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Fast start-up anammox process using Acyl-homoserine lactones (AHLs) containing supernatant

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ABSTRACT

N-dodecanoyl homoserine lactone (C_{12}-HSL) was detected in the supernatant of an anammox granular sludge reactor (AGSR). C_{12}-HSL could enhance the specific anammox activity of anammox biomass. Adding C_{12}-HSL-containing AGSR supernatant into the continuously stirred tank reactors reduced the start-up time of the anammox process from 80 to 66 days. Moreover, the nitrogen loading rate was also enhanced to 1.6 times that of the control reactor. AHLs could increase the secretion of extracellular polymeric substances and anammox obtained better enrichment with the addition of AHLs-containing AGSR supernatant. Denaturing gradient gel electrophoresis analysis further revealed that AHLs played a role in mediating microbial community parameters. In conclusion, adding AHL-containing supernatant could be an effective and economical way to accelerate the start-up of anammox.

Introduction

Anaerobic ammonium oxidation (anammox) is a technology which has been a main focus in wastewater nitrogen removal (Kuenen, 2008). The anammox process can convert ammonium and nitrite into nitrogen gas. Compared to the traditional nitrification–denitrification process, it demands no oxygen or organic matter (Jetten et al., 2001). These advantages suggested a wide use of the anammox process in the future. However, the slow growth rate of anammox bacteria was the choke point on application (Strous et al., 1999). It is very difficult to start up an anammox reactor from conventional activated sludge.

Since the start-up of the anammox process refers to the accumulation of anammox bacteria from low concentration to high, clarifying the community constitution and microbial interactions in the sludge are important. The cell-to-cell interaction in bacteria is referred to as quorum sensing (QS) (Boyer and Wisniewski-Dyé, 2009). It is mediated by diffusible signal molecules whose concentrations relate to the density of microorganisms in the vicinity. When the signal molecule concentration reaches a threshold value, the binding of signal molecules and regulatory proteins activates QS-regulated genes to activate relevant phenotypes, such as activity increase, attachment growth and biofilm development (De Clippeleir et al., 2011; Ponnusamy et al., 2009). A number of auto-inducers have been identified so far, and the acylated homoserine lactones (AHLs) have been well studied ones (Fuqua et al., 2001; Miller and Bassler, 2001; Yong and Zhong, 2013).

Many studies have focused on AHL-regulated QS in recent years. The AHLs in aerobic activated sludge could increase the attachment growth of bacteria through enhancing extracellular polymeric substances (EPS) secretion (Ren et al., 2010).

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Fernández et al. (2008) pointed out that attachment growth is beneficial for biomass retention. AHLs could also enhance the tolerance of bacteria to nutrient-lacking environments (Valle et al., 2004) and improve the biomass activity (De Clippeleir et al., 2011; Yong et al., 2014). Furthermore, C_{12}-HSL detected in an oxygen-limited autotrophic nitrification/denitrification biofilm could significantly improve the specific anoxic ammonium oxidation rate (De Clippeleir et al., 2011). All these characteristics suggested that AHLs might play an important role in the start-up of anammox. Since it was reported that AHL signals existed in the supernatant of anoxic sludge (Feng et al., 2014), an AHL-containing anammox granular sludge reactor (AGSR) supernatant was prepared and added into the influent water to start up the continuously stirred tank reactors (CSTRs) in this study. The objective of our work was to investigate the effects of AHLs on anammox biomass and accelerate the start-up of the anammox process. Results showed that adding AHL-containing anammox supernatant into the reactor is a previously unreported effective and novel way to accelerate the start-up of anammox.

1. Materials and methods

1.1. AHL extraction and LC–MS/MS detection

The supernatant of anammox biomasses was harvested from an AGSR operated for 2 years and filtered through a cellulose filter (pore size of 0. 22 μm). The clarified supernatant was then acidified to pH = 2 with HCl. The supernatant was extracted twice with ethyl acetate (volume ratio 1:1) previously acidified with 0.5% formic acid. The organic phase was firstly concentrated to 400 μL and evaporated with nitrogen flow to dryness. The dried extracts were dissolved with 50 μL methanol and 150 μL 0.01% aqueous formic acid. The AHLs in the supernatant and standard C_{12}-HSL (Sigma) were extracted and analyzed by high performance liquid chromatography coupled with tandem mass spectrometry (HPLC–MS/MS; Q Exactive, ThermoFisher, USA) as described by Haydee de Clippeleir et al. (2011). The relative content of AHL signal molecules was detected with *Agrobacterium tumefaciens* KYC55 according to Miller and Bassler (2001).

1.2. Anammox activity tests

The effects of AHL signal molecules on the anammox activity were tested in six sets (S1–6) of batch tests. Anammox bacteria with mixed liquor volatile suspended solids (MLVSS) concentration of 0.3 g/L was cultivated with the required amounts of mineral medium and trace elements dissolved in different liquids. The culture medium of anammox bacteria was composed as follows: 100 mg/L NH_4^+-N and 132 mg/L NO_2^--N were added to the mineral medium in the form of NH_4Cl and $NaNO_2$ (Sinopharm Chemical Reagent co., Ltd., China). The mineral medium contained (g/L): $KHCO_3$ 1.25, KH_2PO_4 0.025, $CaCl{\cdot}H_2O$ 0.3, $MgSO_4{\cdot}7H_2O$, $FeSO_4$ 0.00625, EDTA 0.00625, and 1.25 mL/L trace elements solution. The trace element solution contained (g/L): EDTA 15, $ZnSO_4{\cdot}7H_2O$ 0.43, $CoCl_2{\cdot}6H_2O$ 0.24, $MnCl_2{\cdot}4H_2O$ 0.99, $CuSO_4{\cdot}5H_2O$ 0.25, $NaMoO_4{\cdot}2H_2O$ 0.22, $NiCl_2{\cdot}2H_2O$ 0.19, $NaSeO_4{\cdot}10H_2O$ 0.21, H_3BO_4 0.014, and $NaWO_4{\cdot}2H_2O$ 0.050 (Sinopharm Chemical Reagent Co., Ltd., China) (Van de Graaf et al., 1996).

The anammox bacteria used in batch tests (S1–S6) were obtained from an anammox reactor in our lab which has operated for 3 years. Batch tests were designed as follows: S1, AGSR supernatant; S2, AGSR supernatant + porcine kidney acylase I (1 g/L, Sigma); S3, tap water + C_{12}-HSL (10 mg/L); S4, 2 × medium + tap water; S5, tap water (Control); S6, no biomasses + supernatant. Among these tests, porcine kidney acylase, a kind of AHL degradation enzyme, was added in S2 to exclude the effects of C_{12}-HSL in the AGSR supernatant. To exclude the effects of residual nutrients on anammox activity, twice the concentration of mineral medium was added in S4.

The bottles were flushed with N_2 for 10 min at the flow rate of 400 mL/min. The detected dissolved oxygen in the bottles was below 1 mg/L in all cases. All serum bottles were tightly sealed with rubber caps to avoid any external O_2 influence and incubated at 35°C at 150 r/min. NH_4^+-N and NO_2^--N concentrations were colorimetrically measured using Nessler's reagent and N-Naphthylethylenediamine, according to the standard methods for the examination of water and wastewater (APHA, 1995). Each test was replicated 3 times. Specific anammox activity (SAA) was estimated by measuring the decrease of nitrogen concentration per unit biomass concentration per day.

1.3. Prepared synthetic wastewater and start-up performance

Three 1.5 L CSTRs (R1, R2, R3) were used to study the effects of AHLs on the start-up of the anammox process. The influent water was prepared as follows: R1, AHL-containing AGSR supernatant; R2, 1:1 (volume) AGSR supernatant and tap water; R3, tap water. Mineral medium and trace elements were added artificially as described in Section 1.2. The initial concentrations of NH_4^+-N and NO_2^--N were 50 mg/L and 66 mg/L. The flow rate of the influent water was 62.5 mL/hr and the hydraulic retention time was 24 hr. The inoculated aerobic activated sludge was obtained from Lingshuihe Wastewater Treatment Plant (Dalian, China) with the concentration of 3.3 g MLVSS/L. The effluent water was monitored every 2 days. Influent NH_4^+-N and NO_2^--N concentrations were 50 mg/L and 66 mg/L, increased stepwise once the effluent nitrogen was below 10 mg/L. To avoid the accumulation of nitrogen in CSTRs, when the detected effluent NO_2^--N was higher than 20 mg/L (Zheng et al., 2016), the influent NH_4^+-N and NO_2^--N concentrations would also be adjusted to 50 mg/L and 66 mg/L, artificially decreased stepwise. The NLR of the CSTRs were calculated as:

$$\mathrm{NLR} = \frac{\left[\mathrm{NH_4^+{-}N_{(\,inf)}} + \mathrm{NO_2^-{-}N_{(\,inf)}}\right] \times 24\ \mathrm{hr} \times 62.5\ \mathrm{mL/hr_{(flow\ rate)}}}{\left[1.5\mathrm{m^3_{(CSTRs\ volume)}} \times 1\ \mathrm{day}\right]}$$

1.4. EPS extraction and denaturing gradient gel electrophoresis (DGGE) analysis

EPS in reactors were extracted according to the procedure of Yang and Li (2009). The extracellular protein and polysaccharide contents were measured by the Coomassie Brilliant Blue method (Bradford, 1976) and Anthrone method (Lever, 1972). The genomic DNA was extracted using the FastDNA spin kit

for soil (MP Biomedicals, USA). The 16S DNA genes for all bacteria were amplified using the primer pair GC341F and 907R. DGGE analysis was performed according to Valle et al. (2004).

1.5. *Fluorescence in situ hybridization (FISH) analysis*

The sludge samples were analyzed by FISH in this investigation. FISH analysis was performed according to the standard hybridization protocol (Amann, 1995; Third et al., 2001). The probes used in this experiment are listed in Table S1 (Supporting information). The EUB338 (Amann, 1995) and AMX820 (Schmid et al., 2005) probes were purchased from TaKaRa Company (Dalian, China). An epifluorescence microscope (Olympus FV1000, Japan) was used for image acquisition.

2. Results

2.1. *Detection and identification of AHLs in AGSR biomass supernatant*

The AHL signal molecules in the supernatant of the AGSR were detected with HPLC–MS/MS. Only one AHL was detected in the AGSR supernatant, whose chromatographic retention time and fragmentation pattern were identical to those of the C_{12}-HSL standard (Appendix A Fig. S1). No other peaks observed in the MS spectrum of the supernatant extract contained the characteristic homoserine lactone product ion (*m*/z 102), indicating that no other AHLs were present in the AGSR supernatant. The result confirmed that C_{12}-HSL was the only QS signal at a detectable level in the supernatant according to our assay.

2.2. *Anammox activity tests*

To confirm the effects of AHLs on anammox, the SAAs were measured in batch tests (S1–S6) (Fig. 1). Since the start-up of anammox is a process of activation and accumulation of anammox bacteria from a very limited concentration, the effect of AHLs on a low concentration of anammox biomass (0.3 g MLVSS/L) was studied. According to the results, the SAA with AGSR supernatant (S1) and C_{12}-HSL (S3) added were significantly improved. The SAA increased from 94.84 mg N/(g VSS d) in S5 (control) to 128.78 mg N/(g VSS d) (S1) and 139.27 mg N/(g VSS d) (S3). However, the group adding AHLs degradation enzyme with porcine kidney acylase I (S2, 92.96 mg N/(g VSS d)) was similar to that in S5 (94.84 mg N/(g VSS d)). Porcine kidney acylase I was added into the AGSR supernatant to further confirm its AHL degradation function. Results in Appendix A Fig. S2 show that almost all the AHLs in the AGSR supernatant were degraded. Since the residual nutrients in the supernatant might enhance the SAA (Dexiang et al., 2008; Qiao et al., 2012, 2013), twice the concentration of mineral medium and trace elements were added in S4. However, no significant changes of SAA were observed, so the effects of residual nutrients on SAA were excluded. To further exclude the effects of chemical and biological compounds in the supernatant, AGSR supernatant was added in S6, but in the absence of anammox biomass. Almost no nitrogen was removed in S6, which showed that no bacteria or enzymes in the supernatant were active in removing ammonium or nitrite. The results above indicated that AHLs in the supernatant of AGSR were the main reason for the improvement of the SAA.

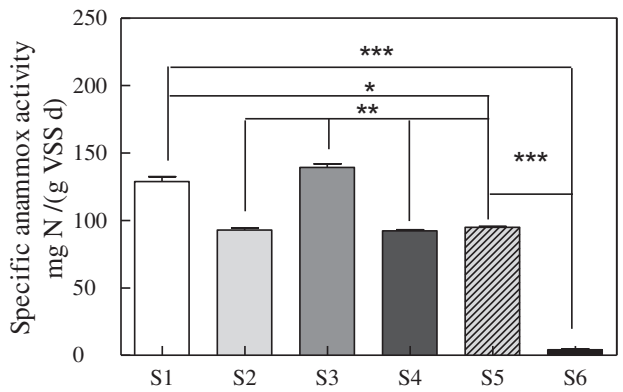

Fig. 1 – Comparison of specific anammox activity. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ were considered significant.

2.3. *Reactor performance*

The CSTRs were operated for 3 months. The relative contents of AHLs in influent water of R1 and R2 were 9.48 Miller units and 6.50 Miller units. Almost no AHLs were detected in the influent water of R3 (Appendix A Fig. S3). According to the effluent ammonium concentration, the experimental period could be divided into four stages: cell lysing phase, lag phase, activity elevation phase and stationary phase.

As shown in Fig. 2, the cell lysing phases in the reactors were all 12 days. In the cell lysing phase, the cells that could not adapt to the given conditions experienced lysis from aerobic bacteria. This caused breakdown of the organic nitrogen to ammonia. As a result, the ammonium concentration rose even higher than that in influent water. No anammox activity appeared in this phase. Denitrifying bacteria predominated the community so that nitrite was mostly consumed.

The anammox activity appeared in the lag phase, with both ammonium and nitrite removed simultaneously. In this stage, most aerobic bacteria had been eliminated and anammox biomasses were gradually enriched. Moreover, the prolonged start-up time of R3 was reflected in this phase. The lag phases of R1 and R2 were from 13 to 42 days. However, the lag phase of R3 was from 13 to 56 days. The NH_4^+-N and NO_2^--N removal efficiency increased faster in R1 and R2 than in R3. At the end of this phase, the NH_4^+-N and NO_2^--N removal efficiency of R1 and R2 was 100% and 95.05%. However, the NH_4^+-N and NO_2^--N removal efficiency of R3 was 69.72%, with effluent nitrogen concentration approaching 20 mg/L. In this case, the start-up time of R3 was prolonged to the 56th day, and the NH_4^+-N and NO_2^--N removal efficiency reached 94.92%.

In the activity elevation phase, anammox bacteria multiplied rapidly so that continuous and higher nitrogen concentration influent water should be applied. The influent nitrogen concentration was increased stepwise once the effluent nitrogen was below 10 mg/L. The activity elevation

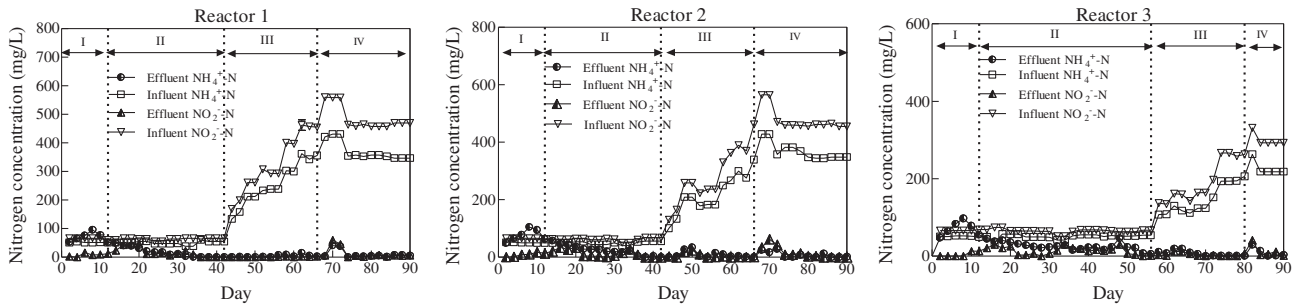

Fig. 2 – Reactors performance of Reactor 1, with influent water prepared from C_{12}-HSL containing AGSR biomass supernatant; Reactor 2, with influent water prepared from 1:1 ratio of C_{12}-HSL containing AGSR biomass supernatant and tap water; and Reactor 3, prepared from no C_{12}-HSL containing tap water. Phase I:cell lysing phase; Phase II: lag phase; Phase III: activity elevation phase; Phase IV: stationary phase. HSL: homoserine lactone; AGSR: anammox granular sludge reactor.

phase in all reactors lasted for 24 days. However, the NLR of R1 and R2 (0.8 mg N/(m^3·day)) rose more rapidly and was higher than that of R3 (0.5 mg N/(m^3·day)). Moreover, the NH_4^+-N and NO_2^--N removal efficiencies were all above 90%. At this point, the reactors were successfully started up. The start-up time in R1 and R2 (66 days) was only 82.5% of that in R3 (80 days).

In the stationary phase, the anammox process exhibited good stability. The NLR could not be enhanced due to the space limitations of the reactors. The NLR in R1 and R2 were kept at 0.8 mg N/(m^3·day), and the NLR in R3 was 0.5 mg N/(m^3·day). The NH_4^+-N and NO_2^--N removal efficiency was 98%, 97% and 96%, respectively.

2.4. EPS content in each reactor

EPS is composed of proteins and polysaccharides. The EPS content was analyzed on the 90th day. As shown in Fig. 3, AHL-containing AGSR supernatant induced the secretion of EPS in R1 and R2. The enhancement of EPS was reflected in the increased secretion of both protein and polysaccharides. The protein contents in R1, R2 and R3 were 67.08, 59.33 and 50.25 mg/g VSS. The polysaccharides contents in R1, R2 and R3 were 35.38, 28.63 and 23.80 mg/g VSS.

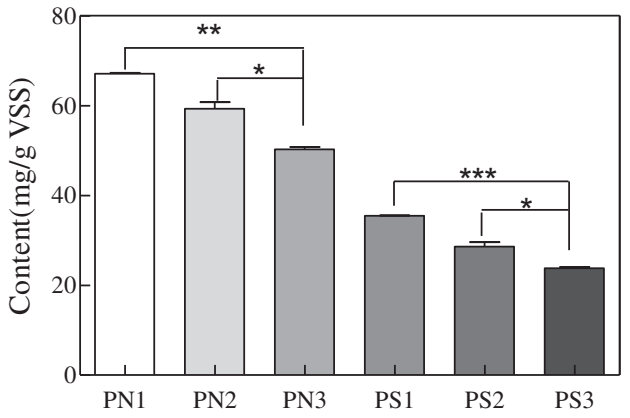

Fig. 3 – Polysaccharides and proteins contents of anammox in each reactor, $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ were considered significant. PN1, protein content in R1; PN2, protein content in R2; PN3, protein content in R3. PS1, polysaccharides content in R1; PS2, polysaccharides content in R2; PS3, polysaccharides content in R3.

2.5. Microbial community analysis

After the long-term operation, DGGE analysis was performed to evaluate the succession of microbial communities in the three reactors (Table 1). In the DGGE fingerprints (Fig. 4), bands 1, 6 were related to *Planctomycetales*. The similarity was 98% and 97%. Besides *Planctomycetales*, the microbial community also consisted of some strains related to *Pseudomonas aeruginosa*, *Proteobacterium*, Nitrospira and an uncultured bacterium clone. The reactors where AHL-containing AGSR supernatant was added experienced community evolution in the reactors such that bands 2, 3 were effectively enriched in R1 and R2. Bands 4, 5 of R3 were eliminated in R1. Moreover, the *Planctomycetales* in inoculated sludge effectively survived after the long operation period. The prominent intensity of band 6 (anammox bacteria) in Fig. 4 further provided evidence that anammox bacteria had successfully accumulated in the reactors. FISH analysis (Appendix A, Fig. S4) showed that, compared with R3, anammox bacteria experienced better enrichment in R1 and R2. The results above indicated that adding AHL-containing AGSR supernatant was beneficial for biomass competition and differentiation into anammox.

3. Discussion

In this investigation, C_{12}-HSL was detected in the supernatant of an AGSR by HPLC–MS/MS. This kind of AHL could improve the SAA of anammox. According to bioassay results of AHLs in Appendix A Fig. S3. AHLs could be added by preparing the influent water from AHL-containing anammox supernatant. Degradation tests after 32 hr showed that although the C_{12}-HSL content significantly decreased within the 32 hr, the residual amounts of C_{12}-HSL detected in AGSR supernatant and R1, R2 and R3 were still 5.55 Miller units, 5.13 Miller units and 3.64 Miller units. Furthermore, the influent water of CSTRS was prepared daily during the performance. We considered that the degradation of C_{12}-HSL in the CSTR influent water could be ignored.

Table 1 – Sequence analysis of 16S ribosomal deoxyribonucleic acid (rDNA) gene fragment denaturing gradient gel electrophoresis (DGGE) bands in reactors.

DGGE band	Closest species	Similarity	Accession no.
1	Uncultured planctomycete clone 5GA_Pla_HKP_50 16S ribosomal ribonucleic acid (RNA) gene, partial sequence	98%	GQ356196.1
2	Uncultured bacterium clone BP U4C 1b03 16S ribosomal RNA gene, partial sequence	99%	GQ182309.1
3	*Pseudomonas aeruginosa* isolate 20070709005 class I integron, partial sequence	100%	FJ157995.1
4	*Denitratisoma oestradiolicum* clone 20b_2 16S ribosomal RNA gene, partial sequence	97%	KF810120.1
5	Uncultured beta proteobacterium partial 16S rRNA gene, isolate high lake 3–6	95%	AM182319.1
6	Uncultured anaerobic ammonium-oxidizing bacterium partial 16S rRNA gene, clone amx 5	97%	FM945331.1
7	Uncultured *Nitrospira* sp. clone 54 16S ribosomal RNA gene, partial sequence	99%	HQ424566

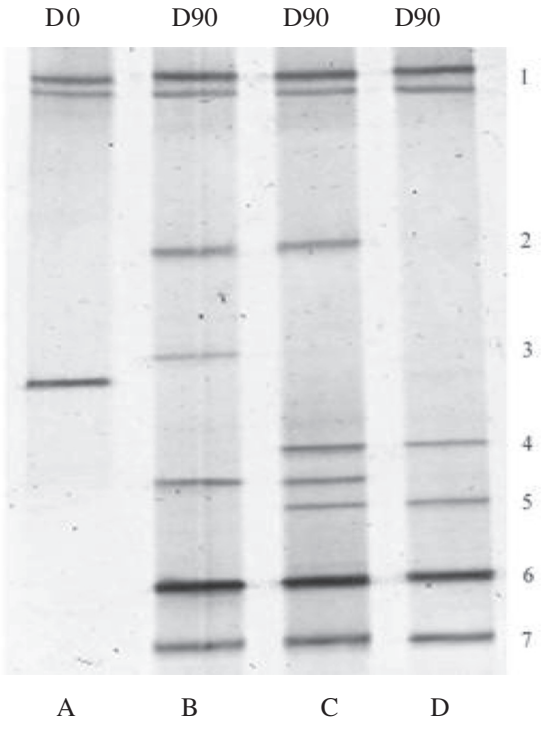

Fig. 4 – Denaturing gradient gel electrophoresis (DGGE) analysis of PCR amplified 16S ribosomal deoxyribonucleic acid (rDNA) gene fragments of microbial in the three reactors (A) inoculated sludge; (B) sludge in Reactor 1 operated for 90 days; (C) sludge in Reactor 2 operated for 90 days; (D) sludge in Reactor 3 operated for 90 days.

According to the results of long term operation, AHL-containing supernatant effectively accelerated the start-up of the anammox process. AHL-containing supernatant affected the biomasses in CSTRs from three aspects: Firstly, it was shown in batch tests that AHLs could improve the SAA. Secondly, AHL-containing anammox supernatant could induce the secretion of EPS. This is consistent with the previous reports that EPS secretion was regulated by AHL-mediated QS (Lv et al., 2014). It was reported that the increase of protein in EPS could enhance the cell surface hydrophobicity and sustain resistance to toxic shock (Yu et al., 2009). The AHL-mediated EPS could also alter the physicochemical characteristics of the cellular surface and induce microbial attachment growth and granulation (Lv et al., 2014; Sheng et al., 2010; Yu et al., 2009). Thus, we speculate that the AHL-mediated EPS secretion of anammox could enhance its ability to sustain in the new environment and prevent it from washing out of the reactors. Finally, AHL-containing AGSR supernatant could result in a difference in the shift of microbial communities (Fig. 4). FISH analysis showed that anammox bacteria were more enriched in AHL-containing reactors (Supporting information, Fig. S4). This kind of AHL-mediated community evolution was also observed in other reports. Valle et al. (2004) presented the community evolution process of the samples with and without AHL treatments by DGGE. They found that adding AHLs into the culture was beneficial for sustaining the functional bacteria. All these altered characteristics of biomasses in CSTRs would affect the start-up of the anammox process.

During the investigation, it was observed that, although the AHL concentration of the influent water in R1 was higher than that in R2, there was not much difference between the performances of these two reactors. This is consistent with our batch experiment results (Appendix A Fig. S5) that AHLs could trigger the QS characteristics of anammox. However, more AHL signal might not necessarily further enhance the activity of anammox. This phenomenon was also reported in other studies. Valle et al. (2004) observed that 2 and 20 μmol/L AHL treatments could both sustain the function of the community under starvation. Moreover, the 2 and 20 μmol/L AHL treatments generated identical changes in community composition.

In this study, C_{12}-HSL was detected in the supernatant of an AGSR. Influent water was prepared from the AHL-containing supernatant of the AGSR. With this kind of influent water, the start-up period of the reactor could be reduced by 14 days. The NLR could also enhance performance to a higher level. Additionally, AHL-containing supernatant induced the increased secretion of EPS and anammox obtained better enrichment. In conclusion, the methods in our experiment have the advantage of low cost and easy operation. Adding AHL-containing supernatant to the influent water could be a potential low cost and easy-to-operate way to accelerate the start-up of the anammox process.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2017.03.025.

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