Identification of the release and effects of AHLs in anammox culture for bacteria communication

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1. Introduction

Anammox is a process that combines ammonium and nitrite to form nitrogen gas. It requires no Chemical Oxygen Demand (COD), reduces oxygen demand and produces low quantities of sludge compared with the traditional method [1]. The nitrogen removal rate as high as 5 kg N m⁻³ d⁻¹ of the anammox system [2], in addition to the advantages mentioned above, demonstrate that nitrogen removal in wastewater treatment processes will save energy and provide lots of benefits [3]. However, because anammox bacteria grow slowly (doubling time is 10–14 days) and are greatly affected by several environmental conditions, such as...
temperature and organics in surroundings [4–6], anammox processes often require long start-up periods. Numerous solutions have been presented to improve the activity of anammox bacteria and shorten the reactor start-up period [7], such as modifying the carrier to provide a better growing environment for the bacteria and choosing the suitable bioreactor type. However, these solutions are unable to essentially regulate the bacterial metabolism. To improve the activity, growth rate and competitive strength of anammox bacteria, the promotions of the expression of several key genes in the anammox bacteria are very important.

A milestone observation of bacterial ecology is that bacteria can communicate with one another by a system called quorum sensing (QS) [8], which regulates gene expression in response to fluctuations in the cell population density [9]. The bacteria which have QS system produce quorum signaling molecules and then release them to their surroundings. When the cell population density increased to a certain value, the signals concentration reached a threshold value, and the binding of signal molecules and regulatory proteins activates quorum sensing-regulated genes to activate relevant phenotypes. Generally, signal molecules of Gram-negative bacteria are AHLs [9]. AHLs-based QS-regulated phenotypes, such as symbiosis, competence, conjugation, antibiotic production and virulence, have been studied in medicine and toxicology [10]. Recently, the biofilm formation, activity and growth rates of bacteria for the wastewater treatment have been investigated because they provide information that is required to solve problems related to water treatment [11]. Previous studies have verified the systems in anammox bacteria have been confirmed. First, the activity of anammox bacteria has an apparent density-dependent phenomenon. It has been discovered that anammox bacteria do not take on activity until the cell concentration is higher than 10^{10}– 10^{11} cell mL^{-1} [15]. This is similar to Vibrio fischeri, the bioluminescence of which could be inspired when the population density reaches a threshold concentration. V. fischeri are typical bacteria regulated by QS system [16]. Moreover, the aggregation of anammox bacteria is similar to the biofilm formation. AHLs-based QS system has been shown to influence biofilm formation of Gram-negative bacteria in water and wastewater systems [17,18]. During anammox reactor operation, the anammox bacteria were frequently found to form biofilm or attach to the container wall. In the activated sludge reactor, high activity anammox bacteria are easy to aggregate as red granules settling in the bottom of the reactor [19]. In addition, the existence of the genes coding for the quorum sensing compounds has been evidenced by Strous et al. [20], where the meta-genome of anammox bacteria was shown. It has been confirmed that four gene clusters encode fatty acid biosynthesis of Kuenenia stuttgartiensis, two of which have functions to encode fatty acid biosynthesis and 5-adenosyl methionine (SAM) radical enzymes (significant biosynthetic pathway for AHLs signals) [8,21]. Even though increasing evidences have indicated that anammox bacteria might have QS regulation systems, the direct evidences for the anammox bacteria communication by QS systems are remained to be explored. Therefore, the potential existence of QS system in anammox bacteria and its effects are very urgent to be investigated, which might be useful to regulate the growth rate and activity of anammox bacteria with the final aim to realize the fast start-up of anammox process.

In this study, three different AHLs were identified in the anammox culture supernatant and culture extraction by applying AHLs biosensor and high-performance liquid chromatography (HPLC). To estimate the potential effects of C_{6}-HSL, C_{9}-HSL and C_{12}-HSL on the regulation of anammox bacteria behavior, batch tests were conducted by adding exogenous AHLs. Moreover, the probable working mechanism of exogenous signals on anammox culture was proposed by real-time analyzing the AHLs concentration after its addition to the system.

2. Materials and methods

2.1. Inoculum bacteria and chemical analysis

The anammox culture used in this study was mainly composed of Candidatus Brocadia fulgida, which accounted for around 80% of the total population [22]. According to standard methods set out by the American Public Health Association [23], the concentrations of NH_{4}^{+}-N were measured by preliminary distillation step and titrimetric method, NO_{3}^{-}-N were determined by Ultraviolet Spectrophotometric Screening Method, NO_{2}^{-}-N were measured by Colorimetric Method, and Volatile Suspended Solids (VSS) were defined under the conditions that suspended solids were dried at 103–105 °C for 12 h and volatile solids were ignited at 550 °C for 2 h. pH was obtained by pH meter (Mettler, Switzerland).

2.2. Reactor operation

Initial experiment was conducted in a 3.0 L bio-fermentor inoculated with anammox bacteria. Cultivation medium, as described previously [24], were added to the fermentor to keep certain concentrations suitable for bacteria activation (the initial concentration of NH_{4}^{+}-N and NO_{3}^{-}-N were kept among 80–100 mg L^{-1}). The solutions were purged with a gas mixture of N_{2}–CO_{2} (95/5%) to exclude the dissolved oxygen (DO) and keep the anaerobic conditions for anammox bacteria. pH, DO and temperature in the fermentor were measured online and controlled at 7.8–8.2, 0–0.05 mg L^{-1} and 37 ± 1 °C, respectively. The stirring speed of standard six-blade disk impeller was set at 100 rpm to produce shear force and improve gas–liquid transfer. The initial solids concentration for the reactor operation was 0.58 g VSS/L. The experiment was conducted in a 3.0 L bio-fermentor with bulk volume of 2.5 L. HRT was shortened from 48 h (0–340 day of the operation) to 24 h (340–400 day of the operation) with improvement of bacterial activity during these days. When the concentration of NO_{2}^{-}-N was under 20 mg L^{-1}, the used medium was discarded, new medium was added and one cultivation cycle was finished, which indicted the operations were continuous. During the 400 days' operation, the nitrogen removal performance, activity and VSS were recorded every 30 day. Every data was measured for three times to get the average value.

2.3. AHLs standard

AHLs structures all have the homoserine lactone ring moiety and acyl side-chain of different length, degree of substitution, and saturation. The acyl side-chain length (n) is typically 4–14 carbons in length. AHLs standards (C_{6}–HSL, C_{9}–HSL, C_{12}–HSL) were purchased from Sigma–Aldrich.
2.4. Extraction of AHLs

The culture supernatant (300 mL) was harvested, filtered through a 0.22 μm cellulose filter to remove bacteria and extracted with an equal volume of ethyl acetate and 0.1% (v/v) formic acid [25]. The solvent extracts were evaporated using a rotary evaporator (Zhengjie, RE-52AA) at 40 °C, and reconstituted in 1 mL ethyl acetate, stored at −20 °C.

AHLs extraction from the anammox bacteria was described below. The biomass was homogenized in a 100 mL serum bottle, and 1 mL mixture was extracted using a syringe. The supernatant was removed by centrifugation at 4000 rpm for 2 min, and the bacteria were obtained and dispersed in methanol. The biomass was completely disrupted using sonication at 300 W for 12 min and filtered through a 0.22 μm syringe filter. The solvent extracts were dried under a nitrogen flow at 40 °C, reconstituted in 400 μL of methanol for concentration and stored at −20 °C.

2.5. AHLs reporter plate assays

*Agrobacterium tumefaciens* A136 (pCF218) (pCF372) is a biosensor strain used in agar plate-based biosassays because it hydrolyzes 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) into a blue pigment in the presence of AHLs, carbon chains ranging from 6 to 14 carbons in length [26]. Prior to the assay, the bacteria was grown in sterilized Luria–Bertani (LB) broth medium with 20 g/L tryptone, 5 g/L yeast extract, 5 g/L glucose, 5 g/L NaCl, 20 mg/L ampicillin, and 50 mg/L spectinomycin. For each Petri dish, 10 μL of a 10 mg/mL AHL solution was applied to a HPLC column (Zorbax SB-Aq 4.6 × 250, 5 μm) with a defined concentration and incubated overnight at 30 °C. The blue pigment was examined to identify the existence of AHLs in the anammox culture supernatant.

2.6. HPLC analysis

HPLC analysis was applied to identify various kinds of AHLs in anammox culture supernatant and bacteria. Briefly, 20 μL solutions were applied to a HPLC column (Zorbax SB-Aq 4.6 × 250, 5 μm), eluted with a linear gradient of methanol in water (20–100%) over a 30-min period at a flow rate of 1 mL min⁻¹ and monitored at 210 nm with DAD detector. For the bacteria extraction contained low concentration of AHLs, the input dosage for HPLC analysis was increased to 100 μL to enhance the AHL peaks.

2.7. Batch test

The NH₄⁺-N and NO₂⁻-N removal profile of the anammox culture were determined in four 100 mL anaerobic serum bottles. The biomass was washed and re-suspended in the cultivation medium. Incubation temperature was kept at 37 °C and anaerobic condition was maintained. The initial biomass was approximately 0.025 g VSS for each bottle. After an experiment cycle, which last for 20–25 h depending on the bacteria activities, the supernatant was poured and fresh medium was added. Samples were collected per 5 h using a syringe. The four bottles were incubated three experimental cycles until the bacterial activity stabilized. After that, C₅-HSL, C₆-HSL and C₁₂-HSL (30 mg L⁻¹) were added to the three bottles respectively. The batch tests with the exogenous addition of AHLs were conducted for three experimental cycles to get the average value. Finally, the nitrogen removal rate, the NO₂⁻-N removal/NH₄⁺-N removal and the bacterial activity were measured and calculated to determine and compare the various effects. Bacterial VSS was measured in every begin (S₀) and the end (S₁) of experiment cycle, and the growth rate of bacteria was defined as the specific growth rate (u = (S₁ − S₀)/S₀; t is the time of one experiment cycle). The test was calculated by comparing the changes of NH₄⁺-N removal rate, bacterial activity, bacterial growth rate and NO₂⁻-N removal/NH₄⁺-N removal of the anammox culture after the introduction of C₆-HSL, C₈-HSL and C₁₂-HSL. Every experiment was repeated for three times and the significance level of data was analyzed through Independent-Samples T Test.

2.8. Real-time PCR analysis

DNA was extracted using a Fast DNA spin kit (Applied Biosystems, USA) according to the manufacturer’s instructions. Real-time PCR assays were conducted as follows. The primers for anammox and eubacteria were 694F (forward primer)–960R (reverse primer) [27] and 314F (forward primer)–518R (reverse primer) [28], respectively. Each PCR working solution (20 μL) contained 12.5 μL of SYBR Green PCR master mix (Applied Biosystems, USA), 1 μL each of forward and reverse primers, 3 μL of template DNA (the DNA extraction was diluted 100 times) and 2.5 μL of ddH₂O. PCR amplification and detection were performed with an ABI Prism 7000 sequence detection system (Applied Biosystems, USA). The PCR temperature program for both anammox and eubacterial DNA extracts was initiated with 2 min at 50 °C and 10 min at 95 °C followed by 30 cycles of 10 s at 95 °C and 1 min at 59 °C.

3. Results and discussion

### 3.1. Bio-fermentor operation profile for anammox bacteria cultivation

The bio-fermentor was operated for 400 days after inoculating 1.74 g VSS anammox bacteria. The reactor nitrogen removal performance was measured and showed in Fig. 1(a). During the 400 days’ operation, the nitrogen removal rate (NRR) was increased from 20 mg N L⁻¹ day⁻¹ to 650 mg N L⁻¹ day⁻¹, indicating the efficient bacteria cultivation and enrichment. The curve was similar to previous reports [29], namely including phase I with initially steady nitrogen removal performance and phase II with rapidly increased nitrogen removal performance. To further identify the apparent divergence of these two phases, the bacteria VSS and nitrogen removal activity during the operation were measured and recorded in Fig. 1(b). Besides the nitrogen removal performance, it clearly shows the abrupt increase of the bacteria VSS and bacteria activity in phase II of the bio-fermentor operation, from day 350 to day 400. Compared to the bacteria behavior in phase I of the operation, it seems that bacteria status was changed and all the bacteria became more active in phase II. It is very strange about what happened in this phase. Actually, this is a typical anammox reactor start-up performance [29]. However, the involved mechanism for the abrupt increase in phase II is significantly worthy to be further investigated, which might be useful to accelerate anammox reactor start-up and solve the key restricted problem for anammox process application.

The phenomenon during the anammox reactor start-up indicates both of the activity and growth rate of anammox bacteria depend on the bacteria amounts. From this, QS system for the bacteria communication comes to our view as the key points to regulate the bacteria behavior. Its identification and verification need further investigation.
3.2. Identification of AHLs release in anammox culture

In order to provide the direct evidence about the existence of QS system in anammox bacteria, we firstly tried to detect the release of AHLs in anammox culture. Because QS-regulated bacteria release AHLs into the surroundings, it is necessary to detect AHLs in the supernatant. At first, the agar-plate based bioassay was applied by using biosensor *A. tumefaciens* A136 to identify the QS signals in the supernatant from the fermentation at the end of the cultivation cycle on the day 400 of the operation period. As results, the bacteria could efficiently display blue color in the C6-HSL, C8-HSL and C12-HSL test wells in the agar-plate. These AHLs signals have been detected in several bacterial communities in water treatment facilities [12,30]. The most worthy to be noticed is that, the concentrated supernatant of anammox culture also showed a blue color in the test wells, strongly verifying the presence of AHLs in the supernatant of anammox culture.

With the aim to further clarify the AHLs species in the supernatant of anammox culture, especially whether it contains C6-HSL, C8-HSL and C12-HSL, HPLC was explored in this investigation. The C6-HSL, C8-HSL and C12-HSL standard were used and the key retention times were determined as 16.02, 29.39 and 44.51 min in the HPLC fingerprint, respectively (Fig. 2). Here we use “Standard addition method” to identify the existence of signals in the concentrated culture supernatant. Standard addition method is an analysis approach used in analytical chemistry, which was conducted by directly adding standard to the analyzed sample with the aim to reduce the interference effects on the analysis results [31]. The sample “standards” are actually “mixture of standards and concentrated culture supernatant”. By comparing the spectrogram peaks of this sample to that of “concentrated culture supernatant”, we can see the peak at 44.51 min clearly increased after dosage C12-HSL. Thus, the peak at 44.51 min in Fig. 2(c) represented C12-HSL. Other peaks came from the concentrated culture supernatant, which have no relationship with C12-HSL. In this part, we mainly paid attention to the release of C12-HSL by the biomass, so we did not check other peaks. For the anammox culture supernatant, the corresponding peaks also appeared, suggesting the simultaneous existence of C6-HSL, C8-HSL and C12-HSL here. We established a linear relationship between peak area and AHLs concentration to quantify the concentration of AHLs in the extraction. At this extraction time, it was calculated that C6-HSL, C8-HSL and C12-HSL in the supernatant was 0.33, 0.18 and 0.33 mg L\(^{-1}\), respectively.

3.3. Effects of AHLs on anammox culture

Although we obtained the direct evidence about the appear release of QS signals in anammox culture, more crucial question about whether these AHLs affect anammox bacteria profile is still necessary to be revealed. It is very important to clarify the important roles of QS communication system in anammox culture. To confirm the effect of the identified AHLs in anammox culture, exogenous C6-HSL, C8-HSL and C12-HSL with concentration of 30 mg L\(^{-1}\) (30 mg L\(^{-1}\) was chosen as the target concentration due to solubility limitations of C12-HSL) were added to Vials B, C and D, respectively. Vial A was considered as control sample without
any addition of exogenous signals. As expected, NH₄-N removal rate, bacterial growth rate, activity and NO₂-N removal/NH₄-N removal of the anammox culture were visibly affected upon their addition, which was clearly present in Fig. 3.

The NH₄-N removal rate was most worthy to be concerned since it could directly impress the performance of anammox reactor. Thereby, effects of the exogenous signaling molecules C₀-HSL, C₈-HSL and C₁₂-HSL on the NH₄-N removal rate were firstly investigated. The quantitative analysis results were clearly in different levels and showed in Fig. 3(a). For the original anammox culture (Vial A), the NH₄-N removal rate was a little increased from 63 mg NH₄-N L⁻¹ day⁻¹ to 71 mg NH₄-N L⁻¹ day⁻¹ after the tested few days. A comparison of the anammox culture treated with different signaling molecules showed significant difference. Every experiment was repeated for three times for the statistical test. The significant (p < 0.05) increase of NH₄-N removal rate occurred when cultivating with signaling molecules C₀-HSL (Vial B) and C₈-HSL (Vial C), with change from 55 mg NH₄-N L⁻¹ day⁻¹ to 74 mg NH₄-N L⁻¹ day⁻¹ and 60 mg NH₄-N L⁻¹ day⁻¹ to 72 mg NH₄-N L⁻¹ day⁻¹ on the average, respectively, resulting in the increased NH₄-N removal performance by 35% and 20%, respectively. However, the nitrogen removal performance of anammox culture when incubated with C₁₂-HSL (Vial D) did not have significant difference (p > 0.05) with that of the control sample without any exogenous AHLs.

The effects of exogenous AHLs on the activities of anammox bacteria were also analyzed (Fig. 3(b)). In the control experiment, the calculated activity was a little increased from the average value of 239 mg NH₄-N g VSS⁻¹ day⁻¹ to 258 mg NH₄-N g VSS⁻¹ day⁻¹ after the incubation. Specifically, a prominent increase of bacterial activity occurred when incubated with 30 mg L⁻¹ C₀-HSL, in which significant (p < 0.05) increase occurred from the average value of 208 mg NH₄-N g VSS⁻¹ day⁻¹ to 247 mg NH₄-N g VSS⁻¹ day⁻¹ after the addition of the signaling molecules. The nearly 20% enhancement indicated the positive effects of C₀-HSL on anammox activity. Addition of C₀-HSL also produced a notable increase of the bacteria activity. However, when incubated with C₁₂-HSL, the bacterial activity significantly decreased (p < 0.005) comparing with the control sample without any exogenous AHLs, suggesting that C₁₂-HSL produced the negative effects on anammox activity.

The variance of bacteria growth rate also indicated the effects of signaling molecules on anammox culture (Fig. 3(c)). The cultivation without exogenous signaling molecules expressed an average growth rate of 0.0027 h⁻¹. Addition of C₀-HSL produced a significant increase of the bacteria special growth rate from 0.0025 h⁻¹ to 0.0032 h⁻¹, meaning 28% increase. The maximum growth-promoting effects were observed when the culture incubated with C₁₂-HSL, which was identified as 0.0038 h⁻¹ with 32% increase contrast to the control sample according to the analysis. The potential effects of signaling molecules on accelerating bacteria growth rate were verified and the signaling molecular, especially C₀-HSL and C₁₂-HSL, was thus regarded to act on accelerating the growth.

Although both of the signaling molecules C₀-HSL and C₁₂-HSL could increase the bacteria growth rate, there is an important question about which bacteria species could be accelerated in the culture community. To identify it, the nitrogen removal profile of the NO₂-N removal/NH₄-N removal was calculated and the results are shown in Fig. 3(d). The ratio of NO₂-N removal and NH₄-N removal has been verified to be 1.32 for anammox reaction. After the dosage of C₁₂-HSL, we found remarkable increase of the NO₂-N removal/NH₄-N removal from 1.23 to 1.64, implying the potential occurrence of the growth of other bacteria and other nitrogen removal process to remove NO₂-N. With the supply of C₀-HSL and C₁₂-HSL, the ratio kept almost stable at 1.32 [29], indicating the functional bacteria (anammox bacteria) kept unchanged. The variation of anammox bacteria percentage in the consortia after supplying C₀-HSL, C₈-HSL and C₁₂-HSL could be verified by RT-PCR analysis.

For the purpose to further identify, RT-PCR was performed to count the change of anammox percentage in the culture community after the addition of exogenous signals (Fig. 4). Signaling molecules were verified to have profound effects on the anammox percentages in the culture, namely the disturbing of bacteria inter-species communication by signaling molecules also break the steady and balance of the original microbiological community. Notably, when following supply of C₁₂-HSL, we found remarkable reduction of anammox percentage from 81% to 58%, indicating the potential growth of other bacteria. This clear decrease of anammox percentage was identified as important effects of C₁₂-HSL on anammox culture. In contrast, it seems that the C₀-HSL brought a little increased anammox percentage from 81% to 88%, compared to the control experiment. It could be easily understood that the quick growth of anammox bacteria obtained by C₀-HSL and correspondingly high nitrogen removal performance. The whole process seems as biomass dependent, which has also been reported by other studies [15]. These results are in agreement with the theory that the AHL signals are the quorum sensing systems and they could exert more effects at higher biomass concentration.

The different signals for inter-species communication were confirmed to capably lead to different behaviors of anammox culture. From the study here, we can attest that C₀-HSL has important functions to increase the anammox bacteria activity and growth rate. C₀-HSL and C₈-HSL have positive effects on anammox culture and significantly increase the nitrogen removal performance.

The dosage of C₁₂-HSL resulted in the evident increase of bacteria total amount (VSS). According to the calculation formula \( u = (S₁ - S₀) / (S₀t) \), the growth rate of the whole bacteria community increased after the introduction of C₁₂-HSL (Fig. 3). Meanwhile, the RT-PCR analysis results (Fig. 4) showed that the dosage of C₁₂-HSL decreased anammox bacteria percentage in the bacteria community. Combined with the nitrogen removal results, it could be inferred that some heterotrophic bacteria grew, which inevitably decreased anammox activity. Thus, although the dosage of C₁₂-HSL could increase the growth rate of the whole bacteria community, the anammox activity decreased since it accelerated the growth of heterotrophic bacteria.

These results are a little discrepant with other study carried out by De Clippeleir et al. [14], who demonstrated that C₁₂-HSL was useful to increase the nitrogen removal performance of OLAND biomass. Actually, the response of different bacteria cultures to AHLs might be different. These effects are also determined by bacteria community composition and biomass concentration. The addition of C₁₂-HSL promoted the growth of heterotrophic bacteria in this study with the appear phenomena about the outstanding increase of NO₂ removal/NH₄ removal, suggesting some bacteria in the anammox cultures in this study might have the ability to utilize C₁₂-HSL to remove nitrite. C₁₂-HSL has opposite effects on anammox culture comparing to C₀-HSL and C₈-HSL, because it prominently inspired the growth of heterotrophic bacteria in the anammox culture.

In all, the advantage of having QS system in anammox bacteria was huge. On one hand, it is well-known that the slow growth rate of anammox bacteria and corresponding long start-up period of anammox process was the main limiting factor for the application of anammox process. According to the results proposed in this study, anammox bacterial growth rate, activity and nitrogen removal rate could be increased and then start-up period could be shortened by the introduction of AHLs. This provided a promising solution for the existing problem of anammox process. On the other hand, the verification of the existence of QS system in anammox bacteria could well explain the intrinsic characteristics of anammox bacteria, such as bacteria aggregation and density-
dependent activity (Anammox bacteria do not take on activity until the cell concentration is higher than $10^{10}$–$10^{11}$ cell mL$^{-1}$).

### 3.4. Induced release of AHLs by exogenous AHLs

With the aim to make clear how the exogenous signals affect anammox culture, C$_6$-HSL was added to the medium of anammox culture and the C$_6$-HSL was tracked by real-time monitoring the concentrations of this signal in the biomass and supernatant. The result is shown in Fig. 5. Surprisingly, the exogenous signal C$_6$-HSL could not sustain in the supernatant of anammox culture for long time. It was rapidly degraded and concentration of C$_6$-HSL in the supernatant decreased in the first 12 h from 35 mg L$^{-1}$ to 10 mg L$^{-1}$ with a good linear correlation ($R^2 = 0.97$). In fact, the degradation of AHLs depends on the pH of the supernatant and is caused by chemical degradation and bacteria degradation. It was reported that 3-oxo-C$_6$-HSL became unstable in a pH range of 7–8 without other interference factors.[32] The pH of 7.8–8.2 of the medium was regulated for anammox bacteria growth. So, it was also confirmed in this study that the degradation of C$_6$-HSL could be performed in the anammox culture medium even without bacteria (Fig. 6a). In addition, the degradation was accelerated under the effects of bacteria (Fig. 6b).

In this case, we all wondered how the exogenous signals worked after their addition. Through the analysis of C$_6$-HSL concentration in the biomass (Fig. 5), we can see although very low amount of C$_6$-HSL was initially detected in the biomass, after the addition of exogenous C$_6$-HSL, the amount of C$_6$-HSL in the biomass increased significantly. This result is similar to that occurs during QS regulation. The increasing concentration of AHLs in the surroundings (the supernatant) reached the threshold value of the QS system and activated the expression of related genes, such as

![Fig. 3. Effects of exogenous C$_6$-HSL, C$_8$-HSL and C$_{12}$-HSL (30 mg/L) on the (a) NH$_4^+$-N removal rate, (b) activity, (c) growth rate and (d) NO$_2^-$-N/NO$_3^-$-N (ratio of nitrite nitrogen and ammonia nitrogen removal) of anammox bacteria. The experiments were carried out for three times to get the average values at the time points before (red) and after (black) the addition of AHLs. One or two asterisks indicate a significant increase compared to no treatment with either ($p < 0.05$) or ($p < 0.01$), respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)](image)

![Fig. 4. Effects of exogenous C$_6$-HSL, C$_8$-HSL and C$_{12}$-HSL (30 mg/L) on the proportion of anammox bacteria in the community. The proportions of anammox bacteria were measured three times before (black) and after (red) the addition of AHLs by RT-PCR. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)](image)

![Fig. 5. The variation of C$_6$-HSL concentrations in supernatant and biomass. The concentration of C$_6$-HSL in biomass over long cultivation times is represented by three phases: (1) initial phase, (2) increasing phase, and (3) stable phase.](image)
as LuxI, the gene encoding AHL synthase [8], which could release more AHLs. Therefore, the amount of C6-HSL in the biomass inevitably increased. For the following 12–32 h, a reduced degradation rate of C6-HSL in the supernatant was observed (Fig. 5), corresponding to the increased concentration of C6-HSL in the biomass. The stable amount of C6-HSL in the biomass is attributed to the counterbalance of degradation and production. Taken all together, the addition of exogenous C6-HSL has crucial roles in enhancing the production of endogenous C6-HSL by biomass, and then to accelerate the nitrogen removal process.

4. Conclusion

In this study, the potential existence of QS communication system in anammox culture was verified by the definite release of QS signals C6-HSL and C12-HSL and the obvious effects of them on anammox bacteria behavior. C6-HSL could increase anammox bacteria activity and growth rate, and C6-HSL only activate on the promotion of anammox bacteria activity. However, addition of C12-HSL decreased anammox activity (Fig. 3). The function pathway that exogenous AHIls activated the bacteria by inducing the production of endogenous signals released by anammox culture was proposed finally, useful to provide a new approach to accelerate the anammox process start-up.

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References


Fig. 6. HPLC analysis of the degradation of C6-HSL in cultivation medium (a) without anammox culture and (b) with anammox culture.


D.C. Harris, Quantitative Chemical Analysis, Macmillan, 2010.