Community analysis of nitrifying bacteria in an advanced and compact Gappei-Johkasou by FISH and PCR-DGGE


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Abstract Fluorescent in situ hybridization (FISH) method with 16S rRNA-targeted oligonucleotide probes was used for quantitative estimation of ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) in a Johkasou. Although the occupation ratios of AOB and NOB increased as nitrification progressed, about one month later, the occupation ratios decreased, despite showing good nitrification ability. Furthermore, even when urea was added to the feeding wastewater to raise the amount of T-N, the occupation ratios of both nitrifying bacteria remained constant. For further investigation, denaturing gradient gel electrophoresis (DGGE) was used to study the community structure of AOB in the Johkasou. As a result, DGGE band patterns and following sequence analysis revealed that the community structure of AOB was complicated and changed during this experiment. It was suggested that even if the occupation ratio of AOB to eubacteria was constant, the majorities of AOB were changed through temperature and load fluctuation. The combination of FISH and PCR-DGGE provides new information that was not available by conventional cultivation-based methods.

Keywords Domestic wastewater; FISH; Johkasou; microbial community; nitrifying bacteria; PCR-DGGE

Introduction

In enclosed water bodies such as lakes and inland seas, water bloom and red tide have occurred with increases in nutrient salts such as nitrogen and phosphorus. Various forms of nitrogen allow the depletion of dissolved oxygen in lakes and marshes, stimulate the growth of aquatic organisms such as algae and plants, cause the growth of toxic algae and affect the suitability of the water for a drinking use. Therefore, the removal of nitrogen compounds is essential to preserve water environment. Recently, an advanced and compact Gappei-Johkasou (a small onsite wastewater treatment system) has become a centre of attraction due to its high ability to remove nitrogen. Since Johkasou is inexpensive to set up and its running cost is economical, it is very useful for nitrogen removal from domestic wastewater on a small scale.

Biological nitrification, which is a two-step process of the oxidation of ammonium (NH₄⁺) to nitrite (NO₂⁻) and nitrite to nitrate (NO₃⁻), is principally carried out by the chemolithoautotrophic ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB). Although the nitrification process has been intensively studied during the past several decades, it is still difficult to keep stable nitrification performance in engineering systems. This is mainly caused by the low growth rates of nitrifying bacteria and their sensitivity to toxic shocks, pH and temperature fluctuation. In general, monitoring the number and the physiological activity of the nitrifying bacteria is necessary for the improvement of nitrogen removal from wastewater. In addition, for the purpose of improving the reliability of nitrification performance in the Johkasou, it is also important to monitor the microbial community of nitrifying bacteria in the Johkasou.
So far, numerous cultivation-based methods such as the MPN (most probable number) method have been used to isolate and identify bacteria from the Johkasou. However, it has been increasingly appreciated that plating or MPN techniques are always selective and therefore cannot yield sufficient documentation of the true community structure. Consequently, in the last decade, several molecular methods such as FISH and PCR-DGGE have been developed to quantify effective microbes and analyze the community structure without bias due to cultivation. In particular, the combination analysis of FISH and PCR-DGGE is highly effective to study the microbial community. We here apply this combined approach to activated sludge in an advanced and compact Gappei-Johkasou to elucidate the community structure of the nitrifying bacteria.

Materials and methods

The advanced and compact Gappei-Johkasou

Anaerobic-aerobic activated sludge process with membrane filtration was applied in an advanced and compact Gappei-Johkasou, which was the onsite individual treatment facility (Figure 1). This Johkasou consisted of a flow equalization tank (1.2 m$^3$), an anaerobic reactor (0.4 m$^3$) and an aerobic reactor (0.4 m$^3$). The volume of influent wastewater was 1.2 m$^3$ day$^{-1}$. The volume of the circulated wastewater was adjusted to three times that of the influent. Treated water was drawn from an aerobic reactor intermittently (draw 6 min at interval of 3 min) through the membrane module. The concentration of mixed-liquor suspended solids (MLSS) in the aeration reactor was kept about 10,000–15,000 mg l$^{-1}$ during this operation. This process makes it possible to raise loading capacity per unit because the activated sludge density was very high due to the effective separation of sludge and effluent water by the membrane module.

Sampling

Activated sludge samples were collected at 36, 76, 118, 154, 174, 295 and 426 days after starting operation from the aerobic reactor equipped with a membrane module. The samples were transferred into sterile plastic tubes with equivalent ethanol and transported into the laboratory on ice for FISH and PCR-DGGE analysis. For FISH analysis, collected samples were washed with PBS (phosphate-buffered saline) and immediately fixed as described below. For DGGE analysis, the collected samples were washed with TE (Tris/EDTA) buffer and stored at –20°C.

Oligonucleotide probes and in situ hybridization

The following three rRNA-targeted oligonucleotides were used: (1) eubacteria specific probe (Amann et al., 1990), abbreviated EUB338; (2) ammonia oxidizing β-subclass Proteobacteria specific probe (Mobarry et al., 1996), abbreviated NSO190; and (3) all hitherto sequenced Nitrobacter species specific probe (Wagner et al., 1996), abbreviated NIT3. These oligonucleotides were synthesized and labeled with FITC (fluorescein isothiocyanate) or XRTIC (X-rhodamine isothiocyanate) by TaKaRa Biotechnology (Dalian) Co., Ltd.

Figure 1 Scheme of treatment process of the advanced and compact Gappei-Johkasou
Fixative solution consisting of 4% paraformaldehyde in phosphate-buffered saline was prepared immediately prior to use. For in situ hybridization, collected samples were fixed by adding 3 volumes of fixative solution to 1 volume of cell suspension and kept at 4°C for 2 h (Amann, 1995). For quantitative analysis, the fixed cells were dispersed by an ultrasonic generator. Subsequently, the cells were spotted on gelatin-coated slides and dehydrated by sequential washes in 50, 80 and 98% ethanol (3 min each). Hybridization solution (40% (for NSO190 and NIT3) or 20% (for EUB338) formamide, 0.9 M NaCl, 20 mM Tris (pH 7.2) and 0.01% sodium dodecyl sulfate (SDS)) containing 25 ng of oligonucleotide probe was added to each hybridization well. The slides were incubated with hybridization solution for 2 h at 46°C in an isotonically equilibrated humid chamber. The hybridization solution was then removed by flushing the slide with several millilitres of washing solution (NaCl 25 MM (for NSO190 and NIT3) or 170 MM (for EUB338), 20 mM sodium phosphate (pH 7.0), 0.01% SDS). Then, the slide was immersed in washing solution at 48°C for 20 min. The slides were briefly rinsed with distilled water, and dried in the dark.

Microscopy and digital image analysis

The samples were mounted in antifade reagent and fluorescence was detected with a fluorescence microscope (OLYMPUS) equipped with a high-pressure mercury bulb and blue and green filter set. In obtained fluorescent photomicrographs, each area of fluorescence derived from the specific oligonucleotide probes was measured by a package software (Mac SCOPE Ver. 2.32). Each area of AOB and NOB specific fluorescence was compared with the area of eubacteria specific fluorescence, and then the occupation ratios of AOB and NOB to all eubacteria were calculated.

DNA extraction

DNA was extracted from a 0.5 g (wet weight) activated sludge at 154 and 426 days after starting operation. The activated sludge was harvested by centrifugation at 10,000 g for 10 min. The harvested cells were sonicated for 30 s in a sucrose-lysis buffer (0.3 M sucrose, 0.7 M NaCl, 40 mM EDTA and 50 mM TrisHCl). After sonication, it was centrifuged at 2,000 g for 10 min. The supernatant was incubated at 55°C in the presence of SDS, proteinase K and hexadecylmethylammonium bromide. DNA was extracted by applying phenol, chloroform and isoamyl alcohol and precipitated by the addition of ethanol and sodium acetate.

PCR

The partial sequence of 16S rDNA from AOB was amplified by nested PCR. In the first PCR, pA and pH primers (Edwards et al., 1989) were used to amplify almost full sequences of eubacterial 16S rDNA. The CTO primers (Kowalchuk et al., 1997) used in the second PCR were designed to amplify partial rDNA sequences (465bp) from β -subdivision AOB while excluding other taxa for which sequences are available. A 40-nucleotide GC-rich sequence called GC clamp was attached to the 5’ end of the forward primer to improve the detection of sequence variation in amplified DNA fragments by subsequent DGGE (Muyzer et al., 1993). PCR was carried out in a total volume of 50 µl on a GeneAmp PCR system 9700 (Applied Biosystems). PCR mixtures used for the initial and secondary amplifications were prepared in accordance with the manufacturer’s recommendations in a total volume of 50 µl by using 0.2 µM each primer, 200 µM each deoxynucleoside triphosphate, 1 mM MgCl₂, PCR buffer for KOD, 2.0 U of KOD DNA polymerase (Toyobo) and sterile water. Thermal cycling for the initial amplification was carried out by an initial denaturation step at 94°C for 4 min, followed by 30 cycles of denaturation step at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 2 min. Cycling was completed by a
final elongation step at 72°C for 7 min. Thermal cycling for the secondary amplification was carried out by an initial denaturation step at 94°C for 4 min, followed by 35 cycles of denaturation step at 94°C for 1 min, annealing at 57°C for 1 min, and elongation at 72°C for 1 min. Cycling was completed by a final elongation step at 72°C for 4 min. The presence and sizes of the amplification products were determined by agarose (2%) gel electrophoresis of the reaction product. Ethidium bromide stained bands were digitally recorded with a Kodak DC120 Zoom Digital Camera.

**DGGE**

PCR products were resolved on a DGGE gel using a D-CODE Multi-system (Bio-Rad). Polyacrylamide gels (8% polyacrylamide, 38 to 50% denaturant) were poured using a gradient maker (Bio-Rad). The gels were run for 4.0 h at 130 V and 60°C. After electrophoresis, gels were soaked for 30 min in SYBR green nucleic acid gel stain (1:10,000 dilution; molecular probes) and photographed on an UV transillumination table with a DC120 zoom digital camera (Kodak).

**Sequence analysis of DGGE bands**

Each selected DGGE band was excised with sterile tips for sequence analysis and placed in sterile vials. DNA extraction from gels was performed with a QIAEX II DNA extraction kit (Qiagen), and PCR was carried out using the CTO primers (without GC clamp) as described before. The PCR products were used as template DNA in a cycle sequencing reaction with a BigDye Terminator cycle sequencing kit (Applied Biosystems) in accordance with the manufacturer’s instructions. Sequencing of 16S rDNA fragments was done with an ABI PRISM 377 DNA sequencing system (Applied Biosystems).

**Results and discussion**

**Performance of the advanced and compact Gappei-Johkasou**

The advanced and compact Gappei-Johkasou showed high nitrification performance (Figure 2). Up to 150 days after starting operation, the T-N concentration at the inlet was approximately 30 mg 1⁻¹. The sharp decrease of NH₄-N concentration at the outlet was observed at day 50 and after that NH₄-N concentration at the outlet was kept below 1 mg 1⁻¹. These results suggested that AOB and NOB populations proliferated as nitrification progressed.

Although after day 150, urea was added to feeding wastewater to raise T-N concentration, NH₄-N was hardly detected at outlet until day 250. On the other hand, from day 270 to day 295 in winter season, water temperature dropped to nearly 10°C, and consequently, effluent NH₄-N concentration increased to 30–40 mg 1⁻¹. These results showed the border of water temperature that reduces nitrification ability in this Johkasou was nearly 10°C. From day 310, cover sheets were used to pack the Johkasou and avoid a drop in water temperature, and as a result, nitrification was progressed.

**Calculation of the occupation ratio by digital image analysis of FISH**

The digital image analysis could be used to calculate the occupation ratios of AOB and NOB to eubacteria (Figure 3). During the early days of the operation, influent T-N concentration was low and the occupation ratios of AOB and NOB to eubacteria were low (2.5% and 7.9%, respectively). At day 76, NH₄-N concentration at the outlet decreased to below 1 mg 1⁻¹, and the occupation ratios of AOB and NOB increased to 24% and 17%, respectively, which were the highest values in this operation. This result is in good agreement with the presumption described above, and showed that this activated sludge with membrane filtration process could maintain microbes, including nitrifying bacteria, at high
density. About one month later, T-N load was as high as $5.4 \times 10^{-3}$ kg-T-N kg-MLSS$^{-1}$ day$^{-1}$ and the concentration of NH$_4$-N at the outlet was below 1 mg l$^{-1}$. Interestingly, slight decrease in the occupation ratios of AOB and NOB (6.4% and 5.4%, respectively, at day 118) was observed in spite of the high nitrification ability. This change of occupation ratios may have been caused by an excessive proliferation of nitrifying bacteria as nitrification progressed, and nitrifying bacteria decreased to appropriate number for influent load, afterwards.

Since day 50, NH$_4$-N concentration at the outlet had been hardly detected, except for days 270–295. In this period, effluent ammonia concentration increased to 30–40 mg l$^{-1}$, even though, the transition of the occupation ratios of AOB and NOB was not observed (2.7% and 4.2%, respectively at day 295). In a word, the occupation ratios of nitrifying bacteria did not decrease very much, in spite of drastic fluctuations in the nitrogen load per cell. Since MLSS of each sample was almost the same value, the result suggested that the decline of nitrification ability was not caused by decrease of number and occupation ratio of nitrifying bacteria but caused by decrease of their activity or change of dominant species.

**DGGE and sequence analysis of AOB in Johkasou**

To clarify the community structure of AOB, DNA was extracted from activated sludge in the Johkasou at days 154 and 426. DGGE analysis was performed with products obtained with CTO primer set with GC clamp. As a result, several bands were detected and the band pattern was not agreed with each other (Figure 4). The varied DGGE band patterns showed that AOB community structures in the collected two samples were complex and evidently different. Consequently, it was suggested that majorities of AOB community were changed during this period.

To identify the species that compose AOB community, sequence analysis of DGGE bands was carried out. The neighbor-joining tree (Figure 5) shows the relationship of the sequences obtained from bands excised from DGGE gels after PCR amplification of $\beta$-subgroup AOB from DNA extracted directly from the Johkasou. At day 426, one of the major species (DGGE Band 2) showed high similarity to *Nitrosomonas europaea*, and that was also observed at day 154. However, other two major species (DGGE Band 3 and 7) that showed high similarity to *Nitrosospira* sp. and *Nitrosomonas ureae*, respectively, were not observed at day 154.

Considering that the water temperature varied from 32°C to 22°C between day 154 and day 426, it was found that the microbial community structure of AOB in this Johkasou was affected by water temperature.
Conclusions

1. During this operation, effluent NH₄-N concentration of almost all samples was below 1 mg l⁻¹, and the high nitrification ability of this advanced and compact Gappei-Johkasou was confirmed.

2. As a result of FISH analysis, it was found that the occupation ratios of AOB and NOB to eubacteria showed little relationship with nitrification performance.

3. As a result of DGGE analysis, a complicated AOB community in the Johkasou was indicated and transition of the community structure was observed during this experiment. Therefore, it was suggested that majorities of AOB were changed in the Johkasou while keeping the occupation ratio constant.

**Figure 4** DGGE band pattern of the β-subgroup ammonia-oxidizing bacteria in the Johkasou

**Figure 5** Neighbor joining tree of the sequences obtained from DGGE bands of the β-subgroup ammonia-oxidizing bacteria from DNA extracted directly from Johkasou. The scale is 10% estimated change. The numbers of bootstrap value of 1,000 replicate trees support the branching order.
4. As a result of sequence analysis, several major species of AOB were identified. Therefore, it was found that AOB community in this Johkasou comprised several species such as *Nitrosomonas europaea*, *Nitrosospira* sp. and *Nitrosomonas ureae*.

**References**


