Urban Ecosystem. Environmental Science & Technology 47, 6047-6051

Bolger AM, Lohse M, Usadel B (2014): Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114-2120

Campos JL, Garrido JM, Mendez R, Lema JM (2001): Effect of two broad-spectrum antibiotics on activity and stability of continuous nitrifying system. Applied Biochemistry And Biotechnology 95, 1-10

Chen JC-y, Tyler AD (2020): Systematic evaluation of supervised machine learning for sample origin prediction using metagenomic sequencing data. Biology Direct 15

Chen L, Zhai Y, van der Mark E, Liu G, van der Meer W, Medema G (2021): Microbial community assembly and metabolic function in top layers of slow sand filters for drinking water production. Journal Of Cleaner Production 294

Farazaki M, Gikas P (2019): Nitrification-denitrification of municipal wastewater without recirculation, using encapsulated microorganisms. Journal Of Environmental Management 242, 258-265

Feng S, Zhang XJ, Chen C, Yang ZY (2012): Research Status and Prospect of GAC-sand Dual Media Filters Used in Drinking Water Treatment. China Water & Wastewater 28, 16-19

Feng Z, Sun Y, Li T, Meng F, Wu G (2019): Operational pattern affects nitritation, microbial community and quorum sensing in nitrifying wastewater treatment systems. Science Of the Total Environment 677, 456-465

Fowler SJ, Palomo A, Dechesne A, Mines PD, Smets BF (2017): Comammox Nitrospira are abundant ammonia oxidizers in diverse groundwater-fed rapid sand filter communities. Environmental Microbiology

- Fu Q, Zheng BH, Zhao XR, Wang LJ, Liu CM (2012): Ammonia pollution characteristics of centralized drinking water sources in China. *Journal Of Environmental Sciences* 24, 1739-1743
- Gao S, Zhou G, Rees RM, Cao W (2020): Green manuring inhibits nitrification in a typical paddy soil by changing the contributions of ammonia-oxidizing archaea and bacteria. *Applied Soil Ecology* 156
- Gu Q, Wu Q, Zhang J, Guo W, Wu H, Sun M (2016): Community Analysis and Recovery of Phenol-degrading Bacteria from Drinking Water Biofilters. *Frontiers In Microbiology* 7
- Hink L, Gubry-Rangin C, Nicol GW, Prosser JI (2018): The consequences of niche and physiological differentiation of archaeal and bacterial ammonia oxidisers for nitrous oxide emissions. *Isme Journal* 12, 1084-1093
- Hirotsugu F, Asami K, Norisuke U, Kengo M, Satoshi T (2015): Selective isolation of ammonia-oxidizing bacteria from autotrophic nitrifying granules by applying cell-sorting and sub-culturing of microcolonies. *Frontiers in microbiology* 6, 1159
- Hu J, Zhao Y, Yang W, Wang J, Liu H, Zheng P, Hu B (2020a): Surface ammonium loading rate shifts ammonia-oxidizing communities in surface water-fed rapid sand filters. *Fems Microbiology Ecology* 96
- Hu WC, Liang JS, Ju F, Wang QJ, Liu RP, Bai YH, Liu HJ, Qu JH (2020b): Metagenomics Unravels Differential Microbiome Composition and Metabolic Potential in Rapid Sand Filters Purifying Surface Water Versus Groundwater. *Environmental Science & Technology* 54, 5197-5206
- Huang T-L, Zhou S-L, Zhang H-H, Bai S-Y, He X-X, Yang X (2015): Nitrogen Removal Characteristics of a Newly Isolated Indigenous Aerobic Denitrifier from Oligotrophic Drinking Water Reservoir, *Zoogloea* sp N299. *International Journal Of Molecular Sciences* 16, 10038-10060
- Huang XJ, Chong Y, Jiu-Pai NI, Zhen-Lun LI (2016): Isolation, Identification and Characteristics of a *Rhodopseudomonas* with High Ammonia-nitrogen Removal Efficiency. *Environmental Science*
- Hyatt D, Chen G-L, LoCascio PF, Land ML, Larimer FW, Hauser LJ (2010): Prodigal: prokaryotic gene recognition and translation initiation site identification. *Bmc Bioinformatics* 11
- Jiang B, Zhang B, Li L, Zhao Y, Shi Y, Jiang Q, Jia L (2020): Analysis of microbial community structure and diversity in surrounding rock soil of different waste dump sites in fushun western opencast mine. *Chemosphere*, 128777-128777
- Joo HS, Hirai M, Shoda M (2005): Characteristics of ammonium removal by heterotrophic nitrification-aerobic denitrification by *Alcaligenes faecalis* no. 4. *Journal Of Bioscience And Bioengineering* 100, 184-191
- Kallscheuer N, Moreira C, Airs R, Llewellyn CA, Wiegand S, Jogler C, Lage OM (2019): Pink- and orange-pigmented Planctomycetes produce saproxanthin-type carotenoids including a rare C-45 carotenoid.

Kang DD, Li F, Kirton E, Thomas A, Egan R, An H, Wang Z (2019): MetaBAT 2: an adaptive binning algorithm for robust and efficient genome reconstruction from metagenome assemblies. *Peerj* 7

Kits KD, Sedlacek CJ, Lebedeva EV, Han P, Bulaev A, Pjevac P, Daebeler A, Romano S, Albertsen M, Stein LY, Daims H, Wagner M (2017): Kinetic analysis of a complete nitrifier reveals an oligotrophic lifestyle. *Nature* 549, 269-+

Kong Q-X, Wang X-W, Jin M, Shen Z-Q, Li J-W (2006): Development and application of a novel and effective screening method for aerobic denitrifying bacteria. *Fems Microbiology Letters* 260, 150-155

Lang X, Li Q, Ji M, Yan G, Guo S (2020): Isolation and niche characteristics in simultaneous nitrification and denitrification application of an aerobic denitrifier, *Acinetobacter* sp. YS2. *Bioresource Technology* 302

Lee CO, Boe-Hansen R, Musovic S, Smets B, Albrechtsen H-J, Binning P (2014): Effects of dynamic operating conditions on nitrification in biological rapid sand filters for drinking water treatment. *Water Research* 64, 226-236

Lee MD (2019): GToTree: a user-friendly workflow for phylogenomics. *Bioinformatics* 35, 4162-4164

Li W, Shi C, Yu Y, Ruan Y, Kong D, Lv X, Xu P, Awasthi MK, Dong M (2021): Interrelationships between tetracyclines and nitrogen cycling processes mediated by microorganisms: A review. *Bioresource technology* 319, 124036-124036

Lin J, Chen N, Yuan X, Tian Q, Hu A, Zheng Y (2020): Impacts of human disturbance on the biogeochemical nitrogen cycle in a subtropical river system revealed by nitrifier and denitrifier genes. *Science Of the Total Environment* 746

Ma B, LaPara TM, Hozalski RM (2020a): Microbiome of Drinking Water Biofilters is Influenced by Environmental Factors and Engineering Decisions but has Little Influence on the Microbiome of the Filtrate. *Environmental Science & Technology* 54, 11526-11535

Ma Y, Du X, Liu Y, Zhang T, Wang Y, Zhang S (2020b): Characterization of the bacterial communities associated with biofilters in two full-scale recirculating aquaculture systems. *Journal Of Oceanology And Limnology*

Ma Y, Yu Z, Du X, Zhang T, Wang N, Tao W (2020c): Characterization of Bacterial Community, Ammonia-Oxidizing Bacteria, and *Nitrospira* During the Operation of a Commercial-Scale Recirculating Aquaculture System for Culturing Pufferfish *Takifugu rubripes*. *Journal Of Ocean University Of China* 19, 1399-1408

Ming H, Zhang H, Chen Q, Wang Y, Su J, Zhao X, Fan J (2020): Abundance and community structure of ammonium monooxygenase (*amoA*) genes in the wet season of liaohhe estuary sediments. *Continental*

Moreno-Vivian C, Cabello P, Martinez-Luque M, Blasco R, Castillo F (1999): Prokaryotic nitrate reduction: Molecular properties and functional distinction among bacterial nitrate reductases. *J. Bacteriol.* 181, 6573-6584

Niu J, Kasuga I, Kurisu F, Furumai H (2019): Growth competition between ammonia-oxidizing archaea and bacteria for ammonium and urea in a biological activated carbon filter used for drinking water treatment. *Environmental Science-Water Research & Technology* 5, 231-238

Oh S, Hammes F, Liu WT (2017): Metagenomic characterization of biofilter microbial communities in a full-scale drinking water treatment plant. *Water Research* 128, 278

Palomo A, Fowler SJ, Gulay A, Rasmussen S, Sicheritz-Ponten T, Smets BF (2016a): Metagenomic analysis of rapid gravity sand filter microbial communities suggests novel physiology of *Nitrospira* spp. *Isme Journal* 10, 2569-2581

Palomo A, Jane FS, Gálvez A, Rasmussen S, Sicheritz-Ponten T, Smets BF (2016b): Metagenomic analysis of rapid gravity sand filter microbial communities suggests novel physiology of *Nitrospira* spp. *Isme Journal* 10, 2569-2581

Parks DH, Imelfort M, Skennerton CT, Hugenholtz P, Tyson GW (2015): CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Research* 25, 1043-1055

Pinto AJ, Marcus DN, Ijaz UZ, Dick GJ, Raskin L (2016a): Metagenomic Evidence for the Presence of Comammox *Nitrospira*-Like Bacteria in a Drinking Water System. *Mosphere* 1

Pinto AJ, Marcus DN, Ijaz UZ, Santos QMB-dl, Dick GJ, Raskin L (2016b): Metagenomic Evidence for the Presence of Comammox *Nitrospira*-Like Bacteria in a Drinking Water System. *Mosphere* 1

Poghosyan L, Koch H, Frank J, van Kessel MAHJ, Cremers G, van Alen T, Jetten MSM, Op den Camp HJM, Lucker S (2020): Metagenomic profiling of ammonia- and methane-oxidizing microorganisms in two sequential rapid sand filters. *Water Research* 185

Potgieter SC, Dai Z, Venter SN, Sigudu M, Pinto AJ (2020): Microbial Nitrogen Metabolism in Chloraminated Drinking Water Reservoirs. *Mosphere* 5

Prodan A, Tremaroli V, Brolin H, Zwinderman AH, Nieuwdorp M, Levin E (2020): Comparing bioinformatic pipelines for microbial 16S rRNA amplicon sequencing. *Plos One* 15, 19

Shitu A, Zhu S, Qi W, Tadda MA, Liu D, Ye Z (2020): Performance of novel sponge biocarrier in MBBR treating recirculating aquaculture systems wastewater: Microbial community and kinetic study. *Journal Of Environmental Management* 275

- Song Z, Zhang X, Sun F, Ngo HH, Guo W, Wen H, Li C, Zhang Z (2020): Specific microbial diversity and functional gene (AOB amoA) analysis of a sponge-based aerobic nitrifying moving bed biofilm reactor exposed to typical pharmaceuticals. *Science Of the Total Environment* 742
- Sun W, Xia C, Xu M, Guo J, Wang A, Sun G (2013): Distribution and Abundance of Archaeal and Bacterial Ammonia Oxidizers in the Sediments of the Dongjiang River, a Drinking Water Supply for Hong Kong. *Microbes Environ.* 28, 457-465
- Tang M, Liu Y, He J, Wang Z, Wu Z, Ji D (2021): In situ continuous hourly observations of wintertime nitrate, sulfate and ammonium in a megacity in the North China plain from 2014 to 2019: Temporal variation, chemical formation and regional transport. *Chemosphere* 262
- Tatari K, Musovic S, Gulay A, Dechesne A, Albrechtsen H-J, Smets BF (2017): Density and distribution of nitrifying guilds in rapid sand filters for drinking water production: Dominance of *Nitrospira* spp. *Water Research* 127, 239-248
- Terry LG, Summers RS (2018): Biodegradable organic matter and rapid-rate biofilter performance: A review. *Water Research* 128, 234-245
- Tu Q, Lin L, Cheng L, Deng Y, He Z (2019): NCycDB: a curated integrative database for fast and accurate metagenomic profiling of nitrogen cycling genes. *Bioinformatics* 35, 1040-1048
- Van Kessel MAHJ, Speth DR, Albertsen M, Nielsen PH, Op dC, Huub J. M., Kartal B, Jetten MSM, Lückers S (2015): Complete nitrification by a single microorganism. *Nature* 528
- Vet WWJMD, Dinkla IJT, Muyzer G, Rietveld LC, Loosdrecht MCMV (2009): Molecular characterization of microbial populations in groundwater sources and sand filters for drinking water production. *Water research* 43, 182-194
- Wang L, Kent LC, Dang H, Hanson TE, Klotz MG (2016): D1FHS, the Type Strain of the Ammonia-Oxidizing Bacterium *Nitrosococcus wardia* spec. nov.: Enrichment, Isolation, Phylogenetic, and Growth Physiological Characterization. *Frontiers in Microbiology* 7, 512
- Wang Y, Ma L, Mao Y, Jiang X, Xia Y, Yu K, Li B, Zhang T (2017): Comammox in drinking water systems. *Water Research* 116, 332-341
- Wang Y, Zhang W, Zhang Z, Wang W, Xu S, He X (2021): Isolation, identification and characterization of phenolic acid-degrading bacteria from soil. *Journal Of Applied Microbiology* 131, 208-220
- Wielen PWJJvd, Voost S, Kooij DVD (2009): Ammonia-oxidizing bacteria and archaea in groundwater treatment and drinking water distribution systems. *Applied & Environmental Microbiology* 75, 4687-95
- Wu Z, Huang S, Yang Y, Xu F, Zhang Y, Jiang R (2013): Isolation of an Aerobic Denitrifying Bacterial Strain from a Biofilter for Removal of Nitrogen Oxide. *Aerosol And Air Quality Research* 13, 1126-1132

- Xiao H-W, Wu J-F, Luo L, Liu C, Xie Y-J, Xiao H-Y (2020): Enhanced biomass burning as a source of aerosol ammonium over cities in central China in autumn. *Environmental Pollution* 266
- Yang X, Liu L, Wang S (2019): A strategy of high-efficient nitrogen removal by an ammonia-oxidizing bacterium consortium. *Bioresource Technology* 275, 216-224
- Yuan K, Li S, Zhong F (2020): Treatment of coking wastewater in biofilm-based bioaugmentation process: Biofilm formation and microbial community analysis. *Journal Of Hazardous Materials* 400
- Zhang D, Li W, Huang X, Wen Q, Miao L (2013): Removal of ammonium in surface water at low temperature by a newly isolated *Microbacterium* sp. strain SFA13. *Bioresource Technology* 137, 147-152
- Zhang X, Han H, Zheng X, Yu T, Chen Y (2019): Tetracycline-induced effects on the nitrogen transformations in sediments: Roles of adsorption behavior and bacterial activity. *Science Of the Total Environment* 695
- Zhang Y, Chen L, Sun R, Dai T, Tian J, Zheng W, Wen D (2016): Temporal and spatial changes of microbial community in an industrial effluent receiving area in Hangzhou Bay. *Journal Of Environmental Sciences* 44, 57-68
- Zhou S, Sun Y, Huang T, Cheng Y, Yang X, Zhou Z, Li Y, Li Z, Cui J, Xiao L (2020): Reservoir water stratification and mixing affects microbial community structure and functional community composition in a stratified drinking reservoir. *Journal Of Environmental Management* 267
- Zhu L, Ding W, Feng L-j, Kong Y, Xu J, Xu X-y (2012): Isolation of aerobic denitrifiers and characterization for their potential application in the bioremediation of oligotrophic ecosystem. *Bioresource Technology* 108, 1-7
- Zhu W (2008): Screening and Identification on High Effective Microorganisms Degrading Ammonia-nitrogen and Determination on Its Degradation Ability. *Journal of Anhui Agricultural Sciences*

Figures

Figure 1

Water quality parameters of the influent and effluent water samples from sand filters

Figure 2

Diversity and composition of bacterial communities in sand filters (SFs) at the phylum level (A) and genus level (B). Phyla or genera with a relative abundance of less than 0.03 are presented as 'others'.

Figure 3

PCR amplification products of *amoA* of ammonia-oxidising archaea (A), ammonia-oxidising bacteria (B), and complete ammonia oxidisers (C) in sand filters (SFs).

Figure 4

Abundance of ammonia-oxidising archaea (AOA), ammonia-oxidising bacteria (AOB), and complete ammonia oxidisers (comammox) amoA genes in sand filters SF1, SF2, and SF3.

Figure 5

Taxonomic assignment of 85 MAGs reconstructed from sand filters SF2 (A) and SF3 (B) metagenomes. The phylogenomic tree was constructed based on 48 single-copy core genes.

Figure 6

Gene circles of three reference genomes of *Candidatus Nitrospira inopinata* (CNI), *Candidatus Nitrospira nitrificans* (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).

Figure 7

Key nitrogen-cycling genes in five *Nitrospira defluvii*-like bins of the present study and a published *N. defluvii* (BioProject Accession: PRJNA640378)

Figure 8

Heterotrophic nitrification(A, C) and aerobic denitrification (B,D) performances of *Pseudomonas* sp. DW-5. OD600, optical density at 600 nm.

Figure 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.

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