

Full Length Research Paper

Dynamics of bacterial community structure in a full-scale wastewater treatment plant with anoxic-oxic configuration using 16S rDNA PCR-DGGE fingerprints

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The microbial community composition and dominant bacterial populations in anoxic-oxic activated sludge from a full-scale wastewater treatment plant (WWTP), were investigated with polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) coupled with sequence analysis of 16S rRNA gene fragments from dominant bands. The bacterial ecological distribution at different parts of each reactor, the dynamics of the bacterial community structure over the total process course and temporal dynamics were obtained by comparing the DGGE patterns. Differences were noted between three sections of the reactors and also two reactors within the entire process of the system. Temporal variations in bacterial composition of the activated sludge in each reactor were responded to their circumstances. The prominent DGGE bands were excised and sequenced in order to identify the predominantly present and active bacterial populations. Here, combined with phylogenetic analysis of the DGGE fingerprints, the four major bacterial lineages can be revealed: α , β , γ - *Proteobacteria*, and the phylum *Firmicutes* (low G+C Gram-positive). The 16S rDNA sequence analysis indicated that the DGGE bands of dominant bacterial from this plant harbored sequences of possible nitrogen remover with potential aerobic denitrification / heterotrophic nitrification.

Key words: Community structure, PCR-DGGE, 16S ribosomal DNA, wastewater treatment plant (WWTP); activated sludge.

INTRODUCTION

Untreated or insufficiently treated wastewaters cause several problems, such as eutrophication, oxygen con-

sumption, and toxicity, when discharged to environment. The activated sludge process used to treat wastewater involves the biological degradation of organic material. Different operational systems have been developed to optimize nitrogen (N) and phosphorus (P) removal from wastewater, making it a widely used technology for biological and advanced wastewater treatment today. Removal of nitrogen compounds from wastewater can be accomplished by a combination of nitrification and denitrification in wastewater treatment (Schmidt et al., 2002).

Although the engineering aspects of the anoxic-oxic system were well understood, little was known about the composition of the microbial community responsible for the process. The diversity and identification of the dominant bacterium was limited with culture-dependent

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Abbreviations: PCR, Polymerase chain reaction; DGGE, denaturing gradient gel electrophoresis; WWTP, wastewater treatment plant; SND, simultaneous nitrification and denitrification; EBPR, enhanced biological phosphate removal; T-RFLP, terminal restriction fragment length polymorphism; ARDRA, amplified ribosomal DNA restriction analysis; SBRs, sequencing batch reactors; TE, tris ethylene diamine tetraacetic acid; DO, dissolved oxygen; COD, chemically dissolved oxygen; HRT, hydraulic retention time; MLSS, mixed liquor suspended solid.

methods due to the low recovery (as percentage of total cell counts less than 5%) and culture contamination by heterotrophic bacteria. Moreover, culture-dependent methods are biased by the selection of species which obviously do not represent the real dominance structure. Recent developments in molecular ecology have provided new molecular techniques that make it feasible to investigate complex microbial communities, overcoming the problems associated with the traditional cultivation dependent methods (Amann et al., 1995).

Denaturing gradient gel electrophoresis (DGGE) technique based on 16S rDNA gene permit investigation of the spatial and temporal variability of the population in environment (Muyzer et al., 1993; Myers, 1987). This technique can provide information on the predominant species in a community and analyze multiple samples simultaneously (Miller et al., 1999). Comparative analyses with nucleotide databases and phylogenetic reconstruction of the amplified 16S rRNA genes from DNA fragments excised from DGGE gels allowed the identification of organisms affected by the population changes.

Knowledge of the dynamics and structural diversity of microbial communities in wastewater treatment plant, and an understanding of the contributions of major members of these communities to overall degradation, are likely to provide unprecedented control over the biological treatment of domestic effluent.

In wastewater treatment systems (full- and lab-scale) there appear to be selection for either predominance of a single bacterial population or several different bacterial populations occur together. It was suggested that different plants might support different populations and different levels of species richness (Rowan et al., 2003; Hallin et al., 2005; Dytczak et al., 2008). In addition, different environmental conditions during long-term operation should result in the selection of different microbial populations. It might be assumed that the system under different operation situations may harbor different bacterial community compositions. Moreover, until now, most of the studies concerning bacterial community structure for successful nitrogen and phosphate removal were focused on lab-scale sequencing batch reactor (Lee et al., 2002), there is still some lack of knowledge about the bacterial community structure in continuous-flow operational processes and in the full-scale wastewater treatment plants (WWTPs). Since the conditions in the full-scale WWTP are so different from a lab-scale one, it might be expected that the composition of the microbial community might also be different. Based on the two aspects, this study aimed to obtain an over-view of the bacterial diversity, community structure and its phylogenetics in the full-scale anoxic-oxic WWTP under two operating conditions by using DGGE and 16S rRNA techniques. The study hopes to add to the body of knowledge and information on the diversity and dynamics of the microorganisms that are commonly present or potentially functional in this type of full-scale system.

MATERIALS AND METHODS

Experimental wastewater treatment system

The activated sludge samples were collected from a WWTP located in Liaoning, China, which receives a mixed wastewater of domestic and industrial origin (agrochemicals and speciality chemicals). The wastewater is treated with the anoxic-oxic treatment process and suspended chain moving aeration technique. Three reactors from the WWTP were studied-two biological reactors ('An' for anoxic and 'O' for oxic) by which nitrification-denitrification with little phosphorus removal was achieved, and the return sludge reactor (R), which are used to allow sludge biomass return to anoxic reactor from the settler. Two operating conditions were designed for optimization of treatment efficiency in this study, that is, stage one with the return sludge ratio of activated sludge (r) was 100% and the average DO (dissolved oxygen) concentration was 1.2 mg/L; stage two r was 150% and the average DO was 1.8 mg/L.

The major parameters obtained from the original WWTP system are: Hydraulic retention time (HRT), 10 h; Mixed liquor suspended solid (MLSS), 3000 mg/L; Sludge age, 15 days; chemically dissolved oxygen (COD) removal with 85% and NH_4^+ -N removal with 80 - 93%.

Sampling and preparation

Wastewater samples were collected in triplicate from the anoxic, oxic and sludge return reactors of the system. Activated sludge samples for DNA extraction were collected in sterile Falcon tubes and frozen at -20°C for immediate processing. Three individually well-mixed activated samples were taken from entrance (En), middle (M) and outlet part (Ou) in anoxic and oxic bioreactors at the same bioreactor depth to monitor the bacteria community structure dynamics between different parts of each reactor. Sludge from the anoxic and oxic bioreactors was respectively collected at three days interval over two weeks to analyze the short-term bacteria community dynamics. In addition, samples were taken under two different operation conditions in order to explore the structure changes. All analytical assays (COD and NH_4^+ -N) were performed according to Standard Methods (APHA, 1998).

The bacterial biomass was harvested by centrifugation of approximately 100 ml well mixed samples at $10,000 \times g$ for 10 min. The pellets were washed using 10 ml TENP buffer solution (50 mM Tris-base, 20 mM EDTA, 100 mM NaCl, 0.01 g/ml polyvinylpyrrolidone, pH 8.0) and PBS buffer solution (8.00 g NaCl, 0.20 g KCl, 1.44 g Na_2HPO_4 , and 0.24 g KH_2PO_4 , added to 1000 ml sterile distilled water, pH 8.0).

DNA extraction

The genomic DNA of each sample was extracted with a modified method described previously (Bourrain et al., 1999). DNA concentrations were determined spectrophotometrically by measuring the A_{260} . The quality of extracted DNA was checked by standard agarose electrophoresis.

PCR amplification of 16S rDNA

Polymerase chain reaction (PCR) amplification of bacterial 16S rRNA gene fragments was performed using primer 968F-GC (5'-CGCCCGGGGCGCGCCCGGGCGGGGCGGGGGCACGGGGGGAACGCGAAGAACCCTTAC-3') and primer 1401R (5'-CGGTGTGTACAAGGCCCGGGAACG-3') for the Domain Bacteria (Heuer et al., 1997), corresponding to positions 968 and 1401 in the 16S rDNA of *Escherichia coli*, with a 40 bp-GC-rich sequence (5'-

CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGG (G- 3') attached to the 5' end of the forward primer to stabilize the melting behavior of the DNA fragments. PCR mixtures had a final volume of 50 μ l which contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 20 pmol of each primer, 2.0 μ l of the DNA template and 2 U of Taq DNA polymerase (Takara Biotechnology, Dalian, Co., Ltd.). PCR amplification was conducted in a DNA engine dyad Peltier thermal cycler (BIO-RAD, USA).

To increase the specificity of the amplification and to reduce the formation of spurious by-products (Don, 1991), Hot start PCR was performed at 94°C for 4 min for activation of the polymerase and a touchdown PCR (Muyzer et al., 1995) was carried out as follows: The annealing temperature was initially set at 65°C and then decreased by 0.5°C every cycle until 55°C, followed by primer extension at 72°C for 2 min. Next, 15 additional cycles were carried out at 55°C for 1 min (primer annealing), followed by denaturation at 94°C for 1 min and primer extension at 72°C for 2 min. Finally, an extension step was carried out at 72°C for 10 min.

Analysis of PCR products by DGGE

DGGE of the PCR amplified 16S rDNA was carried out using the DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). DGGE were applied onto 8% (w/v) polyacrylamide gels in 0.5 × TAE (20 mM Tris-acetate; pH = 7.4, 10 mM acetate, 0.5 mM Na₂EDTA) with a denaturing gradient ranging from 30 - 60%. One hundred percent of denaturant corresponded to 7 M urea and 40% (w/v) formamide (Muyzer et al., 1993). Electrophoresis was performed for 17 h at 130 V. The temperature was set at a constant of 60°C. After electrophoresis, polyacrylamide gel was stained with Genefinder (Takara Biotechnology, Dalian, Co., Ltd.) for 30 min, and then visualized on UV transilluminator (BIO-RAD Universal Hood, Segrate (Milan), Italy) with a digital camera (DC120 ZOOM, Kodak, USA). Gel images were stored by using the Imager System (Ampligene). Reproducibility of patterns was confirmed by repeated DGGE analyses using the same DNA extracts.

Dice coefficient of similarity (Cs, %) was introduced to study the microbial community similarity between the bioreactors. $C_s = 2j / (a + b)$, where j is the number of common bands between samples A and B; a and b are the total number of bands in samples A and B, respectively. This coefficient ranges from 0 (no common bands) to 100% (identical bands patterns) (Dice, 1945). Diversity was assessed, following log transformation, using Simpson's index of diversity based on the following formula: $D = 1 - \sum (n/N)^2$ where D is Simpson's index of diversity; n and N are intensity of each individual band and sum total intensity of all bands in the DGGE profile, respectively. Evenness was assessed using Simpson's index of evenness based on the following formula: $E_D = D / (1-1/M)$, where E_D is Simpson's index of evenness, D is Simpson's index of diversity, and M is total number of bands in DGGE profile. Significance was determined based on triplicate readings using an ANOVA.

Recovery of bands from DGGE gels and sequence analysis

Prominent DGGE bands were selected and excised for nucleotide sequence determination. For each fragment selected, only the middle portion was carefully excised from the gel using a sterile razor under UV illumination, and the DNA was eluted overnight at 4°C in a 1.5-ml tube containing 100 μ l TE buffer. The supernatant solution was used as template in PCR using the former protocol. Before DNA sequencing, the recovered DGGE bands were run on a DGGE gel to confirm the presence of a single band and its relative position compared with the initial run. This step was repeated at

least two times until the band appeared to be single to obtain a pure DNA product for sequencing. Individual DNA fragments were PCR-amplified with the forward primer 968F without the GC clamp and the reverse primer 1401R. Thereafter, the amplicons were purified using a QIA quick PCR Purification Kit (Qiagen, USA) and then the 16S rDNA regions were sequenced by Genecore BioTechnologies Co., Ltd. (Shanghai, China).

Phylogenetic analysis

The sequences obtained from the DGGE were analyzed in comparison with the 16S rDNA sequences in the GenBank database by using the basic local alignment search tool (BLAST, National Centre for Biotechnology Information, US National Library of Medicine) to retrieve similar sequences and phylogenetically related species. The alignment was calculated by the neighbor-joining method according to the Kimura's two-parameter model of Saitou and Nei (1987) using Clustal X. With this evolutionary distance matrix, phylogenetic affiliation of the sequences was further analyzed and a phylogenetic tree was plotted by Mega3.1 program (Kumar et al., 2004). Bootstrap analysis for 1000 replicates was performed to estimate the confidence of tree topologies. The partial 16S rDNA sequences determined during the present study have been deposited with NCBI database.

RESULTS AND DISCUSSION

DGGE profiles of sludge sample at different parts in anoxic and oxic reactor

The results showed that considerable differences in bacterial DGGE data were observed between different sections of the bioreactors (Figure 1, lanes 1 - 6). More bands were detected in samples from the entrance of anoxic (Figure 1, lane 1) and oxic reactor (Figure 1, lane 4) than those at the middle and out sections of the two corresponding reactors (Figure 1, lanes 2 - 3 and 5 - 6). Interestingly, the similarity values (Cs, %) of the bands appeared in the samples collected from the middle and the outlet areas were very high both for anoxic and oxic reactor (e.g. A-M versus A-Ou, Cs = 97.7; O-M versus O-Ou, Cs = 92.6) whereas, the values between the entrance and the middle or outlet were relative low (e.g. A-En versus A-M, Cs=72.0; A-En versus A-Ou, Cs = 73.7; O-En versus O-M, Cs = 78.4; O-En versus O-Ou, Cs = 76.3; Table 1). However, the similarity value between the middle and the outlet in the two reactors were respectively almost more than 70%. This may be attributable to the more homogenous conditions engendered by the vigorous mixing and recycle regime in the middle and rear section of these reactors. It could be contributed to the stratification that exists in unmixed part of the reactors as the waste is degraded and the changes in composition as it passes through the basin (Bishop and Kinner, 1986). The influent into the anoxic basin contained a high amount of organic matter, and as the waste is degraded, the level of organic matter will be reduced and concomitantly oxygen will be consumed. In the presence of high levels of organic matter, heterotrophs

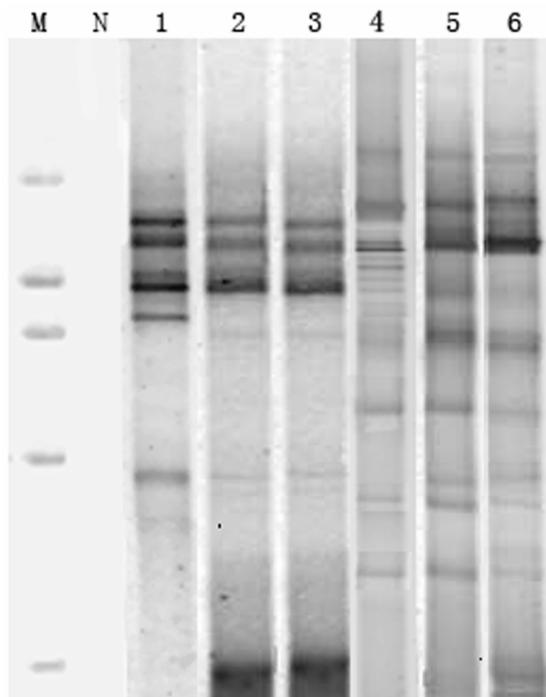


Figure 1. DGGE file generated from the activated sludge samples from different sites in two reactors for PCR amplification of the V6-V8 region of the 16S rDNA gene. M: Marker; N: Negative control; Lane1-3: three samples from Anoxic reactor, A-En, A-M, A-Ou; Lane 4 - 6: three samples from Oxidic reactor, O-En, O-M, O-Ou.

can out-compete autotrophic groups, such as ammonia oxidisers for oxygen (van Niel et al., 1993) and ammonia (Hanaki, 1990; Verhagen and Laanbroek, 1991). As the organic matter declines, the number of heterotrophs decreases and consequently functional microbial group can proliferate. Hence there should be a lower number of heterotrophs and a higher number of some functional groups dominating the system after the entrance part of the anoxic basin (Bishop and Kinner, 1986). Moreover, improved aeration condition caused the major group abundance in the oxidic reactor.

Short time dynamics in DGGE profiles in anoxic and oxidic bioreactors

The short time dynamics of the bacteria community structure were monitored from the three-days-interval activated sludge. The microbial community structure and the dominate bacterial did not change significantly (Figure 2). The temporal variation between the five analyzed samples from each reactor was only low but clearly expressed in the abundance of distinct peaks. This relative stable community structure might stem from relative stable flow rate and composition of influent from

each reactor, and consequently no outer impact influenced the ecological distribution notably, but only inter-specific reactions occurred.

Differences between the oxidic and anoxic reactors in A/O configuration WWTP

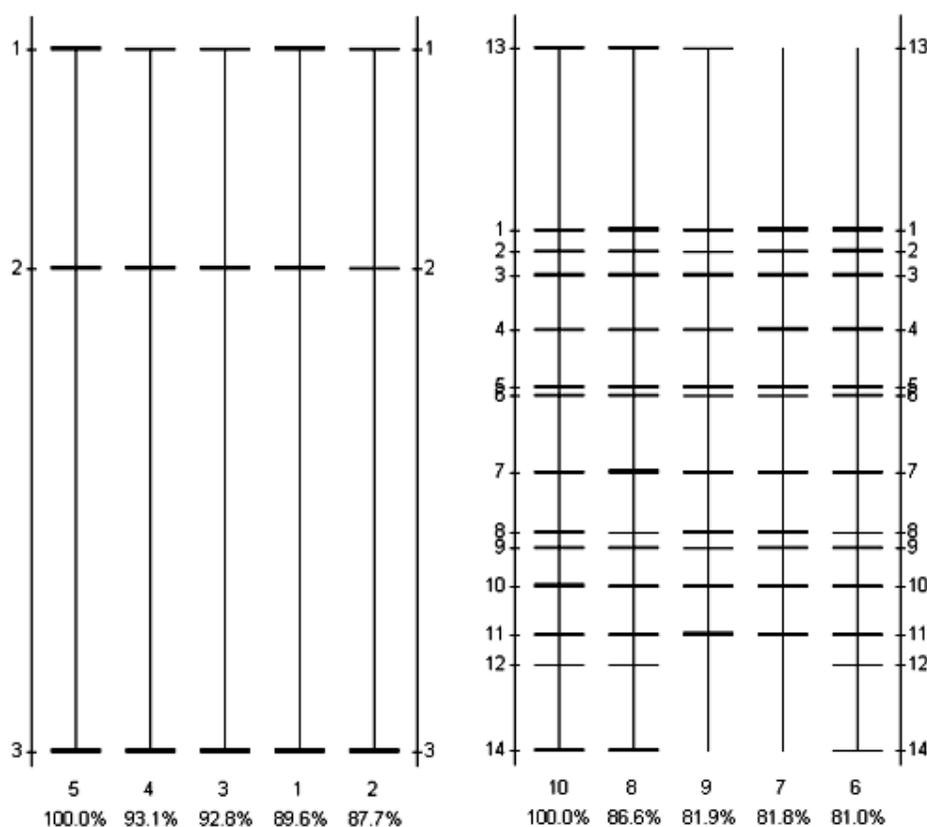
Analysis of duplicate samples by DGGE revealed that the profiles obtained were reproducible. Visual comparison of the DGGE profiles of bacterial 16S rDNA gene fragments from the WWTP revealed some different populations between the reactors (Figure 3). Analysis of triplicate DGGE profiles derived from anoxic and oxidic basin templates detected a total of 19 bands. Of these 19 bands, 10 were reproducibly detected across duplicates in either oxidic or anoxic basin. Of these 10 bands, 4 were common to both oxidic and anoxic reactor profiles. There were 5 bands appearing across all two replicates unique to the profile of oxidic reactor and one band appearing across all two replicates unique to the anoxic one. Simpson's indices of diversity and evenness were calculated for each replicate for each sludge basin. Sludge from oxidic basin generated a DGGE profile significantly ($p < 0.01$) more diverse and even than sludge from anoxic basin (Figure 4).

The level of aeration appears to have an effect on the bacterial populations present as suggested by Rowan et al., (2003). The anoxic-oxidic activated sludge plant is mainly composed of three linked basins, namely; anoxic, oxidic bioreactors and settler, each of which is optimized for different processes. In spite of sewage and sludge feeding to the following one, these three basins were relatively separated from each other, unlike the sequencing batch reactors (SBRs), e.g. simultaneous nitrification-denitrification achievement with or without enhanced biological phosphorus removal just in one reactor. Some microorganisms, adapted to the condition, (oxygen and organic carbon resource) would grow fast and out-compete with other groups; hence peculiar community structure conformed under particular operation mode. In addition, only those groups that were able to grow under the conditions was found to be active, while the rest might exhibit active until it reaches the other reactor, or probably they not be detected due to the low abundance (Gich et al., 2000). This is evident by a visual comparison of the banding pattern of each of the three steps (Figure 5, lanes 1 - 3). The anoxic reactor harboured a lower detectable diversity of bacteria compared to the oxidic one. In particular, some bands that migrated further in the DGGE gel in samples from the oxidic reactor were not noted in the anoxic reactor. Nevertheless both reactors appeared to have some common predominant population (marked c, d, e, i, in Figure 5, lanes 1 - 4).

This finding is consistent with a previous study in which significant differences in the microbial community structure were found by terminal restriction fragment length

Table 1. Dice coefficients (Cs, %) comparing the similarities of PCR-DGGE fingerprints (Figure 1) from anoxic and oxic reactor.

	A-En	A-M	A-Ou	O-En	O-M	O-Ou
A-En	100	72.0	73.7	-	-	-
A-M	72.0	100	97.7	-	-	-
A-Ou	73.7	97.7	100	-	-	-
O-En	-	-	-	100	78.4	76.3
O-M	-	-	-	78.4	100	92.6
O-Ou	-	-	-	76.3	92.6	100

**Figure 2.** Bacterial DGGE patterns of anoxic and oxic reactor sludge taken at continuous time period at 3 day interval from the full-scale wastewater treatment system. Lane 1-5: five samples from Anoxic reactor; Lane 6-10: five samples from Oxic reactor.

polymorphism (T-RFLP) and comparative 16S rDNA analysis in two laboratory plants (Eschenhagen et al., 2003). The result is also similar with that of Ma et al., (2006), who applied FISH analysis to reveal the difference in microbial community between A/O and the –O– reactors, but is not consistent with that of Ehlers and Cloete (1999) who used protein fingerprints to evaluate the differences between the microbial community structures among P-removing, non-P-removing and N-removing systems. It was reported that there were probably no significant changes of the microbial communities between oxic and anoxic reactors using amplified ribo-

somal DNA restriction analysis (ARDRA) (Gich et al., 2000). These differences may be due to the absence of most rigorous validation probes that could cover all the functional groups involved in full-scale processes.

Spatial dynamics for bacterial community structure within the entire process under two different stages

DGGE profiles indicated that although the system was fed the same wastewater influent with stable composition, the system harboured distinct bacterial composition

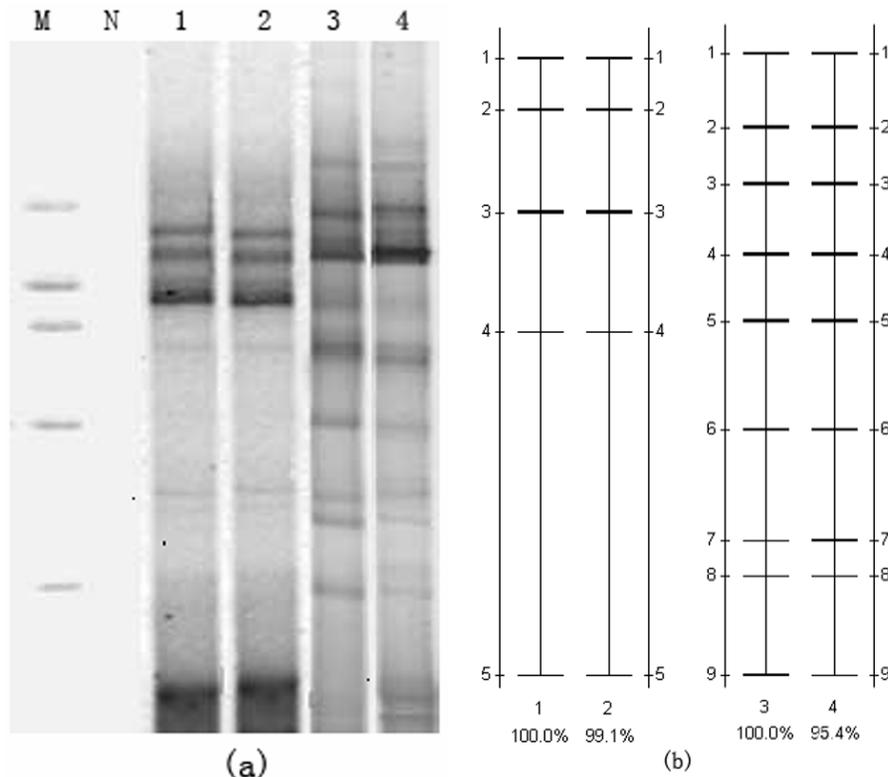


Figure 3. DGGE files and lane comparison generated from the activated sludge samples from different sites in one reactor for PCR amplification of the V6-V8 region of the 16S rDNA gene. (a) DGGE fingerprint file. M: Marker; N: Negative control; Lane1-2: DGGE fingerprints of two replicates from Anoxic reactor activated sludge, A1, A2; Lane 3-4: DGGE fingerprints of two replicates from oxic reactor activated sludge, O1, O2. (b) Lane comparison for different reactor samples.

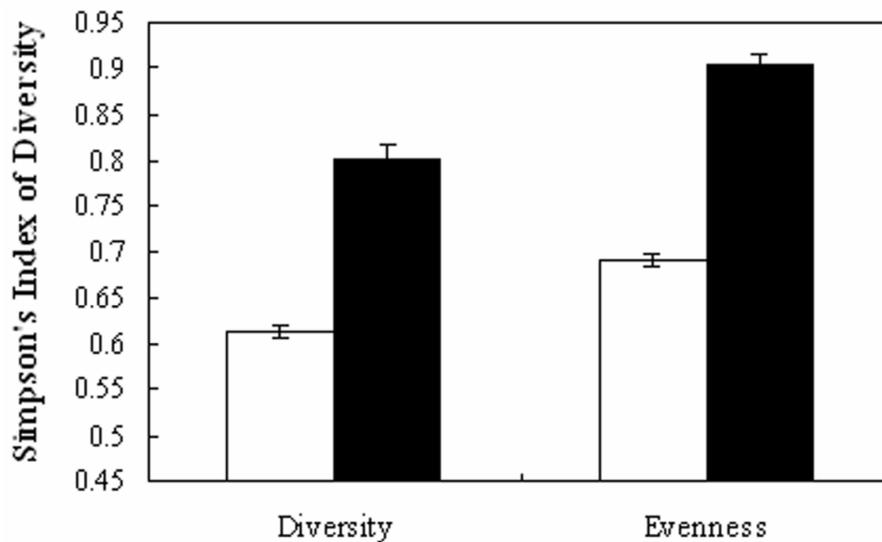


Figure 4. Diversity and evenness of the bacterial communities in anoxic and oxic basin, as derived from denaturing gradient gel electrophoresis (DGGE) profiles and analysis by Simpson's index. The anoxic basin community (white bars) is both less diverse and even than the less functional oxic basin community (black bars). Diversity and evenness indices were determined from triplicate DGGE profiles and averaged. Error bars represent standard deviation across triplicates.

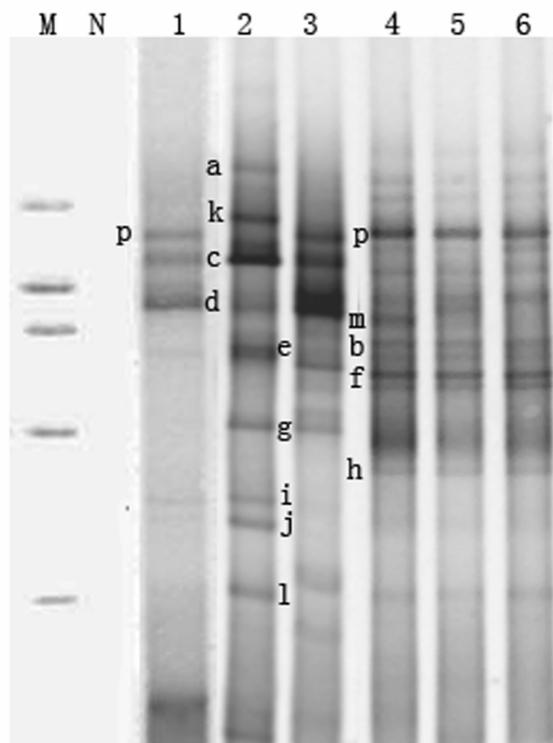


Figure 5. PCR-DGGE fingerprints produced from each reactor samples through the entire process. Letters indicate excised bands from which sequences were determined. M: Marker; N: Negative control; Lane 1-3: Anoxic (An1), Oxidic reactor (O1) and Sludge Return tank (R1) under stage one, Lane 4-6: Anoxic (An2), Oxidic reactor (O2) and Sludge Return tank (R2) under stage two.

under different operational process (Figure 5). The reactors had a lower detectable diversity of bacteria in stage two (Figure 5, lanes 4 - 6) than in stage one (Figure 5, lanes 1 - 3). After increasing sludge biomass amount (the sludge returning ratio was increased from 100 to 150%) from settler to anoxic reactor, and increasing the oxygen concentration (average DO concentration was added to 1.8 from 1.2 mg/L in stage one), DGGE community fingerprint (that is, band numbers and intensity) significantly changed: The positions of the major bands shifted in particular, a number of clear and dense bands that were noted in the DGGE gel in stage one, were observed separate in stage two (Figure 5, band d and m), suggesting an evolution process of the dominant species after the condition changes. When more sludge biomass was partially recirculated and the aeration was improved (Figure 5, lanes 4 - 6), some populations represented by band (a, k, e, g, i, j) almost disappeared, indicating the probable reduction in population. At the same time, bands (c), (d) and (l) weakened, indicating a possible decrease of those bacterial populations. Nevertheless, some bands (p, f, and h) were observed in details in stage two.

Analysis of sequence data

The denaturant gradient used was 40 - 60% from top to bottom. The results of homology search and the origin of the closet relative for the sequences obtained were showed in Table 2. Phylogenetic analysis of 14 distinct groups by DGGE analysis revealed that the predominant fragment prevailing in this biological system were affiliated with 4 phyla or classes of the domain Bacteria: α , β , γ -*Proteobacteria*, and the phylum *Firmicutes* (low G+C Gram-positive). Most corresponded to *Proteobacteria* and only a small fraction corresponded to Gram-positive group.

The recovered fragment sequence of band (a) (Figure 5, lane 2) has a high similarity (Cs = 99%) to an uncultured α -*Proteobacteria* bacterium clone, which was isolated along the Changjiang River by using DGGE (Sekiguchi et al., 2002) This genus was involved in phosphate removal wastewater process (Hiraishi, 1991).

The 16S rDNA sequence of band (k) was close to that of uncultured *Alcanivorax* sp. clone 8-1, members included in bromoamine acid removal (Qu et al., 2005). While member of *Simplicispira limi* sp. nov. affiliated with β -*Proteobacteria* was represented as thick band in most DGGE lanes except the sample O1, that is, band (p) (Figure 1). The organism is a novel species of the genus of *Simplicispira*, Gram-negative, oxidase-positive and catalase-positive, capable of citrate utilizing and nitrate reducing, which was isolated from sludge performing EBPR in a laboratory-scale SBR (Lu et al., 2007).

Band (c) has 100% similarity on the 16S rRNA gene level with *Psychrobacter* sp., which was isolated from an NPnEO-contaminated wastewater treatment plant and had decompose activity to 4-*n*-NP (Di Gioia et al., 2008). *Psychrobacter* is a relatively recent genus whose closest phylogenetic relatives are members of *Moraxella* and *Acinetobacter* genera (Di Gioia et al., 2008; Bowman et al, 1996). Similarly, band (d) can be affiliated to the genus of Moraxellaceae (*Acinetobacter* sp., 100% sequence similarity). Members of this genus are responsible for process of N dechlorination of Aroclor 1260 in the JN cultures (Bedard et al., 2007).

Band (e) could be matched with 100% similarity to an uncultured bacterium 5.9 from environmental sample (Terenius et al., 2008). It is probably that the corresponding organism of band (e) is a new species directly or indirectly involved in industrial wastewater treatment.

The three intense bands in the DGGE profile of O1 sludge, named i, j and l, can be affiliated to γ -*Proteobacteria*, and are grouped into *Pseudomonas* genera respectively (Table 2). Band (i) is 100% similar to *Pseudomonas borbori* from a nitrifying inoculum (Vanparys, 2006). Band (j) is 100% similar with *Pseudomonas stutzeri* strain ZWLR2-1 which was isolated from chloronitrobenzenes-contaminated soil using 2-CNB as a sole source of carbon, nitrogen and energy (Liu et al., 2005). *P. stutzeri* was previously reported to relate to aerobic denitrification (Su et al., 2001). Similarly,

Table 2. Sequence analysis of dominant bands excised from DGGE gels derived from bacterial 16S rDNA gene amplicons (% - percentage of similarity between the 16S rRNA gene of dominant band and the closest relative in the NCBI database).

DGGE Band ^a	Closest relative and accession number	Origin of closest match	Similarity %
a (EU663562)	Uncultured bacterium clone CR98-24-20 (AF428826)	Environmental sample, China: Changjiang River	99
b (EU663547)	Uncultured bacterium H93 (EU529735)	EBPR system, Australia (unpublished)	99
c (EU663548)	<i>Psychrobacter</i> sp. BCcN16A2 (DQ976371)	NPnEO-contaminated wastewater treatment plant, Italian	100
d (EU663552)	<i>Acinetobacter</i> sp. JN18_V5_C (EF059532)	Sediment-free PCB-dechlorinating enrichment culture	100
e (EU663549)	<i>Pantoea agglomerans</i> strain CONC7 (EU275357)	Sediment from tannery waste treatment plant (unpublished)	100
	Uncultured bacterium clone (EF179831)	Environmental sample	100
f (EU663563)	Uncultured beta proteobacterium clone Orbal D7 (AF450461)	Full-scale aerated-anoxic wastewater treatment	98
	<i>Dechloromonas denitrificans</i> (AJ318917)	Earthworm gut	97
g (EU663557)	<i>Exiguobacterium aurantiacum</i> strain DSM 6208 (DQ019166)	Siberian permafrost	100
h (EU663564)	<i>Aeromonas</i> sp. MM.2.5 (EF550574)	Slaughterhouse wastewater treatment plant	100
i (EU663555)	<i>P. borbori</i> (AM114527)	Nitrifying inoculum, Gent, Belgium	100
j (EU663551)	<i>P. stutzeri</i> strain ZWLR2-1 (AY647159)	Chloronitrobenzenes-contaminated soil	100
k (EU663553)	Uncultured <i>Alcanivorax</i> sp. clone 8-1 (AY755368)	Bioaugmented sequencing batch reactors for bromoamine acid removal	100
l (EU663554)	<i>P. suwonensis</i> strain 4M1 (AY927994)	Cotton waste composts	100
m (EU663556)	<i>Acinetobacter</i> sp. G3DM-29 (EU037279)	Chromium contaminated soil, Gujarat, India.	99
p (EU663561)	<i>S. limi</i> sp. nov. (DQ372987)	Sludge performing EBPR in a laboratory-scale SBR	98

^a Name of the DGGE bands in Figure 5.

The database entry with the highest level of identity is shown. When the most similar sequence was the sequence of an unidentified bacterium or environmental clone, the value for the most closely related identified bacterium is also given.

band (l) is 100% similar with validly published *Pseudoxanthomonas suwonensis* strain 4M1 having nitrate reduction ability (Weon et al., 2006).

In addition to the *Proteobacteria* group, member of *Bacillaceae* were detectable within the DGGE patterns of O1 sample represented as band (g), but the band was less dense in R1 than in O1 (Figure 5), suggesting it was active microbial consortium in oxic and return sludge reactor. It is clearly affiliated to an *Exiguobacterium* species with 100% similarity, which was isolated from Siberian permafrost, and was positive for nitrate reduction (Rodrigues et al., 2006). *Bacillus* was able to conduct simultaneously aerobic nitrification/denitrification (Kim et al., 2005). It is tempting to speculate that (you used this phrase in line 309, may be reconstruct this sentence) the corresponding organisms band (g) represented are directly or indirectly involved in nitrogen removal in this WWTP.

During stage two (lanes 4 - 6), there were two most intense bands within the DGGE patterns of all samples. Among which, band (f) is 98% similar to an uncultured bacterium from a scale aerated-anoxic wastewater treatment. A numerical domination of *Rhodocyclus*-positive cells has been observed in EBPR plants (Zilles et al., 2002). In addition, Crocetti et al., (2000) reported that an important group of PAOs in EBPR sludges are bacteria

closely related to *Rhodocyclus*. The cultured species band (f) is 97% similar to a *Dechloromonas* denitrifier affiliated within the β -*Proteobacteria* from earthworm gut (Ihssen et al., 2003). It implied that the members band (f) represented may be essential to nitrogen and phosphate removal in this WWTP.

Band (m) is 99% similar with *Acinetobacter* sp. G3DM-29 from chromium contaminated soil (Desai et al., 2009). However, band (m) has three alkaline base differences with band (d). Band (b) could not be matched with a cultivated strain published in the database, but is most relative (with 99% sequence similarity) with an uncultured bacterium H93 isolated from an EBPR system (unpublished). Band (h) has 100% similarity with *Aeromonas* sp. MM.2 isolated from a slaughterhouse wastewater treatment plant (Alexandra et al., 2007). *Aeromonas* are widespread in the mixed liquor of activated sludge plant and members of this genus have been reported for their capacity for organic matter degradation and nitrate reduction to nitrite (Nsabimana et al., 1999).

Phylogenetic affiliations

The differences in the results of the phylogenetic analysis

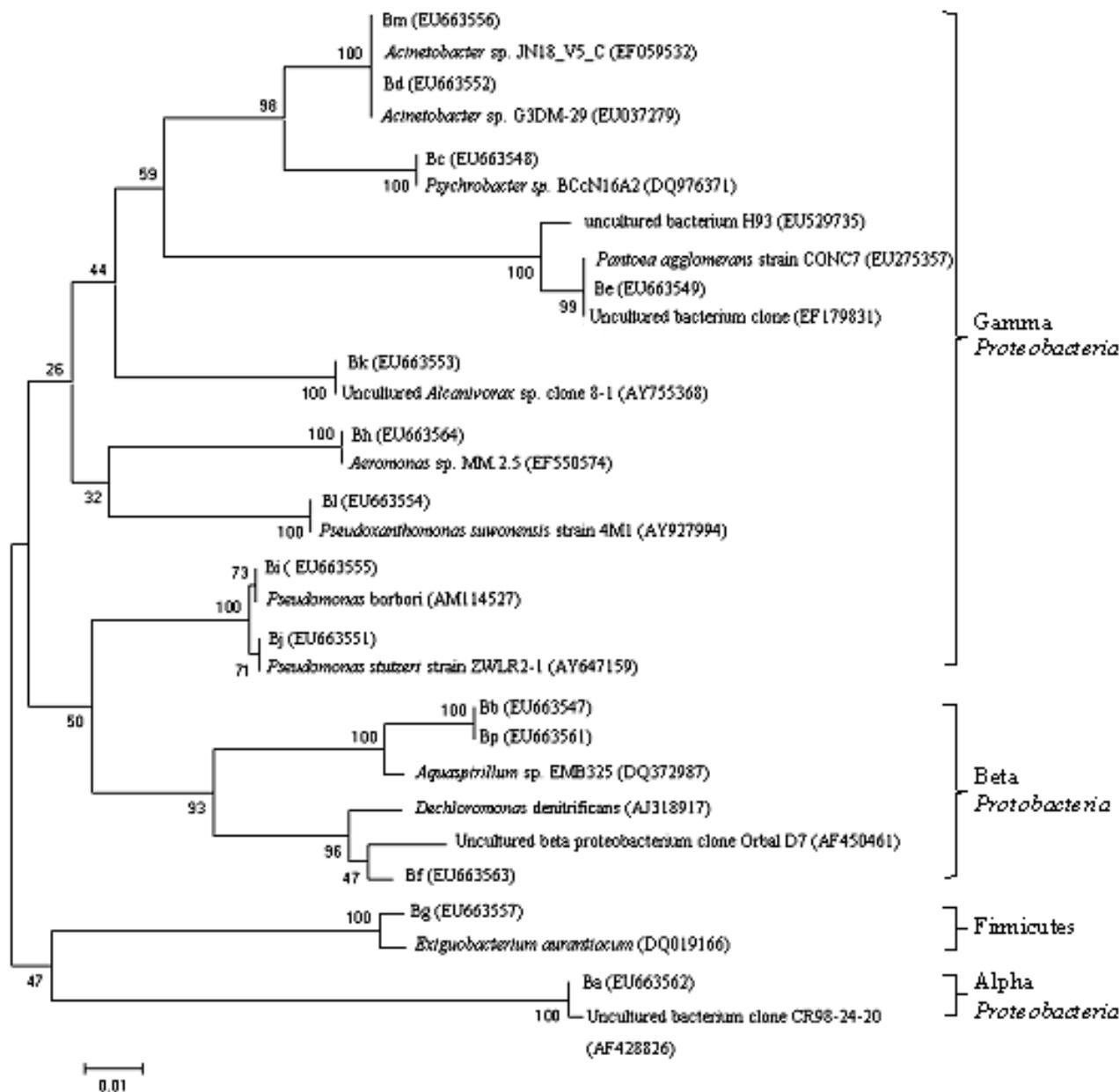


Figure 6. Neighbor-joining phylogenetic tree based on partial 16S rDNA sequences derived from DGGE bands. The tree was constructed by using distance matrix methods with Kimura's two-parameter model. Bootstrap values indicate areas of stable tree topology based on 1,000 bootstrap resamplings. The scale bar represents substitutions per site. The information in parentheses indicates accession numbers. Codes of the DGGE bands of this study are shown with the first character 'B' indicating 'band', and the second character denoting the name of the excised band. Bacterial clusters are depicted on the right side of the tree.

within the entire system could be summarized into two points (Figure 6): (i) Microbial close to the *Pseudomonas* group affiliated with γ -*Proteobacteria* was much more abundant in oxic reactor than in anoxic and return sludge reactor (band i, j and l), (ii) Microbial close to the Comamo-nadaceae (band p) and Rhodocyclaceae (band f) group affiliated with β -*Proteobacteria* were respectively detected not only in oxic one but in anoxic and return

sludge reactor. We considered that the diversity of the established resident population of *Pseudomonas* species in oxic tank was not only influenced by *Pseudomonas* strains supplied by the influent to oxic tank, but depended essentially on the conditions prevailing in the activated sludge. Furthermore, the stronger competition for nutrients and oxygen of heterotrophic population than the nitrifying population may also contribute to the abundant

distribution of the heterotrophic *Pseudomonas* group in oxic reactor (Stenstrom and Song, 1991). Moreover, the mixed liquor suspended solids (MLSS) was 3000 ± 89 mg/L, under which heterotrophic and nitrifying microorganisms do not exist in the suspension as dispersed individuals, but as flocs (Stenstrom and Song, 1991). It suggested that heterotrophic nitrification may contribute significantly in conversion of ammonia to nitrite besides autotrophic bacteria. These results are in accordance with the observation by Jetten et al., (1997).

It is remarkable that β -*Proteobacteria* were found to be more prevalent group during stage two than in stage one (Figure 5, lane 4 - 6). The probable reason may be the perturbation caused by the local condition change in each reactor. This perturbation may be the cause of both composition and diversity changes of *Pseudomonas* and β -*Proteobacteria*. New conditions prevailing in the aeration basin would favour bacterial strains which tolerate that situation, causing the *Pseudomonas* to decrease in oxic reactor, on the other hand, more sludge returning to anoxic reactor led to the similar bacterial community distribution between anoxic and return sludge reactor and dominance of β -*Proteobacteria* in both reactors. As suggested by Nsabimanai et al., (1999), this change may be due either to the community composition of return sludge biomass, or its activity or both. Finally, the competitions of other organisms present in activated sludge, especially protozoa, probably interfere through change in their number and composition. Further work is necessary to test these hypotheses.

These observations revealed that different environmental conditions during long-term operation, selected different dominant microbial populations. It suggested that the system under different operation situations in the same system may harbor different bacterial community compositions. The result is consistent with the finding of Hallin et al. (2005) who found that ammonia-oxidizing bacteria (AOB) distribution was different in different wastewater treatment plants.

Function of the dominant microbial groups in the anoxic-oxic wastewater treatment system

The β -*Proteobacteria* was considered to have a positive role in nutrient and phosphate removal in activated sludge processes (Bond et al., 1995, 1999; Wagner et al., 1993; Ma et al., 2006).

In this study, *Dechloromonas* genus as denitrificans within β -*Proteobacteria* was recovered from band (f). The second similar genus with band (f) in the database was a *Dechloromonas* sp. RCB having anaerobic benzene oxidation ability with nitrate reduction (Coates et al., 2001). *Dechloromonas* group was more closer to *Rhodocyclus* group, and they are both affiliated within Rhodocyclaceae. Many research have showed that *Rhodocyclus* group from activated sludge is responsible

for the enhanced biological phosphate removal (EBPR) processes (Jeon et al., 2003; Bond et al., 1999).

Horsch et al., (2005) attributed to γ -*Proteobacteria*; a distinctive role in initial membrane colonization since they were more predominant than β - and α -*Proteobacteria* in primary biofilms as compared with mature ones.

Our results are in agreement with many reports. In the last decade, numerous denitrifying bacteria of the genus *Pseudomonas* have been isolated from soil (Tiedje, 1988; Zumft, 1992; Gamble et al., 1977) and marine environments (Ward and Cockcroft, 1993). Recently, bacterial strains capable of removing high organic loads and NH_4^+ -N in anaerobic condition have been isolated (Kim et al., 2002 a,b). Moreover, *Pseudomonas* acting as denitrifier was obtained from a municipal wastewater treatment plant (Heylen et al., 2006) and was found capable of aerobic denitrification (Li et al., 2006). A heterotrophic bacterial strain capable of removing both ammonium and nitrate under anaerobic condition was isolated and identified from a soil and wastewater source (Kim et al., 2007).

Acinetobacter sp. within γ -*Proteobacteria* have been considered important in EBPR (Fuhs and Chen, 1975), but was later found not to perform the key biochemical transformations observed in EBPR sludge (Wagner et al., 1994). The presence of *Acinetobacter* sp. represented by band (d) in this study may be due either to the different habitats in these cases, or different methods used (Eschenhagen et al., 2003) or both. The case was also applied to another band (c), which relates to *Acinetobacter* sp. and affiliated within γ -*Proteobacteria*.

Conclusion

Anoxic-oxic process has been widely used for the biological wastewater treatment in order to eliminate eutrophication and improve the water quantity. This work shows the dominance and importance of most heterotrophic bacteria which belongs to the phylum *Proteobacteria* in an anoxic-oxic suspended chain moving aeration WWTP. This kind of plants especially in the aerating reactor (oxic) harbored sequences of possible nitrogen remover with probable aerobic denitrification/heterotrophic nitrification. The current work implies that under aerobic condition denitrification could also occur and nitrification generate not only with autotrophic organism but heterotrophic one as well, which has broken out of the traditional concept of autotrophic nitrification and anoxic denitrification and are becoming a new technique hotpot in biological nitrogen and phosphate removal technique. e.g. How to regulate these two processes simultaneously occur, namely; simultaneous nitrification and denitrification (SND) to achieve higher nitrogen remove. Actually, this study indicated that these aerobic denitrification/anaerobic nitrification may be occurring in a full-scale WWTP while they were just

neglected or underestimated especially in the strict-controlled lab-scale researches. In addition, anaerobic or anoxic may exist in inner partial aerobic condition, which could favor SND in a full-scale WWTP.

Possible phosphate remover affiliated to β -*Proteobacteria* group was also retrieved; indicating that they may also coexist with the one of nitrogen in nitrogen/phosphate removing system. The abundance of the phylum *Firmicutes* (low G+C Gram-positive) also results the presumption that not only one specific organism is responsible for the anoxic-oxic nitrogen with somewhat phosphate in this plant could be confirmed.

In full-scale WWTP particularly in the cool area, where the temperature may be one of the most limit factors to exploit the full potential of the functional bacteria group, the operation condition such as the sludge returning ratio, DO should be monitored to supply the dominant and functional bacteria with the optimal inhabitations. However, this kind of strategies such as improving the DO may spend higher electricity and economic cost. Suspended chain moving aeration technique may be a way of supplying a plant with aerobic and anaerobic conditions alternation in the aerating reactor for saving cost.

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