Original Research

Quorum sensing systems regulate heterotrophic nitrification-aerobic denitrification by changing the activity of nitrogen-cycling enzymes

Ziqian Zhu, Yang Yang, Anran Fang, Yu Lou, Guojun Xie, Nanqi Ren, Defeng Xing

State Key Laboratory of Urban Water Resource and Environment, School of Environment, Harbin Institute of Technology, Harbin, 150090, China

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ABSTRACT

Heterotrophic nitrification-aerobic denitrification (HNAD) is essential in diverse nitrogen-transforming processes. How HNAD is modulated by quorum sensing (QS) systems is still ambiguous. The QS system in Pseudomonas aeruginosa manipulates colony behavior. Here, we described the influence of the Pseudomonas quinoline signal (PQS) and N-acetylhomoserine lactone (AHL) on HNAD. The HNAD of P. aeruginosa was inhibited by the oversecretion of PQS. AHL- or PQS-deficient P. aeruginosa mutants had a higher ability for nitrogen removal. QS inhibited heterotrophic nitrification mainly via controlling the activity of nitrite oxidoreductase (NXR) and the depressed aerobic denitrification by regulating the catalytic abilities of nitrite oxidoreductase (NOR), nitrite reductase (NIR), and nitrate reductase (NAR). The addition of citrate as the sole carbon source increased the nitrogen removal efficiency compared with other carbon sources. Nitrite, as the sole nitrogen source, could be used entirely with only the moderate concentration of PQS contained. AHL and PQS controlled both nitrification and denitrification, suggesting that QS plays an important role in nitrogen cycle under aerobic conditions.

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1. Introduction

Nitrogen is one of the most significant water pollutants [1]. The current mainstream method of wastewater treatment is biological, i.e., through bacterial nitrification and denitrification [2]. It is commonly assumed that denitrification occurs under oxygen-limited or anoxic environments, whereas nitrification only occurs when the carbon source is scant [3]. Differences in oxygen levels and nutrition factors limit the occurrence of simultaneous denitrification and nitrification. However, heterotrophic-nitrifying bacteria, such as Bacillus spp. [4], Alcaligenes faecalis [5], and Thiophaera pantotropha [6], have the ability of heterotrophic nitrification-aerobic denitrification (HNAD). This could be particularly advantageous in sewage disposal systems, and HNAD microbes may be used to reduce expenses related to anaerobic tanks or limitations regarding their volume [7].

HNAD is a phenomenon known to transform NH₄⁺ to N₂O or N₂, which is significant for bacterial growth under aerobic and organic-rich conditions. In the metabolism of heterotrophic nitrification, substrates are first catalyzed by ammonia monooxygenase (AMO), which oxidizes the ammonia nitrogen into hydroxylamine. Hydroxylamine oxidoreductase (HAO) catalyzes hydroxylamine to yield nitrite, which is then converted into nitrate via the catalysis of the nitrite oxidoreductase (NXR) enzyme [8]. In the aerobic denitrifying stage, nitrate is converted to nitrite by nitrate reductase (NAR) and then converted to NO by nitrite reductase (NIR) [9]. Subsequently, nitrite oxidoreductase (NOR) mediates the conversion of NO to N₂O. In the process of biological nitrogen removal, the nitrogen-containing compound NH₄⁺ is reduced to N₂O and N₂ gas, which is degassed at the water surface, leading to successful nitrogen removal.

Some studies have described new forms of cellular signals, identified as cell-to-cell correspondence signals, regulating most gene expression that adjust cellular group behaviors [10]. Specifically, quorum sensing (QS) is a cell-to-cell communication used by different bacteria to respond to fluctuations in cell population density [11]. These bacteria can synthesize and transmit different signal molecules (called autoinducers) to coordinate their activities. Concomitantly, the QS system will change the expression levels of related genes by detecting the minimum stimulus concentration of specific autoinducers.

* Corresponding author. School of Environment, Harbin Institute of Technology, P.O. Box 2614, 73 Huanghe Road, Nangang District, Harbin, Heilongjiang Province, 150090, China.
E-mail address: dxing@hit.edu.cn (D. Xing).
Pseudomonas aeruginosa uses the 2-heptyl-3-hydroxy-4-quinolone and N-acetyl-l-homoserine lactone (AHL) signal molecules to manipulate gene expression according to the stimulus received by the external environment [12]. P. aeruginosa has at least two kinds of signal molecules involved in the QS systems: those regulated by acyl-homoserine lactone (the LasI-LasR, Las; and RhlI-RhlR, Rhl) and those regulated by quinoline [13]. The Pseudomonas quinoline system (PQS) controls the synthesis and reception of the quinoline signal. Las instructs the course of synthesizing and accepting the 3-oxo-C12-HSL (N-(3-oxododecanoyl)-l-homoserine lactone) AHL signal while Rhl commands the process of synthesizing and accepting another C4-HSL (N-butyryl-l-homoserine lactone) AHL signal.

Previous studies have indicated the close correlation between the biological nitrogen-recycling and QS systems [14]. It has been shown that AHL inhibits bacterial anaerobic denitrification [15], and PQS also suppresses anaerobic denitrification [16]. Additionally, some ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) have been verified to generate AHL [17]. A recent study has shown that the concentration of AHL molecules depends upon nutrient conditions and the operation period of nitrifying biofilm reactors [18]. Nitrogen removal in the biofilm reactors declines with an increased concentration of exogenous AHL [19]. Moreover, quorum quenching (QQ) of Nitrobacter winogradskyi has proven that NOx fluxes in nitrification could be regulated by AHL [20]. However, the impact of QS on the HNAD pathway and the QS regulation mechanism involved have not been clearly studied. A previous study indicated a discrepancy in the regulation of bacterial metabolism between chemically synthesized and naturally secreted signal molecules added in aerobic nitrification systems [21]. Yet, the most current research about QS control has been focused on QS control in P. aeruginosa [22]. The effects of naturally secreted signaling molecules on microbial metabolic regulation are likely different from some of the findings outlined above [17,19]. Owing to the divergent results between natural and synthetic molecule signals added to bioreactors, there is a need to examine the function of autocrine signal molecules in HNAD, simulating the actual nitrogen removal environment of microorganisms.

To reveal the mechanism of regulation of the QS system within HNAD bacteria, this study compared the aerobic nitrogen removal efficiency, aerobic denitrification, and activity of nitrogen cycle-related enzymes of P. aeruginosa wild-type and QS-deficient mutant strains under different conditions.

2. Materials and methods

2.1. Strains

P. aeruginosa wild-type PA01 and QS-deficient mutants (lasI/rhlI, pqsA, and pqsL) were offered by Dr. Tim Tolker-Nielsen (University of Copenhagen) [23]. The lasI/rhlI mutant was created by knocking out lasI (3-oxo-C12-HSL synthase) and rhlI (C4-HSL synthase) genes via insertional mutagenesis, which generated fewer AHL autoinducers [24]. The pqsA mutant was constructed by knocking out pqsA gene-encoding proteins with homology to acyl-coenzyme A (acyl-CoA) ligases [25], which was inadequate in producing PQS. The pqsL mutant tended to overproduce PQS signal molecule by transposon mutagenesis of the pqsL gene encoding the mono-oxygenase of PQS [26].

2.2. Media and cultivation conditions

Luria–Bertani (LB) medium was used as a basal medium for pre-cultivation of P. aeruginosa strains. To assay the bacterial HNAD ability and its related enzymatic activities, all pre-cultivated strains were inoculated into the LB medium supplemented with 0.042 M NH4Cl (LB-NH4Cl medium). The removal efficiency of NH4+ might be biased due to the interference of organic nitrogen in the LB-NH4Cl medium. To investigate the impact of nitrogen resource on the performance of QS regulation, we measured the nitrogen removal rates of strains in the sole nitrogen resource mediums as follows (per liter): 5.0 g sodium citrate, 0.110 g KH2PO4·3H2O, 0.10 g MgSO4·7H2O, 0.006 g FeSO4·7H2O, 1.5 g NH4Cl (120 mg L−1 nitrogen content), 0.02 g CaCl2·2H2O, and 1 mL trace element solution (containing 1 g MnSO4·7H2O, 0.2 g CoCl2·6H2O, 2 g ZnSO4·7H2O, 1 g H2BO3, 1 g Na2MoO4·2H2O, 1 g CuSO4·5H2O, and 3.5 g EDTA; pH = 7.0). To test the regulation of HNAD by QS under different nitrogen sources, the nitrogen source was replaced with 120 mg L−1 NaNO2 or NaNO3 in the nutrient medium.

To test the QS regulation under different carbon sources, the sole carbon source was then replaced (sodium succinate or sodium oxalate, which contained 360 mg g−1 carbon) in the medium mentioned above, with NH4Cl as the sole nitrogen resource [27,28]. All strains (0.1 mL) were inoculated in 50 mL LB medium and pre-cultivated (160 rpm, 12 h, 30 °C). The absorbance at 600 nm (OD600) of the culture solution was detected to adapt all cultures to an OD600 = 0.5 at the same growth level. Afterward, the pre-cultivated strains in the LB medium were centrifuged (3000 rpm, 10 min) and resuspended in phosphate-buffered saline (PBS; pH = 7.1) three times. Then, they were inoculated to each testing flask under different culture conditions. The flasks were rotated on a shaker (160 rpm, 30 °C, 24 h) to maintain a homogenous culture. Meanwhile, samples were collected from the Erlenmeyer flasks at 4 h intervals to measure the OD600 and the concentrations of ammonia (NH4+), hydroxylamine (NH2OH), nitrite (NO2−), and nitrate (NO3−). Each test was performed in triplicate.

2.3. Extraction of the crude enzyme

The crude enzyme was extracted as previously described [16]. The bacteria grown in LB-NH4Cl medium were collected from 10 mL solutions sixth hour after incubation (mid-logarithmic phase) and centrifuged at 4 °C (7000 rpm, 10 min). PBS (containing 10% glycerol, 100 mM, pH = 7.1) was then used to rinse bacterial cells twice. Cells were resuspended in the same buffer and ultrasonicated (300 W, on 10 s, off 10 s, 20 min) in an ice bath. The ultrasonicated cells were centrifuged (2000 rpm, 10 min), then re-centrifuged for 60 min at 10,400 rpm to remove intact cells. Supernatants were then collected from the same PBS buffer with 10% glycerol as the membrane part of the crude enzyme solutions.

2.4. Activity measurement of nitorgen cycle enzymes

Catalyzing abilities of six key enzymes, i.e., ammonia mono-oxygenase (AMO), hydroxylamine oxidoreductase (HAO), nitrite oxidoreductase (NXR), nitrate reductase (NAR), nitrite reductase (NIR), and nitric oxide reductase (NOR), were measured when oxygen was present. Protein concentrations in the cell fractions were determined using the BCA Protein Quantitation Kit (Sangon Biotech Co. Ltd., Shanghai, China) according to the manufacturer’s guidelines. All assays were terminated by heating the solution to 100 °C for 15 min [16].

The activity of AMO was evaluated by detecting its nitrite product [29]. The 20 mL reaction mixture for AMO activity determination included the membrane fraction of the crude enzyme solutions, 2 mM ammonium sulfate, and PBS buffer (10 mM, pH
This reaction was carried out (30 min, 30 °C) by adding ammonium sulfate.

The activity of HAO was measured by determining the disappearance of potassium ferricyanide [30]. The 20 ml HAO determination reaction mixture contained soluble crude enzyme solutions, 1 mM potassium ferricyanide, 10 mM Tris-HCl buffer (pH 7.1), and 0.11 mM cytochrome C. The reaction was carried out for 15 min at 30 °C by adding hydroxylyamine.

NXR activity was measured by detecting nitrite consumption [31]. The 20 ml mixture for NXR activity determination included the membrane fraction of crude enzyme solutions and 3.6 mL of 0.01 M PBS (containing 1 mM NaNO2, pH = 7.4). This reaction was carried out for 30 min at 30 °C by adding NaNO2.

Twenty milliliters of reaction mixtures for NAR assays included a reduced form of nicotinamide adenine dinucleotide (NADH) as an electron donor and 10 mM NaNO3. The reaction was initiated by adding NADH at 30 °C for 15 min.

The activity of NIR was measured by assaying nitrite consumption as previously described [32]. The mixture (400 µl) contained 200 µM NaNO2, 100 µM NADH, the soluble fraction of crude enzyme solutions, and 200 µM phenazine methosulfate. The reaction was conducted at 30 °C for 10 min.

The activity of NOR was evaluated by measuring its nitrous oxide product using gas chromatography [33]. One hundred milliliters of 20 mM sodium nitroprusside, serving as the NO donor, was added into the 900 µl reaction mixtures, containing phenazine methosulfate (0.2 mM), NADH (4 mM), and the membrane fraction of crude enzyme solutions. This reaction took 6 min at 30 °C.

The enzyme activity unit (U) is defined as the quantity of enzyme which catalyzes the transition of 1 µM zymolyte per minute. The specific activity (U mg⁻¹) was identified as the quantity per enzyme unit, which was divided by the protein concentration (mg) according to previous studies [34].

2.5. Analytical methods

Growth curves of bacteria were monitored on the OD600 by the Bioscreen C Microbiology Growth Analysis System (Lab Systems, Finland). N2O concentration was detected with a gas chromatograph (GC-8AIT; Shimadzu; USA) [16]. The concentrations of ammonia (NH4⁺), hydroxylamine (NH₂OH), nitrite (NO₂⁻), and nitrate (NO₃⁻) were measured using a UVVis spectrophotometer (723 PC; Jinghua Instruments Co. Ltd.; China) according to the Nessler’s reagent colorimetry method, bromocresol purple colorimetric method, hydrochloride naphtholblue colorimetric method, and 4-amino benzene sulphonate colorimetric method, respectively [35]. The nitrogen removal rate was calculated based on the maximum slope of the trend line of nitrogen concentration, which stands for nitrogen removal divided by the time used. Statistical analysis of variance (one-way ANOVA) was carried out by using SPSS platform with 12 numbers based on triplicate assays.

3. Results

3.1. Effect of QS on the bacterial growth of P. aeruginosa

The pqsA mutant culture in LB medium showed a higher OD600 (1.90) compared with other strains (~1.70; Fig. 1A). When bacteria were cultivated with LB-NH₄Cl medium, there were differences in growth trends detected among the PAO1, lasI, hhl, pqsA, and pqsL mutants (Fig. 1B). Specifically, the arrival of the log phase repressing a pqsL cell OD600 of 1.20 in the stationary phases, whereas both lasI and hhl enhanced OD600 values to 1.7. This suggests that the lack of specific genes regulating AHL and PQS production is beneficial to the growth of P. aeruginosa.

PQS could suppress bacterial growth primarily in their stationary log phases with the LB-NH₄Cl medium, whereas that phenomenon was not apparent using the LB medium (Fig. 1A-B). The addition of NH₄Cl could deplete the oxygen rapidly because of ammonoxidation, and thus the bacteria in the LB-NH₄Cl medium would spend more oxygen than the bacteria in the sole LB medium. Based on those distinct situations, PQS might have inhibited bacterial growth only under oxygen-limited conditions.

3.2. Effect of QS on the HNAD process of P. aeruginosa

In the LB-NH₄Cl medium, the bacteria consumed NH₄⁺ until a concentration of 580 mg L⁻¹ remained at first (Fig. 2A). Because of the organic nitrogen degradation, the NH₄⁺ concentration of pqsL increased to 587 mg L⁻¹ after 16 h. The ammonium concentration decreased to 584 mg L⁻¹ in the pqsL culture, implying repression of NH₄⁺ consumption ability by PQS secretion. Conversely, lasI and hhl promoted NH₄⁺ consumption (4.38 mg L⁻¹ h⁻¹ and 4.24 mg L⁻¹ h⁻¹, respectively), indicating that the nitrification process is negatively affected by both signal molecules through the QS system.

The nitrate concentration gradually reached its highest (4.60 mg L⁻¹) at 12 h and afterward declined to its lowest (2.67 mg L⁻¹; Fig. 2C). The NO₂⁻ content began to decrease because of the aerobic denitrification behavior in P. aeruginosa (12–24 h). In this process, the rate of NO₂⁻ removal of pqsL was the lowest (0.042 mg L⁻¹ h⁻¹), whereas the quorum-induced inhibitory mutant pqsL had a considerably higher NO₂⁻ removal rate than pqsL, reaching 0.105 mg L⁻¹ h⁻¹. In addition, the rate of NO₂⁻ removal by the mutant lasI/lhll lacking AHL was the highest (0.155 mg L⁻¹ h⁻¹) among the strains. Experimental data showed that the presence of the PQS and AHL signal molecules inhibited the aerobic denitrification of cells and hindered the removal of NO₂⁻.

Removal rates of NO₂⁻ in mutants pqsL and pqsL were 0.027 mg L⁻¹ h⁻¹ and 0.043 mg L⁻¹ h⁻¹, respectively, which were much lower than those obtained by other strains (Fig. 2B). This nitrite reduction, which was observed in the LB-NH₄Cl medium, indicated that insufficient or excessive secretion of PQS affected NO₂⁻ transformation negatively during HNAD, which might imply that only a moderate PQS concentration enhances nitrite reduction. There was no NH₃OH detected for the entire process, which might be owing to the unstable nature of hydroxylamine and its rapid transformation speed to the next intermediate nitrite. These results showed that both AHL and PQS signal molecules could suppress nitrogen removal efficiency during HNAD.

3.3. Effect of QS on the HNAD related enzymes’ activities of P. aeruginosa

The protein concentration of crude enzyme extract of pqsL (541.6 mg/g dry cell weight) was higher than that of mutants lasI and hhl and pqsL (429.7–502.6 mg/g dry cell weight; Fig. 3). Activities of the AMO and HAO of the pqsL mutant were 0.0072 U/mg and 0.0093 U/mg (Fig. 4A-B), respectively, but its NXR activity was 0.0017 U/mg, which was only 66% of PAO1 (Fig. 4C). Though higher activities of AMO and HAO of pqsL caused rapid NH₄⁺ → NO₂⁻ transformation in the culture, the lower NXR activity of pqsL could result in the deficient capability of heterotrophic nitrification. This may explain the depressed NO₂⁻ removal rate and the inadequate ammonia removal ability of pqsL. In contrast, the NXR activity of the pqsA with insufficient PQS secretion was significantly enhanced (2.82 times, 0.0085 U/mg) compared with PAO1. Furthermore, the AMO and HAO activities of pqsA were reduced to 0.0015 and 0.0062 U/mg, respectively. By combining the results of heterotrophic nitrification enzymatic activities with the profile of NH₃OH removal, it
was evident that the NXR enzyme transition was the most crucial step during the heterotrophic nitrification process; the undetected NH$_2$OH in the media also supported this observation. Additionally, the activity of AMO (0.0079 U/mg) and NXR (0.005 U/mg) of the lasRhlI mutant with deficient AHL secretion was distinctively higher compared with that obtained by PAO1 (0.0038 and 0.0026 U/mg, respectively), whereas HAO activity decreased slightly to 0.0057 U/mg when AHL was insufficient. These results implied that QS regulated the capability of nitrification by controlling the activity of enzymes. Specifically, the expression of PQS signaling molecules might have limited the catalyzing activity of NXR; therefore, the ability of nitrification declined. In addition, AHL also adjusted the state of nitrification by regulating the activity of AMO and NXR. The lack of AHL and PQS could promote the activity of NXR, and thus enhance nitrification.

The low activity of NAR, NIR, and NOR of the pqsL mutant with excessive PQS secretion was 0.014, 0.014, and 0.002 U/mg, respectively, and their activity in PAO1 was only 75%, 82%, and 86%, respectively (Fig. 5). Conversely, the enzyme activity of the pqsA without the gene coding for PQS secretion was enhanced to 0.021, 0.028, and 0.0027 U/mg. Similar results were observed in the AHL-deficient mutant (lasRhlI), which increased to 0.026, 0.019, and 0.0031 U/mg. Bacterial types insufficient of signal molecules had higher enzyme activities than PAO1. The variations in enzyme activities were due to the presence of PQS and AHL; therefore, denitrification was affected by the QS system. Specifically, the presence of signal molecules might have inhibited the activity of related enzymes to curb denitrifying effects.

Fig. 1. Effect of the quorum sensing system on aerobic growth. LB medium (A), LB-NH$_4$Cl medium (B). Three independent experiments were carried out, and representative data are shown.

Fig. 2. Effect of the quorum sensing system on the HNAD process of P. aeruginosa in LB-NH$_4$Cl medium. NH$_4^+$ reduction (A), NO$_2^-$ production (B), and NO$_3^-$ production (C) of different strains. The error bars represent the standard deviation based on triplicate assays.

Fig. 3. Comparison between the protein content of crude enzyme extractions in P. aeruginosa strains (PAO1, lasRhlI, pqsA, pqsL). Protein content was measured in cells cultured for 6 h. Different lowercase letters on the columns indicate a significant difference between 12 groups based on triplicate assays (one-way ANOVA, $P < 0.05$).
3.4. The influence of QS on nitrogen cycling

When NH₄Cl as the sole nitrogen source was supplemented to the nutrient medium, the growth of PAO1, lasIrhlI, pqsA, and pqsL arrived at the stationary phase within 18 h, as the OD₆₀₀ increased to 0.820, 1.183, 1.140, and 0.815, respectively (Fig. 6A). Inorganic nitrogen was removed with the growth of cells, and the concentrations of ammonia declined sharply within 24 h. The lasIrhlI and pqsA showed a higher removal efficiency of ammonia (86.8% and 85.5%, respectively) after 24 h with removal rates of 5.84 and 5.12 mg L⁻¹ h⁻¹, respectively. On the other hand, pqsL had the lowest ammonia elimination ratio (37.2%) at a rate of 3.72 mg L⁻¹ h⁻¹.

To investigate the involvement of QS in nitrite transformation, the single nitrogen resource was replaced by nitrite (Fig. 6B). The OD₆₀₀ of pqsA and pqsL mutants decreased to 0.73 and 0.53, respectively, below that of PAO1 and lasIrhlI (0.95 and 1.22, respectively). The nitrite removal ratio declined significantly from 100% of lasIrhlI to 38% of pqsL; pqsA also had a lower nitrite removal rate (57%), revealing similar negative trends as pqsL.

The effect on aerobic denitrification of P. aeruginosa was also determined using nitrate as the sole nitrogen source. All strains grew well and their OD₆₀₀ reached to more than 0.9 after 24 h of culture, with lasIrhlI showing the highest OD₆₀₀ growth status (1.15) among the four types (Fig. 6C). Although cell biomass of the pqsL mutant was similar to that of PAO1, its aerobic denitrification capability was only 58.3% compared with that of PAO1. More than 83% of nitrate was consumed by lasIrhlI with a removal rate of 8.35 mg L⁻¹ h⁻¹ after 24 h of cultivation, demonstrating its nitrate removal ability. Further, pqsA displayed a similar removal trend to lasIrhlI, as the nitrate removal ratio was 70% with a removal rate of 8.42 mg L⁻¹ h⁻¹. In summary, the aerobic denitrification ability ranking of strains was consistent with the HNAD experiment, which used LB-NH₄Cl as the medium. The result indicated that the presence of signal molecules could inhibit the aerobic denitrification of P. aeruginosa, reducing the removal efficiency of NO₃⁻ in water, and that the lack of specific QS regulation genes promoted aerobic denitrification.

3.5. Citrate was the superior carbon source for HNAD of P. aeruginosa

The influence of various carbon substrates on the growth of strains and ammonium utilization by P. aeruginosa was also examined (Fig. 7). P. aeruginosa did not use sodium acetate for growth well since the ammonia removal rate was the lowest among all strains. When citrate was used as the sole carbon source, the highest NH₄⁺ removal rate was 86.8% acquired by mutant lasIrhlI. Specifically, more than 50.0% of ammonium was removed by all strains under those conditions, suggesting that citrate was the
ANOVA, represent the standard deviation based on triplicate assays. Different lowercase letters on the columns indicate a significant difference between 12 groups based on triplicate assays (one-way ANOVA, \( P < 0.05 \)).

Fig. 6. The nitrogen removal rate of strains in the medium with single inorganic nitrogen. NH\(_4\) (A), NO\(_2\) (B), NO\(_3\) (C). Activities were measured in cells cultured for 24 h. Error bars represent the standard deviation based on triplicate assays. Different lowercase letters on the columns indicate a significant difference between 12 groups based on triplicate assays (one-way ANOVA, \( P < 0.05 \)).

Fig. 7. NH\(_4\) removal rate of strains cultivated with different organic carbon sources. Activities were measured in cells cultured for 24 h. Error bars represent the standard deviation based on triplicate assays. Different lowercase letters on the columns indicate a significant difference between 12 groups based on triplicate assays (one-way ANOVA, \( P < 0.05 \)).

Our study showed that AHL and PQS had negative effects on the ammonia removal process (Fig. 2). Previous studies have also reported that QS is involved in autotrophic nitrogen removal. For instance, AHL could promote ammonia removal by autotrophic nitrifying bacteria and anaerobic ammonia-oxidation activated sludge \([22,36,37]\). The exogenous AHL also resulted in the change of nitrifying bacteria and anaerobic ammonia-oxidation activated sludge, whereas PQS could promote ammonia removal, whereas high concentrations of PQS may adjust the activity of NXR, NAR, NIR, and NOR.

4. Discussion

Our study showed that AHL and PQS had negative effects on the ammonia removal process (Fig. 2). Previous studies have also reported that QS is involved in autotrophic nitrogen removal. For instance, AHL could promote ammonia removal by autotrophic nitrifying bacteria and anaerobic ammonia-oxidation activated sludge \([22,36,37]\). The exogenous AHL also resulted in the change of nitrifying bacteria, whereas high concentrations of PQS may adjust the activity of NXR, NAR, NIR, and NOR.
via chelation with iron ions in active centers of enzymes. PQS could also prevent iron ions from working as electron acceptors in the solution by combining with them. Furthermore, PQS has been reported to be a powerful antioxidant able to inhibit the oxidation of molecules [43], such as ammonia, which could be one of the reasons for the declined ammonia removal efficiency of pqsl. pqsl showed higher activities of AMO and HAO than pqsa, but its removal of NH₄⁺ was lower than that of pqsa (Figs. 2 and 3). The divergence between enzyme activity and nitrogen removal rate may be due to the temporal changes in activities of AMO and HAO that do not fully reflect total nitrogen removal. However, the expression of nitrogen-transforming enzymes needs further investigation based on transcriptomic and proteomic approaches to elucidate the correlations between enzymes, QS molecules, and nitrogen cycling.

Furthermore, AHL seemed to have a more significant impact than PQS in coordinating bacterial growth, heterotrophic nitrification, and aerobic denitrification, implying that Las or Rhl regulates the function as the hierarchical control in the QS system [13]. Owing to the inhibitory effect of QS signal molecules on the nitrogen cycling pathway of HNAD, a signal molecule quencher, signal molecule-degrading bacteria, or enzymes may be used to improve the HNAD process of bacteria in nitrogen-containing wastewater treatment [44,45]. According to our research, the lack of AHL had a greater effect in promoting AMO enzyme activity than PQS, but the reduction of PQS displayed better performance than AHL in terms of enhancing HAO enzyme activity. Thus, a sequential QP approach (SQQA) of AHL QQ in ammonoxidation and PQS QQ in nitrification was proposed to enhance HNAD in practical applications. However, the SQQA needs further verification in future studies.

5. Conclusions

QS is able to regulate the HNAD process in P. aeruginosa. Here we highlight QS adjustments to bacterial growth, nitrogen removal efficiency, related enzymatic activities, and nutrient limitations by investigating P. aeruginosa wild-type and QS-deficient mutant strains under different conditions. Both the nitrogen removal efficiency of heterotrophic nitrification and aerobic denitrification decreased to the lowest ammonia and nitrate removal rates of 3.72 mg L⁻¹ h⁻¹ and 1.55 mg L⁻¹ h⁻¹, respectively, with the over-secretion of PQS. The absence of AHL and PQS could improve ammonia removal efficiency to the maximum rate of 5.84 and 5.12 mg L⁻¹ h⁻¹, respectively, and nitrate consumption rates to 8.35 mg L⁻¹ h⁻¹ and 8.42 mg L⁻¹ h⁻¹, respectively, by controlling NXR, NAR, NIR, and NOR enzyme activities during HNAD. The capacity for NO₂⁻ removal was enhanced only with the appropriate PQS concentration. Our results clearly indicate that the role of AHL and PQS might have been previously underestimated. With molecular tools regulating QS, the nitrogen removal capacities of HNAD in bacteria can be improved through the adjustment of a series of key enzymatic activities.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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