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Comparison of microbial community structure in a biological nutrient removal process at various stages of operation

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ABSTRACT

In this study, the modified five-stage Bardenpho process was used for the treatment of domestic wastewater in a pilot-scale reactor with an active volume of 8.6 m^3. The hydraulic retention time of the process was 16 h and the sludge retention time was 15 d. The removal efficiencies for chemical oxygen demand, total Kjeldahl nitrogen, ammonia nitrogen (NH_3–N), total phosphorus, phosphate phosphorus (PO_4-P), suspended solids, and volatile suspended solids were obtained as 87 ± 5%; 86 ± 12%; 93 ± 14%; 89 ± 9%; 88 ± 8%; 94 ± 4%, and 94 ± 4%, respectively. The microbial community was also determined in the process by PCR–DGGE–Sequencing molecular techniques. While *Nitrosomonas* sp., *Nitrosospira* sp., *Nitrosomonas europaea*, *Dechloromonas* sp., *Candidatus Accumulibacter* sp., *Bacteroidetes bacterium*, *Firmicutes* were observed during the start-up period, *Nitrosomonas* sp., *Nitrosospira* sp., *N. europaea*, *Dechloromonas* sp., *C. accumulibacter* sp. were observed after the steady-state period.

Keywords: Biological nutrient removal; Community structure; Domestic wastewater; Modified Bardenpho

1. Introduction

The removal of nitrogen and phosphorous from municipal wastewater prior to discharge to the receiving bodies has been recognized as a necessity for years. Nowadays, treatment practices have shifted towards the biological methods, which provide low-cost means of achieving lower effluent concentrations, rather than chemical techniques [1–3]. Biological treatment techniques are far more superior to other techniques in a number of other aspects including: (i) their effectiveness in reducing toxicity of wastewater, (ii) their flexibility during operation, (iii) reduced generation of sludge, (iv) opportunity of improving sludge settling properties, (v) abatement of oxygen requirement, and (vi) opportunity of simultaneously removing nitrogen and phosphorous [4]. Activated sludge systems are used widely for biological and advanced biological treatment purposes [5–8].

Anaerobic, anoxic, and aerobic stages are typical in biological nutrient removal (BNR) processes, respectively, for nitrification, denitrification, and phosphorus removal [9]. In light of recent advances in wastewater treatment technologies, new techniques must be developed to achieve better treatment performances. These new techniques must offer simple design and

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equipment needs, high treatment efficiencies, and low costs of investment and operation [10]. Additionally, a number of limiting factors such as land availability and cost play an important role in the selection of treatment systems [11].

Although a great number of treatment techniques have been developed for the treatment of municipal wastewater, it is vital to improve the existing processes or to develop new ones appropriate for the applied wastewater since municipal wastewater characteristics depend on both location and time. In recent years, some modified bioreactors were designed and investigated for domestic wastewater treatment. Current literature lists that a great number of treatment processes have been applied for carbon and nutrient removal from wastewater including: upflow anaerobic sludge blanket—activated sludge (UASB-AS), upflow anaerobic sludge blanket—sequencing batch reactor (UASB-SBR) [12], sequencing batch reactor (SBR) [13], anaerobic/anoxic/oxic biological aerated filter (A^2O-BAF) [14], anoxic/oxic (AO), A^2O, modified University of Cape Town (modified UCT) [15], cascade-feed UCT [1], cascade-feed A^2O [16], anaerobic/anoxic/aerobic—membrane bioreactor (A^2O-MBR) [17], and five-stage Bardenpho [18]. The first three tanks in the Bardenpho process perform the same duty as in the A^2/O process while the second anoxic–aerobic tank couple aims at further treatment. In the Bardenpho process, two-thirds of influent nitrogen concentration is removed in the first anoxic tank of the sequence [19]. The second anoxic tank is responsible for the removal of nitrate by denitrification process, while no treatment objectives are set for the second aerobic tank. The role of this tank is to strip remaining nitrogen gas by aeration [20]. Therefore, the second aerobic tank is designed with a smaller volume [21]. Sludge disposal does not pose a big problem since no chemicals are used and operating costs are lower.

The process con depends on several factors including wastewater characteristics, economic considerations, and discharge limits [22]. Although investment costs are higher compared to other BNR processes, the Bardenpho process is preferred in regions where strict limitations apply for effluent nitrogen concentrations [23]. The Bardenpho process is also preferred for achieving high levels of nitrogen and phosphorous removal with less chemical use [24]. Effluent concentrations of TN and TP were measured as 1.0 and 0.2 mg/L, respectively, in the Cape Coral Wastewater Treatment Plant in which a modified five-stage Bardenpho process was employed. On the other hand, the Medford Lakes Wastewater Treatment Plant achieves effluent concentrations of 2.6 and 0.09 mg/L, respectively, for TN and TP [25]. Due to the extended sludge retention time, the process offers several additional advantages including enhanced carbon oxidation capacity, good sludge settling properties [26], and reduced sludge production rates [27].

Knowledge on how the microbial population within the treatment system evolves depending on the operational parameters and wastewater characteristics offers valuable information on how to improve treatment efficiency [28]. Although biological methods have been widely used for years, current literature still lacks detailed information about microbial diversity in treatment plants due to methodological limitations till the last decade. During the last decade, however, research has focused on the determination of microbial population in wastewater treatment plants by a number of molecular methods including Polymerase Chain Reaction (PCR), Denaturing Gradient Gel Electrophoresis (DGGE) [29], cloning, slot-blotting hybridization, Fluorescent *In Situ* Hybridization (FISH) [29], and single-strand conformation polymorphism. Additionally, PCR and DGGE methods are used together to monitor microbial diversity in a number of research studies [30].

Research has shown that a great number of parameters influence the structure of the microbial community within the process. Liu et al. [7] reported that microbial community structures were very similar to each other in two processes (A^2O and AO) that differ much in treatment performance and sludge characteristics. In municipal wastewater treatment plants, the dominant phylum is usually Proteobacteria (especially β-Proteobacteria) followed by Bacteriodetes and Actinobacteria. Carvalho et al. [31] reported that *Accumulibacter* was dominant in the reactor when acetate and propionate were used as carbon sources. On the other hand, Whang et al. [32] reported *Nitrosomonas Marina* as ammonia-oxidizing bacteria and *Nitrospira* as nitrite-oxidizing bacteria for a process including the nitrification–denitrification steps operated at 0.3–0.6 d of hydraulic retention time (HRT) and 20 d of SRT (sludge retention times).

This paper aims to evaluate the microbial community assessment by molecular techniques based on 16S rRNA during the start-up and steady-state period. Considering the fact that most of the papers report microbial communities during steady-state period of processes, this paper provides valuable information on the change and evolution of microbial species from the start-up of the process. The results also offer natural selection of the microbial diversity in the process depending on the operational conditions. Moreover, the study also investigates carbon and nutrient removal from municipal wastewater by a BNR process, referred to as the modified five-stage Bardenpho process.

2. Materials and methods

2.1. Modified five-stage Bardenpho process

A pilot-scale treatment plant with an active volume of 8.6 m^3 was operated in the following configuration: primary sedimentation tank (0.25 m^3), distribution tank (0.25 m^3), anaerobic tank (AN—0.5 m^3), first-stage anoxic tank (AO1—1.4 m^3), first-stage aerobic tank (O1—1.7 m^3), second-stage anoxic tank (AO2—1.4 m^3), second-stage aerobic tank (O2—1.7 m^3), and final sedimentation tank (1.4 m^3) [33]. In this study, the first- and second-stage aerobic tanks were of the same volume, which aims to improve the removal efficiencies for both carbon and nitrogen. The main difference of this pilot-scale process from the conventional five-stage Bardenpho process is that the volumes of the aerobic1 and aerobic2 tanks were equal and that an internal recirculation was added from aerobic2 to anoxic2. The purpose of this modification was to improve the treatment performance by setting a treatment objective for aerobic2 tank and recirculating the nitrate-containing effluent to the anoxic2.

The mixed-liquor suspended solids' (MLSS) concentration was kept between 4,500 and 5,500 mg L^{-1} during the study. The HRT was 16 h and the return activated sludge (RAS) ratio was 0.80. The sludge retention time was 15 d. Internal recycle ratios (IR1 and IR2) were kept the same around 450%. Fig. 1 shows the flowchart of the process. The pilot-scale reactor was inoculated with sludge taken from the RAS line of Atakoy Advanced Biological Wastewater Treatment Plant of Istanbul Water and Sewerage Administration (Turkey), and was fed with municipal wastewater from effluent of grit removal unit in the same plant.

2.2. Analytical methods

During the operation, two samples were taken once a week from influent and effluent of the pilot-scale plant, which were analyzed for chemical oxygen demand (COD), total Kjeldahl nitrogen (TKN), NH_3-N (ammonia nitrogen), total phosphorus (TP), PO_4-P (phosphate phosphorus), suspended solids (SS), and volatile suspended solids (VSS) by Standard Methods [34]. Triplicate analyses were performed for each parameter.

2.3. Microbial analyses

Microbial analyses were completed in four stages: nucleic acid extraction, PCR, DGGE, and nucleic acid sequencing. First, nucleic acids were extracted from the samples taken from anaerobic, anoxic1, aerobic1, anoxic2, and aerobic2 tanks of the pilot-scale reactor. The extracts were stored at −20°C. PCR method was employed to enrich 16S rRNA genes of DNA extracts, followed by DGGE and DNA sequencing to determine the microbial community [35].

A Power Soil DNA Isolation Kit (MOBIO Laboratories) was used for isolating DNAs in samples taken from biological treatment units in the pilot-scale plant. To obtain the mixed community DNAs for DGGE analysis, sample DNA extracted from the activated sludge was used for first PCR amplification (initial denaturation for 3 min at 94°C, 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 55°C and extension for 2 min at 72°C, with a final extension for 5 min at 72°C) of the 16S rRNA gene fragment region, at positions ~27 (27F-forward; 5′-AGAGTTTGATCCTGGCTCAG-3′) and ~1,492 (1492r- reverse; 5′-GGYTACCTTGTTACGACTT-3′) (*Escherichia coli* numbering), were conducted by modifying the procedure given in Lee et al. [36]. For DGGE analysis, PCR primers against the V2 region (357–518, *E. coli* numbering) were used for the amplification of the 16S rRNA gene. The second PCR primers were, 357F-GC (5′-CGCCCGCCGCGCGCGGC GGGCGGGGCGGGGGCA CGGGGGGCCTACGGGAGGCAGCAG-3′), which contains a GC-rich clamp and R518 (5′-ATTACC-GCGGCTGCTGG-3′), which is specific for most bacteria, *Archaea* and *Eucarya* [24]. First-step PCR amplification was performed with

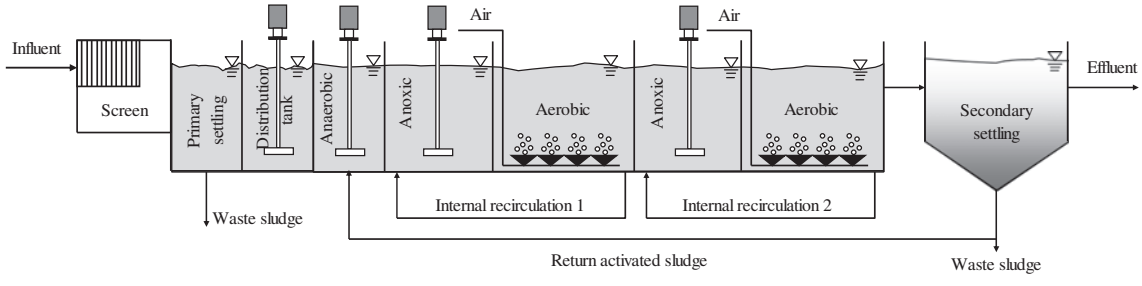

Fig. 1. Schematic representation of modified five-stage Bardenpho process.

5 μL of 10 x reaction buffer, 1.5 μL of 50 mM $MgCl_2$, 1 μL of 40 mM dNTPs, 1 μL of 10 μM primers, and 1.2 μL of 2U/μL Polymerase (FINNZYMES, DyNAzyme™II), 6 μL of each DNA extract, and up to 50 μL of sterile Millipore water. In the second-step PCR amplification, each PCR mixture was prepared with a total volume of 50 μL containing 6 μL of each second step PCR product to 1 μL of 10 μM primers, 5 μL of 10 x reaction buffer (TriseHCl, pH 8.8), 1.5 μL of 50 mM $MgCl_2$, 1 μL of 40 mM dNTPs, 1.2 μL of 2U/μL Polymerase (FINNZYMES, DyNAzyme™II), 0.5 μL of 20 mg/mL bovine serum albumin, and 32.8 μL sterile purified water. The PCR cycling was performed with a BIO-RAD Mycycler Thermal Cycler System. The temperature-cycling conditions were as follows: After a pre-incubation at 94˚C for 3 min, a total of 30 cycles were performed at 94˚C for 30 s, T_A for 30 s and 72˚C for 45 s. In the first 20 cycles, the T_A was decreased by 0.5˚C, stepwise, every two cycles, from 65˚C in the first cycle, to 55˚C by the 20th. In the last 10 cycles, the T_A was 55˚C. The cycling was followed by 10 min of final extension at 72˚C. The profiles of the PCR-amplified DNA were obtained by DGGE, which was performed using 8% polyacrylamide gels (ratio of acrylamide to bisacrylamide, 37.1:1) with denaturing gradient from 25 to 65% (90% denaturing solution contains 7 M urea and 40% formamide) in 1xTAE at 60˚C and 60 volts for 30 min, followed by 60˚C at 120 volts for a period of four hours, using the Dcode mutation detection system (Bio-Rad, USA). The gel was stained with Sybr-Gold (1,000 x concentration) for 30 min and visualized on a UV transilluminator. The bands in DGGE gel were cut and eluted in 20 μL of sterile H_2O overnight. DNA sequences were determined by means of reamplification of bands following similar second-step PCR protocol, with the exception of primer and without GC-clamp. A Nucleic Acid Extraction Kit (GF-1) was used to purify the third-step PCR (after DGGE) products after which the electrophoresis was carried out to assess the quality of the purification process. Sequence data were analyzed by database searches in GenBank using the BLAST program. A phylogenetic tree was constructed by the neighbor-joining method using the Unipro UGENE v.1.9.1.

3. Results and discussion

3.1. Pilot plant performance

The pilot-scale reactor was operated for a period of 18 weeks during the study. The reactor was assumed to reach steady-state period when the time series of removal efficiencies reached a satisfactory hill on the chart (Fig. 2). The process reached steady-state period starting at the end of the eighth week. The bacterial performance is under the influence of a great number of parameters related with physical and environmental conditions including plant capacity, ambient temperature, filamentous growth, equipment breakdown, pH, salinity, increased fatty concentrations, presence of toxic wastewater components, and higher molecules of petrochemical origin [37].

Several events took place related with internal recirculation and return-activated sludge pumps. Also, sludge lines were clogged on a few occasions. The unsteady-state period lasted for longer than estimated due to unexpected equipment failure. The influent and effluent pollutant concentrations as well as the removal efficiencies under unsteady-state and steady-state periods are shown in Table 1. The results from the pilot-scale study suggest that the modified five-stage Bardenpho process can be used efficiently for carbon and nutrient removal from municipal wastewater. Values presented were calculated using results of analyses for 20 samples. Although removal efficiencies were lower compared to steady-state, removal efficiencies during the unsteady-state were also highly satisfactory.

Removal efficiencies at each step of the process are shown in Fig. 3. Increasing the volume of the aerobic2 tank from 0.5 m^3 to 1.7 m^3 led to an increase in removal efficiencies in addition to its primary objective of preventing anaerobic conditions in aerobic2 effluent. Assuming no removal takes place in the second aerobic stage of conventional 5-stage Bardenpho process, any additional removal of contaminants

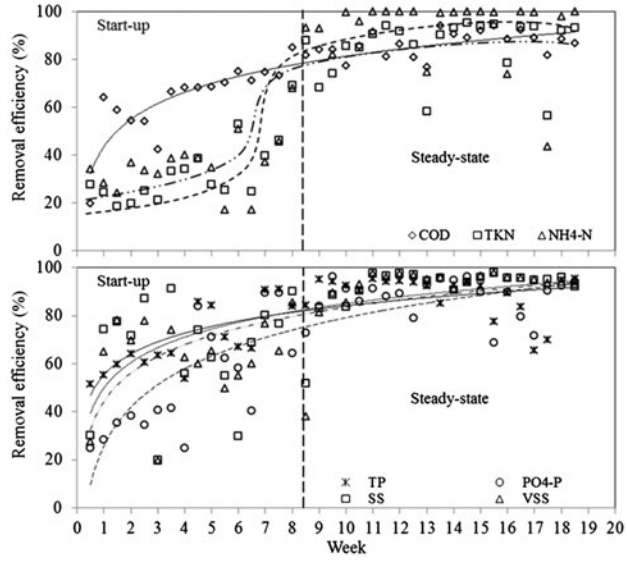

Fig. 2. Time series of removal efficiencies for pollution parameters [26,33].

Table 1
Influent and effluent concentrations as well as removal efficiencies in pilot-scale treatment plant [26,33]

	Influent							
	Mean value (mg L^{-1})	Min.	Q1	Q2	Q3	Max.	Effluent (mg L^{-1})	Efficiency (%)
Start-up period								
COD	685 ± 135	465	595	690	760	930	235 ± 85	64 ± 16
TKN	83 ± 8	69	77	81	89	100	52.9 ± 16.9	36 ± 19
NH_4^+-N	58 ± 8	45	51	57	65	68	35.3 ± 11.9	39 ± 18
TP	7.6 ± 0.4	6.8	7.2	7.6	7.9	8.4	2.3 ± 1.0	70 ± 13
PO_4^{3-}-P	3.3 ± 0.5	2.5	2.9	3.2	3.5	4.4	1.5 ± 0.7	53 ± 22
SS	390 ± 195	119	264	365	445	879	115 ± 65	65 ± 22
VSS	240 ± 75	92	202	243	279	402	85 ± 30	60 ± 18
Steady-state period								
COD	615 ± 85	485	554	610	675	805	80 ± 30	87 ± 5
TKN	76 ± 16	39	70	76	85	99	10.1 ± 8.1	86 ± 12
NH_4^+-N	47 ± 7	34	40	48	51	55	3.6 ± 7.3	93 ± 14
TP	8.2 ± 0.8	6.5	7.8	8.1	8.4	10.3	0.9 ± 0.7	89 ± 9
PO_4^{3-}-P	3.6 ± 0.9	1.7	3.1	3.5	4.3	4.9	0.4 ± 0.3	88 ± 8
SS	265 ± 80	132	217	240	326	426	15 ± 8	94 ± 4
VSS	200 ± 55	93	174	197	226	322	10 ± 5	94 ± 4

Notes: Values are based on 20 samples for both start-up and steady-state periods with triplicate measurement for each parameter in each sample.

in this stage can be considered as a benefit from increasing this tank's volume. Based on data from the second anoxic and aerobic tank effluents, increased volume has lead to resulting increase in removal efficiencies in the range of 2–13% on average for NH_4-N and COD, respectively. Therefore, a detectable improvement of the process was performed by increasing aerobic2 volume.

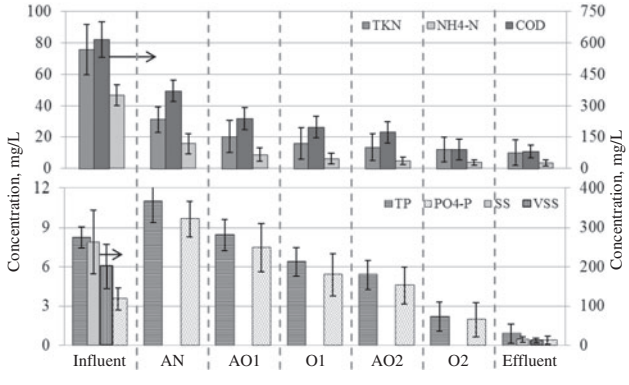

Fig. 3. Change in COD, TKN, NH_4^+, NH_4^+-N, TP, PO_4^{3-}-P, SS, and VSS concentrations between anaerobic, anoxic1, aerobic1, anoxic2, aerobic2, and final sedimentation stages.

3.2. Microbial community composition

In order to investigate the microbial community changes during the start-up and steady-state periods, the PCR–DGGE method was applied to the five-stage BNR system. The results are shown in Fig. 4. Fig. 4 shows the DGGE patterns of the amplified partial 16 s RNA genes.

The nucleic acid sequences were used to estimate microbial species using the BLAST software from http://www.ncbi.nlm.nih.gov and the results are shown in Table 2.

The first four bands of DGGE samples from the modified five-stage Bardenpho process (Fig. 3) were identified as *Nitrosomonas europaea*, *Nitrosomonas* sp., and uncultured *Nitrospira* sp., which are of β-*Proteobacteria* and are known as the genera responsible for the ammonium oxidation in municipal wastewater treatment plants [38,40]. The first, the second, and the fourth bands were identified during both start-up and steady-state periods, while the third band was monitored only for the steady-state period. It was evident that the number of ammonium-oxidizing bacteria increased after the steady-state period was reached, which resulted in detectable increase in ammonium removal efficiency within the pilot-scale plant. On the other hand, the ninth band was identified as uncultured *Bacteriodetes bacterium* only for the start-up

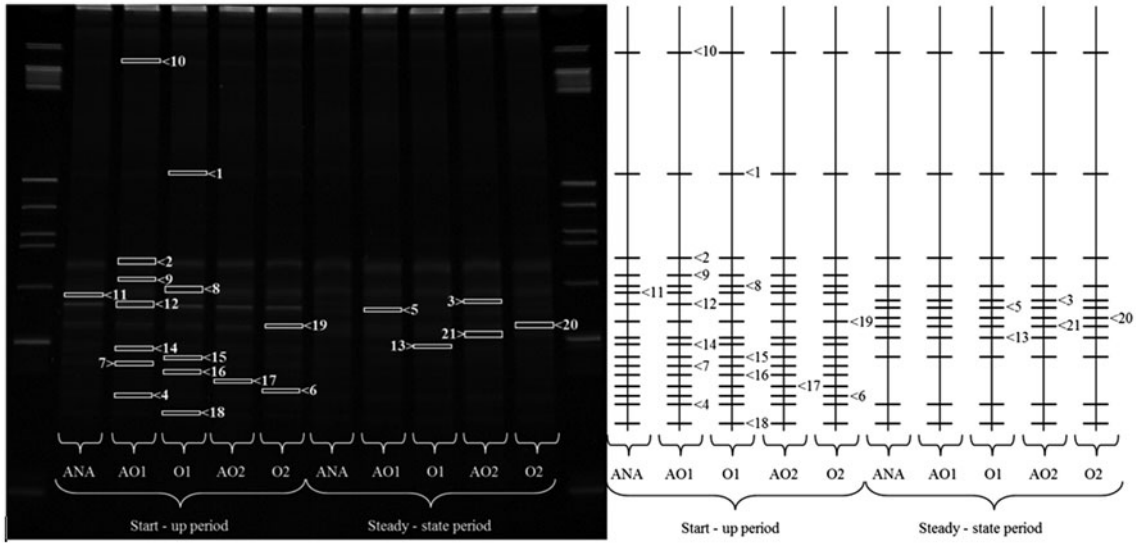

Fig. 4. Recognized bands after DGGE.

Table 2
Microbial species in modified five-stage Bardenpho process

Band number	Accession number	Micro-organism name	SA	SS	Organism group	Similarity (%)	Refs.
Nitrifying micro-organisms							
1	AL954747	*Nitrosomonas europaea*	+	+	Betaproteobacteria	100	[38]
2	CP002876	*Nitrosomonas* sp.	+	+	Betaproteobacteria	94	[39]
3	EU670847	*Nitrosomonas* sp.	–	+	Betaproteobacteria	89	[39]
4	FJ483764	*Uncultured Nitrosospira* sp.	+	+	Betaproteobacteria	86	[40]
Denitrifying micro-organisms							
5	FJ525543	*Uncultured Dechloromonas* sp.	–	+	Betaproteobacteria	87	[41]
6	EU809571	*Uncultured Dechloromonas* sp.	+	–	Betaproteobacteria	90	[42]
7	EF491067	*Uncultured Firmicutes*	+	–	*Firmicutes*	94	[43]
Micro-organisms responsible for phosphorous removal							
8	JN679133	*Uncultured Candidatus Accumulibacter* sp.	+	+	Betaproteobacteria	87	[44]
Filamentous micro-organisms							
9	AP011630	*Uncultured Bacteroidetes bacterium*	+	–	Bacteroidetes	93	[45]
Unidentified micro-organisms							
10	HQ520189	*Uncultured bacterium*	+	+	Bacteria	86	[39]
11	HQ891360	*Uncultured bacterium*	+	–	Bacteria	85	[46]
12	FN827206	*Uncultured bacterium*	+	–	Bacteria	87	[47]
13	HQ523864	*Uncultured bacterium*	+	+	Bacteria	89	[39]
14	GU513185	*Uncultured bacterium*	+	–	Bacteria	88	[48]
15	HQ492658	*Uncultured bacterium*	+	+	Bacteria	90	[39]
16	HQ911043	*Uncultured bacterium*	+	–	Bacteria	98	[49]
17	FJ660528	*Uncultured bacterium*	+	–	Bacteria	100	[50]
18	GU527725	*Uncultured bacterium*	+	+	Bacteria	83	[48]

Notes: SA: Start-up period; SS: Steady-state period.

period. After the steady-state period was reached, these species were not detected. The reason for this is that *Bacteriodetes bacterium* is known to cause filamentous sludge bulking in final sedimentation tanks [45] and it explains the lower treatment efficiencies during the start-up period. This is also supported by the SVI measurements that were 144 ± 7 mL/g for the start-up period and 99 ± 23 mL/g for the steady-state period during the operation.

The eighth band was identified as uncultured *Candidatus accumulibacter* sp. These species are of β-*Proteobacteria* and are known as responsible for phosphorous removal in wastewater treatment plants [44,51]. These species were detected during both the start-up and the steady-state periods, which explain the relatively higher phosphorous removal efficiencies than expected during the start-up period.

The genus uncultured *Dechloromonas* sp. was identified for the fifth and the sixth bands. These species are known as the denitrifying micro-organisms of β-*Proteobacteria* [40,41]. The fifth band was monitored under the steady-state period, while the sixth band was identified in samples taken during the start-up period. Uncultured *Firmicutes*, the seventh band, were detected only in the start-up period. These species are known to be responsible for the denitrification process in wastewater treatment plants [43,52–54].

Bands 10 through 18 from the pilot-scale plant samples were the uncultured species that were detected in wastewater treatment plants in a number of previous research studies [47–49,55]. The 19th, 20th, and 21st bands were not correlated with any species. The phylogenetic trees that were obtained for both the start-up and the steady-state periods are shown in Fig. 5(a) and (b), respectively.

The results from the study are summarized in the following paragraphs :

(1) In the modified five-stage Bardenpho process, increasing the aerobic2 volume leads to an increase in treatment performance and highly satisfactory removal efficiencies compared with

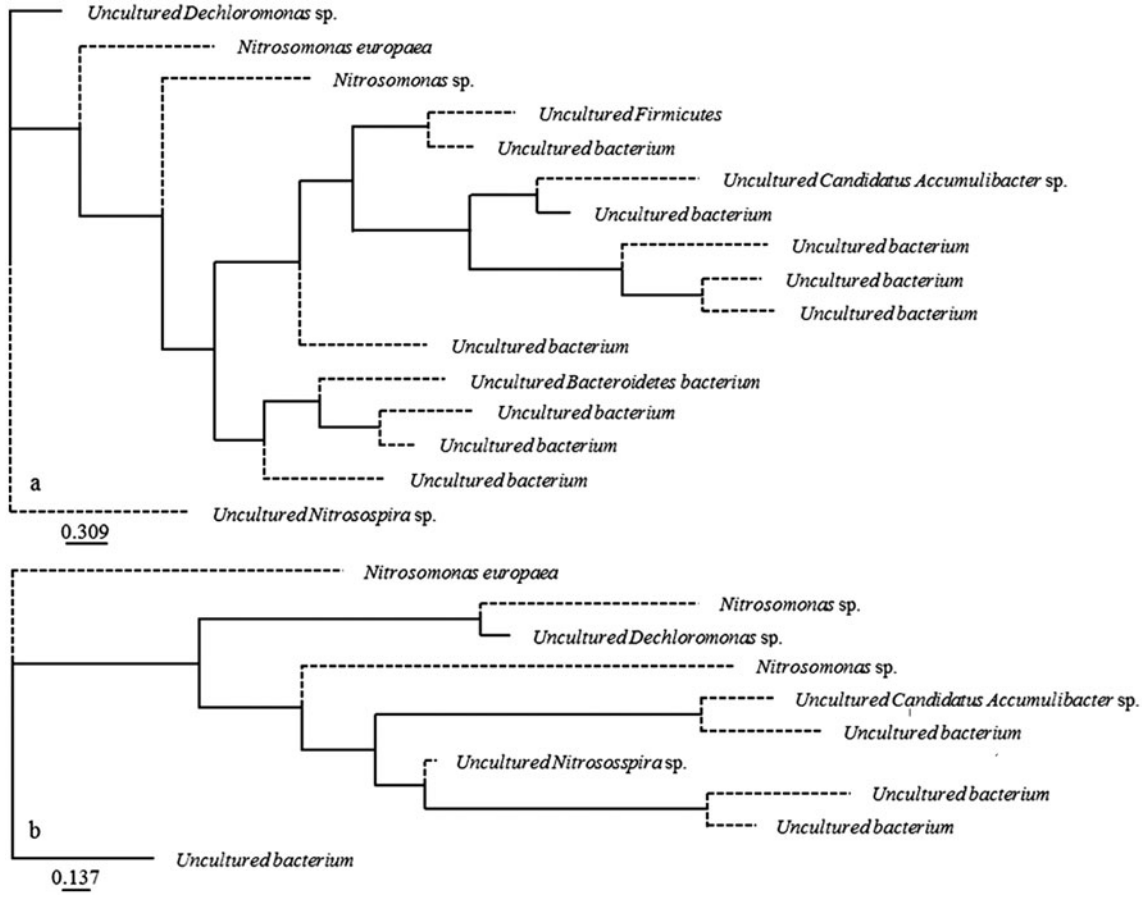

Fig. 5. Phylogenetic trees in modified five-stage Bardenpho process: (a) during the start-up period and (b) under steady-state period.

Table 3
Comparison of results from modified five-stage Bardenpho process with literature data

Reactor type	Wastewater	V–SRT–HRT	Removal efficiency (%)	Refs.
This study	Municipal WWTP	8.6 m^{3-15} d-16 h	COD = 87 TP = 89 PO_4-P = 88 TKN = 86 NH_4^+-N = 93 TN = 82 SS = 94 VSS = 94	[26]
ABR-MBR (CAMBR)	Synthetic + municipal	15 L-90 d-10 h	COD = 89 NH_4^+-N = 98 TN = 65 TP = 83	[56]
Anaerobic–aerobic fixed-bed reactor	Municipal	1572 L-12 h	COD = 92 TN = 55 TSS = 71 NH_4^+-N = 68	[57]
MFSF	Municipal (Tianyu Qingyuan WWTP)	0.067 m^{3-15} d-8.7 h	COD = 78.9 TP = 86.11 NH_4^+- N = 98.31 TN = 70.24	[58]
step-feed UCT	Municipal (Gaobeidian WWTP)	0.34 m^{3-10} d-8 h	COD = 81.9 NH_4^+- N = 85.3 PO_4-P = 63.6	[59]
IMT—A^2O	Wuxi Campus	0.265 m^{3-21} d-16 h	COD = 86 TP = 93 NH_4^+-N = 91 TN = 80	[60]
AOA	Synthetic	43 L-10 d-8 h	TN = 70.3 NH_4^+- N = 93 PO_4-P = 87.3	[61]

Notes: This study: Modified 5-stage Bardenpho process; ABR-MBR (CAMBR): Combined anaerobic baffled reactor—membrane bioreactor; MSFS: Modified four step-feed reactor; Step-feed UCT: Step-feed University of Cape Town; IMT-A^2O: Integrated multi-tank anaerobic–anoxic–oxic; AOA: Anaerobic/aerobic/anoxic.

the literature data (Table 3). The second aerobic tank in the five-stage Bardenpho process could confidently be set for further aerobic treatment in addition to its primary objective of preventing anaerobic conditions before final sedimentation.

(2) PCR–DGGE-sequencing procedures pointed out that *N. europaea*, *Nitrosomonas* sp., and uncultured *Nitrosospira* sp. played an important role in ammonium oxidation during the start-up period. Under the steady-state period, the number of nitrifying organisms as well as the nitrogen removal efficiency increased. Uncultured *Bacteriodetes bacterium*, which is encountered in the start-up period, is known as one of the most important filamentous

micro-organisms that cause filamentous sludge bulking in final sedimentation tanks. These species were not detected under steady-state period, as expected. Uncultured *C. accumulibacter* sp., which are responsible for phosphorous removal in wastewater treatment plants, were identified in both the start-up and steady-state periods. Uncultured *Dechloromonas* sp. and uncultured *Firmicutes* were identified as denitrifying species. On the other hand, a number of uncultured bacteria were identified during both the start-up and the steady-state periods, which were previously detected in wastewater treatment plants. The detected species were in agreement with the literature data.

(3) The incoming municipal wastewater from the Atakoy Advanced Biological Wastewater Treatment Plant of Istanbul Water and Wastewater Administration (Turkey) is classified as medium-high strength wastewater compared to the literature data. The results of the study suggest that the new process developed by modifying the conventional five-stage Bardenpho process showed satisfactory performance in treating medium-high strength wastewater. Since the study is performed using real wastewater in an on-site pilot-scale treatment plant, the results offer a valuable source of information for future process designs.

4. Conclusions

The modified five-stage Bardenpho process used in this study can be preferred confidently for BNR to obtain satisfactory performance. Steady-state removal efficiencies for COD, TKN, NH_4^+-N, TP, PO_4^{3-}-P, SS, and VSS were measured as 87 ± 5%, 86 ± 12%, 93 ± 14%, 89 ± 9%, 88 ± 8%, 94 ± 4%, 94 ± 4%, respectively. The results of this study show that second aerobic tank in the modified five-stage Bardenpho process increased the efficiency of the process. The PCR–DGGE method applied here has provided insights regarding the structures and dynamics of bacterial communities from a sewage treatment plant under both the start-up and steady-state period. The results showed that the microbial community structure of activated sludge experienced significant changes between the start-up and steady-state periods. Although more species were identified in the start-up period and less in the steady-state, it was shown that the species responsible for the treatment remained in activated sludge while others, such as filamentous bacteria, were eliminated after the start-up period. SVI measurements also support this finding. In addition, the number of nitrification bacteria increased in the steady-state period which is evident with higher removal efficiencies of nitrogen in this period. Also, the uncultured bacterium species detected in this study must be clearly identified by proper methods. This way, the species responsible for the treatment can be identified and the findings can positively impact current literature.

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