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Community analysis of ammonia oxidizer in the oxygen-limited nitritation stage of OLAND system by DGGE of PCR amplified 16S rDNA fragments and FISH

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Abstract: OLAND (oxygen limited autotrophic nitrification and denitrification) nitrogen removal system was constructed by coupling with oxygen limited nitritation stage and anaerobic ammonium oxidation stage. Ammonia oxidizer, as a kind of key bacteria in N cycle, plays an important role at the oxygen limited nitritation stage of OLAND nitrogen removal system. In this study, specific amplification of 16S rDNA fragment of ammonia oxidizer by nested PCR, separation of mixed PCR samples by denaturing gradient gel electrophoresis (DGGE), and the quantification of ammonia oxidizer by fluorescence *in situ* hybridization (FISH) were combined to investigate the shifts of community composition and quantity of ammonia oxidizer of the oxygen limited nitritation stage in OLAND system. It showed that the community composition of ammonia oxidizer changed drastically when dissolved oxygen was decreased gradually, and the dominant ammonia oxidizer of the steady nitrite accumulation stage were completely different from that of the early stage of oxygen limited nitritation identified by DGGE. It was concluded that the *Nitrosomonas* may be the dominant genus of ammonia oxidizer at the oxygen limited nitritation stage of OLAND system characterized by nested PCR-DGGE and FISH, and the percentage of *Nitrosomonas* was 72.5% ±0.8% of ammonia oxidizer at the steady nitrite accumulation stage detected by FISH.

Keywords: OLAND; nitritation; ammonia oxidizer; DGGE; FISH

Introduction

Sustainable wastewater treatment systems are being developed that minimize energy consumption, CO_2 emission, and sludge production. However, these systems typically yield effluents rich in ammonium-nitrogen (NH_4^+-N) and poor in biodegradable organic carbon, thereby making them less suitable for biological N removal through the conventional nitrification-denitrification sequence.

Recently, many cost-effective biological nitrogen removal systems with less input of energy and chemicals, suitable for the treatment of the wastewater with high ammonium-nitrogen (NH_4^+-N) and low chemical oxygen demand (COD), have been exploited, such as SHARON (Hellinga, 1998), ANAMMOX (Jetten, 1999), CANON (Sliekers, 2002) and OLAND (Kuai, 1998) and so on. OLAND, as an efficient biological nitrogen removal system, was a new combined system based on partial nitrification of ammonia to nitrite, together with anaerobic ammonium oxidation. Such system has no need for external carbon addition, hardly any sludge production, and uses less energy and oxygen than conventional systems. In this system, the key point is to achieve steady partial nitrification (nitrite accumulation) by controlling oxygen supply (Wyffels, 2003). Partial nitrification stage of OLAND system refers to the oxidation of ammonium to nitrite (nitritation), however, further oxidation of nitrite to nitrate (nitratation) was blocked by controlling oxygen supply. Nitritation is carried out by aerobic autotrophic bacteria-ammonia oxidizer, mainly *Nitosomonas*, *Nitrosospira* and *Nitrosococcus* species (Focht, 1977). In this paper, the community composition and quantity of the ammonia oxidizer were studied by three kinds of molecular biological techniques-PCR, DGGE (Muyzer, 1998) and FISH (Amann, 1998). The results of molecular identification of ammonia oxidizer provided more feasible explanation for achieving steady nitrite accumulation at the oxygen limited nitrification stage of OLAND system, therefore, lay a solid basis for facilitating the fast and steady nitrite accumulation of OLAND nitrogen removal system.

1 Materials and methods

1.1 Sampling from oxygen limited nitritation stage of OLAND

Eight activated sludge samples — 0 d (inoculum sludge), 15, 22, 24, 26, 30, 34 and 67 d (raw wastewater from Deurne Wastewater Treatment Plant of Belgium as influent) were taken from the first stage reactor (oxygen limited nitritation stage) of OLAND over time with the

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decrease of aeration rate.

1.2 DNA extraction, purification and cell fixation

The extraction and purification of total community DNA were performed as described by Boon (Boon, 2000). The resulted total DNA was stored at −20℃ for PCR amplification.

Cell fixation was performed according to a modified method of Amann (Amann, 1990). About 2 ml sludge sample from the nitritation stage reactor was harvested by centrifugation. Then the precipitant was resuspended in 1 volume phosphate-buffered saline (PBS) and 3 volumes freshly prepared cold 4% paraformaldehyde (PFA), fixed at 4℃ for 3 h. After washing twice with 1 × PBS, the centrifuged cells were suspended in a solution of 50% PBS and 50% ethanol, and the mixture was stored at −20℃ for subsequent hybridization and microscopy.

1.3 16S rDNA nested PCR amplification and denaturing gradient gel electrophoresis (DGGE)

In order to increase the sensitivity and facilitate the DGGE of fragments of the same length, a nested-PCR technique was applied. In the first round PCR phylum-specific primers of ammonia oxidizer were used. During the second round PCR, the obtained fragments were re-amplified using the bacteria universal primers P338f + GC (a 30 bp 5' GC-clamp was added for DGGE analysis) and P518r (Boon, 2002). Since the group of ammonia oxidizer is the domain of Bacteria, the primers P338f and P518r used in the second PCR round should re-amplify all fragments obtained after the first round PCR (Boon, 2001—2002). After PCR, samples were stored at 4℃ (few hours) or −20℃ (d) for DGGE analysis. All the primers and PCR conditions are listed in Table 1.

Table 1 Primers of ammonia oxidizer used in nested PCR

Primer	PCR condition						Reference
	Cycles	Denaturation		Annealing		Elongtion	
		℃	min	℃	min	℃	min	
First round PCR								
CTO189ABf, CTO189Cf, CTO654r	35	94	1	57	1	72	2	Kowalchuk, 1998
Second round PCR								
P338f + GC, P518r	30	95	1	53	1	72	2	Øvreas, 1997

DGGE, based on the protocol of Muyzer (Muyzer, 1993), was performed using the Bio-Rad D Gene System (Bio-Rad, Hercules, CA, USA). The PCR products of the second round were loaded onto 8% (w/v) polyacrylamide gels in 1 × TAE (20 mmol/L tris, 10 mmol/L acetate, 0.5 mmol/L EDTA, pH 7.4). The polyacrylamide gels were made with denaturing gradient ranging from 45% to 60%, where 100% denaturant contains 7 mol/L urea and 40% formamide. Electrophoresis was run for 16 h at 60℃, 38 V. After the electrophoresis, the gels were stained for 10 min in SYBR Green I nucleic acid gel stain (1 : 10000 dilution, FMC BioProducts, Rockland, USA). The stained gel was immediately photographed on an UV transillumination table with a Video Camera Module (Vilbert Lourmat, Marne-la Vallee, France).

1.4 Fluorescence *in situ* hybridization (FISH) and microscopy

Fluorescence *in situ* hybridization involves the use of fluorescently labelled nucleic acid probes to target ribosomal RNA (rRNA) within morphologically intact cells. Single bacterial cells with fluorochrome-labelled probes hybridized to their rRNA can be identified within mixed populations with fluorescence microscopy. This is done through selective targeting of regions of rRNA, which consists of evolutionarily conserved and variable nucleotide regions. Thus, by choosing the appropriate rRNA probe sequence, FISH can be used to detect all bacterial cells (a universal probe) or a single population of cells (a strain specific probe). The number of target rRNA copies within a cell is dependent on metabolic activity. This technique can be useful in detection of airborne bacteria, such as *Pseudomonas aeruginosa* and *Escherichia coli*, and in the quantification and identification of organisms.

Fixed cells 2—10 μl were suspended in PBS/ethanol (50 :50, v/v), then were applied on the well of slides coated with gelatin, and then dried at 45℃ and sequentially dehydrated in a solution of 50%, 80% and 100% ethanol (v/v) for 3 min each. To start the hybridization, 9 μl of hybridization buffer and 1 μl of a fluorescently labelled probe were added to the well. Hybridization was conducted for 1.5—3 h at 46℃ in a hybridization oven (Biozyme, The Netherlands).

Following hybridization, a stringent washing step was performed for 10 min at 48℃ in the washing buffer with appropriate NaCl concentration, 1 mol/L tris-HCl (pH 8.0), 0.5 mol/L EDTA and 10% SDS. The samples were counter-stained with DAPI (4,6-diamidino- 2-pheylindole) at a final concentration of 10 mg/L for 10 min on ice and mounted in Citifluor (Citifluor, London, UK) (Egli, 2001).

The probes used in this study, their target sites and target organisms as well as optimal formamide concentration in the hybridization buffers are given in Table 2. Probes were purchased as Cy3, Cy5 and 5 (6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS) labeled derivatives (Eurogentec, Seraing, Belgium). Samples were analyzed by standard epifluorescence microscopy on a Zeiss Axioskop II microscope (Carl Zeiss, Jena, Germany). The microscope was equipped with a Peltier cooled single chip digital colour

CCD camera (Hamamatsu Orca IIIm, Hamamatsu, Massy Cedex, France) connected to a PC to obtain digital images. Images analysis was performed with Microimage 4.0 (Media Cybernetics, Silver Spring, USA).

Table 2 Specific probes of ammonia oxidizer used in this study

Probe	OPD designation	Specificity	Sequence(5'-3')	Target site	Reference
NSO190	S-P-Betao-190-a-A-19	*Ammonia oxidizer of β-proteobacteria*	CGATCCCTGCTTTTCTCC	190-208	
NSM156	S-G-Nsm-156-a-A-19	*Genus Nitrosomonas*	TATTAGCACATCTTTCGAT	156-174	(Mobarry, 1996)
NSV443	S-F-Nsp-0444-a-A-19	*Nitrosospira-cluster*	CCGTGACCGTTTCGTTCCG	444-462	

2 Results and discussion

2.1 Operation of oxygen limited nitritation stage of OLAND

In order to obtain stable nitrite accumulation, oxygen supply was controlled by decreasing the aeration rate from 1600 to 25 ml/min step by step, the nitrogen compounds concentration of effluent from this stage were determined with the drop of dissolved oxygen. The relationship between aeration rate and dissolved oxygen is listed in Fig.1, and the N compounds composition of effluent are given in Fig.2.

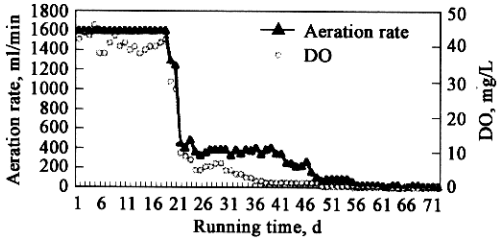

Fig. 1 The relationship between aeration rate and dissolved oxygen at oxygen limited nitritation stage of OLAND

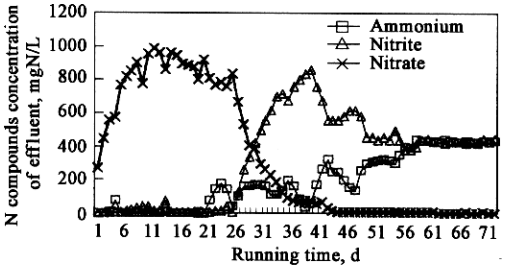

Fig. 2 The N compounds composition of effluent at oxygen limited nitrification stage of OLAND

Fig. 1 and 2 show that the dissolved oxygen decreased proportionally with the drop of aeration rate, and the concentration of nitrogen compounds of effluent were changed dramatically when the aeration rate was decreased gradually. At the early stage of the oxygen limited nitritation, enough aeration was exerted, ammonium was converted to nitrate thoroughly, almost no nitrite accumulation. When the reactor was running at day 20, aeration rate was decreased sharp, nitrite begins to accumulate dramatically at day 25, simultaneously the ammonium was going up and nitrate was going down. With the drop of dissolved oxygen continuously, the N compounds composition of effluent were changing completely-nitrite was accumulating, ammonium was increasing and the ratio of them in effluent was going to be 1:1, nitrate was decreasing step by step and reached to < 10 mg/L.

2.2 Analysis of nested PCR-DGGE of ammonia oxidizer

With the drop of dissolved oxygen, N compounds composition of effluent were changed a lot, it can be found that ammonia oxidizer, which oxidizes ammonia to nitrite, have a high affinity to oxygen, it can be accustomed to a very low oxygen environment. Otherwise nitrite oxidizer which oxidize nitrite to nitrate were inhibited by low oxygen. But with the decrease of dissolved oxygen, how the microbial community of ammonia oxidizer at the oxygen limited nitrification stage shift must be very interesting for explaining how to facilitate the fast accumulation of nitrite. In this study the shifts of ammonia oxidizer at the oxygen limited nitrification stage was studied by nested PCR-DGGE when dissolved oxygen was decreased step by step.

Based on Fig. 3 and 4, it was found that the nested primers used for amplifying 16S rDNA fragment of ammonia oxidizer was effective, the 465 bp specific PCR fragment of first round PCR was obtained, and the PCR fragment of second round PCR was 220 bp (30 bp GC clamp was included).

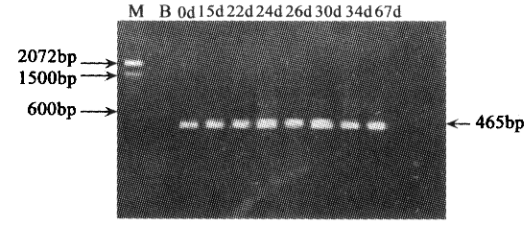

Fig. 3 The first round PCR products of ammonia oxidizer from 0 d—67 d activated sludge by using CTO primer
M—100 bp marker; B—blank(no DNA template)

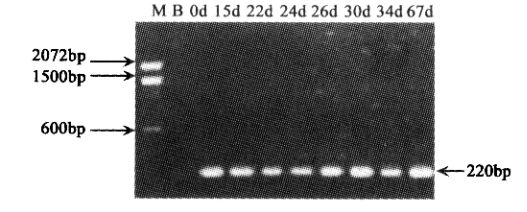

Fig. 4 The second round PCR products of ammonia oxidizer from 0 d—67 d activated sludge by using universal bacteria primer with GC clamp
M—100 bp marker; B—blank(no DNA template)

The PCR products with GC-clamp of second round PCR

were developed for DGGE analysis. From Fig. 5, it was showed that the shift of ammonia oxidizer was very obvious with the decrease of DO, and its microbial community composition was not very complicated at the oxygen limited nitritation stage of OLAND. Band A was dominant specie in inoculum sludge, and it passed away at the steady nitrite accumulation stage(67 d); band B was absent in inoculum sludge, but it was showing up at 24 d and becoming dominant specie of ammonia oxidizer in 67 d, so it can be concluded that the functional ammonia oxidizer of the beginning was completely different from that of the steady nitrite accumulation stage. If the characteristics of species A and B were further studied, it will be very meaningful for facilitating the fast and steady nitrite accumulation. The cloning and sequencing of band A, B and C were in process.

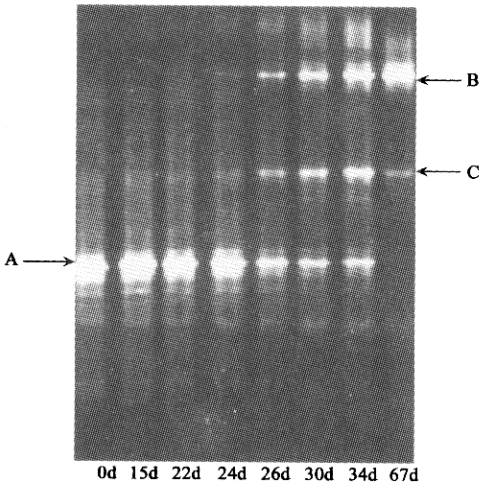

Fig. 5 The DGGE pattern of ammonia oxidizer with the decrease of DO at oxygen limited nitritation stage of OLAND

2.3 Analysis of fluorescence *in situ* hybridization

In this study, three specific probes were selected to identify and quantify the ammonia oxidizer and its two main genus—*nitrosomonas* and *nitrosospira*. Group specific probe of ammonia oxidizer-NSO190 was applied to identify its changes of distribution and quantity with the decrease of DO at oxygen limited nitritation stage of OLAND, simultaneously genus specific probe of *nitrosomonas* and *nitrosospira*-NSM156 and NSV443 were used to determine which genus is the dominant of ammonia oxidizer at oxygen limited nitritation stage of OLAND.

Fig.6 shows that the microbial community composition and distribution was completely different at the early stage from that of at the steady nitrite accumulation stage, at the early stage most of the ammonia oxidizer exist as filamentous and consortia, while they exist as spherical and rod bacteria or cluster at the steady nitrite accumulation stage. The FISH photomicrographs, 20 random fields respectively from the samples of inoculum sludge and 67 d, were analyzed with digital analysis software Microimage 4.0, it was concluded that quantity of ammonia oxidizer was 85% ± 0.2% at the beginning of start up(inoculum sludge), and then with the decrease of dissolved oxygen, it decreased to 70% ± 0.5% when the nitrite accumulation was stable. The photomicrographs(omitted) showed that *nitrosomonas* is the dominant genus of ammonia oxidizer in oxygen limited nitritation stage of OLAND, and *nitrosospira* was not detectable at this stage, the same detection result from inoculum sludge. The FISH photomicrographs, 20 fields from the samples 67 d, were analyzed with digital analysis software Microimage 4.0, the statistic data showed that the percentage of *nitrosmonas* is about 72.5% ± 0.8% of ammonia oxidizer at the steady nitrite accumulation stage of OLAND.

3 Conclusions

The community composition and quantity of ammonia oxidizer of the oxygen-limited nitritation stage of OLAND system were identified by several molecular biology techniques - PCR, DGGE and FISH in this study. It was concluded that the dominant genus of ammonia oxidizer is *nitrosomonas* at

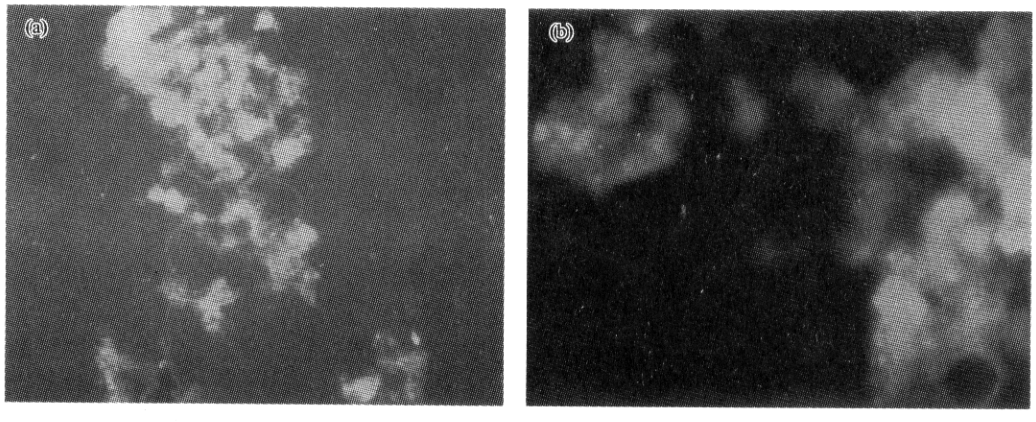

Fig. 6 Detection of ammonia oxidizer in oxygen limited nitritation stage of OLAND for (a) and (b) by epifluorescence microscopy, 20 random fields were checked to quantify the ammonia oxidizer analyzed by microimage 4.0 software
a. Hybridization of a sample from inoculum sludge with FLUOS labelled rRNA probe-NSO190; b. hybridization of a sample from activated sludge of running 67 d of oxygen limited nitritation stage with FLUOS labelled rRNA probe-NSO190, all photomicrographs were taken at × 630 magnification

oxygen limited nitritation stage, and the percentage of it analyzed by digital analysis software is about 72.5% ± 0.8% in ammonia oxidizer. The dominant species of ammonia oxidizer identified by DGGE may be related to *nitrosomonas* that was detected by FISH at oxygen limited nitritation stage of OLAND. And the quantity of ammonia oxidizer determined by FISH decreased from 85% ± 0.2% to 70 ± 0.5% with the accumulation of nitrite gradually.

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References:

Amann R I, Binder B J, Olson R J *et al*., 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations[J]. Appl Environ Microbiol, 56: 1919—1925.

Amann R I, Lemmer H, Wagner M, 1998. Monitoring the community structure of wastewater treatment plants: a comparison of old and new techniques[J]. FEMS Microbiol Ecol, 24: 205—215.

Boon N, Goris J, De Vos P *et al*., 2000. Bioaugmentation of activated sludge by an indigenous 3-chloroaniline degrading comamonas testosteroni strain, I2gfp [J]. Appl Environ Microbiol, 66: 2906—2913.

Boon N, 2001—2002. Bioaugmentation of activated sludge reactors to enhance chloroaniline removal[D]. Ph. D thesis of Gent University. 151—154.

Boon N, De Windt W, Verstraete W *et al*., 2002. Evaluation of nested PCR-DGGE (denaturing gradient gel electrophoresis) with group-specific 16S rRNA primers for the analysis of bacterial communities from different wastewater treatment plants[J]. FEMS Microbiol Ecol, 39: 101—112.

Egli K, Fanger U, Alvarez P J J *et al*., 2001. Enrichment and characterization of an anammox bacterium form a rotating biological contactor treating ammonium-rich leachate[J]. Arch Microbiol, 175: 198—207.

Focht D D, Verstraete W, 1977. Biochemical ecology of nitrification and denitrification[J]. Adv Microb Ecol, 1: 135—214.

Hellinga C, Schellen A A J C, Mulder J W *et al*., 1998. The Sharon process: an innovative method for nitrogen removal from ammonium-rich waste water [J]. Wat Sci Tech, 37(9): 135—142.

Jetten M S M, Strous M, van de Pas-schoonen K T *et al*., 1999. The anaerobic oxidation of ammonium[J]. FEMS Microbiol Reviews, 22: 421—437.

Kowalchuk G A, Bodelier P L E, Heilig G H J *et al*., 1998. Community analysis of ammonium-oxidizing bacterium, in relation to oxygen availability in soils and root-oxygenated sediments, using PCR, DGGE and oligonucleotide probe hybridisation[J]. FEMS Microbiol Ecol, 24: 339—350.

Kuai L, Verstraete W, 1998. Ammonium removal by the oxygen limited autotrophic nitrification denitrification (OLAND) system[J]. Appl Environ Microbiol, 64(11): 4500—4506.

Mobarry B K, Wagner M, Urbain V *et al*., 1996. Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria[J]. Appl Environ Microbiol, 62: 2156—2162.

Muyzer G, de Waal E C, Uitterlinden A, 1993. Profiling of complex microbial populations using denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA[J]. Appl Environ Microbiol, 59: 695—700.

Muyzer G, 1998. DGGE/TGGE— a method for identifying genes from natural ecosystems[J]. Curr Opin. Microbiol, 2: 317—322.

Øvreas L, Forney L, Daae F L *et al*., 1997. Distribution of bacterioplankton in Meromictic Lake Saelevannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA[J]. Appl Environ Microbiol, 63: 3367—3373.

Slickers A O, Derwort N, Gomez J L C *et al*., 2002. Completely autotrophic nitrogen removal over nitrite in one single reactor[J]. Wat Res, 36: 2475—2482.

Wyffels S, Boeckx P, Pynaert K *et al*., 2003. Sustained nitrite accumulation in a membrane-assisted bioreactor (MBR) for the treatment of ammonium rich wastewater[J]. J Chem Technol Biotechnol, 78: 412—419.

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