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ORIGINAL ARTICLE

Biological nutrient removal with limited organic matter using a novel anaerobic–anoxic/oxic multi-phased activated sludge process

Rusul Naseer [a,b], **Saad Abualhail** [a,b], **Lu Xiwu** [a,*]

[a] *School of Energy and Environment, Department of Environmental Science and Engineering, Southeast University, Nanjing 210096, China*
[b] *Faculty of Engineering ,University of Basrah ,Basra ,Iraq*

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Nitrite accumulation rate (NAR);
SND;
PCR assay;
Microorganisms

Abstract An anaerobic–anoxic/oxic (A2/O) multi-phased biological process called "phased isolation tank step feed technology (PITSF)" was developed to force the oscillation of organic and nutrient concentrations in process reactors. PITSF can be operated safely with a limited carbon source in terms of low carbon requirements and aeration costs whereas NAR was achieved over 95% in the last aerobic zone through a combination of short HRT and low DO levels. PCR assay was used for X_{AB} quantification to correlate X_{AB} numbers with nutrient removal. PCR assays showed, high NAR was achieved at X_{AB} population 5.2×10^8 cells/g MLVSS in response to complete and partial nitrification process. It was exhibited that low DO with short HRT promoted X_{AB} growth.

Abbreviations: PITSF, phased isolation tank step feed; A2/O, anaerobic–anoxic/oxic; SBR, sequence batch reactor; NAR, nitrite accumulation rate; SND, simultaneous nitrification and denitrification; MLVSS, mixed liquor volatile suspended solid; X_{AB}, ammonia-oxidizing bacteria; X_{NOB}, nitrite oxidize bacteria; X_{PAOs}, phosphate accumulating organisms; X_{DPAOs}, denitrifying phosphorus organisms; X_H, heterotrophic organisms; PCR, poly chain reaction; PLC, programmable logic control; FISH, fluorescence in situ hybridization; SRT, sludge retention time; HRT, hydraulic retention time; COD, chemical oxygen demand; TP, total phosphorus; VFA, volatile fatty acids; TN, total nitrogen; $NH_4^+ - N$, ammonia nitrogen; $NO_3^- - N$, nitrate nitrogen; $NO_2^- - N$, nitrite nitrogen; PHA, poly-hydroxyl-alkonates; DO, dissolved oxygen; OUR, oxygen uptake rate.

* Corresponding author. Tel.: +86 13260780581; fax: +86 2583792614.
E-mail address: rusalsaad@yahoo.com (L. Xiwu).

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Simultaneous nitrification and denitrification (SND) via nitrate were observed obviously, SND rate was between 69–72%, at a low DO level of 0.5 mg/l in the first aerobic tank during main phases and the removal efficiency of TN, $NH_4^+ - N$, COD, TP was 84.7 .97, 88.3 and 96% respectively. The removal efficiencies of TN, $NH_4^+ - N$, and TP at low C/N ratio and DO level were 84.2, 98.5 and 96.9% respectively which were approximately equal to the complete nitrification–denitrification with the addition of external carbon sources at a normal DO level of (1.5–2.5 mg/l).

1. Introduction

Ammonia-oxidizing bacteria (X_{AB}) first oxidize ammonia to nitrite and subsequently nitrite-oxidizing bacteria (X_{NOB}) oxidize nitrite to nitrate. Because of the slow growth rate of X_{AB}, their high sensitivity to many environmental factors, and their inability to outcompete heterotrophic organisms (X_H) is a rate-limiting step of nitrogen removal in biological treatment systems (Wagner et al., 1995). For this reason, a better understanding of the ecology and microbiology of X_{AB} in wastewater treatment systems is necessary to enhance treatment performance in a proposed process of PITSF technology. Nitrite–N is an intermediate in both nitrification and denitrification where nutrient removal via nitrite pathway may yield up to a 25% reduction in aeration and 40% reduction in organic matter requirements (Blackburne et al., 2008). It is advantageous when the wastewater contains a relatively low level of carbon source. In addition, lower sludge production and higher denitrification rate were observed in nitrogen removal via nitrite nitrite (Van et al., 2001; Tokutomi, 2004).). Moreover, the control of oxidation of ammonia to nitrite instead of nitrate is the key to achieve the process of partial nitrification to nitrite. Therefore, nitrite oxidizing bacteria (X_{NOB}) have to be inhibited or eliminated while ammonia oxidizing bacteria (X_{AB}) have to become the dominant nitrifying bacteria (Hellinga et al., 1998; Yoo et al., 1999). The factors affecting nitrite build-up and the community structure are such as higher temperature, higher free ammonia (Keller and Vadivelu, 2007; Peng et al., 2008; Park and Bae, 2009; Grunditz et al., 1998) inhibitor, and low dissolved oxygen (DO) concentration (Ruiz et al., 2006; Keller et al., 2008). Control of hydraulic retention time (HRT) or aeration time has been investigated (Gao et al., 2009; Wu et al., 2007). Consequently, control of DO concentration, HRT and sludge retention time seems to be the available method to establish nitritation. X_{AB} have a stronger affinity to oxygen than X_{NOB} at low DO concentrations, which causes nitrite build-up as well as savings in aeration cost (Garrido et al., 1997; Ruiza et al., 2002). In this study, PITSF is designed as the same to A2/O in spatial structure and to SBR in control strategy and also has its own property. It has been verified, in full-scale studies, that this BNR process of using an SBR is cost-effective as compared with continuous flow processes (Guo et al., 2009). On the other hand, earlier studies recommended that control of aeration time in sequencing batch reactor process was an essential method to wash out X_{NOB}. Presently, most of the studies on NAR focus on the SBR process (Lemairea et al., 2006; Yang et al., 2007). Only a few studies were carried out on achieving nutrient removal via nitrite in a continuous flow for treatment of domestic wastewater with high ammonia concentrations (Lemairea et al., 2006; Peng et al., 2008; Ruiz et al., 2003) and anoxic–aerobic (A/O) processes have been carried out. Therefore, in this study, the influence of operation time, SRT, DO, HRT on X_{NOB} growth and nitrite accumulation rates were investigated in a continuous flow of PITSF process to build a high rate of nitrite accumulation where a short HRT was applied to avoid excessive aeration and inhibit X_{NOB} growth, both low DO levels and nitritation promote the occurrence of SND via nitrite, which would further reduce carbon requirements. Simultaneous nitrification and denitrification (SND) implies that nitrification and denitrification take place concurrently in the same reaction zone under the same overall operating conditions. SND is of particular interest in saving anoxic volume and in treatment of wastewaters with low C/N ratio (Guo et al., 2009). SND is accompanied by the inhibition of the second step of nitrification where the saving of organic energy is reached up to 40% (Abeling and Seyfried, 1993). In this study, SND process and partial nitrification via nitrite were investigated at ambient temperature in a new process of PITSF technology where removal of nutrient via nitrite will be achieved by reducing the activity of nitrobacteria and correspondingly giving the nitrosomonas species growth advantages. Recently, developed molecular tools include sequence analysis of the 16S rRNA and amoA genes to reveal XAB populations and communities in various environments. An application of specific PCR amplification provides clarification of the XAB community in detail (George and Bodelier, 1998; Fjellbirkeland et al., 2001) whereas PCR-based quantification techniques allow accurate calculation of XAB populations in the environments (Okano and Hristova, 2003; Harms et al., 2003). A fluorescent dye for the quantification of double stranded DNA (Vitzthuma et al., 1999) or fluorescence labeled probe (Okano et al., 2004) is used for quantitative PCR real-time throughout measuring fluorescence that emanate continuously during the amplification reaction. An external standard curve is an important requirement for quantification, which shows the relationship between DNA threshold cycle values and copy numbers. The standard curve was prepared in the previous studies using genomic DNA extracted from pure culture of target bacteria (Okano et al., 2004), the plasmid carrying target gene or commission DNA fragment (Harms et al., 2003). This study aims to (1) investigate a new technology of biological treatment, PITSF was proposed to enhance nutrient removal at low C/N ratio. (2) investigate the influence of operation parameters on nitrite accumulation rate (NAR) in PITSF system and find out the crucial factors to achieve NAR of domestic wastewater at ambient temperatures through investigating changes of microbial activities. (3) determine the mechanism of SND phenomena particularly with regard to the effect of DO concentration in a PITSF process (4) find the total number of bacteria by polymerase chain reaction (PCR) quantification technique and develop real-time PCR assays for X_{AB} quantification to correlate X_{AB} numbers with nutrient removal via nitrite (5) analyze the kinetics of different

microorganisms in each zone of PITSF reactor which include heterotrophic organism; phosphate accumulating organism, nitrite oxidize bacteria, and autotrophic microorganism using oxygen uptake rate OUR.

2. Materials and methods

2.1. Reactor system

PITSF technology is composed of six tanks, separated by baffles with a working 0.5 ton/day. The first five tank sequence in terms of any tank was typically operated under different environment state conditions (anaerobic–anoxic–aerobic) based on phase type while the last tank operated as settler. The effective water depth in a system is 700 mm and plane dimensions are $860 \times 535 \times 905\ mm^3$.The main parts of a pilot plant utilized in this study include the main body which is a rectangular box, an air compressor for aeration, pre-static pumps to adjust the water flow rate, air flow meter to achieve the desired DO concentration according to the experimental condition requirement, mechanical agitation mixers providing mixing in the anaerobic and anoxic zones to keep the biomass in suspension, PLC programmable logic control, display screen, inlet wastewater electromagnetic valves, outlet water electromagnetic valves, aeration, sludge return electromagnetic valves, sludge discharge electromagnetic valves, and plastic pipes and others. The principal diagram of pilot plant with all major components is shown in Fig. 1.

2.2. Experimental operating procedure

An operation cycle is composed of two half-cycles with same running schemes Fig. 2. It is divided into six phases named as phase I, II and III during the first half cycle and phase IV, V and VI during the second half cycle. Step feed influent was pumped into both anoxic and anaerobic zone depending on the biological reaction. The influent flow distribution ratio for the two reactors was 1:2. During the first half-cycle, step feed of a raw wastewater was pumped into both tank one and tank two during phase I and from tank one and tank three during phase II while it is pumped just from tank two during phase III. Then, it flowed continually into tank five. During the second half-cycle, step feed influent was pumped into both tank five and tank four during phase IV and from tank three and tank five during phase V while it is pumped just from tank four during VI. The recirculation of mixed liquor was completed automated through direction change at each half cycle without additional sludge and mixed liquor returns so that this process need not require equipment to return mixed liquor. This is the main difference between our technology and other common activated sludge process technologies. Consequently, this system is effective for reducing energy consumption. Time and environmental state condition were controlled during each phase to achieve the function of A2/O process in the PITSF system. The PITSF system was operated for 7 months including six successive runs. The experimental purpose of run mode No. 1 was to investigate if nutrient removal via nitrite could be achieved by control of DO level at 0.5 mg/l and HRT at 9.1 h. Run mode No. 2 was implemented to further improve TN and TP and NH_4–N removal at low DO and HRT was (11) h with added acetate sodium as carbon sources for denitrification. Run mode No.3 was implemented to investigate nutrient removal, SND, and NAR through a combination of normal DO levels (1.5–2) mg/l with short HRT of 9.1 h and without the addition of external carbon sources. Run mode No.4 was performed at the same condition as that of run mode No.1 with the addition of external carbon sources for denitrification process.

2.3. Wastewater composition and seeding sludge

Raw wastewater from a campus main manhole was pumped into a storing tank for sedimentation, and then fed into the reactor. The seed sludge was taken from the Wuxi treatment plant which utilizes a typical change in the environmental state condition (anaerobic, anoxic, or aerobic) to treat municipal wastewater and performs simultaneous nitrification and denitrification well. COD infiltrated was between 150 and 250 mg/l, of which readily biodegradable substrate (S_S), inert

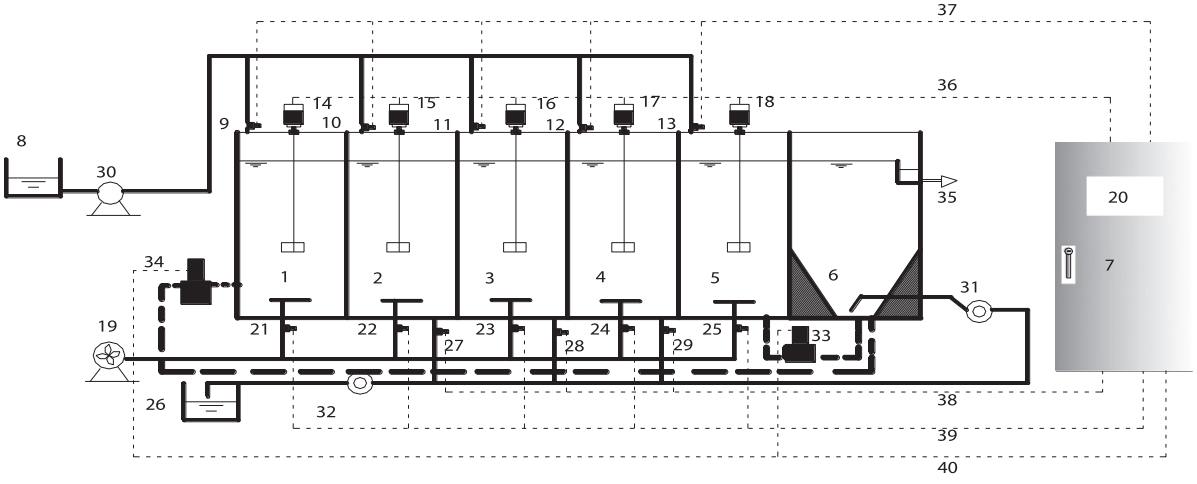


Figure 1 Configuration of PITSF system with all main parts. 1, 2, 3, 4, 5 – five tank, 6 – settling tank, 7- PLC programmable logic controller, 8 – air compressor, 9- inlet reservoir 10 – inlet pipe, 9,10, 11, 12 ,13 – inlet electromagnetic valve, 14,15,16,17,18 – mixer, 21, 22 ,23, 24 ,25 – aeration electromagnetic valve 26- excess sludge tank 27, 28, 29 – sludge return valve, 30- inlet pre-static pump ,31-sludge recycle control meter, 32- excess sludge control meter 33, 34 – sludge discharge valves ,35- effluent , 36- electrical mixer line, 37- electrical inlet valve line 38- electrical sludge return valve line, 39-electrical aeration valve line ,40- electrical sludge discharge valve line.

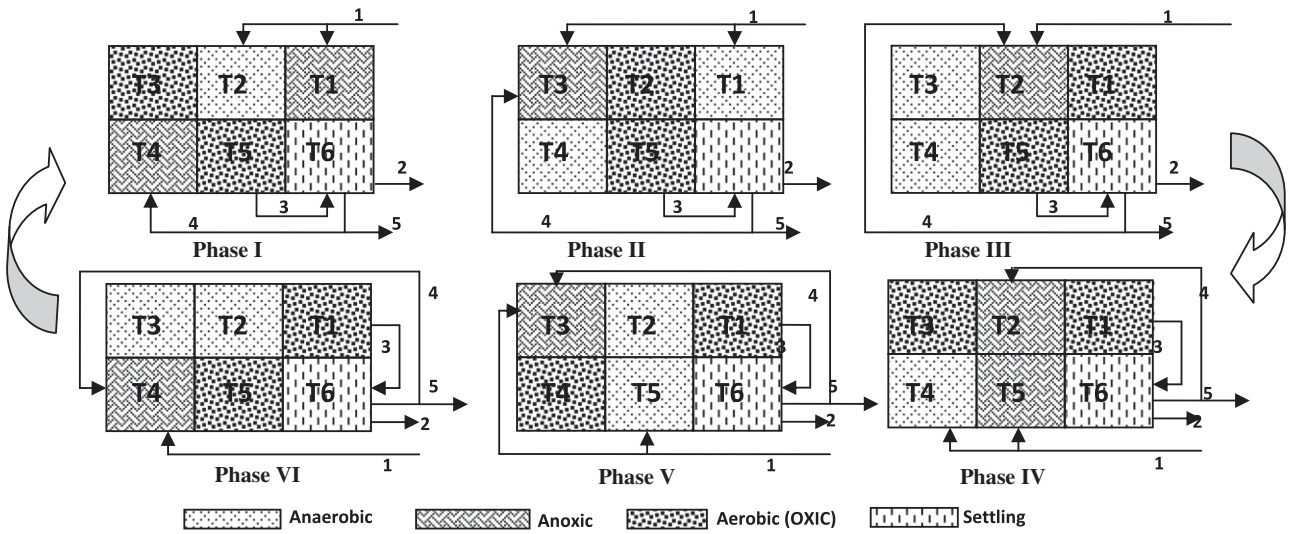


Figure 2 Run scheme of PITSF activated sludge system. T1, T2, T3, T4, T5, T6 –six tanks, 1-step feed of raw wastewater, 2-outlet water, 3-continuous flow of mixed liquor to tank six, 4-sludge recycle , 5-excess sludge.

Table 1 Characteristics of the raw wastewater.

Contents	Min/Max	Average
TN (mg/L)	49.5–64.62	55.4
NH–N (mg/L)	39.6–44.8	41.6
TP (mg/L)	3.73–4.92	3.88
COD (mg/L)	110.4–270	125.3
C/N	1.67–3.98	2.33
pH	6.97–7.23	7.18
VFA (SA) (mg/L)	67–89	71
Readily biodegradable substrate	261–195	233.5
SS (mg/L) biodegradable substrate		
Inert soluble organic material SI (mg/L)	24–26	25.2
Oxygen S_O	0	0

soluble organic material (S_I), slowly biodegradable particulate substrate (X_S) and inert particulate organic material (X_I) accounted for about 35, 2, 40 and 10%, respectively. Average influent COD to nitrogen ratio (C/N) was only about 2.33, and thus the organic carbon source was typically limiting. The raw wastewater composition is shown in Table 1.

2.4. Analytical methods

The Analytical methods for COD, $NH_4^+ - N$,$NO_2^- - N$,$NO_3^- - N$, and TN were analyzed according to standard methods (S.E.P.A. Chinese, 2002). $NO_2^- - N$ and $NO_3^- - N$ were analyzed by the IC method (Metrohm 761 compact IC equipped with metrosep asupp 5 column) while TN was analyzed by analytikjena AG multi N/C 3000. DO and pH were measured on-line using DO/pH meters. Volatile fatty acid (VFA) was analyzed using gas chromatograph (GC). MLVSS and MLSS were measured according to the standard methods (APHA, 1998).

2.5. Optimum operation parameters

The process exhibited good performance with different operation conditions including hydraulic retention time (HRT), sludge retention time (SRT), and aeration amount owing to the influence of these parameters on the removal efficiency. Total influent flow rate was 22 L/h, sludge retention time (SRT) was 13 days and the aeration rate is 0.15 m^3/h. The sludge return ratio was set at 30% of influent flow rate. The operation time for all six phases is 3, 2.5, 2, 3, 2.5, 2 h, respectively. The ambient temperature was (20–23) °C. Furthermore, (MLSS) concentration was between 2260–3000 mg/l. The total HRT for the three phases were calculated according to the following formulas:

$$\begin{aligned} HRT_{total} = {} & HRT_1 \times (\text{Phase I time/Half cycle time}) \\ & + HRT_2 \times (\text{Phase II time/Half cycle time}) \\ & + HRT_3 \times (\text{Phase III time/Half cycle time}) \end{aligned} \quad (1)$$

where, $HRT_1 = (V_T) + (V_T – V_1)/0.5$ Q; $HRT_2 = (V_T) + (V_T - V_1 - V_2)/0.5$ Q; $HRT_3 = (V_T) + (V_T – V_1)/0.5$ Q; VT is the total volume of pilot plant; and Q is the flow rate of influent.

3. Results

3.1. Real-time PCR assay for quantification of Autotrophic bacteria (X_{AB})

In this study, PCR quantification with oligonucleotides probes was used to reveal total bacterial numbers, total X_{AB} and total X_{NOB} numbers. The real-time polymerase chain reaction (PCR) was developed for X_{AB} quantification to correlate X_{AB} numbers with nutrient removal via nitrite.

3.1.1. Sludge sample community and XAB cultures

Sludge samples were collected from the last aerobic zone in phased isolation tank step feed reactor at 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, 360, 390 410, 420, and 450 min. MLVSS were determined in the sampling day. 2 ml of mixed liquid was washed twice, centrifuged it and then resuspended the pellet in 3×PBS, and then centrifuged at 14,000g for 4 min. The supernatant was eliminated and the

Table 2 A list of 16S rDNA targeted-oligonucleotide probes used in this study.

Probe	Sequence(5′–3′)	16s- rDNA target site [b]
TMP 5- FAM[a] and 3-TAMRA	CAACTAGCTAATCAGRCATCRGCCGCTC	226–253
RT1r	CGTCCTCTCAGACCARCTACTG	283–304
CTO 189fA/B	GGAGRAAAGCAGGGGATCG	189–207
CTO 189fC	GGAGGAAAGTAGGGGATCG	652–669

[a] FAM, 6-carboxyfluorescein; TAMRA, 6-carboxy-tetramethylrhodamine.
[b] 16S rDNA position according to *Escherichia coli* numbering.

pellet was stored at −25 °C. Real-time PCR quantification of total bacteria 16S rDNA genes was implemented using primers and probes CTO 189f and RT1r and the Taq Man probe TMP1. The oligonucleotide sequences of the primers and the Taq Man probe are shown in Table 2.

3.1.2. Ammonia-oxidizing bacteria cultures

Genomic DNA extracted from enriched autotrophic bacteria (X_{AB}) communities was used as standard DNA for polymerase chain reaction analysis. The X_{AB}-enriched culture has occurred by converting activated sludge with good nitrification into conical flask and cultivation in a batch mode at 29 °C and mixing at 140 rpm. The medium included, 1 g K2HPO4; 3 g NaHCO3; 2 g chloride sodium; 2.3 g (NH4)2SO4, 0.6 g FeSO4; and 0.6 g MgSO47H2O per liter. After 28 days, NAR is steady at 95% which represents the ratio of $NO_2^- - N$ concentration to the sum of $NO_3^- - N$ and $NO_2^- - N$ in the effluent and a stable $NH_4^- - N$ removal over 96% .

3.1.3. DNA extraction for 16S r DNA quantification of X_{AB} and standard curve preparation

DNA was extracted directly from 2 ml of MLSS samples using fast-DNA SPIN kits for soil (Bio 101, Vista, CA, USA). At the initial step, 1 ml of sodium phosphate buffer solution was mixed to the samples, and then the tube was kept for 20 s on frost. The product from DNA extraction was verified by electrophoresis in 1% agarose (TaKaRa LO3, Tokyo, Japan). The three extracts of DNA were mixed before the DNA was analyzed in order to minimize the variations in DNA extraction. The extracted DNA from enriched X_{AB} culture was 10-fold diluted in pasteurized water and PCR was conveyed in a 50 µl reaction mixture using a PCR kit (TaKaRa Ex Taq) which is included in 4 µl dNTP (2.5 mM), CTO 189f C° (10 µ mol/L), 5 µl × Ex Taq buffer (magnesium), 1 µl forward primer CTO 189fA/B and, 1 µl reverse primer RT1r (10 µmol/L) 0.25 µl TaKaRa EX Taq (5U/µl), 1 µl DNA template, and 37.75 µl ddH2O (31). The operation of PCR amplification was as follows: 180 s at 94 °C, 120 at 50 °C followed by 45 cycles consisting of 35 s at 95 °C, 60 s at 55 °C, and 40 s at 72 °C and a final cycle consisting of 240 s at 72 °C. The PCR products were envisaged after electrophoresis in 3% agarose. The DNA sequence 116-bp bands were excised which are included in agarose gel slices. Meanwhile, the DNA was amplified and then purified using Takara Agarose Gel DNA Purification Kit Ver.2.0. (TaKaRa). A second round of PCR reamplification was produced from the purified target of DNA and the resulting products were purified as before. A spectrophotometer was used to determine DNA concentration, and DNA copy numbers were eliminated. In this work, the standard DNA was predicted using ten-fold serial dilutions of DNA of known copy numbers. Every one of the dilutions was real-time PCR quantified in triplicate. The real-time PCR mixture was organized in a total volume of 25 µl using the TaKaRa Premix Ex Taq kit, containing 0.85 µl forward primer CTO 189fA/B and CTO 189f °C (10 l mol/L), 13.5 µl 10 Ex Taq Buffer (magnesium); 0.85 µl reverse primer RT1r (10 lmol/L); 1 µl TMP1; 1 µl standard DNA; 0.5 µl ROX Reference Dye II; and 8.5 µl ddH2O. PCR amplification was performed in an ABI Prism SDS 7000 instrument under conditions of 120 s at 50 °C and 30 at 95 °C followed by 40 cycles of 25 s at 95 °C and 60 s at 60 °C. DNA concentration assessed by PCR reamplification was 15.2 ng/µl y measured with a spectrophotometer. This value was changed to a DNA copy number of 1.78×10^{11} copies/µl. Serial 10× fold dilutions of DNA with identified copy numbers were used to generate a standard curve ($y = -3.99x + 65.36$), which reveals the relationship between DNA copy numbers and threshold cycle (C_t) values with high correlation. Fig. 3 concluded that the standard curve was reliable for observation of the X_{AB} by 16S rDNA. Table 3 shows the total bacterial number, total X_{AB} and total X_{NOB} numbers in PITSF system in response to optimum operating parameters which are calculated by PCR quantification. The variations of X_{AB} population with different operation conditions were specified using real-time (PCR).

3.2. PHA test

The variation of PHA in each zone of a new system (PITSF) was analyzed according to the method that is described in (Braunegg et al.,1978). In the initial step, Duplicate 20 ml samples of MLSS were obtained and immediately centrifuged at 4 °C. Then, the cold sludge pellet was lyophilized. After that, the pellet was added to the tube closed with a Teflon-lined screw cap for drying. Two ml of sulfuric acid 3% methanol and 2 ml of chloroform were added to the tube. This was digested for 1200 min in an oven at 104 °C. At the second step, once the sample had cooled at 25 °C, 1 ml of water was added and the tube contents were shaken for 600 s. The chloroform

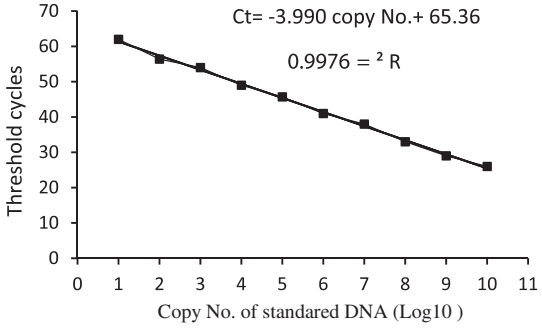


Figure 3 Standard curve for real-time PCR assays.

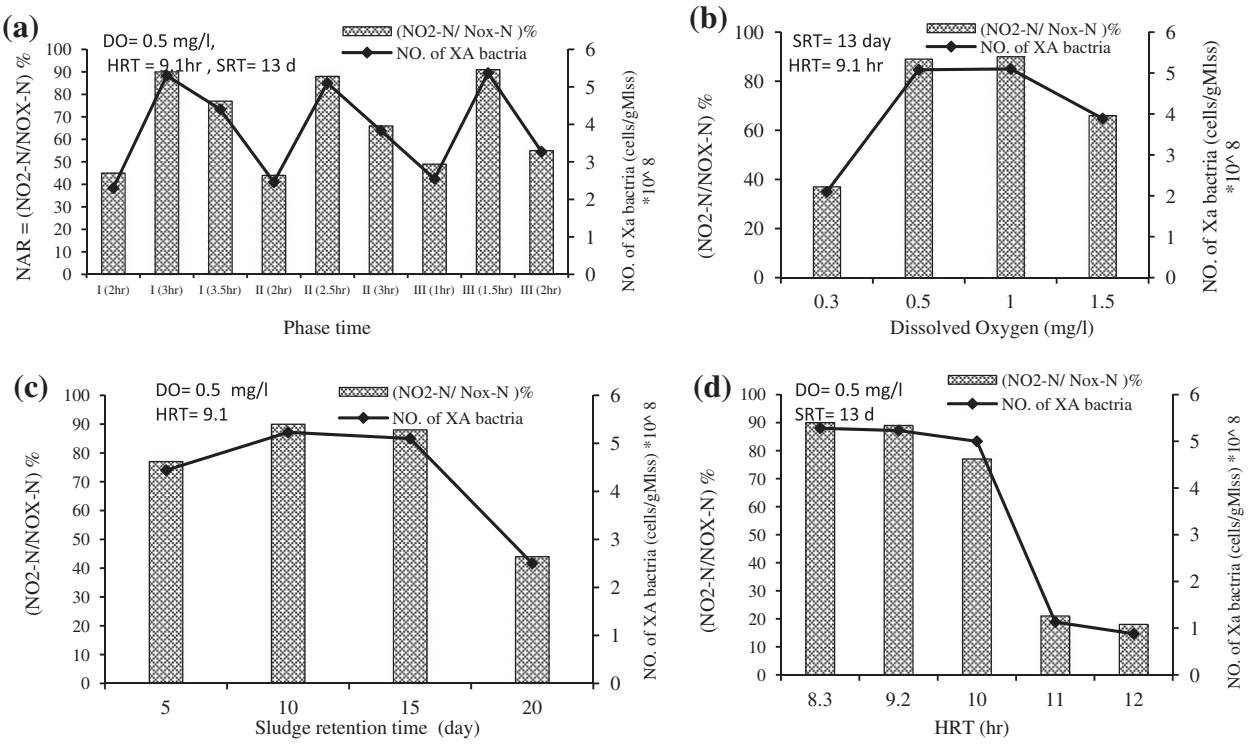


Figure 4 Investigation results of NAR and XAB quantification using real-time PCR with different operation parameters.

Table 3 Number of microorganisms in sludge.

Microorganism type	Sludge activity in the PITSF reactor	
	Value $\times 10^7$ Cell /mL	Average 10^7 Cell/mL
Total number of bacteria	681–977	786
Nitrite-oxidizing bacteria	6.21–8.30	7.70
Ammonia-oxidizing bacteria	73.7–92.2	83.1
PAOs	58.5–83.1	73.2

content remained to the bottom of the tube, and this was drawn off for GC examination. The digested product was exposed on a Varian 3400 GC fitted with a 1.8-m Alltech 0.2% Carbowax 1500 on Graphpac-GC 80/100 mesh stainless steel column. The column temperature was 170 °C and the inoculation temperature was 180 °C. PHA was measured by comparison to a standard consisting of a copolymer of the above-described alkanoates.

3.3. Microbial activity and OUR batch-tests

Oxygen uptake rate (OUR) was determined in this study to evaluate the microbial activity of different microbial functional groups including X_H, X_{PAOs} and X_{NOB}. Oxygen uptake rate (OUR) refers to the amount of oxygen used by a unit mass of active sludge in a unit of time. A certain quantity of MLSS sample was taken from the PITSF reactor and added into OUR chambers. In order to reduce DO concentration, 2% mol/L of $NaClO_3$ solution was added into the sample after approximately 3 min, and OUR was then determined. The difference in the value between the two OUR revealed the activity of nitrifying bacteria. After 3 min of additional DO reduction, 5 mg/L of allylthiourea (ATU) was injected into the composite sample and OUR was recorded again. In order to evaluate the active biomass of X_H; and X_{NOB}, different types of OUR values should be considered which include (OUR_T, OUR_{XH}, and OUR_{XNOB}) .The determination of OUR of X_H and X_{NOB} were based on the subsequent addition of allylthiourea (ATU) and NaClO3, selective inhibitors of X_{NOB} and X_H to the MLSS sample. When determining OUR_T, no inhibitor was added.

3.4. Simultaneous nitrification and denitrification (SND) in PITSF reactor

Simultaneous nitrification and denitrification (SND via nitrate) were observed obviously in the first aerobic tanks of PITSF reactor during the main phases, phase I and II. Experiments were carried out to investigate the SND phenomena with low C/N of 2.66 by measuring nitrogenous compounds in tank three during phase I and tank two during phase II at different DO levels, HRT of 10.2 h, and ambient temperature (20–23) °C. The analysis of tank three pollutant concentration with time during phase I showed that $NO_3^- - N$ decreased to 2.4 mg/l within 60 min due to low DO level at this period ($\leqslant$0.5 mg/l) which meets the requirement of denitrification process. So, $NO_3^- - N$ is used as the electron accepter instead of DO. Owing to high SND rate, NO_x–N accumulation rate increased slowly where the disappearance of $NH_4^+ - N$ was obviously larger than NO_x–N formation as shown in Fig. 5a. The accumulation rate of NO_x–N was increased rapidly at the DO level of (1.5–2.5) mg/l where the $NO_3^- - N$ formation was

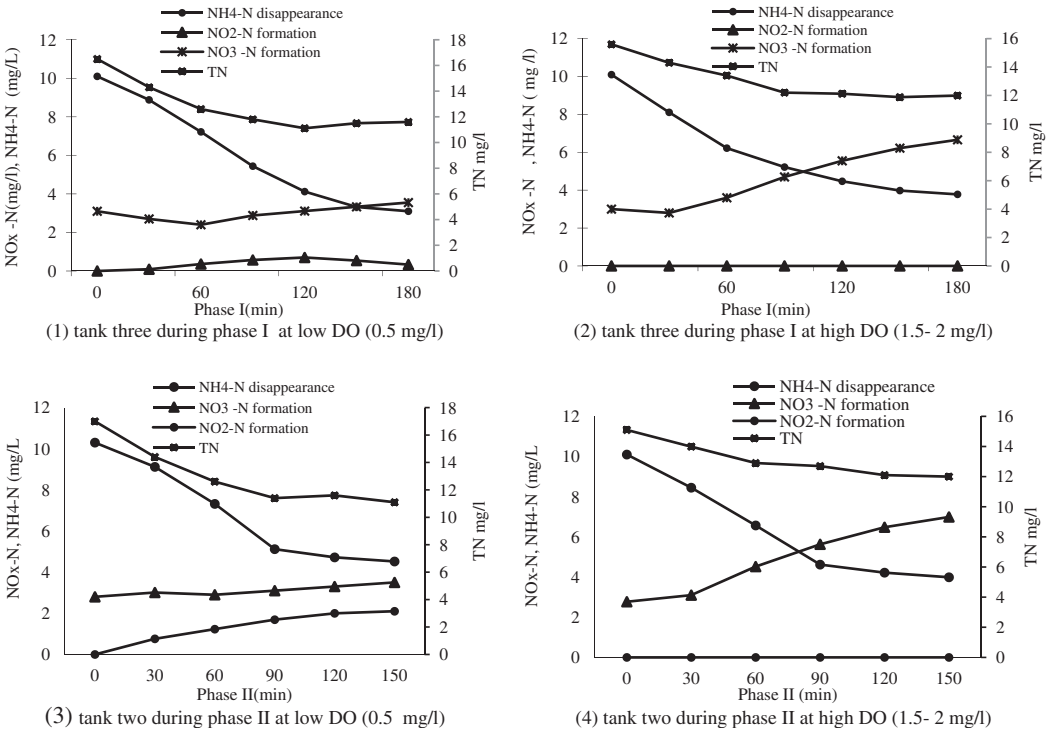


Figure 5 NOx–N formation with total-N disappearance in tank two and tank three during phase II and I respectively.

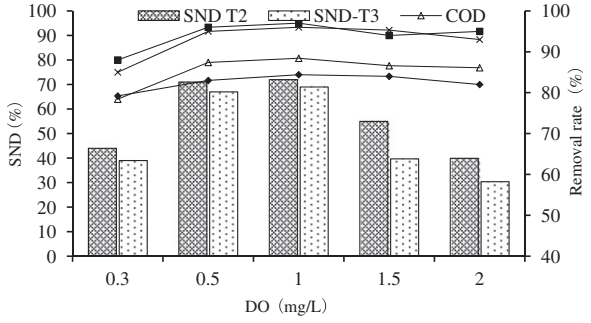


Figure 6 SND rate and nutrient removal rate under different aeration intensities during a first half cycle.

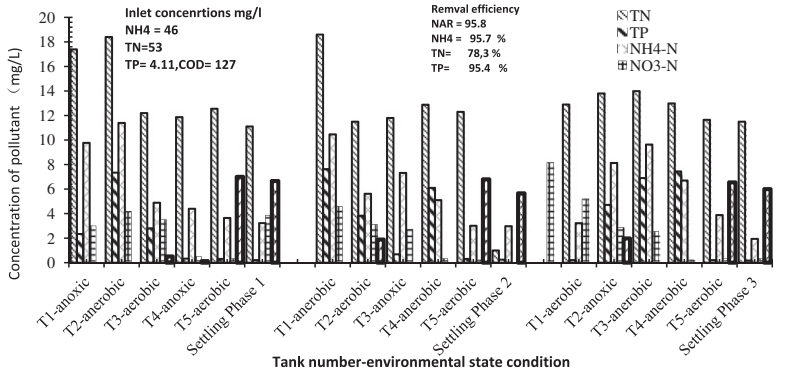
larger than $NH_4^+ - N$ consumption as shown in Fig. 5b. As a result, low DO is conducive to simultaneous nitrification with denitrification while high DO is conducive to nitrification without denitrification. Fig. 5c and d shows, the variation of SND in tank two during phase II. It was found, TN and $NH_4^+ - N$ were rapidly declined during 90 min by nitrification without denitrification while TN and $NH_4^+ - N$ were slowly declined at period 90–150 min due to high SND rate whereas SND rate was about 71% at the DO level of 0.5 mg/l while it was decreased to about 40% at the DO level of 2 mg/l as shown in Fig. 6. It can be seen readily from Fig. 5c and d, the accumulation rate of NO_x–N was decreased at the low DO level that identifies the occurrence of SND phenomena in tank two.

$$SND\% = (1 - NO_x^- - N_{remained}/NH_4^+ - N_{oxidized}) \times 100 \qquad (2)$$

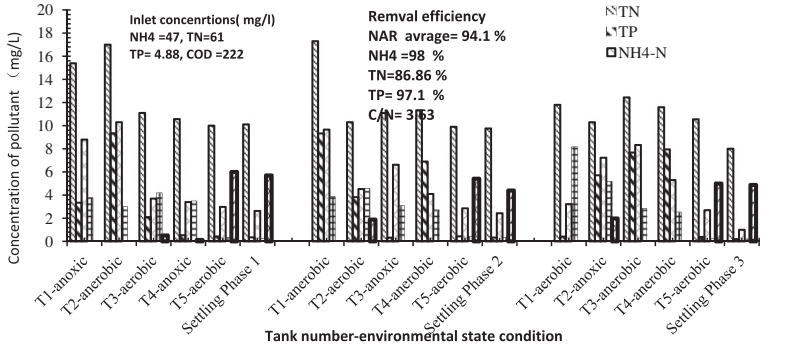
Where $NH_4^+ - N_{oxidized} = (NH_4^+ - N_{influent}) - (NH_4^+ - N_{effluent})$ and NO_x–$N_{remained} = NO_x$–$N_{effluent}$.

3.5. Nutrient removal with limited carbon source

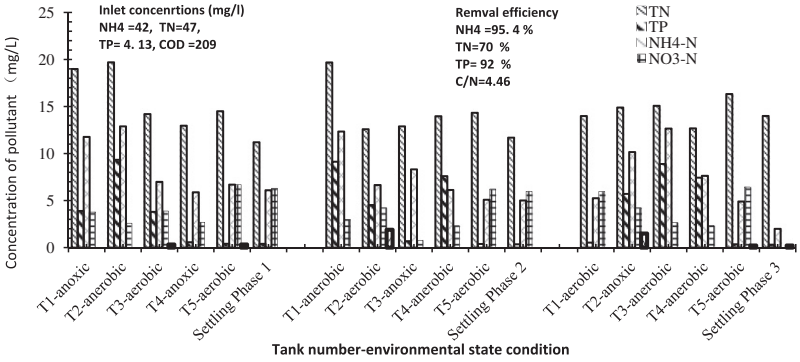
Fig. 7 presents the TP, TN and $NH_4^+ - N$ removal throughout the experimental period in the different runs.$NO_2^- - N$ and $NO_3^- - N$ concentrations along the system in the different phases and different runs were also measured to gain a better insight into nitrogen removal. In run mode No.1, short HRT of 9.1hr, was calculated from Eq. (1), was applied with low DO concentration of 0.5 mg/L. The total removal efficiency was 95.4, 78.3 and 95.7 of TP, TN and $NH4^+$–N, respectively. The average of nitrite accumulation rate during the first half cycle was over 95%, which implied that X_{NOB} activities were successfully suppressed due to a low DO concentration. In comparison to the operation in run mode No. 2, acetate sodium was supplied as an external carbon source for denitrification to enhance TN removal, thus TN removal reached to 86% and ammonia removal was above 97% (Fig. 7b). However, the short HRT of 9.1hr resulted in poor ammonia removal. Thus, in run mode No. 2 HRT was slightly increased to 11 h through increasing the influent flow rates to improve $NH_4^+ - N$ removal. During this period, an average NAR during the first half cycle was stabilized at 94.1%. This outcome was possibly due to suppression or reduction of X_{NOB} through low DO combined with short HRT control. Although, the effluent of total nitrogen in run mode No. 1 was less than 15 mg /l that met the requirement of emission standard level A ‘‘(GB18918–2002)’’. In addition to that, Fig. 7 depicted that the total phosphorus was also removed by anoxic environmental condition in tank four and tank three during phase I and II respectively. The proposed explanation for this observation was the co-existence of denitrifying phosphorus organism (X_{DPAOs}), capable of using nitrate instead of oxygen as electron acceptor in the anoxic phase. In run mode No. 3, experiments were carried out



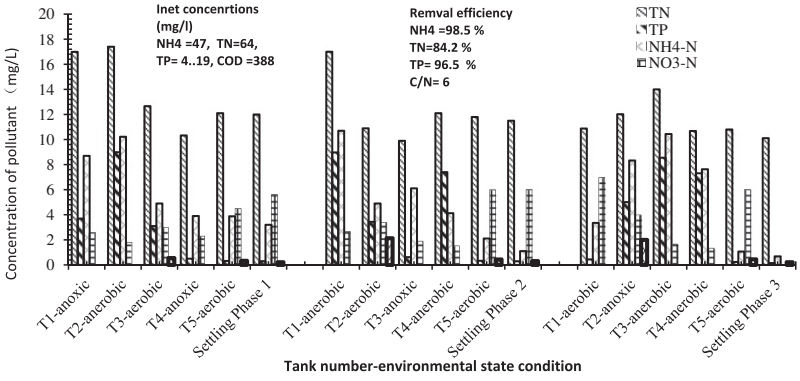
(a) **Run mode no.1**



(b) **Run mode no.2**



(c) **Run mode no.3**



(d) **Run mode no.4**

Figure 7 TN,$NH_4^+ - N$,$NO_2^- - N$,$NO_3^- - N$, and TP concentration along PITSF reactor during phase I, II, and III.

with a normal DO concentration of 1.5–2.5 mg/l and HRT was reduced from 11 h to 9.1 h; ammonia–N and TN removal rate were reduced to 95.2% and 70%, respectively. In run mode No. 4, TN, TP and ammonia-N removal was improved as shown in Fig. 7d by adding NaAc as a carbon source (increased C/N to 6). Based on the seed sludge with complete nitrification and

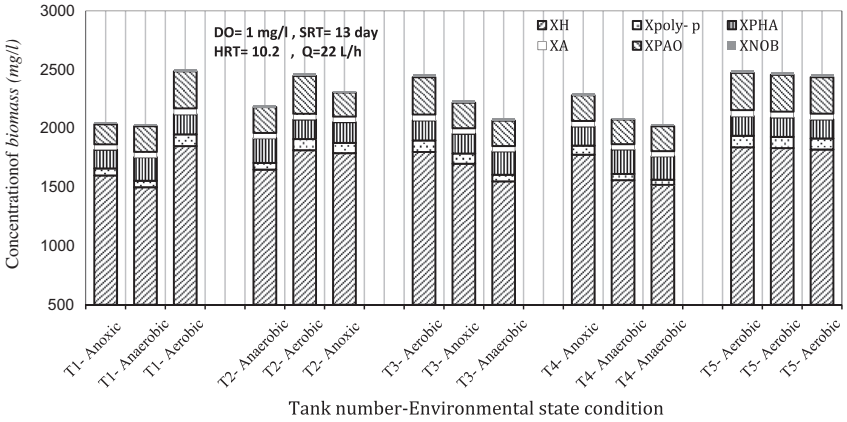


Figure 8 Variations of biomass in each tank during a first half cycle.

denitrification, NAR was so little during run modes No. 3 and No.4 as shown in Fig. 7c and d.

3.6. Variations of biomass in PITSF process

In this study, the variations of microorganisms were evaluated experimentally in each tank during the first half cycle. According to Fig. 8, the X_H; X_{PAOs}; X_{AB}, and X_{NOB} concentrations were 1840 –1520, 322–170, 52– 45.3, and 23.7–15.3 mg/l in PITSF process. As shown in Fig. 8, X_H; X_{PAO}; X_{AB} and X_{NOB} decreased in the anaerobic tank because of the lysis reaction. Then the heterotrophic organism XH; phosphate accumulating organism X_{PAO}, nitrite oxidize bacteria X_{NOB} and autotrophic microorganism XAB increased in the aerobic tanks due to aerobic growth. The heterotrophic organism X_H; phosphate accumulating organism X_{PAOs}, and autotrophic microorganism X_{AB} increased in quantities by about 23, 44, 10 and 43.7% due to change in the environmental state condition from anoxic and anaerobic to aerobic condition. In tank two, firstly, X_H; X_{PAOs}; X_{AB} and X_{NOB} increased in quantities by about 10, 46.3, 11 and 39%, owing to change in the environmental state condition from anaerobic to aerobic. After that, biomass concentration decreased in quantities by about 1.3, 36, 4 and 32%, respectively due to change in the environmental state condition from aerobic to anoxic in which the step feeding influent flowed. Meanwhile, X_H; X_{PAO}; X_{AB} and X_{NOB} decreased in quantities by about 5.5, 31.5, 5.3 and 24.4%, owing to change in the environmental state condition from aerobic to anoxic in tank three. It can be seen readily from Fig. 8 that the biomass concentrations in tank three during phase II were higher than phase III due to step feed location and sludge recycle. In tank four, X_H; X_{PAOs}; X_{AB} and X_{NOB} decreased gradually by about 12, 3.6, 5.6 and 8%, owing to change in the environmental state condition from anoxic to anaerobic. Biomass concentrations in tank five did not change significantly, and the system remained stable.

4. Discussion

4.1. Mechanisms of nitritation in PITSF process

The variations of X_{AB} population with different operation conditions were specified using real-time (PCR). The continuous flow phased isolation tank showed good complete nitrification at 3, 2.5 and 2 h for phase I, II and III, respectively as shown in Fig. 4a. Therefore, PITSF system was set at this time during all experimental processes. Fig. 4b investigates the influence of dissolved oxygen concentration on nitrite accumulation rates. It is illustrated that XAB population increased slightly from 2.1×10^8 cells/g MLVSS at low DO concentration (0.3) mg /l to 5.01×10^8 cells/g MLVSS at 0.5 mg/l, subsequently, nitration deteriorated at DO 1.5 mg /l leading to a decrease X_{AB} to 3.89×10^8 cells/g MLVSS. The X_{AB} population had a clear correlation with nitrite accumulation rates. Fig. 4,c shows the experimental results of NAR investigation with different SRT controls. Short SRT is helpful for the quick start-up of nitrification .The NAR was 77% on 5 days of sludge retention time, while the calculated value was 90% at 10 days in which the mixed liquor suspended solids were constant (2988 mg/l). The MLSS decreased day after day in the experiment when the SRT was 5 days. The value was only 900 mg/l; so that the reactor had to be stopped. The decrease of MLSS led to increase the $NH_4^- - N$ loading and promote the nitrite accumulation. Thus, short SRT is a good condition for NAR process. Moreover, if the SRT is too short (5 days), the system was unstable, so it is not recommended. With larger SRT, longer time is needed to achieve high nitrification and the NAR is lower during the operation. Thus SRT of 10 to 15 d is more suitable in this study where X_{AB} population stabilized at about 5×10^8 cells/g MLVSS. These results indicate that the X_{AB} population tended to be stable during steady nitritation. Consequently, longer SRT may significantly decrease the nitrification process. The probable reasons are as follows: (1) Removal of XAB through sludge wastage is essentially slower when applying longer SRT. (2) Both X_{NOB} and X_{AB} are grown during the aerated period of the cycle. Although a few X_{NOB} exist in the system, they grew quickly under suitable conditions. It can be seen readily in Fig. 4, NAR gradually ncreased as HRT was reduced. It is concluded that the XAB population reached 5.28×10^8 cells/g MLVSS at 8.3 h corresponding to a nitrite accumulation rate of 90%. Subsequently, nitration deteriorated at HRT greater than 9.1 h leading to a decrease X_{AB} to 8.8×10^7 cells/g MLVSS at HRT 11hr and NAR of only 18%. Consequently, high flow rate is a good condition for controlling the nitration process at low level dissolved oxygen.

4.2. SND in PITSF reactor

The achievement of SND phenomena is highly beneficial for the treatment of domestic wastewater in FITSF reactor, it reduced the potential to save the costs for a second anoxic state condition through reducing its time or at least reducing its aeration flux. In addition, one of the efficient SND conditions was to minimize the decrement of carbon source by oxidation at low C/N wastewater whereas under aerobic conditions with good SND rate, the carbon source was firstly consumed by heterotrophic organisms while the denitrifying heterotrophic organisms could only use carbon sources from endogenous respiration. The removal efficiencies of TN, $NH_4^- - N$, COD, TP were 84.7 .97, 88.3 and 96% respectively with optimum SND rate between 69–72% at a low DO level of 0.5 mg/l (Fig. 6).

4.3. Low DO concentration with limited carbon source

The oxygen affinity constant of X_{AB} and X_{NOB} is 0.5 mg/L and 1.5 mg/L, respectively, indicating X_{AB} having a stronger affinity with DO than NOB (Peters et al., 2004). Especially at low DO concentrations, the specific growth rate of X_{AB} is higher than that of X_{NOB} (Blackburne et al., 2008). In this study, X_{NOB} activities were selectively inhibited under a long-term operation for 4 months (run1 and run2) with low DO levels of 0.5 mg/L whereas X_{AB} dominance was enhanced, leading to a nitrite build-up. In addition to that, PITSF process can be operated safely with limited carbon sources in terms of lower carbon requirements and reduced aeration costs whereas the total nutrient removal efficiencies of TN, $NH_4^- - N$, and TP at low C/N ratio and DO level of 0.5 mg/l were approximately equal to the complete nitrification–denitrification with the addition of external carbon sources at a normal DO level of 1.5–2.5 mg/l. The achievement of nutrient removal via nitrite in PITSF process is highly beneficial for the treatment of domestic wastewater with low C/N whereas nitrite accumulation rate (NAR) in the last aerobic zone was stabilized over 95% at low DO of 0.5 mg/l and short HRT of 9.1 h (Fig. 7).

4.4. Variations of biomass in PITSF process

In this study, the variations of microorganisms were evaluated experimentally in each tank during the first half cycle. According to experimental investigation, X_H; X_{PAO}; X_{AB}, and X_{NOB} decreased in the anaerobic tanks because of the lysis reaction. Then the X_H; X_{PAOs}; X_{AB}, and X_{NOB} increased in the aerobic tanks due to aerobic growth. Although the biomass of X_{AB} and X_{NOB} varied in each tank, the ratio of total nitrifying species to total active biomass was about 2.8–3% in each tank. In this study, the disadvantages of the developed BNR processes were improved by reconfiguring the process without mixed liquor and sludge recirculation. This was done by configuring the process into six-tank with variable environmental state condition, anaerobic/anoxic, aerobic in each tank to achieve optimum removal of phosphorus and nitrogen. In PITSF, the intake location changing of raw wastewater was also used to direct the influent into the anoxic zone as an external carbon source for denitrification process. So, the heterotrophic organism of the X_H; X_{PAOs}; and X_{AB} decreased in the anoxic tank due to the dilution effect of the flow (Fig. 8). In addition to that, the dilution effect of the influent, the X_{AB} also decreased due to the negative growth rate resulted from lysis reaction in the anoxic tank.

5. Conclusion

The achievement of partial nitrification via nitrite (NAR) is highly beneficial for the treatment of domestic wastewater with low organic content in terms of lower carbon requirements and reduced aeration costs .Also, the occurrence of SND phenomena is important to reduce the quantity of aeration and the time of a next anoxic state condition. In the present study, PCR assay showed that high NAR was achieved at X_{AB} population 5.2×10^8 cells/g MLVSS whereas NAR in the last aerobic zone was stabilized over 95% throughout a combination of a low DO level of 0.4 mg/l and a short HRT of 9.1hr. SND was observed obviously with a high rate of (69–72%) and a low DO level of 0.5 mg/l in the first aerobic tanks during main phases whereas the removal efficiencies of TN, NH_4–N, COD, TP were 84.7 .97, 88.3 and 96% respectively. The total nutrient removal efficiencies of TN, $NH_4^+ - N$, and TP with low C/N ratio and DO level were 86.2, 97 and 96.1% respectively which was approximately equal to complete nitrification–denitrification with the addition of external carbon sources at high DO levels of (1.5–2.5) mg/l.

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