Ecological Engineering 110 (2018) 48–53

Contents lists available at ScienceDirect

Ecological Engineering

journal homepage: www.elsevier.com/locate/ecoleng

Research Paper

Nitrite-dependent methane-oxidizing bacteria seasonally and spatially shift in a constructed wetland used for treating river water

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ARTICLE INFO

Keywords:
Constructed wetland
Candidatus Methylomirabilis oxyfera
Methanotroph
Microbial community

ABSTRACT

Nitrite-dependent anaerobic methane oxidation (n-damo) process, performed by "*Candidatus Methylomirabilis oxyfera*" (*M. oxyfera*), can be an important methane sink in wetland anoxic layers. However, little is known about the distribution and variability of *M. oxyfera*-like bacteria in constructed wetlands (CWs). The current study investigated spatial and seasonal dynamics of *M. oxyfera*-like bacteria in a free water surface flow CW (FWSF-CW) used to improve river water quality and explored the influences of wetland environmental variables. In the FWSF-CW, *M. oxyfera*-like bacterial abundance was found to change considerably among both sampling sites and seasons, ranged between 8.99×10^5 and 4.34×10^6 16S rRNA gene copies per gram dry sediment/soil. Considerable spatial and seasonal variations of *M. oxyfera*-like bacterial richness, diversity, and community structure were also observed in the FWSF-CW. In addition, both nutrients and temperature were the key factors determining the community structure of wetland *M. oxyfera*-like bacteria.

1. Introduction

Wetlands are the largest natural source of greenhouse gas methane (CH_4), accounting for a significant part of the global atmospheric flux (Chowdhury and Dick, 2013; Zhu et al., 2012). Aerobic methane oxidation, carried out by methanotrophic bacteria (mainly belonging to phylum *Proteobacteria*), was usually considered as a key microbial methane sink in wetland ecosystems (Chowdhury and Dick, 2013; Yun et al., 2015; Zhu et al., 2012). The recent discovery of nitrite-dependent anaerobic methane oxidation (n-damo) process, assumed to be performed by "*Candidatus Methylomirabilis oxyfera*" (*M. oxyfera*) (affiliated within NC10 phylum) through an "intraaerobic" pathway (Ettwig et al., 2010), reveals a potential mitigation of methane in anoxic environments. Several previous studies have confirmed that n-damo process can also be an important methane sink in the anoxic layers of wetlands, with potential n-damo activities of 0.31–5.43 nmol of CO_2 per gram of dry soil per day (Hu et al., 2014; Shen et al., 2015). The presence of *M. oxyfera*-like bacteria has been detected in natural wetlands (Chen et al., 2015; Han and Gu, 2013; Hu et al., 2014; Zhu et al., 2012), an urban wetland (Hu et al., 2014; Shen et al., 2015), and vertical subsurface flow constructed wetlands (VSF-CWs) used for treating river water (Yang et al., 2017). Zhu et al. (2015) further reported the ubiquity of *M. oxyfera*-like bacteria in Chinese wetland ecosystems. These previous studies suggested that the community of *M. oxyfera*-like bacteria could vary among sampling sites, wetland layers, and sampling times. So far, the environmental factors driving the variability of wetland *M. oxyfera*-like bacteria remain poorly known. pH might be a determinant of the community diversity of *M. oxyfera*-like bacteria (Zhu et al., 2015), while ammonium and nitrite nitrogen played important roles in structuring the community of wetland *M. oxyfera*-like bacteria (Chen et al., 2015). However, different environmental factors might collectively regulate the distribution of wetland *M. oxyfera*-like bacteria (Zhu et al., 2015).

Free water surface flow CWs (FWSF-CWs) have become a favorite option to treat polluted surface water (Dzakpasu et al., 2015; Hernandez-Crespo et al., 2016; Li et al., 2017). To date, information about the distribution of *M. oxyfera*-like bacteria in FWSF-CWs is still lacking. Therefore, the main objectives of the present study were to evaluate spatial and temporal dynamics of *M. oxyfera*-like bacteria in an FWSF-CW used for treating river water and explore the influences of wetland environmental variables.

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http://dx.doi.org/10.1016/j.ecoleng.2017.10.005
Received 13 July 2017; Received in revised form 30 September 2017; Accepted 6 October 2017

2. Materials and methods

2.1. Study sites and sampling

The studied FWSF-CW (with a total surface area of nearly 0.47 km^2), used for the treatment of polluted river water entering Lake Erhai, was located in subtropical Dali City (southwest China). The local region had an annual average air temperature of 15.7 °C and an annual average precipitation of 1000 mm. In consistence with our previous study (Li et al., 2017), eight sampling sites (A–H) (25°56′42″–25°57′11″ N, 100°6′0″–100°6′9″ E) and three seasons (spring, summer and winter) were selected. Sediment sites A–F were inundated during both dry and wet seasons, while soil sites G and H were flooded only during wet seasons (summer and autumn). The dominant plant species in these sites were water hyacinth, reed, watermilfoil, penny grass, duckweed, water-lily, alfalfa, and cattail, respectively. In accordance with our previous study (Li et al., 2017), the obtained sediment/soil samples from the FWSF-CW were denoted as A–H, in correspondence with the sampling site, and SP, SU, or WI, in correspondence with the sampling time (spring, summer, or winter, respectively). During winter, no sample at site B was obtained due to inaccessibility, so no sample was denoted as "BWI". The values of wetland sediment/soil temperature, pH, oxidation and reduction potential (ORP), ammonia nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N), nitrite nitrogen (NO_2^--N), total nitrogen (TN), total phosphorus (TP), and total organic carbon (TOC) were 10.6–27.8 °C, 7.06–7.99, −59.9 to 5.6 mv, 1.29–34.28 mg/kg, 0.42–187.61 mg/kg, 0.01–1.26 mg/kg, 647.36–2564.06 mg/kg, 46.69–1411.36 mg/kg, and 5.10–44.85 g/kg, respectively (Table S1) (Li et al., 2017).

2.2. Quantitative PCR

Genomic DNA from each FWSF-CW sediment/soil sample (0.5 g, dry weight) was extracted using the PowerSoil™ DNA extraction kit (MO BIO Laboratories, Carlsbad, CA, USA), and the DNA quality and quantity were evaluated by 1.2% agarose gel electrophoresis and using a biophotometer (NanoDrop™ 2000 spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA). The density of *M. oxyfera*-like bacteria in FWSF-CW sediment/soil was assessed by quantitative PCR (qPCR) using the specific bacterial primers qP1F/qP1R (Ettwig et al., 2009), using the PCR program: denaturation at 95 °C for 10 min; 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, followed by elongation at 72 °C for 30 s; and finally elongation at 72 °C for 5 min. A standard curve was constructed with a series of 10-fold dilutions of plasmid DNA harboring *M. oxyfera*-like bacterial 16S rRNA gene. The results with amplification efficiency > 95% and correlation coefficient > 0.98 were adopted (Wang et al., 2016). Negative controls containing no template DNA were also performed.

2.3. Clone library analysis

The *pmoA* gene of *M. oxyfera*-like bacteria in FWSF-CW sediment/soil was amplified with a nested approach (first-step primer sets A189_b/cmo682 and second-step primer sets cmo182/cmo568) (Luesken et al., 2011). The PCR was performed as previously described (Long et al., 2017a,b). The resulting amplicons were purified using the TIANquick Mini Purification Kit (Tiangen Biotech (Beijing) Co., LTD, China). The purified amplicons were ligated into the *pEASY*®-T1 Cloning Vector and then transformed into Trans1-T1 Phage Resistant Chemically Competent Cell (TransGen Biotech Co., Ltd, Beijing, China). Positive clones were randomly selected and then sequenced at Sino-GenoMax Co., Ltd. (Beijing, China). Chimeras were detected and removed using UCHIME (Edgar et al., 2011). A total of 521 chimera-free *pmoA* gene sequences were retrieved from the studied FWSF-CW and were deposited in the GenBank database (accession numbers: MF419820–MF420340). The obtained wetland *pmoA* gene sequences in the present study were grouped into operational taxonomic units (OTUs) with ≥97% similarity. The OTU-based community α-diversity indices (Chao1 richness estimator and Shannon diversity) were generated with the MOTHUR program (Schloss et al., 2009). Phylogenetic analysis of the FWSF-CW sediment/soil *pmoA* gene sequences and their close matches reported in the GenBank database was further performed with the MEGA 6.0 software (Tamura et al., 2013) based on the neighbor-joining method. The phylogeny was visualized using the online tool Interactive Tree Of Life (iTOL) v3 (http://itol.embl.de) (Letunic and Bork, 2016). Moreover, weighted Unifrac distance was calculated with *R* library GUniFrac, and hierarchical clustering analysis of FWSF-CW sediment/soil samples was further performed with the *R* software (version i386, 3.3.2).

2.4. Statistical analysis

Differences in the densities of *M. oxyfera*-like bacteria among FWSF-CW sediment/soil samples were tested at a 5% significance level with one-way analysis of variance. Spearman's rank correlation analysis was conducted to determine the correlations of FWSF-CW sediment/soil physicochemical variables with the abundance, richness and diversity of *M. oxyfera*-like bacteria were performed using SPSS 20.0 software (IBM, Armonk, NY, USA). The CANOCO 4.5 software was used to discriminate the relationships between community composition of *M. oxyfera*-like bacteria and wetland environmental variables using redundancy analysis (RDA).

3. Results

3.1. M. oxyfera-like bacterial abundance

So far, there has been no suitable qPCR primers to quantify the *pmoA* gene of *M. oxyfera*-like bacteria; thus, the abundance of FWSF-CW sediment/soil n-damo organisms was estimated by targeting *M. oxyfera*-like bacterial 16S rRNA gene (Zhu et al., 2015). In this study, *M. oxyfera*-like bacterial 16S rRNA gene in the sediment at site E during winter was not successfully amplified using the primers qP1F/qP1R. In the FWSF-CW, the number of *M. oxyfera*-like bacterial 16S rRNA gene ranged between 8.99×10^5 and 4.34×10^6 copies per gram dry sediment/soil (Fig. 1). Among the sediment/soil samples obtained during different seasons, significant differences in *M. oxyfera*-like bacterial abundance were detected at sampling sites B, C, F, G, and H ($P < 0.05$), but no significant difference at sampling sites A, D, and E ($P > 0.05$). This suggested that the seasonal variability of *M. oxyfera*-like bacterial abundance in the FWSF-CW was site-specific. Moreover, during a given season, significant differences in *M. oxyfera*-like bacterial abundance could be observed among the sediment samples (or

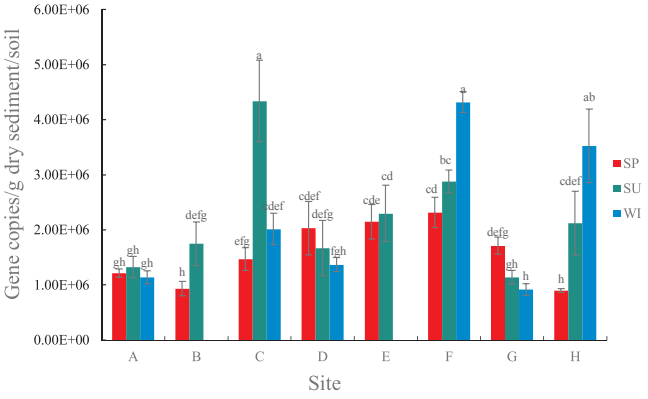

Fig. 1. Abundance of *M. oxyfera*-like bacteria in soils and sediments of the FWSF-CW. Vertical bars indicate standard deviations. Different letters above the columns indicate the significant difference ($P < 0.05$).