Review

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A critical review on the effects of antibiotics on anammox process in wastewater

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Abstract: Anaerobic ammonium oxidation (anammox) has recently become of significant interest due to its capability for cost-effective nitrogen elimination from wastewater. However, anaerobic ammonia-oxidizing bacteria (AnAOB) are sensitive to environmental changes and toxic substances. In particular, the presence of antibiotics in wastewater, which is considered unfavorable to the anammox process, has become a growing concern. Therefore, it is necessary to evaluate the effects of these inhibitors to acquire information on the applicability of the anammox process. Hence, this review summarizes our knowledge of the effects of commonly detected antibiotics in water matrices, including fluoroquinolone, macrolide, β -lactam, chloramphenicol, tetracycline, sulfonamide, glycopeptide, and aminoglycoside, on the anammox process. According to the literature, the presence of antibiotics in wastewater could partially or completely inhibit anammox reactions, in which antibiotics targeting protein synthesis or DNA replication (excluding aminoglycoside) were the most effective against the AnAOB strains.

Keywords: anammox; antibiotics; microbial community; nitrogen removal; specific anammox activity.

1 Introduction

In recent years, excess release of nitrogen into waters has become a progressively important problem, and nitrogen should be eliminated from effluents before their discharge into the environment. Conventionally, nitrogen is removed from wastewater by the biological nitrification–denitrification (N&DN) process. In this process, firstly ammonium is biologically oxidized to nitrate through nitrite under oxic conditions (nitrification: $\text{NH}_4^+ \rightarrow \text{NO}_2^- \rightarrow \text{NO}_3^-$), and then nitrate is biologically transformed to nitrogen gas under anoxic conditions in the presence of organic carbon compounds (denitrification: $\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{N}_2$) (Noophan et al. 2012).

However, nowadays, the anaerobic ammonium oxidation (anammox) process has become an innovative and more sustainable alternative for the removal of nitrogen from NH_4^+ -rich wastewater (Van Hulle et al. 2010), which was first discovered in a denitrifying fluidized bed reactor in 1995 (Mulder et al. 1995).

Anammox is a biological process in which ammonium is directly oxidized by autotrophic anammox bacteria into nitrogen gas under anoxic conditions with nitrite as the electron acceptor, which can simply be shown as Equation (1) (Tsushima et al. 2007).

AnAOB belong to the deep-branching lineage of *Planctomycetales*. So far, *Candidatus Kuenenia stuttgartiensis* and *Candidatus Brocadia anammoxidans* (freshwater species) and *Candidatus Scalindua sorokinii*, *Candidatus Scalindua wagneri*, and *Candidatus Scalindua brodae* (marine species) have been suggested as AnAOB. In addition, a mixotrophic anammox bacterium *Candidatus Anammoxglobus propionicus* was recently proposed (Tsushima et al. 2007).

Anammox bacteria, unlike most prokaryotes, have a rather complex cell structure (Figure 1A) (van Niftrik and Jetten 2012). Basically, their cell structure is composed of three membrane systems. The outermost membrane, along with a thin layer of peptidoglycan, builds the cell wall, which might be coated by an S-layer protein lattice (van Teeseling et al. 2014, 2015). The second membrane

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Figure 1: (A) Cellular compartmentalization in anammox bacteria. Left: schematic overview, right: transmission electron microscopy image of a cell of the anammox bacterium *Kuenenia stuttgartiensis*. Within the central organelle, the anammoxosome, tubule-like structures are found (white arrow heads) as well as electron-dense iron-rich particles (black arrowheads). Scale bar, 500 nm. (B) C17-C20 ladderane lipids containing 3–5 cyclobutane rings, with or without a cyclohexane ring. Fatty acids are esterified with methanol or the glycerol backbone, and alcohols are ether-linked to glycerol, all in different combinations. Adapted from the study by Kartal and Keltjens (2016).

layer covers the cytoplasm, creating a periplasmic area in between these two outer membrane layers, like in Gram-negative bacteria. The cytoplasm includes the nucleoid (DNA), translation, transcription, and household machinery, as well as anabolic functions. A major part of the cell consists of a vacuolar cell organelle, the anammoxosome, which is completely surrounded by the third membrane layer (Neumann et al. 2014; van Niftrik et al. 2004). Inside this organelle, the anammox metabolism occurs (de Almeida et al. 2015). Unlike other prokaryotes, anammox bacteria, have special lipids, so-called “ladderane” lipids, in their cellular membrane (Figure 1B). These membrane ladderane lipids possess cyclobutane/cyclohexane ring formations, creating a highly impermeable anammoxosome membrane in comparison with other non-anammox bacterial membranes (Damsté et al. 2002; van Niftrik et al. 2004).

In brief, anammox is a three-step reaction with nitric oxide (NO) and hydrazine as intermediates: initially, nitrite is reduced to NO, then the generated NO reacts with ammonium to produce hydrazine by using the hydrazine synthase enzyme as a catalyst, and eventually hydrazine is oxidized to N_2 (Kartal et al. 2011). The detailed metabolism of anammox is shown in Figure 2.

The stoichiometry of the anammox reaction, involving the transformation of NH_4^+ and NO_2^- into free nitrogen (N_2)

Figure 2: Possible biochemical pathway and cellular localization of the enzyme systems involved in anammox reaction (Ni and Zhang 2013).

and nitrate (NO_3^-), is shown in Equation (2). The ratio of ammonium consumption to nitrite consumption to nitrate production is 1:1.146:0.161 (Ibrahim et al. 2016).

Since the inhibitory impact of nitrite on anammox is more severe than that of ammonium, in order to assure that nitrite is a limiting substrate, the concentrations of nitrite and ammonium are supplied as needed with a molar ratio of 1:1 (Shi et al. 2017).

In comparison with conventional N&DN, the anammox process provides important advantages such as 60% reduction in oxygen demand (aeration), no requirement for organic carbon supply, less/no N_2O production, 90% lower sludge generation (which leads to reducing the sludge treatment cost) (Ali and Okabe 2015), less energy consumption (Shi et al. 2017), higher nitrogen removal rate (NRR), fewer operational costs, and smaller space demand (Jin et al. 2012).

Unlike ammonium, nitrite is not common in wastewater; therefore, recently, new methods have been introduced on the basis of the metabolism of autotrophic bacteria for nitrogen removal from wastewaters (Zhang et al. 2008). In all of these autotrophic approaches, partial nitrification is needed before the anammox process, where about 50% of the ammonium in the influent is oxidized to nitrite. Subsequently, the remaining ammonium will be transformed into N_2 gas by anammox bacteria through the anammox process, with nitrite as a terminal electron acceptor under anoxic conditions (Gonzalez-Martinez et al. 2014). This combination is recognized as partial nitrification (PN) and anammox (PN&A) (Xing and Jin 2018).

PN:

Anammox:

Partial nitrification has some advantages over total nitrification-based technologies, including a 25% reduction in aeration, 30% decrease in biomass, and 20% lower CO_2 ejection (Gonzalez-Martinez et al. 2014). The PN&A process can be applied separately in two different bioreactors (partial nitrification/anammox technique) or inside the same bioreactor [Completely Autotrophic Nitrogen-removal Over Nitrate (CANON), aerobic/anoxic DEamMONification process (DEMON) techniques] (Rodriguez-Sanchez et al. 2017). Up to now, anammox-based processes have successfully been employed for the treatment of wastewater with high nitrogen levels under mesophilic conditions, and more than 200 full-scale plants have been constructed across the world (Ali and Okabe 2015; Cao et al. 2017; Lackner et al. 2014).

However, since anaerobic ammonia-oxidizing bacteria (AnAOB) have a slow growth rate with low cell yield (Strous et al. 1998), the presence of the inhibitory materials commonly found in nitrogen-rich wastewater, such as free nitrous acid (FNA), free ammonia (FA), dissolved oxygen (DO) (Xing and Jin 2018), and other inhibitors including antibiotics (Yang et al. 2013), organic matter (Molinuevo et al. 2009), and heavy metals (Hamidian et al. 2019; Mansouri et al. 2013; Mojoudi et al. 2018; Zhang et al. 2016a), has restricted the application and industrialization of anammox-based processes (Jin et al. 2012). Moreover, AnAOB have a high susceptibility to environmental changes such as pH and temperature, which make their cultivation extremely difficult (Jin et al. 2012). Hence, the anammox process has principally been applied for the treatment of low chemical oxygen demand (COD)-containing and ammonium-rich wastewaters such as tomato-processing effluent, sludge digester liquor, and landfill leachate (Joss et al. 2009; van der Star et al. 2007). Generally, for wastewater with low C/N ratios, the anammox process is an appropriate method, while at C/N ratios beyond 1, anammox bacteria cannot compete with heterotrophic denitrifying bacteria (Breisha and Winter 2010). In fact, this process has not yet been used for the treatment of domestic wastewater, probably because of the high C/N ratio and low quality of the effluent water (Ali and Okabe 2015).

To date, several studies have shown that the presence of antibiotics in wastewater can directly put selective pressure on bacteria, contributing to alteration of the bacterial community structure and subsequent disturbance of the stability and efficiency of biological wastewater treatment systems because of their severe bacteriostatic impacts (Deng et al. 2012; Strous et al. 2006). The inhibitory impacts of operational conditions, environmental stresses, and some toxic substances on anammox performance have been investigated in the literature (Cho et al. 2020; Gonzalez-Martinez et al. 2018b; Hu et al. 2013a; Jin et al. 2012; Ma et al. 2016; Rodriguez-Sanchez et al. 2014; Roose-Amsaleg and Laverman 2016); however, these studies have not provided compelling evidence to clarify the difficulties posed by antibiotics. Hence, in this review paper, we have summarized and interpreted the effects of antibiotics on the performance of the anammox process in wastewater treatment.

2 Effects of antibiotics on anammox process

2.1 Effect on pH

Generally, in an anammox reactor, the effluent pH increases due to the utilization of H^+ when AnAOB uses nitrite as the electron acceptor to oxidize ammonia, which is regarded as the main reason for pH variations in the anammox process (Jin et al. 2012). The optimum pH and temperature for AnAOB to achieve high anammox activity have been reported as pH ranging from 6.4 to 8.3 and temperature varying from 20 to 43 °C (Breisha and Winter 2010).

Other obstacles to achieving successful operation of an anammox system are FA and FNA inhibition (Jin et al. 2012). The presence of FA and FNA is pH-dependent in the anammox system (Park and Bae 2009), in which at low pH, the FA concentration decreases but FNA concentration increases; on the other hand, at high pH, FA concentration increases but the FNA concentration is reduced, which consequently leads to either FNA or FA inhibition (Jin et al. 2012).

FA can more easily diffuse into biological cells through the lipid membrane than NH_4^+ . In the mechanism of FA suppression, the FA affects the pH of the intracellular compartment after entering the cells (intracellular pH < extracellular pH). Low or high pH damages the transmembrane capability and changes the selectivity of the substance exchange between the cells and the exterior

environment; this will disturb the enzyme synthesis, contributing to cell deactivation or even death (Jin et al. 2012; Liu et al. 2019b).

According to several investigations, inhibition by NO_2^- , which is mainly owing to the toxic impacts of FNA, could suppress anammox bacteria at certain concentrations (Dasgupta et al. 2017; Kang et al. 2018; Miao et al. 2019; Puyol et al. 2014). In the mechanism of FNA inhibition, NO_2^- serves as an uncoupling agent to pass through the membrane of prokaryotic cells; the permeability expansion of the cell membrane influences the enzymes involved in the transfer of electrons and protons, thereby causing the inhibition of adenosine triphosphate (ATP) synthesis. Ultimately, the ATPase-catalyzed reaction will be affected and the biological activity will be reduced (Duan et al. 2019; He et al. 2018).

Therefore, pH could impact the process not only directly, but also indirectly through its relationship with FA and FNA. Hence, the influent pH should be adjusted to neutral to achieve a stable process. Nevertheless, the presence of antibiotics may affect the effluent pH. For example, Zhang et al. (2014) reported that oxytetracycline (OTC) shock (155–1731 mg/L) led to a significant increasing trend in pH during the shock period along with lower substrate removal efficiencies. If the reaction pH stayed at a very high level for a longer time, because of FA inhibition, the bioactivity of the reaction would decline, leading to instability in the process (Zhang et al. 2014). The probable explanation for the increase of pH is that the anammox community was suppressed by OTC and the conversion of nitrogen compounds was not completely performed (Zhang et al. 2014). OTC is a lipophilic substance and can easily diffuse into the bacterial membrane or passively pass through porin channels in the cell membrane (Riond and Riviere 1988). Hence, the inhibitory effects of toxic substances on anammox bacteria could result from damage to the cell membrane integrity and permeability (Ramos et al. 2015; Yang et al. 2013). In addition, OTC acts by inhibiting protein synthesis in bacteria (Jafari Ozumchelouei et al. 2020; Pickens and Tang 2010), thus negatively influencing the abundance of functional genes and proteins associated with nitrogen removal, such as hydrazine synthase gene and heme c. An increasing trend in pH was also observed in pharmaceutical wastewater during the anammox process, probably owing to the presence of some inhibitory substances such as kitasamycin, although the pH range (8.1–8.36) in the reactor remained below the toxic threshold (Tang et al. 2011).

However, Beneragama et al. (2013) reported that the addition of OTC in manure had no impact on pH during the

digestion process (Beneragama et al. 2013). Erythromycin and norfloxacin antibiotics (long-term, 0.001–100 mg/L) were also reported to have no significant effect on pH during the anammox process (Zhang et al. 2018c, 2019c), which could be attributed to the stabilization of the whole community.

In contrast to the previous studies, Shi et al. (2017) stated that after inoculation of the effluent with OTC, the pH tended to decrease; it continued to decline until reaching a minimum pH of 7.52 on day 55, even though there was no OTC addition during days 53–58. The reason might be because the metabolic pathways of AnAOB were impaired, contributing to loss of the capability of the bacteria to consume H^+ and convert NH_4^+ (Shi et al. 2017).

In conclusion, the influence of antibiotics on pH during the anammox process is controversial; however, anammox processes should be sufficiently controlled to minimize pH fluctuations.

2.2 Effect on stoichiometric ratio

In a balanced biochemical anammox reaction, theoretically, the stoichiometric ratios of R_S ($R_S = \text{NO}_2^-$ -N conversion/ NH_4^+ -N depletion) and R_P ($R_P = \text{NO}_3^-$ -N production/ NH_4^+ -N depletion) are 1.32 and 0.26, respectively (Strous et al. 1999b), suggesting stable operation of the anammox system. The variation in the stoichiometric ratio depends on the substrate concentration, operating conditions, reactor configuration, and the type of AnAOB (Yang et al. 2013). Additionally, in the literature, it was shown that the presence of antibiotics could affect the stoichiometric ratio of the anammox reaction, in which higher antibiotic concentrations exerted stronger impacts.

For instance, Shi et al. (2017) demonstrated that the introduction of OTC contributed to changes in the stoichiometric ratio. Their results revealed a slight increase in R_S and R_P after the addition of 1 mg/L OTC ($p > 0.5$). However, as the OTC dosage reached 2 mg/L, a significant surplus of R_S and R_P was observed ($p < 0.03$). These synchronous deviations in R_S and R_P may be attributed to abnormalities in the anammox metabolic pathways from exposure to moderate concentrations of OTC, which continued despite discontinuation of OTC addition (Shi et al. 2017).

Yang et al. (2013) reported that the values of R_S and R_P for the anammox process without OTC stress were 1.17 ± 0.07 and 0.16 ± 0.01 , respectively, which were both close to the values reported by Strous et al. (1998). However, under a high OTC stress (50 mg/L), the stoichiometric

ratios changed and the values of R_S and R_P during days 28–42 were 0.98 ± 0.21 and 0.18 ± 0.05 , respectively. The low and unstable value of R_S indicated that the presence of OTC in the influent resulted in changes in bioreaction properties. Also, one potential biochemical reaction, which is the ammonification of organic substances stemming from the cell lysis, might take place within the reactor (Yang et al. 2013).

However, the inhibitory effects of antibiotics on the stoichiometric ratio of the anammox process could be reversible. For example, Zhang et al. (2014) showed that OTC shock caused the value of R_S to diverge from the theoretical value, while R_P was relatively constant at 0.26 in the anammox reaction. In addition, at moderate and high levels of OTC, R_S experienced more deviation to a value of 1.32, which might be due to impairment of anammox metabolism, inhibition of anammox microbials by OTC, and incomplete conversion of nitrogen compounds. After discontinuing the OTC shock, however, the anammox performance was recovered and the stoichiometric ratio became relatively close to the theoretical value, but still fluctuated because the recovery of the performance was time-consuming (Zhang et al. 2014).

In another study, Zhang et al. (2019c), while testing the effect of erythromycin (ERY) on the anammox process, reported that after the addition of low concentrations of ERY (≤ 1 mg/L), the values of R_S and R_P fluctuated but were still around the baseline, suggesting that the performance in the anammox system was stable (Zhang et al. 2019c). ERY, a representative class of macrolide antibiotics, acts generally via protein synthesis suppression. It inhibits the translocation of peptidyl-tRNA by adhering to the 23S rRNA molecule in the 50S ribosomal subunit (Alighardashi et al. 2009). However, most Gram-negative bacteria are rather resistant to erythromycin because of the relative impermeability of the Gram-negative membrane and the hydrophobicity of the antibiotic (Pechère 2001). Nevertheless, in the ensuing operations, with the increase of ERY dosage (10–100 mg/L), R_S and R_P started to diverge from the initial values and became more variable than in previous phases, which demonstrated that anammox metabolism was actually impaired in the subsequent phases. Meanwhile, the performance of the reactor also started to deteriorate as the ERY level increased. When the addition of ERY was discontinued, R_S and R_P tended to recover to the initial levels, revealing the reversible nature of the inhibitory effect of ERY on anammox metabolism (Zhang et al. 2019c).

In summary, the presence of antibiotics can disturb the stoichiometric ratio of the anammox reaction, in which the trends of changes in R_P and R_S may be similar or different. Moreover, a higher level of antibiotics was found to have a

more severe influence on the stoichiometric ratios. Besides, it was shown that stoichiometric ratios can be recovered to their initial values over time after the discontinuation of antibiotic addition.

2.3 Effect on nitrogen removal

The total nitrogen removal efficiency (TNRE) and nitrogen removal rate (NRR) have been considered as indicators of the performance of the anammox process. TNRE and NRR can be affected by the presence of antibiotics in anaerobic treatment processes, with the effects strongly related to the concentrations and types of antibiotics as well as the exposure time; hence, nitrogen removal can be slightly or completely inhibited.

Generally, high concentrations of antibiotics (mg/L) and long exposure time (days) have a stronger impact on anammox performance, which can be somewhat recovered after the removal of antibiotics, with or without reinoculation, depending on the level of deactivation of the biomass and the abundance of bacteria. For instance, Collado et al. (2013) demonstrated that an exposure concentration of 50 μ g/L sulfamethoxazole did not influence the reactor performance of a Sequential Batch Reactor (SBR) (Collado et al. 2013). The reason might be that with exposure to low antibiotic concentrations in wastewater treatment processes, bacteria can maintain metabolic activity through shifts in the bacterial community (Theriot et al. 2014) or the expression of resistance genes (Walters et al. 2003).

Tetracycline at concentrations ≤ 0.3 mg/L was found to exert no significant impact on anammox performance, demonstrating that the anammox process could resist the influence of low levels of tetracycline, while the extracellular polymeric substance (EPS) content slightly increased. Thus, EPS might contribute to the stable operation of the reactor during the initial phases. However, a remarkable deterioration in nitrogen removal performance took place at a tetracycline level of 1 mg/L due to cell lysis. Adjusting the dosage back to 0.5 mg/L successfully alleviated the inhibition, and NRE returned to the prior level after 10 days, suggesting that the nitrogen removal performance could quickly recover from the stress of high-level tetracycline (Fan et al. 2019).

Additionally, various investigations have indicated that antibiotic inhibition increases with prolonged exposure time. For example, Yao et al. (2018) showed that AnAOB were less sensitive to chlortetracycline stress than ammonium oxidizing bacteria (AOB) initially, but after 10 days, a synchronous increase of NH_4^+ -N and NO_2^- -N

was found. The accumulation of NO_2^- -N suggested that chlortetracycline remarkably suppressed AnAOB activity with elevated exposure time, as this antibiotic is a growth inhibitor (Yao et al. 2018).

Yang et al. (2013), while studying the acute and chronic impact of OTC on the anammox process, obtained the half-maximal inhibitory concentration (IC_{50}) of 517.5 mg/L in batch experiments, indicating that low levels of OTC had no effect on anammox in a short period, while in a long-term test, 50 mg/L OTC significantly reduced the NRR from 12.4 to 2 kg N/m³ d within 26 days, demonstrating severe suppression of anammox performance by OTC due to cell lysis and growth inhibition of AnAOB. Although the recovery of anammox performance inhibited by OTC would be difficult, adding fresh anammox sludge as a biocatalyst would be an effective approach (Yang et al. 2013).

Shi et al. (2017) indicated that the addition of 1 mg/L OTC caused the TNRE to decrease from 92% (control) to 82.7%, while NRR became stable at 5.32 ± 1.05 kg N/m³ d, suggesting that anammox sludge could accommodate a low concentration of OTC and also that the AnAOB experienced a period of latency. However, the anammox process was considerably suppressed within three weeks by the presence of moderate concentrations of OTC (2 mg/L) in which TNRE significantly reduced to 19.7%, and NRR decreased to 1.20 ± 0.09 kg N/m³ d due to the inhibition of the growth of anammox microorganisms by OTC. After the removal of OTC from the influent, the nitrogen removal capacity recovered after a long time, and the abundance of resistance genes slowly decreased via the efflux pumping mechanism (Shi et al. 2017).

Zhang et al. (2018c) reported that the addition of 0.001–50 mg/L norfloxacin (NOR) could be somewhat tolerated by anammox systems, in which the NRR was first reduced to 0.220 from the initial value of 0.345 due to NOR inhibition of protein synthesis during AnAOB growth, then recovered to $0.354 \text{ kg m}^{-3} \text{ d}^{-1}$ after the acclimatization of AnAOB to NOR. As the NOR concentration was elevated to 100 mg/L, the NRR significantly decreased by almost 53.4% of the control NRR and did not recover during the whole experiment. The irreversible inhibition threshold of NOR on anammox ranged from 50 to 100 mg/L (Zhang et al. 2018c). Norfloxacin is a broad-spectrum antibiotic in the quinolone family and has bacteriolytic activity. It acts by blocking the DNA gyrase enzyme, which is responsible for bacterial DNA synthesis and repair (Amorim et al. 2014). NOR could prevent proper DNA replication, and has remarkable inhibitory effects on bacteria, particularly Gram-negative bacteria (Zheng et al. 2016). After discontinuing NOR feeding, NRR was restored to approximately the initial level, indicating that NOR had no

cumulative impact on the system and that the occurrence of resistance genes gradually decreased through the efflux pumping mechanism (Zhang et al. 2018c).

The tolerance of the anammox granules in batch tests could be attributed to their multiple-layer structure with two different regions. The outer region, which is kept together by the easily extractable extracellular polymeric substances (EPS), is dispersible because the molecules bind via weak interactions, i.e., EPS ion bridging through multivalent ions and van der Waals forces. However, the interior region, which comprised rather compact EPS, is stable, and the molecules interact strongly via polymer entanglement. Therefore, antibiotics could be efficiently adsorbed on the EPS (Xu et al. 2013), decreasing the risk of antibiotics coming in direct contact with AnAOB in the short-term test. Moreover, the duration of the exposure time may be a crucial factor since these antimicrobials are growth inhibitors. Additionally, the long doubling time of AnAOB makes it difficult to assess their inhibitory impacts in short-term (several hours) batch studies (Hu et al. 2013b).

Zhang et al. (2019c) stated that TNRE and NRR were initially inhibited when the system was subjected to ERY in the range of 0.001–1 mg/L, but was immediately restored after 12 and 10 days, respectively. The possible reason was that AnAOB could entirely withstand the invasion of ERY at low levels (≤ 1 mg/L) by secreting more EPS, which is a compact layer that covers the bacteria cells. ERY could be adsorbed effectively on the EPS, which hinders the penetration of these antimicrobial agents into the bacteria cells. Thus, the EPS-based matrix serves as a barrier, which inhibits antibiotics from reaching their targets. However, increasing concentrations of ERY led to a significant reduction in TNRE and NRR, by 40 and 41% for 10 mg/L ERY, 50 and 50% for 50 mg/L ERY, 51 and 51% for 100 mg/L ERY, respectively, without recovery. One probable explanation was that EPS could not prevent antibiotics from penetration into the microbial cells, although EPS was still secreted. In other words, AnAOB reached their maximum antibiotic-resistant capacity and EPS secretory amount. As the ERY concentration increased, the deterioration of the anammox system continued because more antibiotics diffused into the cell and suppressed the bioactivity owing to the reduction in EPS. However, the deterioration rate gradually decreased in each phase. It can be hypothesized that AnAOB resisted the biotoxicity of ERY to some extent via gene mutations and an increase in the abundance of resistance genes. When no ERY was supplied, the anammox performance rapidly recovered, although the complete recovery of AnAOB was time-consuming (Zhang et al. 2019c).

Similar results were obtained for the impact of spiramycin (a macrolide antibiotic) on the anammox system. Low concentrations of spiramycin (0.5 mg/L) did not appreciably influence the nitrogen removal performance, whereas high spiramycin concentrations (5 mg/L) exerted inhibitory impacts, which required different time periods to restore (Jing-Wu et al. 2020).

Interestingly, several studies have demonstrated that the inhibitory effect of antibiotics toward anammox bacteria in granular anammox reactors could be counteracted by increasing the biomass (Sguanci et al. 2017). In this regard, findings reveal that the presence of non-anammox biomass could promote the resistance of anammox bacteria to antibiotics in mixed-culture systems. Generally, bioaugmentation is obtained by introducing selected/acclimatized organisms (pure cultures or aggregates) or native/allochthonous/genetically modified microorganisms into nonadapted or stressed systems to promote bioactivity and improve an unstable process (Zhang et al. 2017a).

Sludge addition has been considered as a powerful approach to restoring the nitrogen removal efficiency of the process, because the addition of fresh anammox sludge to the reactor can lead to improvement of the living conditions of AnAOB (Tang et al. 2011; Yang and Jin 2013).

Jin et al. (2014) showed that bioaugmentation helped the anammox performance to recover after oxytetracycline shock (518 mg/L, 1 h) with a restoration time of 38 h (Jin et al. 2014). In fact, bioaugmentation played a significant role in restoring the anammox performance in different ways: (1) Adsorption: as bioaugmentation was applied, more OTC was adsorbed, which diminished the inhibition and restored the deteriorated performance. (2) Biodegradation: the bioaugmentation efficacy might be partly due to the elevated biodegradation of OTC (Jin et al. 2014). (3) Augmentation of the predominant consortia and improvement of their corresponding activity: the impact of bioaugmentation could primarily be due to the predominance of ammonia oxidizing bacteria (AOB) in the nitrifier community, remarkably increasing the AOB activity (Bartolí et al. 2011). Besides, bioaugmentation could alleviate inhibition and decrease harm by delayed enhancement of the heme c content. (4) Quorum-sensing impact: a perfect match was achieved between the added biomass and the native microorganisms (Jin et al. 2014).

However, occasionally, the suppression induced by antibiotics on the anammox system becomes irreversible. For example, Fernandez et al. (2009) showed that in a long-term experiment, the continuous addition of 50 mg/L tetracycline hydrochloride in an Anammox Sequential Batch Reactor (SBR) system led to a significant reduction in

the nitrogen removal efficiency. However, after the removal of antibiotics on day 70, the reactor efficiency was not recovered due to biomass deactivation (Fernandez et al. 2009) derived from the bacteriostatic effect of tetracycline hydrochloride on bacteria by adhering to the bacterial 30S ribosomal subunit and preventing incoming aminoacyl tRNA from binding to the ribosome acceptor site, or by adhering to bacterial 50S ribosomal subunit and altering the cytoplasmic membrane leading to the leakage of intracellular components from bacterial cells (Chukwudi 2016).

Notably, sometimes exposure to low levels of antibiotics may result in increasing the nitrogen removal performance. For example, Meng et al. (2019) reported that exposure to low levels of tetracycline (TC) (1–100 µg/L) slightly enhanced the nitrogen removal efficiency, which might be owing to elevated heme c content or multiplied relative abundance of anammox bacteria and denitrifiers, whereas exposure to a high TC level (1000 µg/L) contributed to poor anammox performance (Meng et al. 2019). About 1 mg/L OTC was also observed to improve the ammonia removal efficiency from 62.9% (control) to 76.6% in the partial nitrification process, suggesting the promotion of ammonia oxidation (Zhang et al. 2020b).

It has been reported that some communities of “non-anammox” microorganisms are regularly found with anammox bacteria in these environments, such as ammonia oxidizing bacteria (AOB), fermentation bacteria, and denitrifiers (Zhang et al. 2017b). Thus, although the anammox performance was suppressed by tetracycline stress, the metabolic processes carried out by the aforementioned functional bacteria for the removal of nitrogen did not change (Zhang et al. 2018b).

There are indications that in the presence of greater quantities of organic substances, denitrifying bacteria can enhance the total nitrogen removal in anammox reactors, since the nitrate generated during the anammox metabolism can be consumed by denitrifiers (Pereira et al. 2017). Most members of *Ignavibacteriaceae*, *Bacteroidaceae*, and *Bacteroides* families have been considered as fermentation bacteria (Cao et al. 2016a; Wang et al. 2017). These bacteria can convert organic matter, such as antibiotics and extracellular polymeric substances secreted by anammox sludge, into short-chain volatile fatty acids and alcohols, which provides the heterotrophic denitrifying bacteria with electron donors. Therefore, nitrogen elimination in anammox systems is the result of the collaboration between these fermentative bacteria and anammox bacteria via a cometabolic process (Gonzalez-Gil et al. 2015). Furthermore, the activity of certain bacteria has been shown to be promoted when they are exposed to a threshold level of

antibiotics, thereby benefiting the survival of sensitive bacteria in unfavorable environments and subsequently improving wastewater treatment performance (Chung et al. 2018; Du et al. 2018b). Generally, highly diverse bacterial communities are considered to be responsible for stable ecosystems (Xia et al. 2018).

The influence on anammox performance has been also shown to be related to the type of antibiotics. For instance, Zhang et al. (2015b) reported that in terms of nitrogen removal performance, the inhibition order of tested antibiotics on the anammox process was florfenicol > amoxicillin > sulfamethazine, suggesting that AnAOB were more susceptible to florfenicol than others. These differences were attributed to the differences in the bacterial target sites suppressed by these antibiotics (Zhang et al. 2015b). Florfenicol belongs to the class of chloramphenicol-type, broad-spectrum antibiotics that suppress protein synthesis via transpeptidase inhibition and further disturb the function of the 50S ribosomal subunit (Ding et al. 2015). Amoxicillin, a representative class of β -lactams, is a broad-spectrum and bacteriolytic antibiotic in the family of aminopenicillin and disrupts cell wall synthesis by suppressing the cross-linking of peptidoglycan by adhesion to penicillin-binding proteins (PBPs) (Hu et al. 2013b). Sulfamethazine is a broad-spectrum sulfonamide antibiotic that interferes with purine metabolism by inhibiting the synthesis of nucleic acids (Lotti et al. 2012). In Zhang et al.'s study, AnAOB were susceptible to florfenicol and the decreased NRR was attributed to protein synthesis inhibition during AnAOB growth, as shown by the low heme c content. Amoxicillin inhibition might be owing to cell wall damage, which leads to an osmotic pressure imbalance. For sulfamethazine, at concentrations up to 200 mg/L, no inhibitory impacts and no significant damage to cell structures were observed in continuous-flow anammox reactors, mainly because of functional redundancy (Zhang et al. 2015b).

Cao et al. (2016b) also showed that a low level of tetracycline could stimulate the growth of ammonia oxidizers, because the low tetracycline dosage could be used as a nitrogen and carbon source for the growth of ammonia oxidizers (Cao et al. 2016b); however, a low level of ofloxacin did not have any impact on the ammonia oxidizers in dewatered sludge (Xia et al. 2019), which might be due to the distinct structures of the two antibiotics. The amino group in the tetracycline structure may be better for use as a nutrient source for ammonia oxidizers in comparison with ofloxacin, which lacks an amino group. Zhang et al. (2015a) also demonstrated that the number of ammonia oxidizers in activated sludge could

be considerably increased with the addition of spiramycin rather than oxytetracycline (Zhang et al. 2015a).

The performance of partial-nitritation anammox was also investigated in multifarious studies. For example, Yao et al. (2018) evaluated the short- and long-term influences of chlortetracycline (CTC) on the nitritation-anammox process. The IC_{50} of CTC on the nitritation-anammox process was 278.91 mg/L in a short period of 12 h, suggesting that low levels of CTC had no significant impact on nitritation-anammox in a short time, as this antibiotic is a growth inhibitor. The results from continuous experiments revealed that the NRR remarkably declined from 0.61 to 0.25 kg N/m³ d within 14 days on addition of 60 mg/L CTC owing to reduction in the relative abundances of AOB and AnAOB in the reactor. Although the inhibition of nitritation-anammox performance by CTC was irreversible, with the addition of fresh anammox sludge into the reactor, the NRR recovered to 0.09 ± 0.03 kg N/m³ d (Yao et al. 2018).

The effect of different dosages of ciprofloxacin (0, 100, and 350 ng/L) on the performance of a partial-nitritation bioreactor was also investigated. According to the results, at a concentration of 100 ng/L ciprofloxacin, the system achieved a more efficient removal of ammonium than the desired 50% ammonium-50% nitrite, suggesting the acclimatization of the microbial communities to low dosages of this antibiotic (Gonzalez-Martinez et al. 2014). Ciprofloxacin has been reported to bind strongly to bio-solids because of its high octanol-water partition coefficient (Wu et al. 2009; Wunder et al. 2011). This raises the possibility of antibiotic elimination in the bioreactor as a result of the sorption characteristics of the biomass, even though a biotransformation process and the ability of some antibiotic degrader microorganisms such as *Commamonas* sp., which consume ciprofloxacin as a carbon source, cannot be dismissed. However, high concentrations of ciprofloxacin (350 ng/L) exerted an inhibitory impact on the microbial communities (e.g., a significant reduction in AOB) in a partial-nitritation bioreactor, leading to a considerable reduction in ammonium oxidation by 70% during the first few days. When the system became stable, ammonium conversion was restored, although the final performance was 30% lower than that at the start of the experiment (Gonzalez-Martinez et al. 2014), indicating that the addition of pharmaceuticals affects the structure of granular biomass (Rodriguez-Sanchez et al. 2017) such that the presence of antibiotics creates more compact granules. Besides, the addition of ciprofloxacin to the system led to a decrease in biomass by about 40% for 100 ng/L and 60% for 350 ng/L, although at the end of the test, the biomass

had increased by about 5% for the lower level and 80% for the higher level (Gonzalez-Martinez et al. 2014).

The results of some studies regarding antibiotic impacts on nitrogen removal performance in the anammox process are shown in Table 1.

To date, few experiments have been conducted to assess the individual and interactive inhibitory influences of antibiotics on anammox performance, while combined antibiotics usually have a more severe impact than those of individuals. These effects can be worsened with increasing concentration and exposure time and can be recoverable or irrecoverable. For instance, Ghosh et al. (2009) observed no impact on ammonia oxidation for individual antibiotics, including three macrolides: azithromycin, clarithromycin, and roxithromycin; five sulfonamides: sulfadimethoxine, sulfadiazine, sulfamerazine, sulfamethoxazole, and sulfamonomethoxine; five quinolones: ciprofloxacin, enrofloxacin, levofloxacin, nalidixic acid, and norfloxacin; and others: tetracycline, lincomycin, salinomycin, and trimethoprim (below 0.05 mg/L), although antibiotics in mixed condition revealed a stronger effect on bacterial ammonia oxidation even at the same dosage (Ghosh et al. 2009). Sguanci et al. (2017) observed a synergistic impact for the joint toxicity of enrofloxacin and doxycycline on anammox activity at concentrations of 25, 50, and 75 mg/L of both antibiotics for 24, 48, and 72 h of exposure, which deteriorated with higher dosages and longer exposure time (Sguanci et al. 2017).

Synergistic interactions between heavy metals and antibiotics have also been recorded in several studies. Based on their concentrations, heavy metals can be inhibitory or even toxic in biochemical reactions (Şengör et al. 2009). This impact is primarily owing to the chemical interaction between heavy metals and intra- and extracellular enzymes, affecting the structure and activity of enzymes (Li and Fang 2007).

Fan et al. (2019) demonstrated that the joint toxicity of tetracycline and zinc with a total dosage of ≥ 3 mg/L led to remarkable decreases in the nitrogen removal performance, suggesting the presence of a synergistic interaction between zinc and tetracycline; about 30 d was required for performance to recover after the reduction of both inhibitor doses, whereas it could quickly return to the previous level within 10 d from the shock of high concentrations of tetracycline. This suggested that the joint inhibition altered the microbial structure, which plausibly required more time to restore (Fan et al. 2019).

Zhang et al. (2020b) reported that 1 mg/L copper oxide nanoparticles (CuO NPs) and OTC increased the ammonia removal efficiency (ARE) from 62.9% (control) to 68.9 and 76.6%, respectively. However, the joint effects of CuO NPs

and OTC significantly suppressed the partial nitrification and diminished the ARE to 51.0%. This was owing to the formation of a complex between CuO NPs and OTC that disturbed the metabolic activity and suppressed the cell growth (Zhang et al. 2020b). A similar result was observed in the partial nitrification process when it was exposed to a combination of CuO NPs and sulfamethoxazole, suggesting synergetic suppression by these two inhibitors (Zhang et al. 2020a). It was speculated that the composite compound formed between CuO NPs and the antibiotic could readily diffuse into the microbial cell, and then chemically bind to enzymes, which subsequently could disrupt the metabolic activity and suppress the microbial growth in the PN system.

Synergistic joint toxicity was also reported between roxithromycin and Cu(II) (Guo et al. 2016; Zhang et al. 2012) as well as copper nanoparticles and OTC (Cheng et al. 2020). The addition of combined Cu(II)/OTC was also found to cause considerable nitrite accumulation and worsen the settling features of granular sludge in the anammox process (Zhang et al. 2016b). However, Yang and Jin (2012) observed that the joint toxicity of oxytetracycline and copper (II) on an anammox mixed culture was antagonistic due to OTC-metal ion complexes (Yang and Jin 2012). These conflicts may be attributed to variations in the experimental biomass and in the kind of antibiotic applied.

Although the activity and performance of an anammox system were initially stressed by exposure to copper (II) and OTC for a long period of time (about 200 days) with respective concentrations of 0.5–5 and 6–50 mg/L, they were recoverable, and the restoration process required a relatively short period of time (51–56 days) (Zhang et al. 2013a).

Xing and Jin (2018), while examining the individual and interactive inhibitory short-term effects of copper (II), zinc (II), sulfamethazine (SMZ), and oxytetracycline (OTC) on the partial nitrification (PN) of mixed cultures in the nitrogen removal processes, showed that by increasing the content of heavy metals and antibiotics, the specific respiration rates of PN sludge would be reduced. The joint toxicities of heavy metals (Zn^{2+} and Cu^{2+}) and antibiotics (OTC and SMZ) in the PN mixed culture were mainly synergistic, with the exception of an antagonistic interaction between Zn^{2+} and Cu^{2+} . In joint toxicity tests, the importance of the inhibitory impact of Zn^{2+} , Cu^{2+} , OTC, and SMZ on the activity of nitrifying bacteria varied in decreasing order according to $\text{SMZ} > \text{Cu}^{2+} > \text{Zn}^{2+} > \text{OTC}$. Furthermore, all experiments showed that inhibition increased with increasing exposure time. The probable reasons might be that these inhibitors were initially accumulated on the surface of the PN sludge and then adhered to extracellular

Table 1: Impact of antibiotics on nitrogen removal performance.

Antibiotic	Concentration (mg/L)	Mode of action	Exposure time	NRR inhibition	TNRE inhibition	Recovery after antibiotic termination	References
Erythromycin	10	Short-term	48 h	46%	–	–	Alighardashi et al. 2009
	≥20	Short-term	48 h	≥76%	–	–	
Erythromycin	0.001	Long-term	35–64 d	11.76%	14.35%	After 30 d	Zhang et al. 2019c
	1	Long-term	65–94 d	19.41%	22.63%		
	10	Long-term	95–124 d	41%	40%		
	50	Long-term	125–154 d	50%	50%		
	100	Long-term	155–184 d	51%	51%		
Spiramycin	0.5	Long-term	1–150 d	No inhibition	No inhibition	–	Jing-Wu et al. 2020
	1	Long-term	151–170 d	45%	36.5%	After 15 d	
	3	Long-term	196–215 d	45%	35.4%	After 10 d	
	5	Long-term	225–245 d	17%	17.3%	Quickly	
Kitasamycin	Real wastewater	Short-term	5 h	–	No inhibition	Recovery after reinoculation	Tang et al. 2011
		Short-term	10 d	–	No inhibition		
		Long-term	35 d	–	42.9–75.1%		
		Long-term	170 d	–	64.1–77.1%		
Tetracycline	5	Long-term	120 d	–	60%		Du et al. 2018a
Tetracycline	0–0.3	Long-term	1–70 d	No inhibition	No inhibition	120 < d < 200	Fan et al. 2019
	0.5	Long-term	71–90 d	10%	20%	–	
	1	Long-term	91–120 d	55%	57.2%	After 10 d	
	1.5	Long-term	211–258 d	34%	42%	–	
Oxytetracycline	1	Long-term	21 d	3%	10%	After 90 d	Shi et al. 2017
	2	Long-term	184 d	78%	78.5%		
Oxytetracycline	2	Long-term	30 d	No inhibition	No inhibition	–	Zhang et al. 2018b
			72–108 d	2%	4%		
			109–138 d	30%	27%		
			139–160 d	60%	70%		
Chlortetracycline	20–800	Short-term	Several h	No inhibition	No inhibition	No recovery	Yao et al. 2018
	60	Long-term	71–84 d	9%	25%		
	20	Long-term	85–94 d	46%	52%		
Tetracycline hydrochloride	0.1–1	Long-term	243 d	–	No inhibition	–	Zhu et al. 2017
	1–2	Long-term	243–275 d	–	33%		
Sulfamethoxazole	0.1–1	Long-term	243 d	–	No inhibition	–	Zhu et al. 2017
	1–2	Long-term	243–275 d	–	33%		
Sulfamethazine	5	Long-term	14–18 d	No inhibition	No inhibition	–	Zhang et al. 2015b
	20	Long-term	19–27 d	2%	No inhibition		
	30	Long-term	28–50 d	2%	No inhibition		
	80	Long-term	51–65 d	17%	15%		
	100	Long-term	66–80 d	15%	9%		
	200	Long-term	81–92 d	28%	18%		

Table 1: (continued)

Antibiotic	Concentration (mg/L)	Mode of action	Exposure time	NRR inhibition	TNRE inhibition	Recovery after antibiotic termination	References
Amoxicillin	10	Long-term	14–26 d	No inhibition	No inhibition	-	Zhang et al. 2015b
	30	Long-term	27–41 d	2%	57.8%		
	60	Long-term	42–57 d	No inhibition	No inhibition		
	80	Long-term	58–80 d	40%	38%		
	150	Long-term	81–92 d	60%	52%		
Norfloxacin	0.001–50	Long-term	30–154 d	27.5%	-	After 30 d	Zhang et al. 2018c
	100	Long-term	155–184 d	53.4%	-		
Florfenicol	10	Long-term	14–26 d	No inhibition	No inhibition	After 22 d	Zhang et al. 2015b
	20	Long-term	27–30 d	36%	37%		
	15	Long-term	31–52 d	58%	34%		
	10	Long-term	53–60 d	38%	30%		
	5	Long-term	61–69 d	56%	53%		

-, not mentioned.

or intracellular enzymes, and eventually led to the inactivation of ammonia oxidation and electron transfer processes (Xing and Jin 2018).

A mixture of azithromycin, norfloxacin, trimethoprim, and sulfamethoxazole (13, 2, 10, and 5 mg/L, respectively) was found to cause a rapid decrease in nitrogen removal efficiency in an anaerobic digestion reactor, reaching a value of 15–20% at days 71–77, along with a deep alteration in the bacterial community, diversity, and structure of the granular biomass. However, as time passed, the nitrogen removal of the system gradually increased, until it remained in the range of 40–50% from day 95 to day 120 (Rodriguez-Sanchez et al. 2017). The impact of the aforementioned antibiotics in a partial nitrification bioreactor was also shown to cause a significant reduction in ammonium oxidation efficiency (from 50 to 5%) and in biomass concentration, although the ammonium oxidation efficiency could recover to around 30% after antibiotic adaptation (Gonzalez-Martinez et al. 2018a).

2.4 Effect on specific anammox activity

The impact on the anammox activity highly depends on the type of anammox sludge, the inoculation biomass, the operation conditions, and the presence of inhibitors. Specific anammox activity (SAA) can be calculated as follows, which is applied to reflect the AnAOB bioactivity:

$$SSA = \frac{([TN]_{\text{initial}} - [TN]_{\text{ending}}) \times V_{CT}}{m_{\text{biofilm}} \times t} \quad (5)$$

where t : retention time, TN: Total nitrogen (TN) was calculated as the sum of NH_4^+ -N, NO_2^- -N, and NO_3^- -N, CT: centrifuge tube, and m : mass.

The presence of antibiotics can completely or partially inhibit the SAA depending on the level and kind of antibiotic as well as the retention time. The antibiotic inhibition might be reversible or irreversible depending on the level of suppression. For example, Lotti et al. (2012) found that the inhibiting impact of exposure to sulfathiazole and oxytetracycline on the SAA increased with higher dosages and longer exposure time. The results of short-term exposure indicated an insignificant loss of activity after 24 h at dosages up to 100 mg/L of oxytetracycline ($\text{IC}_{50} = 1,100$ mg/L) and sulfathiazole ($\text{IC}_{50} = 650$ mg/L), while after 14 days of exposure to 100 mg/L of oxytetracycline and sulfathiazole, the SAA reduced to 75 and 50% relative to the control, respectively (Lotti et al. 2012). Sulfonamides and tetracyclines impose a biostatic impact on bacteria, suppressing the bacterial synthesis of nucleic acids and proteins, respectively (Thiele-Bruhn and Beck 2005). The probable

reason for the stronger inhibitory impact of sulfathiazole than that of oxytetracycline might be that the sorption capability of tetracyclines is higher than that of sulfonamides (Petrovic et al. 2007), and strong adsorption leads to a smaller and delayed antibiotic effect on microorganisms (Thiele-Bruhn and Beck 2005).

However, even short-term exposure to low concentrations of antibiotics could affect the anammox process. For instance, Phanwilai et al. (2020) reported that the addition of 5–100 mg/L chloramphenicol (CAP) inhibited anammox reactions in both suspended- and attached-growth reactors in short-term studies, in which the higher CAP levels led to greater suppression over this time, suggesting that even low CAP levels would influence anammox activities, but the inhibition was almost completely reversible in both systems. In long-term investigations, with daily additions of 6 mg/L CAP, the anammox activities decreased to baseline levels of 18 and 17% on day 41 and 27 in both systems, respectively, in which the suppression was irreversible upon fresh nutrient loading without CAP addition in both reactors (Phanwilai et al. 2020). Chloramphenicol is a bacteriostatic antibiotic due to its ability to inhibit protein synthesis, but it may also be bactericidal at high concentrations or against highly sensitive microorganisms. Chloramphenicol is highly lipid-soluble, enabling it to pass through the bacterial cell membrane. It stops bacterial growth by adhering to the bacterial ribosome (blocking peptidyl transferase) and suppressing protein synthesis (Maddison et al. 2008; Moffa and Brook 2015). Nevertheless, with lower daily addition of CAP (100–1000 µg/L), the anammox activities in both reactors stayed stable for two weeks (Phanwilai et al. 2020), indicating bacterial adaptation through either the expression of resistance genes (Walters et al. 2003) or alterations in bacterial communities (Theriot et al. 2014) when exposed to low levels of antibiotics.

Zhang et al. (2014) also reported that OTC shock (155–1731 mg/L) caused SAA and heme c content to decrease by 1.4% and 17.6–29.4%, respectively, suggesting that OTC stress affected the sludge properties and the process stability (Zhang et al. 2014).

In terms of antibiotic type, Sguanci et al. (2017) found that doxycycline and enrofloxacin had stronger short-term inhibitory impacts on anammox activity than that of tiamulin, with estimated IC_{50} values of 920, 665, 157 mg/L for tiamulin, doxycycline, and enrofloxacin, respectively; besides, the long-term toxicity of doxycycline was observed to be more severe than that of enrofloxacin due to the faster mechanism of action (Sguanci et al. 2017). Alvarino et al. (2014) also highlighted the susceptibility of anammox bacteria to doxycycline, indicating the more toxic nature of

doxycycline. In this study, exposure to 100 mg/L doxycycline diminished the anammox activity by 47.6% (Alvarino et al. 2014).

The short- and long-term effects of NOR and erythromycin (ERY) in trace concentrations (1 µg/L) on anammox biofilms were assessed by Zhang et al. (2019d). Their findings revealed that short- and long-term exposure to NOR contributed to decreases of 2 and 30% in SAA, indicating the inhibition by NOR at trace concentrations. These results might owe to the high susceptibility of AnAOB to NOR, although short-term exposure had an insignificant impact. However, 1 µg/L ERY very slightly affected the anammox process for both short- and long-term exposure. This discrepancy could be explained by the distinct activities of the two antibiotics in wastewater and the generation of ARGs. It was reported that the long-term effect of NOR could not lead to the evolution of any ARGs in anammox systems to protect the cells by resisting or decomposing the antibiotic, while ERY induced amplification of two ARGs, *ermB* and *mphA*. Both genes targeted the ERY antibiotic, in which *ermB* encoded the defense mechanism to withstand ERY inside the cell, and *mphA* could deactivate ERY (Zhang et al. 2019d).

However, the anammox sludge may be restored after the disappearance of antibiotics. For instance, Shi et al. (2017) showed that the addition of 2 mg/L OTC resulted in a notable and synchronous decrease in SAA of 81.3% and heme c content of 50.1%, which strongly suppressed anammox activity. However, after the elimination of OTC, the anammox sludge recovered, indicating that the activity could be restored even after OTC stress (Shi et al. 2017).

Exposure to 0.001–50 mg/L norfloxacin (NOR) led to a reduction in SAA from 10.84 to 7.56 mg h⁻¹ g⁻¹ SS within 30 d, and then an enhancement of SAA up to 11.01 mg h⁻¹ g⁻¹ SS due to the acclimation of AnAOB to NOR, and also the successful resistance of the anammox biofilm to the low levels of NOR. However, as the NOR level increased to 100 mg/L, the SAA significantly diminished to 5.18 mg h⁻¹ g⁻¹ SS during 30 d, indicating the strong inhibitory effect of NOR on autotrophic nitrogen removal bacteria. After termination of NOR feeding, the SAA rose to 9.86 mg h⁻¹ g⁻¹ SS, implying the recovery of bioactivity in the absence of NOR and a notable self-healing capability of anammox systems (Zhang et al. 2018c).

However, sometimes the antibiotic inhibition of anammox activity is irrecoverable. For example, Noophan et al. (2012) reported that the anammox activity was thoroughly inhibited via daily addition of 5 ± 3.5 mg/L of OTC to an anammox sequencing batch reactor within five weeks

due to a remarkable decrease in the population of the anammox culture (Noophan et al. 2012).

Fernandez et al. (2009) indicated that chloramphenicol dosages of 250, 500, and 1000 mg/L resulted in declines in SAA by 40, 60, and 80%, respectively, in batch tests. The SAA decreased by 30, 40, 60, 60, and 80% with tetracycline hydrochloride concentrations of 100, 200, 250, 500, and 1000 mg/L, respectively. According to the data, both antibiotics strongly inhibited SAA, and SAA inhibition increased as the antibiotic dosage increased. At lower antibiotic dosages, it was shown the inhibitory effects of tetracycline hydrochloride ($EC_{50} = 94$ mg/L) were stronger than those caused by chloramphenicol ($EC_{50} = 420$ mg/L); however, at concentrations higher than 500 mg/L, the impacts of both compounds were similar. In a long-term assay, the SAA of the biomass declined by 80% due to exposure to 20 mg/L chloramphenicol. Similar results were found when 50 mg/L of tetracycline hydrochloride was continuously fed, in which SAA decreased by 60%. It was also demonstrated that the inhibition by chloramphenicol is reversible and that the system can restore its SAA without a reinoculation, although the recovery of SAA up to 59% of its primary value required 2 months. However, since long-term exposure to tetracycline hydrochloride led to the deactivation of the biomass, the recovery of SAA in the reactor required reinoculation (Fernandez et al. 2009).

Therefore, both short- and long-term inhibition tests have demonstrated that antibiotics can exert inhibitory impacts on anammox performance, with the exception of a few studies reporting no significant effect or even stimulatory impacts; however, the inhibition could be recoverable depending on the deactivation level.

In terms of combined toxicity, Zhang et al. (2019a) showed that the SAA was inhibited when the oxytetracycline (OTC) or sulfamethoxazole (SMX) or OTC + SMX dosage was raised from 0.1 to 0.5 mg/L, in which the highest suppression of the SAA was observed at 0.5 mg/L OTC + SMX, indicating the existence of a synergistic interaction between OTC and SMX. However, as the concentrations were raised from 0.5 to 1.0 mg/L, the SAA enhanced to some extent, suggesting that the anammox bacteria could tolerate higher levels of antibiotics (Zhang et al. 2019a). A synergetic impact on the anammox biomass with a 90% reduction in SAA was also observed when a mixture of *p*-nitrophenol, *o*-cresol, and quinoline (8 mg/L of each substance) was applied (Ramos et al. 2015).

Table 2 shows the results of some studies that evaluated the inhibitory effects of antibiotics on anammox activity.

2.5 Effect on heme c and EPS contents

Heme c is a component of some enzymes, which is abundant in anammox bacteria cells and involved in energy metabolism pathways such as hydrazine synthesis (HZS), hydroxylamine oxidoreductase (HAO), and hydrazine oxidase (HZO). Heme proteins have been reported to comprise 20% of the cellular protein in anammox bacteria (Jetten et al. 2009). Heme c plays a crucial role in the storage and transfer of electrons during the anammox process (Kleingardner and Bren 2015); therefore, the heme c content is typically considered as an indicator of the anammox activity (Ma et al. 2019).

The content of heme c is associated with the anammox activity, such that anammox sludge with high activity has a bright red color (Zhang et al. 2014). It was shown the presence of antibiotics can affect heme c content in the anammox process and that higher levels of antibiotics may have more serious impacts. For instance, Yang et al. (2013) stated that the presence of OTC caused the color of the sludge in the anammox system to turn black, representing a remarkable inhibition of anammox activity owing to the loss of heme c (Yang et al. 2013).

It was reported that the heme c content of an anammox reactor exposed to low dosages of TC was initially augmented, but then diminished as the TC level was raised. A good correlation between the heme c content and anammox abundance was also observed, demonstrating that the heme c content is a potential indicator for determining the activity of anammox microorganisms (Meng et al. 2019).

Antibiotics in combination may exert stronger impacts on heme c. For instance, it was shown that as the concentrations of OTC and SMX increased in separate experiments, the heme c content of anammox granules decreased. However, the response of heme c content to OTC + SMX was varied: 0.5 mg/L OTC + SMX promoted the heme c content, while 1.0 mg/L OTC + SMX remarkably reduced the heme c content. These findings might be because of the higher resistance of anammox consortium to OTC + SMX than OTC and SMX (Zhang et al. 2019a).

Nonetheless, the heme c content can be recovered after a long period of time. For example, although the introduction of Cu and OTC slightly reduced the heme c content due to deceleration of AnAOB growth rates, after long-term acclimation to the presence of Cu and OTC, the heme c content of an anammox reactor gradually elevated to 1.2-fold more than the initial content, implying the ability of AnAOB to grow normally under OTC + Cu stress with the help of acclimation (Zhang et al. 2016b).

Table 2: Antibiotic inhibition of anammox activity.

Antibiotic	Type	Concentration (mg/L)	Mode of action	Exposure time	Activity inhibition	References
Chloramphenicol	Chloramphenicol	20	Batch	–	(36 ± 5)%	Van de Graaf et al. 1995
Chloramphenicol	Chloramphenicol	200	Batch	–	(a) For the first 3 days: (98 ± 2)% (b) After the first 3 days: (68 ± 10)%	Van de Graaf et al. 1995
Chloramphenicol	Chloramphenicol	1	Batch	–	No inhibition	Dapena-Mora et al. 2007
Chloramphenicol	Chloramphenicol	5, 10, 20, 50, 100	Batch (attached)	7 h	16–27%	Phanwilai et al. 2020
Chloramphenicol	Chloramphenicol	5, 10, 20, 50, 100	Batch (suspended)	7 h	8–60%	Phanwilai et al. 2020
Chloramphenicol	Chloramphenicol	6	Continuous (attached)	27 d	82%	Phanwilai et al. 2020
Chloramphenicol	Chloramphenicol	6	Continuous (suspended)	41 d	85%	Phanwilai et al. 2020
Chloramphenicol	Chloramphenicol	250	Batch	–	40%	Fernandez et al. 2009
		500			60%	
		1000			80%	
Chloramphenicol	Chloramphenicol	20	Continuous	38 d	80%	Fernandez et al. 2009
Penicillin	β-lactam	1	Batch	–	17% (36 ± 10)%	Van de Graaf et al. 1995
		100				
Ampicillin	β-lactam	400	Batch	–	71% (94 ± 4)%	Van de Graaf et al. 1995
		800				
Chlortetracycline	Tetracycline	20–800	Batch	12 h	IC ₅₀ = 278.91 mg/L	Yao et al. 2018
Chlortetracycline	Tetracycline	60	Continuous	14 d	60%	Yao et al. 2018
Oxytetracycline	Tetracycline	2	Continuous	120 d	40%	Zhang et al. 2018b
Oxytetracycline	Tetracycline	100–1000	Batch	30 h	0–50%	Lotti et al. 2012
Oxytetracycline	Tetracycline	1	Continuous	11–31 d	22%	Shi et al. 2017
		2		32–52 d	81.3%	
Oxytetracycline	Tetracycline	100	Continuous	14 d	25%	Lotti et al. 2012
		500		14 d	50%	
Oxytetracycline	Tetracycline	5	Continuous	35 d	Complete inhibition	Noophan et al. 2012
Oxytetracycline	Tetracycline	2	Continuous	46 d	91%	Zhang et al. 2019b
				108 d	99%	
				138 d	74%	
				160 d	83%	
Tetracycline hydrochloride	Tetracycline	100	Batch	–	30%	Fernandez et al. 2009
		200			40%	
		250			60%	
		500			60%	
		1000			80%	
Tetracycline hydrochloride	Tetracycline	50	Continuous	70 d	60%	Fernandez et al. 2009
Doxycycline	Tetracycline	5, 10, 50, 100	Batch	24 h	5–17%	Sguanci et al. 2017
				48 h	5–22%	
Doxycycline	Tetracycline	50, 100	Continuous	9–12 d	20–40%	Sguanci et al. 2017

Table 2: (continued)

Antibiotic	Type	Concentration (mg/L)	Mode of action	Exposure time	Activity inhibition	References
Doxycycline	Tetracycline	50 100 250 400	Batch	–	No inhibition 47.6% 65.1% 83.2%	Alvarino et al. 2014
Sulfathiazole	Sulfonamide	100–1000	Batch	30 h	0–70%	Lotti et al. 2012
Sulfathiazole	Sulfonamide	100 500	Continuous	14 d 14 d	50% 80%	Lotti et al. 2012
Erythromycin	Macrolide	0.001 1 10 50 100	Continuous	35–64 d 65–94 d 95–124 d 125–154 d 155–184 d	No inhibition No inhibition 15% 30% 32%	Zhang et al. 2019c
Erythromycin	Macrolide	0.001	Batch	4 h	1.7%	Zhang et al. 2019c
Erythromycin	Macrolide	0.001	Continuous	30 d	1.7%	Zhang et al. 2019c
Enrofloxacin	Fluoroquinolone	25, 50, 100, 200	Batch	24 h 48 h	5–55% 5–60%	Sguanci et al. 2017
Enrofloxacin	Fluoroquinolone	50 100 200	Continuous	9–12 d	10–1% 40–30% 55–80%	Sguanci et al. 2017
Norfloxacin	Fluoroquinolone	0.001	Batch	4 h	2.2%	Zhang et al. 2019c
Norfloxacin	Fluoroquinolone	0.001	Continuous	30 d	30%	Zhang et al. 2019c
Norfloxacin	Fluoroquinolone	0.001–50 100	Continuous	30 d	30% 52%	Zhang et al. 2018c

–, not mentioned.

Extracellular polymeric substances (EPS) secreted by microorganisms in response to harsh environmental stress chiefly comprised polysaccharides, proteins, humic acids, nucleic acids, and other organic macromolecules (Hou et al. 2017), and can affect activated sludge properties (Sheng et al. 2010). The characteristics and structures of these biomacromolecules are extremely complex owing to their different functional groups, including hydroxyl, carboxyl, phosphate groups, aldehyde, amide, and benzyl, which are likely to interact with each other and join these macromolecules, resulting in creating more intricate spatial structures (Jia et al. 2017). These functional groups can also lessen the exterior toxicity through redox or adsorption reactions with exogenous toxic substances (Pi et al. 2019; Yan et al. 2017; Zhang et al. 2018a).

EPS have a considerable impact on the physicochemical characteristics of microbial aggregates, including settling capability, stability (Yang and Li 2009), and adsorption attributes (Hu et al. 2007). Furthermore, the elimination and migration of antibiotics can be significantly affected by their adsorption on EPS (Xu et al. 2013). Singh et al. (2010) indicated that reaction with or sorption to EPS could reduce the penetration of some antibiotics through biofilms, leading to decreased exposure of the bacterial cells to the antibiotics (Singh et al. 2010).

Nevertheless, antibiotic exposure has been found to exert impacts on the properties and structure of EPS as well as the level of EPS, in which a low concentration of antibiotics promotes the EPS content while higher dosages of antibiotics reduce the EPS content.

Exposure to a low level of TC was found to lead to the enhancement of EPS contents (Meng et al. 2019). The increased generation of EPS, recognized as the EPS-based defense, serves as a protective “cocoon” to postpone the permeation of toxic materials into the cell body, thus preventing antimicrobials from reaching their targets (Li and Yu 2014; Zhang et al. 2015b, 2018d). However, high levels of antibiotics damage cell structures in anaerobic bioreactors, contributing to cell lysis and a decrease in EPS content (Højby et al. 2010; Li and Yu 2014; Zhang et al. 2015b, 2016b, 2018d). For example, with the increase of TC dosage to 1000 µg/L, the EPS content remarkably dwindled, illustrating the inconstancy of the anammox consortium, specifically the systemic instability (Meng et al. 2019).

The EPS content in anammox reactors exposed to sulfamethazine (SMZ) and sulfadimethoxine (SDM) was observed to rise when the level of SMZ or SDM was lower than 3 mg/L as a response to stress conditions, while it diminished when the SMZ or SDM level was ≥ 5 mg/L, indicating the instability of the granular sludge owing to

disruption by cell lysis and subsequent reduction of the sludge resistance in anammox reactors (Du et al. 2018b).

Liu et al. (2019a) observed that the addition of tetracycline reduced the hydrophobicity of the microenvironment, which consequently facilitated the extensional degree of peptide chains in proteins (Liu et al. 2019a). A previous investigation showed that the hydrophobicity of proteins is a critical factor in promoting the aggregation of AnAOB and maintaining a stable granule structure (Hou et al. 2015). As the tetracycline dosage was augmented (≥ 50 mg/L), the peptide chains of proteins expanded to diminish the hydrophobicity, which decreased the granular stability, indicating that the binding sites were entirely occupied and the peptide chain structure was considerably damaged. Besides, the humic acids in EPS can interact with tetracycline, probably owing to the presence of $-\text{COO}$ groups as binding sites (Liu et al. 2019a).

Zhang et al. (2018c) investigated EPS contents, including protein (PRO) and polysaccharide (PS), under NOR stress. PS and PRO are hydrophilic and hydrophobic components within EPS, respectively. A decrease in PRO/PS contributes to poor hydrophobicity, which might decrease the flocculation capacity of sludge. According to the results, the PRO significantly decreased by 69% because the addition of NOR restricted PRO secretion, then it slowly increased to 2.7-fold higher than the initial value due to the acclimatization to NOR and the secretion of more EPS, mostly PRO, to guard the microbial cells. However, as the NOR level was enhanced beyond the resistance capacity of the biofilm, the PRO contents in EPS declined because of AnAOB bioactivity suppression, but upon the termination of NOR feeding, the PRO level rose up to higher than the initial level. PS in EPS also showed the same trend as that of PRO in each phase, whereas the PRO/PS ratio showed an opposite trend, suggesting that PRO in the anammox consortia had more important effects on resisting NOR antibiotics (Zhang et al. 2018c).

Li et al. (2020a) also reported that NOR (0–30 mg/L) promoted the production of EPS in activated sludge, which surrounded microorganisms and decreased NOR toxicity. Under the influence of norfloxacin, the PRO content was found to be higher than the PS content (Li et al. 2020a). The elevation of PRO can reduce the electrostatic repulsion among the microbial cells by enhancing the hydrophobicity and the net-negative surface charge of the cell surface (Basuvaraj et al. 2015).

The addition of TC (10–50 mg/L) was also observed to increase the contents of PRO and PS in EPS. Since TC could disrupt the integrity of the cell membrane, some intracellular PRO-like and PS-like compounds were released to the outside of the cell as the TC concentration increased,

leading to the increment of PRO and PS contents in EPS. Additionally, owing to the presence of some functional groups such as carboxyl and amino, PRO and PS could bind TC and the interaction could reduce the toxicity of TC to bacteria. These also induced bacteria to secrete more PRO and PS in EPS. The PRO/PS ratios in EPS increased with the increase in TC concentration, demonstrating that the defense mechanism of EPS against TC was predominated by proteins (Wang et al. 2018).

Therefore, generally, the interaction of antibiotics with the EPS matrix leads to overall biofilm resistance, but the resistance is limited and depends on the antibiotic mode of action, level, and exposure time. It was also indicated that antibiotics mainly interacted with the PRO in EPS through chemical binding (Xu et al. 2013).

It was observed that EPS contents in anammox reactors decreased considerably at 0.1 mg/L of OTC, SMX, and OTC + SMX, then increased in the presence of 0.5 mg/L of the aforementioned antibiotics, in which the impact of OTC on EPS was greater than that of SMX and OTC + SMX, but eventually declined with 1.0 mg/L of the aforementioned antibiotics, demonstrating that the response of EPS to antibiotic interference was limited (Zhang et al. 2019a).

2.6 Effect on microbial community

An anammox granule is an aggregate composed of large numbers of heterotrophic denitrifiers and fermentative bacteria in addition to AnAOB. Put another way, an anammox granule is an ecological niche with a specific metabolic network. Since anammox is a reaction dominated by AnAOB, the number of AnAOB is associated with the operational performance of the anammox process (Strous et al. 1999b). Therefore, the maintenance of the population of AnAOB, as the key functional bacteria in anammox reactors, is essential for achieving stable performance (Zhang et al. 2019d).

Anammox bacteria are associated with the bacterial phylum *Planctomycetes* (Strous et al. 1999). *Planctomycetes* are distinguished by characteristics that are unusual among bacteria, including a complicated system of internal membrane invaginations (Boedeker et al. 2017; Lage et al. 2013), sterol synthesis (Pearson et al. 2003), membrane coat-like proteins (Santarella-Mellwig et al. 2010), and crateriform structures of the outer membrane (Boedeker et al. 2017; Fuerst 1995). Additionally, anammox bacteria are well-known for their special membrane ladderane lipids that make the anammoxosome membrane, inside which the anammox process is performed, more highly impermeable than other known non-anammox

bacterial membranes (Roose-Amsaleg and Laverman 2016; van Niftrik et al. 2004). Moreover, the outer membrane serves as an extra protective measure that significantly restricts the passive permeation of charged, hydrophobic, and hydrophilic molecules (Nikaido 2003; Pagès 2004).

Therefore, anammox bacteria are expected to be more tolerant to antibiotics because of their distinct cell structure, although this relies on the antibiotic mode of action (Strous et al. 1999a). It has been reported that antibiotics present in wastewater can directly put selective pressure on bacteria, leading to alteration of the bacterial community structure (Xiong et al. 2015). Nonetheless, few studies have evaluated the behavior of anammox bacteria in the presence of antibiotics.

Godinho et al. (2019) revealed that all tested *Planctomycetes* were resistant to β -lactams, glycopeptides, and aminoglycosides. According to their findings, antibiotics targeting protein synthesis or DNA replication (except for aminoglycoside) were the most effective against the studied strains, and chloramphenicol, clindamycin, and ciprofloxacin showed the highest effectiveness (Godinho et al. 2019). The resistance of *Planctomycetes* to glycopeptides seems inherent because these molecules are usually incapable of passing the outer membrane in Gram-negative bacteria and consequently cannot reach their target of action (Blair et al. 2015). Resistance to β -lactams was thought to be owing to the lack of peptidoglycan in *Planctomycete* cell walls, an assumption recently confirmed to be incorrect (Jeske et al. 2015; van Teeseling et al. 2015). Thus, resistance mechanisms such as β -lactamase development and/or multidrug-resistance efflux pumps may account for the resistance (Aghnati and Drancourt 2016; Faria et al. 2018).

Anammox bacteria were found to be insensitive to penicillin G-Na. Penicillin G-Na in concentrations ranging from 0 to 100 mg/L did not inhibit anammox bacteria (Van de Graaf et al. 1995). Some other investigations demonstrated that even 1000 mg/L penicillin G did not have inhibitory effects on the activity of anammox bacteria (Güven et al. 2005; Jetten et al. 1998). However, Hu et al. (2013b) found that β -lactam antibiotics could inhibit the anammox bacteria at high concentrations and long exposure times. According to their results, penicillin G (0.5, 1, and 5 g/L) reversibly suppressed the growth of anammox bacterium *Kuenenia stuttgartiensis* in long-term experiments due to the inhibition of the cross-linking of peptidoglycan by binding to penicillin-binding proteins (PBPs), while it did not have any observable impacts on the activity in short-term experiments, which might be because these antibiotics are growth inhibitors (Hu et al. 2013b).

Since the target of the antibiotic chloramphenicol is disturbance of the function of 50S ribosome, anammox bacteria are plausibly vulnerable to chloramphenicol. It was reported that 200 mg/L chloramphenicol could completely inhibit anammox bacteria activity (Van de Graaf et al. 1995); however, other researchers indicated that 0–1000 mg/L chloramphenicol did not suppress the activity of anammox bacteria (Dapena-Mora et al. 2007; Jetten et al. 1998). These discrepancies might be related to the different enrichment conditions used.

Planctomycetes showed a varied sensitivity to aminoglycosides and tetracyclines, which might be related to their mechanism of action (Godinho et al. 2019). Although both antibiotics target the 16S rRNA (A-site), tetracyclines influence the delivery of tRNAs to the A-site while aminoglycosides influence the ensuing translocation of the mRNA-tRNA complex through the ribosome (Wilson 2014). *Planctomycetes* were also observed to have variable resistance to sulfamethoxazole and trimethoprim. These antibiotics suppress DNA synthesis by inhibiting dihydropteroate synthase encoded by the *folP* gene and dihydrofolate reductase encoded by the *folA* gene. Thus, the absence of the *folA* gene in some tested strains may explain their resistance to sulfamethoxazole and trimethoprim (Cayrou et al. 2010). However, quinolone derivatives are considered broad-spectrum efflux pump inhibitors, leading to an increase in the intracellular accumulation of the antibiotic after their introduction to bacterial culture (Mahamoud et al. 2007).

For example, Zhang et al. (2019d) showed that in trace dosages (1 µg/L), NOR could considerably inhibit microbial activity, while ERY had no detectable impacts on microbial activity, suggesting the considerable resistance of AnAOB to ERY, while they were more susceptible to NOR because NOR exposure could not lead to the emergence of ARGs to protect the cell, but ERY could effectively induce ARGs such as *ermB* and *mphA*. Both NOR and ERY led to an increase in the biodiversity through increasing the OTU numbers, while NOR reduced the OTUs related to *Candidatus Kuenenia* from 4.31 to 1.87% (Zhang et al. 2019d).

Previous studies have reported that *Proteobacteria*, especially *Betaproteobacteria*, were dominant in wastewater treatment systems containing OTC (Chen et al. 2019; Liu et al. 2012; Zhang et al. 2018b) or high concentrations of antibiotics (Deng et al. 2012) owing to the high antibiotic resistance of *Proteobacteria* (Goñi-Urriza et al. 2000; Xia et al. 2012) and their ability to consume OTC as a carbon/energy source (Duan et al. 2017).

Therefore, it can be concluded that the degree of anammox performance inhibition is correlated with the type of antibiotic.

Generally, bacteria can tolerate low antibiotic concentrations, but medium and higher antibiotic concentrations can lead to cell death and disappearance of some inadapted microbiota in the anammox reactor. Surveys on the elimination pathway of toxic organics in anammox processes have demonstrated that toxic organics can be expelled from cells via efflux pumping mechanisms (Shi et al. 2017), and can be decomposed by heterotrophic bacteria (Pereira et al. 2014; Zhang et al. 2018c). Moreover, the biomass degradation of AnAOB that contributes to the organic carbon resource or the soluble bacterial products can be favorable for heterotrophic bacteria (Claus et al. 2000). These might explain why bacteria can remain active in the anammox process with exposure to low antibiotic levels.

For instance, the microbial community diversity was reported to increase when exposed to trace levels of TC due to the emergence of tetracycline-resistant denitrification bacteria under exposure to subinhibitory levels of tetracycline, but decrease when exposed to higher levels of TC (Li et al. 2020b; Selvam et al. 2012; Zhang et al. 2013b). Du et al. (2018b), while investigating the long-term effects of sulfamethazine (SMZ) and sulfadimethoxine (SDM) on the anammox process, reported that anammox bacteria could endure and adapt to low concentrations of SDM and SMZ (less than 3 mg/L) because of extracellular polymeric substances. At dosages between 5 and 7 mg/L, SDM had inhibitory impacts on the growth of AnAOB and on the abundance of *Candidatus Brocadia*, which declined from 2.57 to 0.39%. At concentrations ranging from 5 to 9 mg/L, SMZ suppressed the denitrification process much more strongly than SDM, which contributed to higher accumulation of nitrite and nitrate. At the level of 9 mg/L, both SDM and SM strongly suppressed anammox bacteria, which resulted in ammonia accumulation (Du et al. 2018b).

In another study, it was shown that exposure to low TC dosages altered the abundance of anammox bacteria, e.g., an augmentation in *Candidatus Jettenia* abundance occurred from $2.20 \pm 0.97\%$ (0–10 µg/L) to $12.13 \pm 1.66\%$ (100 µg/L). Similarly, the genus *Denitratisoma*, the most abundant denitrification bacteria, also had higher occurrence at a TC concentration of 100 µg/L ($15.60 \pm 6.42\%$) than other TC dosages. These findings demonstrate the capability of these bacteria to tolerate or adapt to low TC exposure. In contrast, exposure to a high TC dosage (1000 µg/L) resulted in a decrease in the abundance of anammox bacteria and denitrifiers ($1.53 \pm 0.64\%$ and $8.18 \pm 0.63\%$, respectively) but an elevated abundance in the nitrifier population ($8.07 \pm 1.21\%$) (Meng et al. 2019). Likewise, in the presence of 100 ng/L ciprofloxacin, the relative abundance of AOB initially declined, then slightly

increased; however, higher concentrations of ciprofloxacin (up to 350 ng/L) eliminated the AOB dominance, in favor of *Comamonas* sp., which has been reported to be ciprofloxacin-resistant (Gonzalez-Martinez et al. 2014).

Interestingly, research showed that inhibition of anammox bacteria by antibiotics could be reduced to some extent. For instance, Zhang et al. (2019c) demonstrated that AnAOB could thoroughly resist low dosages of ERY (≤ 1 mg/L) due to the protection provided by the compact EPS layer. In fact, ERY could be efficiently adsorbed on the EPS layer, postponing the permeation of these antibiotics into the bacterial bodies. However, when the ERY dosage was elevated to 10 mg/L, remarkable inhibition was observed, although the inhibiting trend became slower with the increase in ERY dosage, representing a better survival strategy for AnAOB. It was also shown that the abundance of AnAOB recovered to lower than the initial level after removal of the antibiotic (Zhang et al. 2019c).

Inhibitory substances use distinct modes or act on distinct targets, but their joint toxicities may be independent, additive, synergistic, or antagonistic, making it necessary to consider the joint toxicity of these compounds. For instance, individual acute toxicity tests showed that the IC_{50} values of penicillin G-Na (C), chloramphenicol (E), and kanamycin sulfate (F) were 5114.4 (4946.4–5282.4), 409.9 (333.7–486.1), and 5254.1 (3934.4–6573.8) mg/L, respectively, suggesting that the toxicities varied in the order $E > C > F$. However, the joint acute toxicity of $C + E$, $C + F$, and $E + F$ were 2203.6 (958.8–3448.4), 6970.1 (5585.8–8354.4), and 2968.3 (2604.0–3332.6) mg/L, respectively, suggesting that the joint toxicities of the biocomponents were independent, synergetic, and additive, respectively (Ding et al. 2015).

The inhibition of anammox microorganisms by OTC + SMX was reported to be more severe than that of OTC or SMX separately, indicating a synergistic interaction between OTC and SMX (Zhang et al. 2019a). The synergistic impact of tetracycline and zinc in the anammox process inhibited *Planctomycetes* (represented by *Candidatus Kuenenia*), although it still remained the dominant species. Moreover, *Caldilinea* (affiliating to *Chloroflexi*) was an abundant species during the inhibitory period, representing its potential resistance to both inhibitors. These results indicated that anammox could be suppressed by metals and antibiotics, but it had the ability to eliminate nitrogen from wastewaters containing both of them within a concentration threshold (Fan et al. 2019).

The addition of a mixture of azithromycin, norfloxacin, trimethoprim, and sulfamethoxazole antibiotics was found to cause significant changes in the bacterial community structure of the CANON bioreactor over time, in which the

relative abundance of ammonium oxidizing bacteria *Nitrosomonas* and *Candidatus Brocadiales* decreased from 40 to 3%, while several species appeared, including *Alcaligenes aquatilis*, *Ochrobactrum antropii*, *Paracoccus versutus*, and *Acidovorax ebreus* (Gonzalez-Martinez et al. 2018a).

Regarding the long-term suppression of antibiotics, AnAOB have shown antibiotic resistance, as observed by the restoration of SAA levels over an extended period (Zhang et al. 2015). This antibiotic resistance was mainly ascribed to the elevated EPS content, which was capable of protecting the microorganisms from environmental changes. First, AnAOB were enclosed in a hydrated matrix of PS and PRO, creating a compact layer like a cocoon, which hindered the permeation of these antimicrobial agents into the anammox granule cells. Hence, the EPS-based matrix served as a barrier, preventing antibiotics from reaching their targets. Additionally, the interaction of the antibiotics with the EPS matrix led to overall granule resistance. The binding process took place spontaneously, with hydrophobicity as the driving force (Xu et al. 2013). However, the response and binding ability of AnAOB varied for different classes of antibiotics. These behaviors can be regarded as AnAOB defense strategies to decrease antibiotic uptake. Other conventional mechanisms such as adaptive stress responses, active efflux, the formation of persister cells, and quorum sensing could also contribute to the emergence of antibiotic resistance (Høiby et al. 2010).

Therefore, antibiotics in wastewater may exert a direct impact on antibiotic-resistance genes (ARGs) because bioreactors provide breeding conditions for the selection, metastasis, and spread of ARGs among various bacteria (Aydin et al. 2015; Ma et al. 2011; Munir et al. 2011). The tolerance of AnAOB during extended exposure may cause an increment in the occurrence of resistance genes (Guo et al. 2015). Even a relatively low concentration of antibiotics could lead to the emergence of ARGs in anaerobic SBRs (Aydin et al. 2015).

Generally, microorganisms can gain resistance to antibiotics through different mechanisms: (1) The disturbance of the activity of antibiotics by degrading the antibiotics or replacing the active group; (2) The incapability of antibiotics to be integrated, with modification of the antibiotic target to show endurance; (3) The discharge of antibiotics from the cell by antibiotic efflux pumps, decreasing intracellular antibiotic levels and showing resistance; (4) Other resistance mechanisms, including the development of polysaccharides on the cell membrane to decrease the diffusion of antibiotic into the cell (Chopra and Roberts 2001).

For instance, Shi et al. (2017) reported the presence of the efflux pump antibiotic-resistance genes *tetA*, *tetB*, and *tetC* and the enzymatic modification gene *tetX* in anammox mixed culture under OTC stress. The abundance of *tetA* rose from 1.03 to 2.51%. The occurrence of *tetB* and *tetC* doubled, while there were no significant changes in the occurrence of *tetX*. Hence, it can be concluded that AnAOB resisted the toxicity of OTC via the efflux pumping mechanism. After the discontinuation of OTC, the nitrogen removal capacity of the anammox reactor was restored, and the occurrence of resistance genes decreased slowly (Shi et al. 2017).

Although some studies have evaluated the occurrence and possible transfer of ARGs in wastewater treatment plants (Boopathy 2017; Ghosh et al. 2009; Naquin et al. 2015; Zhang et al. 2009), investigation of the antibiotic resistance, dynamics of ARG transfer in nitrogen removal processes, and nitrogen assimilatory bacteria has not been sufficient.

In conclusion, although it is expected that anammox bacteria should be more resistant to antibiotics because of their specific cell structure, compelling evidence from previous investigations has indicated that anammox activity is considerably inhibited by antibiotics. The presence of high concentrations of antibiotics in wastewater has been found to lead to remarkable changes in microbial communities because of the severe bacteriostatic impacts of the antibiotics, leading to the death of inadaptable AnAOB, although the effects of antibiotic suppression can partly be alleviated after discontinuation of antibiotics. However, at low levels of antibiotics, the activity of anammox bacteria may remain stable. In fact, the bacteria have been assumed to be able to adapt to low antibiotic dosages and regain stable nitrogen removal efficiency after self-adaptation. Additionally, antibiotic inhibition has been demonstrated to increase as the exposure time increases, and long-term antibiotic exposure has been reported to significantly reduce the bacterial diversity and richness. Furthermore, the joint toxicity of antibiotics has been shown to be more severe than that of individual antibiotics because of synergic, antagonistic or additive interactions. In addition, microbial communities may develop and express antibiotic-resistance genes to lessen the adverse impacts of antibiotics.

3 Conclusion

The presence of antibiotics in wastewater has been found to inhibit anammox reactions; hence, assessment of the toxic impacts of antibiotics on microorganisms involved in the anammox process should be given more attention,

which can improve our knowledge of the elimination mechanisms of pollutants through biological treatments and also to improve their removal efficiencies from wastewater. The inhibitory impacts of these substances are related to the anammox species, exposure dosage and time, type of antibiotic, and operating conditions of the anammox process. According to various studies, high levels of antibiotics and prolonged exposure time impose more serious effects on anammox performance. However, after the discharge of antibiotics, anammox performance can be recovered to some degree, with or without sludge addition, which might be hard at times even if the reactor operates at a low antibiotic level within a long recovery period. In fact, AnAOB can tolerate and adapt to low levels of antibiotics in short-term exposure, but a high level of antibiotics can lead to the death of inadaptable AnAOB during long-term exposure, reducing anammox performance. To date, the toxic effects of only a few types of antibiotics in wastewater have been evaluated, including β -lactams, macrolides, sulfonamides, fluoroquinolones, aminoglycosides, and tetracyclines, and these studies mainly focused on the potential inhibitory impacts of individual antibiotics despite the fact that antibiotics do not exist in swine wastes individually, but may be present with many other kinds of antibiotics and noxious contaminants like heavy metals. Therefore, much more research on other types of antibiotics commonly detected in wastewater as well as on their combined toxicity with other inhibitors, including both organic and inorganic toxic substances due to their synergistic and/or antagonistic effects, should be carried out in the future. Besides, a more standardized technique for assessing the impact of environmentally relevant concentrations of antibiotics at chronic levels (simulating in situ conditions) on the anammox process is required to investigate the capacity of anammox bacteria to grow in real conditions. Additionally, owing to the development and distribution of antibiotic-resistance genes in wastewater treatment plants, more research is required to examine the relationships between antibiotics, microorganisms, and antibiotic-resistance genes to reduce the dangers of ARGs in biological treatment processes. It has been found that the interaction of antibiotics with the EPS matrix leads to overall biofilm resistance. However, regarding anammox performance, the relation between EPS and the abundance of ARGs has not been completely addressed. Whether EPS, ARGs, or their interaction lead to the recovery of suppressed performance is not also obvious and requires further evaluation. Moreover, for practical applications, the metabolic and genetic features of anammox bacteria as well as the relationship between the synergy and competition of functional microorganisms in the

anammox sludge need to be scientifically assessed to guarantee the stability of anammox systems.

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