An integrated meta-omics approach reveals the different response mechanisms of two anammox bacteria towards fluoroquinolone antibiotics

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Research Article

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Abstract

**Background:** The emerging fluoroquinolone antibiotics (FQs) are highly influential in nitrogen removal from livestock wastewater. However, beyond the capability of nitrogen removal, little is known about the molecular mechanism (e.g., shift of core metabolism and energy allocation) of anaerobic ammonium-oxidizing bacteria (AnAOB) under continuous FQ stress.

**Results:** This study investigated the effects of ciprofloxacin, ofloxacin and their mixture at concentrations detected in livestock wastewater on an anammox community in membrane bioreactors. It was found 20 μg/L FQs promoted nitrogen removal efficiency and community stability. Integrated meta-omics analysis revealed varied gene expression patterns between the two dominant AnAOB, *Candidatus Brocadia sapporoensis* (B AnAOB) and *Candidatus Kuenenia stuttgartiensis* (K AnAOB). The nitrogen metabolic processes were bolstered in B AnAOB, while those involved in anammox pathway of K AnAOB were inhibited. This difference was tentatively attributed to the up-regulation of reactive oxygen species scavenger genes (*ccp* and *dxf*) and FQ resistance gene (*qnrB72*) in B AnAOB. Importantly, most enhanced core biosynthesis/metabolism and close cross-feeding of B AnAOB with accompanying bacteria were also likely to contribute to higher levels of biomass yield and metabolism activity under FQ stress.

**Conclusions:** This finding suggests that B AnAOB has the advantage of higher nitrogen metabolism capacity over K AnAOB in livestock wastewater containing FQs, which is helpful for efficient and stable nitrogen removal by anammox community.

**Highlights**

- 20 μg/L FQs promoted nitrogen removal from the anammox system.
- B and K AnAOB were dominant anammox consortia but responded differently to FQs.
- The biomass of B AnAOB was significantly increased by FQs.
- The ROS scavenger and FQ resistance genes were expressed in B AnAOB under FQ stress.
- Core biosynthesis and metabolism processes were up-regulated by FQs in B AnAOB.

**Background**

Livestock wastewater has recently become the primary source of agricultural pollution, and the third major source of water pollution after industrial and domestic wastewater, which is characterized by high concentrations of ammonium and residual antibiotics. In swine wastewater, the concentrations of ammonium and total nitrogen were found ranging from a few hundred to 2,050 and 5,500 mg/L, respectively [1–3]. Fluoroquinolone antibiotics (FQs), one class of the most commonly used veterinary antibiotics in livestock farms, had been detected at increasing frequency in livestock wastewater. Surveys have revealed that the concentrations of FQs in swine wastewater were ranging from 0.028 to 111 μg/L [4–6]. As a highly efficient, cost-effective, and environmentally friendly technology for nitrogen removal, anaerobic ammonium oxidation (anammox) has been widely used to treat wastewater containing high...
concentrations of ammonia nitrogen, which is technically and economically feasible in nitrogen removal from livestock wastewater [7, 8]. The continuous stress of FQs, though inevitably affects the nitrogen removal performance of microorganisms in anammox system [9], has not been investigated in great depth yet.

Two anaerobic ammonium-oxidizing bacteria (AnAOB) genera, Candidatus Brocadia and Candidatus Kuenenia (B and K AnAOB), were widely detected in wastewater treatment processes and enriched in laboratory anammox setups [10, 11]. Some studies reported that B AnAOB could be well adapted to antibiotic stress, e.g., gaining a survival advantage in the presence of 1 µg/L clarithromycin [12], and adapting to sulfamethazine of less than 7 mg/L [13]. Others reported that K AnAOB was more adaptable to antibiotics, with the relative abundance decreased first and then increased at norfloxacin concentrations from 0 to 50 mg/L [14]. However, the molecular responses of both B and K AnAOB to FQs, e.g., energy allocation, expression of core metabolic pathways, remains rarely explored in anammox processes. In previous studies, enzyme and metabolomic analyses showed that FQs at concentrations detected in livestock wastewater (20 µg/L) enhanced ammonia assimilation, hydrazine dehydrogenation, and nitrite reduction, and up-regulated the metabolisms of various amino acids, purines, and pyrimidines, as well as carbon fixation processes, contributing to more efficient metabolic activities [15]. Therefore, exploring the response mechanisms of B and K AnAOB to FQs at concentrations detected in livestock ecosystem is helpful to find the dominant microorganisms suitable for nitrogen removal from livestock wastewater. Moreover, cross-feeding is an important nutritional interaction between AnAOB and other symbiotic bacteria, which is involved in nitrogen loop, vitamins, cofactors, and amino acid exchanges etc. [16]. Cross-feeding of amino acids, cofactors and organics in anammox consortia may regulate and change the activity of AnAOB [17, 18]. Yet, no study has examined the cross-feeding mechanism of microbial community to prolonged periods of FQ exposure in anammox systems.

The present study aimed to reveal the transcript response mechanism of B and K AnAOB and the shift in the cross-feeding relationship of anammox consortia exposed to FQs at concentrations detected in livestock wastewater in long-term experiments. Four anammox membrane bioreactors (MBRs) with B and K AnAOB as the dominant microbes were exposed to ciprofloxacin (CIP), ofloxacin (OFL), and their mixture (MIX) of 10–20 µg/L for 120 d, respectively. Genome-resolved metagenomic and transcriptomic analyses were conducted to unveil functional gene expression variations in specific microbes and cross-feeding relationship shifts. Our findings prove that FQs at concentrations detected in livestock wastewater improve nitrogen removal efficiency, and further suggest that B AnAOB has the advantage of higher nitrogen removal capacity over K AnAOB in livestock wastewater containing FQs, which may promote the practical application of more stable anammox technology.

**Methods**

**The MBR setup and operation**
The study was carried out in four 1.5 L MBRs running for 120 d. The MBR configuration is shown in Fig. S1 and detailed operations were provided in our previous study [19]. Briefly, seed anammox sludge was taken from a 5 L anammox MBR and distributed evenly into four reactors with an initial biomass of approximately 4 g/L. The structure and operating conditions of the four 1.5 L MBRs are identical to those of the 5 L MBR. Simulated nitrogenous wastewater [19] was used as medium and the influent concentrations of nitrite and ammonium were kept at 700 mg N/L with NaNO₂ and NH₄Cl as nitrogen sources, respectively. CIP, OFL and MIX were added at concentrations detected in livestock wastewater (Table S1). The wastewater medium was fed into the MBRs continuously using a pump with a hydraulic retention time of 24 h, and completely mixed by a mechanical stirrer at 100 rpm. N₂ was sparged into the medium prior to use for 20 min to remove dissolved oxygen. The temperature and pH were kept at 35 ± 1 ºC and 6.8–8.0. A control (CON) MBR was set up without FQs.

**Chemical analyses**

Influent and effluent samples were collected for chemical analyses through filtration with 0.22-µm membrane filters two or three times a week. The concentrations of total nitrogen (TN), nitrite (NO₂⁻-N), ammonium (NH₄⁺-N), nitrate (NO₃⁻-N) and chemical oxygen demand (COD) were then measured using standard spectrophotometric methods (APHA, 2012).

**Characterization of anammox sludge**

Anammox sludge samples were taken from the MBRs on days 1, 60 and 120. The granular sludge particle size was determined using a laser diffraction particle size analyzer (Mastersizer 3000, Malvern Panalytical, UK). Mixed liquid volatile suspended solids (MLVSS) were determined every month using the standard methods (APHA, 2012). Extracellular polymeric substances (EPS) was extracted using modified cation exchange resin method (CER, DOWEX™ MARATHON™, CA) (Text S1) [20]. The polysaccharides (PS) [21] and proteins (PN) [22] in EPS were determined using the bicinchoninic acid assay and the anthrone method, respectively. Enzyme activities of nitrite reductase (NIR), hydrazine synthetase (HZS), hydrazine dehydrogenase (HDH), nitrate reductase (NAR), and nitric oxide synthase (NOS) were measured using the enzyme-linked immunosorbent assay kit (Enzyme-linked Biotechnology Co., Ltd., China).

**Extraction and Sequencing of DNA and RNA**

Biomass was sampled on days 1 and 120 before and after exposure to 20 µg/L FQs from four MBRs for DNA extraction using the DNeasy PowerSoil Pro Kit (QIAGEN, Germany). Three consecutive biomass samples were taken on days 1 and 120 from the OFL, CIP and MIX-exposed groups for total RNA extraction using RNeasy PowerSoil Total RNA Kit (QIAGEN, Germany). DNA and RNA quality was assessed prior to library preparation and sequenced on an Illumina NovaSeq 6000 platform generating 150-bp paired-end reads (Guangdong Magigene Biotechnology Co., Ltd., Guangzhou, China) (Text S2).

**Meta-omics Analyses**
Metagenomic analyses were conducted based on our previous studies [19]. Details are provided in Text S3. Briefly, clean reads were assembled using MEGAHIT v.1.2.2 [23] The filtered contigs were binned with BASALT (Binning Across a Series of AssemBlies Toolkit) [24] to obtain metagenome-assembled genomes (MAGs). The MAGs with completeness – 5 * contamination ≥ 50% [25] were retained to infer potential metabolism functionalities. And then the MAGs were assigned taxonomic classifications and relative abundance was calculated [26, 27]. Prodigal v.2.6.2 [28] was used to predict open reading frames (ORFs) from the MAGs. Predicted amino-acid sequences were annotated against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database via the BLASTP program with an E-value cutoff of 10^{-5}. Metabolic pathways were constructed using the KEGG Mapper. To identify putative antibiotic resistance genes (ARGs), the ORFs were aligned against the Comprehensive Antibiotic Resistance Database using the Resistance Gene Identifier [29]. An ORF was identified as an ARG if the best identity was above 80%. The taxonomic annotations of ARGs were then retrieved to track their potential microbial hosts.

Trimomatic v. 0.36 [30] was used to acquire clean data from the raw data of transcriptomics. Clean reads were mapped to the NCBI SILVA databases. rRNA total sequences were removed by SortMeRNA [31]. The remaining total RNA sequences were mapped back onto the MAGs and the RPKM of each gene was calculated to represent the expression level. To exclude the bias in the comparison of gene expression levels caused by cell growth, the number of mapped reads of a certain ORF against mapped reads of total ORFs from a certain genome was normalized for RPKM value calculation with the following equation [32]:

\[ RPKM = \frac{\text{number of mapped reads of gene } a}{\text{total mapped reads of genome } A \ast \text{length of the gene}} \ast 10^9 \]

where gene a is present in genome A. The normalized RPKM value helps more precisely compare gene expression levels among genes from the same genome, rather than the whole community. T test and fold change (FC) analysis were used to identify differentially expressed genes (DEGs). \( P \) values were corrected by the Benjamini-Hochberg method. The DESeq2 package [33] in R v.4.0.5 was used to visualize DEGs in volcano maps with \( P < 0.05 \) and \( |\log_2(\text{FC})| > 1 \). The DEGs were subjected to enrichment analysis using the KEGG databases by the clusterProfiler package [34] in R v.4.0.5.

Results and discussion

Promotion effects of FQs on nitrogen removal in anammox system

Nitrogen removal performance of the anammox systems was promoted by FQs of 10 µg/L in the first 60 d and by FQs of 20 µg/L from 61 d onwards (Fig. 1). The COD concentration was stable (Fig. S2). During 1–60 and 61–120 d, the TN removal efficiency of FQs-exposed groups was steadily 2–3% and 3–5% higher than that of the CON group, respectively (Fig. 1A). The TN removal efficiency was raised by FQs,
especially by 20 µg/L CIP which had a more significantly positive effect on nitrite removal in the long-term run.

It has been widely reported that nitrite was an inhibitor of anammox consortia, which would lead to the instability of the anammox system [10, 35]. The initial effluent NO$_2^-$-N concentration in the four MBRs was about 10 mg N/L (Fig. 1B). In the absence of FQs, the average concentration of effluent NO$_2^-$-N was 18 mg N/L during 60–120 d with fluctuation in nitrogen removal performance in the CON group. Upon exposure to FQs, the NO$_2^-$-N concentration gradually decreased to negligible within 20 d, and the effluent ammonia nitrogen concentration was accompanied by a decrease (Fig. 1C), indicating that 10–20 µg/L FQs could mitigate nitrite inhibition to enhance nitrogen removal efficiency. This finding is consistent with the previous study, in which the nitritation efficiency was 20% higher with 100 ng/L CIP [36]. In addition, Kong et al. [37] observed an increase of 7.1% in specific anammox activity after 30 d of exposure to oxytetracycline. Therefore, it is critical to understand such a promotion scenario, as it may be closely associated with biomass or microbial community adaptation in anammox systems.

Characteristics of anammox sludge in response to FQs

The anammox sludge was characterized to explore the reasons for improving nitrogen removal performance of the anammox system. The MLVSS values increased by 20%, 10% and 14% after exposure to CIP, OFL and MIX for 120 d, respectively, while slight decrease was noted in the CON group (Fig. 2A). A previous study also found that microbial biomass was 5% higher in a partial nitritation reactor fed with CIP of 100 ng/L [36]. It has been also reported that excessive EPS secretion might be a protection mechanism under antibiotic shocks [38], so the decrease in EPS content suggests that 20 µg/L FQs may help microorganisms release from substrate stress (Fig. 2B). In the secreted EPS, the PN/PS ratio informs the aggregation capacity and surface hydrophobicity of the anammox sludge, and the higher its ratio is, the weaker sludge aggregation [39]. In our study, on the whole, the PN/PS ratio increased with prolonged FQ exposure. Correspondingly, the particle size of anammox sludge decreased after FQ exposure (Fig. 2C). Previous study reported that K AnAOB preferred to grow in larger granules [40]. Therefore, long-term FQ exposure may lead to the reduction of K AnAOB in smaller granules, but the response of the microbial community still needs to be further explored.

Shift of the functional bacteria community in response to FQs

To precisely link the shift of microbial community to FQ exposure, metagenomic analysis was conducted to characterize the microbial functions throughout the whole reactor operation periods. A total of 132 MAGs were recovered with metagenomic binning from eight samples (Table S2). These high-quality MAGs were further analyzed and taxonomically classified based on phylogenetic analysis of taxonomic marker genes (Fig. 3A). The metabolic inference of these high-quality MAGs was then conducted to determine the nitrogen metabolic potential of the bacterial communities. Six MAGs belonging to AnAOB
were taxonomically classified as B, K and J AnAOB, i.e., Ca. Brocadia, Ca. Kuenenia and Ca. Jettenia, respectively. B (AMXB1) and K (3AMXK1 and 3AMXK2) AnAOB were dominant microorganisms in our MBRs with a total relative abundance of higher than 35%. Other than the AnAOB, 71 MAGs were found with potential DN functions and 71 MAGs were found with DNRA functional genes.

FQs were found to exert a selective effect on specific microorganisms based on correlation analysis (Fig. 3B). The relative abundance of B AnAOB increased by 14%, 18% and 13% in the CIP, OFL and MIX-exposed groups, which was consistently twice or more as high as that in the CON group (7%). This increase was positively correlated to both CIP and OFL exposure with rho values of 0.65 and 0.51. On the contrary, the relative abundance of K AnAOB decreased in FQs-exposed groups as compared with the CON group. This reduction was negatively associated with both CIP and OFL exposure, with rho values of −0.33 and −0.05, respectively. This also suggests B AnAOB is more adaptive to CIP and OFL stress than K AnAOB in the present study. Previous studies also demonstrated that B AnAOB could adapt to sulfamethazine of 7 mg/L [13] and clarithromycin of 1 µg/L [12]. Similarly, stress adaptation of B AnAOB to FQs was considered to occur in our anammox MBRs. The higher abundance of B AnAOB may consequently result in higher nitrogen metabolism. Moreover, the relative abundances of DNRA and DN bacteria showed a negative correlation with FQ exposure, indicating that prolonged exposure of FQs was not conducive to the survival of these microorganisms. This observation was consistent with that long-term exposure of norfloxacin caused significant and irreversible suppression of cell growth to DN bacteria [14]. Therefore, the AnAOB communities occupied an ecological niche under 20 µg/L FQ stress in a more efficient and steadier anammox process for the long-term run.

**Different nitrogen metabolism responses of B and K AnAOB to FQs**

The anammox pathway was found to play a key role in the nitrogen removal by AnAOB, in which process the enzyme activity of HDH was significantly increased after exposure to 20 µg/L FQs (Fig. 4A). Meta-genomic and meta-transcriptomic results further revealed the transcriptional regulation of specific bacterial responses to 20 µg/L FQs (Fig. 4B). The regulating genes of anammox co-existed with those of DNRA and ammonia assimilation pathways, but responded differently in nitrogen metabolism of B and K AnAOB upon exposure to FQs (Fig. 4C and 4D). In B AnAOB, the up-regulation of hdh gene which was in agreement with the significant increase in HDH activity, together with up-regulation of the hao gene, induced enhancement of the anammox pathway. In K AnAOB, on the contrary, the nirS and hdh genes were significantly down-regulated in both CIP and MIX-exposed groups, inferring the weaker nitrogen metabolism in anammox pathway. It’s worth noting that the up-regulated genes of narGH and nrfA/nirB for nitrate and nitrite reduction indicates the activation of DNRA pathway in B AnAOB. Previous studies have also revealed the DNRA pathway was conducted in AnAOB grown on organic carbon like formate, acetate and propionate as electron donors [41, 42]. This unique physiological feature is particularly advantageous for B AnAOB to gain their ecological competence, possibly by increasing metabolic processes in the presence of organic FQs as external carbon sources [43]. Glutamate synthase (gltD) and
glutamate dehydrogenase (*gdhA*) genes involved in the ammonia assimilation pathway were generally up-regulated in B AnAOB, while no alteration in K AnAOB after exposure to FQs. L-glutamate is considered a more critical metabolite as a linkage between the carbon and nitrogen cycles. It is the direct product of ammonia assimilation, which subsequently serves as a primary precursor in multiple biosynthesis pathways including carbon sequestration, amino acid metabolism, purine and pyrimidine metabolism [44].

Furthermore, in B AnAOB (Fig. 4C), the increased activity of genes mediating nitrite (*nirC*) and ammonium transport (*amt*) indicated that extracellular substrates could be quickly and efficiently adsorbed by bacterial cells from wastewater, thus promoting intracellular nitrogen metabolism. NAD and cytochrome c play a vital role in electron transfer such as from ammonium and nitrite into nitrogen in the biochemical reaction of the anammox system [45, 46]. The up-regulation of *nuoB/ndh* and *qcrA* genes, which mediated the NADH dehydrogenase and cytochrome c reductase, might contribute to the enhanced capacity of electron transfer in B AnAOB. Meanwhile, the *atpB* and *atpG* genes mediating ATP synthesis were up-regulated in the oxidative phosphorylation pathway, indicating more energy was generated and used for active biochemistry reactions after FQ exposure. On the contrary, the down-regulation of *nirC* under CIP and MIX stress was not conducive to nitrite intake and nitrogen removal by K AnAOB (Fig. 4D). This suggests it is more conducive to enhance the nitrogen metabolism of B AnAOB than that of K AnAOB under FQ stress.

**Other different core metabolism responses of B and K AnAOB to FQs**

In addition to nitrogen metabolism, the microbial core metabolic pathways of carbon fixation, and metabolism of carbohydrate, lipids, lipopolysaccharides, amino acids, nucleotides, cofactors, vitamins and energy were also significantly changed by 20 µg/L FQs (Fig. 5A). The DEGs, which were involved in ABC transport, cell cycle and DNA replication that regulated protein synthesis, DNA or RNA synthesis, cell stabilization and mass transfer, responded correspondingly (Fig. 5B), thereby altering cell proliferation of AnAOB.

It is worth noting that gene expression responded differently in B and K AnAOB. In B AnAOB, the carbon fixation capacity was up-regulated, while those of K AnAOB and other major microorganisms were down-regulated (Fig. 5A). Previous studies have found that nitrogen metabolism was closely coupled to carbon fixation, which significantly influenced nitrogen transformation in partial nitrification-anammox process [47]. AnAOB could complete the Wood-Ljungdahl pathway to transform inorganic carbon into organic carbon, in which Mo(VI) is involved in the hydrogen transfer process in NADH [48]. The Wood-Ljungdahl pathway and *modAC* mediating molybdate transport system were up-regulated in B AnAOB after FQ exposure, indicating the enhanced activity of carbon fixation (Fig. 5A and 5B). And the acetyl CoA from Wood-Ljungdahl pathway is known to enter the TCA cycle, which is a central metabolic hub that supports the production of ATP and provides precursors for many biosynthetic pathways. It can fulfill the requirements of energy, biosynthesis and maintaining redox balance for AnAOB.
The ability to biosynthesize lipids, polysaccharides, and amino acids were improved in B AnAOB, but was impaired in K AnAOB (Fig. 5A). The activity of aminoacyl-tRNA biosynthesis mediating amino acid translation processes was enhanced in B AnAOB after FQ exposure. Aminoacyl-tRNA is mainly responsible for delivering the amino acids to the ribosome for incorporation into the polypeptide chain being produced [49]. A similar study demonstrated that aminoacyl-tRNA biosynthesis was active in Escherichia coli exposed to CIP [50]. These results suggest a higher capability of protein synthesis in B AnAOB. The elevated contents of proteins, together with polysaccharides and lipids in larger quantities as important components of cell walls and membranes, are conducive to higher levels of biomass production [51]. Moreover, the nucleotide metabolism was up-regulated in B AnAOB, providing the necessary raw materials for DNA replication. The up-regulation of ATP synthesis implied that a more energetic status was generated after FQ exposure, which could be deemed as more competent to provide energy for metabolic processes in B AnAOB.

It was noted that AnAOB relied on metabolites from other microbes to survive, making material exchange between microorganisms particularly important. Based on the core metabolic and biosynthetic pathways of the dominant MAGs, a complex cross-feeding relationship was found among 3CHL2 (p_Chloroflexi; g_SHND01), 3PRO16 (p_Proteobacteria; s_Desulfovibrio desulfuricans), BAC2 (p_Bacteroidetes; s_IGN3 sp900696555), and AnAOB (Fig. 5C). AnAOB expressed glycogen biosynthesis pathway, in which glycogen was synthesized to feed other bacteria in anammox consortia such as BAC2 and 3CHL2. AMXB1 and 3CHL2 had no potential to generate acetate, but needed acetate provided by other bacteria to synthesize acetyl CoA and thereafter to perform other metabolic activities. In addition, metabolic pathways of phosphatidyl ethanolamine (PE), phosphatidylcholine (PC) and fatty acid synthesis were expressed in AnAOB and BAC2, but there were no complete pathways of lipid synthesis in 3CHL2 and 3PRO16. Similar results were obtained for nucleotide biosynthesis pathways. All nucleotide biosynthesis pathways were expressed in AnAOB, while BAC2, 3CHL2 and 3PRO16 lacked some modules for UMP, IMP, GDP, GTP, CDP and CTP biosynthesis. In amino acid metabolism, AnAOB synthesized most amino acids except alanine, threonine, tyrosine, methionine, histidine, and phenylalanine, harboring the ability to feed amino acids to symbiotic bacteria. 3CHL2 was capable of providing threonine, methionine, and histidine, while BAC2 could provide alanine and histidine, and 3PRO16 provided alanine, tyrosine and phenylalanine. Altogether, they formed a complete cross-feeding of amino acids within anammox consortia. Similar to the expression of metabolic pathways of amino acids, AnAOB expressed most of the cofactor and vitamin metabolism pathways. Biotin and NAD played important roles in fatty acid synthesis and cell energy metabolism [52, 53]. Siroheme is an intermediate in the synthesis of heme and heme d1 in prokaryotes and vital in nitrite reduction [54]. Lipoic acid, as a coenzyme, is involved in acyl group transfer in substance metabolism and can eliminate free radicals to mitigate cell aging [55]. These cofactors and vitamins are crucial to the nitrogen metabolism and normal life activities of AnAOB cells. Meanwhile, AnAOB were cross fed by accompanying consortia of 3CHL2, BAC2, and 3PRO16, providing vital components such as isoprenoid, pyridoxal-P, molybdenum and tetrahydrofolate for bacterial plasma membrane formation, electron transport, oxidative phosphorylation, and carbon fixation [16, 56, 57]. Besides, as one of the largest superfamilies of transmembrane matter transporters, ABC transporters play
an essential role in the high-affinity uptake of various nutrients [58], thereby promoting nutrient uptake by bacteria. The DEG expression of ABC transporters was up-regulated in B AnAOB while down-regulated in K AnAOB (Fig. 5B), indicating that B AnAOB was more capable of competing for limited nutrients. Consequently, 20 µg/L FQs significantly induced higher levels of B AnAOB biomass, simultaneously promoting metabolic efficiency for enhanced nitrite and ammonium removal from wastewater.

The reason for the different metabolism responses between B and K AnAOB to FQs

Activation of the reactive oxygen species (ROS) scavenger genes may help B AnAOB release from oxidative stress. Excessively high concentrations of nitrite and antibiotic may affect the synthesis of nucleotides, proteins and lipids of microorganisms by producing ROS [59, 60]. Increased levels of intracellular ROS can inhibit cell growth or even cause death if they are not scavenged immediately [61]. Pyrazolone derivative and taurine have been shown to relieve oxidative stress [62, 63]. Therefore, we focused on the analysis of ROS scavenging genes of B and K AnAOB, including the genes {ccp, dxf and sod2 (Fig. 6A). Significantly up-regulated of these ROS scavenger genes were observed in B AnAOB upon FQ exposure. In the CIP, OFL and MIX-exposed groups, the expression of gene ccp was 3.2, 3.9 and 4.2 times as high as that in the CON group, which might help B AnAOB encode cytochrome c peroxidase to scavenge hydrogen peroxide in periplasm [64]. The gene dfx expressed 3.1, 3.1 and 4.1 times as high as that in the CON group, which may be response to reduce superoxide to hydrogen peroxide by encoding desulfuroferdoxin [65]. The gene sod2 encoded superoxide dismutase, which cleared harmful superoxide anion radicals [66], and its expression had no regularity under FQ stress. However, no obvious changes were noted in the expression patterns of ROS scavenger genes in K AnAOB. This may be the reason for differential response mechanisms of nitrogen metabolism to FQs in B and K AnAOB.

In addition, ARGs played a positive role in alleviating antibiotic stress [67, 68]. ARG \textit{qnrB72} found in B AnAOB was 2.8, 2.2, 4.6 times higher in CIP, OFL and MIX-exposed groups than that in the CON group, which might increase the chance of cell survival and metabolism. All other three FQ resistance genes of \textit{qnrA1}, \textit{qnrVC6} and \textit{qnrS2} changed with small variation in K AnAOB (Fig. 6B). Therefore, the different expression levels of ARGs may also be the cause of the different metabolic responses between B and K AnAOB.

Conclusions

In this study, it was found that both the efficiency and stability of nitrogen removal could be promoted in the anammox system by FQs at environmentally detected concentrations. Metagenomics and metatranscriptomics analyses revealed that FQs regulated bacterial nitrogen metabolism by altering the physiological and biochemical features of AnAOB, simultaneously by bolstering the metabolism activities of anammox, DNRA and ammonia assimilation. Notably, most core biosynthesis and metabolism of B AnAOB were upregulated, and close cross-feeding of amino acids, cofactors and organics by symbiotic bacteria might contribute to higher levels of biomass yield and metabolism activity. The up-regulated
genes for ROS scavenger and FQ resistance were presumed to be prompters of B AnAOB outperformance. These results reveal the potential roles of B AnAOB that are confronted by a heavy nitrogen load and higher emerging frequency of FQ contaminant in livestock wastewater. The obtained biological information is to provide the environmental engineers with a crucial clue to cultivate B AnAOB for nitrogen removal from livestock wastewater, which can guide towards enhancing the efficiency and stability of anammox processes in the long-term application of practical livestock wastewater treatment.

Declarations

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Authors’ contributions

X.Q., H.T., and L.Z. designed the research project. X.Q. analyzed all the data and wrote the manuscript. F.F., C.F., R.W., Y.C., S.Z., and X.W. contributed to the operation of the reactor. K.Y., L.D., Y.Y., N.X., and L.Z. contributed substantially to manuscript revisions. All authors read and approved the final manuscript.

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Availability of data and materials

All raw sequences of meta-omics were deposited at the National Center for Biotechnology Information (NCBI) Sequence Read Archive under the accession number PRJNA901366.

Ethics approval and consent to participate

The manuscript does not report data collected from humans and animals.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.
References


**Figures**
Figure 1

Performance of the four anammox MBRs in removing (A) total nitrogen, (B) nitrite, and (C) ammonium.

Figure 2
The characteristics of anammox sludge exposed to different FQs. (A) MLVSS, (B) EPS content, and (C) particle size of anammox sludge in four MBRs. The red and black marks represent the significance of EPS content and PN/PS ratio changes relative to day 1, respectively. (* : \( P < 0.05 \), ** : \( P < 0.01 \), *** : \( P < 0.001 \) by \( t \)-test).

Composition of the anammox community. (A) Phylogenomic reconstruction of MAGs from the anammox communities. A phylogenetic tree was generated by the maximum likelihood (inferred with IQ-tree and the LG + F + R8 model) with 1,000 bootstrap replications. The circle of grey to purple represents the abundance of MAGs. The genes in various metabolic pathways are marked with colored circles by iTOL version 4.4.2. (B) A heatmap of correlations between FQs and the different classifications of microorganisms including dominant AnAOB, DNRA and DN bacteria (matrix values are Rho values of Pearson correlation coefficient).
Figure 4

(A) Activities of nitrogen removal enzymes. The asterisks represent the significance of relative changes in enzyme activity to day 1. (*) \( P < 0.05 \), (**) \( P < 0.01 \), and (***) \( P < 0.001 \) by \( t \)-test. (B) Fold change of DEGs involved in nitrogen metabolism of AnAOB for the FQs-exposed groups compared with the CON group (\( P < 0.05 \) by \( t \)-test). Bars represent log\(_2\) transformed fold intensity and directionality. Transcriptional
The regulation of nitrogen metabolism in (C) B AnAOB and (D) K AnAOB. Colored dots from left to right represent samples exposed to CIP, OFL and MIX, respectively.

**Figure 5**

Enrichment analysis of the KEGG pathways in B AnAOB. (A) The relative gene transcription associated with core metabolism and biosynthesis in domain genomes (> 1%). Transcriptomic expression of...
pathways was relativized by the average RPKM (log$_2$) value of genes in the related pathways. The white box represents the incomplete pathways. (*: $P < 0.05$, **: $P < 0.01$, and ***: $P < 0.001$ by $t$-test). (B) Fold change of DEGs for ABC transporters, cell cycle and DNA replication in the FQs-exposed groups compared with the CON group. The size and color of the dots represent log$_2$ transformed fold intensity and directionality. (C) The interspecies interaction associated with expressed core metabolic pathways in anammox consortia. Colored squares from left to right represent samples exposed to CIP, OFL and MIX, respectively.

![Figure 6](image1.png)

**Figure 6**

Fold change of (A) ROS scavenger gene and (B) FQ resistant gene expression in different AnAOB. The bars represent the folding strength and directivity of the transformation through log2.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- floatimage1.png
- SupplementaryInformationA.docx
- SupplementaryInformationB.xlsx