Microbial community comparison of different biological processes for treating the same sewage

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Abstract The microbial communities, including ammonia-oxidizing bacterial (AOB), eubacterial, actinomycetic and yeast communities, were investigated in two different systems by PCR-DGGE (denaturing gradient gel electrophoresis) using amplified 16S rRNA gene fragments of bacteria and 26S rRNA gene fragments of yeast. The two systems, which used an anoxic-anaerobic-aerobic process (A₂O) and an anoxic-aerobic process (AO), respectively, received identical sewage, operated under the same conditions and demonstrated similar treatment performance. The AOB communities of the two systems showed almost identical structures corresponding to similar ammonium removal, while bacterial, actinomycetic and yeast communities demonstrated obvious differences. The A₂O system showed richer eubacterial, actinomycetic and yeast communities than the AO system. FISH results showed that the AOB cells in the A₂O system made up $3.6 \pm 0.2\%$ of the total bacterial population, while those in the AO system accounted for $1.9 \pm 0.2\%$. Thus the existence of an anaerobic environment in the A₂O system resulted in a marked increase in biodiversity.

Keywords Activated sludge · Community analysis · PCR-DGGE · Sewage treatment · Yeast

Abbreviations

FISH	Fluorescence in situ hybridization
DGGE	Denaturing gradient gel electrophoresis
AOB	Ammonia-oxidizing bacteria
A ₂ O	Anoxic-anaerobic-aerobic process
AO	Anoxic-aerobic process
DO	Dissolved oxygen
HRT	Hydraulic retention time
SRT	Solid residence time
MLSS	Mixed liquor suspended solid
SS	Suspended solid
SVI	Sludge volume index
TN	Total nitrogen
TP	Total phosphorus

Introduction

Activated sludge is a very complicated system, comprised of various populations including heterotrophic bacteria, autotrophic bacteria, fungi and protozoa (Madoni et al. 1993). The relationship between the microbial population composition and the treatment performance of activated sludge processes has long attracted the attention of microbial ecologists and environmental engineers, as this information might be useful for the proper design and operation of biological wastewater treatment systems. Protozoa have been studied and utilized as an important indicator for judging process performance and effluent quality since the 1970s, because these large sized microorganisms can be directly observed and identified under a microscope (Curds and Cockburn 1970; Curds 1982;

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Chen et al. 2004). However, protozoa cannot live in activated sludge systems without other smaller microorganisms as their food. Research on the smaller microorganisms has been hampered by the crude, labor-intensive and biased traditional methods, because a majority of them are not cultivatable using standard techniques (Amann et al. 1995).

The recently developed molecular biological techniques, such as denaturing gradient gel electrophoresis of polymerase chain reaction (PCR-DGGE), fluorescent in situ hybridization (FISH), etc., have made it possible to look into the microbial ecology in wastewater treatment systems without relying on cultural techniques (Paniagua-Michel et al. 2005; Zheng et al. 2005). With the help of these novel techniques, the composition and population dynamics of ammonia-oxidizing bacteria (AOB) have been investigated by many researchers, because ammonia oxidation is a rate-limiting step for biological nitrogen removal (Wagner et al. 1995). Although some results are contradictory, it is now a general view that most of the AOB in the engineered ammonia-rich systems belong to the genera *Nitrosomonas* or *Nitrospira* within the class betaproteobacteria (Ballinger et al. 1998). Environmental factors like salinity, ammonia levels, etc., have been found to affect the AOB composition (Bollmann and Laanbroek 2002; Rowan et al. 2003). Research has also focused on other specific microbial populations of activated sludge, such as actinomycetes because these bacteria play important roles in sludge bulking and foaming (Madoni et al. 2000; Davenport et al. 2000).

All of the above efforts provide us a much better view of the microbial ecology in activated sludge systems. However, most of the previous studies have only focused on specific bacterial populations, such as AOB and actinomycetes, in activated sludge. Since activated sludge is a complicated ecological system harboring many different groups of microorganisms and these different groups are related with each other, it is important to analyse the composition and structure of microbial communities of different groups. Only with a comprehensive understanding of the microbial ecology in activated sludge is it possible to establish a more efficient biological wastewater treatment system and operational strategy.

In this study, microbial communities of two different systems, an anoxic-anaerobic-aerobic process (A_2O) and an anoxic-aerobic process (AO), respectively, receiving identical sewage and having similar treatment performance, were determined using group-specific PCR-DGGE and subsequent sequence analysis of rRNA genes. The community structures of eubacteria,

AOB, actinomycetes, and yeast in the two systems were investigated to evaluate the effects of different designs on microbial populations. FISH was used for the determination of the ratio of AOB in each system with the probe NSO190.

Materials and methods

Objective wastewater treatment plant

An A_2O and an AO process were respectively adopted in a sewage treatment plant in the eastern suburbs of Beijing, each with a treatment capacity of 500,000 tonnes/day (raw water flow rate for each process, 350,000–400,000 tonnes/day). The schematic diagram for the A_2O and AO processes are shown in Figs. 1 and 2, respectively. The A_2O system was reconstructed from another AO system series by changing 16.3% of the aeration tank into an anaerobic compartment. Raw sewage consisted of about 60% municipal wastewater and 40% industrial wastewater. In the A_2O system, 10% of raw sewage flows into the anoxic compartment, while the remaining 90% flows into the anaerobic compartment. The hydraulic retention times (HRTs) of the anoxic, anaerobic and aerobic compartments were 1.5, 1.5, and 6.0 h, respectively. In the AO system, the HRTs of the anoxic and aerobic compartments were 1.5 and 7.5 h, respectively. The mixed-liquor suspended solid (MLSS) of the two systems was about 2300 mg/l, and other operation conditions, such as dissolved oxygen (2.0 mg/l), pH (6.6), solid residence time (SRT, 6–12 days) and total HRT (9.0 h), were almost the same. The sludge recycling ratio was 100%, and the internal recycling ratio was 150–200%. The average water temperature during the sampling period was 19°C. The influent and effluent characteristics of the two systems are listed in Table 1. The COD, BOD, NH_4^+-N , TN, TP, and SS were analysed according to Standard Methods for the Examination of Water and Wastewater (APHA, 1985). The flow charts of the A_2O and the AO process are shown as Figs. 1 and 2 respectively.

Fig. 1 Flow chart of the anoxic-anaerobic-aerobic process

Fig. 2 Flow chart of the AO

Samples of activated sludge

Activated sludge samples were collected three times from the aeration tanks of the two systems during the summer (April, May, June 2004). Samples for FISH and DNA extraction were placed in different sterile polypropylene tubes, respectively, and taken back to the laboratory in an hour. For DNA extraction, the sludge from approximately 50 mg (wet weight) of biomass was transferred into a 2.0-ml Eppendorf tube and centrifuged at $14,000 \times g$ for 10 min. The supernatant was removed, and the pellets were stored at -20°C until DNA extraction. The MLSS concentrations were determined on the day of sampling.

DNA extraction of bacteria and yeast

Bacteria DNA was extracted from samples in triplicate using the method of Miller et al. (1999) with a small modification at the initial step: 1 ml phosphate buffer salt solution (100 mM NaH_2PO_4 , pH 8.0) was added to and mixed with the sample, and then the tube was sonicated for 30 s on ice.

Yeast DNA was extracted also in triplicate according to the protocol of Schloss et al. (2003)

The product from DNA extraction was verified by electrophoresis in 0.8% agarose.

PCR and DGGE

All the primers used and PCR conditions are listed in Table 2. The final concentrations of different components in the mastermix were: 15 pmol of each primer, 200 μM of each deoxynucleoside triphosphate, $1 \times$ reaction buffer, 1.25 U/50 μl of Taq DNA Polymerase (Promega, USA), 400 ng/ μl of bovine serum albumin, and DNase- and RNase-free filter-sterilized water.

For bacterial population analysis, the general primers GC-338f and P518r were used to directly amplify the 16S rRNA gene of bacteria by adding 2 μl of extracted DNA to 48 μl PCR mastermix.

The D1 region of the 26S rRNA gene of yeast was amplified by PCR using the forward primers GC-NL1 and a reverse primer LS2 according to the protocol of Cocolin et al. (2000).

For analysis of AOB and actinomycetic populations in activated sludge, a nested PCR technique was used in order to increase the sensitivity (Boon et al. 2002). In the first round, 1 μl of extracted DNA was added to 24 μl of PCR mastermix, and different primers were used, each with their own corresponding PCR protocol. During the second PCR round, 1 μl of amplified product from the first round was added to 49 μl of the PCR mixture and then reamplified by using their own corresponding primers and PCR protocol, shown in Table 2.

The DGGE analysis was basically performed as first described by Muyzer et al. (1993), using a DCode System apparatus (Bio-Rad Co., USA). The PCR products of the second round were loaded onto 10% (w/v) polyacrylamide gels (bisacrylamide: acrylamide = 37.5:1) in $1 \times$ TAE buffer. The polyacrylamide gels were made with the denaturing gradient ranging from 38% to 50% denaturant (where 100% denaturant contains 7 M urea and 40% formamide). The gels were run at 60°C for 5.5 h at a constant voltage of 120 V. After electrophoresis, the gels were stained with ethidium bromide for 15 min and photographed on a u.v. transillumination table with a Gel Documentation System (Gel Doc 2000TM, Bio-Rad Laboratories-Segrate, Milan, Italy).

Sequence analysis of bands excised from DGGE gels

Bands chosen for sequence analysis were carefully excised from the DGGE gel with a sterile scalpel, and then placed in 2-ml sterilized screw-cap polypropylene tubes, with 60 μl TE buffer. The DNA was allowed to passively diffuse into water at 4°C overnight. 5 μl of the eluate was used as template DNA for a PCR performed with the primers and conditions described above for environmental samples. The PCR products

Table 1 Influent and effluent characteristics of the two systems in April 2004

Item	COD _{Cr} (mg/l)	BOD (mg/l)	NH ₄ ⁺ -N (mg/l)	TN (mg/l)	TP (mg/l)	SS (mg/l)
Raw sewage	310–400	180–200	35.6–47.5	49.0–62.0	5.2–6.6	260–350
A ₂ O effluent	30.2–35.6	8.5–13.4	11.0–25.0	36.0–41.0	0.6–3.8	8.0–12.0
AO effluent	33.1–37.6	9.3–12.5	8.6–17.8	33.6–38.5	5.3–5.9	10.5–13.5

Table 2 PCR primers used in this study

Target	Primers	Number of cycles	PCR conditions						References
			Denaturation		Annealing		Elongation		
			°C	min	°C	min	°C	min	
<i>First PCR round</i>									
Actinomycetes	F243, R1378r	35	95	1	63	1	72	2	Heuer et al. (1997)
Ammonia oxidizers	CTO189fAB, CTO189Fc, CTO654r	35	95	1	57	1	72	1	Kowalchuk et al. (1998)
<i>Second PCR round</i>									
Bacteria	GC-P338f, P518r	30	95	1	55	1	72	1	Òvreas et al. (1997)
Yeast	GC-NL1, LS2	30	95	1	55	1	72	1	Cocolin et al. (2000)

Before each run of cycles, the temperature was held at 95°C for 5 min, and after each run the temperature was kept at 72°C for 10 min for final template elongation

(40 µl) were sent to Shanghai Genecore Biotechnology Company for sequence analysis.

FISH analysis and cell counting

Before hybridization, the samples were dispersed into individual cells by ultrasonication, then immediately processed and fixed in 4% paraformaldehyde for 3 h at 4°C. After that, the biomass was washed with phosphate buffered saline (pH 7.4) and stored at a 1:1 ratio of phosphate-buffered saline and 100% ethanol at –20°C.

All hybridizations were performed at 46°C for 120 min as described by Manz et al. (1992). The 16S rRNA-targeted oligonucleotide probes and the stringency used in this study are listed in Table 3. After hybridization, the slides were mounted with Citifluor (Ted Pella Inc., Redding, CA, USA) to avoid bleaching and examined with an Axioskop 2 epifluorescent

microscope (Carl Zeiss, Jena, Germany). All image processing and analysis were performed with the standard software package provided by Zeiss (Axio Vision 4.1).

Three probes are listed in Table 3. Nso190, Nsv443, and NEU were used for detecting AOB. DAPI stained bacteria were counted as the total bacterial number. Cell counts were determined by enumerating at least 1,000 cells per view and more than 10 fields were obtained for each sample.

Results

DGGE analysis results

DGGE gels for analysing eubacteria, actinomycetes, AOB, and yeast of the two systems in April 2004 are shown in Figs. 3–6, respectively, and the samples

Table 3 Oligonucleotide probes

Probe	Specificity	Probe sequence (5'–3')	Target site (16S rRNA positions)	% FA	NaCl (mM)	References
EUB338	Bacteria	GCTGCCTCCCGTAGGAGT	338–355	20	225	Amann et al. (1990)
Nso190	Ammonia-oxidizing β -subclass <i>Proteobacteria</i>	CGATCCCCTGCTTTTCTCC	190–208	55	20	Mobarry et al. (1996)
Nsv443	<i>Nitrosospira</i> spp.	CCGTGACCGTTTCGTTCCG	444–462	30	112	Mobarry et al. (1996)
NEU	Halophilic and halotolerant members of the genus <i>Nitrosomonas</i>	CCCCTCTGCTGCACTCTA	653–670	40	56	Mobarry et al. (1996)

Fig. 3 DGGE analysis of bacteria communities and lane comparison in the two systems. The left side shows the DGGE patterns of activated sludge samples taken from the aeration tanks of the A₂O system and the AO system, respectively. The right side gives the analysis results of lane comparison from the DGGE patterns using Quantity One 4.3.0 software. Bands 1 and 2 of the A₂O system were excised from DGGE gel for sequencing

collected in May and June 2004 also gave similar results (data not shown). It was found that the two systems harbored quite different microbial communities,

Fig. 4 DGGE analysis of actinomycetic communities and lane comparison in the two systems. The left side shows the DGGE patterns of activated sludge samples taken from aeration tanks of the A₂O system and the AO system, respectively. The right side gives the analysis results of lane comparison from the DGGE patterns using Quantity One 4.3.0 software

Fig. 5 DGGE analysis of AOB communities and lane comparison in the two systems. The left side shows the DGGE patterns of activated sludge samples taken from the aeration tanks of the A₂O system and the AO system, respectively. The right side gives the analysis results of lane comparison from the DGGE patterns using Quantity One 4.3.0 software

and the similarities for bacteria, actinomycetes and yeast in the two systems were 55.1%, 53.0% and 52.8%, respectively, although they received identical sewage and showed similar treatment performance. The band numbers for bacteria, actinomycetes and yeast in the A₂O system were 15, 10 and 26, compared with 11, 7 and 20 in the AO system, indicating that the A₂O system tends to harbor richer community structures of these groups.

The two main bands of bacteria which only existed in the A₂O system were excised from the DGGE gel and sent for sequencing. The sequences reported in this study are available in GenBank under accession nos. AY338406 and AY338407.

The two systems, however, contained similar AOB compositions. The similarity of the two systems was 77.5%. The main bands were excised from the DGGE gel and sent for sequencing. The sequences reported in this study are available in GenBank under accession no. DQ196226 to DQ196234. Sequence analysis of 16S rRNA genes revealed that all bands were closely related to *Nitrosomonas* spp.

Sequence analysis results of yeast bands

Major bands of the DGGE gel were excised from the A₂O lane in Fig. 6 and sent for sequencing. These sequence data have been submitted to the GenBank

Fig. 6 DGGE analysis of yeast communities and lane comparison in the two systems. The left side shows the DGGE patterns of activated sludge samples taken from aeration tanks of the A₂O system and the AO system, respectively. The right side gives the analysis results of lane comparison from the DGGE patterns using Quantity One 4.3.0 software

databases under accession number DQ105591 to DQ105609 and are shown in Table 4. It is interesting that there were many kinds of yeast, such as *Candida maltosa*, *Malassezia* sp., *Basidiomycete* yeast sp. etc., appeared in activated sludge and both systems demonstrated very diverse compositions.

FISH analysis of AOB in activated sludge

In this study, multiple probes were used for the analysis of AOB in the two systems. Probes Nsv443, NEU, and

Nso190 were respectively used for detecting bacteria belonging to the *Nitrosospira* cluster, and the halophilic and halotolerant *Nitrosomonas* cluster, and the total AOB (Table 3). All samples collected in April, May and June 2004 gave similar results. It was found that in the two systems, the genus *Nitrosomonas* were accounted for about 1.8% (the A₂O system) and 1.6% (the AO system) respectively. The *Nitrosospira* proportions in the two systems were 2.2% (the A₂O system) and 1.2% (the AO system) respectively. Hybridizations with probe Nso190 showed that the total AOB number in the A₂O system on average accounted for $3.6 \pm 0.2\%$ of the total bacterial population, while that in the AO system was only $1.9 \pm 0.2\%$.

Discussion

Before reconstruction, the two systems were of the same design. One of the two systems was reconstructed into an A₂O system by changing a part of the aeration basin into an anaerobic basin to enhance the phosphate removal efficiency. After reconstruction, the A₂O system had a smaller aeration compartment than the AO system. However, the two systems still received identical sewage. As shown in Table 1 the two systems demonstrated almost identical performance in the removals of COD, BOD and SS, while the AO system had a slightly higher ammonia removal and the A₂O system had a higher phosphate removal.

Even though the systems received identical sewage, and in spite of the quite similar constitutions, operational conditions, and treatment performance, the two systems demonstrated quite different microbial

Table 4 Sequence analysis of yeast bands excised from DGGE gels of the A₂O system

DGGE band	Accession number	% Identity	Most closely related bacterial sequence
1	DQ105591	100%	Uncultured organism clone ASt-35
2	DQ205592	94%	Uncultured organism clone ASt-35
3	DQ105593	95%	Uncultured organism clone ASt-1
4	DQ105594	86%	Uncultured mycorrhiza clone 1287
5	DQ105595	96%	Uncultured organism clone ASt-1
6	DQ105596	96%	Uncultured organism clone ASt-35
7	DQ205597	97%	Uncultured organism clone ASt-1
8	DQ105598	94%	Uncultured fungus clone FF36
9	DQ105599	100%	<i>Candida maltosa</i>
10	DQ105600	97%	Uncultured organism clone ASt-35
12	DQ105601	98%	<i>Entyloma ranunculi-repentis</i>
13	DQ105602	97%	<i>Malassezia</i> sp.
14	DQ105603	97%	<i>Entyloma lobeliae</i>
15	DQ105604	98%	<i>Entyloma fuscum</i>
16	DQ105605	100%	<i>Geotrichum fragrans</i>
17	DQ105606	100%	<i>Dipodascus tetrasperma</i>
18	DQ105607	91%	<i>Candida</i> sp.
20	DQ105608	96%	<i>Basidiomycete</i> yeast sp.
21	DQ105609	98%	<i>Plasmidiophora brassicae</i>

community structures except for AOB. It is interesting that the A₂O system had much richer compositions of bacterial, actinomycotic, and yeast populations. The richer community structures of these populations were obviously related to the creation of the anaerobic compartment. Most of the increased species in the A₂O system might be the ones that tend to grow under anaerobic environments. Phosphate accumulation organisms (PAOs) should be the one category of population that increased through the creation of an anaerobic compartment. The A₂O process is an enhanced biological phosphorus removal (EBPR) process and PAOs play the key role in phosphorus removal in EBPR systems (Okunuki et al. 2004). Table 1 indicates that the AO system had almost no phosphate removal ability. The A₂O system, on the other hand, had a TP removal of about 50%, which should be attributed to the possible accumulation of PAOs. The sequencing results of bacterial DGGE bands indicate that the two dominant populations (AY338406, AY338407) appearing solely in the A₂O system belong to the gamma proteobacteria. Bacteria of the gamma proteobacteria have been considered to be related with phosphate removal (Wentzel 1988; Kavanaugh and Randall 1994).

The relatively low TP removal in the A₂O system, on the other hand, was perhaps related to the relatively high residual nitrate coming from the anoxic compartment. The nitrate concentration of mixed liquor in the anoxic compartment was as high as 15.9 mg/l, which could be used as sufficient electron acceptors for denitrifying bacteria to utilize organic substrates. The competition between denitrifying bacteria and PAOs for organic compounds might be the main reason of the low TP removal (Hascoet and Florentz 1985; Kuba et al. 1994).

It is interesting that the two systems had a very rich yeast population. Yeast tends to occur in environments with high salt and/or substrate concentrations, such as the boundary between the terrestrial environment and the ocean (Gadanhó et al. 2003), and can grow well under low moisture and low pH conditions relative to bacteria (Chigusa et al. 1989). Organic substrates can foster yeast growth. However, yeast has been traditionally used in high load, high salt, and high substrate concentration processes such as the fermentation, brewing and soy sauce industries. Recent studies show that yeasts have advantages in treating high strength oily wastewater and monosodium glutamate-manufacturing wastewater over the conventional bacteria-dominating processes (Yang et al. 2003, 2005). Its existence and ecological role in activated sludge, however, has long been neglected. The yeast DGGE

patterns in Fig. 6 and the sequencing results in Table 4 show that many yeast populations including *Candida* sp., *Malassezia* sp. and *Basidiomycete* sp. etc. existed in both systems. The exact roles and the ecological significance of yeasts in wastewater treatment processes, however, are still unknown and need further research.

Actinomycetes in activated sludge have recently become the focus of research because they are believed to play an important role in sludge bulking and foaming in activated sludge plants (Madoni et al. 2000; Davenport et al. 2000). Their hydrophobic cell surface is supposed to support adherence and stabilization of interfaces and thus promote sludge flotation, leading to a higher SVI (Gisi et al. 1998). The average SVIs of the two systems were 150 l/g (A₂O) and 100 l/g (AO) respectively. The richer actinomycete population in the A₂O system suggests that some actinomycetes might be related to its higher SVI. Apart from these negative roles, actinomycetes are active in the decomposition of organic materials (McCarthy 1987). The diversity of actinomycetes in the two systems suggests that some actinomycetes might also play an important role in the removal of organic substance. Further investigation, however, is required to draw a conclusion.

Of the four populations analysed, only AOB communities demonstrated a clear similarity (77.5%) between the two processes, suggesting that the introduction of the anaerobic compartment did not change the richness of AOB populations markedly. However, as shown in the FISH analysis results, the proportion of AOB to total bacterial numbers in the two systems was quite different ($3.6 \pm 0.2\%$ for A₂O system and $1.9 \pm 0.2\%$ for AO system). Obviously, the A₂O system had much more AOB cells than the AO system. Previous study (Limpiyakorn et al. 2005) showed that SRT mainly influenced the total numbers of AOB in activated sludge systems. But in this study, the two systems had similar SRT as well as MLSS. Further studies are necessary to carry out in order to explain this phenomenon completely.

Conclusion

The addition of an anaerobic compartment in the A₂O system had an important impact on microbial communities and biodiversity, leading to much richer microbial community structures of bacterial, actinomycetic and yeast groups. The band numbers for bacteria, actinomycetes and yeast in the A₂O system were 15, 10 and 26, compared with 11, 7 and 20 in the AO system. The structure of the AOB group in the two systems, on the other hand, demonstrated a relatively

high similarity. The rich yeast compositions suggested that yeast might play some important roles in pollutant removal.

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