

A review on anammox process for the treatment of antibiotic-containing wastewater: Linking effects with corresponding mechanisms

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HIGHLIGHTS

- Anammox is promising for nitrogen removal from antibiotic-containing wastewater.
- Most antibiotics could inhibit the anammox performance and activity.
- Antibiotic pressure promoted the increase in antibiotic resistance genes (ARGs).
- Antibiotic-resistance mechanisms of anammox bacteria are speculated.

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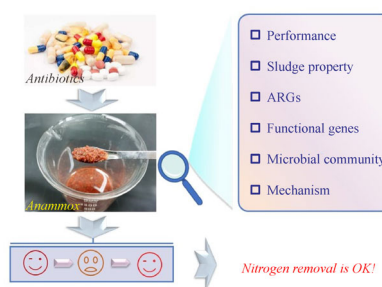
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ABSTRACT

Antibiotic is widely present in the effluent from livestock husbandry and the pharmaceutical industry. Antibiotics in wastewater usually have high biological toxicity and even promote the occurrence and transmission of antibiotic resistant bacteria and antibiotic resistance genes. Moreover, most antibiotic-containing wastewater contains high concentration of ammonia nitrogen. Improper treatment will lead to high risk to the surrounding environment and even human health. The anaerobic ammonium oxidation (anammox) with great economic benefit and good treatment effect is a promising process to remove nitrogen from antibiotic-containing wastewater. However, antibiotic inhibition has been observed in anammox applications. Therefore, a comprehensive overview of the single and combined effects of various antibiotics on the anammox system is conducted in this review with a focus on nitrogen removal performance, sludge properties, microbial community, antibiotic resistance genes and anammox-involved functional genes. Additionally, the influencing mechanism of antibiotics on anammox consortia is summarized. Remaining problems and future research needs are also proposed based on the presented summary. This review provides a better understanding of the influences of antibiotics on anammox and offers a direction to remove nitrogen from antibiotic-containing wastewater by the anammox process.

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1 Introduction

The anaerobic ammonia oxidation (anammox) process is a new wastewater treatment technology that has been rapidly developed in recent years. Till 2015, there were 114 full-scale anammox sewage treatment plants around the world (Ali and Okabe, 2015). The anammox reaction relies on the special physiologic metabolic mechanism of anammox

bacteria and converts nitrite (NO_2^-) and ammonia (NH_4^+) into nitrogen (N_2) under anoxic conditions (Mulder et al., 1995). Compared with traditional nitrification/denitrification processes, no requirements of aeration and exogenous carbon sources makes anammox process can save operating cost, reduce energy demand and greenhouse gas production by more than 60% and 25%, respectively (Tang et al., 2010; Ma et al., 2016; Cogert et al., 2019), which realizes the practical application of carbon biotransformation processes in energy production (Jin et al., 2012; Qu et al., 2019). Moreover, anammox bacteria grow slowly and have low cell yield, so the residual sludge is less,

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which saves the treatment and disposal cost of residual sludge (Chen et al., 2013). To date, the highest reported value of the nitrogen removal rate (NRR) in the laboratory scale anammox up-flow anaerobic sludge bed (UASB) reactors is 74.3–76.7 kg N/(m³·d) (Tang et al., 2011). In addition, some studies may focus on some coupling processes, such as partial nitrification-anammox, partial denitrification-anammox etc. (Johansson et al., 2017; Zhang et al., 2017; Zhang et al., 2019a). Thus, the anammox process has great potential to remove nitrogen by microbial.

Antibiotics have been widely utilized in the medical industry, livestock husbandry and aquaculture, because they have inhibitory effect on the growth, reproduction and metabolism of harmful bacteria (Brown et al., 2006; Liu et al., 2009; Wang et al., 2019b). However, antibiotics cannot be wholly assimilated or metabolized and are finally discharged into surrounding environments in the form of crude drugs or metabolites through urine and feces (Massé et al., 2014). Inevitably, antibiotics residues have been detected in many wastewaters. It was reported that the concentrations of antibiotics are 10⁻⁴ and 10² µg/L in treated municipal sewage and hospital wastewaters, 10⁻¹ and 10⁴ µg/L in industrial wastewater, 10⁻⁴ and 10³ µg/L in raw surface waters from ponds, lakes and rivers (de Cazes et al., 2014), and 10² µg/L in landfill leachate (Topal and Arslan Topal, 2016). Typical antibiotics detected in water include tetracycline (TC), oxytetracycline (OTC), erythromycin (ERY), clarithromycin, metronidazole, sulfamethoxazole (SMX), enrofloxacin, ciprofloxacin (CIP) and norfloxacin (NOR) (Valcárcel et al., 2011; Dafouz et al., 2018). The presence of antibiotics in aquatic environments may increase antibiotic resistance genes (ARGs) that have long environmental persistence in aquatic microorganisms (Ronquillo and Hernandez, 2017). In addition, mobile genetic elements can carry and spread multiple ARGs (Lin et al., 2015). Furthermore, it confirmed that sewage treatment plants had become the main reservoir of ARGs (Xu et al., 2015). Once ARGs enter the drinking water pipeline, they may directly seed on the pipeline biofilm and are hazardous to human health by affecting the safety of tap water (Wan et al., 2019). Thus, ARGs have become a worldwide concern and bring potential threats.

The traditional treatment of antibiotic wastewater mainly includes advanced oxidation technologies such as chlorine (Qiang et al., 2006), Fenton's reagent (Ben et al., 2009), ozone (Wang et al., 2018), and ultraviolet light processes (Hu et al., 2016a). However, biopharmaceutical wastewater and livestock wastewater often contain high concentrations of ammonia nitrogen (Gagné et al., 2006; Huang et al., 2010). Thus, these processes are not efficient for nutrients removal and consume large amounts of energy and even inevitably produce byproducts that cause secondary pollution to the environment. In recent years, anammox has been applied in the treatment of antibiotic-

containing wastewater. Anammox and partial nitrification achieved a good performance in the treatment of piggery wastewaters, which contained large amounts of antibiotics (Suto et al., 2017). However, it has been demonstrated that antibiotics have adverse effects on the nitrogen removal by the anammox process, such as OTC (Shi et al., 2017), ERY (Zhang et al., 2019d), and chloramphenicol (CAP) (Fernández et al., 2009).

Although the anammox process has the advantages of low cost, high efficiency and environment-friendly, an inhibitory phenomenon of antibiotics on anammox has still been observed under various conditions. Furthermore, the influencing mechanisms of antibiotics on anammox bacteria remain unclear. Therefore, this review systematically summarizes the inhibitory effects and corresponding mechanisms of antibiotics on anammox, aiming to provide theoretical guidance for the anammox-based treatment of antibiotic-containing wastewater and the establishment of antibiotic-transmission-blocking technology.

2 Single effects of antibiotics on anammox

2.1 Effects of antibiotics on anammox performance

Most anaerobic microorganisms are affected by the presence of antibiotics (Aydin et al., 2016). Anammox has also been reported to be inevitably interfered by different types and concentrations of antibiotics. Relevant studies mainly focused on the anammox granular sludge in laboratory scale SBR and UASB reactors fed with synthetic wastewaters. The influence thresholds of different antibiotics on anammox process are different. The specific effects of antibiotics on the anammox system for nitrogen removal performance are listed in Table 1.

Tetracyclines are widely used in aquaculture (Huang et al., 2017). After a long-term adaptation to TC of 100 µg/L (a concentration in natural environments), the anammox process showed a satisfactory nitrogen removal efficiency in a laboratory scale up-flow column anammox reactor, while at 1000 µg/L (a concentration in industrial wastewaters), the process performance deteriorated (Meng et al., 2019). However, the tolerance of partial nitrification sludge to TC was 150 mg/L in a long-term study (Xu et al., 2019). At a concentration of 1 mg/L, OTC had little effect on anammox performance for 20 days, while after the level of OTC progressively increased to 2 mg/L, the performance of anammox dropped quickly in 5 days in a laboratory scale UASB reactor (Shi et al., 2017). Furthermore, Yang et al. (2013) found that the IC₅₀ of OTC on anammox system in the short-term was about 520 mg/L, but only 50 mg/L OTC could affect the nitrogen removal performance of anammox within 7 days in the long term test, and this deterioration caused by OTC was challenging to reverse. The transient impact of OTC on

Table 1 The effects of antibiotics on the performance of the anammox process.

Reactor	Antibiotic	Antibiotic concentration	Operating days	NLR (kg N/(m ³ ·d))		NRR (kg N/(m ³ ·d))		Reference
				Before adding	After adding	Before adding	After adding	
UASB	Oxytetracycline	0.1–1 mg/L	156	–	–	20.0	14.3±2.9	Zhang et al. (2019a)
	Sulfamethoxazole						13.2±2.7	
	Oxytetracycline and Sulfamethoxazole						15.2±1.9	
UASB	Oxytetracycline	1 and 2 mg/L	42	–	–	5.50	1.20±0.09	Shi et al. (2017)
UAF	Erythromycin	0.01–50 mg/L	120	–	–	0.34	0.17	Zhang et al. (2019b)
Biofilm reactor	Tetracycline	10 µg/L	200–306	0.89±0.06	–	0.56±0.05	0.56±0.04	Meng et al. (2019)
		100 µg/L	307–418				0.64±0.04	
		1000 µg/L	419–518				0.49±0.04	
FBAS	Chlortetracycline	60 mg/L	71–84	0.75±0.05	0.85±0.02	0.57±0.12	0.48±0.10	Yao et al. (2018)
		20 mg/L	85–94	0.85±0.02	0.32±0.00	0.48±0.10	0.11±0.02	
UASB	Oxytetracycline	50 mg/L	26	–	–	12.4	2	Yang et al. (2013)
UASB	Oxytetracycline	6, 12.5, 25, 50 mg/L	57	–	–	12.4±1.3	5.0±5.5	Zhang et al. (2013)
Cylindrical Bio-filter	Norfloxacin	0.001, 1, 10, 50, 100 mg/L	150	–	–	0.345	0.198	Zhang et al. (2018c)
UASB	Tetracycline	0.1, 0.2, 0.3, 0.5, 1.0 mg/L	110	7.25±0.22	–	7.20±0.16	3.2	Fan et al. (2019)

anammox was also evaluated in a UASB reactor, and the results confirmed that the NRR decreased from 12.1 to 4.04 kg N/(m³·d) (Zhang et al., 2014). After successive suppression by 60 and 20 mg/L chlortetracycline (CTC), the average total nitrogen removal efficiency (TNRE) of an integrated fixed-biofilm and activated sludge reactor ranged from 75.6%±12.4% to 36.1%±8.1%. The performance of the reactor was difficult to recover at a low CTC level even after a long operational period (Yao et al., 2018). Moreover, a study on the inhibitory effect of two different veterinary pharmaceuticals declared that the anammox process was sensitive to 400 mg/L acetaminophen (PAR) and doxycycline (DOX), resulting in an inhibition of 61% and 83%, respectively (Alvarino et al., 2014).

Used in humans as medicine for healthcare purposes and in animals for growth promotion, sulfonamides are frequently found in pharmaceutical wastewater and livestock wastewater (Baran et al., 2011). It was found that low levels (< 3 mg/L) of sulfadimethoxine (SDM) and sulfamethazine (SM) did not affect the growth of anammox bacteria from a municipal sewage treatment plant, but 5–7 mg/L SDM would disrupt the anammox system. However, compared with SDM, 5–9 mg/L SM resulted in severer inhibition on anammox bacteria due to higher accumulation of nitrite (Du et al., 2018).

CAP and penicillin are also two widely used antibiotics. In a batch test, van de Graaf et al. (1995) found that 200 mg/L CAP and 800 mg/L ampicillin inhibited anammox activity from the denitrifying fluidized bed reactor by more than 95%. Similarly, Fernández et al. (2009) found that CAP between 250 and 1000 mg/L had a serious impact on the activity of anammox in batch tests. They also observed that adding 20 mg/L CAP to a sequential batch reactor (SBR) for 20 days caused a 25% decrease in the nitrogen removal efficiency. It was recently demonstrated that as the concentration increased, the disruption of anammox bacteria under two ranges of CAP concentrations (100–1000 µg/L and 5–100 mg/L) was inhibited more severely (Phanwilai et al., 2020). It can be seen from the above studies that as the concentration of CAP increases, the inhibitory effect of CAP on anammox microbes is more serious. Anammox granules in high-loaded laboratory scale UASB reactors were also inhibited by 10 mg/L florfenicol (FF), 60 mg/L amoxicillin (AMX) and 100 mg/L SM (Zhang et al., 2015).

In addition to the above types of antibiotics, other commonly used antibiotics pose varying degrees of influence on the anammox system. It was reported that several wastewater treatment systems were influenced by NOR and ERY (Amorim et al., 2014; Du et al., 2016; Hu et al., 2016b). Related research investigated the effect of NOR and ERY at 1 µg/L added to anammox biofilms for 30 days and found that the former caused severe disruption of microbial activity, while the latter inhibited microbial activity slightly (Zhang et al., 2019e). Another study based on an upflow anaerobic biological filter (UAF) demon-

strated that an ERY lower than 1 mg/L might cause a transient suppression of anammox performance. In comparison, when the ERY was higher than 10 mg/L, the inhibiting trend became slower as the concentration of the ERY increased, but the recovery time became longer. After 150 days of dosing in a laboratory scale UASB reactor, the TNRE decreased from 85.7% to 40.4% (Zhang et al., 2019d).

In summary, the inhibitory concentrations of different antibiotics on anammox are different. The influence of high concentration tetracyclines on anammox is generally irreversible, for example OTC and CTC. By contrast with CAP, tetracyclines and sulfonamides showed more significant disruptions on anammox granules. However, the types of reactors used in relevant researches were limited, and almost all the influents were synthetic wastewaters. In addition to the types of antibiotics, the different degrees of inhibition might also be attributed to the bacterial community, reactor configuration and operational conditions. Therefore, more researches are needed to reveal the general rule of the response of anammox to antibiotics.

2.2 Effects of antibiotics on sludge properties

2.2.1 Bioactivity

Both short- and long-term effects on anammox activity have been studied based on different antibiotics at various concentrations (Table 2). From the current research, anammox bacteria are susceptible to antibiotics. After 5 mg/L OTC was added to an SBR reactor for five weeks, the anammox system was almost completely collapsed (Noophan et al., 2012). Exposure to 1 and 2 mg/L OTC in a UASB reactor inoculated with anammox sludge caused the specific anammox activity (SAA) to decrease by 81.3% after 42 days (Shi et al., 2017). When exposed to 10 mg/L tetracycline hydrochloride (TCH), SAA was sharply reduced by 60% in continuous tests (Fernández et al., 2009). After adding 100 mg/L CTC for 7 days, SAA was virtually unchanged, but the value decreased to 75% after 14 days (Lotti et al., 2012).

2.2.2 Extracellular polymeric substances

Extracellular polymeric substances (EPS) can respond to environmental stress, such as heavy metals, nanoparticles and antibiotics, and play a pivotal role in the treatment of wastewater for the removal of organic micropollutants (Zhang et al., 2014; Zhang et al., 2018a; Zhang et al., 2018d). In addition, adsorption by EPS greatly influences the removal and migration of antibiotics (Xu et al., 2013). Cells secrete EPS, which provide a protective mechanism by forming a network structure (Kunacheva and Stuckey, 2014). The main components of EPS are proteins (PN) and polysaccharides (PS). PN contains many hydrophilic

Table 2 Summary of the specific anammox activity in the presence of antibiotics

Reactor	Antibiotic	Antibiotic concentration	SAA	Operating days	Reference
SBR	Chloramphenicol	6 mg/L	0.528–0.096 mg N/(mg VSS·d)	41	Phanwilai et al. (2020)
SBR	Chloramphenicol Tetracycline hydrochloride	20 mg/L 50 mg/L	0.25–0.05 g N/(g VSS·d)	20	Fernández et al. (2009)
Cylindrical Biofilter	Norfloraxacin Erythromycin	1 µg/L	10.8–7.56 mg/(g SS·h) 10.83–10.65 mg/(g SS·h)	30	Zhang et al. (2019c)
UASB	Amoxicillin Florfenicol Sulfamethazine	10, 30, 60, 80, 150 mg/L 10, 20, 15, 10, 5 mg/L 5, 20, 30, 80, 100, 200 mg/L	427.7 to ~500 mg TN/(g VSS·d) 430.4 to ~450 mg TN/(g VSS·d) 502.9 to ~450 mg TN/(g VSS·d)	80	Zhang et al. (2015)
UASB	Oxytetracycline	6, 12.5, 25, 50 mg/L	16.6– 6.4±6.6 mg TN/(g VSS·h)	57	Zhang et al. (2013)
Cylindrical Biofilter	Norfloraxacin	0.001, 1, 10, 50, 100 mg/L	10.84 –7.56 mg/(g SS·h)	150	Zhang et al. (2018c)
UASB	Spiramycin	0.5 mg/L 5 mg/L	92.7±7.7–85.1 mg N/(g VSS·d) 92.7±7.7 to 47.19±14.1 mg N/(g VSS·d)	65	Wu et al. (2020)
UASB	Oxytetracycline	1 and 2 mg/L	347.7±5.3–58.76 mg N/(g VSS·d)	42	Shi et al. (2017)

functional groups, such as hydroxyl and carboxyl groups, which can provide numerous adsorption binding sites (Sheng et al., 2010). Therefore, EPS acts as a defense layer (Zhang et al., 2014) and absorbs sulfonamide and tetracycline antibiotics (Hou et al., 2016). Because of adsorption by EPS, with the increasing concentrations of OTC and SMX, the EPS content tended to decrease, then increase, and then repeatedly, but the final content was higher than that of without antibiotics (Zhang et al., 2019b). However, with the increasing concentrations from 0.001 to 100 mg/L of ERY, the trend of EPS content was exactly opposite to those of OTC and SMX. The level of EPS changed from 76.8 to 94.71 mg/g-SS (Zhang et al., 2019d). Du et al. (2018) also observed that higher than 5 mg/L SDM and SM would lead to an EPS concentration decline, while less than 3 mg/L had the opposite effect. The reason may be that hazardous substances at low concentrations can cause the microorganism to secrete EPS (Jia et al., 2017). Overall, long-term acclimatization may enable the development of a better survival strategy for EPS and reduce the antibiotic stress on anammox biomass by providing a protective ‘cocoon’ (Liang et al., 2015), but overloading of antibiotics will render this protection strategy ineffective (Zhang et al., 2015).

2.2.3 Heme c

Heme c, as an indispensable part of hydrazine-oxidizing enzyme (HZO) and hydroxylamine oxidoreductase (HAO), is closely implicated in anammox bacteria metabolism (Guo et al., 2015b). To some extent, the proportion of heme c can reflect the NRR and SAA (Sabine Marie et al., 2015). The heme c showed a downward trend as TC and SMX progressively increased from 0.1 to

1.0 mg/L. However, the heme c content fluctuated in response to OTC + SMX. These differences may be attributed to the different resistance properties to anammox consortia when OTC and SMX function separately and jointly (Zhang et al., 2019b). Shi et al. (2017) indicated that the characteristics of sludge were degraded and that the stability of the UASB reactor was broken by 2 mg/L OTC. In a biofilm reactor, increased the content of heme c and EPS were raised after adding 1, 10 and 100 µg/L TC, whereas 1000 µg/L TC reduced the levels of heme c and EPS (Meng et al., 2019).

2.2.4 Granule properties

TCH and CAP did not change the size distribution and physical properties of anammox granules, and sludge settleability was also not affected by either antibiotic (Fernández et al., 2009). Consistent with the results of this reference, Liu et al. (2015) investigated the response of aerobic granules to SM, OTC, and CIP at trace concentrations and found that the average granule size, settling properties and physical strength exposed to antibiotics had almost no significant changes. After inhibition by OTC, the color of the anammox sludge turned black, the activity and settling ability were damaged, and the quantity of irregular cells was small (Yang et al., 2013).

Based on the above analysis, SAA, heme c, and EPS usually exhibit the same change trend. Low concentrations of antibiotics will stimulate the secretion of EPS, which prevents antibiotics from contacting with microbial cells. This protective effect will no longer be efficient if the antibiotic concentration is too high and leads to the deterioration of the particle characteristics of the anammox sludge. However, the tetracyclines, sulfonamides and CAP

studied so far have little effect on the granule properties of anammox though the color of the anammox sludge was changed.

2.3 Effects of antibiotics on the microbial community

Many studies have demonstrated that anammox bacterial communities are highly susceptible to antibiotics. However, the anammox system can also resist the harm caused by some antibiotics to some degree. The responses of different anammox bacteria to antibiotics are listed in Table 3.

Without adding antibiotics into the anammox system, the dominant phyla were Planctomycetes and Proteobacteria. Meanwhile, Firmicutes existed in all samples, although the abundance was low. Gammaproteobacteria was also detected with an average abundance of 0.88%–1.79% (Zhang et al., 2019b; Zhang et al., 2019d). It suggested that Proteobacteria might contain antibiotic resistant bacteria (ARB) (Shi et al., 2013), Firmicutes contains many tetracycline ARB (Ghosh and LaPara, 2007), and Gammaproteobacteria could resist a high concentration of antibiotics (Zhu et al., 2017). Moreover, Betaproteobacteria was the dominant bacterial community with a high antibiotic resistance ability in OTC-containing or other antibiotic wastewater treatments (Deng et al., 2012; Liu et al., 2012). The same results appeared in an anaerobic digestion process; ARG-bearing bacteria were mainly associated with Proteobacteria and its class, Betaproteobacteria (Wang et al., 2019a). Thus, anammox sludge has a tolerance to antibiotics owing to the existence of resistant microorganisms.

In a report by Zhang et al. (2018c), the dominant genus in an anammox system was *Candidatus Kuenenia*, which is a typical resistant bacterium. Long-term adaptation to OTC and SMX might improve the tolerance of *Candidatus Kuenenia* to antibiotics in a laboratory scale continuous stirred tank reactor (CSTR) (Tian et al., 2018). The genus *Nitrosomonas* can protect anammox bacteria by utilizing oxygen to oxidize some ammonia to nitrite. The genus *Rhizobium* is a Gram-negative nitrogen-fixing bacterium widely present in the soil that also exists in anammox systems. Zhang et al. (2019e) proved that addition of 1 µg/L NOR in an anammox system for 30 days caused a 2.44% decrease in the abundance of *Candidatus Kuenenia* and an 0.47% increase in the abundance of *Nitrosomonas*, while the same concentration of ERY showed no significant effect on the microbial community. Additionally, high levels of ERY reduced the relative abundance of *Candidatus Kuenenia* by 18.4% in addition to causing changes in *Nitrospira*, *Acidovorax*, *Denitratisoma* and *Desulfovibrio* related to the nitrogen cycle. After long-term acclimatization to NOR for 150 days, the relative abundance of *Candidatus Kuenenia*-related anammox bacteria ultimately improved from the initial 20.7% to 25.3% (Zhang et al., 2018c). 5–7 mg/L SDM caused the abundance of *Candidatus Brocadia* to reduce by 2.2% (Du et al., 2018). TC caused *Candidatus Jettenia* and *Denitratisoma*, which were the key functional bacteria, to become enriched at 100 µg/L but disrupted at 1000 µg/L, suggesting that the microbial community could adjust to low concentrations of TC and maintain nitrogen removal in a stable state (Meng et al., 2019). In contrast, Collado et al. (2013) found that a low concentration of 50 µg/L SMX

Table 3 The dynamic variations of different AnAOB in anammox systems under antibiotics pressure.

Antibiotic	Antibiotic concentration	Microbial species	Varieties of relative abundance	Reference
Oxytetracycline	0.1–1 mg/L	Planctomycetes and Proteobacteria	Decreased 26.2%	Zhang et al. (2019a)
Sulfamethoxazole			Decreased 15.9%	
Tetracycline	100 µg/L	Planctomycetes	Increased 7.11%	Meng et al. (2019)
		Proteobacteria	Increased 2.20%	
	1000 µg/L	Planctomycetes	Decreased 10.12%	
		Proteobacteria	Decreased 16.91%	
Norfloxacin	1 µg/L	Planctomycetes	Decreased 2.44%	Zhang et al. (2019c)
		Arenimonas	0	
Erythromycin		Planctomycetes	Decreased 0.01%	
		Arenimonas	Increased 4.54%	
Sulfadimethoxine	5–7 mg/L	<i>Candidatus Brocadia</i>	Decreased 2.18%	Du et al. (2018)
Norfloxacin	0.001, 1, 10, 50, 100 mg/L	<i>Candidatus Kuenenia</i>	Increased 4.54%	Zhang et al. (2018c)
Tetracycline	0.1–1.5 mg/L	Planctomycetes, Proteobacteria and Chloroflexi	Decreased 10%	Fan et al. (2019)
Erythromycin	0.01–100 mg/L	Proteobacteria	Increased 43.22%	Zhang et al. (2019b)
		Planctomycetes	Decreased 21.91%	

decreased microbial diversity. This may be attributed to the antibacterial effects of these two antibiotics are different.

Generally, Planctomycetes and Proteobacteria showed better tolerance to antibiotic pressure. With the long-term addition of antibiotics, functional bacteria like anammox bacteria were inhibited by antibiotics. In contrast, the abundance of other nitrifying bacteria like *Nitrosomonas* slightly increased, which makes compensation for the nitrogen removal performance in the system (Zhang et al., 2019e).

2.4 Effects of antibiotics on the fate of ARGs in anammox systems

Antibiotics in wastewater can affect the production and migration of ARGs. Related studies have indicated that ARGs are difficult to eliminate, and their antimicrobial properties have a potential negative impact on living things and the environment (Song et al., 2011). Moreover, because the content of ARGs has a certain effect on the anammox and denitrification process, the application of pig manure in farmland will increase the potential ecological risk (Rahman et al., 2018).

In biological wastewater treatment systems, tetracyclines are potent drivers of ARGs (Gao et al., 2012). Typical anti-tetracycline genes include *tetA*, *tetB*, *tetC* and *tetX*, among which *tetA*, *tetB* and *tetC* are efflux pump genes and *tetX* is an enzymatic modification gene. After 6 weeks of exposure to 1–2 mg/L OTC, the gene with the highest abundance was *tetA*, while *tetB* had the lowest abundance (Shi et al., 2017). Efflux pumps can reduce susceptibility to heavy metals and antibiotics by squeezing harmful substances out of cells (Martinez et al., 2009). Zhang et al. (2019b) tested the absolute abundance of *tetG*, *tetM*, *tetX*, *int11*, and *sul2* after exposure to OTC, SMX and OTC + SMX, and the most abundant was *tetX*, whose absolute abundance was 3.03×10^6 , 2.80×10^6 and 2.03×10^6 copies/mg in the different antibiotics, respectively. Neither of the genes targeting NOR was detected after exposure to NOR and ERY for one month. Still, targeting genes of ERY (*ermB* and *mphA*) were detected, and the results showed that NOR had a negative effect on the nitrogen removal process and that ERY slightly impacted the anammox system (Zhang et al., 2019e). In verifying the variation in eight types of tetracycline resistance genes in an SBR reactor, Zhang et al. (2013) indicated that 100 µg/L TC could enhance ARG proliferation in activated sludge.

2.5 Effects of antibiotics on functional genes in anammox systems

A hypothetical metabolic pathway for anammox bacteria includes three steps: 1) nitrite is converted into nitric oxide by the catalysis of cytochrome *cd1* nitrite/nitrogen oxide oxidoreductase (*nirS*); 2) nitric oxide is bound to

ammonium under the action of hydrazine synthase (*hzs*) to form the completed hydrazine; and 3) the hydrazine is oxidized by hydrazine dehydrogenase (*hdh*), finally generating dinitrogen gas (Kartal et al., 2011). With TC and SMX concentrations ranging from 0 to 1.0 mg/L, *nirS*, *hzsA*, and *hdh* are the most sensitive to SMX in anammox systems, followed by OTC and OTC + SMX (Zhang et al., 2019b). At the mRNA level, the absolute abundance of *nirS*, *hzs*, and *hdh* gradually decreased when anammox bacteria were exposed to 2 mg/L OTC (Zhang et al., 2019c). The abundance of *hzsA* did not change significantly as the concentration of SDM ranged from 1 mg/L to 5 mg/L. However, the abundance of *hzsA* decreased sharply from approximately 1.1×10^6 to 3.0×10^5 copies/ng-DNA under the pressure of 7 mg/L SDM, while the abundance of *hzo* increased significantly at 1 mg/L. Unlike SDM, the effect of SM concentration on *hzsA* and *hzo* lagged, so anammox bacteria are more sensitive to SDM (Du et al., 2018). Similarly, Fan et al. (2019) found that the abundance of these three functional genes increased primarily and then decreased, eventually dropping below those of the control group as the concentrations of Zn(II) and TC increased on an anammox system. In contrast, the expression of *nirS*, *hzsA*, and *hdh* at the transcriptional level increased under nitrite shock, and the authors speculated that this increase might be a compensation mechanism for the reduction of enzyme activity in *Candidatus* *Kuenenia stuttgartiensis* (Wang et al., 2016). Zhi and Ji (2014) hold the view that microbial-mediated ecological processes may be assessed by the relative abundance of functional genes. However, the impact of antibiotics on functional genes in anammox systems remains limited; thus, more research needs to be done.

2.6 Influencing mechanism of antibiotics

Different concentrations of multiple antibiotics have different effects on anammox systems, but their effect mechanisms may be disparate. Tetracyclines, CAP and streptomycin can interfere with the synthesis of ribosomal proteins. Sulfonamides prevent bacteria from synthesizing nucleic acids, inhibiting the growth of bacteria. Penicillin can interfere with the synthesis of the cell wall.

Levin-Reisman et al. (2017) demonstrated that bacteria growing slowly develop antibiotic resistance more easily than those growing quickly. In the presence of trace TC, slow-growing microorganisms involved in the nitrification process are more likely to produce TC resistance genes (Liu et al., 2018). The doubling time of anammox bacteria was about 11–14 days, so they are typical slow-growing bacteria (Strous et al., 1998). The possible tolerance mechanism of anammox bacteria to OTC and SMX may include four parts (Fig. 1): 1) A protective layer of EPS. 2) Potential resistant species because of the aggregation of dominant microbes. 3) Functional gene regulation in the dominant microbes to adapt to external disturbances.

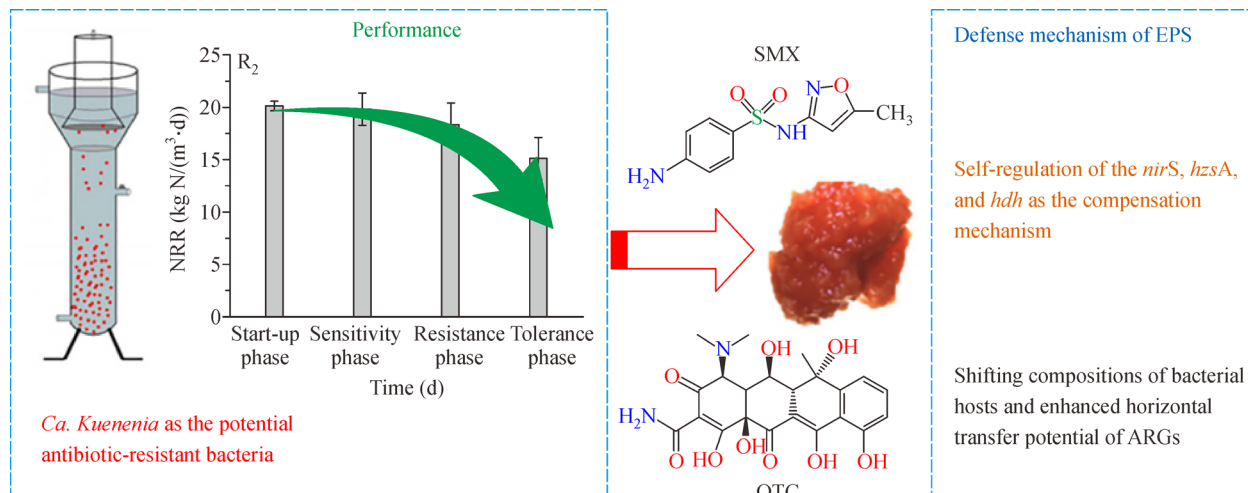


Fig. 1 Possible tolerance mechanism of AnAOB to OTC and SMX, adapted from (Zhang et al., 2019b) with permission.

4) Progressively increasing tolerance of anammox to OTC and SMX by the succession of bacterial hosts and horizontal transfer of ARGs. Furthermore, from the changes in ARGs, it can be inferred that anammox bacteria rely on the efflux pump mechanism to resist OTC (Shi et al., 2017). AMX, a broad-spectrum antibiotic of the aminopenicillin family, prevents peptidoglycan and penicillin binding proteins from cross-linking to obstruct the synthesis of the cell wall (Hu et al., 2013). The metabolism of purine and synthesis of nucleic acid are interfered with by SM (Lotti et al., 2012). FF inhibits the synthesis of proteins by inhibiting the function of the 50S ribosomal subunit and transpeptidase (Ding et al., 2015). The cell wall of anammox bacteria contains functionally similar components to peptidoglycan, so anammox systems were inhibited by AXM, SM and FF as mentioned in Section 2.1 (Zhang et al., 2015). CAP is often used to resist infections stimulated by Gram-negative bacteria, but it can inhibit prokaryotic cells as well as eukaryotic cells. CAP disrupts protein synthesis by preventing the formation of peptide bonds (Dapena-Mora et al., 2007). Moreover, related research illuminated that the degree of anammox activity effected by CAP was dependent on the enrichment status of the culture (van de Graaf et al., 1996). Therefore, some studies have found CAP did not inhibit the anammox process. However, despite good sludge characteristics, some studies still confirmed that CAP inhibited the reaction; this phenomenon may be because CAP reached the inhibitory concentration mentioned in Section 2.1.

Regarding the influencing mechanism of NOR and ERY on anammox systems at low doses, it could be concluded that NOR could not protect cells by inducing target genes to resist and degrade antibiotics; however, ERY could effectively induce the expression of *ermB* and *mphA* to protect the anammox system (Zhang et al., 2019e). In fact, *ermB* is a cellular protection gene that can stimulate cell membranes to produce polysaccharides to prevent anti-

biotics from entering cells (Zhu et al., 2013). Moreover, *mphA* is an inactivation gene that can chemically modify macrolide antibiotics such as ERY and degrade or replace the active groups in antibiotics, thus inactivating them by expressing synthetic oxidoreductase (Guo et al., 2015a). In addition, long-term addition of ERY inhibited an anammox system primarily via interference with the synthesis of protein (Zhang et al., 2019d) and combination with 23S rRNA molecule in the 50S ribosomal subunit to inhibit peptidyl-tRNA translocation (Alighardashi et al., 2009).

Based on the above studies, antibiotics mainly affect the synthesis of nucleic acids or corresponding proteins to hinder the performance of the anammox process. However, the long-term existence of antibiotics will lead to the occurrence of ARGs. Ribosome protective genes can stimulate anammox bacteria to secrete more EPS and prevent antibiotics from damaging the cells. The inactivated genes can degrade or replace the active groups of antibiotics, thus inactivating antibiotics. Even though, the in-depth research on the resistance mechanism of antibiotics to anammox is still limited, which needs to be further studied.

3 Combined effects of antibiotics and other contaminants on anammox

3.1 Combined effects of various antibiotics

In addition to single effects, some studies have also described combined effects of multiple antibiotics on anammox consortia. Zhang et al. (2019b) indicated that the performance of the anammox system deteriorated gradually as the OTC and SMX dosage gradually increased from 0.5 to 1.0 mg/L, and the inhibition of OTC + SMX was lower than that of OTC and SMX functioning independently. However, in this study, EPS did not change

significantly, possibly because SM, sulfamerazine, sulfadiazine, and tetracyclines can be adsorbed by hydrophobic regions of EPS (Hou et al., 2016). Another study reported that the acute toxicity of single antibiotic from big to small on the anammox system was polymyxin B sulfate, CAP, penicillin G-Na(C) and kanamycin sulfate. In contrast, the combined effects of these antibiotics were polymyxin B sulfate + CAP, penicillin G-Na + CAP, CAP + kanamycin sulfate, polymyxin B sulfate + kanamycin sulfate, penicillin G-Na + polymyxin B sulfate and penicillin G-Na + kanamycin sulfate (Ding et al., 2015). Furthermore, the addition of NOR, azithromycin, SMX and trimethoprim (TMP) had a noticeable impact on the performance, bacterial community and substrate composition of the completely autotrophic nitrogen removal over nitrite (CANON) process. Moreover, the loss of ammonium-oxidizing bacteria (AOB) resulted in a feeble nitrogen removal efficiency after the addition of antibiotics (Rodriguez-Sanchez et al., 2017).

3.2 Combined effects of antibiotics and heavy metals

The joint effects of Zn(II) and TC on anammox were studied in a UASB reactor for 258 days (Fan et al., 2019). When the concentrations of Zn(II) and TC were 0–2.26 mg/L and 0–0.5 mg/L, the performance of the reactor was less affected. When the total concentration of Zn(II) and TC was above 3 mg/L, a severe decrease of NRE occurred, which was stronger than that of single inhibition. Yang and Jin (2012) indicated that the inhibitory of 2.5 mg/L Cu(II) began to remit as the OTC concentration increased, which proved that OTC might alleviate the toxicity of Cu(II) when they coexist. Similarly, as the level of CuNPs and OTC was 0.5 mg/L, the anammox process has a good nitrogen removal ability. Still, but 5.0 mg/L CuNPs and 2.0 mg/L OTC severely inhibited the anammox system was severely inhibited when the concentrations of CuNPs and OTC were 5.0 mg/L and 2.0 mg/L, respectively (Cheng et al., 2020). Another study indicated that the joint application of CuONPs (1 mg/L) and OTC (1 mg/L) decreased the ammonia removal efficiency to 51.0% during the partial nitrification process, which was attributed to the formation of the complex CuONPs-OTC, which can lead to disorder of the cell metabolism and retardation of cell growth (Zhang et al., 2020). In a study of the single and combined influence of Cu(II), Zn(II), OTC and SMZ on partial nitrification, Xing and Jin (2018) found that the effects of antibiotics and heavy metals were synergistic.

3.3 Combined influencing mechanism of various antibiotics and heavy metals

At present, there are few reports on the mechanism of the combined disruption of different antibiotics, while research on the combined influencing mechanism of antibiotics and

heavy metals mainly focuses on the co-selective mechanism shown in Fig. 2, which includes the following: (a) a co-resistance mechanism: antibiotic and heavy metal resistance genes are located in the same vector, such as a plasmid, integron or transposon, so that bacteria can develop synergistic resistance to antibiotics and heavy metals; (b) a cross-resistance mechanism: efflux pumps act in the copresence of heavy metals and antibiotics. When different antibiotics and heavy metals attack the same target, they can start or share a common cell pathway to obtain their respective targets; and (c) a coregulation mechanism: a two-component system under antibiotic or heavy metal stimulation. A series of transcription and translation response systems in bacteria will respond to antibiotics and heavy metals under any pressure applied.

Antibiotics and heavy metals are often present in wastewater simultaneously, especially in aquaculture wastewater. Ding et al. (2019) verified that Zn(II), Cu(II), Cd(II), and Cr(II) drove the co-selection of antibiotic resistance in a water environment, which is related to a coupling mechanism of physiologic genetic co-resistance in Fig. 2(a) and cross-resistance in Fig. 2(b). In addition, Xu et al. (2017) declared that, in addition to antibiotic stress, the selective stress of heavy metals was positively correlated with the abundance of ARGs, suggesting a co-selection mechanism between heavy metals and ARGs in bacterial populations. Baker-Austin et al. (2006) found the co-resistance mechanisms of a TC and Zn(II) resistance system. Obviously, resistance genes for both TC and heavy metals could be present in the same mobile genetic element. Perron et al. (2004) found that the *mex* operon caused quinolone, TMP, and CAP resistance expression, the *czc* operon led to the expression of metal efflux, and the simultaneous action of these two processes caused imipenem resistance, as shown in Fig. 2(c).

In addition to the co-selective mechanism, metal ions could form complexes with OTC because of the electron-donor groups of OTC (Gu et al., 2007). The composite formed by Cu(II) and OTC is water-soluble and this characteristic is very stable. Moreover, due to the electrostatic attraction, the composite can be combined on the surface of granules, thereby forming Cu(II)-OTC-granule complex structure (Song et al., 2011). Thus, the overall toxicity of OTC and Cu(II) was reduced; that is, OTC and Cu(II) played an antagonistic role in the anammox consortia when joined together (Yang and Jin, 2012). Additionally, the mechanisms of penicillin G-Na, polymyxin B sulfate and kanamycin sulfate combined with each other were independent; polymyxin B sulfate and CAP were additive, CAP and kanamycin sulfate were additive, and penicillin G-Na and CAP were synergistic (Ding et al., 2015).

In brief, the combined influencing mechanism of antibiotics and heavy metals on anammox mainly concentrated on the co-selection and synergistic resistance.

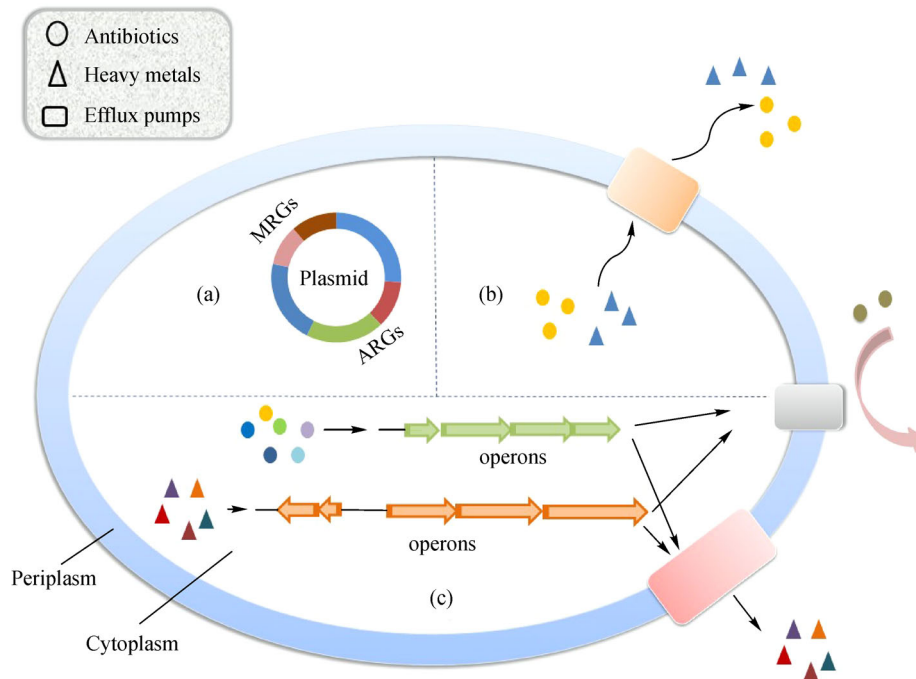


Fig. 2 Co-selection mechanism of antibiotic and metal resistant: (a) co-resistance mechanism; (b) cross-resistance mechanism; (c) co-regulation mechanism. Circles, triangles, and rectangles represent antibiotics, heavy metals and efflux pumps, respectively. Different colors show different substances mentioned above.

While, a small number of studies believed that antibiotics and heavy metals exhibited antagonistic and independent effects on the anammox process.

4 Mitigation strategy for antibiotic inhibition on anammox

Most trace antibiotics have no influence on nitrogen removal because of biodegradation and biosorption by anammox consortia (de Graaff et al., 2011; Alvarino et al., 2015). However, anammox bacteria have a long generation cycle and is very sensitive to the surrounding environment (Strous et al., 1998). Moreover, the precipitation, adsorption and accumulation of antibiotics in sludge will lead to continuous suppression of anammox bacteria. As a result, even if biocatalysts are added, it also takes a long time for the suppressed anammox system to recover.

When the activity was inhibited, it was efficacious to add anammox sludge to recover it (Jin et al., 2012). Yao et al. (2018) suggested that adding anammox sludge can effectively restore a deteriorated anammox system because it may stimulate quorum sensing (QS) in anammox bacteria. The anammox system can eliminate the effect of antibiotics through adsorption, and the removal of ERY was closely associated with the anammox reaction rate (Alvarino et al., 2015). The stress of OTC on anammox can be mitigated by bio-augmentation (BA) because BA mode regulation may gradually acclimate the microbial commu-

nities and maintain system stability sequentially. The main reason for performance stabilization is that functional bacteria are potential antibiotic-resistant species (Zhang et al., 2018b). Jin et al. (2014) suggested that BA is a feasible method to enhance the capacity of anammox recovery and that the critical operating parameters of BA are bio-augmentation dosage (BAD) and bio-augmentation time (BAT).

5 Future perspectives

As one of the most promising technologies for nitrogen removal from wastewater, it is necessary to understand the impact of antibiotics on the anammox process because different antibiotics may have various activities in the wastewater system. The type and concentration of antibiotics, or even the presence of coexisting heavy metals or other inhibitors, will disturb the performance of the anammox system to varying degrees. To achieve efficient treatment of wastewater rich in nitrogen and antibiotics through the anammox process, future studies should focus on the following aspects:

i) In-depth studies on antibiotics and other substances based on the anammox system. Currently, the combined effects of different antibiotics or antibiotics and organics are still rarely reported. Moreover, Yin et al. (2020) discovered that stress reactions caused by photocatalytic oxidation could accelerate the evolution of antibiotic

resistance in bacteria. In addition to photocatalytic oxidation, other environmental factors, such as heat or starvation, may also accelerate the development of bacterial resistance, which is beneficial for the biological treatment of antibiotic wastewater. Therefore, the above aspects are practically significant and need further research.

ii) Removal of antibiotics and their effects on the anammox system. There are two main ways to remove antibiotics by microorganisms: one method is pretreatment by advanced oxidation and other methods. In this approach, it is necessary to pay attention to the effects of oxidation intermediates on microorganisms, including hydroxide radicals and ozone; the second removal method is to couple antibiotic-degrading bacteria in the sludge system. At this time, the long-term stability of the system and the interactions between microflora should be considered. Additionally, some antibiotics may be converted into more toxic intermediate metabolites, increasing the risk to functional microorganisms and the environment. Thus, the realization of anammox system for antibiotic removal has both application prospects and challenges, and deserves further study.

iii) The generation, transmission and ecological environmental assessment of ARGs are crucial. The reuse of treated wastewater is a growing global trend, but the uninterrupted usage of antibiotics is causing antibiotic resistance to become complicated, making it difficult to predict the risk of ARGs in the environment (Singh et al., 2019). In addition, the information regarding possible causes of ARGs is incomplete, and other probable mechanisms that cause antibiotic resistance, such as adaptive stress response, active efflux, QS, and persistent cells formation, need further study (Høiby et al., 2010; Liang et al., 2015).

iv) Enrichment and cultivation of the dominant bacteria and appropriate selection of sludge morphology. High concentrations of microbes could seal off many toxic substances to ensure reactor stability (de Vrieze et al., 2015). Therefore, based on the influence of antibiotics on the anammox microbial community, the dominant bacteria (especially functional bacteria like *Nitrosomonas* involved in the nitrogen cycle) can be selected for enrichment and culture to reduce or eliminate the inhibition of anammox by antibiotics. Generally, granular sludge has the strongest resistance, followed by biofilms, and floc sludge has the weakest resistance. However, once granular sludge disintegrates under antibiotic shock events, recovery may be highly time-consuming. In our opinion, biofilms are the recommended form. However, the current studies mainly focus on anammox granular sludge, so more investigations on biofilms should be carried out for the above verification.

v) More attention should be paid to combined processes. The anammox process is generally coupled with other processes, such as partial nitrification and partial denitrification. In fact, the tolerance of multi-process systems to

antibiotics may vary, and the effects on microbial balance can also be different.

vi) The feasibility treated real antibiotic-containing wastewaters and verification using the anammox process. Previous studies mainly used synthetic wastewaters and were performed in laboratory scale systems. To achieve the practical application of anammox, more efforts should be paid to pilot-scale or full-scale work with real wastewaters, in which the issues of scale-up can be solved.

Abbreviations

Anammox	Anaerobic ammonium oxidation
ARGs	Antibiotic resistance genes
MRGs	Metal resistance genes
NRR	Nitrogen removal rate
TC	Tetracycline
OTC	Oxytetracycline
CTC	Chlortetracycline
PAR	Acetaminophen
DOX	Doxycycline
SDM	Sulfadimethoxine
SM	Sulfamethazine
CAP	Chloramphenicol
SAA	Specific anammox activity
SBR	Sequential batch reactor
FBAS	Integrated fixed-biofilm and activated sludge
CSTR	Continuous stirred tank reactor
FF	Florfenicol
AMX	Amoxicillin
CIP	Ciprofloxacin
NOR	Norfloxacin
ERY	Erythromycin
UAF	Up-flow anaerobic biological filter
TNRE	Total nitrogen removal efficiency
EPS	Extracellular polymeric substances
PN	Protein
PS	Polysaccharide
SMX	Sulfamethoxazole
HZO	Hydrazine-oxidizing enzyme
HAO	Hydroxylamine oxidoreductase
TCH	Tetracycline hydrochloride
ARB	Antibiotic resistant bacteria
AOB	Ammonium oxidizing bacteria
QS	Quorum sensing
BA	Bio-augmentation
BAD	Bio-augmentation dosage
BAT	Bio-augmentation time
TMP	Trimethoprim

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