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# Effect of submerged plant species on $CH_4$ flux and methanogenic community dynamics in a full-scale constructed wetland

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ABSTRACT

Annual monitoring of $CH_4$ and associated microorganisms from four submerged plant species (*Potamogeton crispus*, *Ceratophyllum demersum*, *Myriophyllum spicatum* and *Hydrilla verticillata*) was conducted in a full-scale constructed wetland (CW). Plant species had a significant impact on $CH_4$ emissions. The highest $CH_4$ flux (5.7 mg $m^{-2}$ $h^{-1}$) came from *P. crispus*, and the lowest flux (1.6 mg $m^{-2}$ $h^{-1}$) was from *M. spicatum*. The dissolved oxygen (DO) and nitrogen of the rhizosphere was significantly affected by plant species, which were the most important factors affecting $CH_4$ flux and methanogenic communities. Changes of DO level caused by plant species were more significant than temperature changes. Real-time quantitative PCR (q-PCR) analysis showed that the number of methanogens and methanotrophs varied with plant species and seasons, whereas the total number of eubacteria was less affected ($p > 0.05$). The variation of TN and $NO_2$-N caused by plant species resulted in the differentiation of methanogen populations. Each plant species was characterized by a specific group of methanogens. Illumina sequencing showed that the dominant methanogenic populations from *P. crispus* and *C. demersum* were hydrogenotrophic archaea of the *Methanoregula*, while those from *M. spicatum* and *H. verticillata* were hydrogenotrophic archaea of the *Methanobacterium*. Both plant species and seasons affected the percentage of acetoclastic archaea of the *Methanosarcina* and *Methanosaeta*. This study indicates that the submerged plant species are important parameters in the production of $CH_4$ emissions in CWs. The proper arrangement of submerged plant species in CWs is vital to maximize the environmental benefit.

## 1. Introduction

Constructed wetlands (CWs) are man-made versions of natural wetlands (Arroyo et al., 2015). CWs have been widely used in ecological engineering as a low-cost alternative to conventional secondary or tertiary wastewater treatment (Deklein and Werf, 2014; Maucieri et al., 2016; Vanderzaag et al., 2010). CWs have become increasingly important in wastewater treatment and landscape engineering due to their high efficiency and low cost. However, greenhouse gases, including methane, carbon dioxide, and nitrous oxide, are formed under anoxic conditions when CWs are inundated (Chiemchaisri et al., 2009).

The atmospheric concentration of methane ($CH_4$) was 1803 ppb in 2011, exceeding the pre-industrial level by 150% (Hu et al., 2017). Although $CH_4$ concentration in the atmosphere is lower than $CO_2$, its global warming potential over the next 100 years is 298 times more potent than $CO_2$ (Beringer et al., 2013). $CH_4$ in CWs is at a lower concentration than natural wetlands (Liu and Ding, 2011), but its global warming potential is significant since CWs are employed extensively in many countries. In China, CWs are commonly used to prevent inundation of inland urban areas.

Many studies have focused on greenhouse gas (GHG) fluxes from natural wetlands (Borah and Baruah, 2016; Cao et al., 2008; Kayranli et al., 2010; Kim et al., 2008; Xu et al., 2014). Studies on $CH_4$ emissions from CWs have mainly focused on the relationship between wastewater treatment efficiency and $CH_4$ emissions (Deklein and Werf, 2014; Maucieri et al., 2016; Vanderzaag et al., 2010; Yan et al., 2012; Zhang et al., 2011). Plant community type clearly affects the quantity of $CH_4$ emitted (Marínmuñiz et al., 2015). One mechanism is by plant influence on the redox potential in the rhizosphere zone and differing rates of oxygen transport via plant aerenchyma from the rhizosphere (Philippot et al., 2009). Additionally, organic inputs differ between plant species, and these are associated with observed differences in $CH_4$

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emissions (Hernández and Mitsch, 2007; Marínmuñiz et al., 2015). Some plants can also produce $CH_4$ under aerobic conditions (Bruhn et al., 2012; Wishkerman et al., 2011). Most studies on $CH_4$ emissions from wetlands have focused on natural systems and emergent plants, rather than constructed wetlands and submerged plants. In natural freshwater wetlands, graminoids and their associated rhizosphere production sites emit lower amounts of $CH_4$ than sedges due to different transport mechanisms via aerenchyma (Hernández et al., 2017). Understanding of plant effects on $CH_4$ emissions now include transformation mechanisms of $CH_4$ in different plants and $CH_4$ oxidation mechanisms in the rhizosphere (Borah and Baruah, 2016; Martin and Moseman-Valtierra, 2017; Ndanga et al., 2016). However, there is a dearth of information about $CH_4$ transport mechanism in submerged plant species and associated microbial processes, as the literatures focuses on emergent plant species (Niu et al., 2015; Sutton-Grier and Megonigal, 2011).

Microorganisms play key roles in CWs and determine the rate of biogeochemical processes (Godin et al., 2012). Microorganisms are regarded as the cornerstone of CWs (Serrano-silva et al., 2014). $CH_4$ flux is a balance between $CH_4$ production, transport and exchange to the atmosphere (Peralta et al., 2013). Methanogens and methanotrophs are microorganisms responsible for methane production and oxidation, respectively (Ramakrishnan et al., 2001). Methanogenesis is the terminal step of anaerobic decomposition pathways and is completed by members of the phylum Euryarchaea living in anaerobic conditions (Godin et al., 2012; Watanabe et al., 2013). Analysis of microorganism community composition and abundance is important for understanding the process of $CH_4$ emission in CWs. Methanogens and methanotrophs can be characterized by the functional genes *mcr*A and *pmo*A, respectively (Kolb et al., 2003). The *mcr*A gene encodes the K-subunit of methyl coenzyme M reductase, a key catabolic enzyme of methanogens. The *pmoA* gene encodes the active-site polypeptide of particulate methane monooxygenase, a key enzyme in methane oxidation (Ramakrishnan et al., 2001). The *mcr*A and *pmo*A genes are commonly used as phylogenetic markers for quantification of methanogens and methanotrophs. The next generation sequencing (NGS) technique is effective for characterizing microbial communities (Arroyo et al., 2015; Mao et al., 2015). The effect of submerged plant species and associated microorganisms on $CH_4$ emissions is unclear and requires investigation.

This study focused on plant-mediated microbial processes. There appears to be no other studies that have investigated the effects of submerged plants on $CH_4$ emissions and their influence on $CH_4$ emissions associated with methanogenic community composition, particularly in CWs. This study investigated the following: (1) the effects of $CH_4$ emissions from submerged wetland plants and (2) the drivers affecting microbial processes. We hypothesized that $CH_4$ emissions would be affected by the species of submerged plants, and that there would be different methanogen communities and pathways of methanogenesis among the submerged plant species.

## 2. Materials and methods

### 2.1. Study site and CW description

The study area is located in the southwest Chengdu plain, Sichuan province, China (N 30°24′ 14″, 103°51′50″E). This area has a subtropical monsoon climate, and the annual mean temperature is 16.2 °C. The mean elevation is 830 m and the terrain is flat. Sewage from nearby regions goes to a wastewater treatment facility. Then, the effluent is sent to the CW areas covered with different vegetation types. Four submerged plants (*Potamogeton crispus*, *Ceratophyllum demersum*, *Myriophyllum spicatum* and *Hydrilla verticillata*) commonly used in China and other Asian countries were selected for study. The plants grow in 20–30 cm deep open water. The water level has no obvious change over the year.

### 2.2. Sample collection and $CH_4$ measurement

Methane was sampled once a week from May 2016 to April 2017. The methane flux was measured using the static chamber technique. Plexiglas chambers (50 cm × 50 cm × 50 cm) were used. The methane sampling process was described in previous reports (Duan et al., 2007; Xu et al., 2014). The static chambers were installed into the selected microcosms. To avoid disturbance, the chambers were immobilized once established in the study areas. The chambers were installed at 10 cm below the wetland substrate. The sampling time was between 8:00 am and 11:00 am to minimize the influence of diurnal variation. Gas samples (50 mL) were extracted from the chamber at 0, 15 and 30 min using polyurethane syringes and stored in gas sampling bags (Delin Ltd., Dalian, China). Chamber temperature was measured during the sampling process. Three replicates of the experimental unit were conducted for each plant. $CH_4$ was determined on a gas chromatograph (Agilent 7890, CA, USA) with a flame ionization detector (FID) and HP-5MS capillary column (30 m × 0.32 mm × 0.25 μm; Agilent Co., CA, USA). The operation parameters were as follows: an injector temperature of 150 °C, a column temperature of 80 °C, a FID temperature of 200 °C. The carrier gas was $N_2$ (99.999%).

### 2.3. Water sampling and characterization

Water samples and methane samples were collected on the same days. After completion of gas collection, rhizospheric water samples were collected. The depth of the rhizosphere zone sampled was around 5–10 cm near the rhizosphere. Nine samples from three sites were collected (3 sites per plant species). Dissolved oxygen (DO) and pH of the rhizosphere was measured in situ using a DO meter (HI 98186, Beijing, China) and a pH meter (Mettler Toledo, Shanghai, China), respectively. Three samples from the rhizosphere zone were collected and mixed into a composite sample. Water samples were collected by using a water sample bottle and the composite sample was 150 mL. Samples were stored on ice in coolers during transportation. Total carbon (TC) and total nitrogen (TN) was analyzed using an Auto Analyzer multi N/C 2100 (Analytikjena AG, Jena, Germany). Total phosphorus (TP) was determined by an Auto Analyzer AQ2 (Seal Analytical, Southampton, UK) 0.2 μl of each sample was injected for analysis.

### 2.4. DNA extraction and q-PCR

DNA was extracted using a Power Soil DNA Isolation Kit (Mo Bio Laboratories Inc., USA). Primers 341-f 5′-CCTACG GGA GGCAGCAG-3′ and 341-r (5′-ATTCCG CGCCTGGCA-3′) (Kim et al., 2008), *mcr*A-f (5′-GGTGGTGTMGGATTCACACARTAYGCWACAGC-3′) and *mcr*A-r (5′-TTCATT GCRTAGTTWGGRTAGTT-3′) (Juottonen et al., 2006), A189-f (5′-GGNGACTGG GACTTCTGG-3′) and mb661-r (5′-CCGGMGCAACG-TCYTTACC-3′) (Kolb et al., 2003) were used for 16S rRNA gene, *mcr*A gene and *pmo*A gene amplification respectively. PCR amplification was performed in a 50 μl reaction volume containing 5 μl of Taq buffer, 1 μl of genomic DNA (10 ng), 1.5 μl of each primer (0.3 μM), 4 μl of each dNTP (0.2 mM), and 0.5 μl (1.5 units) of Taq polymerase (Takara Biotechnology Co. Ltd., Beijing, China). The copy numbers of bacterial 16S rRNA gene, methanogenic *mcr*A gene and methanotrophic *pom*A gene were determined by q-PCR on an ABI 7500 real-time PCR system instrument (Applied Biosystems, CA, USA). The 20 μl of q-PCR reaction mixtures contained 10 μl SYBR Premix Ex Taq™ II (Takara Biotechnology Co. Ltd., Beijing, China), 0.6 μl of each primer (0.3 μM), 1 μl of genomic DNA and 7.8 μl $ddH_2O$. The plasmid standard curve was obtained with tenfold serial dilutions of the plasmid DNA. All real-time PCR standards and samples were treated in triplicate. The procedures were as follows: preheated at 95 °C for 5 min, then denatured at 95 °C for 30 s, annealed at 57 °C for 1 min, extended at 95 °C for 10 s for 30 cycles, then extended at 72 °C for 8 min. The threshold cycle (Ct) values determined were plotted against the logarithm of the initial gene copy