TITLE PAGE

Title:

Low-dissolved-oxygen nitrification in tropical sewage: an investigation on potential, performance and functional microbial community

Short Title:

Low-DO nitrification in tropical sewage

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Abstract

Intensive aeration for nitrification is a major energy consumer in sewage treatment plants (STPs). Low-dissolved-oxygen (low-DO) nitrification has the potential to lower the aeration demand. However, the applicability of low-DO nitrification in the tropical climate is not well-understood. In this study, the potential of low-DO nitrification in tropical setting was first examined using batch kinetic experiments. Subsequently, the performance of low-DO nitrification was investigated in a lab-scale sequential batch reactor (SBR) for 42 days using real tropical sewage. The batch kinetic experiments showed that the seed sludge has a relatively high oxygen affinity. Thus, the rate of nitrification was not significantly reduced at low DO concentrations (0.5 mg/L). During the operation of the low-DO nitrification SBR, 90% of NH_4-N was removed. The active low-DO nitrification was mainly attributed to the limited biodegradable organics in the sewage. Fluorescence in-situ hybridisation and 16S rRNA amplicon sequencing revealed the nitrifiers were related to *Nitrospira* genus and *Nitrosomonadaceae* family. Phylogenetic analysis suggests 47% of the Operational Taxonomic Units (OTUs) in *Nitrospira* genus is closely related to a comammox bacteria. This study has demonstrated active low-DO nitrification in tropical setting, which is a more sustainable process that could significantly reduce the energy footprint of STPs.

Keywords

Activated sludge; ammonia-oxidizing bacteria; biological nitrogen removal; nitrification; *Nitrospira*; tropical climate

INTRODUCTION

Malaysia is a tropical country rich in water resources. However, many of these water resources are polluted with excessive levels of nitrogen. The major nitrogenous pollutant is ammoniacal nitrogen (NH_4-N), which is mainly discharged to the environment through inadequately treated

sewage and agricultural-based industries. Consequently, NH_4-N removal from sewage is important if the water quality is to be preserved. For example, high ammonia concentration may cause fish toxicity in aquatic environment. Therefore, the Environmental Quality (Sewage) Regulation 2009 (DOE 2009) was enacted to limit NH_4-N concentration discharged from sewage treatment plants (STPs) to 5 mg/L.

Ammonia removal from sewage is usually achieved by nitrification. Nitrification is typically considered a two-step process in which ammonia is oxidised into nitrite in the first step. Nitrite is then oxidised into nitrate in the second step. The theoretical oxygen demand for this process is 4.57 g O_2/g NH_4-N (Tchobanoglous *et al.* 2014). Thus, a high dissolved oxygen (DO) level (> 2 mg/L) is recommended to ensure complete nitrification. Maintaining a high DO level in the aerobic tank requires a large amount of energy.

There are several proposed processes with the potential to reduce the energy consumption of an STP including, Single reactor High activity Ammonia Removal Over Nitrite (SHARON), Anaerobic ammonium oxidation (Anammox) and low-DO nitrification. Low-DO nitrification has been widely studied in lab-scale and pilot-scale reactors (Hanaki *et al.* 1990; Bellucci *et al.* 2011; Arnaldos *et al.* 2013). Bellucci *et al.* (2011) and Arnaldos *et al.* (2013) reported complete NH_4-N oxidation at low DO concentrations but produced contradictory results. Bellucci *et al.* (2011) did not find any difference in the NH_4-N oxidation rate between their low DO (0.5 mg/L) and high DO (3 mg/L) reactors. On the other hand, Arnaldos *et al.* (2013) reported a NH_4-N oxidation rate in low-DO reactor (0.1 mg/L) half of that in their saturated DO reactor. Hanaki *et al.* (1990) has also studied the interaction between DO, organic loading and nitrification activity. They found that the observed oxygen half-saturation constant for nitrifiers increased when carbon source was added. This suggests organic loading may inhibit nitrification at low DO concentrations.

The microbial community structure of the nitrifying sludge at low DO concentrations is believed to differ from conventional high DO nitrifying community. Active nitrification in low DO condition has been linked to an increase in oxygen affinity in both ammonia-oxidizing bacteria (AOB) and nitrite-oxidising bacteria (NOB) (Daebal *et al.* 2007; Arnaldos *et al.* 2013; Keene *et al.* 2017). Arnaldos *et al.* (2013) and Fitzgerald *et al.* (2015) found that *Nitrosomonas sp.* were the abundant AOB in low DO condition. Daebal *et al.* (2007) reported that the NOB could develop an affinity for oxygen that is equal or higher than AOB. Low DO condition have also been found to change the composition of NOB by enriching *Nitrospira*-like organisms. *Nitrospira* are considered to be K-strategists due to their competitive advantage for oxygen over *Nitrobacter*-related NOB, which is an r-strategist (Liu & Wang 2013).

The foregoing studies were all undertaken in temperate regions. The biological nitrogen removal process is not well-understood in the tropical region, where the sewage temperature is around 30°C (Ong *et al.* 2013). High temperature (30°C) is known to accelerate the rate of microbial metabolisms, consequently the rate of biological reactions is also increased. This study aims to both take advantage of high rate of nitrification in tropical climate and address the knowledge gap of nitrification in the tropics by investigating the efficiency of ammonia removal from real sewage at low DO concentrations. Batch kinetic experiments were first conducted to evaluate the effect of DO on specific ammonia uptake rate (SAUR). After the potential of low-DO nitrification was validated by batch experiments, the performance of NH_4-N removal efficiency in a low-DO nitrification sequential batch reactor (SBR) was assessed. In addition, 16S rRNA amplicon sequencing and fluorescence in-situ hybridisation (FISH) were used to investigate the microbial community structure of the low-DO nitrifying sludge.

METHODS

Sampling of seed sludge and sewage

Grab samples of return activated sludge (RAS) and sewage after preliminary treatment were acquired from a municipal STP in Kuala Lumpur, Malaysia, henceforth referred to as STP A. The plant is operating with extended aeration system combined with preanoxic tank, while the preliminary treatment in STP A includes bar screen and aerated grit chamber. The samples were stored at 4°C prior to use.

Batch kinetic experiments of DO effect

The procedure of kinetic experiments is adapted from van Loosdrecht *et al.* (2016). A 1-L jar test was used for the batch kinetic experiments. The reaction mixture for the experiment was made up of concentrated sludge from STP A, tap water, NH_4Cl solution and Na_2CO_3 solution. The initial concentrations of NH_4-N and alkalinity in the reaction mixture were 20 mg/L and 200 mg/L as $CaCO_3$ respectively. The pH of the mixture was adjusted to between 7.5 and 8.0 at the beginning of kinetic experiment. The kinetic experiment was performed at DO set points of 0 mg/L, 0.25 mg/L, 1.25 mg/L, 2.25 mg/L, 3.25 mg/L, 5.25 mg/L and 6.5 mg/L. Compressed air was sparged into the reaction mixture and a solenoid valve that connects to DO sensor was used to control the DO level in the reaction mixture. Mixed liquor samples were taken periodically for anion and cation analyses.

SBR operation for low-DO nitrification

The working volume of the SBR was 2 L. Seed sludge obtained from STP A was inoculated into the SBR to achieve initial total suspended solid (TSS) concentration of around 2500 mg/L. The initial TSS concentration of 2500 mg/L was selected based on typical MLSS concentration range suggested by Tchobanoglous *et al.* (2014). Sewage obtained from STP A was fed into

the SBR as influent. The SBR was operated in 6-hour cycle, including 5 minutes filling phase; 300 minutes reaction phase; 50 minutes settling phase; 4 minutes decanting phase and 1 minute idling phase. Overhead stirring mechanism was used for both mixing and aeration to maintain low DO condition in the reactor. The impeller designed rotational speed was 300 rpm to ensure that the DO concentration during the 300-minute reaction phase was lower than 0.5 mg/L. The SBR was operated with a hydraulic retention time (HRT) of 15 hours and SRT of 20 days, which corresponds to effluent withdrawal rate of 0.8 L/cycle and sludge wastage rate of 50 mL/cycle. However, HRT was shortened to 10 hours (effluent withdrawal rate = 1.2 L/cycle) after 21 days of reactor operation due to excessive accumulation of NO_3-N in the reactor. The DO concentration, temperature and pH were monitored online using InPro6850i DO probe coupled with M300 Process 1-channel 1/2 DIN DO monitor (Mettler-Toledo, U.S.) and Ceragel CPS71D digital pH sensor (Endress + Hauser, Germany). Mixed liquor samples were taken from the reactor at regular interval for chemical analyses listed in the next subsection.

Chemical analyses

The TSS and volatile suspended solid (VSS) concentrations of the samples collected from SBR were analysed in accordance to the standard method (APHA 1998). Mixed liquor samples from SBR and batch kinetic experiments were filtered through 0.2-μm membrane filter immediately. The filtered samples were analysed for nitrite ion (NO_2^-), nitrate ion (NO_3^-) and ammonium ion (NH_4^+) concentrations using 861 Advanced Compact Ion Chromatography (Metrohm, Switzerland). The total nitrogen (TN) concentration of the samples was analysed using TOC-V CSN total organic carbon analyser coupled with TNM-1 nitrogen measuring unit (Shimadzu, Japan). The samples were filtered through 0.45-μm membrane filter prior to TN analysis. In addition, chemical oxygen demand (COD) was analysed using high range COD test kit with DRB 200 COD digester (Hach, U.S.).

DNA extraction and library preparation for 16S rRNA amplicon sequencing

Sludge samples were collected weekly for DNA extraction using NucleoSpin Soil DNA Extraction Kit (Macherey-Nagel, Germany). All the DNA samples (day 0, 7, 14, 21, 28, 35 and 42) was then amplified using FastStart High Fidelity PCR System (Roche Diagnostics Ltd., UK). The V4 and V5 regions of the 16S rRNA genes were amplified using a pair of barcoded universal primers F515/R926 targeting both bacteria and archaea (F515: **5'**- GTG CCA GCM GCC GCG GTA A **-3'**; R926: **5'**- CCG TCA ATT CCT TTR AGT TT **-3'**). Each DNA template was amplified in a 50 μL PCR reaction mixture, which consists of 1.0 μL of each primer set (10 μM), 6.5 μL of High Fidelity PCR Master (Sigma-Aldrich, UK), 40.5 μL molecular grade water (Sigma-Aldrich, UK) and 1.0 μL of DNA extract. The PCR amplification was performed in a thermocycler Techne TC-5000 (Bibby Scientific, UK) with initial denaturation at 95°C for 2 min. Subsequently, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and elongation at 72°C for 45 seconds were carried out, followed by final elongation at 72°C for 7 min.

PCR amplified product was purified using AGENCOURT AMPure XP beads (Beckman Coulter, Ireland). The purified PCR samples were then quantified using Qubit™ dsDNA HS Assay Kits with the use of Qubit® 2.0 Fluorometer (Invitrogen, U.S.). Prior to amplicon sequencing, all the purified and quantified PCR samples were diluted to 500 pM each and pooled together. Size selection on the pooled samples was performed using Pippin Prep System (Sage Science, U.S.) as recommended for Ion Torrent workflow. Clonal amplification of the DNA fragments onto Ion Sphere™ Particles (ISP) and ISPs enrichment were performed using Ion OneTouch 2 (Thermo Fisher Scientific, U.S.) prior to amplicon sequencing on Ion Torrent Personal Genome Machine (PGM™) (Thermo Fisher Scientific, U.S.).

Post-sequencing bioinformatics analyses

The **.fasta* and **.qual* files were processed using Quantitative Insights Into Microbial Ecology (QIIME) v1.8.0 open-source software package with the workflow described by Caporaso *et al.* (2010). The barcoded sequences were first demultiplexed and trimmed with the minimum average quality score of 20 and minimum sequence length of 100. Open reference operational taxonomic unit (OTU) picking of the sequence data was then performed by clusteringsequences into OTUs based on 97% similarity using uclust method. Sequence similarity of 97% is widely accepted to represent taxonomic relatedness down to species level (Schloss & Handelsman 2005). Subsequently, the picked OTUs were aligned against the latest GreenGenes database using PyNAST algorithm. The aligned sequences generated was filtered to eliminate recurring alignment gap at the same position. Chimeric sequences in the aligned sequences were also being identified using ChimeraSlayer method and filtered. The filtered and aligned sequences was then used for core diversity analyses in QIIME to generate taxa plots. In addition, reference sequences were downloaded from GenBank database and aligned with the 16S rRNA fragment sequences. Phylogenetic analysis of the 16S rRNA fragment sequences was performed by aligning the 16S rRNA gene sequences using MUSCLE algorithm and constructing phylogenetic trees with neighbour-joining method using MEGA7 (Kumar *et al.* 2016).

The nucleotide sequences have been deposited into GenBank under accession numbers SRX3284361:SRX3284366.

Fluorescence in-situ hybridisation (FISH)

Cell fixation was carried out using 4% paraformaldehyde solution as described by Amann *et al.* (1990). Three sludge samples were collected on day 7, day 24 and day 42 of the SBR operation, respectively. The fixed cells were all hybridised with EUB338 MIX, which consists

of equi-molar concentrations of EUB 338, EUB 338 II and EUB338 III (Amann *et al.* 1990; Daims *et al.* 1999) to target all the bacteria.

Other FISH probes targeting both AOB and NOB are listed in Table 1. The probe EUB338 MIX was attached with 6-FAM fluorophore, while all other probes have Cy-3 fluorophore attached to the probes. The hybridised slides were viewed under DM2500 fluorescence microscope (Leica Microsystems, Germany), images were captured using DFC310 FX cooled charged-coupled device (CCD) digital colour camera (Leica Microsystems, Germany).

Table 1. FISH probes applied in molecular analyses of fixed sludge samples targeting nitrifying bacteria

Probe Name	Sequence (5' – 3')	% FA Conc.	Specificity	Reference
Nso1225	CGC CAT TGT ATT ACG TGT GA	35	Betaproteobacterial AOB	Mobarry *et al.* (1996)
Ntspa662	GGA ATT CCG CGC TCC TCT	35	Genus *Nitrospira (NOB)*	Daims *et al.* (2001a)
NIT3	CCT GTG CTC CAG GCT CCG	40	Genus *Nitrobacter (NOB)*	Wagner *et al.* (1996)

SAUR and kinetic modelling of DO effect

The value of ammonia uptake rate (AUR) was calculated from the slope of the time profile of NH_4-N or NO_x-N (NO_2-N + NO_3-N) obtained by kinetic experiments. AUR was divided by the VSS concentration to determine the value of SAUR for each kinetic experiment.

Saturation kinetic model was used to simulate the effect of DO on SAUR (Tchobanoglous *et al.* 2014), as shown in eq. (1),

$$SAUR = \frac{SAUR_{max} \cdot DO}{K_O + DO} \quad (1)$$

where $SAUR_{max}$ is the maximum SAUR and K_O is the half-saturation constant of oxygen. $SAUR_{max}$ and K_O were determined by performing nonlinear regression in MATLAB (v7.3, The Math Works Inc., Natick, MA, USA) by minimising squared error of regression line.

RESULTS AND DISCUSSION

Effect of DO on seed sludge nitrification performance

The relationship between SAUR and DO is described by saturation kinetics (Figure 1). From the nonlinear regression, the value of K_O of the sludge is 0.22 mg/L. The values of K_O for nitrifiers reported in the literatures range from 0.10 to 1.0 mg/L (Manser *et al.* 2005; Keene *et al.* 2017). Hence, the seed sludge in this work may have a relatively high affinity for oxygen. Low DO condition does not significantly reduce the SAUR, the rate being 70% of the maximum at 0.5 mg O_2/L.

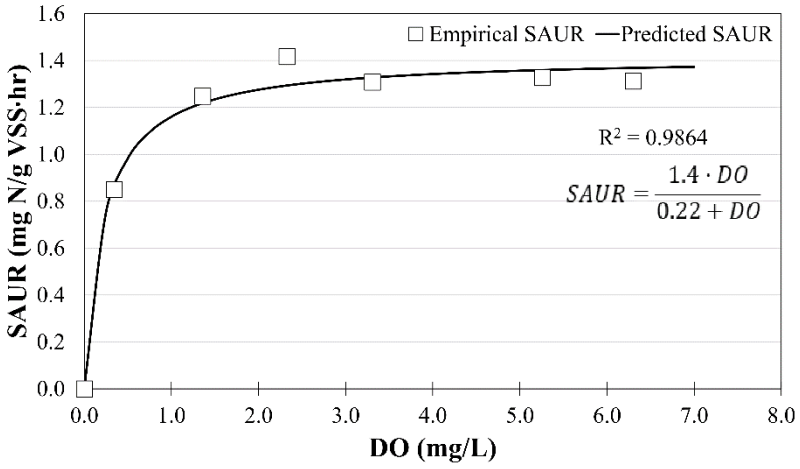


Figure 1. The effect of DO on SAUR of the seed sludge from STP A. The square markers denote the SAUR data determined from batch kinetic experiments while the solid line represents the saturation kinetic model fitted to the data.

The maximum SAUR is estimated to be 1.4 mg N/g VSS·hr (Figure 1); in the lower range of literatures' values, which range between 1 and 3 mg N/g VSS·hr (Arnaldos *et al.* 2013; Yang *et al.* 2016).

The curve fitting using saturation kinetics suggests that nitrifiers in the seed sludge have high oxygen affinity, which matches with the survival strategies of K-strategists, such as *Nitrosospira sp.* and *Nitrospira sp.* (Tchobanoglous *et al.* 2014). *Nitrospira sp.* was reported to be abundant in full-scale STPs and in low-DO reactors (Liu & Wang 2013; Yang *et al.* 2016). In the real plant, pockets of low-DO zones are found in the macro-environment of the aerobic tank (Daigger & Littleton 2014). The non-uniform DO distribution in the macro-environment may be favourable for the growth of K-strategist nitrifiers.

The batch experiment in this section indicates that operating a nitrification reactor at low DO condition is, in principle, feasible. However, the conditions were artificial (a synthetic medium with only ammonia and alkalinity as feed). Therefore we further examine the effect of low DO in an SBR fed with real wastewater.

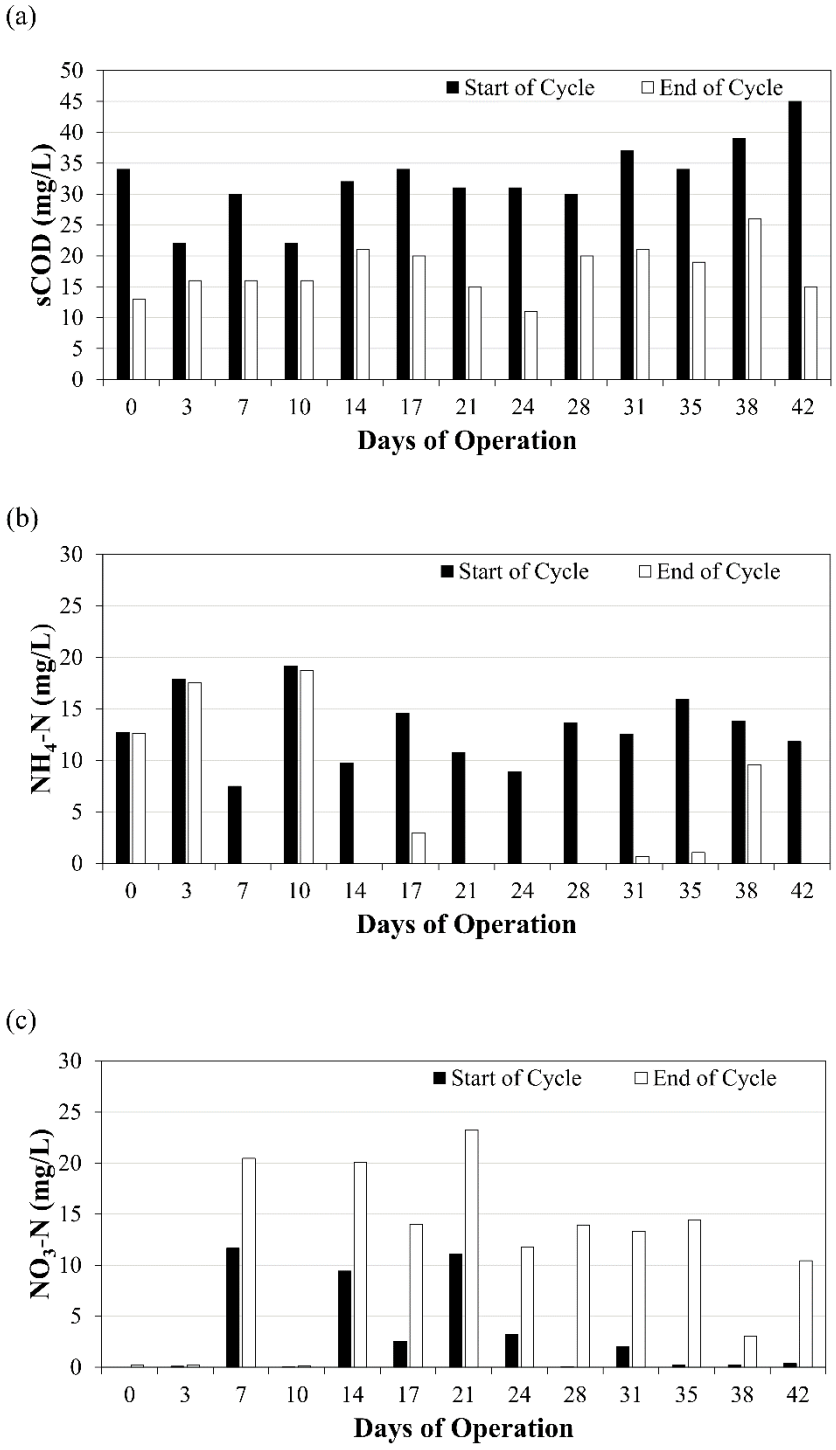
Performance of low-DO nitrification SBR

Sewage characteristics

The COD and TN concentrations of the sewage were characteristics of a low-strength wastewater (Tchobanoglous *et al.* 2014). The total COD (tCOD), soluble COD (sCOD), TN and NH_4-N were 280 ± 24 mg/L, 71 ± 9 mg/L, 23 ± 3 mg/L and 17 ± 3 mg/L respectively. The sewage NO_2-N and NO_3-N were < 0.5 mg/L at all times. The tCOD-to-nitrogen (COD/N) ratio of the sewage was approximately 12, which is lower than the typical range of 25 to 35 (Tchobanoglous *et al.* 2014).

238 *COD and NH_4-N removal efficiency*

239 The SBR was operated for 42 days at DO concentration below 0.5 mg/L. The initial TSS and
240 VSS concentrations were 2400 mg/L and 1800 mg/L respectively. Both TSS and VSS
241 concentrations reduced in the first 14 days of SBR operation, after which both concentrations
242 were maintained at 1500 ± 200 mg/L and 1300 ± 160 mg/L respectively. The sCOD removal
243 and nitrification were both stable after 14 days of SBR operation (Figure 2). The reactor
244 typically removed sCOD and NH_4-N adequately. The effluent sCOD was 18 ± 5 mg/L (Figure
245 2(a)) and the residual organic matter might represent small fraction of non-biodegradable COD
246 (Figure S1(d)). Effluent NH_4-N was often less than 2 mg/L (Figure 2(b)). The one peak of
247 effluent ammonia on day 38 was associated with the failure of the impeller.



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249 **Figure 2.** Profiles of (a) sCOD, (b) NH_4-N, (c) NO_3-N throughout low-DO nitrification SBR

250 operation.

251 The adaptation period in this study (14 days) is much shorter than 25 and 140 days reported by

252 Fitzgerald *et al.* (2015) and Arnaldos *et al.* (2013) respectively. The rapid appearance of active

nitrification could be caused by either the more rapid growth of nitrifiers at warmer temperature or the high proportion of suitably adapted organisms in the seed sludge. Using a simple model of AOB adaptation developed by Ofiţeru and Curtis (2009), the adaptation of AOB to low DO condition was simulated. The simulation suggests that the adaptation is more sensitive to the initial AOB concentrations than the nitrifiers' maximum growth rate. Presumably the seed sludge used in low-DO nitrification studies by Arnaldos *et al.* (2013) and Fitzgerald *et al.* (2015) were from conventional aerobic sludges and so a longer acclimation period was required for the adaptation of aerobic sludge in low DO condition.

Low COD concentration in the sewage may have contributed to the active nitrification in low-DO condition by reducing the oxygen competition between heterotrophs and nitrifiers. Satoh *et al.* (2000) reported a decreased in AOB population relative to heterotrophs when biodegradable organics was introduced, suggesting that heterotrophs may outcompete AOB for oxygen. Hanaki *et al.* (1990) also suggested that addition of COD would hamper nitrification performance by encouraging heterotrophs' growth.

Despite the low DO concentrations, all the ammonia was converted into nitrate (Figure 2(c)). Accumulation of nitrite was not detected in a typical SBR cycle (Figure S1(c)). Low DO level is conventionally associated with the accumulation of nitrite in the nitrification process. However, more recent studies showed that low-DO nitrification could exert selection pressure on certain lineages of NOB that possess high oxygen affinity, thus making NOB a better competitor of oxygen than AOB (Daebal *et al.* 2007; Liu & Wang 2013). Consequently, nitrite consumption by NOB is always higher than its production by AOB. Furthermore, high free ammonia (FA) concentration is a significant factor of nitrite accumulation. FA concentration approximately 9 mg N/L is sufficient to inhibit oxidation of nitrite to nitrate (Tchobanoglous *et al.* 2014). FA concentration in the sewage of this study was less than 0.5 mg/L based on acid

dissociation of ammonia. Hence, both increased oxygen affinity of NOB and negligible FA concentration might have contributed to complete oxidation of ammonia to nitrate.

Interestingly, Figure 2(c) shows that the NO_3-N at the beginning of the cycle was negligible between day 28 and day 42, which suggests a loss of NO_3-N in the reactor. The theoretical NO_3-N at the start of an SBR cycle should be 5 mg/L if denitrification did not occur. Denitrification obviously did not occur during the reaction phase because the increase in NO_3-N is equivalent to the NH_4-N reduction (Figure 2(b) and Figure 2(c)). Thus, denitrification might have occurred during the settling phase. Significant denitrification in clarification step has been reported by previous studies (Siegrist *et al.* 1995; Mikola *et al.* 2014). The condition in the settling phase was favourable for denitrification because of the anoxic environment, the decay of biomass was found to provide the COD required for denitrification (Siegrist *et al.* 1995). The detailed profiling of NO_3-N in the settling phase would be useful to determine the efficiency of denitrification in future works.

Rate of nitrification

The specific rate of accumulation of NH_4-N and NO_3-N were 1.8 ± 0.4 mg N/g VSS·hr though low DO concentration was maintained (Figure 3). The rate of NH_4-N uptake at steady-state low-DO nitrification reactor operation (> 14 days; Figure 3) appeared to be 1.3 times higher than that of the seed sludge (Figure 1). A modest increase in rate of nitrification is expected as the sludge adapts to low DO condition. When compared with literature, Yang *et al.* (2016) reported 3 mg N/g VSS·hr of NH_4-N uptake at DO concentration between 1 and 2 mg/L. Low-DO nitrification study conducted by Arnaldos *et al.* (2013) also reported nitrification rate close to 4 mg N/g TSS·hr at DO concentration of 0.1 mg/L. Thus, the rate of nitrification in this work is still relatively low. One of the reasons could be the high nitrifier abundance in other studies (Arnaldos *et al.* 2013; Yang *et al.* 2016). For example, Arnaldos *et al.* (2013) found that

nitrifiers constituted nearly half of the total bacteria in their reactor. Also, Yang *et al.* (2016) reported nitrifiers' abundance in the order of 10^{10} copies/g VSS, which is significantly higher than the typical abundance (10^6 to 10^8 copies/mL) published elsewhere (Bellucci *et al.* 2011; Fitzgerald *et al.* 2015).

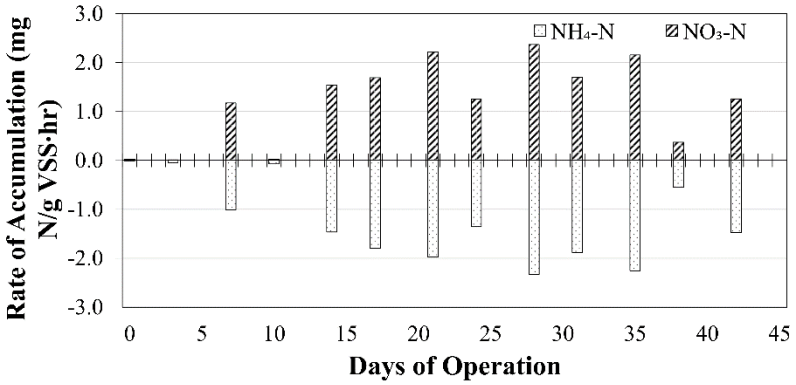

Figure 3. Profiles of rates of accumulation for NH_4-N and NO_3-N throughout SBR operation.

Proportional abundance of nitrifiers and denitrifiers in low-DO nitrification SBR

16S rRNA amplicon sequencing data identified more than 746 known taxonomies but only 20 taxonomies were present in more than 1% throughout the SBR operation. The top three most abundant organisms in the sludge are *Saprospiraceae* family (13 ± 2%), *Sphingobacteriales* order (5 ± 1%) and *envOPS12* order (3 ± 0.5%). Figure 4 shows the four most abundant taxonomies that associate with nitrification and denitrification. The information was extracted from the taxa plots generated by core diversity analysis in QIIME.

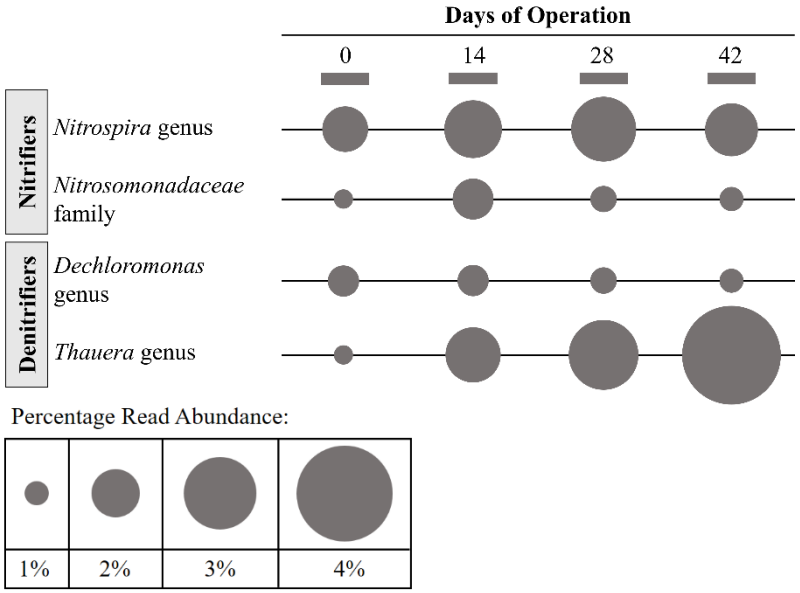

Figure 4. Population dynamics of significant OTUs present in the low-DO nitrification reactor.

The microbes related to the family *Nitrosomonadaceae* and genus *Nitrospira* were found to be the most abundant AOB and NOB respectively. *Nitrobacter*-related NOB were not detected by 16S rRNA amplicon sequencing. FISH analysis supports the presence of *Nitrosomonadaceae*-related AOB and *Nitrospira*-related NOB detected by 16S rRNA amplicon sequencing (Figure S2).

Genus *Nitrospira* and family *Nitrosomonadaceae* each represent 1% to 3% of total biomass. The family *Nitrosomonadaceae* is known to be able to oxidise ammonia autotrophically to nitrite. Members of the genus *Nitrospira* are known to be chemolithoautotrophic aerobic NOB. *Nitrospira*-related NOB has been described as K-strategists organisms that survive in low substrate concentrations. Strains related to *Nitrospira sp.* have been observed in other nitrification studies (Yang *et al.* 2016). For instance, Yang *et al.* (2016) has observed the coexistence of large population of *Nitrospira sp.* with AOB in reduced DO aerobic tanks of a water reclamation plant in Singapore.

To further examine the diversity and phylogeny of the unidentified *Nitrospira sp.*, a phylogenetic tree was constructed for all the 224 OTUs within the *Nitrospira* genus (Figure

5(a)). Most of the OTUs are clustered into two equally divided clades, termed Clade A and Clade B here. The average sequence similarity within each clade is 98 ± 2% based on p-distance matrix, which suggests OTUs in each clade could be related down to species level (sequence similarity > 97%). Thus, a representative sequence from each clade was generated based on the highest frequency of nucleotide base at each position. The representative sequences were aligned with other known *Nitrospira* 16S rRNA-coding sequences to construct a phylogenetic tree (Figure 5(b)). Figure 5(b) shows that Clade A (52% of total OTUs) of the *Nitrospira sp.* detected in this study is closely related to *Ca. Nitrospira defluvii*, while Clade B (47% of total OTUs) is closely related to one of the comammox strains, *Ca. Nitrospira nitrosa* (van Kessel *et al.* 2015).

Ca. Nitrospira defluvii is classified into sublineage I of *Nitrospira sp.* based on 16S rRNA phylogeny and is an important NOB in sewage treatment (Daims *et al.* 2001b; Lücker *et al.* 2010). Lücker *et al.* (2010) found that the key enzyme nitrite oxidoreductase (nxr) in *Ca. Nitrospira defluvii* differs significantly with other *Nitrospira sp.* but shares the closest homolog with an anammox bacteria (*Ca. Kuenenia stuttgartiensis*). *Ca. Nitrospira defluvii* also lacks protection mechanism against oxidative stress commonly present in *Nitrospira* (Lücker *et al.* 2010). Thus, *Ca. Nitrospira defluvii* may be an important NOB in low-DO nitrifying systems.

van Kessel *et al.* (2015) identified some strains of the genus *Nitrospira* as comammox bacteria, so called because of their ability to completely oxidise ammonia to nitrate. The BLAST alignment of the representative 16S rRNA gene sequence of Clade B in this work and *Ca. Nitrospira nitrosa* showed 99% sequence identity. Therefore, the presence of comammox-related *Nitrospira sp.* supports the earlier finding on complete oxidation of ammonia to nitrate in the low-DO nitrification reactor. The other two strains of comammox bacteria, *Ca. Nitrospira inopinata* and *Ca. Nitrospira nitrificans* are phylogenetically distant from the

354 members of the *Nitrospira sp.* detected in this study (Daims *et al.* 2015; van Kessel *et al.* 2015).

355 Thus, these two comammox strains are not included in Figure 5(b).

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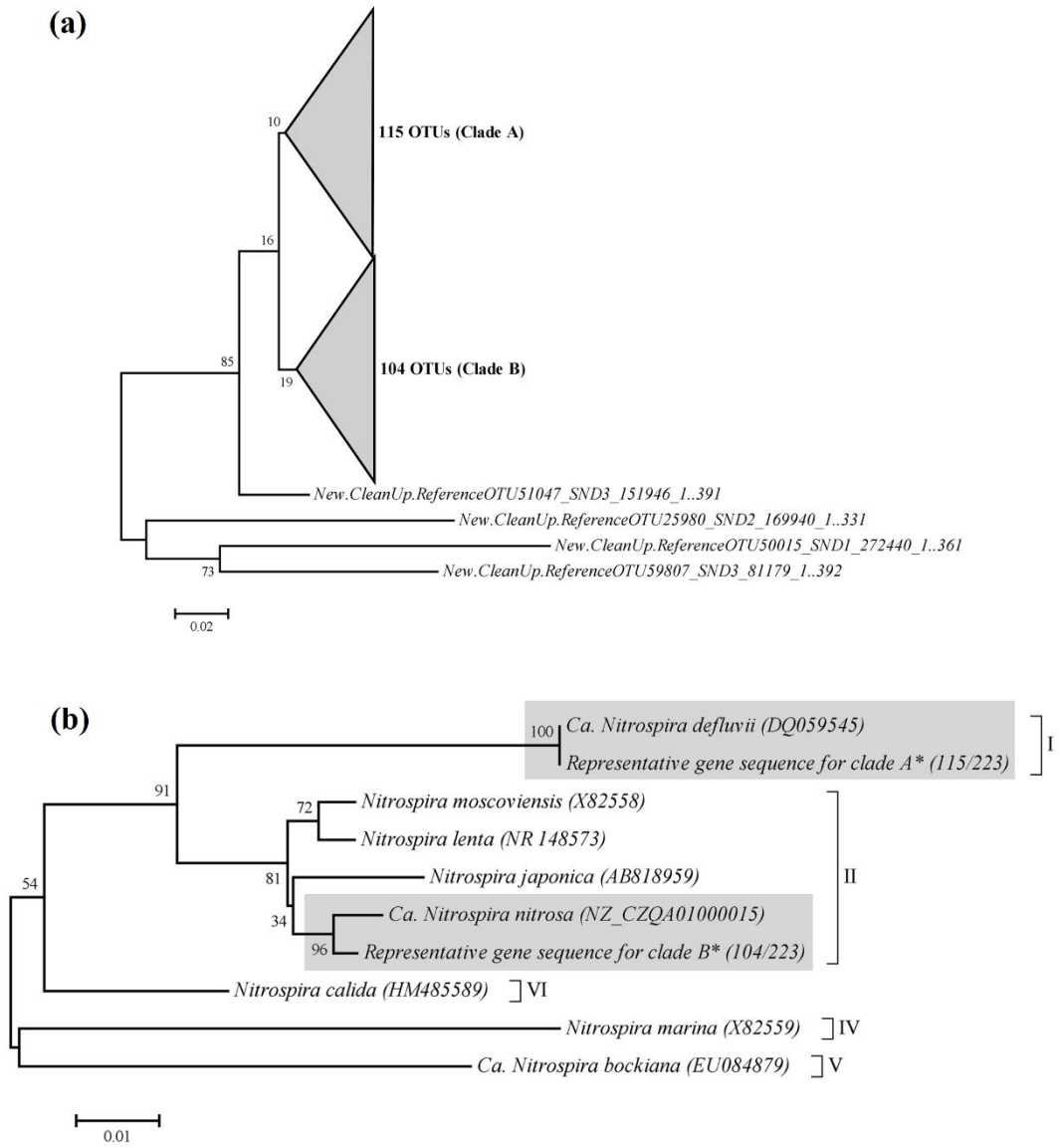

Figure 5. Neighbour-joining phylogenetic tree of the *Nitrospira sp.* 16S rRNA gene sequences showing the relationship (a) amongst all 223 OTUs belong to *Nitrospira* genus in this study, and (b) between the representative sequences of *Nitrospira sp.* (marked with *) obtained from low-DO nitrification reactor and reference sequences in the GenBank database. Numbers in parenthesis indicate the frequencies of OTUs exhibiting the same 16S rRNA gene sequences. Bootstrap values are shown in percentages of 1000 replicates. The defined sublineages of the genus *Nitrospira* (Daims *et al.* 2001b) were indicated by roman numerals adjacent to the square brackets. The shaded branches indicate the closest phylogenetic relationship between the present study sequence and the available reference sequence.

The role of comammox bacteria in wastewater treatment system is still uncertain (van Kessel *et al.* 2015; Chao *et al.* 2016; Gonzalez-Martinez *et al.* 2016). Phylogenetic analyses conducted by van Kessel *et al.* (2015) suggested that *Ca. Nitrospira nitrosa* was present in engineered systems, including wastewater treatment plant and drinking water distribution system. Conversely, both Chao *et al.* (2016) and Gonzalez-Martinez *et al.* (2016) inferred that comammox-like bacteria probably do not play an important role because of their low abundance (< 0.1%) in wastewater treatment plants. Chao *et al.* (2016) hypothesised that the operating conditions in wastewater treatment plants are not favourable for the growth of comammox bacteria as they are known to proliferate in low-substrate (< 10 NH_4^+ mg/L, < 22 mg NO_2^-/L) and hypoxic conditions (< 0.1 mg O_2/L) (van Kessel *et al.* 2015). In this study, the low-substrate and the low-DO environment in the nitrification reactor could promote the growth of comammox-related bacteria. Besides, the comammox-related *Nitrospira sp.* was also detected in the seed sludge of the low-DO nitrification reactor, suggesting their potential role in nitrification in the tropical STPs. Further research is required to clarify the role of comammox bacteria in the tropical wastewater treatment systems.

Denitrifying bacteria were present in the low-DO nitrifying sludge. Denitrifiers related to the genera *Dechloromonas* and *Thauera* were present with average abundances of 1.3% and 2.8% respectively. Interestingly, the genus *Thauera* was present in less than 1% in the inoculum but gradually enriched to beyond 4%. The presence of denitrifying community in this study may have contributed to the possible denitrification activity in the settling phase. The possible reason for the absence of denitrification activity during the 300-minute reaction phase could be that the denitrifiers preferentially use oxygen as an electron acceptor. Thus, further study is required to achieve nitrogen removal from tropical sewage via nitrification and denitrification in low-DO environment.

Energy savings of low-DO nitrification

The amount of energy reduction to operate a low-DO nitrification process (0.5 mg/L) relative to conventional nitrification process (2 mg/L) was estimated based on oxygen transfer rate to maintain biological activities and bulk DO level in the tropical sewage (30ºC) (Tchobanoglous *et al.* 2014). The calculation assumes surface aerator was used to provide aeration. The estimated energy reduction is 23% of the energy required to operate the process at 2 mg O_2/L. Keene *et al.* (2017) reported estimated energy reduction of the similar range (25%) when operating a biological nutrient removal process at 0.33 mg O_2/L relative to higher DO concentrations (0.9 – 4.3 mg/L). The reduction in aeration energy requirement offers a strategy to improve the energy efficiency of STPs.

CONCLUSIONS

Efficient removal of ammonia from tropical sewage was achieved in low-DO condition (< 0.5 mg O_2/L) through batch kinetic experiments and lab-scale SBR operation. Short adaptation period of the seed sludge to low-DO condition was observed, possibly due to the high proportional abundance of AOB in the seed sludge. Operating a low-DO nitrification process was estimated to reduce 23% of the aeration energy requirement when compared to conventional nitrification process (2 mg O_2/L).

AOB and NOB related to family *Nitrosomonadaceae* and genus *Nitrospira,* respectively were the dominant nitrifiers in the low-DO nitrifying system. The unidentified members of the genus *Nitrospira* detected in this study were closely related to *Ca. Nitrospira defluvii* and *Ca. Nitrospira nitrosa* (comammox organism). More investigations to quantify nitrifiers and comammox organisms in the sludge will be required. Denitrifiers were also detected in the low-DO nitrification reactor, suggesting the possibility to achieve nitrogen removal from tropical sewage in future study.

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