Journal of Environmental Sciences 98 (2020) 179–185

Contents lists available at ScienceDirect

Journal of Environmental Sciences

journal homepage: www.elsevier.com/locate/jes

JES JOURNAL OF ENVIRONMENTAL SCIENCES www.jesc.ac.cn

Bioaugmentation and quorum sensing disruption as solutions to increase nitrate removal in sequencing batch reactors treating nitrate-rich wastewater

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ARTICLE INFO

Article history:
Received 7 April 2020
Revised 3 June 2020
Accepted 3 June 2020

Keywords:
Bioaugmentation
Denitrification
Nitrate removal
Quorum sensing inhibitor
Curcumin

ABSTRACT

Bioaugmentation of denitrifying bacteria can serve as a promising technique to improve nutrient removal during wastewater treatment. While denitrification inhibition by bacterial quorum sensing (QS) in *Pseudomonas aeruginosa* has been indicated, the application of bacterial QS disruption to improve nitrate removal from wastewater has not been investigated. In this study, the effect of bioaugmentation of *P. aeruginosa* SD-1 on nitrate removal in sequencing batch reactors that treat nitrate rich wastewater was assessed. Additionally, the potential of a quorum sensing inhibitor (QSI) to improve denitrification following bacterial bioaugmentation was evaluated. Curcumin, a natural plant extract, was used as a QSI. The chemical oxygen demand (COD) and initial nitrate concentration of the influent were 700±20 mg/L and 200±10 mg/L respectively, and their respective concentrations in the effluent were 56.9±3.2 mg/L and 9.0±3.2 mg/L. Thus, the results revealed that bioaugmentation of *P. aeruginosa* SD-1 resulted in an increased nitrate removal to 82%±1%. Further, nitrate was almost completely removed following the addition of the QSI, and activities of nitrate reductase and nitrite reductase increased by 88%±2% and 74%±2% respectively. The nitrogen mass balance indicated that aerobic denitrification was employed as the main pathway for nitrogen removal in the reactors. The results imply that bioaugmentation and modulation of QS in denitrifying bacteria, through the use of a QSI, can enhance nitrate removal during wastewater treatment.

Introduction

Nitrogen compounds discharged into the aquatic environment can result in eutrophication of rivers, deterioration of water sources, and serve as a human health hazard. Galloway et al. (2014) identified nitrate pollution as a critical environmental issue. Nitrate pollution of soil, surface water, and groundwater is prevalent in areas associated with agriculture, livestock farming, and specific industries. Long term consumption of groundwater with excessive nitrates can be detrimental to human health and has been associated with methemoglobinemia and cancer (Sandor et al., 2001). Therefore, nitrate concentration discharged in the wastewater has to be reduced.

Several biological, physical, and chemical processes have been adopted to treat wastewater containing high nitrate levels (Ahn et al., 2008; Prüsse, 2001; Rivett et al., 2008). Although biological means are considered the most economical and sustainable, traditional biological nitrogen removal processes require complicated operating conditions. However, Zhang et al. (2017) observed that aerobic denitrifying bacteria can be used to achieve simultaneous nitrification and denitrification in an aerated tank, thereby reducing investment and operational complexity. Nitrate removal using specific bacteria such as *Pseudomonas aeruginosa* (ATCC 9027), also known as *P. aeruginosa* SD-1 (Appendix A **Fig. S1b**), under aerobic conditions has been studied (Chen et al., 2003). However, aerobic denitrifying bacteria are not abundant in the natural environment and are less likely to form dominant populations (Wang et al., 2017). Bioaugmentation is a biological method, wherein specific functional strains are introduced into activated sludge to successfully colonize the bacteria and enhance the efficiency of pollutant removal (Herrero and Stuckey, 2015). Pei et al. (2016) found that addition of the denitrifying bacterium *Paenibacillus* sp. XP1 to a pilot scale reed constructed wetland increased the nitrate-nitrogen (NO_3^--N) and total nitrogen removal rates in the secondary effluent of rural domestic wastewater. Further, Fu et al. (2019) noted

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https://doi.org/10.1016/j.jes.2020.06.007
1001-0742/

that addition of salt tolerant denitrifying strains to constructed wetlands enhanced denitrification during saline wastewater treatment. Bioaugmentation had been used to increase nitrate removal in denitrification reactors (Du et al., 2017; Ji et al., 2014). Yao et al. (2013) noted that combination of heterotrophic nitrification and aerobic denitrification used for bioaugmentation enhanced nitrogen removal at low temperatures. Multiple studies have investigated factors such as the selection of inoculated strains and dynamics of bacterial communities for the construction and operation of bioaugmentation systems (Liu et al., 2018; Medhi et al., 2017).

Further, quorum sensing (QS) is the most widely explored cell to cell communication mechanism, wherein small chemical signaling molecules are involved in chemical communications between microbes. The signal molecule binds to its specific receptor when its concentration attains a threshold, thereby regulating the expression of related genes and physiological behaviors (Chernin et al., 2011). Toyofuku et al. (2007) reported that *las* and *rhl* QS systems negatively regulated denitrification under anaerobic conditions. Wang et al. (2017) reported that in mediums containing nitrate as the nitrogen source, *las* and *rhl* QS deficient mutant of *P. aeruginosa* SD-1 exhibited higher utilization of nitrate than the wild strains. Additionally, Schuster et al. (2003) reported that the gene expression of nitrate reductase NAP (enzyme of periplasmic nitrate reductase) increased two to four folds during aerobic growth in *lasR* and *rhlR* mutants of *P. aeruginosa* PAO-MW1. These observations illustrated that *las* and *rhl* QS systems exerted an inhibitory effect on denitrification and could serve as an effective approach to remove nitrate.

AHLs (*N*-acyl-homoserine lactones) play an important role in QS as a language of microbial communication. QS disruption blocks the exchange of information between microorganisms and can be realized by different mechanisms: (i) promoting AHL degeneration, (ii) limiting AHL cognate receptor protein or AHL synthase activity, (iii) blocking QS signal molecule synthesis, and (iv) using synthetic molecules to mimic signal molecules (Kalia, 2013). Further, quorum quenching enzymes (QQE), enzymes capable of inactivating QS signals, and quorum sensing inhibitors (QSI), chemicals capable of interrupting QS pathways to interfere with the expression of QS controlled genes, can be used (Fong et al., 2018). Use of QS disruption in wastewater treatment to improve efficiency of wastewater treatment and biofouling control has been investigated (Jiang et al., 2013; Yeon et al., 2009). However, studies that utilize QS disruption to enhance denitrification have not been conducted. Curcumin is a substance extracted from the root of turmeric *(Curcuma longa L)*. Studies have shown that curcumin inhibits *las* and *rhl* QS systems by interfering with the synthesis of signal molecules, such as attenuating virulence factors (Rudrappa and Bais, 2008). Lade et al. (2017) observed that curcumin inhibited membrane biofouling but did not increase the influent organic loading rate. Further, preliminary experimentation during the study confirmed that curcumin could increase the removal of nitrate without affecting *P. aeruginosa* SD-1 growth (Appendix A **Fig. S1a**). Therefore, curcumin was used as a QSI to improve aerobic denitrification in this study. The effect of bioaugmentation with *P. aeruginosa* SD-1 on nitrate removal in synthetic wastewater was explored using sequencing batch reactors. Further, the mechanism employed by the QSI to suppress the QS pathway of *P. aeruginosa* SD-1 and achieve improved denitrification, was evaluated.

1. Materials and methods

1.1. Activated sludge and bacterial strain

The activated sludge used in this study was obtained from the Qige wastewater treatment plant in Hangzhou, China. The basic characteristics of the activated sludge are listed in Appendix A **Table S1**. *P. aeruginosa* SD-1, a highly efficient denitrifying strain, was preserved in our laboratory. The strain was activated by adding 3-morpholinopropanesulfonic acid (MOPS), a biological buffer with pH 6.5–7.9, to 100 mL Luria-Bertani (LB) liquid medium, to obtain a final MOPS concentration of 50 mmol/L. *P. aeruginosa* SD-1 was grown in a 250 mL conical flask with 150 mL LB-MOPS at 37 °C for 12 hr until it reached the logarithmic growth phase. Next, the cultures were centrifuged at 7000 × *g* for 5 min at room temperature and washed three times using sterile water. The pellet was resuspended in 150 mL sterile water and utilized for bioaugmentation system inoculation.

1.2. Reactor operation and monitoring

Twelve identical lab scale sequencing batch reactors with individual working volume of 1.0 L were operated. The reactors comprised of PVC cylinders with height to diameter ratio of 4:1. The seeding activated sludge was centrifuged and washed three times using 0.9% NaCl solution, and added to the reactor with approximately 3 g/L of mixed liquid suspended solids (MLSS). Synthetic wastewater, which contained 0.74 g dextrose monohydrate, 1.24 g $NaNO_3$, 0.22 g $NaHCO_3$, and 0.022 g KH_2PO_4 in 1 L tap water, was utilized. The chemical oxygen demand (COD) of the influent was 700±20 mg/L, initial nitrate concentration was 200±10 mg/L, and influent carbon-to-nitrogen (C/N) ratio was nearly 3.5. All reactors were operated with a cycle time of 24 h, comprising 10 min of influent feeding, 21 hr of aeration, 2.5 hr of settling, 10 min of decanting, and 10 min of idle time. During each cycle, 700 mL of the supernatant was withdrawn from the reactor after the settling phase, and 700 mL fresh wastewater was added to the reactor during the feeding phase, resulting in a hydraulic retention time of 30 hr. The sludge was wasted regularly at the idle stage to maintain the MLSS level at 3 g/L and solid retention time of 7 days. The pH was adjusted to 7.5 using 2 mol/L NaOH or 2 mol/L HCl, and temperature was maintained at 30±2 °C. Reactors were provided with air through an aerator equipped with an on/off control system to maintain a dissolved oxygen concentration of around 2–3 mg/L.

The reactors were divided into four groups of triplicates, and operated for 31 days. The operational conditions of the reactors are listed in Table 1. The operation of reactors was divided into period I (day 1–15) representing bioaugmentation and period II (day 16–31) representing QS disruption.

1.3. Bioaugmentation and quorum sensing disruption

During period I (bioaugmentation), 3% (*V/V*) washed *P. aeruginosa* SD-1 cells (OD600 = 2.000±0.1) were inoculated in the first eight cycles in the BA reactors. During period II (QS disruption), curcumin (Aladdin, Shanghai, China) was added to the QSI reactors during every cycle from days 16 to day 31. After each addition, the final concentration of curcumin in the reactors was 6.0 μg/mL.

1.4. Reverse transcriptase polymerase chain reaction

On day 31, activated sludge was collected in 2 mL centrifuge tubes and centrifuged at 13,000 × *g* for 5 min at 4 °C, to obtain sludge samples (Zhang et al., 2015). The total RNA in the sludge sample was extracted using a Bacterial Total RNA Isolation Kit B518625 (Sangon, Shanghai, China). The extracted RNA sample was purified using a DNase I (RNase-free) kit to obtain a DNA-free RNA sample. RNA was then reverse transcribed into cDNA using a TransScript kit AT341-01 (Transgen, Shanghai, China) and *napA* gene was amplified by PCR to verify if the activated sludge had been colonized by the strain. Primers for PCR were designed using Primer-BLAST,

Table 1 – Treatment of each experimental groups.

Reactor name	Period I (Day 1–15) Bioaugmentation	Period II (Day 16–31) Inhibition of QS
	Pseudomonas aeruginosa SD-1[a]	Quorum sensing inhibitor [b] (Curcumin)
Non-bioaugmented reactor (non-BA)	-	-
Non-bioaugmented reactor+ Quorum sensing inhibitor (non-BA+QSI)	-	+
Bioaugmented reactor (BA)	+	-
Bioaugmented reactor+ Quorum sensing inhibitor (BA+QSI)	+	+

" + " indicates addition and " - " indicates no addition.
[a] *Pseudomonas aeruginosa* SD-1 was inoculated into per cycle and added a total of 8 times in 3% (v/v) washed SD-1 cells (OD600 = 2.000 ± 0.1).
[b] Curcumin was added from the 16th day of reactor operation.

which used Primer3 to design specific primers (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primers used were forward primer (5′-AGCCGATCAGTTGGGAACAG-3′) and reverse primer (5′-CATGGTCCAGAACGACACCA-3′). The *napA* gene, which encodes the periplasmic nitrate reductase NAP, is a key gene involved in nitrate removal during aerobic denitrification by *P. aeruginosa* SD-1. The PCR system contained 12.5 μL PCR mix (Transgen, Shanghai, China), 1 μL each of 4 μmol/L forward and reverse primers, and RNase-free water to attain a total volume of 25 μL, resulting in 50 ng/μL cDNA. The cycling profile used for PCR was as follows: pre-denaturation at 95 °C for 10 min, followed by 35 cycles of 95 °C for 30 sec, 57 °C for 30 sec, 72 °C for 30 sec, and final elongation at 72 °C for 10 min. The PCR product was detected using electrophoresis on 0.5% agarose gel, and observed and photographed under a gel imaging system. Products were sequenced at Sangon (Shanghai, China) and aligned using BLAST in GenBank.

1.5. Analytical methods

During all cycles, the influent was sampled 5 min after introduction in the reactor and effluent was sampled from the withdrawal effluent. Nitrate concentration and COD were respectively determined using the brucine method (Nicholas and Nason, 1957) and "Standard Methods for the Examination of Water and Wastewater" (Raji et al., 2015) to measure reactor performance.

1.6. Nitrogen mass balance analysis

Culture solutions were collected on day 31 to analyze the nitrogen mass balance in the reactors. The solutions were centrifuged at 8000 r/min for 10 min, supernatants were filtered using 0.22 μm membrane filters, and used to determine TN, NO_3^--N, NO_2^--N, and ammonium concentrations. Nitrogen assimilation was calculated on the basis of the amount of sludge growth (Li and Irvin, 2007).

1.7. Analysis of quorum sensing signals

To measure the QS signal, 2 mL of the culture was extracted three times, using the same volume of ethyl acetate (containing 0.1% acetic acid). Next, 6 mL of the extract was dried with nitrogen. The signal molecules were redissolved using 1 mL ethyl acetate prior to the assay. Finally, *N*-3-oxopododecanoyl homoserine lactone (3-oxo-C_{12}-HSL) and *N*-butyryl homoserine lactone (C_4-HSL) were quantified using a bioassay strain (Pearson et al., 1994, 1995).

1.8. Determination of denitrifying enzyme activity

Samples were extracted on day 31 and washed three times using 0.01 M phosphate buffer (pH 7.4) prior to measurement. Next, the resuspended pellets were sonicated at 20 kHz and 4 °C, for 5 min to disintegrate the cell structure of the activated sludge. Subsequently, the debris was centrifuged at 12,000 × *g* and 4 °C for 10 min. Crude extracts from the supernatant were obtained to determine the enzymatic activities, using bovine serum albumin as the standard (Lowry et al., 1951).

Periplasmic nitrate and nitrate reductase (NAP and NIR) enzyme activities were determined using methyl viologen as an electron donor. Firstly, 300 µL of the crude extract was added to initiate the reaction in a cuvette containing 0.01 mol/L phosphate buffer (pH 7.4), 1 mmol/L $NaNO_3$ or 1 mmol/L $NaNO_2$, 1 mmol/L methyl viologen, and 5 mmol/L sodium hyposulfite ($Na_2S_2O_4$) to attain a final volume of 2 mL. Next, samples were incubated at 30 °C for 30 min and the produced nitrite was measured using a NAP activity assay or the reduced nitrite was determined using a NIR activity assay. The specific activities of NAP or NIR were expressed as the produced μmol nitrite/min•mg protein or the reduced μmol nitrite/min•mg protein (Chen et al., 2018).

1.9. Real-time quantitative RT-PCR

The RT-PCR primers used in this study are listed in Appendix A **Table S2**. The primers were designed using online NCBI primer design software and specificity of the primer was checked using BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). RNA was extracted from the reactor on day 31 as described in 2.4. The residual DNA in total RNA was removed using DNase I and the concentration of total RNA was determined spectrophotometrically using a Nanodrop system (Nanodrop, 2000, Thermo, USA). RT-qPCR was performed using a TransScript kit AT341-01 (Transgen, Shanghai, China) and Top Green qPCR SuperMix (Transgen, Shanghai, China). The total reaction volume was 20 μL, which included 10 μL qPCR mix, 1 μL forward primer, 1 μL reverse primer, 1 μg cDNA, and ddH_2O. Cycle conditions were as follows: initial denaturation for 10 min at 95 °C followed by 40 cycles of 95 °C for 10 sec, annealing at 57 °C for 30 sec, and extension at 72 °C for 15 sec. The expression of target genes was normalized to the expression of an internal control gene (*proC*), which yielded a $2^{-\Delta\Delta Ct}$ value.

1.10. Statistical analysis

All data were expressed as mean value ± standard error. Statistical analyses consisted of one-way ANOVA, which was conducted using SPSS software (version 22.0). A $p < 0.05$ was considered as statistically significant.

2. Results and discussion

2.1. Survival of P. aeruginosa SD-1 in the reactors

Bioaugmentation and colonization of a strain is considered successful if a gene of the inoculated strain is detected after a long time period following inoculation (Wang et al., 2013). The amount of *napA* gene in the BA system and BA+QSI system on day 15 was 20.4±1.0 and 20.9±2.1 copies of *napA*/copies of 16S gene, respectively, confirming that *P. aeruginosa* SD-1 had colonized successfully in the reactors. The target fragment of *napA* gene was about 400 bp following amplification in this study. As shown in Appendix

A **Fig. S2**, a target fragment was detectable in the BA and BA+QSI reactors after 31 days. At this time, the amount of *napA* gene in the BA system and BA+QSI system was 17.6±1.3 and 15.9±1.8 copies of *napA*/copies of 16S gene, respectively. Further, constant addition of the QSI curcumin did not affect the survival of *P. aeruginosa* SD-1. The survival of the denitrifying bacteria *P. aeruginosa* SD-1 in the reactors despite the addition of QSI (Appendix A **Fig. S2**) can be attributed to the fact that an effective QSI neither inhibits the growth of bacteria nor produces any toxic side effects (Choo et al., 2006; Musthafa et al., 2010). Contrarily, gene *napA* was not detected in the non-BA reactors as *P. aeruginosa* SD-1 was not inoculated.

2.2. Reactor performance after bioaugmentation

2.2.1. COD and nitrate removal

The nitrate levels and COD removal rate in the reactors for 31 days is illustrated in Fig. 1. The nitrate removal efficiency in non-BA reactors stabilized at about 56%±2%. The nitrate removal efficiency in the BA reactors was higher than the non-BA reactors after the initial inoculation of *P. aeruginosa* SD-1 on day 1; this difference was prominent after the third inoculation. After eight days, the nitrate removal efficiency in the BA reactors was stable in the range of 81%–83% from day 9 to day 31, which indicated that *P. aeruginosa* SD-1 had successfully colonized in the BA reactors, and the nitrate removal efficiency increased by nearly 30%. As shown in Fig. 1b, bioaugmentation also had an impact on COD removal. COD removal efficiency increased to 92% in the BA reactors after inoculation of *P. aeruginosa* SD-1 and effluent COD concentration was stable at 59.0 mg/L (Fig. 1d), whereas COD removal efficiency was about 85% in the non-BA reactors.

2.2.2. Denitrifying enzyme activities

Increased aerobic denitrification after colonization by *P. aeruginosa* SD-1 in the reactors was identified on the basis of the activities of nitrate reductase NAP, which is preferentially expressed in an aerobic environment and functions under both aerobic and anaerobic conditions (Kuypers et al., 2018), and nitrite reductase NIR. At the end of the experiment, both enzymes were detected in the non-BA reactors at low activity levels (Fig. 2). However, NAP and NIR enzyme activities respectively increased by 260%±6% and 350%±7% in the successfully colonized BA reactors, compared with non-BA reactors. Thus, the results highlighted that bioaugmentation with *P. aeruginosa* SD-1 improved nitrate removal; nitrate removal efficiencies of BA reactors reached nearly 80%, suggesting that *P. aeruginosa* SD-1 can be employed as the denitrifying strain for bioaugmentation.

2.3. Reactors performance after disruption of quorum sensing

2.3.1. Release of AHLs in the reactors

Secretion of *P. aeruginosa* SD-1 related signaling molecules in each reactor was investigated to understand the mechanism used by QS disruption to achieve aerobic denitrification. Fig. 3 illustrates the changes in the concentrations of two signal molecules (3-oxo-C_{12}-HSL and C_4-HSL) in the reactors during the experiment. In the non-BA+QSI reactors, the original ecological balance in the reactor was maintained as *P. aeruginosa* SD-1 was not added. The contents of the two signal molecules in the non-BA+QSI reactors remained low and did not change significantly during the operation. The concentrations of 3-oxo-C_{12}-HSL and C_4-HSL in the BA reactors exhibited similar tendency and a considerable amount of signaling molecules were produced following the inoculation of *P. aeruginosa* SD-1 (Fig. 3). During the process of bioaugmentation, QS can promote the colonization of exogenous strains by increasing EPS secretion and biofilm forming ability, which is achieved

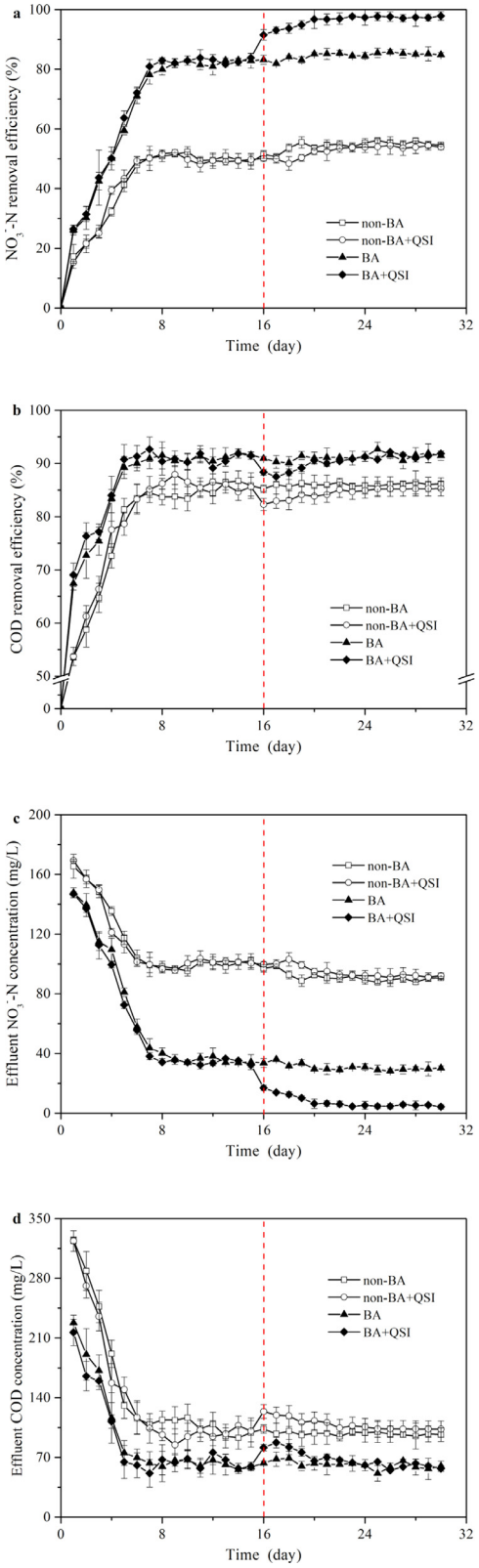

Fig. 1 – Evaluation of nitrate and COD removal in reactors. Removal efficiency of nitrate (a); removal efficiency of COD (b); effluent nitrate (c); effluent COD (d). The red dotted line indicates that the reactor operation period is divided into Period I (Day 1–15) and Period II (Day 16–31). Inoculation of strain SD-1 (from day 1to day 8) in Period I. Addition of QSI (curcumin) from day 16 to day 31 in Period II. Error bars represent the standard deviations of triplicate tests. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.) .

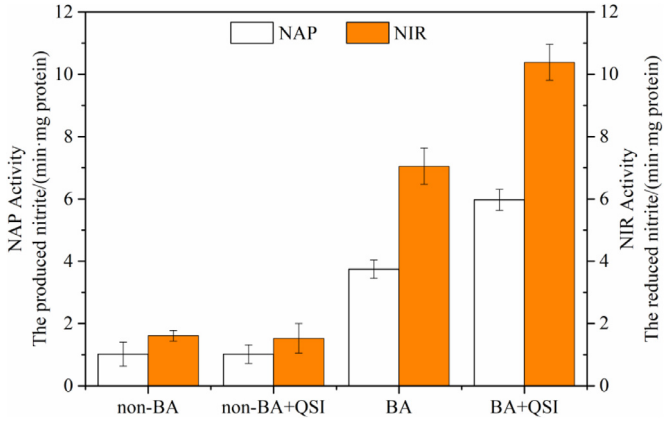

Fig. 2 – Aerobic denitrifying enzyme activity on day 31 of the reactor's operation. The black solid column is NAP and the white solid column is NIR. Error bars represent the standard deviations of triplicate tests.

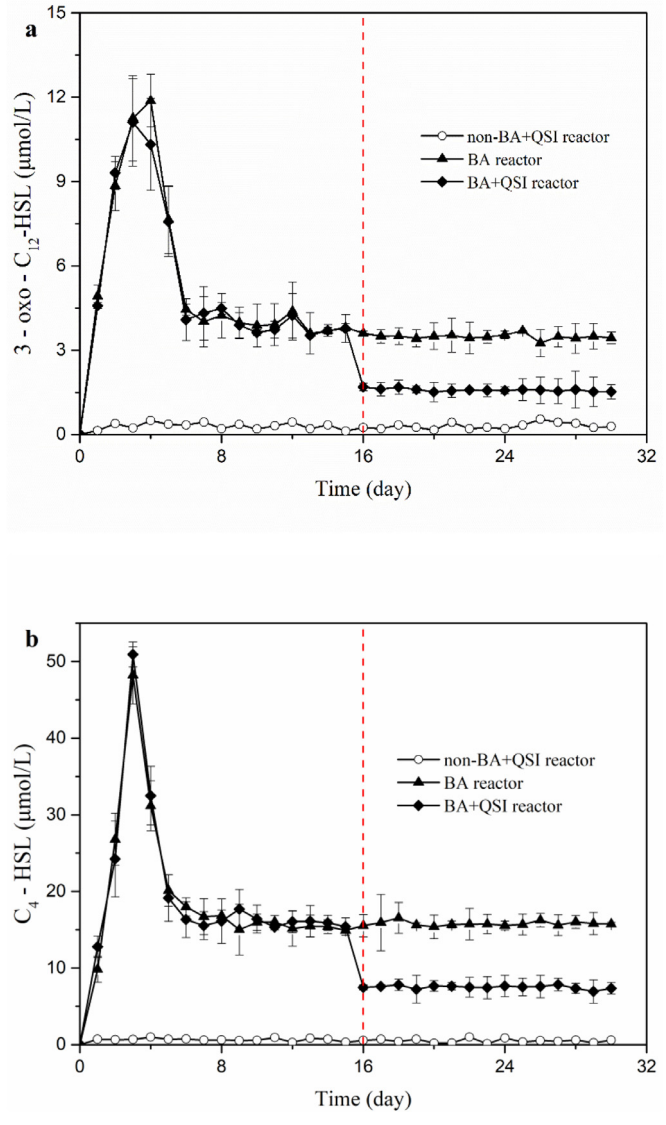

Fig. 3 – The release of AHL from reactors after the addition of curcumin (QSI). Content of 3-oxo-C_{12}-HSL (a); content of C_4-HSL (b). The red dotted line indicates that the reactor operation period is divided into Period I (Day 1–15) and Period II (Day 16–31). Inoculation of strain SD-1 (from day 1to day 8) in Period I. Addition of QSI (curcumin) from day 16 to day 31 in Period II. Error bars represent the standard deviations of triplicate tests. .

by increasing the secretion of signaling molecules (Hooshangi and Bentley, 2008; Marketon et al., 2003; Vu et al., 2009). The balance of the original ecosystem was disturbed due to the inoculation of initial amounts of *P. aeruginosa* SD-1. After the exogenous *P. aeruginosa* SD-1 adapted to the new environment and further strains were not added, the indigenous bacteria in the reactor established a new ecological balance with the exogenous strain, and the secretion of signaling molecules tended to form a new equilibrium (Geske et al., 2008). From day 1 to day 15, the accumulation of AHL in the BA+QSI reactors was consistent with the BA reactors, indicating that *P. aeruginosa* SD-1 had successfully colonized in the reactors.

However, on day 16, the AHL contents were significantly reduced after sludge in the reactor was exposed to 6 μg/mL of curcumin. Subsequently, curcumin was added to the reactors regularly. The concentrations of AHL in the BA+QSI reactors was lower than the BA reactors, and the concentrations of C_4-HSL and 3-oxo-C_{12}-HSL were reduced by about 51% and 57%, respectively. These results indicated that the addition of curcumin successfully inhibited QS in *P. aeruginosa* SD-1.

2.3.2. Nitrates and COD removal

The QSI curcumin promoted the removal of nitrate in the BA+QSI reactors, compared with the BA reactors (Fig. 1c). On day 15, nitrate in the BA+QSI reactors and BA reactors had reduced by nearly 80% and nitrate concentration in the effluent was 40–45 mg/L. However, after the addition of curcumin on day 16 in the BA+QSI reactor, nitrate concentrations in the effluent decreased to 9.0±3.2 mg/L (Fig. 1c). Subsequently, nitrate concentration in the effluent further decreased to about 7.0 mg/L and remained stable. Additionally, on the fifth day of QS disruption, the nitrate removal efficiency increased to 96% (Fig. 1a). In the non-BA+QSI reactors (control group), the QSI did not affect nitrate removal, and nitrate removal efficiency was about 55% in period II.

As shown in Fig. 1b, COD removal efficiency was between 83%–91% before the addition of curcumin in the BA and non-BA reactors. However, it reduced by nearly 5% when curcumin was added to BA+QSI and non-BA+QSI reactors. Nevertheless, COD removal efficiency recovered after five cycles and reached 86±4%. Curcumin is a polyphenol obtained from the herbal and dietary spice turmeric (Sharma et al., 2005), and its addition caused an increase in the initial amount of COD in water, which affected COD removal initially. Moreover, enhancing or quenching AHLs in biological wastewater treatment processes does not have a negative effect on contaminants removal (Jiang et al., 2013; Lade et al., 2017). In membrane bioreactors (MBR), Nam et al. demonstrated QSI could effectively reduce the biofouling without compromising the effluent water quality (Nam et al., 2015).

2.3.3. Mass balance of nitrogen

The mass balance of nitrogen was investigated to explore the pathway of nitrogen conversion in the reactors. Table 2 presents the nitrogen balance analysis of each reactor on day 31. The TN concentration in the influent was represented by the NO_3^--N concentration, which was approximately 207 mg/L, as it was the only form of nitrogen entering the reactors. Table 2 indicates that TN concentration in the effluent of the BA reactors was 31.3±1.8 mg/L; 176.7±1.3 mg/L of nitrogen was removed, of which 17.7±3.6 mg/L of NO_3^--N was converted to biomass (sludge), accounting for 8±1% of the TN removal rate. Nitrogen is an essential component of bacterial cells; a small portion of the nitrogen was assimilated to achieve proper cell function and formation during biological treatment (Kuypers et al., 2018). In addition, a loss of 77%±0.3% of the nitrogen can be attributed to gaseous release (as N_2 or N_2O) by aerobic denitrification. NO_2^--N and ammonia nitrogen were not detected during the experiment. These results indicated that aero-

Table 2 – Nitrogen balances in each reactor in a cycle on day 31.

Reactors	Influent NO_3^--N (mg/L)	Effluent TN (mg/L)	NO_3^--N (mg/L)	Nitrogen Assimilation (mg/L)	TN removal efficiency (%)	Denitrification rate (%)	Nitrogen assimilation rate (%)
non-BA	207.1±3.0	93.4±1.1	93.2±1.5	15.4±2.4	54.7±0.4	47.2±0.7	7.4±1.3
BA	207.2±3.8	31.3±1.8	30.9±2.2	17.7±3.6	84.9±0.7	76.6±0.3	8.2±1.0
non-BA+QSI	207.8±3.3	94.6±2.2	94.1±1.4	14.6±3.7	53.9±1.1	46.9±0.4	7.0±0.6
BA+QSI	207.1±4.6	7.25±3.1	7.03±2.1	17.3±0.3	97.9±1.5	89.6±1.1	8.3±0.0

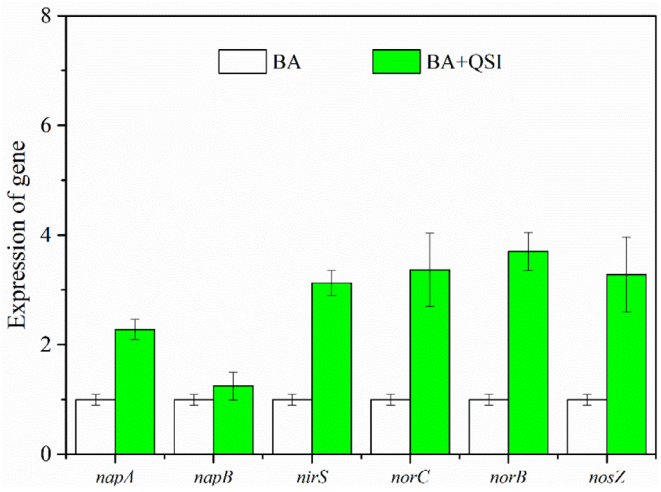

Fig. 4 – Effect of QS disruption on gene expression. The black solid is the BA reactor and the red solid is the BA+QSI reactor with the group sense inhibitor. Error bars represent the standard deviations of triplicate tests. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

bic denitrification was the main pathway employed to remove nitrate in BA reactors, rather than nitrate assimilation by bacteria. The nitrogen balance in each reactor revealed that the denitrification efficiencies in the BA reactors were significantly higher than non-BA reactors. Additionally, bioaugmentation of *P. aeruginosa* SD-1 strains was successful and stable. The denitrification efficiency in the BA+QSI reactors with curcumin increased by 13%±1%, compared with BA reactors, which also confirmed that QS disruption promoted aerobic denitrification in the reactor.

2.3.4. Denitrifying enzyme activities

Fig. 2 depicts the activities of nitrate reductase NAP and nitrite reductase NIR with QS disruption. NAP and NIR enzyme activities in the BA+QSI reactors with curcumin were higher than the BA reactors at the end of the experiment. The NAP and NIR activities in the BA+QSI reactors increased by 88%±2% and 74%±2% ($p<0.05$) respectively, suggesting that QS disruption was beneficial for denitrification. The enhanced activities of key enzymes (especially denitrifying enzymes) were directly related to aerobic denitrification. Although similar results were obtained in pure culture experiments (Schuster et al., 2003; Wang et al., 2017), improved denitrification in an activated sludge process by inhibiting QS has not been reported.

2.3.5. Denitrification gene expression

Enzymes secreted by denitrifying bacteria are encoded and controlled by corresponding functional genes. The transcription of *napA, nirS, norB, norC,* and *nosZ* was up-regulated after QS disruption (Fig. 4). Under aerobic conditions, microbial denitrification consists of four-step reductions catalyzed by NAP, NIR, NOR, and NOS. NAP is a dimer composed of two subunits, NapA and NapB. The *napA* gene encodes the large subunit NapA, while the *napB* gene encodes NapB (Gonzalez et al., 2006; Moreno-Vivián et al., 1999). In addition, NIR, NOR, and NOS are regulated by the *norC, norB,* and *nosZ* genes, respectively. Fig. 4 highlights that *napA* level in the BA+QSI reactors increased by a factor of 2.3 compared with the BA reactors. Similarly, after QS disruption, the expression of *nirS* was higher than the BA reactors, and the mRNA level increased by 3.1-fold. The transcription level of *norC* and *norB* increased by 3.3 and 3.7-fold respectively, and the expression of *nosZ* in the BA+QSI reactors increased by 3.2-fold compared with the BA reactors (Fig. 4). However, there was no significant change in the expression level of *napB* ($p>0.05$).

Curcumin attenuates the *las* and *rhl* QS system responses in *P. aeruginosa* SD-1 through retardation of AHL synthesis; thus, the inhibition of denitrification by QS naturally diminished. After QS disruption, the functional genes encoding key enzymes for denitrification were further quantified using real-time qPCR. An increase in the expression of denitrification genes, including *napA, nirS, norB, norC,* and *nosZ* was observed. The improvement of gene expression resulted from disruption of *las* and *rhl* QS systems, which controlled the transcriptional modulation of denitrification genes (Toyofuku et al., 2007). Further, the expression of *napB* was a notable outlier; *napB* gene exhibited similar expression levels in all experimental groups, and the expression of this gene did not appear to be regulated by QS. Further, *napA* and *napB* play different roles in NAP to encode nitrate sites, and the same set of external factors have different effects on genes of different functions (Oikawa, 2015). Although expression of the *napB* gene in the reactors was similar, the expression of the target gene *napA* in the BA+QSI reactors was higher than the BA reactors. The gene expression results were consistent with results illustrated in Fig. 2. The up-regulated genes suggested that increased denitrification gene expression regulated the synthesis and expression of corresponding NAP and NIR enzymes when curcumin was added, which led to further NO_3^--N removal in the BA+QSI reactors. Studies have indicated that colonization of strains could be hampered by quorum quenching (Zhang et al., 2015). Therefore, during practical application, QSI should be added after successful biological enhancement.

3. Conclusions

The study concluded that bioaugmentation of *P. aeruginosa* SD-1 resulted in the enhancement of nitrate removal by 82%±1%. The nitrate and COD concentrations in the effluent were 90.7±0.8 mg/L and 59.8±6.1 mg/L, respectively. Addition of QSI curcumin following bioaugmentation disrupted the secretion of signal molecules and weakened information exchange between the strains. Consequently, the nitrate in the reactors was almost completely removed; activities of nitrate reductase and nitrite reductase increased by 88%±2% and 74%±2% respectively, and the functional genes encoding denitrification enzymes were up-regulated. Although a combination of bioaugmentation and QS disruption offers potential to ensure complete nitrogen removal, further research is needed before the technique is implemented in practice. The results of this study will serve as valuable aid for further studies focusing on the use of QS disruption for nitrate removal in wastewater treatment.

Acknowledgments

The work was supported by the Natural Science Foundation of Zhejiang Province (nos. LY17E080001 and LQ18E080005) and the China Scholarship Council (no. iCET 2017).

Appendix A. Supplementary data

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jes.2020.06.007.

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