Effect of intermittent aeration cycle on nutrient removal and microbial community in a fluidized bed reactor-membrane bioreactor combo system

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Abstract

Effect of intermittent aeration cycle (IAC=15/45-60/60 min) on nutrient removal and microbial community structure was investigated using a novel fluidized bed reactor-membrane bioreactor (FBR-MBR) combo system. FBR alone was found more efficient for removing PO_4-P (>85%) than NH_4-N (<40%) and chemical oxygen demand (COD<35%). However, in the combo system, COD and NH_4-N removals were almost complete (>98%). Efficient nitrification, stable mixed liquor suspended solid and reduced transmembrane pressure was also achieved. Quantitative real-time polymerase chain reaction results of total bacteria 16S rRNA gene copies per mL of mixed-liquor varied from $(2.48\pm0.42)\times10^9$ initial to $(2.74\pm0.10)\times10^8$, $(6.27\pm0.16)\times10^9$ and $(9.17\pm1.78)\times10^9$ for 15/45, 45/15 and 60/60 min of IACs, respectively. The results of clone library analysis revealed that *Proteobacteria* (59%), *Firmicutes* (12%) and *Bacteroidetes* (11%) were the dominant bacterial group in all samples. Overall, the combo system performs optimum nutrient removal and host stable microbial communities at 45/15 min of IAC.

Keywords: Fluidized bed reactor; Intermittent aeration cycle; Membrane bioreactor; Microbial community; Nutrient removal

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Abbreviations

AOB	ammonia oxidizing bacteria
COD	chemical oxygen demand
DO	dissolved oxygen
EPS	extracellular polymerase substance
FBR	fluidized bed reactor
IAC	intermittent aeration cycle
MBR	membrane bioreactor
MLSS	mixed liquor suspended solid
MLVSS	mixed liquor volatile suspended solid
NE	nitrification efficiency
NOB	nitrite oxidizing bacteria
PCR	polymerase chain reaction
qPCR	quantitative PCR
SMP	soluble microbial product
SND	simultaneous nitrification denitrification activity
TMP	transmembrane pressure

1. Introduction

Enrichment of surface water with nutrients (phosphorus and nitrogen) from municipal wastewater treatment discharges is an important water quality concern due to eutrophication. As a result, regulations of the nitrogen and phosphorus contents from wastewater discharge are becoming increasingly more stringent (Ahmed, 2012). Currently, different technologies have been employed to remove these nutrients from wastewater.

Phosphate removal as struvite ($MgNH_4PO_4.6H_2O$) using fluidized bed reactors (FBRs) has been widely reported in literatures (Adnan et al., 2003; Le Corre et al., 2007; Liu et al., 2008; Guadie et al., 2013a). Phosphorus recovery as struvite is not only solving the problem of eutrophication but also a sustainable option for replacing the stock phosphate that is being depleted. Phosphate reserve depletion has been estimated less than 100 years (Shu et al., 2006), which drew the attention of the scientific community to focus on alternative strategies to obtain

phosphorus(Adnan et al., 2003; Shu et al., 2006; Le Corre et al., 2007). Guadie et al. (2013a) designed a novel three cone-inserted FBR that could achieve an efficient phosphorus removal (>90%) and high quality struvite recovery at low and high phosphorus concentrations. Using an internal recycling reactor, Liu and his colleagues (Liu et al., 2008) also observed 78% phosphorus recovery at low phosphorus concentration. Although FBRs are efficient for phosphorus removal and recovery, parallel nitrogen removal as to the discharge limit was not reported. Using FBR for wastewater treatment, Adnan et al. (2003) and Le Corre et al. (2007) observed low nitrogen (<50%) removal than phosphorus (about 90%). Compared to FBR, membrane bioreactor (MBR) has been found a promising nitrogen removal technology as it can enhance growth of bacteria for nitrification and denitrification process (Duan et al., 2009; Guo et al., 2009; Kornboonraksa et al., 2009; Xia et al., 2010).

Nitrification is completed in two steps: ammonium (NH_4) oxidized into nitrite (NO_2) by ammonia-oxidizing bacteria (AOB), and NO_2 oxidized into nitrate (NO_3) by nitrite-oxidizing bacteria (NOB) (Ahmed, 2012). Although the stoichiometric showed the requirement of 2 mol of oxygen (75% for AOB and 25% for NOB) per mol of nitrogen to be nitrified (Ruiz et al., 2003), there has been several reports that also showed complete nitrification below 2.0 mg/L dissolved oxygen (DO) concentration (Bellucci et al., 2011; Wang et al., 2012). Current research efforts on intermittent aeration cycles (IAC) are leading to reduce the level of DO required for nitrification process. During IAC (on/off), the main advantage has been reported the coexistence of nitrifier and denitrifier microbial communities that can perform simultaneous nitrification-denitrification (SND) activities in a single reactor (Third et al., 2003; Rong et al., 2007; Wang et al., 2012). This is due to DO level fluctuation which creates anoxic/oxic microenvironment during “on” and “off” operations (Huang and Ju, 2007; Guadie et al., 2013b). Depending on DO level in the environment, some microbes were reported to shift their electron accepter from oxygen to other preferential sources which in turn leads metabolic activity shift (Feng et al., 2007; Sadaie et al.,

2007). Using an online nicotinamide adenine dinucleotide (NAD) fluorescence profiles, Huang and Ju (2007) confirmed that the change of metabolic state observed in the reactor was associated with the shift of electron-accepting mechanisms in the sludge's microbial populations. Sadaie et al. (2007) detected *Burkholderiales* which could survive under aerobic and anaerobic conditions, based on electron acceptor shift. Ammonia oxidizers (like *Nitrosomonas europaea* and *Nitrosomonas eutropha*) were also identified a versatile group of microorganisms found in many natural and engineered ecosystems due to their metabolism flexibility (Sofia et al., 2004; Feng et al., 2007).

Apart from oxygen saving and avoiding two reactor configuration, the advantages of SND such as reduced organic substrate requirements for heterotrophic denitrification, lower biomass production, and self-balanced pH were also reported (Rong et al., 2007; Wang et al., 2012). During nitrification process, hydrogen ion (H^+) released causes a pH drop in the reactor. However, this drop counter balanced during denitrification process that produces hydroxyl ion (OH^-) (Rong et al., 2007).

Traditionally, microorganisms in wastewater has been analyzed using microscopic observation and culture dependent techniques, although these methods only estimate 1-15% of the total activated sludge community (Amann et al., 1995). To overcome the limitation of culture dependent methods, recently molecular techniques such as polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), fluorescence in situ hybridization, quantitative PCR (qPCR) and 16S rRNA clone library methods have been applied to the detection of microbial community abundance and diversity at various environmental samples (Duan et al., 2009; Wittebolle et al., 2009; Xia et al., 2010; Zhang et al., 2012).

Although a novel combination of MBR with other systems have been evaluated at laboratory and pilot scale for efficient nutrient and organic matter removal (Guo et al., 2009; Kornboonraksa et al., 2009; Guadie et al., 2013b), parallel microbial community analysis data

have not been available yet. For instance, Guadie and his colleagues (Guadie et al., 2013b) has been designed a novel FBR-MBR combo system and found a promising reactor performance, however; microbial community information has not been provided. As a result, this study was carried out to support the biochemical data with microbial community analysis. Nutrient removal efficiency (targeting struvite recovery in FBR and nitrogen removal in MBR) and microbial community structures has been investigated by considering IACs as a major factor that has been previously showed significant difference in the reactor performance. The abundance and diversity of the microbial community in the MBR system was investigated using qPCR and clone library construction for total bacteria 16S rRNA gene.

2. Materials and Methods

2.1. Wastewater characteristics

The synthetic wastewater used in this study was prepared according to Xia et al. (2010) with some modifications. It contains mg/L: starch (175), glucose (200), peptone (28), urea (64), ammonium chloride (150), potassium dihydrogen phosphate (52.5), magnesium chloride hexahydrate (150) and calcium sulfate dihydrate (50). Trace mineral solution containing (mg/L): $MnCl_2.4H_2O$ (30), H_3BO_3 (300), $CoCl_2.6H_2O$ (10), $CuCl_2.2H_2O$ (10), $NiCl_2.6H_2O$ (10), $ZnCl_2$ (100) and $FeSO_4.7H_2O$ (300) were mixed together and 1 mL/L was used. Sodium hydroxide (NaOH=12.5 mM) was also separately prepared for pH adjustment in the FBR (Guadie et al., 2013a).

2.2. Sludge source

The reactor was inoculated with mixed liquor that was obtained from a secondary clarifier from Quyang wastewater treatment plant (Shanghai, China). The pH, DO, mixed liquor suspended solid (MLSS) and mixed liquor volatile suspended solid (MLVSS) were measured and their average values were found 6.93±0.32, 0.64±0.41 mg/L, 3.62±0.40 g/L and 2.72±0.23

g/L, respectively. In order to make the source uniform, the sample was stored at 4°C using 20% glycerol. When the new running conditions were needed, the stored inoculum was acclimatized before feeding to the MBR.

2.3. Experimental procedures

Previously, simultaneous removal of phosphorus and nitrogen from sewage using a novel combo system of FBR-MBR has been studied by constructing a FBR (about 9.5 L) and two identical MBRs (0.3 m length × 0.1 m width × 0.28 m height) at Tongji University, Shanghai (Guadie et al., 2013b). The same principle and combination also apply in this study (Fig. S1).

Wastewater and NaOH solution were introduced at the bottom part of the FBR using a peristaltic pump. When the solution was fluidized-up crossing the various areas, chemical reactions were observed in the middle part of the reactor. Through time, the bigger precipitates were settled down at the bottom section. Once sufficient amount of crystals stored at the bottom part of the reactor, they were collected by opening the lower valve. The liquid together with very fine precipitates were flow down from the top part of the FBR to the external recycler. Portion of FBR treated effluent was collected in supernatant tank while the rest was returned back to FBR (Fig. S1).

Effluent from the FBR was continuously treated in MBR system at a constant flow rate of 23 mL/min. Hollow fiber polyvinylidene fluoride membrane with an area of 200 cm^2 and a pore size of 0.1 μm was used (Li-tree Company, Suzhou, China). The IAC (on/off) was made through an air diffuser installed directly beneath the membrane module for supplying oxygen to microbial consortia, mixing the liquor, and scouring the membrane surface. The air flow in MBR was controlled at 8 L/h. Intermittent filtration with 10-minutes cycle (8 minutes filtration and 2 minutes pause) was employed. Membrane fouling was monitored with a transmembrane pressure (TMP) gauge (YN-60, Shanghai Weiken).

In order to investigate the performance of FBR-MBR combo system and microbial

communities inside the MBR system, two MBRs (MBR_O and MBR_V which operated at optimum and variable, respectively) were evaluated under optimum IAC (IAC_O) (on/off=45/15 min) and variable IACs (on/off=15/45, 30/30, 60/30 and 60/60 min), based on the previous study (Guadie et al., 2013b). The optimum operation conditions for the FBR-MBRs such as pH_{FBR}, hydraulic retention time (HRT) and flux rate were kept constant 9, 6 h and 70 L/(m^2 h), respectively (Guadie et al., 2013a). Except the sludge used for MLSS analysis, both reactors were operated under no sludge withdrawal condition.

Nitrification efficiencies (NE) and SND activities in the combo system were calculated according to Eq. 1 (Wittebolle et al., 2009) and Eq. 2 (Third et al., 2003), respectively.

$$NE(\%) = 100 - \frac{(NH_4^+ - N)_{eff} + (NO_2^- - \mathrm{N})_{eff}}{(NH_4^+ - N)_{\mathrm{inf}}} \times 100 \quad (1)$$

$$SND = (1 - \frac{C_{NO_x^- produced}}{C_{NH_4^+ - oxidized}}) \times 100\% \quad (2)$$

All experiments were conducted at room temperature (average 25.2±1.8°C). Summary of the operating conditions are shown in Table 1.

Table 1

2.4. Chemical analytic methods

For chemical analysis, influent and effluent samples were collected every two days from feed, supernatant and effluent tanks. The constituents of PO_4-P, NH_4-N, NO_2-N, NO_3-N, Mg, Ca, MLSS and MLVSS were analyzed according to standard methods (APHA et al., 1998). The PO_4-P and NH_4-N were analyzed using UV-visible spectrophotometer (UV-2700, Shimadzu, Japan) while Mg and Ca were measured using flame atomic absorption spectrophotometer (PE-AA400, Perkin Elmer, USA).

Turbidity, DO and pH were measured using turbidimeter (2100N, HACH, USA), DO meter (HQ4d, HACH USA) and pH meter (PHS-29A, HACH, USA), respectively. The

membrane fouling in the MBR and crystal harvested in the FBR were observed using scanning electron microscope, SEM (XL-30, Philips, Netherlands). Moreover, crystal products were characterized using x-ray diffractometer (XRD).

Sludge characteristics such as soluble microbial product (SMP) and extracellular polymerase substance (EPS) were extracted according to Guadie et al. (2013b). To quantify the EPS and SMP contents of carbohydrate and protein, phenol-sulfuric acid (Dubois et al., 1956) and Lowry (Lowry et al., 1951) methods were used, respectively.

2.5. Microbial community analyses

2.5.1. Sample collection and DNA extraction

For total genomic DNA extractions, mixed liquor samples were collected at different IACs (on/off=15/45, 45/15 and 60/60 min). Under IAC_O, more number of samples were collected at day 30, 60, 90, 120 and 150 for both qPCR and clone library construction, aiming for time matter analysis on microbial abundance (Table 1). Forty mL of mixed liquor was collected and centrifuged for 20 min at 12000 g. Then, the supernatants were removed and pellets were resuspended using PBS buffer (mmol/L: Na_2HPO_4 10; KH_2PO_4 2, NaCl 137; KCl 2.7, pH 7.4).

Total genomic DNA of each sample was extracted in duplicate using Fast DNA Spin Kit (MP Biomedical, LLC, France) following the manufacturer's protocol, and the duplicate samples were pooled together and stored at -20°C for subsequent assays.

2.5.2. *Quantitative real-time PCR (qPCR)*

The abundance of bacterial communities in the mixed liquor was quantified by SYBR Green I-based qPCR method using 338f (5'-ACTCCTACGGGAGGCAGC-3') and 536r (5'-GTATTACCGCGGCKGCTG-3') primers. Triplicate reaction tubes for each sample were used. Each 20 µl reaction mixture contained 10 µl SYBR® Premix Ex Taq™ (Takara, Dalian, China), 1 µL template DNA (5–10 ng), 0.4 µL (10 µM) of each primer, 0.4 µL of bovine serum albumin (BSA, 0.8 µg/µL final concentration), 0.4 µL of ROX reference dye (50×) and 7.4 µL of sterile

distilled water. Instead of template DNA, plasmids containing cloned amoA PCR amplicons were used for generating standard curves. The qPCR amplification was carried out in AB7500 qPCR thermocycler (Foster City, USA) using the program 95 °C for 2 min, followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. The result was analyzed using Applied Biostatistics 7500 software (version 2.0.5). The amplification efficiency and correlation coefficients (R^2) were 99.76% and 0.99, respectively. Melting curves were also analyzed to detect the presence of primer dimers.

2.5.3. Clone library analysis

DNA amplification was carried out using 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3') universal bacterial primers. Each 50 μL reaction mixture contained 2 μL templates DNA (5–10 ng), 25 μL Taq PCR Master Mix (Takara, Dalian, China), 2 μL (10 μM) of each primer, 2 μL BSA (0.8 μg/μL final concentration) and 17 μL of distilled water. Amplification was carried out using Mycycler (Bio-Rad, USA) under the following conditions: an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 60 s, annealing at 55 °C for 60 s and extension at 72 °C for 2 min, and a final extension at 72 °C for 10 min prior to cooling at 4°C.

Following PCR amplification, the correctly sized product (~1,465 base pairs) was checked by electrophoresis, using 1% (w/v) agarose gel stained with ethidium bromide. The PCR products were excised and purified from the gel using a BioDev Gel Extraction System Kit (Beijing, China) following the manufacturer's instructions. The purified PCR products were ligated into a cloning vector (PMD^{18}-T vector) (Takara, Dalian, China) following manufacturer's protocol. Ligated PCR products were transformed into the competent cells (*Escherichia coli,* DH5α), on ampicillin-supplemented LB medium. About 100 clones from each (total five) samples were used for Sanger sequencing (BGI, Shanghai, China).

2.5.4. Sequence analysis

In this study, the mothur software package (Schloss et al., 2009) was used to screen and align sequences, calculate distances, assign sequences to operational taxonomic units (OTUs), and calculate the diversity indices. The forward primers were trimmed from the raw sequences using Bioedit software and then aligned using the SILVA bacteria reference alignment (http://www.arb-silva.de/). Chimeric sequences were screened and removed from the subsequent analysis using the chimera.slayer (version 1.22.0) algorithm (Schloss et al., 2009). Quality sequences were assigned into OTUs. Rarefaction and alpha diversity (Shannon-Wiener diversity index and species richness estimator (Chao1)) indices were analyzed in mothur. All richness and diversity comparisons were made after normalizing the number of sequences in each sample. Sequence information was also used for principal component analysis (PCoA) and Jackknife environment clustering using the UniFrac online tool (http://bmf.colorado.edu/unifrac).

2.5.5. Nucleotide sequence accession number

Sequences obtained in this study were submitted to the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) under accession number KF830278-830660.

3. Results and discussion

3.1. Effect of intermittent aeration cycle on FBR-MBR combo performance

3.1.1. Effect of intermittent aeration on pH

As the pH value of 8 to 10 was recommended as the operative range for quality struvite recovery in the FBR (Le Corre et al., 2007; Guadie et al., 2013a), the feed pH (6.5±0.7) was adjusted with NaOH solution to an alkaline range (pH≥9). After FBR treatment, the solution pH was 8.8±0.5 in the supernatant tank, which was lower than the pH in the FBR (p=0.001, n=72) (Fig. 1). Throughout this study, by using Student's t-test a value of $p<0.05$ was considered statistically significant.

Crystal (struvite) formation observed at the middle part of the FBR resulted in a pH drop (produce H^+) in the system (Eq. 3). Using SEM and XRD, harvested crystals were analyzed and confirmed pure struvite (Fig. S2). Struvite recovery in the combo system can be viewed as a unique feature of this study which makes phosphorus management more sustainable (by avoid eutrophication risk and resource depletion). Studies showed that struvite is a slow released fertilizer which can be applied directly to plant growth (Shu et al., 2006; Liu et al., 2008).

$$Mg^{2+} + NH_4^+ + H_nPO_4^{n-3} + 6H_2O \rightarrow MgNH_4PO_4.6H_2O + nH^+ \text{ , } n\text{= 0, 1 and 2} \quad (3)$$

Microorganisms are very vulnerable and sensitive to pH value of the wastewater. Since pH lower than 6.5 or higher than 9.0 could inhibit nitrification completely, the preferential ranges for AOB and NOB are about 7.5–8.5 and 6.5–7.5, respectively (Ruiz et al., 2003; Campos et al., 2007; Rong et al., 2007). Generally, pH adjustment with $NaHCO_3$ needs to be performed during MBR operation. However, in this study, it was found that the combo system was confirmed working in harmony without any pH adjustment, because struvite formation and IAC played a significant pH modification in the system. For example, when pH was measured in MBR_O under non-aeration cycle (off) and aeration cycle (on), results were statistically different (p=6.48E-11<0.05) with pH value of 7.8±0.5 and 7.2±0.4, respectively (Fig. 1). The same significant pH difference was observed in MBR_V system (7.1±0.7 for "on" cycle and 7.4±0.8 for "off" cycle, p=1.06E-05). This could be due to the microbial consortia catabolic activities on starch, glucose, urea and peptone added as a feed which usually converts to simple organic acids and decreased the pH value. The final effluent pH for MBR_O and MBR_V were 7.4±0.5 and 7.2±0.6, respectively, which was in the range of pH discharge limit set by many countries (6-9).

Fig. 1

3.1.2. Effect of intermittent aeration on DO concentration and removal efficiency

As shown in Table 2, under optimal operating condition (FBR-MBR_O), the average DO concentration was 3.6±0.6 mg/L for aeration cycle and 1.5±0.6 mg/L for non-aeration cycle,

which was statistically significant (p<0.05). When the FBR-MBR_V combo system works under different IACs, the average DO concentrations varied from 0.8-6.1 mg/L for aeration cycle and 0.3-3.4 mg/L for non-aeration cycle (p<0.05) (Table 2).

Prior treatment of the raw wastewater with FBR system modified the wastewater characteristics and improved the removal efficiency in MBR system. The removal efficiency of PO_4-P in FBR, and COD and NH_4-N in the MBR was significant (Table 2). When FBR-MBR_O operated at IAC_O, the COD, NH_4-N and PO_4-P removals were 99.4±0.6, 98.7±2.1 and 92.3±4.1, respectively. Varying the IAC in the combo system (FBR-MBR_V) did not show apparent difference in phosphorus removal (91-95%). For instance, at IACs of 60/60 and 15/45 min, the phosphorus removal efficiency showed statistically insignificant change (p=0.83). However, the variation was considerably significant for NH_4-N (64-99%, p=0.002) and COD (83-99.5%, p=0.003) removals. At IAC of 15/45 min, the level of DO was between 0.3 and 0.8 mg/L, which showed significant reduction of NH_4-N (~35%) and COD (~15%) removal efficiency than FBR-MBR_O (Table 2). These results clearly showed that DO is an important factor for microbial degradation of NH_4-N and COD removal than phosphorus, particularly AOB showed more response to the low DO tension. Studies showed that nitrification processes by nitrifiers are more sensitive to environmental conditions (oxygen, substrate, pH and availability of electron acceptors) than carbon oxidation by heterotrophs (Sofia et al., 2004; Campos et al., 2007).

Although extending the aeration cycle higher than 45 min (e.g. IAC=60/60 min) help to assure more efficient NH_4-N and COD removal, the condition was found disturbing the sludge floc structure developed in the MBR system. Since there was no apparent difference in COD (p=0.640) and NH_4-N (p=0.738) removals at 60/60 and 45/15 min IACs, the later operation has been considered as optimum, based on energy saving.

Table 2

As shown in Fig. 2, microbes governing the subsequent conversion of NH_4-N to NO_2-N

and then NO_3-N initially seemed more quiescent than COD degrading microbes. However, after one month operation, the sludge became active and showed a significant reduction of NH_4-N. Throughout the entire experimental process in FBR-MBR_O, the initial NH_4-N and COD concentrations varied from 40-60 (50.7±4.0) mg/L and 250-350 (291.03±27.0) mg/L, respectively (Fig. 2a). The average NH_4-N and COD effluent concentrations were also found less than 5 and 2 mg/L, respectively. The NO_2-N and NO_3-N in the FBR-MBR_O system was 0.06-0.20 (0.15±0.04) mg/L and 4.0-25.0 (8.32±4.6) mg/L, respectively (Table 1). After one month operation, respectively the NE and SND efficiency in FBR-MBR_O were 92-99% (98.1±2.2%) and 75-95% (82.8±11.7%) (Table 1), suggesting that the combo system provides suitable environmental niche for AOB and NOB communities. When the system operated at IAC_O and 60 min "on" aeration cycle, DO concentration was not the limiting factor for nitrification process (Table 2). Generally, DO concentrations greater than 2 mg/L is essential to maintain complete nitrification in biological wastewater treatment plants (Ruiz et al., 2003; Ahmed, 2012; Wang et al., 2012).

The nitrogen and COD removals were consistent with biomass growth in the bioreactor (MBR_O). The MLSS and MLVSS concentrations varied from 3.4-5.2 (4.6±0.7) g/L and 2.7-4.6 (4.0±0.2) g/L, respectively (Fig. 2b). The ratio of MLVSS to MLSS concentration in the MBR system was found to be varying from 0.75-0.92 (0.89±0.03). The ratio was found almost constant and higher after 30 days of reactor operation.

Fig. 2

3.1.3. Effect of intermittent aeration on membrane fouling

To illustrate membrane fouling, EPS and SMP concentrations were quantified as fractions of carbohydrates and proteins. As shown in Fig. 3a, relatively higher EPS protein (EPS_P) was observed at each intermittent cycle, but the difference was insignificant. The same insignificant difference was also observed on SMP protein and carbohydrate (SMP_P and SMP_C). Nevertheless,

the EPS carbohydrate (EPS_C) was significantly varied from 20 to 80 mg/L, depending on IACs (Fig. 3a). The observed EPS_C concentrations of 47.2±1.7 mg/L for IAC 30/30 min and 78.4±2.4 mg/L for IAC 15/45 min were probably the main cause of TMP development (Fig. 3b) that led to fast membrane fouling. The TMP was higher (>50 kPa) at lower IAC (15/45 min) than extended IACs (TMP<10 kPa) (Fig. 3b). At lower aeration cycle (IAC= 15/45 min), the level of DO was <1 mg/L (Table 2) which resulted in low biological organic degradation. This condition could create unfavorable environment for key microbial consortia that play a significant role in reducing the incoming organic feed and polymeric byproducts (Dionisi et al., 2003; Xia et al., 2010). In the absence of DO tension (IACs=45/15, 60/30 and 60/60 min), these microorganisms might be well acclimatized and able to reduce the membrane foulants (Fig. 3a). This might be supported by higher qPCR and *Proteobacteria* results observed in section 3.2 (IACs=45/15 and 60/60 min). Previous studies showed that under favorable conditions, microorganisms could grow well and be capable of consuming more macromolecules such carbohydrate and protein as substrates leading to reduce membrane fouling rate (Masse et al., 2006).

The low MLSS concentration observed in all operations (4<MLSS<10 g/L) suggested that MLSS concentration in the system has not been positively associated with TMP values (Fig. 3b). This could be due to inorganic components (like phosphate) were precipitated as struvite in the FBR and did not accumulate in the MBR system. This result is consistent with the previous study that MLSS concentration (3.0-8.5 mg/L) was not the main cause of TMP raise/membrane fouling (Guadie et al., 2013b).

Fig. 3

3.2. Microbial community analyses

In this study, attempts have been made to support the molecular data with the biochemical results. Total bacterial abundance and diversity were analyzed using qPCR and clone library construction.

3.2.1. Abundance of total bacteria

Real-time qPCR was used to examine the dynamics of the total bacterial 16S rRNA gene copies in the MBRs that were operated at different IACs (Fig. 4). Under IAC_O, the abundance of bacterial 16S rRNA gene copies showed substantial amount of fluctuation over the course of time. The average copy numbers per mL of mixed-liquor increased from $(4.13\pm0.10)\times10^9$ to $(6.27\pm0.16)\times10^9$ (sample 2-6) (Fig. 4). Compared to the initial inoculum $(2.48\pm0.42)\times10^9$ gene copies/mL), all samples showed significant difference ($p<0.05$). However, there was no significant difference between samples collected at day 60 (sample 3) and day 150 (sample 6) ($p=0.139$). This result is consistent with the chemical analysis. As stated earlier, throughout the entered study period complete nitrification was achieved after one month operation, suggesting that the nitrifier communities may be more adapted through time in the combo system and played a significant role for modifying the wastewater characteristic and maintain stable reactor performance. This result is also consistent with other studies. According to Duan et al. (2009), the bacterial numbers showed dynamic changes in the first 20 days, and stabilized after that time. Dionisi and his colleagues (Dionisi et al., 2003) also stated that due to slow grower bacteria biomass increase (i.e. *Nitrospira* cells), complete nitrification has been observed at 20 day sludge age sample than 2, 5 and 10 days of reactor operation.

Samples (7-12) were also collected from FBR-MBR_V combo system at day 30, 60 and 90 from short (IAC=15/45 min) and extended aeration cycle operations (IAC=60/60 min). The 16S rRNA gene copy numbers per mL of mixed-liquor were significantly reduced from $(3.38\pm0.07)\times10^9$ to $(2.74\pm0.10)\times10^8$ (samples 7-9, $p=0.0002$), when the combo system was operated at short aeration cycle (Fig. 4). The results were also consistent with the low NH_4-N and COD removal efficiencies observed at short aeration cycle (64% and 83%, respectively) (Table 2). On the contrary, samples (10-12) that was operated at 60/60 min of IAC showed increase bacterial gene abundance from $(4.87\pm1.15)\times10^9$ to $(9.17\pm1.78)\times10^9$ per mL of mixed-liquor (Fig.

4). Since the bacterial abundance has no apparent difference between 45/15 and 60/60 min of IACs (e.g., p=0.120 for sample 6 and 12), optimizing the combo system to work at 45/15 min aeration cycle could be more sustainable, which is consistent with the chemical analysis.

Fig. 4

3.2.2. Microbial community composition and diversity

After chimeric sequences are removed (<1%), quality sequences were affiliated to different OTUs. As shown in Table 3, a total of 383 and 375 OTUs were observed at 97% and 95% similarity cutoff, respectively. The initial OTUs (72) were higher than the OTUs (66) obtained at 60/60 min of IAC operation. Although further efforts needed to evaluate the microbial biodiversity in the reactor with other molecular techniques, this result may be a clue that the initial microbial inoculum (Quyang wastewater treatment plant) has been affected by the DO difference created in the MBR treatment system (IAC=60/60 min). Moreover, operations at IACs of 15/45 and 45/15 min showed higher OTUs; particularly the later operation showed relatively higher number of OTUs (81-84). At IAC_O, extending the growth period from 90 to 150 days showed almost the same number of OTUs and Shannon-Wiener diversity, which are consistent to qPCR results. However, Chao1 showed higher number at day 150 than 90, indicating extending the growth period could favor species richness than diversity (Table 3) (Dionisi et al., 2003; Sofia et al., 2004).

Shannon-Wiener index (higher numbers imply higher diversity) was found between 4.19-4.43, regardless of IAC and cutoff values (Table 3). This result was found comparable to other similar Shannon-Wiener diversity index reports (0.91-3.38) on wastewater treatment activated sludge (Xia et al., 2010; Duan et al., 2009). The co-existence of more diverse microbial populations in the combo system might offer a wide range of microbial metabolic diversity which could play efficient wastewater removal activity (Table 2). Although microbial diversity observed in this study seemed higher, yet the current sampling effort might be inadequate to

quantify all the microbial diversity found in the combo system. The linearity of the rarefaction curve (95% and 97%) suggested that diversity becomes more in the combo with an increasing number of sample sequences (Fig. S3).

Table 3

Samples from the different IACs were clustered using PCoA and environment clustering through weighted normalized UniFrac analysis (Fig. 5). Out of the total 54% variance observed, 29.12% and 24.89% variations were explained by the first (P1) and second (P2) coordinates, respectively (Fig. 5a). The initial DO where the microbial inoculum first collected (0.64 mg O_2/L) and IAC of 15/45 min (0.30-0.80 mg O_2/L) were almost found in the same range, which can group the two environments on the left side of the first axis. Sample 2 and 3 (IAC_O) and sample 5 (IAC=60/60 min) were also clustered on the right side of the first axis. However, using DO as final electron acceptor, sample 2 (day 90) and 3 (day 150) that was operated at the same IAC_O did not cluster on the same coordinate, instead the former clustered with sample 5 (day 90) as a function of growth period. This result indicates the insignificant difference in DO concentration and removal efficiency observed at extended IACs (Table 2). Normally, community clustering is caused by only one environmental factor at a time (Lozupone and Knight, 2005). This is consistent with the current study in which either DO level or growth period at a time cause for clustering. The Jackknife environment cluster analysis (Fig. 5b) was also consistent with PCoA.

Fig. 5

The phylogenetic classification using the SILVA taxonomy reference file at 80% threshold value showed that all of the sequences were grouped into 14 known bacterial phyla and a small proportion to unclassified sequences (about 5%) (Fig. 6a). In the entire operation, *Proteobacteria* (59%) followed by *Firmicutes* (12%) and *Bacteroidetes* (11%) were the most dominant phyla which together comprised 82% of the total sequences. The phyla *Actinobacteria,*

Chloroflexi, Nitrospirae and *Planctomycetes* were also detected at various operations (Fig. 6a). Interestingly, the phylum *Nitrospirae* which comprise a known NOB group was not detected at IAC of 15/45 min, but identified at IACs of 45/15 min and 60/60 min. This result clearly showed that the nitrifying (i.e. NOB) communities were sensitively responded to the DO concentration resulted from the different IACs. This finding was also consistent with the qPCR results that showed lower abundance (Fig. 4), and relatively poor NH_4-N removal (Table 2) observed at 15/45 min of IAC. Depending on IACs, other seven phyla were also detected. *Acidobacteria, Gemmatimonadates, Verrucomicrobia* and Candidate division-OP10 were detected only at IAC_O while *Chlorobi,* Candidate division-BRC1 and WCHB1-60 identified only at 15/45 min of IAC (Fig. 6a). Most phyla detected in the current study were also reported in previous studies carried on wastewater treatment systems (Liu et al., 2005; Cheng et al., 2008; Xia et al., 2010). The three dominant phyla which have been dominant here also found predominant in others. For example, Cheng et al. (2008) detected mainly *Bacteroidetes* followed by *Proteobacteria*, *Firmicutes* and *Actinobacteria* as dominant groups which together accounted for 93.1%.

During various IACs, community shifts were observed among phyla and unclassified bacteria. Samples taken after three months (day 90) showed that the *Proteobacteria* increased from the initial 31% to 56%, 62% and 63% at the IACs of 15/45, 45/15 and 60/60 min, respectively (Fig. 6a). The increase of *Proteobacteria* with increasing the aeration (''on'') cycle was accompanied by a decrease trend on the other phyla. For instance, *Firmicutes* decreased from the initial 26% to 18% and 4% at IACs of 15/45 and 45/15 min, respectively (Fig. 6a). The same decreasing trends were also observed in *Bacteroidetes* and unclassified bacteria. Moreover, compared 90^{th} and 150^{th} day sample that operated at the same IAC_O, an increased *Proteobacteria* percentage (83%) were observed for the later sampling date which totally unfavorable for *Firmicutes* (undetected) and other phyla, implying longer operation time could favor a specific group of bacteria (i.e. *Proteobacteria*) (Fig. 6a). This result agrees with Sofia et

al. (2004) investigation of microbial community in an A/O submerged membrane bioreactor that observed a complete microbial population shift to *Proteobacteria* at long sludge age.

The community shifts observed among different phyla were also happened at class level on the same phylum. For example, within the predominant phylum *Proteobacteria*, the four major classes such as *Alphaproteobacteria, Betaproteobacteria* (dominant)*, Deltaproteobacteria* and *Gammaproteobacteria* showed significant community shifts depending on aeration cycles (Fig. 6b). When the combo system operated at IAC_O, the *Betaproteobacteria* increased over time from 50% (day 90) to 88.6% (day 150), while the proportion of the other *Proteobacteria* classes decreased (Fig. 6b). The same trend of community dynamics were also observed when the combo system operated at IAC of 60/60 min. Comparing IACs of 45/15 min (day 90 and 150) and 60/60 min (day 90), more proportion of *Betaproteobacteria* community were observed for samples collected at day 150. The 90^{th} day sample for both 45/15 and 60/60 min of IACs exhibited almost proportional *Proteobacteria* population, indicating that DO level was not the limiting factor at these two operations for *Proteobacteria* rather the sludge age. This was consistent with the UniFrac results shown in Fig. 5. Furthermore, at shorter aeration cycle (IAC=15/45 min), the proportion of *Betaprotobacteria* and *Alphaproteobacteria* were equally dominant (36%) followed by *Gammaproteobacteria* (25%) and *Delatprotobacteria* (3%).

In a more specific phylogenetic view, the total sequences were placed in 32 orders and some unclassified bacteria (Fig. 6c), among which 15 bacterial orders were affiliated to *Proteobacteria* (with ≥ 80% similarity) (Table S1). *Burkholderiales* and *Rhodocyclales* from *Betaprotobacteria* and *Rhodospirillales* and *Rhizobiales* from *Alphaproteobacteria* were detected from 3.4-18% of the total sequence orders (Fig. 6c and Table S1). *Burkholderiales*, the dominant order in this study (particularly sample 3), were previously identified in MBR system operated under aerobic-anaerobic condition (Sadaie et al., 2007), which indicate its metabolic flexibility. Members from *Rhodocyclales*, a purple non-sulfur photosynthetic bacteria, was

identified as phosphate accumulating organism that use either oxygen or nitrate as final electron acceptor (Seviour et al., 2003). Members of the phylum *Actinobacteria* such as *Acidimicrobidae and Actinobacteridae* (Gram-positive bacteria with high DNA G+C content) and *Gemmatimonadales* (97% similarity with phylum *Gemmatimonas*) were detected in the current study also reported as a predominate group of bacteria in phosphate-removing activated sludge (Seviour et al., 2003; Liu et al., 2005) (Fig. 6c and Table S1). Interestingly, *Pseudomonadales* and *Aeromonadales* from *Gammaproteobacteria* group were detected in this study had also been previously reported denitrifying bacteria (Seviour et al., 2003; Sofia et al., 2004). The order *Planctomycetales* detected in the current study (Fig. 6c) was also identified as denitrifying bacteria which play anammox (anaerobic ammonia oxidation) activity under anoxic/anaerobic environment (Zhang et al., 2012). Moreover, *Bacillales* (low DNA G+C content and Gram-positive bacteria) from the phylum *Firmicutes,* were also detected (Fig. 6c) and identified previously as denitrifying bacteria (Sofia et al., 2004; Cheng et al., 2008). *Nitrosomonadales* (*Betaproteobacteria*) and *Nitrospirales* (*Nitrospirae*), which has been previously reported as the primary AOB and NOB, respectively in wastewater treatment systems (Sofia et al., 2004; Ahmed, 2012) were identified here (Fig. 6c). In this study, the co-existence of nitrifying and denitrifying communities in a single reactor demonstrated that SND activities occurred in the combo system, which was also supported by the chemical analyses (Tables 1). All *Bacteroidetes* related phylotypes were affiliated to the order *Sphingobacteriales* and *Flavobacteriales* which was consistent with others study (Cheng et al., 2008). The *Sphingobacteriales* groups were known for their interesting catabolic capabilities to degrade a wide variety of environmentally pollutants (Fig. 6c).

Fig. 6

4. Conclusions

In this study, attempts have been made to relate the effect of IACs on nutrient removal and microbial community structure. At IAC_O (on/off=45/15 min), the combo system was able to achieve very good nutrient (PO_4-P=92.3±4.1% and NH_4-N=98.7±2.1%) and organic matter (COD=99.4±0.6%) removals. Particularly, phosphorus recovery in FBR as struvite is a unique sustainable feature of the combo system. The NE (98.1±2.2) and SND activities (82.8±11.7%) were also achieved due to the co-existence of diverse and stable microbial communities in MBR. The phylum *Proteobacteria* (59%), *Firmicutes* (12%) and *Bacteroidetes* (11%) were the dominant bacterial groups, which showed shifts depending on IACs.

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Figure captions

Fig. 1. The pH values (n=72) of feed, FBR, MBR and effluent that operate at optimum (MBR_O) and variable (MBR_V) conditions. IAC-on and IAC-off is used to indicate pH measurements carried out in the MBR under air pump working and pausing periods, respectively. The MBR effluent pH was the combined result that flows during “on” and “off” cycles.

Fig. 2. Performance of FBR-MBR_O combo system through time (a) NH_4-N and COD concentration (b) MLSS and MLVSS concentration.

Fig. 3. Effect of different IAC (a) soluble microbial product (SMP) and extracellular polymerase substance (b) Transmembrane pressure (TMP) and mixed liquor suspended solids (MLSS).

Fig. 4. Total bacterial copy numbers of 16S rRNA gene per mL of mixed-liquor for initial (sample 1), 45/15 min (sample 2, 3, 4, 5 and 6 collected at day 30, 60, 90, 120 and 150, respectively), 15/45 min (sample 7, 8 and 9 collected at day 30, 60 and 90) and 60/60 min (sample 10, 11 and 12 collected at day 30, 60 and 90) aeration cycles for FBR-MBR combo systems as revealed by the SYBR Green I based qPCR method.

Fig. 5. UniFrac analysis using 16S rRNA gene sequences recovered from mixed-liquor (a) P1 and P2 are plots of the first two principal coordinate axes on which the percentages in bracket represents the variation explained by the coordinates. Sample 1 (Initial), sample 2 and 3 (IACO=45/15 min), sample 4 (IAC=15/45 min) and sample 5 (IAC=60/60 min) were sludge samples collected at day 90 except sample 3 (day 150) (b) normalized weighted Jackknife environment clusters of sample 1, 2, 3, 4 and sample 5 (description as (a)).

Fig. 6. Classification of the 16S rRNA gene sequences of clone library from FBR-MBR combo system (a) total phyla detected (b) class level category of *Proteobacteria* (c) order level category of total bacteria. Sample 1 (initial), sample 2 and 3 (IAC_O= 45/15 min), sample 4 (IAC=15/45 min) and sample 5 (IAC= 60/60 min). All samples collected at day 90 except sample 3 (day 150).

Table 1

Summary of operating conditions and results.

Conditions	Unit	FBR-MBR$_O$	FBR-MBR$_V$
IAC (on/off)	min	45/15	15/45, 30/30, 60/30, 60/60
Initial flux rate	L/(m^2×h)	70	70
Hydraulic retention time	h	6	6
Sludge retention time[a]	d	without sludge withdraw	without sludge withdraw
Molecular sampling time[b]			
qPCR	d	0, 30, 60, 90,120,150	0, 60, 90
Clone library	d	0, 90,150	0, 90
Influent COD[c]	mg/L	250-350	250-350
Influent NH_4-N[c]	mg/L	40-60	40-60
Influent PO_4-P[c]	mg/L	10-13	10-13
Effluent COD	mg/L	<2	1-40
Effluent NH_4-N	mg/L	<5	1-15
Effluent PO_4-P	mg/L	<1	<1
Effluent NO_3-N	mg/L	8.32±4.6	<30
Effluent NO_2-N	mg/L	0.15±0.04	<1
MLSS	mg/L	4.6±0.7	3.0-9.2
MLVSS	mg/L	4.0±0.2	3.3-8.4
Nitrification efficiency	%	98.1±2.2	>85
SND	%	82.8±11.7	>50
Turbidity	%	>99.8	>99.6
Temperature	^oC	25.2±1.8	25.2±1.8

[a]=90-150 days, [b] =sample was collected from IACs of 15/45, 45/15 and 60/60 min, [c]=raw feed concentration before FBR treatment (which contribute 27-33%, 37-39% and 85-90% removals of COD, NH_4-N and PO_4-P, respectively)

Table 2

Effect of intermittent aeration cycles on DO concentration (mg/L) and removal efficiency (%) in the combo system.

IAC (on/off) (min)	DO (mg/L)		FBR			FBR-MBR		
	on	off	COD	NH_4-N	PO_4-P	COD	NH_4-N	PO_4-P
60/60	6.1±0.5	3.4±0.2	27.4±5.5	39.0±6.6	89.1±2.0	99.5±0.5	99.1±0.8	91.0±1.8
60/30	4.4±0.7	1.6±0.5	31.7±3.8	38.2±3.2	88.2±2.7	99.0±1.0	99.0±0.5	94.8±1.7
45/15[a]	3.6±0.6	1.5±0.6	28.8±5.8	37.1±10.1	85.4±4.7	99.4±0.6	98.7±2.1	92.3±4.1
30/30	1.5±0.3	0.6±0.3	28.2±8.3	38.6±8.1	90.1±1.0	88.2±2.6	76.7±4.0	93.7±1.5
15/45	0.8±0.2	0.3±0.2	32.7±4.4	37.7±5.4	86.5±3.8	83.3±7.3	64.3±9.0	92.1±1.3

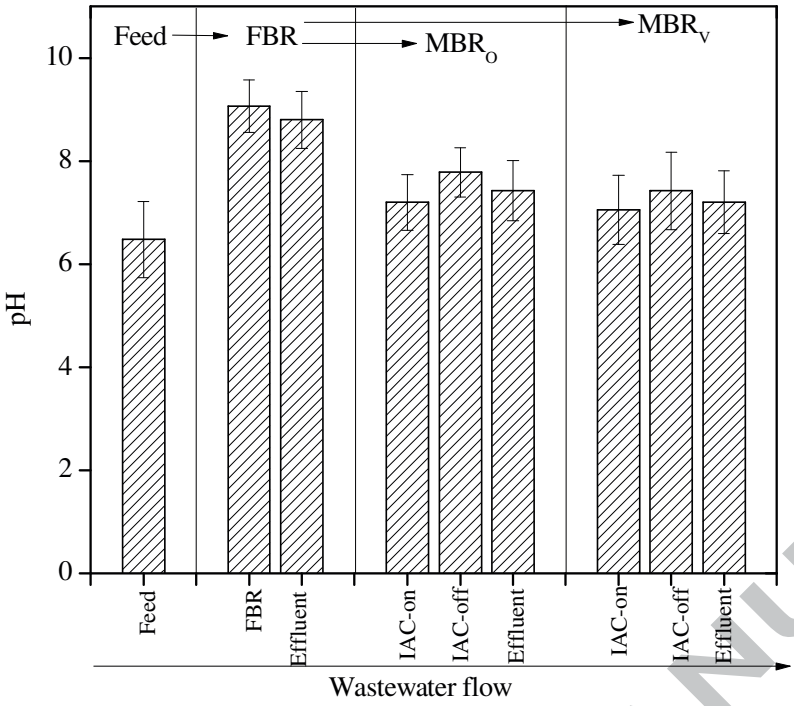
[a]=results obtained from IAC_O

Table 3

Characteristic and diversity estimators for 16S rRNA gene clone libraries derived from initial and experimental samples.

IAC (on/off) (min)	Time (day)	0.03 (97% similarity)			0.05 (95% similarity)		
		OTUs	Chao1	Shannon	OTUs	Chao1	Shannon
-	0	72	2628	4.28	71	1279	4.26
45/15	90	83	1744	4.41	81	832	4.38
45/15	150	84	3570	4.43	83	1744	4.41
15/45	90	78	1541	4.35	74	678	4.28
60/60	90	66	2211	4.19	66	2211	4.19

IAC=intermittent aeration cycle, OTUs=operational taxonomic units, -=IAC operation not considered (initial sample)



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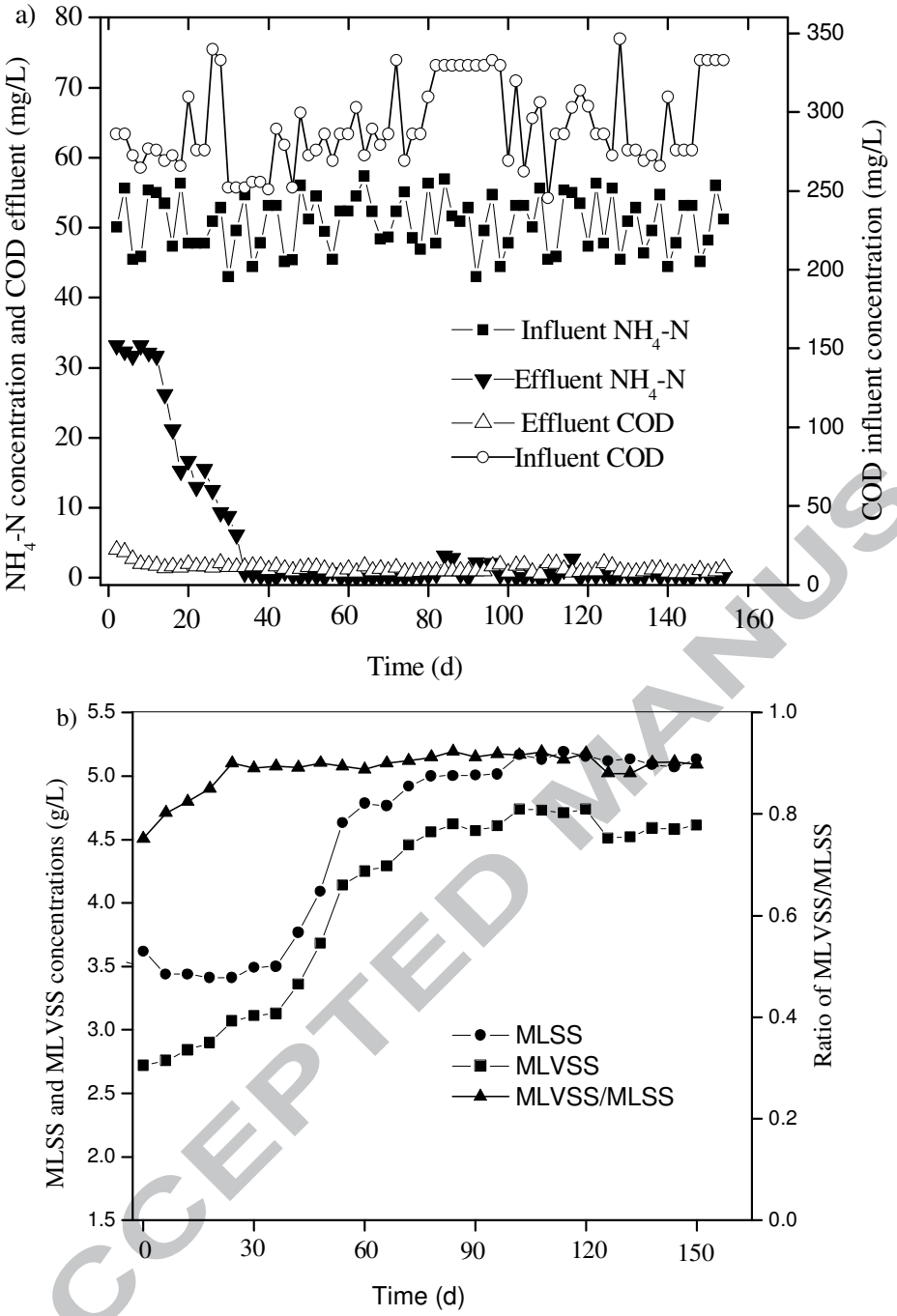
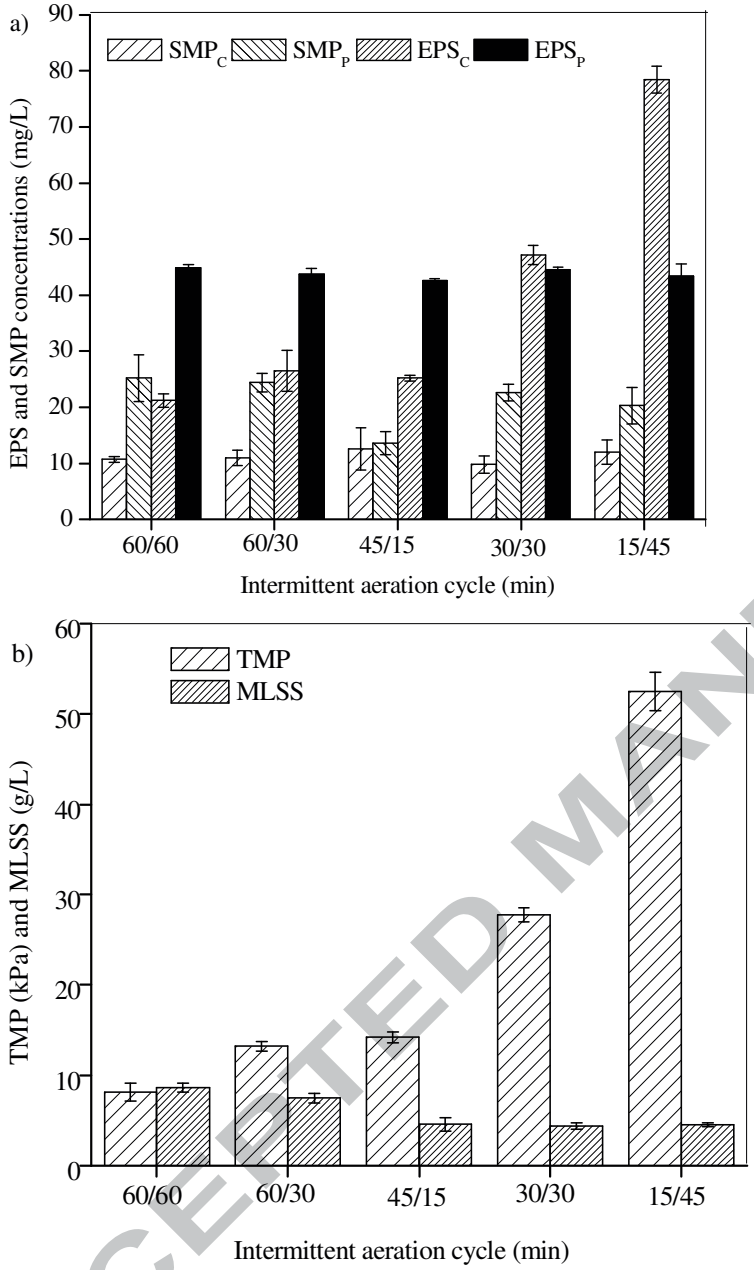


Fig. 2. Performance of FBR-MBR$_O$ combo system through time (a) NH_4-N and COD concentration (b) MLSS and MLVSS concentration.



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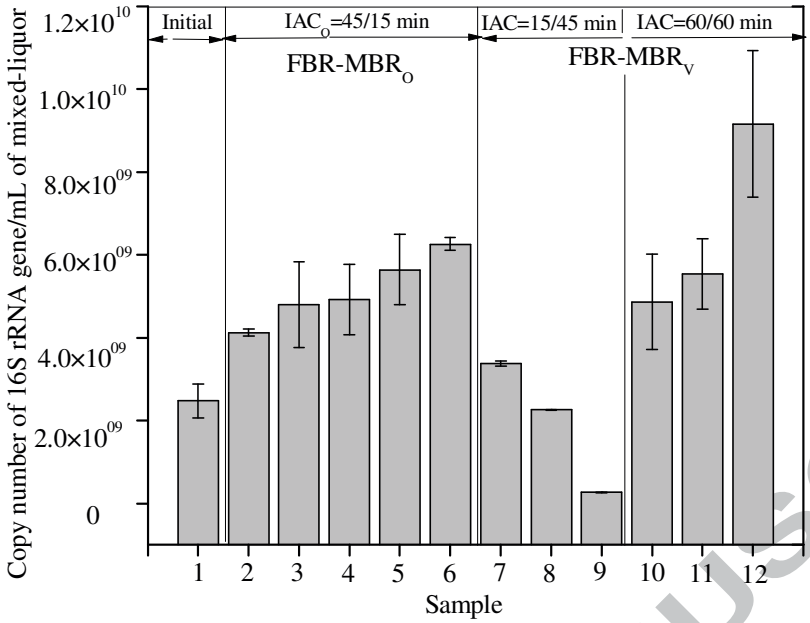

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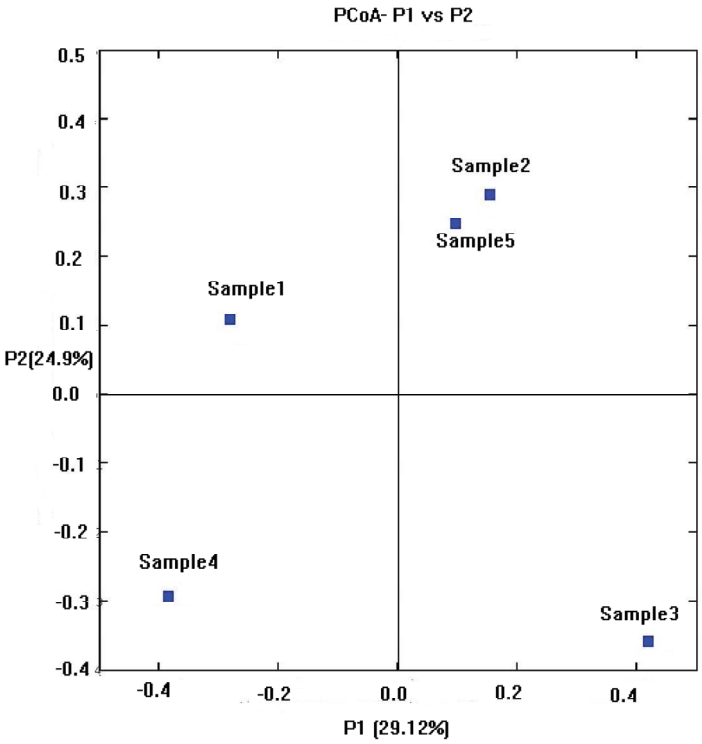
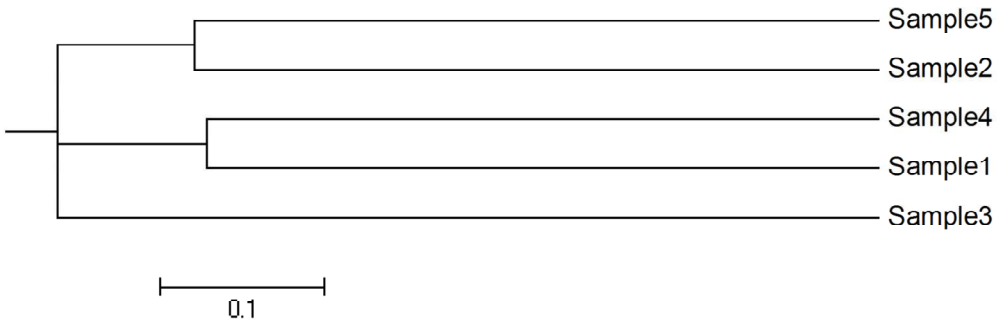
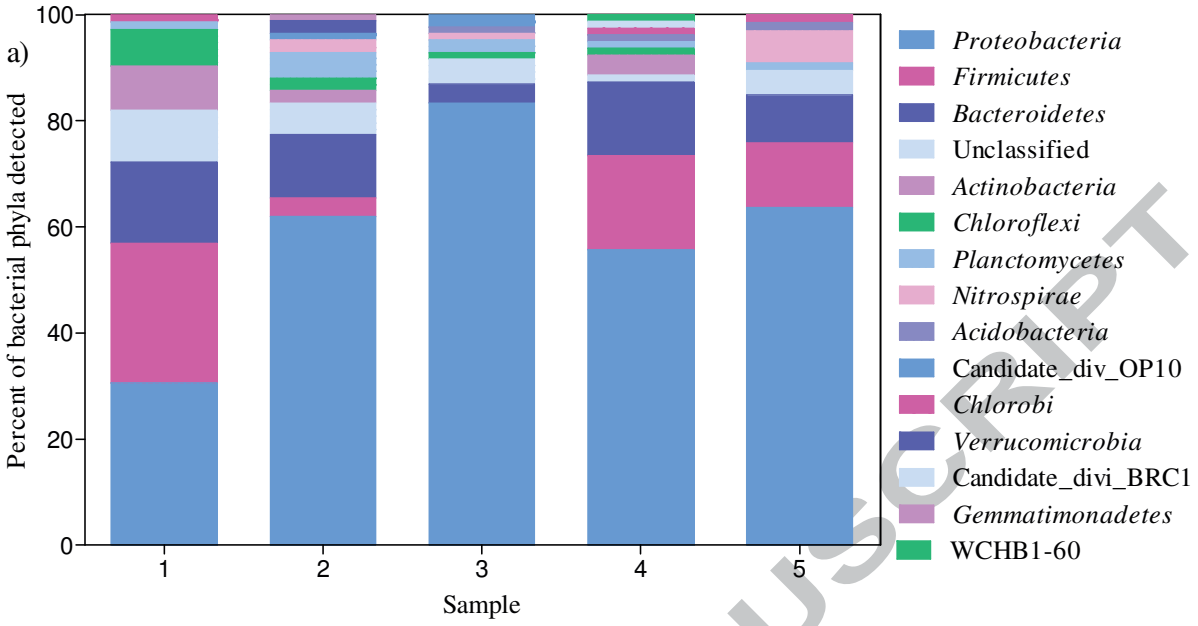


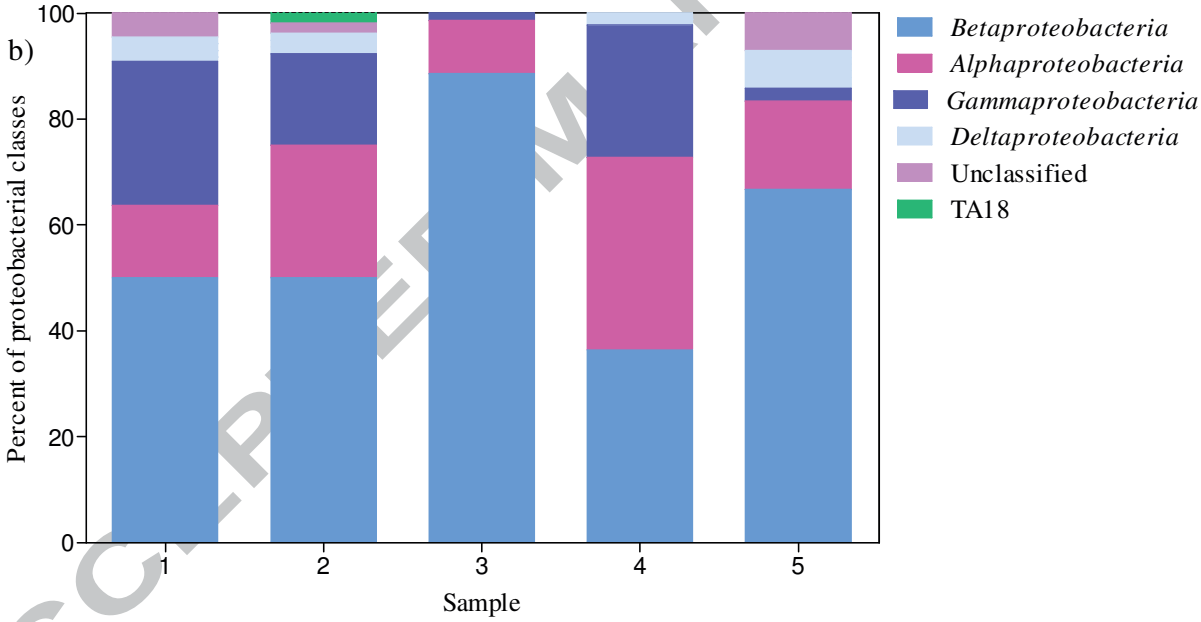
a) Principal coordinate analyses (PCoA)



b) Jackknife

Fig. 5. UniFrac analysis using 16S rRNA gene sequences recovered from mixed-liquor (a) P1 and P2 are plots of the first two principal coordinate axes on which the percentages in bracket represents the variation explained by the coordinates. Sample 1 (Initial), sample 2 and 3 (IACO=45/15 min), sample 4 (IAC=15/45 min) and sample 5 (IAC=60/60 min) were sludge samples collected at day 90 except sample 3 (day 150) (b) normalized weighted Jackknife environment clusters of sample 1, 2, 3, 4 and sample 5 (description as (a)).





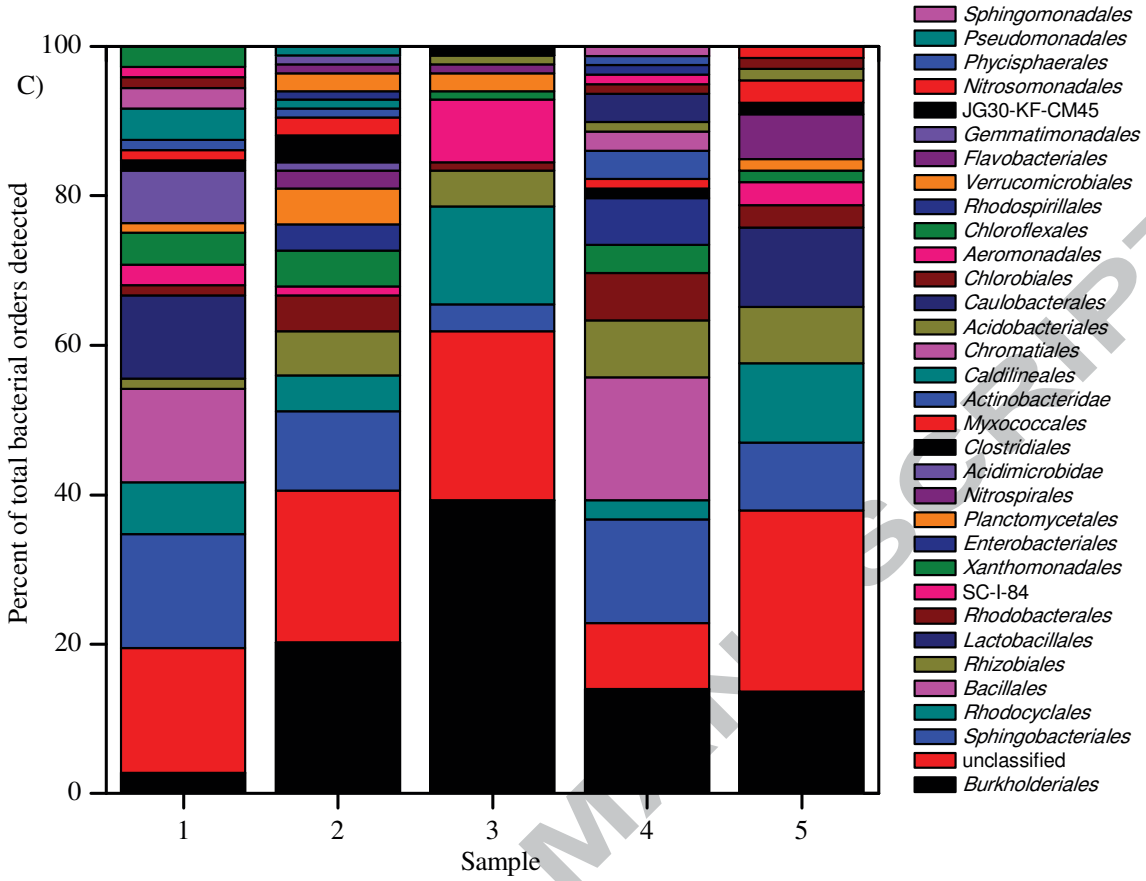


Fig. 6. Classification of the 16S rRNA gene sequences of clone library from FBR-MBR combo system (a) total phyla detected (b) class level category of *Proteobacteria* (c) order level category of total bacteria. Sample 1 (initial), sample 2 and 3 (IAC_O= 45/15 min), sample 4 (IAC=15/45 min) and sample 5 (IAC= 60/60 min). All samples collected at day 90 except sample 3 (day 150).

Highlights

- Complete nitrification, high SND and phosphorus recovery were achieved in FBR-MBR.
- The pH in FBR and MBR were found working in harmony.
- The FBR-MBR combo system was a suitable niche for diverse microbial groups.
- Varying intermittent aeration cycle resulted in microbial community shift.
- *Proteobacteria*, *Firmicutes* and *Bacteroidetes* were the dominant phyla identified.