Determination of Microbial Community in a Pilot Scale Two-Stage Step-Feed Biological Nutrient Removal Process

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Abbreviations and symbols

ANA	Anaerobic tank
AO1	First-stage anoxic tank
AO2	Second-stage anoxic tank
BOD	Biochemical oxygen demand
BSA	Bovine serum albumin
C	Carbon
COD	Chemical oxygen demand
DGGE	Denaturing gradient gel electrophoresis
HRT	Hydraulic retention times
IR	Internal recycle
IR1	Internal recycle from aerobic1 to anoxic1
IR2	Internal recycle from aerobic2 to anoxic2
MLSS	Mixed liquor suspended solids
N	Nitrogen
N_2	Nitrogen gas
NH_3-N	Ammonia nitrogen
NH_4^+-N	Ammonium nitrogen
NO_2^--N	Nitrite nitrogen
NO_3^--N	Nitrate nitrogen
O1	First-stage aerobic tank
O2	Second-stage aerobic tank
PCR	Polymerase chain reaction
PO_4^{3-}-P	Phosphate phosphorus
Q1	Inflow anoxic2 tank
Q2	Inflow anaerobic tank
RAS	Return activated sludge
SRT	Sludge retention time
SS	Suspended solids
STD	Standard deviation
TKN	Total Kjeldahl nitrogen
TN	Total nitrogen
TP	Total phosphorus
VSS	Volatile suspended solids

Abstract
Microbial community was determined in a pilot-scale two-stage step-feed biological nutrient removal system treating municipal wastewater with 10 m^3 d^{-1} capacity. Grit chamber effluent at Istanbul Ataköy Biological Wastewater Treatment Plant was used as influent wastewater. In the pilot plant, the influent wastewater was split into two fractions to anaerobic and anoxic2 tank. *Nitrosomonas*, *Nitrosospira*, *Accumulibacter*, and *Dechloromonas* along with some other uncultured microorganisms were determined in the aerobic, anoxic and anaerobic stages. COD, TN, NH_4^+-N, TP, PO_4^{3-}-P, SS, and VSS removal efficiencies were found to be 86.7%, 80.3%, 92.5%, 89.5%, 87.5%, 94.8%, and 95.0% in average, respectively, at 5000 mg MLSS L^{-1}, 15 days of SRT and 16 hours of HRT. The results indicated that microbial community in the process was quite similar with those in the nutrient removal processes with no step feeding. This process can be used cost-effectively to remove carbon and nutrients from medium-strength municipal wastewaters.

Keywords: Biological nutrient removal, Step feeding, Municipal wastewater, Microbial community, PCR-DGGE

1. Introduction
Due to uncontrolled discharge of wastewaters, septic conditions and offensive odor problems may develop in receiving water bodies. The main polluting parameters in wastewaters that cause nuisance are known as organic pollutants, nitrogen, and phosphorous (Wang et al. 2008). The nitrogenous and phosphorous compounds in wastewaters cause environmental problems such as eutrophication, depletion of dissolved oxygen, or toxicity unless handled properly prior to discharge into receiving waters (Ding et al. 2011; Usharani and Lakshmanaperumalsamy 2010; Zeng et al. 2009; Oehmen et al. 2007; Kim et al. 2005; Sommariva et al. 1996).
Nitrification-denitrification processes are of the most cost-effective methods for the removal of nitrogenous species from municipal wastewaters (Ding et al. 2011; Gupta and Gupta 2001). These processes involves oxidation of ammonia-nitrogen (NH_3-N) to nitrite-nitrogen (NO_2^--N) and nitrate-nitrogen (NO_3^--N), respectively, and then back to nitrite-nitrogen (NO_2^--N) and nitrogen gas (N_2) at considerably high rates. You and Chen (2008) reported that the rate of denitrification for nitrite is 1.5-2 times higher than that of nitrate, in that the rate of sludge generation in nitrite accumulation processes decreased by 33-35% and 55% in nitrification and denitrification stages, respectively.
It is well known that denitrification of wastewaters with high total nitrogen levels requires excessive amounts of organic carbon. As the municipal wastewaters generally contain low carbon to nitrogen (C:N) ratios, it is difficult to achieve a complete removal of total nitrogen in such wastewaters during the anoxic stage (Zeng et al. 2010; Guo et al. 2007). The influent wastewater must have a BOD:TKN (biochemical oxygen demand to total Kjeldahl nitrogen) ratio of 3:1 to ensure denitrification (Jeyanayagam 2005). For wastewaters with low organic carbon contents, an external carbon source might be used to promote nitrogen removal (Kampas et al. 2009; Lim et al. 2008; Thomas et al. 2003). Methanol or acetate can be used as an external carbon source to favor total nitrogen removal in carbon-limited wastewaters. Their excessive use may, however, lead to more accumulation of soluble microbial products in effluent, and thus it might be necessary that these species should be added in appropriate amounts to avoid excessive sludge production (Sattayatewa et al. 2009). Use of an external carbon source also increases operating costs due to more sludge production (Gao et al. 2011). Step feeding, on the other hand, eliminates the need for external carbon and reduces treatment cost. Step feeding is accomplished by splitting the influent wastewater into various stages of the treatment process (Amand 2008). To eliminate the use of external carbon source, the influent wastewater is divided between the anaerobic and anoxic stages. Some previous

studies have showed that step-feed nitrification-denitrification systems offer a number of advantages including higher nutrient removal efficiencies and economic feasibility (Vaiopoulou et al. 2007). Besides, step feeding offers flexible operating opportunities (Amand 2008).

A detailed analysis of microbial community is extremely important to improve the performance of wastewater treatment processes. The microbial population differs significantly with respect to the changes in wastewater characteristics and operating conditions. Understanding the mechanisms involved in biological treatment processes requires a critical evaluation of microbial composition in activated sludge systems (Hesham et al. 2011). Activated sludge typically contains bacteria, protozoa, fungi, metazoan, viruses, and algae. 95% of microbial population in activated sludge is comprised of bacteria (Liu et al. 2007). Of bacteria, species including β- and α-*Proteobacteria* (Ahmed et al. 2008), *Dechloromonas* (Hallin et al. 2006), *Accumulibacter* (Carvalho et al. 2007), *Acinetobacter* (Lin et al. 2003), *Nitrospira*, *Nitrosovibrio*, *Nitrobacter* (Li et al. 2006), *Nitrosomonas* (Whang et al. 2009) are responsible for the various stages of the treatment. The number of species shows an increasing trend with increasing SRT while species such as beta-proteobacteria exist for all SRTs (Duan et al. 2009).

Microbial composition of activated sludge is based on 16S rRNA gene sequence analyses, of which denaturing gradient gel electrophoresis (DGGE) on PCR-amplified partial 16S rRNA sequence has been one of the most widely used techniques for this purpose (Karadag et al. 2013; Sanz and Köchling 2007).

In this study, microbial community was determined in a pilot-scale two-stage step-feed biological nutrient removal process treating the effluent wastewater from the grit chamber unit in Istanbul Ataköy Biological Wastewater Treatment Plant (Turkey). The pilot-scale plant was configured to feed half of the inflow to the second anoxic tank to prevent the need for external carbon source.

2. Methods

2.1. Two-stage step-feed biological nutrient removal process

The pilot-scale, two-stage step-feed biological nutrient removal process used in this study was installed in Ataköy Biological Wastewater Treatment Plant of the Istanbul Water and Sewerage Administration (Istanbul/Turkey). The pilot-scale plant has an active volume of 8.6 m^3 and consists of a primary sedimentation tank (0.25 m^3), an inflow distribution tank (0.25 m^3), an anaerobic tank - ANA (0.5 m^3), a first-stage anoxic tank – AO1 (anoxic1, 1.4 m^3), a first-stage aerobic tank – O1 (aerobic1, 1.7 m^3), a second-stage anoxic tank – AO2 (anoxic2, 1.4 m^3), a second-stage aerobic tank – O2 (aerobic2, 1.7 m^3), and a final sedimentation tank (1.4 m^3). The capacity of the plant is 10 m^3 d^{-1}. The inflow to the system was withdrawn from the effluent of grit removal unit of the full-scale plant. In order to eliminate the need for external carbon source in the second stage, 5 m^3/d of inflow (Q1) was fed to the anoxic2 tank while the other 5 m^3 d^{-1} (Q2) was taken into the anaerobic tank (Q1:Q2= 50-50%) (Manav Demir et al. 2016a). The flow diagram of the pilot-scale plant is shown in Fig. 1. The return activated sludge (RAS) ratio was 80%. Two internal recycle (IR) lines were responsible of returning the nitrate from aerobic1 to anoxic1 (IR1), and from aerobic2 to anoxic2 (IR2). The IR ratios were kept constant around 4.0. The mixed-liquor suspended solids (MLSS) concentration was kept in a range of 4500 to 5500 mg L^{-1} and the sludge retention time (SRT) was 15 days. The process was inoculated with the sludge from the RAS line of the full-scale plant.

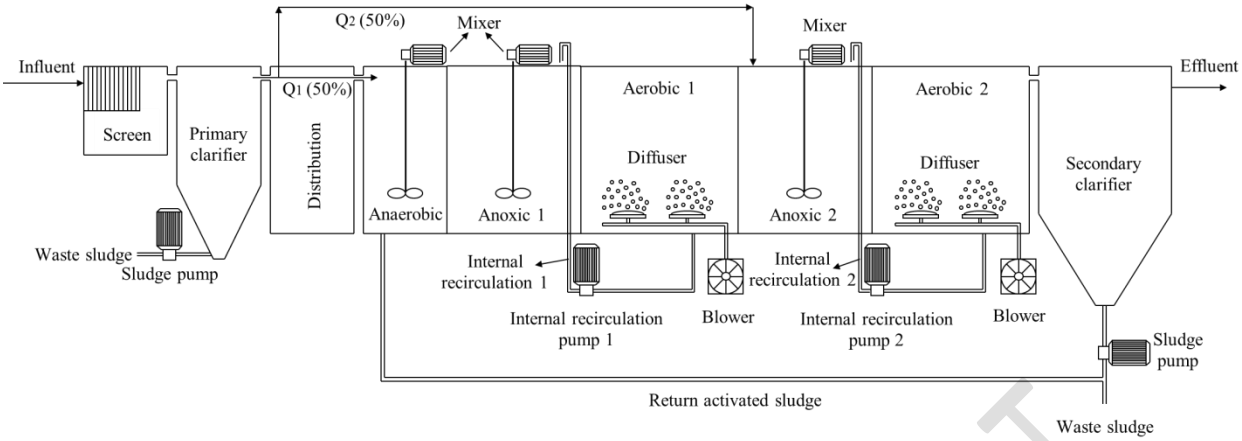

Figure 1. Schematic representation of the pilot-scale plant (adapted from Manav Demir, 2012)

2.2. Wastewater characteristics

The effluent from the grit chamber of the full-scale plant was used in the experimental work. The influent wastewater was periodically analyzed to determine the concentrations of various parameters such as COD, nitrogenous-phosphorus species, suspended solids (SS), and volatile suspended solids (VSS). The results are summarized in Table 1 along with their standard deviations, and minimum-maximum values.

Table 1. The characteristics of raw domestic wastewater (previously published in Manav Demir et al. 2016a)

Parameter, unit	Mean value[a]	STD[b]	Min.	Q1[c]	Q2[d]	Q3[e]	Max.
COD, mg L^{-1}	556	60	420	521	560	600	670
TKN, mg L^{-1}	71.8	6.8	63.4	67.8	71.5	74.7	92.2
NH_4^+-N, mg L^{-1}	44.0	6.7	35.7	39.6	42.5	45.6	59.6
NO_2^--N, mg L^{-1}	0.03	0.02	0.01	0.01	0.03	0.04	0.06
NO_3^--N, mg L^{-1}	0.06	0.04	0.01	0.03	0.06	0.09	0.15
TN, mg L^{-1}	71.9	6.8	63.5	67.8	71.5	74.8	92.3
TP, mg L^{-1}	8.1	0.4	7.4	7.8	8.0	8.4	9.2
PO_4^{3-}-P, mg L^{-1}	4.0	0.5	2.9	3.7	3.9	4.2	5.0
SS, mg L^{-1}	316	47	234	287	322	352	390
VSS, mg L^{-1}	230	33	172	202	234	258	280

[a]Average value in 20 samples, [b]STD: Standard deviation from 20 data points, [c]Q1: First quartile, [d]Q2: Median value, [e]Q3: Third quartile

2.3. Analytical methods

During the steady-state operation of the pilot-scale process, samples were collected from plant influent and effluent twice a week and analyzed for COD, NH_4^+-N, NO_3^--N, NO_2^--N, TP, PO_4^{3-}-P, SS, and VSS using standard methods. The analysis of each sample or parameter was performed in triplicate.

Activated sludge samples were also collected from each stage of the process (anaerobic, anoxic1, aerobic1, anoxic2, and aerobic2), and the microbial communities were determined according to a procedure, described in Manav Demir (2012) and Manav Demir et al. (2016b). Power Soil DNA Isolation Kit (MOBIO Laboratories) was used for DNA isolation. Amplification was performed using BIO-RAD Mycycler Thermal Cycler System with 5µL 10xPCR buffer, 1.5 mM $MgCl_2$, 0.8 mM dNTPmix (deoxynucleotide triphosphate, dATP, dCTP, dGTP, dTTP), 0.2 µM each of primer (27F: 5'-AGAGTTTGATCCTGGCTCAG-3'; 1492r: 5'-GGYTACCTTGTTACGACTT-3'), 0.2 mg/mL BSA (bovine serum albumin), 0.048U/µL Polymerase (FINNZYMES, DyNAzymeTMII), and template DNA as well as sterile Millipore water up to 50 µL. The temperature

program for PCR1 was set as 3 minutes of initial denaturation at 94°C, followed by 30 seconds of denaturation at 94°C, 30 seconds of annealing at 55°C, 2 minutes of extension at 72°C (30 cycles), and finally 5 minutes of final extension at 72°C. The procedure ended at 4°C.

The bacterial primer pairs 357F-GC and R518 (357F-GC: 5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3' and R518: 5'-ATTACCGCGGCTGCTGG-3') were used in the second stage PCR (PCR2). The temperature program in the second stage was adjusted to 3 minutes of initial denaturation at 94°C, followed by 30 seconds of denaturation at 94°C, 30 seconds of annealing at 65°C, and 45 seconds of extension at 72°C (20 cycles with annealing temperature decreased by 0.5°C in every two cycles). The temperature program was extended with 30 seconds of denaturation at 94°C, followed by 30 seconds of annealing at 55°C and 45 seconds of extension at 72°C (10 cycles). The final extension step was 10 minutes at 72°C and the finishing temperature was 4°C. The PCR products were electrophoresed on a 1% (wt/vol) agarose gel. DGGE was performed using a Bio-Rad Dcode mutation detection system (Bio-Rad, USA) with an 8% polyacrylamide gel (ratio of acrylamide to bisacrylamide, 37.1:1) with 25% to 65% denaturing concentrations in 1xTAE buffer as previously described (Bio-Rad Manual, USA). The electrophoresis followed 60 volts at 60°C for 30 minutes and 120 volts at 60°C for 4 hours. The gel was stained with SYBR-Gold (1,000 x concentration) for 30 minutes and visualized on a UV transilluminator. The bands in DGGE gel were cut and eluted in 20 µL of sterile H_2O overnight. Nucleic Acid Extraction Kit (GF-1) was used for purification. Sequence data were analyzed by database searches in GenBank using BLAST software. A phylogenetic tree was constructed by the neighbor-joining method using the Unipro UGENE v.1.9.1.

3. Results

3.1. Pilot Plant Performance

The input COD to the pilot-scale reactor was 556±60 mg L^{-1}. The average COD removal efficiency was observed around 86.7±10.4%. The mean influent and effluent NH_4^+-N concentrations were found to be about 44.0±6.7 mg L^{-1} and 3.0±4.0 mg L^{-1}, respectively, corresponding to a removal efficiency of 92.5±10.1%. The results have also demonstrated that about 80.3±11.0% of total nitrogen (TN) was removed during the treatment. The mean influent/effluent concentrations for NO_2^--N and NO_3^--N were measured as "0.03±0.02/0.55±0.21" and "0.06±0.04/2.08±0.59" mg L^{-1}, respectively.

The initial and final concentrations for total phosphorus (TP) were determined around 8.1 and 0.9 mg L^{-1}, respectively, which corresponds to a removal efficiency of 89.5±6.8%. Similar results were also observed for the removal of phosphate as PO_4^{3-}-P with influent and effluent concentrations of 4.0±0.5 mg L^{-1} and 0.5±0.4 mg L^{-1}, respectively (Manav Demir et al. 2016a). Fig. 2 shows the change of influent and effluent concentrations of COD, SS, TP, PO_4^{3-}-P, NH_4^+-N, TN, NO_3^--N, and NO_2^--N along with their respective removal efficiencies in the whole steady-state period.

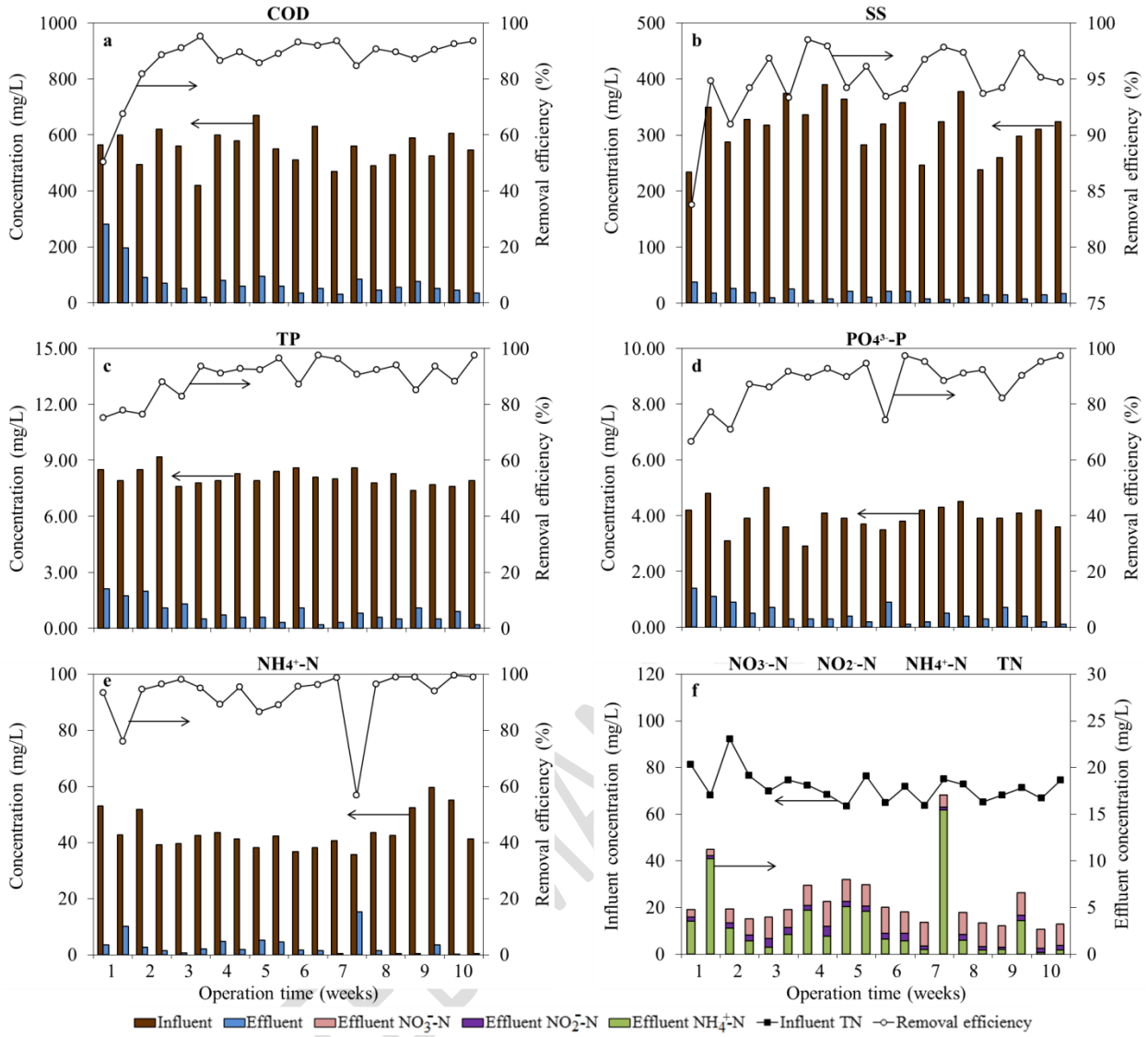

Figure 2. The change of influent and effluent concentrations along with removal efficiencies for (a) COD, (b) SS, (c) TP, (d) PO_4^{3-}-P, (e) NH_4^+-N, (f) TN, NO_3-N, and NO_2-N

The removal efficiencies for species were examined using descriptive statistics as shown in Fig. 3. The lower and upper whiskers in the figure represent the 10th and the 90th percentiles of the time series for the removal efficiencies, respectively. The lower and upper ends of the boxes were 25th and 75th percentiles, while the black lines within the boxes represent the median values. The red lines in the figure correspond to the mean values of the removal efficiencies.

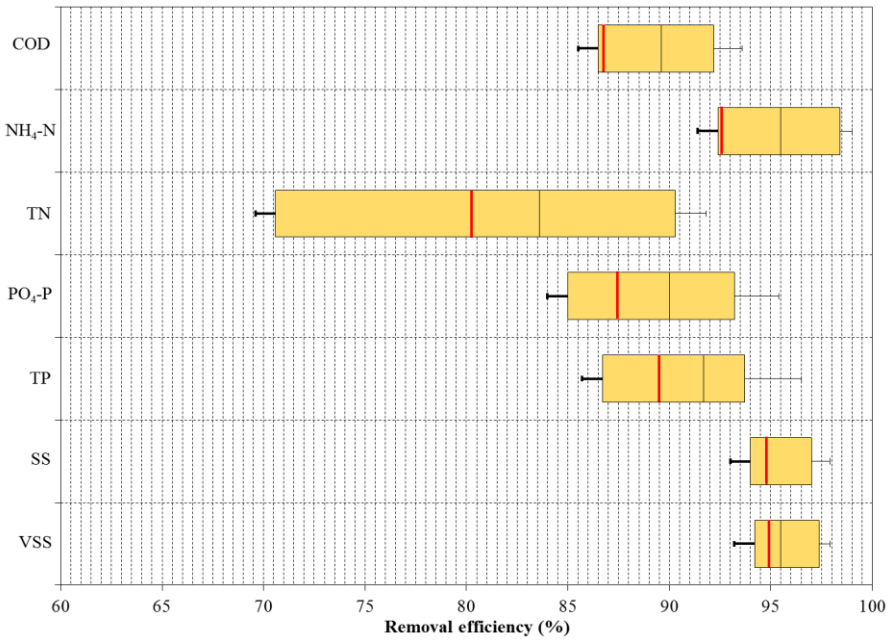

Figure 3. Descriptive statistics of removal efficiencies

While the average COD removal efficiency was around 87%, the median value reached up to 90% indicating that the time series of COD removal efficiencies skewed upwards from the mean value. The 90th percentile value for COD removal efficiency was found to be equal to or higher than 93%. These results clearly imply that a sufficient amount of COD was removed following the treatment of municipal wastewater in the pilot reactor system.

The 10th and 90th percentiles of NH_4^+-N removal efficiencies were in a range of 91% to 99%. The mean value was determined around 95.5%, which was actually lower than the median value. Such a difference between mean and median values indicates that the time series for the NH_4^+-N removal efficiencies are leaning upwards, and thus the system involves an effective nitrification of ammonium. Similarly, time series for TN removal efficiencies was positively skewed, but the removal efficiencies for total nitrogen (≤ 80%) were lower than those obtained for ammonium. This is actually due to the fact that not all ammonium was converted to the nitrogen gas, some of which was accumulated as nitrate during the nitrification-denitrification process. Similar trends were observed for PO_4^{3-}-P and TP removal efficiencies. The 10th and 90th percentiles for PO_4^{3-}-P removal efficiencies were 84% and 95.5%, respectively, while those for TP were 85.5% and 96.5%, respectively. The mean removal efficiencies for both PO_4^{3-}-P and TP were lower than the median values, indicating that the time series were positively skewed.

Finally, descriptive statistics were performed for the data on SS and VSS removal efficiencies to evaluate the settling characteristic of the sludge in secondary sedimentation tank. Results have demonstrated that the 10th percentiles for both parameters were higher than 93%, which means the sludge had a fairly good settling characteristic.

3.2. Microbial community composition

Microbial species were determined in samples of anaerobic, aerobic and anoxic treatment units. A picture of the gel after DGGE procedure is shown in Fig. 4 along with identified bands. The AGCT sequence files were evaluated using the BLAST software (available at http://www.ncbi.nlm.gov/) and identified species are shown in Table 2.

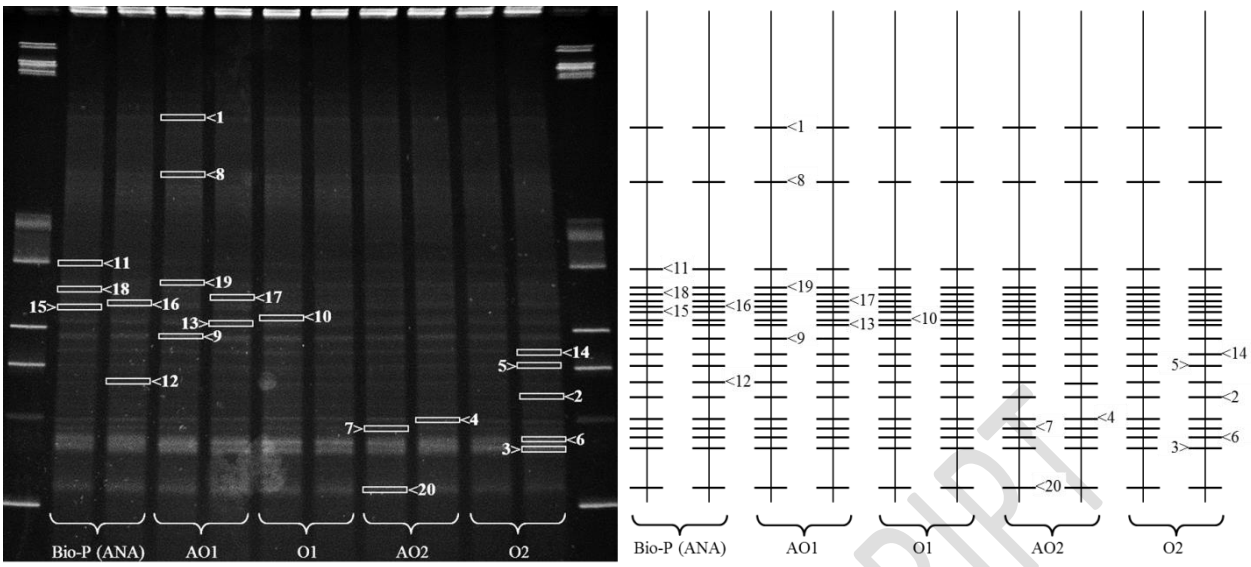

Figure 4. (a) DGGE profiles of 16SrRNA genes for samples (black region is for the gel) and (b) schematic of overall DGGE banding patterns

Table 2. Microbial species in two-stage step-feed biological nutrient removal process

Band number	Accession number	Microorganism name	Organism group	Similarity	Isolation source	Ref.
Nitrifying microorganisms						
1	DQ857301	*Uncultured Nitrosomonas* sp.	Beta-proteobacteria	100%	AS	Geets et al. 2007
2	EU670847	*Nitrosomonas* sp.	Beta-proteobacteria	88%	AS	Kim and Park 2010
3	FJ483764	*Uncultured Nitrosospira* sp.	Beta-proteobacteria	86%	MAS	Wagner et al. 2002
Denitrifying microorganisms						
4	FJ525543	*Uncultured Dechloromonas* sp.	Beta-proteobacteria	89%	AOA	Kondo et al. 2009
Microorganisms responsible for phosphorous removal						
5	JN679133	*Uncultured Candidatus Accumulibacter* sp.	Beta-proteobacteria	87%	MBR	Burow et al. 2007
Unidentified microorganisms						
6	FJ660528	*Uncultured bacterium*	Bacteria	100%	A^2O	Wang et al. 2011
7	HQ492658	*Uncultured bacterium*	Bacteria	92%	AS	Kim and Park 2010
8	EF175888	*Uncultured bacterium*	Bacteria	100%	AS	Hornek et al. 2006
9	AB176864	*Uncultured bacterium*	Bacteria	100%	A^2O	Limpiyakorn et al. 2005
10	HQ467517	*Uncultured bacterium*	Bacteria	88%	AOA	Kondo et al. 2009
11	FJ660550	*Uncultured bacterium*	Bacteria	100%	A^2O	Wang et al. 2011
12	HQ891360	*Uncultured bacterium*	Bacteria	85%	MW	Kang 2010

MBR: Membrane bioreactor; MW: Municipal wastewater; AS: Activated sludge; MAS: Municipal

Band number	Accession number	Microorganism name	Organism group	Similarity	Isolation source	Ref.
activated sludge; A^2O: Anaerobic/anoxic/oxic; AOA: Anaerobic/oxic/anoxic						

Similar bacterial community was identified in all samples. Uncultured *Nitrosomonas* sp. and *Nitrospira* sp. identified for the first, second, and the third bands are of Beta-proteobacteria group and are responsible for the oxidation of ammonia in municipal wastewater treatment plants (Geets et al. 2007; Wagner et al. 2002; Waheed et al. 2013). Some other nitrifying species of beta-proteobacteria are also known to exist in municipal wastewater treatment plants (Wagner and Loy 2002). FISH analyses showed that the microorganisms responsible for nitrification are mainly composed of Beta-proteobacteria (*Nitrosomonas*, *Nitrosococcus mobilis*, *Nitrospira*, *Nitrosovibrio* ve *Nitrosolobus*), among which *Nitrosomonas* sp. dominates the others (Li et al. 2006; Norström et al. 2008; Liang et al. 2010).

The uncultured *Dechloromonas* sp. of Beta-proteobacteria was identified in the 4th band. It has been broadly reported in several studies that such bacteria plays an important role in the denitrification of nitrogenous species in municipal wastewater (Ding et al. 2011; Kondo et al. 2009).

The 5th band was identified as uncultured *Candidatus Accumulibacter* sp. of Beta-proteobacteria. This species were probably responsible for the removal of phosphorous in the pilot plant, as suggested by many other studies (Lopez-Vazquez et al. 2008; Lemaire et al. 2006; Mehandjiyska 1995). In addition, *Candidatus Accumulibacter phosphatis*, listed as a new PAO candidate by Daims et al. (2006) may have taken a part in removing phosphorus from the wastewater.

The bands 6 to 12 were identified as uncultured microorganisms, known also as the activated-sludge bacteria which participate in the biological oxidation of carbonaceous species in municipal wastewaters (Kim and Park 2010; Wang et al. 2011; Hornek et al. 2006; Limpiyakorn et al. 2005; Kang 2010; Kwon et al. 2010; Jin et al. 2011). No species was identified for bands 13 through 20. The sequences of these bands were unfortunately not good enough for identification.

Sequence analyses showed that beta-proteobacteria exist and take part in all stages of treatment in activated sludge in two-stage, step-feed biological nutrient removal process, similar to findings by Miura et al. (2007). Fig. 5 shows the phylogenetic tree obtained for two-stage step-feed biological nutrient removal process used in this study.

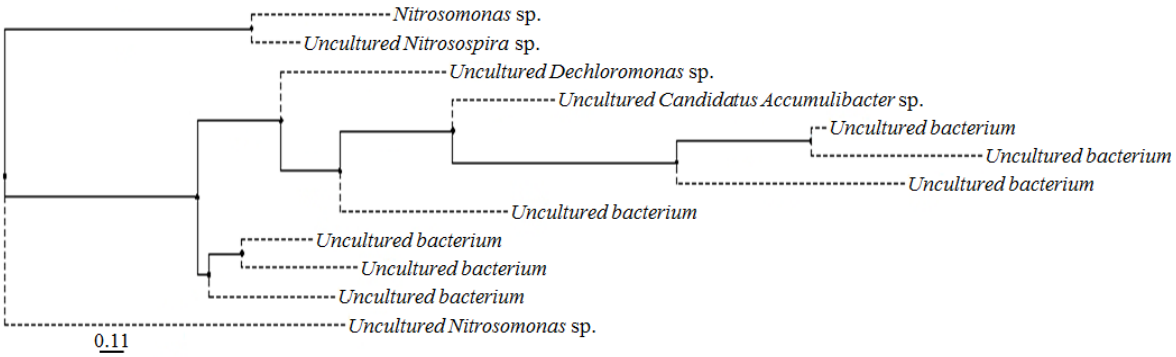

Figure 5. Phylogenetic tree for two-stage step-feed biological nutrient removal process (The scale line indicates 11% difference in nucleotide sequences)

4. Discussion

The results of this study were compared with those of previous works using similar processes for the treatment of municipal wastewaters (Table 3). The observed removal efficiencies of 86.7%, 80.3%, 92.5%, 89.5%, 87.5%, and 95.0% in this study for COD, TN, NH_4^+-N, TP, PO_4^{3-}-P, and SS, respectively, were found to be significantly greater than

those reported in the literature. Such high COD removal efficiencies have been reported in literature for IMT-A^2O (86%) (Abualhail et al. 2013) and A^2O – MBR (94%) (Hu et al. 2013) processes with lower influent COD concentrations (306 mg L^{-1} and 227 mg L^{-1}, respectively). The most important design parameters for activated sludge processes are hydraulic retention time (HRT), sludge retention time (SRT), F/M (food/microorganism) ratio, and mixed liquor suspended solids (MLSS). Of these, HRT is directly proportional to organic load and reactor volume, and SRT is related with the growth rate of microorganisms (Sarria et al. 2011). Literature data suggests the step-feed BNR systems be operated at an HRT between 5-22.5 h, and an SRT between 10-17 days (Vaiopoulou and Aivasidis 2008; Get et al. 2010; Majdi Nasab et al. 2016). In this study, the SRT was 15 days and the HRTs of anaerobic, first-stage anoxic, first-stage aerobic, second-stage anoxic, and second-stage aerobic tanks were 1.2 h, 3.36 h, 4.08 h, 3.36 h, and 4.08 h, respectively. Considering the fact that higher influent COD, TP, PO_4^{3-}-P, NH_4^+-N, and TN concentrations were targeted in this study, the performance of the pilot-scale plant appears to be more than satisfactory.

Apart from HRT and SRT, step-feeding is also an effective and advantageous strategy in BNR system operation. Advantages include (1) operational flexibility, (2) maximization of the use of existing facilities, (3) better handling of peak flows, and (4) robust and stable operation (Bhattarai 2015). When compared with modified five-stage Bardenpho process in the same pilot-scale plant (Manav Demir et al. 2016b), microbial species identified in 2, 3, 4, 5, 6, 7, and 12 bands were the same in two-stage step-feed BNR while those identified in 1, 8, 9, 10, and 11 bands were different in current process. The main reason for this may be (1) the different feeding strategies in two process and (2) seasonal variations in wastewater characteristics. Besides, Duan et al. (2009) reported that the number of microbial species increases with increasing SRT, however, COD removal efficiency is independent of number of species and bacterial counts. Zou and Lu (2016) identified different microbial species for synthetic and real wastewaters in a continuous flow BNR-IC (biological nutrient removal coupled with induced crystallization).

Table 3. Comparison of results from two-stage step-feed biological nutrient removal process with literature data

Reactor type	Wastewater	Influent distribution ratio (%)	V–SRT(d)–HRT(h)	Removal efficiency (%)	Ref.
This study[a]	Municipal WWTP: COD= 555mg L^{-1} NO_3^--N= 0.06mg L^{-1} NH_4^+-N= 44mg L^{-1} TP= 8.1mg L^{-1} TN= 72mg L^{-1}	AN[f] : AO2[g] 50:50%	8.6 m^3-15-16	COD= 86.7% TP= 89.5% NH_4^+-N= 92.5% TN= 80.3%	-
MFSF[b]	Municipal (Tianyu Qingyuan WWTP): COD= 160mg L^{-1} NH_4^+-N= 30.23mg L^{-1} TP= 3.47mg L^{-1} TN= 31.73mg L^{-1}	PAO[h]:AN:AO2:AO3 20:35:35:10%	0.067 m^3-15-8.7	COD= 78.9% TP= 86.11% NH_4^+-N= 98.31% TN= 70.24%	Cao et al. 2013
step-feed UCT[c]	Municipal (Gaobeidian WWTP): COD= 308 mg L^{-1} NO_3^--N= 0.91 mg L^{-1} NH_4^+-N= 51.0 mg L^{-1} PO_4-P= 3.92 mg L^{-1} TN= 52.9 mg L^{-1}	AN:AO2:AO3 40:30:30%	0.34 m^3-10-8	COD= 81.9% NH_4^+-N= 85.3% PO_4-P= 63.6%	Ge et al. 2010
Modified UCT step	Municipal (Gaobeidian WWTP): COD= 254 mg L^{-1}	AN/AO/O/AO/O/AO/O Sinusoidal variation 40:30:30%	0.34 m^3-10-8	NH_4^+-N= 99.2% TN= 83.8%	Ge et al. 2014

feed process	NO_3^--N= 0.45 mg L^{-1} NH_4^+-N= 48.8 mg L^{-1} PO_4-P= 4.73 mg L^{-1} TN= 50.3 mg L^{-1}	AN/A/O Constant one step		NH_4^+-N= 99.1% TN= 74.7%	
		AN/AO/O/AO/O/AO/O Sinusoidal variation 40:30:30%		NH_4^+-N= 99.7% TN= 86.0%	
step-feed UCT	Synthetic: COD= 300mg L^{-1} COD= 500 mg L^{-1}	AN:AO2:AO3 60:25:15%	0.022m^3-no date - 13.8	COD= 95% TP= 78% NH_4^+-N= 95% TN= 93%	Majdi Nasab et al. 2016
A^2O – MBR[d]	Xi'an Campus: COD= 227mg L^{-1} NH_4^+-N= 23.5mg L^{-1} TP= 3.2mg L^{-1} TN= 32.2mg L^{-1}	-	1150 m^3-50-13.8	COD= 94% TP= 91% NH_4^+-N= 91% TN= 73%	Hu et al. 2013
AOA[e]	Synthetic: COD= 300mg L^{-1} NH_4^+-N= 50mg L^{-1} PO_4^{3-}-P= 3.8mg L^{-1}	-	16m^3-20-8	TP= 99% TN= 90%	Liu et al. 2013

[a]Two-stage step-feed biological nutrient removal process, [b]Modified four step-feed reactor, [c] University of Cape Town, [d]Anaerobic–anoxic–oxic membran bioreactor, [e]Anaerobic/aerobic/anoxic – Membran bioreactor, [f]AN: Anaerobic, [g]AO: Anoxic, [h]PAO: Preanoxic

5. Conclusions

The two-stage step-feed biological treatment system seems to be effective for nutrient removal from municipal wastewaters. It offers a cost-effective alternative to wastewater treatment processes in developing countries by eliminating external carbon requirement and reducing soluble microbial products in effluent. The system provided satisfactory removal efficiencies. Sequence analyses showed that beta-proteobacteria exist and operate in all stages of the process. The microbial species responsible for the removal of nutrients were determined as *Nitrosomonas* sp., *Nitrosospira* sp., *Dechloromonas* sp., *Candidatus Accumulibacter* sp., and other uncultured bacteria species in the pilot plant treating the effluent wastewater from the grit chamber unit. Future studies should be conducted for optimization of the process involving different step-feeding configurations, different HRTs, and different SRTs.

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