Curr Microbiol (2013) 66:555–565
DOI 10.1007/s00284-013-0311-z

Comparison of the Bacterial Communities in Anaerobic, Anoxic, and Oxic Chambers of a Pilot A_2O Process Using Pyrosequencing Analysis

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Received: 21 August 2012 / Accepted: 3 January 2013 / Published online: 29 January 2013
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Abstract A_2O process is a sequential wastewater treatment process that uses anaerobic, anoxic, and oxic chambers for nitrogen and phosphorus removal. In this study, the bacterial communities among these chambers were compared, and the diversity of the bacteria involved in nitrogen and phosphorus removal was surveyed. A pilot-scale A_2O process (50 m^3 day^{-1}) was operated for more than 6 months, and bacterial 16S rRNA gene diversity was analyzed using pyrosequencing. A total of 7,447 bacterial sequence reads were obtained from anaerobic (1,546), anoxic (2,158), and oxic (3,743) chambers. Even though there were differences in the atmospheric condition and functionality, no prominent differences could be found in the bacterial community of the three chambers of the pilot A_2O process. All sequence reads, which were taxonomically analyzed using the Eztaxon-e database, were assigned into 638 approved or tentative genera. Among them, about 72.2 % of the taxa were contained in the phyla *Proteobacteria* and *Bacteroidetes*. Phosphate-accumulating bacteria, *Candidatus* Accumulibacter phosphatis, and two other *Accumulibacter* were found to constitute 3.1 % of the identified genera. Ammonia-oxidizing bacteria, *Nitrosomonas oligotropha*, and four other phylotypes in the same family, *Nitrosomonadaceae*, constituted 0.2 and 0.9 %, respectively. Nitrite-oxidizing bacteria, *Nitrospira defluvii*, and other three phylotypes in the same family, *Nitrospiraceae*, constituted 2.5 and 0.1 %, respectively. In addition, *Dokdonella* and a phylotype of the phylum *Chloroflexi*, function in nitrogen and/or phosphate removal of which have not been reported in the A_2O process, constituted the first and third composition among genera at 4.3 and 3.8 %, respectively.

Introduction

Over the past decades, the anaerobic-anoxic–oxic (A_2O) process has been applied for removing organic carbons, nitrogen,

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The GenBank/EMBL/DDBJ accession number for the pyrosequencing data of partial 16S rRNA gene sequences of the bacteria in mixed liquor suspended solids (MLSS) from a pilot A_2O reactor is SRA050633.

Tables representing the diversities of the genus and species in the biomass of anaerobic, anoxic, and oxic chambers in this A_2O process are available online as supplementary material.

Electronic supplementary material The online version of this article (doi:10.1007/s00284-013-0311-z) contains supplementary material, which is available to authorized users.

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and phosphorous from wastewater before discharging it to a receiving water body. In A_2O process, nitrifying and denitrifying bacteria play the major function in nitrogen removal, and phosphate-accumulating organisms (PAOs) are involved in enhanced biological phosphorus removal (EBPR) [41, 50]. Until now, the function of the A_2O process has been characterized with influent compositions, such as COD, free ammonia, total nitrogen (TN), and total phosphorus (TP), and operational conditions, such as hydraulic retention time (HRT), solid residence time (SRT), system pH, temperature, dissolved oxygen (DO), mixed liquor suspended solids (MLSS), total suspended solids (TSS), and food-microorganism (F/M) ratio.

The function of the bacteria in each reactor of the A_2O process mainly determines the removal of nitrogen and phosphorus. Nitrogen is removed through nitrification and denitrification. Nitrification is a process to oxidize ammonia into nitrate via nitrite under aerobic condition, whereas denitrification is a reduction process whereby nitrate is reduced to dinitrogen gas via nitrite, nitric oxide, and nitrous oxide under anoxic condition [16]. The large difference in the oxidation–reduction potentials of nitrification and denitrification hinders the two processes from simultaneously occurring in the same chamber [17]. The ratio of bacteria is influenced by physical factors; for example, Hanaki et al. [19] reported that, in a suspended-growth system, the growth of ammonium-oxidizing bacteria (AOB) increased at low levels of DO but nitrite-oxidizing bacteria (NOB) did not.

Meanwhile, EBPR process is a phosphate-removing process via the circulation of sludge through anaerobic and aerobic phases, and phosphorous is removed by PAOs after the introduction of wastewater into the anaerobic phase [3, 6, 35]. In anaerobic condition, PAOs uptake organic carbon and release intracellularly-stored polyphosphate, and in oxic condition, the PAOs uptake phosphate and accumulate it as polyphosphate [35]. Molecular techniques and enrichment in a lab-scale EBPR process have led to *Candidatus* Accumulibacter phosphatis being as a PAO [13, 22, 33].

Though, bacterial strains determine the performance of an A_2O process, few data relating to bacterial communities have been reported in the process and the differences in respective bacterial diversities of the anaerobic, anoxic, and oxic chambers of the A_2O process. Liu et al. performed fluorescence in situ hybridization (FISH) for the AOB in their A_2O process and found that the AOB accounted for 3.6 % of the total bacterial community in the A_2O system [32].

For a better understanding of the difference of bacterial communities among anaerobic, anoxic, and oxic chambers within A_2O process and of the true diversity of the bacteria involved in nitrogen and phosphorus removal, the bacterial communities in a pilot-scale A_2O process treating 50 m^3 day^{-1} of municipal sewage were intensively studied using pyrosequencing of partial 16S rRNA gene sequences, followed by analysis with the Eztaxon-e database.

Materials and Methods

Operation Condition of Pilot Wastewater Treatment Plant

The bacterial community analysis was performed for the bacterial biomass in a pilot-scale A_2O process that was operated for 6 months to treat 50 m^3 day^{-1} domestic sewage. The volumes of the anaerobic, anoxic, and oxic chambers were 4.17, 4.17, and 8.33 m^3, respectively (Fig. 1). MLSS taken from the aerobic chamber of a well-operated local wastewater treatment plant (WWTP) were used as seeding inoculum. In the beginning, all chambers in this pilot A_2O system were filled with this MLSS. The removal of nitrogen and phosphate in this A_2O system was stabilized after 1.5 months. The HRTs of the anaerobic, anoxic, and oxic chambers were 2, 2, and 4 h, respectively. The overflow of a primary settling tank was used as the influent of the pilot-scale process. The MLSS of the A_2O system was maintained at about 2,000–3,000 mg l^{-1}. The SRT was maintained at 15 days, and the pH was 6.5–7.2. The DO of the oxic zone was maintained at 3.01–3.65 mg l^{-1}. The average water temperature at the time of sampling was 27.8 °C. The COD_{Cr}, NH_4^+-N, TN, and TP were taken from influent (domestic sewage) and effluent (overflow of clarifiers) and analyzed according to Standard Methods [5].

Samples and DNA Extraction

For pyrosequencing analysis, the MLSS used as bacteria biomass samples that were taken out from the anaerobic, anoxic, and oxic chambers simultaneously, and the genomic DNA of the biomass samples was extracted for molecular analysis. The MLSS samples from each chamber were placed in sterile 50-ml polypropylene tubes and taken back to the laboratory within an hour in a cooling box. The tubes were centrifuged, and the supernatant was removed to collect the biomass, which was subsequently transferred into a 2.0-ml Eppendorf tube for DNA extraction. The genomic DNA of the collected biomass was extracted and purified using PowerSoil DNA kit (MO BIO Laboratories Inc., CA) according to manufacturer's instructions. The purified genomic DNA was used as a template for analysis of the bacterial community in each chamber.

PCR, Pyrosequencing, and Sequence Analysis

PCR, pyrosequencing, and sequence analysis were performed at a sequencing service company (Chunlab, Republic of Korea). Purified DNA was amplified with a barcoded fusion universal bacterial primer set. The barcoded primer sets were designed for the amplification of ~450 bp and covered V1 to

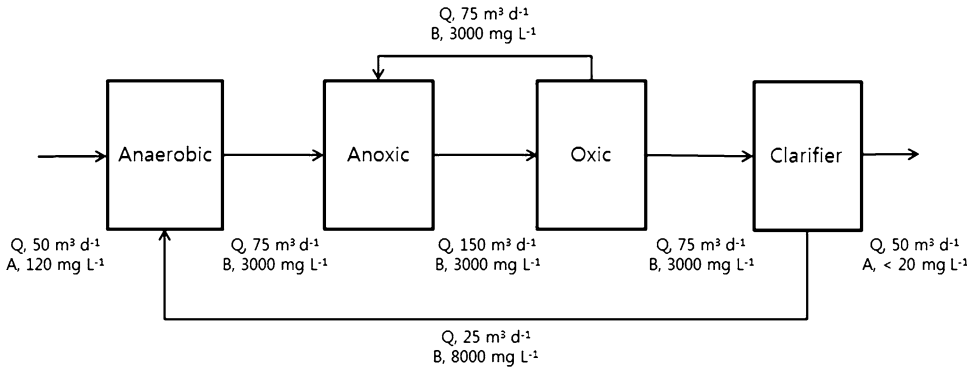

Fig. 1 Schematic diagram of the pilot A_2O process reactor used in this study for the treatment of a municipal wastewater in Seoul, Republic of Korea, showing the position of the anaerobic, anoxic, and oxic chambers (the volumes of each chamber were 4.17, 4.17, and 8.33 m^3, respectively). *Q* flow rate, *A* TSS and *B* MLSS

V3 of 16S rRNA variable regions. The sequence of the forward and reverse primers were 5′-GAG TTT GAT CMT GGC TCA G-3′ and 5′-WTT ACC GCG GCT GCT GG-3′, respectively, and the barcode sequences for bacteria in the anaerobic, anoxic, and oxic chambers of the A_2O process were 5′-ATG TAC ACG-3′, 5′-ACA CAC TAG-3′, and 5′-CTG TAC ATAC-3′, respectively. Template DNA 100 ng was used in a 50 µl PCR reaction. Pyrosequencing was conducted following the method proposed by Lim et al. [31] with the Roche 454 pyrosequencing system (454 GS FLX Titanium). The raw sequence processing and taxonomical assignments were carried out by a service company (Chunlab). Among the obtained reads of partial 16S rRNA gene sequences, non-16S rRNA reads and reads without primer sequences were removed from barcode-sorted reads. The chimera of selected reads was checked using MOTHUR with Bellerophon methods [43]. The checked reads of partial 16S rRNA gene sequences were taxonomically assigned using the Eztaxon-e database [28, http://eztaxon-e.ezbiocloud.net]. According to the Eztaxon-e database, the names of taxon were described both using formal taxonomic names and phylotypes that may be represented in nature but were not cultured. The name of an uncultured taxon was described following Kim et al. [28] and the Eztaxon-e database, in which the names were labeled with an underscore and a specific suffix (e.g., X_s for the phylotypes, X_g for the tentative genus, X_f for the tentative family, and so on, where X represents the GenBank accession number or an arbitrary name). MOTHUR [43] was used to calculate the shared operational taxonomic units (OTUs) of *Candidatus* Accumulibacter by clustering at a distance of 0.03 and to reveal the representative sequences of each shared OTU. The phylogenetic tree was inferred using MEGA5 [49].

Estimation of Species Richness and Diversity

The number of OTUs and rarefaction of the total bacterial communities were analyzed using MOTHUR [43, http://www.mothur.org]. The OTUs, rarefaction analysis, and estimators were calculated at different clustering distances of 0.03 (species), 0.06 (genus), and 0.10 (family) using MOTHUR [43]. The rarefaction analysis was carried out using MOTHUR with processed raw sequences. The calculation of diversity estimator was carried out with normalized random subsets. Each sample was normalized by generating random subsets using CLcommunity (Chunlab). Each random subset contained a total of 1,546 randomly selected bacterial sequence reads. An abundance-based estimator ACE [10], a Chao1 estimator [11], and other indices for each sample were calculated using MOTHUR [43].

Results and Discussion

Bacterial communities were analyzed for MLSS collected from anaerobic, anoxic, and oxic chambers of a pilot-scale A_2O process with a capacity of 50 m^3 d^{-1}, which had been operated to treat municipal sewage for more than 6 months. Influent and effluent at the time of sampling are described in Fig. 2.

Respectively, 1,546, 2,158, and 3,743 reads were obtained from the MLSS of anaerobic, anoxic, and oxic chambers (Table 1). According to the analyses of pyrosequencing data with the EzTaxon-e database, a total of 7,447 reads were taxonomically assigned to 969 species, including 169 known species and 800 tentative phylotypes, which constitute 12.8 and 87.1 % of the total reads, respectively, and were assigned into 638 genera (Supplementary Tables S1, S2).

The microbial diversity indices of the normalized samples from each reactor (Table 2) and the composition of microbial community indicated that community structures of each reactor were not significantly different. The similarity of the microbial community among the different reactors in WWTPs resembles the results of Gich et al., as they reported that the differences in the bacterial community structures were not observed between the oxic and anoxic chambers of each of the three WWTPs located in Girona, Spain [18]. Rarefaction analysis showed that the species did not reach asymptote, so the generated sequence

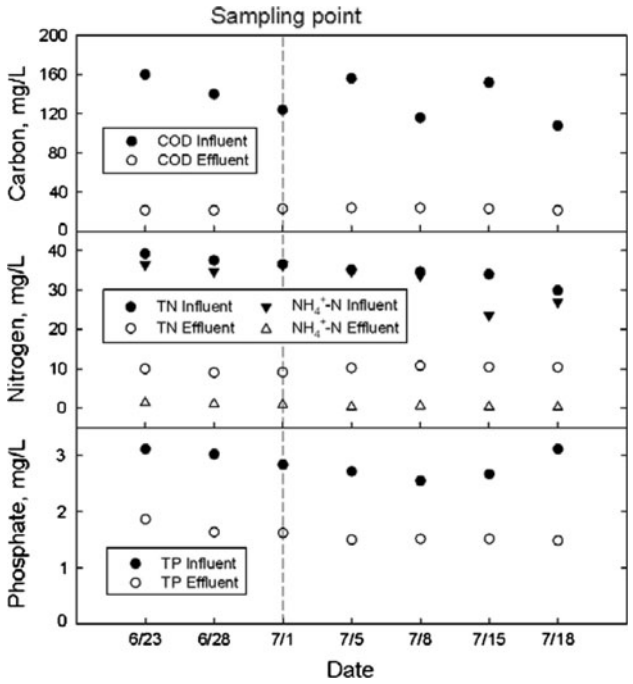

Fig. 2 Characteristics of influent and effluent of the A_2O process at the time of biomass sampling for bacterial community analysis

Table 1 Summary of the pyrosequencing data sets

Reads	Anaerobic	Anoxic	Oxic
Number of reads			
Sorted reads	1,654	2,276	3,979
Dropped reads[a]	27	22	41
Dropped reads[b]	0	0	0
Chimera	81	96	195
Assigned reads	1,546	2,158	3,743
Sequence lengths (base)			
Maximum	545	555	519
Minimum	300	300	300
Average	471	470	471

[a] Dropped by primer

[b] Dropped by BLAST

reads could not fully represent the microbial community of each chamber and that a bit more species could be detected in the A_2O process; however, similar curves were shown from the bacterial diversity of anaerobic, anoxic, and oxic chambers (Fig. 3).

Although the distinct structure of the bacterial community in each chamber was expected, the differentiating flora in the chambers was not observed. However, these pyrosequencing data elucidated the true similarity among the chambers, and the bacterial community did not significantly change between the anaerobic, anoxic, and oxic chambers within a total 8 h of HRTs in this pilot A_2O process.

All 7,447 reads of the A_2O process were assigned to 34 kinds of bacterial phyla. The major phyla, *Proteobacteria*, *Chloroflexi*, and *Bacteroidetes* comprised 52.5–55.1, 17.1–19.7, and 6.3–6.9 % of each community, respectively. The phyla *Chlorobi*, *Acidobacteria*, *Nitrospirae*, *Planctomycetes*, *Actinobactera*, *Firmicutes*, and *Gemmatimonadetes* constituted minor communities; they only accounted for 0.9–3.7 % in all chambers of the A_2O process. The other 24 phyla constituted less than 1 % in all reactors. Compositions of taxa constituting more than 2 % of the total reads are shown in Table 3.

Ding et al. presented the differences in microbial community composition and dominant bacterial population of anoxic and oxic sludge collected from a WWTP by performing PCR-DGGE analysis [14]. They identified 14 sequences from the DGGE band analysis as *Psychrobacter*, *Acinetobacter*, *Pantoea*, *Exigubacterium*, *Aeromonas*, *Pseudomonas*, *Alcanivorax*, and *Simplicispira* [14]; however, these genera were not major reads from anoxic and oxic MLSS in this study. *Psychrobacter*, *Pantoea*, *Exigubacterium*, and *Alcanivorax* were not observed. *Simplicispira limi,* reported as a major band in most DGGE lanes in Ding et al.'s study, was not observed, either [14]. Only three reads were taxonomically assigned to the genus *Simplicispira* in this study.

Of the genera identified in this pilot A_2O process, the genus *Dechloromonas* (4.1 %) and *Candidatus* Accumulibacter (3.1 %) in the family *Rodocyclaceae* constituted 7.2 % of the total 7,447 reads. The presence of *Rodocyclaceae* has been previously reported in EBPR processes. Zilles et al. reported the domination of *Rhodocyclus*-positive cells in EBPR plants [56], Crocetti et al. reported the presence of bacteria closely related to *Rhodocyclus* as an important group of PAOs in EBPR sludges [13], and Jeon et al. reported a 28 % (10/35) presence of a *Rhodocyclus* clone in the aerobic stage sludge of an SBR for phosphorus removal [23].

The genus *Candidatus* Accumulibacter was reported as directly responsible for phosphate accumulation in WWTPs [8], and dominant clones were reported in libraries generated from the biomass of alternating EBPR systems operating with sequential anaerobic and oxic conditions [13, 22]. The genus *Dechloromonas*, which was the second most dominant genus identified in this A_2O process, was also reported as one of the major betaproteobacteria in an SBR for phosphorus removal [2].

Kim et al. [27] reported four *Candidatus* Accumulibacter clades in SBRs for EBPR operated with sodium acetate as the sole carbon source. The four clades, which were designated as Acc-SG1, Acc-SG2, Acc-SG3, and Acc-SG4, constituted 81, 9, 24, and 3 clones, respectively,

Table 2 Indices quantifying the species richness and diversity of the microbial community

MLSS	Cluster distance												
	0.03					0.06				0.10			
	OTU[a]	ACE[b]	Chao1	Shannon	Simpson	OTU	ACE	Chao1	Shannon	Simpson	OTU	ACE	Chao1	Shannon	Simpson
Anaerobic	699	2,397	1,581	6.0161	0.0052	506	1,199	926	5.5096	0.0087	373	766	591	4.9388	0.0204
Anoxic	705	2,937	1,789	6.0254	0.0046	495	1,277	903	5.5117	0.0078	363	782	613	5.0140	0.0150
Oxic	713	3,443	1,972	6.0297	0.0045	508	1,686	1,184	5.5682	0.0082	366	934	760	4.9391	0.0172

[a] Operational taxonomic unit

[b] Abundance-based estimator

in a total of 117 clones [27]. The most dominant Acc-SG1 *Candidatus* Accumulibacter clones contained the type 16S rRNA gene sequence of *Candidatus* Accumulibacter phosphatis clone R6 [22], whereas in this A_2O process, only 16 out of 237 *Candidatus* Accumulibacter reads were classified as *Candidatus* Accumulibacter phosphatis. The 237 reads were compared with the 117 clones presented by Kim et al. [27] using phylogenetic analysis (Table 4, Table 5; Fig. 4). In this A_2O process, 8 shared OTUs (124 reads) belonged to four clades and 12 shared OTUs (113 reads) were not grouped in the four clades. The differences in the compositions and numbers of PAO clades between the SBR operated by Kim et al. [27] and those observed in this study might be due to the differences in the reactor systems (simple SBR vs. A_2O process) and operation conditions such as seeding biomass, feed compositions, and HRT.

Regarding other reported polyphosphate accumulating bacteria, two reads of *Tetrasphaera australiensis* were detected in an anaerobic chamber, but *Tessaracoccus bendigoensis*, *Tetrasphaera japonica*, *Quadricoccus australiensis*, and *Quadrisphaera granulorum* were not detected.

Kämpfer et al. reported that members of the genus *Acinetobacter* accounted for 1–5 % of all bacteria in the oxic and anaerobic zones of a WWTP for enhanced phosphorous removal after they had analyzed in situ rRNA-targeted oligonucleotide probe data [26]. In the previous studies, the genus *Acinetobacter* was generally considered as PAO [20, 37]. However, the genus *Acinetobacter* comprised only 0.1–0.5 % of all bacteria in this study using A_2O process.

Nitrification refers to the two biochemical oxidation processes that sequentially transform ammonia to nitrate via nitrite [9]. The ammonia-oxidizing bacteria (AOB), e.g., the genus *Nitrosomonas* and *Nitrosospira*, and the nitrite-oxidizing bacteria (NOB), e.g., the genus *Nitrobacter* and *Nitrospira*, are involved in microbially-catalyzed nitrification [4]. Table 6 shows the number of AOB and NOB in this pilot A_2O process.

As AOB, the genus *Nitrosomonas* and another uncultured *Nitrosomonadaceae* constituted 0.2–0.7 and 0.4–0.8 %, respectively, but the genus *Nitrosospira*, which is another well-reported AOB [21, 24] was not observed in this A_2O process. The dominance of *Nitrosomonas* in nitrifying biofilms was reported by Okabe et al. [38]. While You et al. reported that the genus *Nitrosospira* was found as a major AOB in their A_2O system by the cloning-DGGE method [55], and Schramm et al. [45] observed the *Nitrosospira* group instead of the genus *Nitrosomonas* as a major AOB in bacterial aggregates from their nitrifying fluidized bed reactor. Liu et al. [32] performed the FISH for the AOB in their A_2O process, and found that the AOB accounted for 3.6 % of the total bacteria population in the A_2O system.

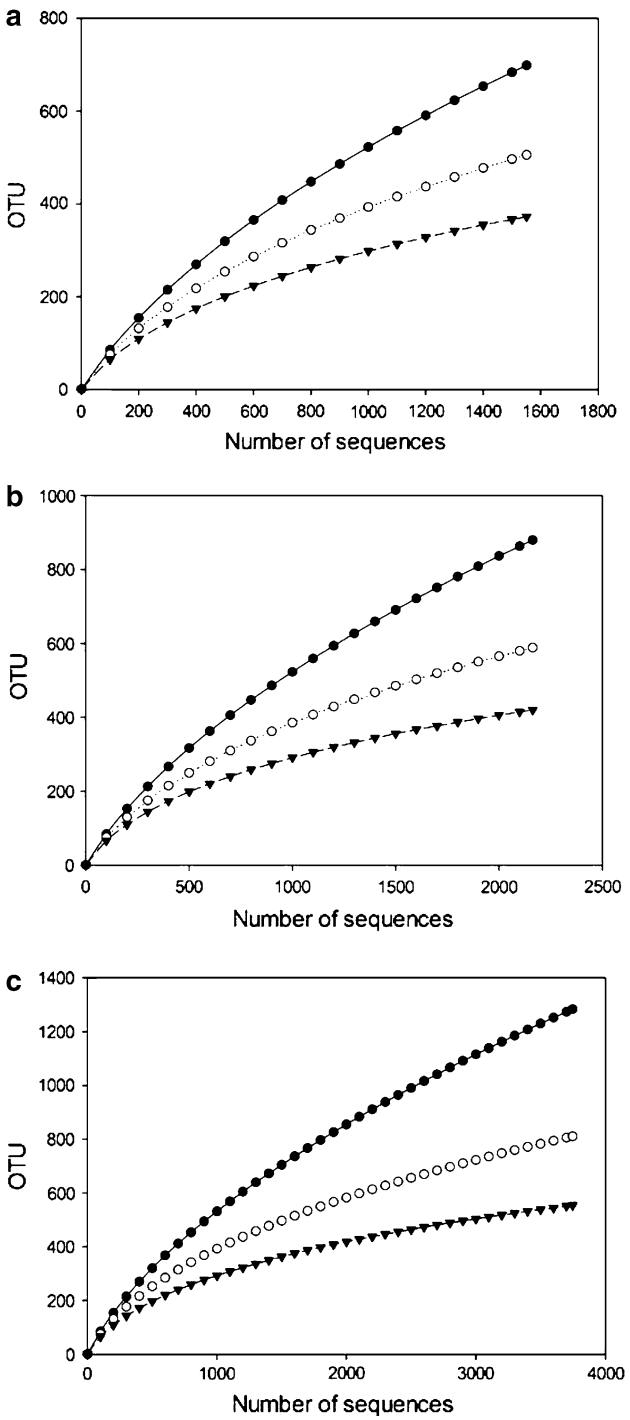

Fig. 3 The effect of the sequencing effort on the estimation of the number of OTUs **a** anaerobic chamber; **b** anoxic chamber; and **c** oxic chamber *black circle* species, *white circle* genus and *black down-pointing triangle* family

Regarding NOBs, the genus *Nitrospira* constituted 2.0–3.0 %, but the genus *Nitrobacter* only constituted 0.05 % of the total bacterial reads (4/7,447) in this A_2O process. The species *Candidatus* Nitrospira defluvii, a NOB contained in the phylum *Nitrospirae* [34], constituted 1.9–2.8 % in this pilot A_2O process. The NOB dominance in this study is consistent with the other reports in the literature: the genus *Nitrospira* as a dominant nitrite oxidizer in WWTPs, nitrifying fluidized bed reactor, and A_2O [25, 45, 55], and the genus *Nitrobacter* as a deficient nitrite oxidizer in nitrifying domestic wastewater biofilms and nitrifying fluidized bed reactor [38, 45].

In the proportion of AOB and NOB, the amount of NOB was higher than AOB in this pilot A_2O process. A similar proportion in nitrifying bacteria was also reported by Dionisi et al.; in their study, the *Nitrosomonas oligotropha*-like ammonia oxidizers and *Nitrospira* spp. in the MLSS collected from an aeration basin of a municipal WWTP represented 0.0033 and 0.39 % of the total bacterial population, respectively [15]. On the contrary, a greater number of AOB than that of NOB was observed from biomass of an A_2O by the cloning-DGGE method [55].

In addition to nitrification and denitrification, nitrogen can be directly removed by an anaerobic ammonium oxidation (ANAMMOX) process in which nitrite and ammonium are converted into dinitrogen gas [30, 51]. All reported ANAMMOX bacteria are contained in the *Planctomycetes*. In this pilot A_2O process, *Planctomycetes*, containing 38 genera level phylotypes, constituted 2.7 % of the total reads. The reads of *Planctomycetes* consisted of five reads of the genus *Pirellula*, which are reported as ANAMMOX bacteria, three reads of *Singulisphaera*, and 178 other reads of 36 phylotypes of unreported genera. These unreported phylotypes of *Planctomycetes* may be involved in the nitrogen removal in this pilot A_2O process.

Interestingly, the genus *Dokdonella* was the most abundant genus in this A_2O process (4.1–5.0 %). In particular, a phylotype FM213038_s constituted 4.1–4.8 %, the largest constituent in the species level. The function of the genus *Dokdonella* in nitrogen and/or phosphate removal in wastewater treatment was not reported. The genus *Dokdonella* contains four validated species, and among them *D. koreensis* and *D. soli* have physiological characteristics that reduces nitrate in aerobic culture condition [53]. Even though the reads of the genus *Dokdonella* detected in this pilot A_2O process were not related with four validated species of the genus *Dokdonella*, the genus may be involved in the nitrogen removal in this A_2O process.

The second major genus *Dechloromonas* (4.0–4.2 %) is a chlorate- and perchlorate-reducing heterotrophic facultative anaerobe, and some of these genera use nitrate as an alternative electron acceptor [1, 12]. The third major genus was a phylotype of *Chloroflexi* (AF234698_g), constituting 3.6–4.2 %. The fourth genus was *Candidatus* Accumulibacter, constituting 3.6–4.2 %. The genera *Sphingopyxis*, *Curvibacter*, *Azospira*, *Piscinibacter*, *Ideonella*, and *Rhizobacter* each constituted 1.0–1.8 % of the total reads. The

Table 3 Composition of taxa constituting >2 % of the total reads

Taxonomic rank	Name	Composition (%)			
		Anaerobic	Anoxic	Oxic	Total
Phylum	*Proteobacteria*	55.1 (852)[1]	55.2 (1,193)	52.5 (1,966)	53.8 (4,011)
Class	*Betaproteobacteria*	28.5 (441)	27.0 (584)	26.1 (977)	26.8 (2,002)
Order	*Burkholderiales*	10.5 (163)	10.4 (226)	9.1 (343)	9.8 (732)
Family	*Sphaerotilus*_f	6.0 (94)	6.2 (134)	4.4 (167)	5.3 (395)
Family	*Comamonadaceae*	3.8 (60)	3.6 (78)	3.9 (149)	3.8 (287)
Order	*Rhodocyclales*	9.9 (153)	9.5 (207)	9.1 (344)	9.4 (704)
Family	*Rhodocyclaceae*	9.9 (153)	9.5 (207)	9.1 (344)	9.4 (704)
Genus	*Dechloromonas*	4.2 (66)	4.0 (87)	4.0 (153)	4.1 (306)
Genus	*Accumulibacter*	3.4 (53)	2.8 (62)	3.2 (122)	3.1 (237)
Class	*Gammaproteobacteria*	11.9 (185)	10.3 (224)	11.3 (425)	11.2 (834)
Order	*Xanthomonadales*	7.0 (109)	6.4 (140)	7.0 (265)	6.9 (514)
Family	*Xanthomonadaceae*	5.6 (88)	5.0 (109)	5.4 (202)	5.3 (399)
Genus	*Dokdonella*	5.0 (78)	4.1 (90)	4.1 (155)	4.3 (323)
Class	*Alphaproteobacteria*	6.3 (98)	9.8 (212)	8.1 (305)	8.2 (615)
Order	*Rhizobiales*	2.3 (37)	4.4 (97)	3.0 (113)	3.3 (247)
Order	*Sphingomonadales*	1.3 (21)	2.4 (53)	2.5 (95)	2.2 (169)
Family	*Sphingomonadaceae*	1.3 (21)	2.4 (53)	2.5 (94)	2.2 (168)
Class	*Deltaproteobacteria*	7.4 (115)	7.3 (158)	6.4 (240)	6.8 (513)
Order	DQ906906_o	2.9 (45)	3.5 (77)	3.1 (116)	3.2 (238)
Order	Myxococcales	3.0 (47)	2.4 (53)	2.2 (83)	2.4 (183)
Phylum	*Bacteroidetes*	18.9 (293)	17.1 (370)	19.7 (738)	18.8 (1,401)
Class	*Sphingobacteria*	9.5 (147)	9.8 (212)	11.2 (421)	10.4 (780)
Order	*Sphingobacteriales*	6.1 (95)	6.2 (134)	6.1 (231)	6.1 (460)
Family	*Chitinophagaceae*	1.8 (29)	1.9 (43)	2.2 (83)	2.0 (155)
Order	EU234264_o	2.2 (34)	2.3 (50)	3.1 (118)	2.7 (202)
Family	EU234264_f	1.4 (22)	2.0 (45)	2.5 (95)	2.1 (162)
Class	*Flavobacteria*	6.6 (102)	4.5 (98)	5.5 (206)	5.4 (406)
Class	*Cytophagia*	2.0 (31)	2.1 (46)	2.0 (76)	2.0 (153)
Phylum	*Chloroflexi*	6.4 (100)	6.3 (138)	6.9 (259)	6.6 (497)
Class	*Anaerolineae*	5.1 (80)	4.4 (96)	5.0 (187)	4.8 (363)
Order	*Anaerolinaeles*	4.7 (73)	3.8 (82)	3.7 (142)	3.9 (297)
Family	*Anaerolinaceae*	4.5 (71)	3.7 (80)	3.6 (138)	3.8 (289)
Genus	AF234698_g	4.2 (66)	3.7 (80)	3.6 (137)	3.8 (283)
Phylum	*Chlorobi*	2.7 (43)	3.7 (81)	3.7 (139)	3.5 (263)
Class	*Ignavibacteriae*	1.5 (24)	2.2 (49)	2.1 (81)	2.0 (154)
Order	*Ignavibacteriales*	1.5 (24)	2.2 (49)	2.1 (81)	2.0 (154)
Family	AY118152_f	1.5 (24)	2.2 (48)	2.1 (81)	2.0 (153)
Phylum	*Acidobacteria*	3.0 (47)	3.2 (69)	2.8 (105)	2.9 (221)
Class	*Solibacteres*	2.5 (40)	2.6 (58)	2.0 (76)	2.3 (174)
Order	FJ479064_o	2.5 (40)	2.6 (58)	1.9 (74)	2.3 (172)
Phylum	*Nitrospirae*	2.9 (45)	2.0 (44)	3.0 (115)	2.7 (204)
Class	*Nitrospira*_c	2.9 (45)	2.0 (44)	3.0 (115)	2.7 (204)
Order	*Nitrospirales*	2.9 (45)	2.0 (44)	3.0 (115)	2.7 (204)
Family	*Nitrospiraceae*	2.9 (45)	2.0 (44)	3.0 (115)	2.7 (204)
Genus	*Nitrospira*_g1	2.9 (45)	2.0 (44)	3.0 (114)	2.7 (203)
Phylum	*Planctomycetes*	2.1 (33)	2.5 (56)	2.5 (97)	2.5 (186)

[1] The value in parenthesis is number of reads in each chamber

Table 4 Comparison of composition of clades of the *Candidatus* Accumulibacter-related 16S rRNA gene clones/reads between groups in SBR and A_2O process

Clades defined by Kim et al. [27]	Composition of clone from SBR[a] (%)	Composition of reads from A_2O (%)			
		Anaerobic	Anoxic	Oxic	Total
Acc-SG1	69.2 (81)[b]	15.1 (8)	4.8 (3)	3.3 (4)	6.3 (15)
Acc-SG2	7.7 (9)	7.5 (4)	6.5 (4)	25.4 (31)	16.5 (39)
Acc-SG3	20.5 (24)	20.8 (11)	17.7 (11)	13.9 (17)	16.5 (39)
Acc-SG4	2.6 (3)	26.4 (14)	25.8 (16)	29.5 (36)	27.8 (66)
Other	0 (0)	30.2 (16)	45.2 (28)	27.9 (34)	32.9 (78)
Total	100 (117)	100 (53)	100 (62)	100 (122)	100 (237)

[a] Data from Kim et al. [27]

[b] The value in parenthesis is number of clone/reads in each chamber

Table 5 Shared OTUs of *Candidatus* Accumulibacter in this pilot A_2O process

	Anaerobic	Anoxic	Oxic	Total	Representative sequence	Similarity (%)	Cluster
OTU01	0[1]	0	1	1	G9IMNKZ01AO1SQ	97.57	A2O-1
OTU02	0	0	6	6	G9IMNKZ01BD2CE	97.91	A2O-2
OTU03	0	0	1	1	G9IMNKZ01A6YEA	96.65	A2O-1
OTU04	0	0	1	1	G9IMNKZ01AYV6T	99.79	ACC-SG4
OTU05	0	0	1	1	G9IMNKZ01A8P8D	97.89	ACC-SG2
OTU06	1	0	3	4	G9IMNKZ01AEOXE	97.18	A2O-1
OTU07	1	0	0	1	G9IMNKZ01A5Q40	96.98	A2O-2
OTU08	1	0	1	2	G9IMNKZ01BKHX4	96.47	ACC-SG2
OTU09	1	0	0	1	G9IMNKZ01BM4KK	94.76	A2O-1
OTU10	1	0	0	1	G9IMNKZ01BQ1WH	96.49	A2O-1
OTU11	3	3	2	8	G9IMNKZ01AXACU	97.59	ACC-SG1
OTU12	1	1	2	4	G9IMNKZ01BXAKG	97.16	ACC-SG3
OTU13	9	9	14	32	G9IMNKZ01A7UP7	97.78	A2O-2
OTU14	1	1	0	2	G9IMNKZ01BZUW7	97.39	A2O-1
OTU15	0	1	1	2	G9IMNKZ01AF9VF	96.23	A2O-2
OTU16	0	1	0	1	G9IMNKZ01AT5H7	95.37	A2O-1
OTU17	3	3	20	26	G9IMNKZ01APA5B	97.30	ACC-SG2
OTU18	11	21	29	61	G9IMNKZ01B08RD	97.78	A2O-1
OTU19	0	1	0	1	G9IMNKZ01AMONG	96.12	ACC-SG2
OTU20	20	21	40	81	G9IMNKZ01ANJW2	100	ACC-SG4
Total	53	62	122	237			

[1] Number of reads

genera *Azospira*, *Ideonella*, and *Rhizobacter* can reduce nitrate, but *Sphingopyxis*, *Curvibacter*, and *Piscinibacter* cannot [36, 46, 48].

Even though denitrification reactions are favorable at anaerobic (or anoxic) than aerobic condition, there are a few reports about aerobic denitrifying bacteria that reduce nitrate even in the presence of a high concentration of O_2 [47]. In addition to *D. koreensis* and *D. soli* [53], some *Pseudomonas* spp. [29], *Alcaligenes* sp. STC1 [39], *Paracoccus denitrificans* (formerly *Thiosphaera pantotropha*) [7, 42], *Microvirgula aerodenitrificans* [40], and *Thaurea mechernichensis* [44] are revealed as aerobic denitrifiers. In this study, only 26 reads for the genus *Thauera* and 6 reads for the genus *Pseudomonas* were detected out of a total of 7,447 reads, and the genera *Alcaligenes*, *Paracoccus*, and *Microvirgula* were not detected at all. Therefore, it seems that the function of known aerobic denitrifiers is not significant in denitrification reactions in our A_2O system.

Meanwhile, You et al. reported that *Streptococcus suis* were the predominant bacteria in the aerobic activated

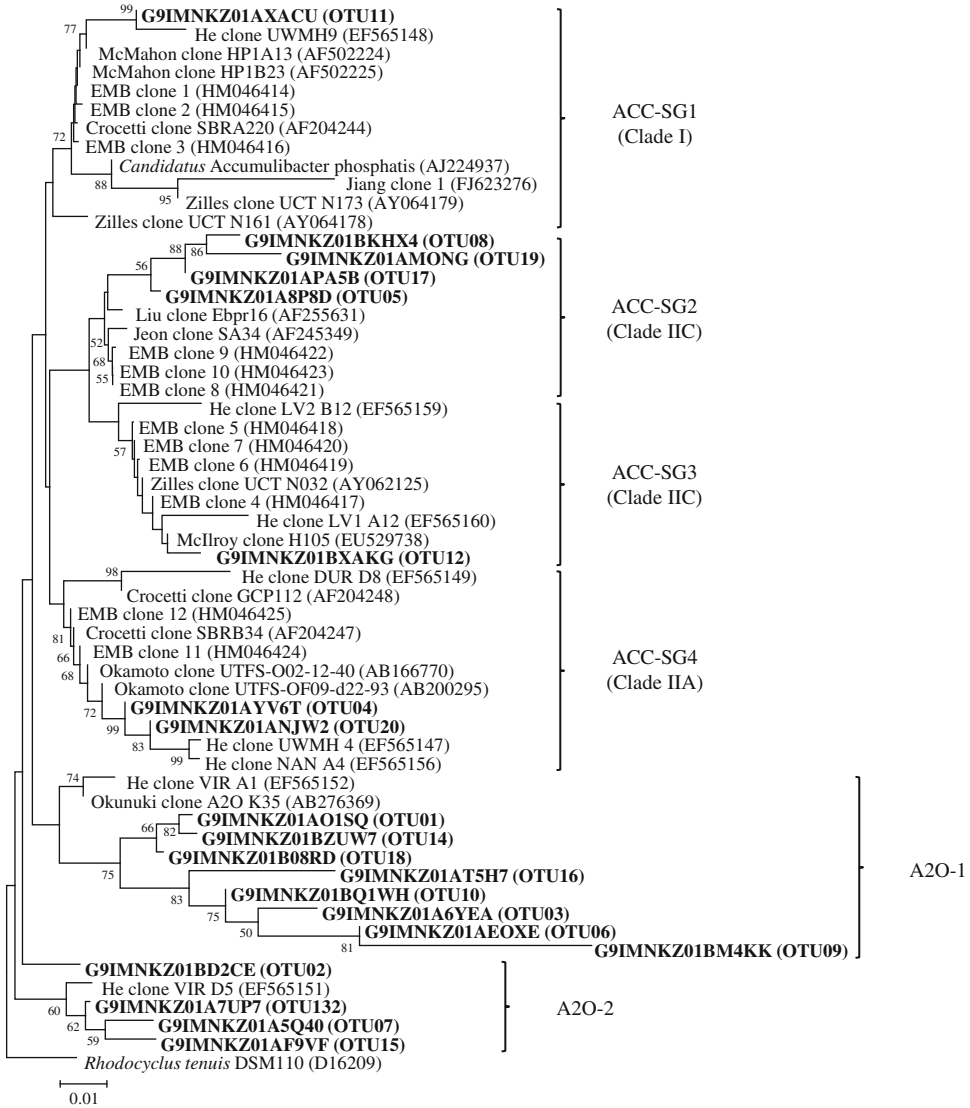

Fig. 4 Neighbor-joining tree of the shared OTUs *of Candidatus* Accumulibacter The representative sequences of the shared OTUs in this study were marked in *bold*. The *numbers* of the node were bootstrap values based on neighbor-joining analyses of 1,000 resampled datasets, and only bootstrap values greater than 50 % were shown in the tree. *Scale bar*, 0.01 nucleotide substitutions per position

Table 6 Composition of nitrifying bacteria in this pilot A_2O process

Nitrifying bacteria	Composition (%)			
	Anaerobic	Anoxic	Aerobic	Total
AOB	1.2 (19)[1]	1.3 (30)	1.1 (42)	1.2 (91)
NOB	2.9 (45)	2.0 (45)	3.1 (118)	2.7 (208)

[1] The value in parenthesis is number of reads in each chamber

sludge of their A_2O system; the abundance of *S. suis* was 16.5 % [54]. In the study of You et al., microbial communities were analyzed using a combined cloning-DGGE method [54]. In this study, only three reads of a phylotype of the genus *Streptococcus* were detected only from the anoxic chamber. Another well-reported autotrophic denitrifier in the A_2O process *Thiobacillus denitrificans* [52] was not found in this study, either. However, the family *Thiobacillaceae* constituted 0.8–1.2 % of the microbial reads obtained from our A_2O process.

Microorganisms determine the function of a biological wastewater treatment process for nitrogen and phosphorus removal. In this study, the pyrosequencing analysis of 16S rRNA gene sequences for biomass from a stable pilot-scale A_2O process showed similarity in the bacterial communities in the anaerobic, anoxic, and oxic chambers, even though the operation conditions for the biological process determine the population of the bacteria. This indicates that the same microbial community might perform a different function under different environmental conditions. In addition, an unexpected diversity of bacteria and unreported *Candidatus* Accumulibacter groups were observed. Finally, the numbers of reads have provided additional questions about some major phylotype, such as the genus *Dokdonella*, and a phylotype of *Chloroflexi*, function in the A_2O process of which was not determined.

Acknowledgments This work was supported by research funding from Hanyang University (HY-2011-T) and the R&D Program of MKE/KEIT [10037331, Development of Core Water Treatment Technologies based on the Intelligent BT-NT-IT Fusion Platform].

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