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Methanotrophic community composition based on *pmoA* genes in dissolved methane recovery and biological oxidation closed downflow hanging sponge reactors

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ABSTRACT

Dissolved methane in the effluent of anaerobic wastewater treatment processes is unrecovered and released into the atmosphere as methane, a greenhouse gas. To prevent methane emissions from effluent, a post-treatment system consisting of two closed downflow hanging sponge (DHS) reactors for the recovery and biological oxidation of dissolved methane was developed. More than 99% of the dissolved methane was completely eliminated using this system under ambient temperatures for 1 year. In this study, the methanotrophic community composition of the two closed DHS reactors was investigated. The performance of the closed DHS reactor was evaluated at different heights of the reactor in summer and winter. The clone libraries and T-RFLP analyses based on the *pmoA* gene revealed that type I and type II methanotrophs were present in the closed DHS reactors. Furthermore, type I methanotrophs showed wide diversity and contained uncultured phylogenetic clusters of methanotrophs (FWs and LWs), while type II methanotrophs were dominated by *Methylocystis*- and *Methylosarcina*-related clusters. The relative abundance of type II methanotrophs increased during winter. The type I methanotroph population dynamically changed with height of the reactor. These results demonstrate the important role of methanotrophs in removal of dissolved methane from upflow anaerobic sludge blanket effluent treating sewage.

1. Introduction

Anaerobic wastewater treatment has been widely applied as an eco-friendly technology in terms of improved energy conservation and reduced carbon dioxide (CO_2) emissions. However, the technology has some disadvantages [1,2]. One of the disadvantages is that the anaerobic wastewater treatment process discharges unrecovered methane as dissolved methane in the effluent. Methane is a greenhouse gas with a 25-fold greater effect on global warming than CO_2 [3]. In the case of low-strength wastewaters such as municipal sewages with low methane production potential, the ratio of dissolved methane to the recovered methane gas significantly increases with decreasing wastewater strength [4,5]. Therefore, new technologies enabling economic recovery or treatment of dissolved methane are needed to reduce greenhouse gas emissions and improve anaerobic wastewater treatment technology.

In recent years, some new technologies to prevent dissolved methane emission have been reported [6–8]. We have also developed a post-treatment system consisting of two closed downflow hanging sponge (DHS) reactors for the recovery and oxidation of dissolved methane from sewage effluents after anaerobic treatment processes [9]. The first stage closed DHS reactor is mainly involved in dissolved methane recovery as burnable gas containing over 30% methane; unrecovered residual dissolved methane is almost completely removed by methane-oxidizing bacteria, or methanotrophs, in the second stage closed DHS reactor. The two continuously closed DHS reactor system efficiently removed organic material and also allowed dissolved methane to be recov-

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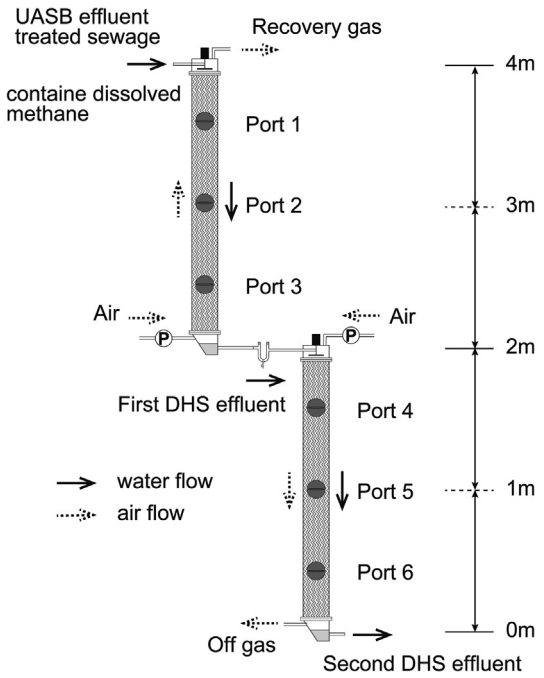

Fig. 1. Two-stage closed downflow hanging sponge (DHS) reactor for dissolved methane recovery and biological dissolved methane oxidation.

ered as a useful burnable gas, preventing the methane from being emitted into the atmosphere.

Methanotrophs are divided into two main groups—type I (*Gamma-proteobacteria*) and type II (*Alpha-proteobacteria*)—based on differences in several biochemical characteristics. The distribution of methanotrophs in many environments has been well studied, as have the competitive and selective factors for type I versus type II methanotrophs [10]. Methanotrophs show great diversity and many uncultured clusters, based on *pmoA* gene (which encodes the α-subunit of particulate methane monooxygenase (pMMO)) sequences, exist in the environment [11]. The two closed DHS reactors contain a gradient of methane, oxygen and other constituents with height of the reactor. The reactors were operated under ambient temperature for 1 year to assess the effects of temperature and concentrations of key constituents on the methanotrophic community. There is considerable interest in identifying and understanding the ecology of methanotrophs playing a key role in preventing methane emissions from the two closed DHS reactors. The results of this study will provide insight into understanding dissolved methane removal and methanotrophic ecology.

In this study, we analyzed the water quality and gas compositions with height of the closed DHS reactors in summer and winter. The methanotrophic community structure of the retained sludge responsible for oxidation of dissolved methane was sequenced based on *pmoA* genes. The diversity and distribution of methanotrophs was investigated by terminal restriction fragment length polymorphism (T-RFLP) analysis of samples from different heights in the closed DHS reactors during both seasons.

2. Material and methods

2.1. Two closed DHS reactors post-treatment system

The two closed DHS reactors for dissolved methane recovery and biological oxidation were installed in series with an upflow anaerobic sludge blanket (UASB) reactor treating actual municipal sewage (Fig. 1). Further details regarding the DHS reactors have been described previously [9]. The first and second closed DHS reactors had the same square columnar structure and size. The volume of sponge containing microbes was 35.2 L, accounting for 44% of the volume of the reactor. The reactors had differences in air supply direction and rate. In the first closed DHS reactor, air was supplied from the bottom of the reactor to recover dissolved methane by physical gasification at 250 L m^{-3} day^{-1} based on the reactor volume. The off-gas with methane was recovered from the top of the reactor. In the second closed DHS reactor, air was fed from the top at a rate of 2500 L m^{-3} day^{-1} to oxidize the unrecovered dissolved methane and complete the wastewater polish-up. The exhaust gas was released from the bottom to the atmosphere. The system was operated under ambient temperature conditions, and the daily average temperature ranged from 10 °C to 28 °C.

2.2. Analytical methods

Chemical oxygen demand (COD), ammonium nitrogen, nitrate, pH, dissolved methane, oxygen gas and methane gas concentrations were measured as previously described [12] to evaluate the methanotrophic conditions with height of the closed DHS reactors during summer and winter. Ammonium and nitrate were analyzed by high-performance liquid chromatography (HPLC; Shimadzu LC 20-ADsp). Gas samples were collected from the top, middle (Port 2 in the first closed DHS reactor or Port 5 in the second closed DHS reactor) and bottom of each closed DHS reactor. The gas composition was analyzed using a gas chromatograph equipped with thermal conductivity detector (GC-TCD; Shimadzu GC-8A).

2.3. Sludge sampling and DNA extraction

Sludge samples were squeezed and collected from the upper, middle, and lower parts of sponges from each reactor on day 110 during summer and day 261 in winter. The collected sludge samples were washed with phosphate buffer. DNA from the washed sludges was extracted using a Fast DNA spin kit for soil (MP Biomedicals, Irvine, CA, USA) according to the manufacturer's instructions.

2.4. Phylogenetic analysis based on pmoA genes

Extracted DNA was used for amplification of *pmoA* gene fragments with the primer pair A189f/mb661 [13]. PCR amplification was performed using the ONE Shot LA PCR MIX (TAKARA BIO, Otsu, Japan). PCR amplification of *pmoA* genes was conducted by subjecting the samples to 5 min of initial denaturation at 94 °C, followed by 25 cycles of 30 s at 94 °C, 30 s at 56 °C and 1 min at 72 °C. The final extension step was at 72 °C for 4 min. The PCR products were electrophoresed on a 1% (w/v) agarose gel, after which they were purified with a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany).

The PCR products were cloned using the TOPO XL PCR Cloning Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cloned *pmoA* genes were randomly picked from each clone library and subjected to sequencing from both ends at the Dragon Genomics Center (Takara Bio, Yokkaichi, Japan). Chimeric sequences were identified and removed using Bellerophon [14]. The *pmoA* gene sequences were classified using the FastGroupII program [15]. Fragments with 98% sequence identity were considered unique operational taxonomic units [16]. A phylogenetic tree was constructed using the *pmoA* database, which includes 6628 reference sequences corresponding to 53 low-level taxa [17], with the ARB program package [18]. Sequences from samples were imported into the ARB program and the phylogenetic tree of translated PmoA amino acid sequences was reconstructed based on the neighbor-joining method implemented in the ARB program. Bootstrap resampling analysis for 1000 replicates was