



Function and potential application of quorum sensing in nitrogen-removing functional bacteria: a review

Feng Luo, Huizhi Hu*, Yirong Liu

Faculty of Resources and Environmental Science, Hubei University, Wuhan 430062, China, Tel. +86-18627100053; email: huhuizhi@126.com (H. Hu), Tel. +86-15107112563; email: luofeng2563@163.com (F. Luo), Tel. +86-15327408045; email: liuyr778@163.com (Y. Liu)

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ABSTRACT

Due to the long generation cycle and sensitivity to environmental factors of nitrogen-removing functional bacteria, the traditional nitrogen removal process is faced with two major problems in practical application: long start-up time and susceptibility to environmental variations. Quorum sensing (QS), a ubiquitous phenomenon in bacteria, has attracted more attention in realizing the inter-cell "communication". Bacteria with the QS mechanism can synthesize and release specific signal molecules to regulate the gene expression and physiologic behavior in response to cell density. This article systematically summarized recent research progresses on QS-related genes, possible regulation mechanisms of nitrogen-removing functional bacteria, and the impacts of exogenous QS signal molecules on nitrogen-removing functional bacteria. Many characteristics of nitrogen removal bacteria have been confirmed to be related to QS, including bacterial activity, growth rate and formation of extracellular polymeric substances, which directly affect the performance of the nitrogen removal process. Specifically, the production and release of nitric oxide and nitrous oxide are identified to be related to QS, which is of great significance to slow down the trend of global warming. This review attempts to clarify the potential solution to the main bottleneck: improving the competitiveness of nitrogen removing bacteria and maintaining high stability when reactor operation fluctuating. Therefore, corresponding regulation strategies and future prospects are suggested on the basis of current studies.

Keywords: Quorum sensing; Nitrogen-removing functional bacteria; Signaling mechanism; Wastewater treatment

1. Introduction

A milestone in bacterial ecology is that *Vibrio fischeri* was found to produce bioluminescence only at high cell density, which reveals that single-celled individuals could communicate with each other by a system called quorum sensing (QS) [1,2]. The bacteria with QS regulation systems could produce signal molecules and then release them into the environment. When the signal molecule's concentration reaches a critical threshold, the signal molecules bind to the regulatory proteins. The expression of related genes

is then triggered to coordinate the corresponding behavior, such as the expression of virulence factors, formation of biofilms, bioluminescence and the production of extracellular polymeric substances (EPSs). For instance, the production of virulence factors (namely protease, pyoverdinin and chitinase) were partially or completely suppressed in *Pseudomonas aeruginosa* cultures grown when QS was inhibited by furanone C-30 [3]. Autoinducer-2 (AI-2) based QS system inhibited biofilm formation by repressing *rbf* expression in *Staphylococcus aureus* [4]. In the marine bioluminescent bacterium *Vibrio fischeri*, the signal molecules bound to a transcription regulator, *luxR*, and then altered

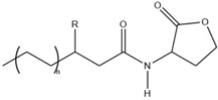
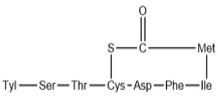
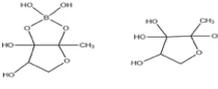
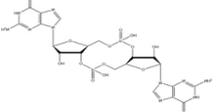
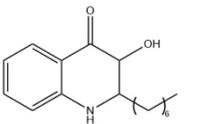
* Corresponding author.

the gene expression to coordinate bioluminescence at high concentrations [5]. It was proved that QS was related to the production of EPS, and the exogenous addition of signal molecules significantly increased the tightly bound extracellular polymeric substance production by 3.5% and 7.8%, respectively [6].

In general, the types of QS signal molecules are discussed according to the different chemical structures. (1) N-acyl-homoserine lactones (AHLs). AHLs are the primary signal molecules secreted by Gram-negative bacteria. Acyl homoserine lactone ring is a common feature of AHLs signaling molecules. The differences in acyl side chain length, carbon-chain skeleton saturation and substituents lead to the diversity and specificity of AHLs [7]. (2) Autoinducer peptide (AIP). AIP secreted by Gram-positive bacteria is recognized to be derived from a longer precursor peptide. The precursor peptide is cleaved and modified with upper lactone and thiolactone loops, sulfided bialanine, and isoprene groups [8]. (3) Autoinducer-2. AI-2 can simultaneously regulate Gram-negative and Gram-positive bacteria to achieve interspecies communication. And AI-2 is synthesized from the precursor 4,5-dihydroxy-2,3-pentanedione [9]. In addition, other types of signal molecules have also been discovered, including *Pseudomonas* quinolone signals (PQSs), diffusible signal factors (DSFs), γ -butyrolactones, and bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) [7,10]. Common signal molecules and their regulatory genes in bacteria are shown in Table 1.

Nitrification and denitrification are two crucial steps in the biological nitrogen removal process, which are essential processes in wastewater treatment systems (Fig. 1). Nitrification is the conversion of ammonia nitrogen into nitrite and nitrate under aerobic conditions. Known bacteria involved in this process include ammonia-oxidizing bacteria (AOB), ammonia-oxidizing archaea (AOA), nitrite-oxidizing bacteria (NOB) and complete ammonium oxidation (Comammox) [15]. Denitrification is the process of reducing nitrate and nitrite to gaseous nitrogen by denitrifying bacteria under anoxic conditions. In addition, ammonium can be directly converted to nitrogen under anoxic or anaerobic conditions by anaerobic ammonium oxidation bacteria (AnAOB) using nitrite nitrogen as an electron acceptor [16,17]. Anaerobic ammonium oxidation (anammox) has received extensive attention due to its high efficiency and low cost in recent years. Most AOB and NOB are aerobic autotrophic bacteria, denitrifying bacteria are anoxic heterotrophic bacteria, while AnAOB is strictly anaerobic bacteria. All the nitrogen-removing functional bacteria grow slowly, and the generation time of anammox bacteria is up to 11 d. Since AOB grows more slowly than NOB, the conversion of ammonia nitrogen to nitrite becomes the rate-limiting step in nitrification processes. Since AOB, NOB and AnAOB are highly environmentally sensitive bacteria with slow growth rates and low assimilation efficiencies, the retention of these autotrophic microorganisms in wastewater treatment becomes the key factor affecting the efficiency of nitrogen removal [18,19]. However, the

Table 1
Common signal molecules and regulatory genes in bacteria

Signals	Chemical structure	Characteristics	Source	Regulatory genes		Bacteria in sewage treatment	Reference
				Synthesis	Reception		
AHLs		Intraspecific signals	Gram-negative bacteria	<i>luxI/luxM</i> <i>hdtS</i>	<i>luxR</i>	AOB, NOB, AnAOB, denitrifying bacteria, etc.	[11]
AIP		Intraspecific signals	Gram-positive bacteria	<i>agrC</i>	<i>agrB agrD</i>	Unknown	[9]
AI-2		Interspecific signals	Gram-negative and Gram-positive bacteria	<i>luxS</i>	<i>luxQ</i>	AnAOB, etc.	[9]
DSF		Intra- and interspecific signals	Gram-negative and Gram-positive bacteria	<i>rpfF</i>	<i>rpfC</i>	<i>Xanthomonas</i>	[12]
c-di-GMP		Intracellular signal	Gram-negative and Gram-positive bacteria	DGCs genes	Unknown	AnAOB	[13]
PQS		Intercellular signal	Gram-negative bacteria	<i>pqsR</i>	<i>pqsABCDE</i>	Denitrifying bacteria	[14]

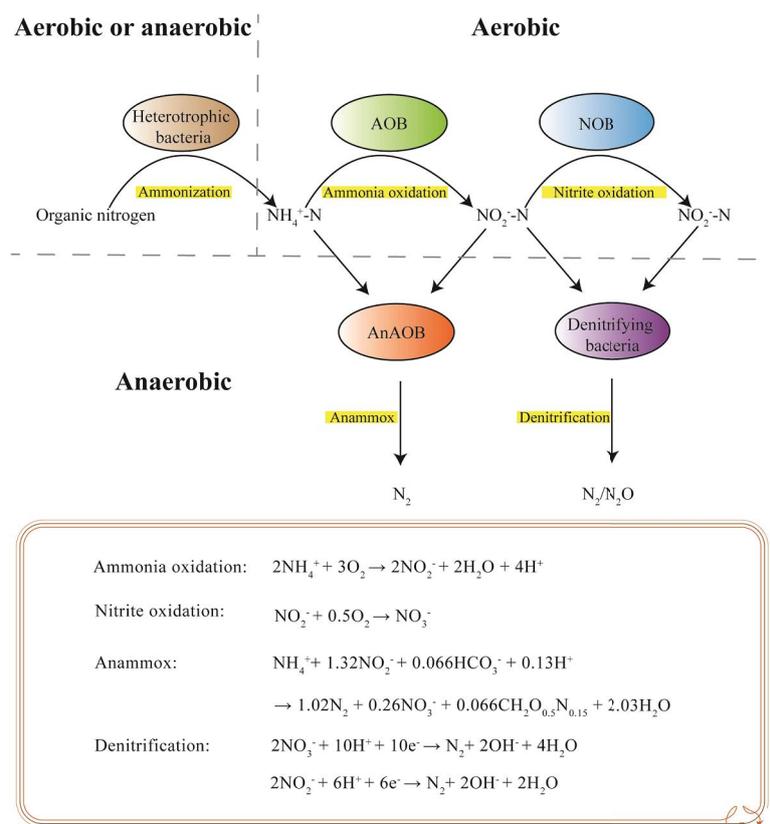


Fig. 1. Biological nitrogen removal process and principle.

formation of biofilms and anammox granules effectively avoids the loss of biomass [6,20]. A biofilm is a special form of bacterial existence. Bacteria secrete polysaccharide matrix, protein and other substances to wrap themselves on the surface of the matrix and form a large number of highly ordered membrane polymers [21]. The existence of biofilm can resist adverse conditions, provide a good environment for the survival of microorganisms, and hold a variety of microorganisms on the surface, which is beneficial to the stability and higher pollutants removal efficiency of the reactor [22]. Granular sludge is a special form of biofilm [23]. Owing to the relatively high cell density in biofilm and granular sludge, QS is possible to be triggered. Subsequently, it was first confirmed that QS accelerated the process of anammox by adding signal molecules [24]. Exogenous signal molecules also affected the growth metabolism of AnAOB by regulating LysoPC (20:0) metabolism and regulated the content of EPS by mediating the synthesis of uridine diphosphate N-acetylgalactosamine [25]. Prior studies have shown that QS can regulate the activities, growth rates and EPS formation of nitrogen-removing functional bacteria. Furthermore, nitrous oxide produced by nitrification and denitrification will destroy stratospheric ozone, and further accelerate global warming [26]. Recent researches revealed that QS affected the activities of enzymes (including nitrate reductase, nitrite reductase, nitrite oxidoreductase, and nitrous oxide reductase) by regulating the key enzyme genes expression, such as *nirS*, *norB*, *norC*, *nosZ*, *nirK* and *ncgABC*, then eventually influenced

the conversion of nitrogen oxide gases (NO, NO₂ and N₂O) and N₂ in nitrogen metabolism [27,28]. The emergence of the QS-based regulation method provides new perspectives for the transformation of nitrate and nitrite. Therefore, it is a valuable topic to study the functional bacteria in the process of nitrogen removal from the perspective of QS.

In the field of wastewater treatment, the first application of QS-based technology was to solve the problem of membrane biofouling. Quorum quenching (QQ) is a kind of antagonistic QS method, which is a process of disrupting QS to inhibit gene expression and bacterial behaviors [29]. In the membrane bioreactors (MBRs) system, membrane biofouling was effectively alleviated by adding an enzyme (acylase) to inhibit the AHL-based QS system [30]. Adding an indigenous QQ bacterium (*Acinetobacter* sp. DKY-1) to MBR can also substantially mitigate membrane fouling via the suppression of the AI-2 based QS system [31]. However, QQ inhibited the removal of ammonia mainly by suppressing the activity of AOB [32]. In granular sludge, QS-based technology has also been applied to accelerate the formation of granular sludge aerobic by regulating EPS to enhance cell adhesion [33,34]. In addition, QS-based technology showed an efficient performance by promoting swarming, EPS production, and biofilm formation in a bioaugmentation system for tobacco wastewater treatment [35]. Many researchers in recent years have paid much attention to the role of QS in AnAOB and attempt to promote performance and stability in the anammox process through QS-based technology [36–40].

This article aims to present the research status of QS in nitrogen-removing functional bacteria, and several prospects and suggestions are conducive to promote the optimization and engineering applications of QS in the nitrogen-removing process.

2. QS in AOB

AOB, as a type of microorganisms widely found in soils, lakes, sediments and oceans, can convert ammonia nitrogen into nitrite in the first step of the nitrification process [41]. Due to the slow growth rate, sensitivity to environmental factors and other limitations of the physiological characteristics of AOB, the ammonia oxidation process has become a rate-limiting step for biological nitrogen removal. By adding N-hexanoyl-L-homoserine lactone (C6-HSL) and N-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL), the average growth rates of biomass were almost twice that of the control, and the bacterial adhesion and nitrogen removal efficiency were also improved [49]. It is demonstrated that QS plays an important role in regulating AOB metabolic behaviors in increasing growth rate, promoting bacterial adhesion and improving nitrogen removal efficiency, which also provides a new perspective for ammonia oxidation process optimization based on QS technology (Table 2) [48–50,52].

As *Nitrosomonas europaea* is one of the most thoroughly studied AOB at the molecular level, with the whole genome sequencing completed in 2003, it has become the most studied AOB on QS [42]. The first report about the phenomenon of QS in *N. europaea* was published in 1997 [43]. It was suggested that the production and accumulation of signal molecules made the *N. europaea* recover rapidly to a level not possible in a relatively low cell density culture. Similarly, the exogenous addition of 3-oxo-C6-HSL signal molecules into low-cell-density

suspended cultures could also recover the growth of *N. europaea* under starvation. Other studies also found that there was no difference in the recovery of *N. europaea* in the presence of N-octanoyl-L-homoserine lactone (C8-HSL) [44]. Then, through thin layer chromatography and gas chromatography–mass spectrometry (GC-MS), the researchers further discovered that *N. europaea* strain Schmidt could produce at least three signal molecules with different chain lengths, including C6-HSL, C8-HSL and N-decanoyl-L-homoserine lactone (C10-HSL) [45]. However, *luxI/luxM* homologs acting on the typical AHLs synthase were not detected in this strain, indicating that there may be another QS regulatory system of AHLs synthesis.

The complete genome analysis revealed that a synthesis protein encoded by *nmuI* gene was in *Nitrosospira multiformis*, which was more than 60% similar to the AHL synthase *lasI* in *Pseudomonas* [46]. Then, two long-chain signal molecules N-tetradecanoyl-L-homoserine lactone (C14-HSL) and N-(3-oxo-tetradecanoyl)-L-homoserine lactone (3-oxo-C14-HSL) were successfully detected after introducing the gene *nmuI* into *Escherichia coli* [47]. By using the genomic database search and experimental identification, it was confirmed that *N. multiformis* (ATCC 25196) and *Nitrosospira briensis* also had *luxI/R* homolog, and the AHL they produced was identified as N-(3-hydroxytetradecanoyl)-L-homoserine lactone (3-OH-C14-HSL) [48]. However, regardless of the culture supernatant of *N. multiformis* or pure-cultured *N. europaea*, AHLs were not discovered, which may be attributed to the existence of some AHLs degradation mechanisms, possible AHL-inactivating activities or other concealed factors that affected the synthesis of AHLs.

Besides AOB in pure culture, QS characteristics of nitrifying bacteria in lab-scale nitrifying reactors were also studied. Six kinds of AHLs were added to autotrophic

Table 2
QS research status of nitrifying bacteria

Type of bacterium	Genus and species	QS ^a	Produced signal molecules	Functions
AOB	<i>Nitrosospira multiformis</i>	+/+	C10-HSL	Unknown
	<i>Nitrosospira briensis</i>	+/+	3-OH-C14-HSL	Unknown
	<i>Nitrosomonas europaea</i>	-/-	/	/
	<i>Nitrosomonas eutropha</i>	-/-	/	/
	<i>Nitrosococcus oceani</i>	-/-	/	/
	<i>Nitrobacter hamburgensis</i>	+/-	/	/
NOB	<i>Nitrobacter vulgaris</i>	+/+	C10:1-HSL	Nitrogen oxides metabolism
	<i>Nitrococcus mobilis</i>	+/-	/	/
	<i>Nitrospina gracilis</i>	-/-	/	/
	<i>Nitrospira defluvii</i>	-/-	/	/
	<i>Nitrospira moscoviensis</i>	+/+	C8-HSL	Unknown
	<i>Nitrobacter winogradskyi</i>	+/+	C7-HSL, C8-HSL, C9-HSL, C10-HSL, C10:1-HSL	Nitrite oxidation, nitrogen oxides metabolism

^aThe data indicate whether the species genome contains a putative AHL synthase (*luxI* homology) and a putative AHL receptor (*luxR* homology), and whether AHL is detected in pure cultures by a bioassay. For example, +/- means that the genome contains QS genes and AHLs are not detected.

nitrifying sludge in the mixed culture of nitrification reactor to investigate the effects of AHLs on cell adhesion, nitrification and sludge granulation [49]. The results showed that the average biomass growth rates of C6-HSL and 3-oxo-C6-HSL supplementation were 0.260 and 0.227 g SS L⁻¹ d⁻¹, respectively, almost double that of the control (0.126 g SS L⁻¹ d⁻¹). By changing the composition of EPS to prevent the loss of slow growth of nitrifying bacteria, 3-oxo-C6-HSL and C6-HSL were also beneficial to promote bacterial adhesion and improve nitrogen removal efficiency, thus accelerating the granulation of nitrifying sludge. Similarly, the cellular extract containing AHL molecules from nitrifying granular sludge was added into nitrifying activated sludge, which improved the cell adhesion and aggregation, increased the microbial activity, and promoted the maximum ammonia removal efficiency by 83.3% after treatment for 30 d [50]. However, the average growth rate of the sludge added the cellular extract just increased 0.01 g SS L⁻¹ d⁻¹, far less than 0.134 g SS L⁻¹ d⁻¹ dosed with C6-HSL [49], which might be due to the low AHLs concentration in the cellular extract. For ammonia oxidation systems with AOA and AOB coexisting, adding AHLs can significantly improve ammonia oxidation rate and the number of *amoA* genes, and AOA microbial community composition changes, but it has no significant effect on AOB community composition, which may be caused by the specificity of AHLs [51]. In nitrifying biofilm systems, C6-HSL and C8-HSL were proved to be related to the activities of AOB and NOB and regulate biofilm formation by affecting the production of EPS [52]. Additionally, in the MBR system, QQ inhibited the removal of ammonia mainly by suppressing the activity of AOB rather than changing the abundance of AOB, further indicating that the activity of nitrifying bacteria was under the regulation of QS [32]. The AHLs have positive effects on the performance of nitrifying systems according to the previous work.

The above studies have rudimentarily revealed the potential regulation mechanism of QS on AOB. On the one hand, QS can regulate the metabolism rate of AOB, improve the growth rate of bacteria, and thus improve the ammonia oxidation rate; on the other hand, QS signal molecules can promote the secretion of EPS to enhance the adhesion of AOB, and assist AOB to resist the influence of environmental factors. However, the regulation mechanism of QS in AOB has not been clarified in current research, and considering other bacteria may also produce and use signal molecules, the experimental results may interfere under the conditions of mixed culture. Thus, future research needs to focus on the QS regulation mechanism in AOB, and explicit how signal molecule works.

3. QS in NOB

NOB, which can oxidize nitrite to nitrate, belongs to Gram-negative bacteria, including four strains: *Nitrobacter*, *Nitrococcus*, *Nitrospira* and *Nitrospina*. The strict growth conditions of NOB lead to the slow growth rate and difficult enrichment and ultimately limit the rate of nitrification process in the wastewater

treatment system. The optimum growth conditions are temperature 25°C ~ 30°C, pH 7.5~8.0, nitrite concentration 2~30 mmol L⁻¹, doubling time 10 h to several days [53]. As previously described, QS of NOB has a certain regulatory effect on nitrification rate, but only a few NOB have been proven to produce AHLs, as shown in Table 2 [48].

Nitrobacter winogradskyi is considered to be a typical species for the study of NOB due to its multiple metabolic capacities, good tolerance to nitrite and wide distribution [18]. Two different signal molecules, C10-HSL and 7,8-*trans*-N-(decanoyl) homoserine lactone (C10:1-HSL), were detected during the batch culture and chemostat culture of *N. winogradskyi* [54]. By sequencing the whole genome of this bacteria, it was inferred that the genome contains AHL synthetase and receptor genes *nwi0626* (*nwiI*) and *nwi0627* (*nwiR*) which were subsequently confirmed to be related to the production of AHLs [54,55]. In 2017, Shen [42] expressed *nwiI* gene heterogenically in *E. coli* and produced eight different signal molecules: N-heptanoyl-L-homoserine lactone (C7-HSL), C8-HSL, N-nonanoyl-L-homoserine lactone (C9-HSL), C10-HSL, C8:1-HSL, C9:1-HSL, C10:1-HSL and C11:1-HSL [42]. The types of signal molecules produced by *N. winogradskyi* under different culture conditions (autotrophic, heterotrophic and mixotrophic culture conditions) were also identified through LC-MS technology. The results showed that AHL signal molecules could be produced under all three culture conditions, but the content of signal molecules (per unit of bacteria) was the highest under autotrophic conditions [42]. In addition, C7-HSL and C10:1-HSL could be produced under mixotrophic culture conditions, but only C10:1-HSL could be detected under both autotrophic and heterotrophic conditions [42]. The different cultural conditions may be the possible reason for the producing AHL different from previous reports [54]. Introducing C10:1-HSL to the culture medium of *N. winogradskyi* resulted in the improvement in nitrite utilization rate and nitrification rate, and the expression of related genes was also affected [42]. Then, the researchers added the AiiA enzyme as a QQ enzyme to the *N. winogradskyi* culture, and transcriptome analysis was used to determine the presumed QS control gene. The results revealed that QS might be related to the nitrite reductase gene *nirK* and its gene cluster *ncgABC*, which mediate the conversion of nitrogen oxide gases (NO, NO₂, and N₂O) in nitrogen metabolism [28]. As a result, the production and consumption of NO_x increased, while the production of N₂O decreased under QS-proficient conditions [28]. These results preliminarily revealed the regulation mechanism and of QS in *N. winogradskyi*.

However, NOB *Nitrobacter hamburgensis* and *Nitrococcus mobilis* do not produce AHLs in pure culture even if they have AHLs synthase gene, indicating that cell density is not the only factor controlling microbial QS, and environmental pressure is also a possible factor [48,56]. Moreover, the same bacteria produce different signal molecules under different conditions, which may be the specific expression of bacteria adapted to different environments [42].

In recent years, complete ammonium oxidation (Comammox), another type of NOB widely distributed in soils, freshwater environments and sewage treatment plants, have attracted much attention due to their ability

to convert ammonia nitrogen to nitrate independently. Meanwhile, Comammox has a higher affinity for ammonia nitrogen compared with most culturable AOA and AOB, so as to better adapt to the extremely low ammonia concentration environment [57–59]. Although some studies have identified that Comammox has genes for AHL synthetic and EPS formation, it is unclear whether QS plays a role in Comammox and which kinds of Comammox relate to QS [52].

Current studies have proved that a few NOB exist QS, and QS can regulate the conversion of nitrate and improve the efficiency of nitrification. Nevertheless, it is worth noting that although some NOB has AHL synthase, no signal molecules have been detected under pure culture conditions. Therefore, more types of NOB need to be studied to further clarify the QS mechanism of NOB in future work. Moreover, whether the QS mechanism in Comammox is different from traditional NOB is waiting to be answered in future studies.

4. QS in AnAOB

Anaerobic ammonium oxidation (anammox) refers to the process in which AnAOB converts nitrite to nitrogen with ammonia nitrogen as electron donor and nitrite nitrogen as an electron acceptor under anoxic or anaerobic conditions [17]. Compared with the traditional nitrogen removal process, the anammox process has become a promising process due to its little sludge production, no organic carbon source requirement and cost-effective [47]. However, the low growth rate and strict environmental requirements of AnAOB limit the application of the anammox process on a large scale. Thus, how to shorten the start-up time of anammox and improve AnAOB activity have become attractive fields in anammox-related researches.

The biological activity of AnAOB could be reflected only when the cell concentration reaches 10^{10} to 10^{11} cells mL^{-1} [60]. This effect of cell density is consistent with the phenomenon of QS in regulating microbial behaviors, suggesting that QS may exist in AnAOB [57]. Subsequently, several signal molecules have been detected in the supernatant of anammox culture, such as C6-HSL, C8-HSL and N-dodecanoyl-L-homoserine lactone (C12-HSL), proving the existence of QS. Exogenous modulation strategies have been shown to effectively regulate the growth and metabolism of AnAOB [61]. Furthermore, genes related to the synthesis of signal molecules have been found in AnAOB from the point of view of genomics, which essentially reveals the QS mechanism in AnAOB.

QS technology provides new insights into the performance optimization of anammox processes. De Clippeleir et al. [24] firstly confirmed that AHL accelerated the process of anaerobic ammonium oxidation by adding C12-HSL, which was successfully detected when studying the rate of anaerobic ammonium oxidation in the oxygen-limited autotrophic nitrification/denitrification (OLAND) biofilm. In another study, the addition of C8-HSL could significantly improve the activity of AnAOB, and C6-HSL can not only enhance the activity of AnAOB, but also

significantly promote its growth rate by 28%, while C12-HSL promoted the growth of heterotrophic bacteria in the community and reduced the activity of AnAOB [61]. In the above two studies, exogenous addition of C12-HSL had the opposite effect on AnAOB, which may be due to the differences in system operating conditions and flora structure, indicating that the exogenous regulation strategy is not a “master key” and needs to be adjusted according to the cultivation conditions. Meanwhile, the large differences in AHL signal molecules function even between closely related strains inferred that dynamic changes in the environment control the maintenance of bacterial communities and thus affect the QS system. In addition, when $150 \mu\text{M}$ C6-HSL was added to the UASB reactor, the activity of AnAOB granular sludge increased by 16% [62]. Apart from increasing the activity of AnAOB through QS regulation, studies had also confirmed that adding 3-oxo-C6-HSL signal molecules could affect the growth metabolism of AnAOB by regulating LysoPC (20:0) metabolism [25]. According to the above research, the improvement of growth rate and activity of AnAOB based on QS technology is expected to fundamentally solve the main bottleneck of long start-up time in the practical application of the anammox process.

In addition, to regulate the growth rate and activity of AnAOB, QS can also affect the formation of EPS. It is necessary to apply efficient biomass retention of AnAOB to keep the stable operation of the anammox process. The formation of biofilms and anammox granules more effective than suspended sludge to avoid the loss of biomass, because the immobilization of AnAOB is significant to resist adverse conditions and maintain a higher biomass density [63]. EPS, as the barrier between bacteria and the environment, is mainly composed of proteins, polysaccharides and nucleic acids, which is crucial for bacteria to cope with environmental pressure [64]. The increase of EPS will promote the formation of AnAOB biofilm and granular sludge, thus shortening the start-up time and maintaining the stable operation of the anammox process system [13,36]. QS could regulate the content of EPS by mediating the synthesis of uridine diphosphate N-acetylgalactosamine (UDP-GlcNAc) and improving the synthesis rate of adenosine triphosphate [20,25]. A previous study showed that the addition of vanillin and porcine kidney enzyme led to the degradation of AHL, resulting in the decrease of denitrification ability of AnAOB, the decrease of protein and polysaccharide content in EPS and the decrease of particle stability, indicating that the inhibition of QS was not conducive to the stability of granular sludge and the treatment effect of anammox process [37]. Meanwhile, based on the addition of 3-oxo-C6-HSL, 3-oxo-C8-HSL and C6-HSL, EPS synthesis was promoted and the aggregation levels of anaerobic bacteria changed when five signaling molecules ($2 \mu\text{M}$ each for 3-oxo-C6-HSL, 3-oxo-C8-HSL, C6-HSL, C8-HSL and C12-HSL) were added to the experiment, reconfirming that QS regulates the production of AnAOB [25]. Apart from AHL-mediated QS, other types of QS have also been found in the anammox process. Boron, as an AI-2 activating factor, was added into the UASB reactor to explore the effect on the anammox process [39]. The results showed that the sludge in both reactors presented granulation after 132 d of

acclimation, and the total nitrogen load of the influent of the reactor with boron was three times that of the reactor without boron, while the nitrogen removal rate of the reactor with boron was 10% higher. Additionally, the volume average particle size of the granular sludge in the reactor with boron was 430.469 μm , slightly larger than that in the reactor without boron (357.122 μm). Moreover, it was shown the higher concentration of AI-2 signal molecules, the higher content of EPS, and more AnAOB enrichment in the reactor with boron. The above results suggested that boron could increase the concentration of AI-2, promote the synthesis of EPS to form granular sludge with larger particle size and tighter structure and shorten the start-up time of the anammox process system [38,39]. Similarly, the c-di-GMP signal molecule was reported to promote the synthesis of EPS and improve the aggregation ability of the anammox colony [13]. These findings have suggested that the formation of EPS and the accumulation of anammox colony can be promoted through QS regulation, thus reducing the environmental sensitivity of AnAOB and enhancing system stability.

Molecular studies have deepened the understanding of QS in AnAOB and provide a new perspective for better researching it. The first evidence of QS in AnAOB at the metabolic level was discovered by Strous et al. [65]. The synthetic genes of acyl-acyl carrier protein (acyl-ACP) and S-adenosyl methionine are necessary biomolecules for AHLs synthesis in *Candidatus Kuenenia stuttgartiensis* [65]. The *htdS* type AHL synthetic genes *jqsI-1* and *jqsI-2* of *Candidatus Jettenia caeni* were successfully expressed in vitro to produce four AHLs, and it was found that AHL could regulate the activity of AnAOB by affecting the expression of *hzsA* gene [40]. In recent years, the diguanylate cyclases (DGCs) genes that synthesize intracellular signal molecule, c-di-GMP, were discovered in the anammox system, and 13 putative genes encoding c-di-GMP were detected in *Candidatus Jettenia caeni* through a variety of protein sequence comparison techniques [13,66]. For interspecific signals, *lsrf*, a AI-2 regulatory genes, and *rpfF*, a DSF synthetic gene, with high abundance in anammox system indicated that AnAOB can also regulate the expression of related genes through AI-2 and DSF mediated QS system, thus inferring that interspecific communication in anammox system relies on complex "language" [66,67].

The deduced QS mechanism in anammox is a hierarchical signaling mechanism system (Fig. 2). In this mechanism, the downstream GRMI-GRMR and EPSI-EPSR QS channels are regulated by the upstream AMXI-AMXR QS channel [68]. In the upstream AMXI-AMXR QS channel, the signal molecules are synthesized by the AMXI protein and released outside the cell under the regulation of *amxI* gene. Due to the different types of AMXI protein, the diversity and specificity of AHLs can be produced with the differences of acyl side chain length, carbon-chain skeleton saturation and substituents. Then, AHL is bound to form the AMXR-AHL protein complex with the receptor protein AMXR regulated by the *amxR* gene. The AMXR-AHL protein complex can not only regulate the expression of AnAOB related genes to achieve anaerobic ammonia oxidation but also positively regulate the expression of related genes in the downstream QS channels [69]. In the downstream QS channels, the GRMI-GRMR

QS channel can positively regulate the expression of genes related to the growth and metabolism of AnAOB through the GRMR-AHL protein complex which is bound by receptor protein GRMR and AHL produced by GRMI protein [25]. The EPSI-EPSR QS channel can regulate the expression of genes related to EPS formation and hydrolysis through the EPSR-AHL protein complex which is formed by receptor protein EPSR and AHL produced by EPSI protein [25]. AnAOB assesses its cell density through the content of AHLs. When the cell density of AnAOB is too low to reach the initiation threshold of QS, the AHLs cannot bind to the AMXR protein and other ways will regulate the growth of AnAOB and EPS synthesis, such as c-di-GMP pathway [13]. At this time, AMXR only performs the duty of activating the enzyme to activate the phosphate channel of the corresponding operon *AmxO*. *AmxO-P* encode *Qrr* small regulatory RNAs (*Qrr* sRNAs) genes, and then *Qrr* sRNAs inhibit *HapR* genes, while *HapR* genes can inhibit c-di-GMP to translate polysaccharide RNA, and subsequently inhibit the formation of biofilms. Therefore, at low cell density, the c-di-GMP pathway regulates the formation of biofilms to increase cell density. The concentration of extracellular AHLs increases with the increase of the proliferation and quantity of AnAOB. When the extracellular AHLs are higher than the threshold, the AMXR-AHL protein complex is formed to trigger anammox, activate the downstream GRMI-GRMR and EPSI-EPSR QS channels and promote the growth of AnAOB and the synthesis of EPS. With the further increase of AnAOB, the excessive AMXR-AHL protein complex in cells will trigger the expression of EPS hydrolyzation-related genes to hydrolyze the excess EPS. Therefore, the biofilm of AnAOB cannot continue to form in high cell density. Through AHL and c-di-GMP mediated QS system, AnAOB can dynamically regulate its growth and metabolism, the synthesis and hydrolysis of EPS to maintain the stability of the AnAOB system.

In summary, QS in AnAOB can make AnAOB better gather in the system, occupy more resources and space, and gradually become the dominant population in the environment. Specifically, it can improve the growth rate and activity of AnAOB, affect the synthesis of EPS, then increase the anammox rate, shorten the start-up time of anammox, and enhance system stability. At the same time, QS was also found to have negative effects on AnAOB activity under different cultural conditions. Moreover, signal molecules have not been detected in pure cultured AnAOB, and this problem is worthy of further study. The discovery of the AnAOB QS mechanism provides a new perspective for the dynamic regulation of bacterial growth and metabolism. However, QS regulation has not been applied to the actual anammox process. On the one hand, the exogenous regulation strategy is too expensive, so it is necessary to explore the methods and techniques for bacteria to continuously produce signal molecules in the environment; on the other hand, the negative effects of QS on the system have not been explored clearly, and different types of signal molecules may have opposite effects, so QS mechanism in AnAOB at the genetic level is urgently needed. Therefore, research should not be limited to the influence of a single type of signal molecule on the system, but also explore the combined regulation mechanisms of multiple types of QS.

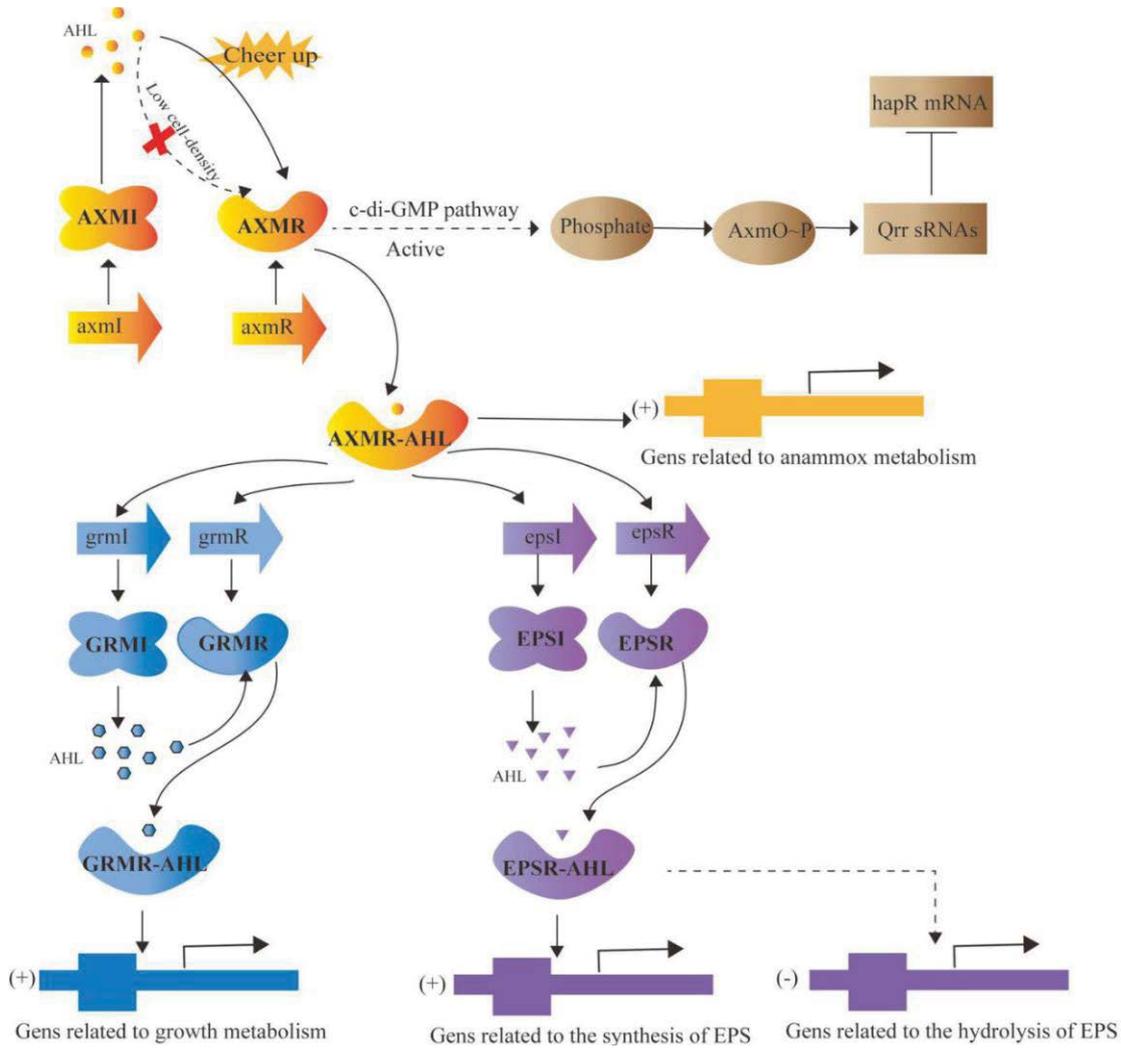


Fig. 2. The hierarchical QS signaling mechanism of AnAOB.

5. QS in denitrifying bacteria

Denitrification is an important part of nitrogen cycle, which completes the conversion of nitrate and nitrite to gaseous nitrogen under anoxic conditions. *P. aeruginosa*, as one of the pathogenic bacteria widely distributed in nature, is the primary target for the most research of QS in denitrifying bacteria at present. It was found that the expression of 350 genes in *P. aeruginosa* was regulated by QS system [70]. The denitrification activity of *P. aeruginosa* PAO1 can be inhibited by exogenous addition of C4-HSL and 3-oxo-C12-HSL [71]. Similarly, PQS also affects the denitrification process by at least two pathways: one pathway depends on the iron-chelating property of PQS, and the other pathway depends on PQS transcriptional regulation mediated by *pqsE* and *pqsR* genes [72]. The addition of PQS into the medium inhibited the production of N₂O, but promoted the accumulation of NO, since the nitrite reductase activity was elevated, while the nitrite oxidoreductase activity was suppressed [72]. Zhu et al. [73] demonstrated that the lack of 3-oxo-C12-HSL, C4-HSL, or PQS, had positive effects

on the ammonia removal and cell growth, whereas high concentrations of PQS suppressed the aerobic denitrifying activity [73]. Moreover, AHL and PQS can regulate denitrification simultaneously was also proved in this study. *P. aeruginosa* was deduced to have four QS systems that regulate its metabolism independently and dependently, namely two AHL-mediated system (*las* and *rhl*), PQS based QS system and QS-dependent system, as well as an orphan *luxR*-type regulator, *qscR* [68,74]. As reported, the three systems of *las*, *rhl* and PQS are in a cascading control relationship to regulate the biofilm formation and membrane vesicles (MVs) production, accordingly forming a dynamic balance (Fig. 3). In the *las* system, 3-oxo-C12-HSL synthesized by *lasI* protein binds to the receptor protein *lasR* regulated by the *lasR* gene, to form the *lasR*-3-oxo-C12-HSL protein complex. Then, the *lasR*-3-oxo-C12-HSL protein complex activates *las*, *rhl* and PQS system. In the PQS system, the *pqsH* gene regulated by *lasR* gene, encodes mono-oxygenase necessary for the transformation of the precursor HHQ into PQS. The complex formed by PQS and its receptor protein *pqsR* can regulate *rhl* and PQS system.

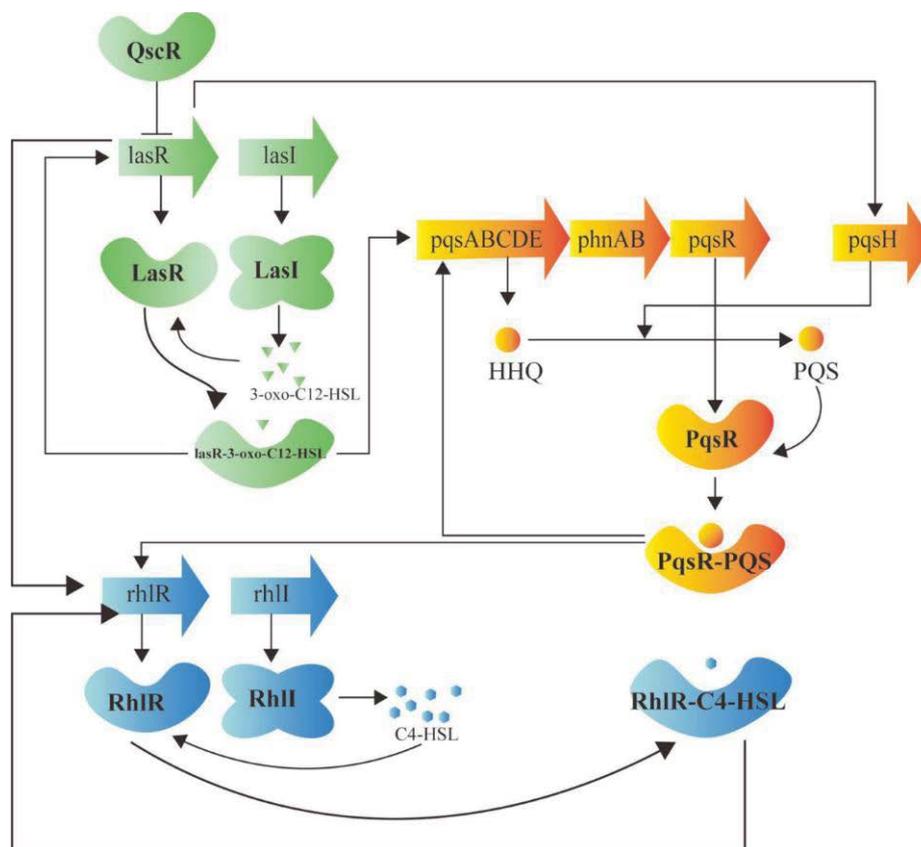


Fig. 3. The three QS systems, *las*, *rhl* and PQS in *P. aeruginosa*.

In the *rhl* system, *rhIR* regulated by *rhIR* gene binds its cognate signal, and the complex autoregulates the *rhl* system [68,75,76].

Paracoccus denitrificans, as a kind of aerobic denitrifying bacteria, have been confirmed to produce C16-HSL. When investigating the influence of exogenous signal molecules on *P. denitrificans* through adding C6-HSL and C14-HSL produced by other bacteria, and self-produced C16-HSL, it was found that the effects of long-chain signal molecules on the growth and denitrification process of *P. denitrificans* were more significant than that of short-chain signal molecules [27]. This may be due to the fact that C16-HSL was produced by *P. denitrificans* themselves, which makes it more responsive to long-chain signal molecules. In addition, *P. denitrificans* can also transmit signals through MVs. The MVs released by *P. denitrificans* can absorb the long-chain AHLs in the environment and transmit the signal to themselves. This signal transduction mechanism may be beneficial for *P. denitrificans* to reach the QS threshold at low cell density [77]. Moreover, exogenous C6-HSL and C14-HSL and C16-HSL significantly suppressed the accumulation activity of N_2O in *P. denitrificans* under aerobic conditions. Conversely, exogenous C6-HSL and C14-HSL and C16-HSL accelerated the production of N_2O under anaerobic culture conditions [27]. By measuring the individual messenger ribonucleic acid (mRNA) levels of nitrate, nitrite, nitric oxide and nitrous oxide reductases, it was found that transcription of the key

enzyme genes in the AHLs-treated sample was repressed under aerobic conditions, while the expression of these genes was increased under anaerobic conditions, which was consistent in the accumulation activity of N_2O [27]. The result suggested that QS affected the enzyme activities of nitrate reductase, nitrite reductase, nitrite oxidoreductase, and nitrous oxide reductase by regulating the related enzyme genes expression, and ultimately affected the transformation of nitrite to nitric oxide and its reversion to nitrous oxide gas. By removing the functionality of the *lasI* and *rhlI* QS system, the mutant *P. aeruginosa* PAO1 had a much higher N_2O removal rate resulting in little N_2O accumulation compared with the wildtype *P. aeruginosa* PAO1 [78]. The above studies indicated that the regulation of denitrification by QS may play an important role in nitrate conversion to decrease N_2O release.

Recent studies preliminarily proved that bacterial activity and denitrification rate can be regulated by the QS system, but the regulation mechanism in the whole denitrification process cannot be clarified due to the insufficient data available. The role of QS in other denitrifying bacteria, the function of different signal molecules in different denitrifying bacteria and the specific regulatory QS mechanism in denitrifying bacteria need to be elucidated. In addition, some new findings are worthy of further study. For example, the discovery of MVs explains that long-chain signal molecules have a more significant effect on *P. denitrificans* than short-chain signal molecules. Whether

this mechanism exists in other QS-mediated bacteria also deserves further exploration.

6. Conclusions and prospects

This article reviews the research progress and potential utilization of QS in four nitrogen-removing functional bacteria, that is, AOB, NOB, AnAOB and denitrifying bacteria. In general, QS can affect the growth and activity of nitrogen-removing functional bacteria, mediate the conversion of nitrate and the formation of EPS by regulating the expression of related genes. The application of the QS strategy to regulate and control nitrogen-removing functional bacteria is expected to strengthen the nitrogen removal system, shorten the start-up time of the anaerobic ammonia oxidation process, improve the nitrogen removal efficiency, and thus enhance the stability of the system. Therefore, the influence of QS on nitrogen-removing functional bacteria is mainly positive and has wide application prospects in wastewater treatment. However, there are still several aspects that urgently await further investigation:

- Evaluation of the negative effects of QS regulation strategy on the system.

QS can affect the physiological behavior and community structure of bacteria, which is beneficial to some bacteria but inevitably inhibits others. Besides, the effect of the same signal molecule is inconsistent in different studies, such as the effect of adding C12-HSL on bacterial activity in AnAOB. This negative effect has not been well-paid attention to. Simultaneously, the influence of QQ on QS-mediated technology needs continuous investigation.

- Effects of by-products in the nitrogen removal process.

The regulation of NO and N₂O emissions by QS during denitrification may play a role in slowing down the Greenhouse Effect. Although some studies have proved that QS is involved in the regulation of nitrogen oxides, the specific way of regulation and indirect effects on other N-cycling processes are still unclear.

- Interaction of multiple signaling molecules.

The research on nitrogen-removing functional bacteria is mostly focused on the intraspecific signal molecules-AHLs, while the research on interspecific signal molecules, such as DSF and AI-2, and their potential roles are rarely involved. An in-depth study of the interaction of multiple signaling molecules at the ecological level is significant for the practical application of QS in full-scale wastewater treatment.

- Cost control of QS strategy.

Adding exogenous signal molecules to the system is a common method based on QS regulation, but the cost is too expensive to be applied on a full scale, therefore the in-depth exploration of embedding technology is expected to solve this problem. At the same time, whether there

are differences between the effects of exogenous signal molecules and that self-produced by bacteria on bacterial metabolism and system needs subsequent analysis.

- In-depth study of QS regulatory mechanism.

Most studies are carried out under the condition of mixed cultures, the essence of signal molecule transformation in the system cannot be precisely explored. The development of pure culture technology has the potential to explore the QS mechanism. However, the pure cultures of nitrogen-removing functional bacteria are difficult to obtain in most traditional methods, so it is necessary to develop pure culture technology.

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Authors' contributions

Feng Luo, Huizhi Hu and Yirong Liu developed the idea of the review. Feng Luo participated in its design and wrote the main part of the manuscript. Yirong Liu contributed to refining the ideas and wrote parts of the manuscript. Huizhi Hu provided critical review and substantially revised the manuscript. All authors read and approved the final manuscript.

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