Potential methane production and molecular characterization of bacterial and archaeal communities in a horizontal subsurface flow constructed wetland under cold and warm seasons

Daniela López[1], Mario Sepúlveda[1], Nathaly Ruiz-Tagle[2], Katherine Sossa[2], Enrica Uggetti[3] and Gladys Vidal[1]

[1]Engineering and Biotechnology Environmental Group, Environmental Science Faculty & Center EULA–Chile, Universidad de Concepción. P.O. Box 160- C, Concepción-Chile. Tel.: +56-041-220 4067. Fax: +56-041-2661033. (E-mail: glvidal@udec.cl).

[2]Biofilm laboratory and Environmental Microbiology, Biotechnology Center, Universidad de Concepción, PO Box 160-C, Concepción, Chile.

[3]GEMMA - Environmental Engineering and Microbiology Research Group, Department of Civil and Environmental Engineering, Universitat Politécnica de Catalunya·Barcelona Tech. c/ Jordi Girona 1-3, Building D1, E-08034 Barcelona, Spain.

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(*)Corresponding author: Dr. Gladys Vidal

E-mail: glvidal@udec.cl

Telephone: 56-41-2204067

Fax: 56-41-2207076

Abstract

Organic matter removal in a horizontal subsurface flow constructed wetland (HSSF) treating wastewater is associated with the presence of bacteria and archaea. These organisms perform anaerobic microbial processes such as methanogenesis, which can lead to methane emissions. The aim of this study was to evaluate methane production and characterize the bacterial and archaeal communities found in HSSFs treating secondary urban wastewater during cold and warm seasons. The pilot system used in this study corresponds to four HSSFs, two planted with *Phragmites australis* (HSSF−Phr) and two planted with *Schoenoplectus californicus* (HSSF−Sch), the monitoring was carried out for 1335 days. Removal efficiencies for organic matter (biological and chemical oxygen demand) and total and volatile suspended solids were evaluated in each HSSF. Moreover, biomass from each HSSF was sampled during warm and cold season, and methane productions determined by Specific Methanogenic Activity assays$_{(maximum)}$ (SMA_m). In the same samples, the quantification and identification of bacteria and archaea were performed. The results showed that the degradation of organic matter (53−67% BOD_5 and 51−62% COD) and suspended solids (85−93%) was not influenced by seasonal conditions or plant species. Potential methane production from HSSF-Sch was between 20−51% higher than from HSSF-Phr. Moreover, potential methane production during warm season was 3.4−42% higher than during cold season. The quantification of microorganisms in HSSFs, determined greater development of bacteria (38%) and archaea (50−57%) during the warm season. In addition, the species *Schoenoplectus californicus* has a larger number of bacteria (4−48%) and archaea (34−43%) than *Phragmites australis*. The identification of microorganisms evidenced the sequences associated with bacteria belong mainly to Firmicutes (42%), Proteobacteria (33%) and Bacteroidetes (25%). The archaea were represented primarily by Methanosarcinales, specifically *Methanosaeta* (75%) and

Methanosarcina (16%). The community structure of the methanogenic archaea in HSSFs did not change throughout the seasons or plant species.

Keywords: horizontal subsurface flow constructed wetland; methane production; archaeal and bacterial communities; molecular characterization; cold and warm season.

1. INTRODUCTION

Horizontal subsurface flow constructed wetlands (HSSFs) have proven to be an efficient ecological technology for wastewater treatment. These systems are effective in the removal of total solids (80−95%) and organic matter (70−89% chemical oxygen demand (COD) and 74–94% biological oxygen demand (BOD_5)) from domestic wastewater (Trang *et al*., 2010; Vera *et al*., 2011).

The mechanisms of organic matter removal in HSSFs are associated with anaerobic microbiological processes, such as sulfate reduction, denitrification and methanogenesis, which account for 90−94% of processes (García *et al.*, 2004). The prevailing conditions in HSSFs are anaerobic, with dissolved oxygen values usually lower than 2 mg/L, and redox potential between -400 to +200 mV (García *et al*., 2010). Specifically, methanogenesis occurs during the mineralization of organic matter in several sequential steps (hydrolysis, acidogenesis, acetogenesis and methanogenesis) (Zhang *et al*., 2012). If the methanogenic route is complete, the metabolic end products will be H_2, CO_2 and CH_4 (Vymazal and Kröpfelová, 2011; Mander *et al*., 2014).

In HSSFs, methane emissions into the atmosphere are the net result of production (methanogenesis) and consumption (oxidation). Methane production and emissions can vary in a wide range (from -375 to 36,792 mg $CH_4/m^2 \cdot d$) (López *et al.*, 2015; Corbella and Puigagut, 2015; de la Varga *et al*., 2015; Carballeira *et al*., 2017). Indeed, the emission rate is affected by a number of factors, including the redox condition, the amount of substrate, the type of plants,

70 the temperature, the mobilization of CH_4, the microbiological community, and the
71 characteristics of certain microbial groups (Wang *et al.*, 2013a; Adrados *et al*., 2014; Niu *et al*.,
72 2015, Mauceri *et al*., 2017).
73 Microbiological communities associated with anaerobic environments and methane production
74 have been identified and quantified in natural wetlands (Lv *et al*., 2014), sediments (lakes,
75 rivers and marine) (Zhang *et al*., 2015), artificial riverine wetlands (Ligi *et al*., 2014) and
76 anaerobic reactors (Niu *et al*., 2015). However, there is still very little information about
77 constructed wetlands (Adrados *et al*., 2014; Mander *et al*., 2014). The first investigations
78 associated with bacterial and archaeal communities in constructed wetlands were carried out by
79 Calheiros et al. (2009) and Adrados et al. (2014), identifying and quantifying only bacterial
80 groups. These authors determined that the main phyla found in HSSFs were Proteobacteria
81 (26%), Bacteroidetes (26%) and Firmicutes (15%). Moreover, He et al. (2014) found that β-
82 Proteobacteria (55%) and γ-Proteobacteria (37%) were the dominant groups in vertical and
83 horizontal constructed wetlands. However, the presence of archaeal communities in constructed
84 wetland systems has not been extensively studied. In this line, it has been shown that microbial
85 communities and methane emissions can be directly influenced by the plant species used (Wang
86 *et al*., 2013b; Zhang *et al*., 2018). Moreover, it has been determined that the dissolved oxygen
87 of the rhizosphere is one of the most important factors affecting CH_4 flux and methanogenic
88 communities (Zhang *et al*., 2018). However, Wang et al. (2015) have shown that the
89 determining factor of microbial diversity in CW is seasonality. Even more, there appears to be
90 no other studies that have investigated the effects of different plants and season of the year
91 about potential methane production and microbiological composition, particularly in
92 methanogenic community. The objective of this study was to evaluate potential methane
93 production and to characterize the bacterial and archaeal communities found in HSSFs planted

with two different species (*Phragmites australis* and *Schoenoplectus californicus*) under cold and warm seasons.

2. MATERIAL AND METHODS

2.1 Pilot plant and sampling strategy

The constructed wetland pilot plant system was located in Hualqui (36°59'26.93" south and 72°56'47.23" west), Biobío Region, Chile. The HSSFs were fed with wastewater from a preliminary treatment that serves a rural community of 20,000 inhabitants.

The influent is primarily treated in sand trap-degreaser, septic tank and pumping tanks, and then uniformly distributed to the constructed wetlands by entering through a perforated distributor pipe (80 mm diameter) placed horizontally and perpendicular to the direction of flow. The HSSFs system consisted of four units, whose characteristics are presented in Table 1 and Figure 1. Table 1 shows the characteristics, operational and control parameters for all the HSSFs units. Figure 1 shows the scheme and side view of pilot system planted with two macrophyte species (*Phragmites australis* and *Schoenoplectus californicus*).

Table 1 and Figure 1

The system was implemented in July 2011, operating and monitored for 1335 days (3.7 years between 2011 and 2015). Two units were planted with *Phragmites australis* (HSSF−Phr) and other two with *Schoenoplectus californicus* (HSSF−Sch), for the sake of comparison, all the results are presented as the average obtained for each plant species.

In order to characterize the physicochemical properties and quantify removal efficiencies of the HSSFs, water samples from the influent and the effluent were collected every 15−30 days and characterized for COD, BOD_5, total suspended solids (TSS), and volatile suspended solids (VSS) (López *et al.,* 2015). The results of characterization of influent and effluent are presented as the average of the spring/summer and fall/winter seasons of the entire experimental period

(3.7 years). Fall/winter and spring/summer seasons will be considered throughout the all text as the cold and warm season, respectively.

Two sampling campaigns were conducted in cold (June 2013) and warm (March 2015) season to extract biomass samples used for methanogenic activity assays and for molecular characterization (López *et al*., 2015). Three sampling points were selected along the HSSF unit (Figure 1). Samples were transported and stored in septic wastewater from HSSF systems in order to maintain similar conditions (Ruiz *et al*., 2010). The biomass adhered to the gravel samples (800−1000 g gravel) was extracted by sonicating the sample for 3 min in a saline solution (0.9%), that was subsequently vortexed for 30 s and used for SMA_m and DNA extraction for molecular characterization. This procedure has been previously tested and applied in other CW systems (Ruiz *et al*., 2010; López *et al*., 2015; Carballeira *et al*., 2017). Results of the SMA_m and molecular characterization are presented as the average of the three samples obtained along the HSSFs.

2.3 Analytical methods

- *Characterization of the influent and effluent.* To characterize the influent and effluent of each HSSF, the samples were filtered through Whatman 0.45 µm membrane pore size filters. The parameters were measured according to the protocols described in the Standard Methods. The BOD_5 and COD were determined via the Winkler and colorimetric methods, respectively. Total suspended solids (TSS) and volatile suspended solids (VSS) were determined by gravimetric methods (APHA, 1998).

- *Specific methanogenic activity tests (maximum) (*SMA_m*).* Methanogenic activity tests were carried out as described in López et al. (2015). Biomass extracted from each zone (with concentrations in the range 0.16−2.8 gVSS/L) was mixed with a volatile fatty acid (VFA) solution (2.0 g/L acetic acid, 0.5 g/L propionic acid and 0.5 g/L butyric acid), nutrients and

142 Na_2S in 100 mL (effective volume) reactors. The analysis was carried out at 35 °C. The
143 methane production was determined by measuring the volume displacement of NaOH (2.5%)
144 by the methane accumulated in the headspace of the flask. It was measured daily to obtain the
145 volume of the methane produced (V_{CH4}) in one day (Vidal and Diez, 2003). Eq. (1) shows the
146 calculation to obtain SMA_m.

$$SMA = \frac{dV_{CH4}}{dt} \times \frac{1}{X_0 \times V_r \times f_1} \qquad \text{Equation 1}$$

147 Where t is time (d), X_0 is the VSS concentration in the reactor (g VSS/mL), V_r is the useful
148 volume of reactor (mL) and f_1 a conversion factor to represent the COD value of the unit of
149 volume of methane at a temperature of 35°C (Soto *et al*., 1993). SMA_m is expressed in g COD
150 g^{-1} SSV·d^{-1} (Sepúlveda *et al*., 2017).
151 On the other hand, the production of methane in the constructed wetland, it was determined
152 accord the equation 2 and it was called potential methane production (PMP).

153 $$PMP = VMP \times X_{CW} \qquad \text{Equation 2}$$

154 Where VPM is the volume of methane production (mL_{CH4}/gVSS·d) and X_{cw} is the
155 microbiological biomass attached (g VSS/m^2). The potential methane production in the
156 constructed wetlands does not consider the loss of methane through oxidation.

157 - *DNA extraction.* The extracted biomass was centrifuged for 5 min at 5000 g. DNA was
158 isolated in duplicate from 500 mg of sample pellet using the Fast DNA™ SPIN Kit for Soil and
159 the FastPrep® Instrument (MP Biomedicals) following the protocol from the manufacturer.
160 - *PCR amplification, DGGE and sequencing of 16S rRNA genes.* The quantification of total
161 (viable and dead) bacteria and archaea was performed by separately targeting the universal

primers of the 16S rRNA gene in bacteria and archaea. Primers used were 341F (5′-CCTACGGGAGGCAGCAG), 907R (5′-ATTACCGCGGCTGCTGG), 341F-GC (5′-GCclamp-GCCTACGGGAGGCAGCAG), 534R (5′-ATT ACC GCG GCT GG) for bacteria, and 344F (5′-ACGGGGCGCAGCAGGCGCGA) and 915R (5′-GTGCTCCCCCGCCAATTCCT) for archaea (Muyzer *et al*., 1993; Lane *et al*., 1985; Raskin *et al*., 1994; Coolen *et al*., 2004). One microliter of isolated DNA was added to 19 µL of a mixture containing MasterMix (LightCycler FastStart DNA Master SYBR Green I, Roche), 0.4 µL of each primer and 8.2 µL of PCR grade water.

Amplification reactions were carried out in a LightCycler 2.0 (Roche). The cycle program included a pre−incubation step for 3 min at 95 °C, followed by 40 cycles of 10 seconds at each 95 °C (denaturation), 10−61 °C (primer annealing) and 15−72 °C (extension) for both, archaea and bacteria, except for the primer annealing (20−58 °C for bacteria). The gene copy number was calculated using LightCycler 4.05 software. The calibration curve was generated with *Escherichia coli* and *Methanosarcina mazei* standard DNA for bacteria and archaea, respectively.

Universal primers for the bacteria and archaea domains were used for the amplification of the 16S rRNA gene for PCR−DGGE performance. The samples that showed the highest gene copy numbers of each HSSF (obtained from q-PCR) were considered for PCR−DGGE. PCR consisted of 1 µL of sample added to a solution of 1x PCR buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 0.5 µL of each primer. Reaction conditions are described in Jopia et al. (2011). One microliter of PCR product was amplified through a nested PCR (n-PCR) reaction with the corresponding primer.

For the DGGE, a biomass sample of the three zones of the HSSF was analyzed. DGGE was performed with 5 to 18 µL of amplified DNA sample using BioRad (Universal Mutation Detection System). Gels were made with 7.5% acrylamide/bis-acrylamide and a denaturant

187 gradient in the range of 40−62% for archaea and 38−65% for bacteria. The assays were carried
188 out at 60 V for 16 h at 60 °C (Valdebenito-Rolack *et al.,* 2011). Gels were stained with $AgNO_3$
189 for pattern fingerprint analysis and with ethidium bromide (5 μg/mL) for band excision. Band
190 pattern analysis was carried out with the Gel-Pro Analyzer, taking in to account bands with
191 intensity peaks of 1% or more. For band pattern comparison, a presence/absence matrix was
192 constructed and analyzed by Bray Curtis similarity using Prime 5 Software.
193 Isolated DNA was subsequently amplified with primers and sequenced (Macrogen Inc. Seúl
194 Korea). Sequences obtained were compared with the sequences stored in the GenBank database
195 using the BLASTn tool.

196 **2.4 Statistical analyses**

197 Statistical analyses were performed using the statistical program INFOSTAT (Di Rienzo *et al*.,
198 2011). Previously, data were subjected to a normality test (Shapiro–Wilk test) to determine the
199 appropriate statistical tests. To compare the HSSFs: (a) data with a normal distribution were
200 analyzed using a paired t-test; (b) data without normal distribution were analyzed with a
201 Wilcoxon test. Furthermore, to compare the influence of the seasons, (a) data with a normal
202 distribution were analyzed by ANOVA; (b) data without a normal distribution were analyzed by
203 a Kruskal–Wallis test. For all statistical tests, the significance level was $\alpha = 0.05$ (Vera *et al*.,
204 2011).

205 **3. RESULTS AND DISCUSSION**

206 **3.1 Organic matter and suspended solids concentrations**

207 Table 2 shows the results of the seasonal physicochemical characterization of the influent,
208 effluent and removal efficiencies for the monitored period. The pollutant concentrations of the
209 influent in the warm and cold seasons showed no significant differences ($p \geq 0.05$). The average
210 organic matter concentrations in the influent (185 mg/L of BOD_5 and 278 mg/L of COD) were

similar to the average values found in previous studies (García *et al*., 2004; Vymazal and Kröpfelová, 2011; Mburu *et al*., 2012). According to average organic matter content, the influent can be defined as a concentrated wastewater (Henze *et al*., 2001).

The biodegradability capacity (BOD_5/COD relation) of the influent varied between 0.72 in cold and 0.61 in warm season. These values were similar to the ones found in other studies, ranging between 0.46−0.66 (García *et al*., 2004; Hijosa-Valsero *et al*., 2012; Mburu *et al*., 2012). According to Tran et al. (2015) and Henze et al. (2001), BOD_5/COD ratio of 0.67 corresponds to a biodegradability capacity of 67%, meaning that a substantial part of the organic matter can be biologically degraded.

Table 2

Concerning the average BOD_5 removal, no significant differences between species were observed ($p \geq 0.05$). In all the units, removals during the warm season (53%-HSSF-Phr and 55%-HSSF-Sch) were 12% lower than in the cold season (65%-HSSF-Phr and 67%-HSSF-Sch), resulting in effluent concentrations from 71 to 76 mg/L (HSSF-Phr and HSSF-Sch average) for cold and warm season, respectively. Similarly, COD removal was on average for both HSSF 61% in cold and 54% in warm season, with effluent concentrations of 100 and 133 mg/L, for respectively. Furthermore, the applied load in warm season (4.74 ± 1.01 $gBOD_5/m^2d$) was similar than in cold season (4.64 ± 1.30 $gBOD_5/m^2$ d).

In general, the removal efficiency of organic matter was on average 16% lower than values determined by other authors, that found average BOD_5 removals of 76% (57−96%) of and COD removals of 73% (44−88%) (García *et al*., 2004; Trang *et al*., 2010; Mburu *et al*., 2012). This deficiency in removing organic matter stems from the high organic loading rates applied to this system (2.7−7.2 g $BOD_5/m^2 \cdot d$), which exceed by 26−72% the 2 g $BOD_5/m^2 \cdot d$ recommended by García et al. (2004). Nevertheless, organic loading rates in the range of 8 to 12 g $COD/m^2 \cdot d$ are still appropriated for an HSSF (García *et al*., 2010; Pedescoll *et al*., 2011). In addition, such

values coincide with the ones determined by Carballeira et al. (2016), who found a reduction in term of BOD_5 efficiencies by increasing the organic load from 2.5 to 4.7 g $BOD_5/m^2 \cdot d$.

The average influent concentrations of TSS and VSS were 268 and 231 mg/L, respectively. Such concentrations were 50−81% higher than values found in literature (García *et al*., 2004; Hijosa-Valsero *et al*., 2012; Mburu *et al*., 2012). This could be attributed to the rural origin of the influent, showing high solids concentration. Besides, removal efficiencies of TSS and VSS showed minor variations (10%), with high removal efficiencies for all the units (85−93%), generating effluents with average concentrations of less than 22 mg/L. No significant differences between species or seasons were observed ($p \geq 0.05$). The removal efficiencies during the operating period were consistent with those found in other studies (74−95% TSS) (Trang *et al.*, 2010; Pedescoll *et a*l., 2011). Such high suspended solids removal can be explained by the installation of primary treatment during the second year of operation maintained the removal efficiencies, reducing the solids loading rates by 50% on average (4.4 g $TSS/m^2 \cdot d$). Besides, the solids loading rates during the monitored period (4.3 to 8.7 g $TSS/m^2 \cdot d$) were near the range applied (3.6−10 g $TSS/m^2 \cdot d$) and recommended (5 g $TSS/m^2 \cdot d$) by others authors to avoid clogging and to obtain solids removal of 4.2 g $TSS/m^2 \cdot d$, corresponding to efficiency levels greater to 78% (Winter and Goetz, 2003; Vymazal, 2005; García *et al*., 2005, Caselles-Osorio *et al*., 2007; Alvarez *et al*., 2008).

On the whole, organic matter and suspended solids removal efficiencies did not show significant seasonal differences ($p \geq 0.05$). This could be attributed to the phenomenon described by Vymazal and Kröpfelová (2011), according which bacterial activity and removal efficiencies were not significantly influenced by temperature (12ºC cold season and 20ºC in warm season see Table 1), especially after few years of operation. Rather, the supply of organic matter and nutrients are the main factors affecting the HSSF efficiency (Huang *et al*., 2005).

3.2 Potential methane production and quantification of bacterial and archaeal communities

Table 3 shows the SMA_m, the amount of attached biomass, the potential methane production and the quantification of bacterial and archaeal communities for HSSF−Phr and HSSF−Sch obtained in the tests performed with the biomass extracted in cold and warm season. The SMA_m of the biomass extracted from HSSF-Phr and HSSF-Sch during the cold season was 0.02 and 0.01 g COD_{CH4}/gVSS·d, being significantly lower ($p < 0.05$) (75 and 80%) than values found during the warm season, with activities of 0.07 and 0.06 g COD_{CH4}/gVSS·d, for HSSF-Phr and HSSF-Sch, respectively. Meanwhile, when comparing both species, HSSF-Phr presents higher SMA_m than HSSF-Sch, both during the cold (33%) and warm season (23%).

The ranges of the SMA_m found in this study were between 3-30 times (63-97%) higher than those evidenced in HSSFs with *Phragmites* by Carballeira et al. (2017) (0.0025-0.0037g CH_4/gVS·d). This could be due to the fact that author worked with a substrate concentration (0.5 g COD / L-acetic acid) 75% lower than the one used in this research (2.0 g/L acetic acid, 0.5 g/L propionic acid and 0.5 g/L butyric acid) and besides they worked at psychrophilic temperatures (25ºC).

Table 3

The average potential methane production of HSSF-Phr were 1434 ± 283 mg CH_4/m^2· d and 1485 ± 86 mg CH_4/m^2·d in cold and warm season, respectively. The potential methane productions of HSSF-Sch were 1799 ± 447 mg CH_4/m^2·d and 3075 ± 1149 mg CH_4/m^2·d in cold and warm season, respectively. On the whole, the methane production found here matched with the values found in other studies, with ranges from 200 to 6480 mg CH_4/m^2·d in HSSFs operated in cold and temperate climates with an organic loading rate of 0.5–20 g BOD_5/m^2·d (Tai *et al.*, 2002; Wang *et al.*, 2013a; Corbella and Puigagut, 2015; López *et al.*, 2015;

285 Carballeira *et al*., 2017). HSSF-Phr showed similar productions in both seasons (variations less
286 than 3%). However, potential methane production for HSSF-Sch was significantly higher (42%)
287 in the warm season compared to the cold season (value $p \leq 0.005$). The higher potential methane
288 production for HSSF-Sch may be linked to the increment of the specific methanogenic activity
289 (approx 80%) and to the increase in the amount of attached biomass (194 g VSS/m^2), that was
290 71% higher in the warm season. In turn, seasonal variations in the SMA_m are in accordance with
291 previous studies, that associate the increase of methane production (3-100 times) to the increase
292 of microbiological activities derived from the highest radiation and temperature in warm
293 seasons (Tai *et al*., 2002; Teiter and Mander, 2005; Ding and Cai, 2007; Waletzko and Mitsch,
294 2014; Maucieri *et al*., 2017). Moreover, Zhu et al. (2007) showed that the methane emissions
295 rate decreased by 12 times over variations of 5.5 ºC, dropping from 9.4 mg $CH_4/m^2 \cdot d$ (15 °C) to
296 0.74 mg $CH_4/m^2 \cdot d$ (9.5 °C) in the HSSFs areas with macrophytes. Similarly, Ding and Cai
297 (2007) determined that *P. australis* methane emissions vary by up to 70 times between the
298 warm season (943 $CH_4/m^2 \cdot d$) and cold season (12.5 mg $CH_4/m^2 \cdot d$). Maucieri et al. (2014)
299 reported up to 98% higher methane emissions in spring season than in fall season, with a
300 correlation between this emissions and radiation of 0.76 (with a significance level of 0.001).

301 Regarding the plant species, greater potential methane production (20−51%) was observed in
302 the wetlands planted with *Schoenoplectus californicus* (1799−3075 mg $CH_4/m^2 \cdot d$) with respect
303 to the one planted with *Phragmites australis* (1434−1485 mg $CH_4/m^2 \cdot d$) in both seasons.
304 The number of bacterial copies found in this study is shown in Table 3. Values ranged between
305 4.18×10^6 and 9.26×10^6 (copies/mgVSS) and coincide with data determined by Zhang et al.
306 (2015), who showed for wetlands built in summer season with *Phragmites australis*, *Arundo*
307 *donax* and *T. orientalis* a number of bacteria of 6.05×10^6 copies/g, 3.55×10^6 copies/g and
308 1.52×10^6 copies/g, respectively. In the case of archaea (Table 3), the quantification of this group
309 found in this study was between 95 and 99% lower than concentrations determined in anaerobic

conditions (sediments) by Zhang et al. (2015), who evidenced archaeal 16S rRNA gene copy numbers of 1.73×10^{6}–3.16×10^{7} copies per gram dry sediment.

In the HSSF-Phr, from cold to warm season, the number of bacteria decreased by 14% and archaea increased by 57%. For the HSSF-Sch during the same period, the number of bacteria and archaea increased by 38 and 50%, respectively. This could explain the increase in methane emissions that were 42% higher in the warm than in the cold season. The increase in the number of copies during the warm season determined in this research is confirmed by Wang et al. (2016); who showed that during summer, the abundance of the total bacteria in support medium was 1.54×10^{5} copies/g and in winter was 7.60×10^{4} copies/g. Besides, the total bacterial abundance on the root surfaces and support medium decreased from summer to winter (Wang *et al*., 2016).

Comparing plant species, the number of copies of the 16S rRNA archaeal genes obtained from the HSSF-Sch was 43 and 34% higher than the number obtained from the HSSF-Phr, during cold and warm season, respectively. The above this could be explained due to the radial oxygenation capacity of *Phragmites australis* (3.94-25.2 g O_2/m^2 d) with respect to *Schoenopectus* sp. (0.94 g O_2/m^2 d) (Liu *et al*., 2016), since methanogenic archaea need preferably anoxic conditions to be able to develop (Christy *et al*., 2014). Moreover, it has been determined that the dissolved oxygen of the rhizosphere is one of the most important factors affecting CH_4 flux and methanogenic communities (Zhang *et al*., 2018). While the number of bacteria showed only a 4% difference in cold season. On the other hand, in the warm season, 48% higher number of bacteria were found in HSSF-Sch compared to HSSF-Phr. The higher amount of archea and bacteria directly and positively affected the potential methane production that was 20-51% higher in HSSF-Sch than in HSSF-Phr.

3.3. Identification of bacterial and archaeal communities

Figure 2 shows the seasonal banding patterns for the 16S rRNA gene PCR amplicons for bacteria and archaea in HSSF−Phr and HSSF−Sch. Clear differences were observed in band position, intensity and band number for the different samples, demonstrating differences in the bacterial and archaeal communities developed in different HSSFs. The DGGE determined number of bands per lane varying from 16 to 17 for bacteria and 10 to 18 for archaea, in which it was possible to identify 12 bands for archaea and bacteria. This coincides with results from Adrados et al. (2014) and Cao et al. (2017) who found lower band richness for archaea with respect to bacteria in HSSFs. On the other hand, a similar number of bands for bacteria was found by Sidrach-Cardona et al. (2015) and Calherios et al. (2009), who determined a number of bands ranging from 14 to 25 in the biomass adhered to the gravel in constructed wetlands planted with *Phragmites* and *Typha*.

Figure 2

In Figure 3, the dendrogram constructed from the band patterns of bacteria and archaea is presented. There is a similarity (51%) among the bacterial communities of the different seasons. Samples from cold season exhibit a similarity between communities in HSSF−Phr and HSSF−Sch (94%) greater than the similarities between bacterial communities in samples from warm season (63%).

On the other hand, the dendrogram of the archaea shows that similarity between communities from HSSF-Phr and HSSF-Sch was higher during the cold (92%) than in the warm (67%) season. At the same time, *Schoenoplectus* in the warm season presents high similarity (80%) with samples obtained during the cold season.

Figure 3

Table 4 and 5 show the most similar sequences found in each extracted DGGE band to those stored in the GenBank database, indicated with numbers from 1 to 24 (1−12 for bacteria and

13−24 for archaea). The sequences of bacteria and archaea belonged mostly (>95%) to non-cultivable microorganisms. Microorganisms found by sequencing are similar to those found by other authors in HSSFs (Adrados *et al.*, 2014; Sidrach−Cardona *et al.*, 2015) and natural wetlands (Lv *et al.*, 2014), who found between 53−80% of sequences belonged to non−cultivable bacteria and archaea.

The sequences associated with bacteria primarily belonged to Firmicutes (42%), Proteobacteria (33%) and Bacteroidetes (25%) phyla (Table 4). The sequences found in this research coincide with the predominant phyla determined by different authors in constructed wetlands (Proteobacteria: 20.1−38.8%, Bacteroidetes: 11.5-41.4% and Firmicutes: 3.6−15%) (Calheiros *et al*., 2009; Adrados *et al*., 2014; Cao *et al*., 2017; Wang *et al*., 2017). Meanwhile, He et al. (2014) found that members of the β-Proteobacteria (55%) and γ-Proteobacteria (37%) were dominant for vertical and horizontal flow constructed wetlands, being present in more than 1/3 of the bed. Besides, in the same study the predominant genera and subgroups were *Clostridium*, Bacteroidetes, β−proteobacteria and γ−proteobacteria, clone sequences mainly affiliated with anaerobic environments, sediment, natural and constructed wetlands and anaerobic reactors (Bouali *et al*., 2014; Lv *et al*., 2014; Adrados *et al*. 2014; Zhang *et al*., 2015; Sidrach−Cardona *et al*, 2015).

Table 4

In this study, Firmicutes (order Clostridiales) and Bacteroidetes were detected in all samples accounting 65% of the total community. These are highly versatile groups capable to participate in various stages of anaerobic degradation (such as; hydrolysis, fermentation, lipase at low temperatures, protease activity and acidogenesis) (Song *et al*., 2010; Lv *et al*., 2014; Kim *et al*., 2015; Lin *et al*., 2016; Gulhane *et al*., 2017); they are frequently found in anaerobic digesters, wastewater treatment plants and natural and constructed wetlands (Adrados *et al*., 2014; Kim *et al*., 2015). *Clostridium*, were found in both macrophytes species and seasons. Indeed, these

384 microbes have the capacity to survive in different climatic conditions, sporulating when
385 environmental conditions become hostile (heat, changes in nutrients status, pH extremes and
386 toxic chemicals) (Gulhane *et al*., 2017). Besides, this group has been determined in association
387 with hydrogenotrophic methanogens in mesophilic conditions (Song *et al*., 2010).
388 On the other hand, in Figure 2 the same number of bacteria band richness was observed for both
389 seasons and macrophyte (17 bands for each season); however, greater species richness was
390 observed in warm season (7 species) than in cold season (4 species). This in turn, coincides with
391 the higher development of adhered biomass (51-71%), number of bacteria (38%) and methane
392 production (4-42%) during the warm season.
393 In turn, 91% of the archaea bands belonged to the Phylum Euryarchaeota, class
394 methanomicrobia and order Methanosarcinales, specifically the genera *Methanosaeta* (75% of
395 total bands) and *Methanosarcina* (16% of total bands). A similar archaeal community
396 composition was described in a biogas plant, anaerobic reactors (Song *et al*., 2010), a river
397 (Zhang *et al*., 2015), rice field soil (Watanabe *et al*. 2006) and natural wetlands (Liu *et al*.,
398 2015).
399 Table 5
400 The phylum and class found were consistent with those found in a phylogenetic analysis by Liu
401 et al. (2015) and Lv et al. (2014) in natural wetland with *Phragmites sp*. These authors revealed
402 between 50-69.4% of the clones were affiliated with Euryarchaeota, dominated by
403 Methanobacteriales (60.7%), Methanomicrobiales (20.2-71%) and Methanosarcinales (17.2%).
404 Methanomicrobiales contribute to a large proportion of the methane emissions in cold and
405 subtropical areas. Methanogenic acetoclastic of genera *Methanosaeta* (34%) and
406 *Methanosarcina* (10%) belong to this class (Liu *et al*., 2015; Lv *et al*., (2014).
407 On the other hand, it has been determined that during mesophilic digestion of wastewater
408 sludge, *Methanosarcina* and *Methanosaeta* were most abundant, accounting for up to 90% of

409 the total archaea present (Traversi *et al*., 2011). It has also been determined a significantly
410 positive correlation between *Methanosarcina* and Methanosaetae with the biogas production
411 rate ($p < 0.01$ and $p < 0.05$) (Traversi *et al*., 2011). This coincides with results found in this
412 study, since increases in SMA_m (75-80%), potential methane production (3.4-42%) and the
413 number of archaeal copies in the biomass (50-57%) were observed in the warm season.
414 The presence of *Methanosaeta concilii* during both seasons is influenced by the temperature of
415 the HSSFs systems (12ºC cold season and 20ºC in warm season). Narihiro et al. (2009)
416 evidenced the abundances of *Methanosaeta* accounted for 5.7–49% of the total archaeal
417 populations in mesophilic processes, and those of *Methanosarcina* represented 42% of the total
418 archaeal populations in thermophilic processes. Song et al. (2010) and Zhang et al. (2012)
419 determined that in anaerobic system from 5−18°C the dominant methanogen was
420 Methanosaetaceae. It has also been reported that Methanomicrobiales-related populations are
421 likely to play important roles in low-temperature (psychrophilic and mesophilic) conditions
422 (Watanabe *et al*., 2006).
423 Watanabe et al. (2006) observed that the community structure of the methanogenic archaea in
424 soils did not change throughout the year, even under oxic conditions. Similarly, in this study it
425 is observed that the archaea community does not change significantly between the different
426 seasons or plant species, with a persistent prevalence of *Methanosaeta concilii*.

427 **4. CONCLUSIONS**

428 -Degradation of organic matter (53−67% BOD_5 and 51−62% COD) and suspended solids (85-
429 90% TSS and 86-93% VSS) in the HSSFs was not influenced by seasonal conditions or plant
430 species.
431 -Potential methane production from HSSF planted with *Schoenoplectus californicus* (1799−3075
432 mg/$CH_4 \cdot m^2 d$) were between 20 and 51% higher than HSSF planted with *Phragmites australis*

(1434−1485 mg/$CH_4 \cdot m^2 d$). Moreover, potential methane production during warm season was 3.4% (HSSF-Phr) and 42% (HSSF-Sch) higher than during cold season.

-The quantification of microorganisms in HSSFs determined greater development of bacteria (38%) and archaea (50−57%) during the warm season. In addition, the species *Schoenoplectus californicus* had a larger number of bacteria (4−48%) and archaea (34−43%) than *Phragmites australis.*

-The quantification and identification of microbial consortia demonstrated the presence of facultative and anaerobic bacteria, represented primarily by Firmicutes (42%), Proteobacteria (33%), and Bacteroidetes (25%). The archaea were represented primarily by Methanosarcinales, specifically, *Methanosaeta* (75%) and *Methanosarcina* (16%). The community structure of the methanogenic archaea in HSSFs did not change throughout the seasons or macrophytes.

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REFERENCES

Adrados, B., Sánchez, O., Arias, C., Becares, E., Garrido, L., Mas, J., Brix, H., Morató, J. 2014. Microbial communities from different types of natural wastewater treatment systems: vertical and horizontal flow constructed wetlands and biofilters. Water Research 55, 304-312.

Álvarez, J. A., Ruíz, I., Soto, M. 2008. Anaerobic digesters as a pretreatment for constructed wetlands. Ecological Engineering 33, 54-67.

American Public Health Association (APHA) 1998. Standard Methods for the Examination of Water and Wastewater, 20th ed. American Public Health Association, Washington, D.C.

Andersen, R., Chapman, S. J., Artz, R. 2013. Microbial communities in natural and disturbed peatlands: a review. Soil Biology and Biochemistry. 57, 979-994.

Borrel, G., Harris, H., Parisot, N., Gaci, N., Tottey, W., Mihajiovski, A., Deane, J., Gribaldo, S., Bardot, O., Peyrataillade, E., Peyret, P., O'Toole, P., Brugère, J. 2013. Genome sequence of "*Candidatus Methanomassiliicoccus intestinalis*", a third Thermoplasmatales-related methanogenic archeon from human feces. Genome announcements 1, e00453-13.

Bouali, M., Zrafi, I., Bakhrouf, A., Chaussonnerie, S., Sghir, A. 2014. Bacterial structure and spatiotemporal distribution in a horizontal subsurface flow constructed wetland. Applied microbiology and biotechnology 98, 3191-3203.

Cadillo-Quiroz, H., Bräuer, S., Yashiro, E., Sun, C., Yavitt, J., Zinder, S. 2006. Vertical profiles of methanogenesis and methanogens in two contrasting acidic peatlands in central New York State, USA. Environmental microbiology 8, 1428-1440.

Calheiros, C., Duque, A., Moura, A., Henriques, I., Correia, A., Rangel, A., Castro, P. 2009. Substrate effect on bacterial communities from constructed wetlands planted with *Typha latifolia* treating industrial wastewater. Ecological Engineering 35, 744-753.

Cao, Q., Wang, H., Chen, X., Wang, R., Liu, J. 2017. Composition and distribution of microbial communities in natural river wetlands and corresponding constructed wetlands. Ecological Engineering 98, 40-48.

Carballeira, T., Ruiz, I., Soto, M. 2016. Effect of plants and surface loading rate on the treatment efficiency of shallow subsurface constructed wetlands. Ecological Engineering 90, 203-214.

Carballeira, T., Ruiz, I., Soto, M. 2017. Methanogenic activity of accumulated solids and gas emissions from planted and unplanted shallow horizontal subsurface flow constructed wetlands. Ecological Engineering 98, 297-306.

Christy, P. M., Gopinath, L. R., Divya, D. 2014. A review on anaerobic decomposition and enhancement of biogas production through enzymes and microorganisms. Renewable and Sustainable Energy Reviews 34, 167-173.

Coolen, M., Hopmans, E., Rijpstra, W., Muyzer, G., 2004. Evolution of the methane cycle in Ace Lake (Antarctica) during the Holocene: response of methangens and methannotrophs to environmental change. Organic Geochemistry 35, 1151-1167.

Corbella, C., Puigagut, J. 2015. Effect of primary treatment and organic loading on methane emissions from horizontal subsurface flow constructed wetlands treating urban wastewater. Ecological Engineering 80, 79-84.

De la Varga, D., Ruiz, I., Álvarez, J. A., Soto, M. 2015. Methane and carbon dioxide emissions from constructed wetlands receiving anaerobically pretreated sewage. Science of The Total Environment 538, 824-833.

Di Rienzo J., Casanoves, F., Balzarini, M., Gonzalez, I. Tableda, M., Robledo, C. 2011. Infostat Statistical software. Infostat group, National University of Córdoba, Argentina 336 pp.

García, J., Aguirre, P., Mujeriego, R., Huang, Y., Ortiz, L., Bayona, J. 2004. Initial contaminant removal performance factors in horizontal flow reed beds used for treating urban wastewater. Water Research 38, 1669-1678.

García, J., Rousseau, L., Morató, J., Lesage, E., Matamoros, V., Bayona, J., 2010. Contaminant Removal Processes in Subsurface-Flow Constructed Wetlands: A review. Critical Reviews in Environmental Science and Technology 40, 561-661.

Gulhane, M., Pandit, P., Khardenavis, A., Singh, D., Purohit, H. 2017. Study of microbial community plasticity for anaerobic digestion of vegetable waste in Anaerobic Baffled Reactor. Renewable Energy 101, 59-66.

He, G., Yi, F., Zhou, S., Lin, J. 2014. Microbial activity and community structure in two terrace-type wetlands constructed for the treatment of domestic wastewater. Ecological Engineering 67, 198-205.

Henze, M., Harremoes, P., la Cour Jansen, J., Arvin, E. 2001. Wastewater treatment: biological and chemical processes. Springer Science Business Media.436 pp.

Hijosa-Valsero, M., Sidrach-Cardona, R., Bécares, E. 2012. Comparison of interannual removal variation of various constructed wetland types. Science of the total environment 430, 174-183.

Huang, Y., Ortiz, L., Aguirre, P., García, J., Mujeriego, R., Bayona, J. 2005. Effect of design parameters in horizontal flow constructed wetland on the behavior of volatile fatty acids and volatile alkylsulfides. Chemosphere 59, 769-777.

Jopia, P., Urrutia, H., Sossa, K., Nocker, A. 2011. Effect of PCR amplicon length on suppressing signals from membrane-compromised cells by propidium monoazide treatment. Journal of microbiological methods 87, 89-95.

Kim, Y., Jang, H., Lee, K., Chantrasakdakul, P., Kim, D., Park, K. 2015. Changes in bacterial and archaeal communities in anaerobic digesters treating different organic wastes. Chemosphere 141, 134-137.

Lane, D., Bernadette, P., Olsen, G., Stahl, D., Sogin, M., Pace, N. 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proceedings of the National Academy of Sciences 82, 6955-6959.

Ligi, T., Oopkaup K, Truu M, Preem J-K, Nõlvak H, Mitsch WJ, Mander Ü, Truu J. 2014. Characterization of bacterial communities in soil and sediment of a created riverine wetland

527 complex using high-throughput 16S rRNA amplicon sequencing. Ecological Engineering 72,
528 50-66.
529 Lin, Q., De Vrieze, J., He, G., Li, X., Li, J. 2016. Temperature regulates methane production
530 through the function centralization of microbial community in anaerobic digestion.
531 Bioresource technology 216, 150-158.
532 Liu, H., Hu, Z., Zhang, J., Ngo, H. H., Guo, W., Liang, S., Wu, H. 2016. Optimizations on
533 supply and distribution of dissolved oxygen in constructed wetlands: a review. Bioresource
534 technology 214, 797-805.
535 Liu, Y., Li, H., Liu, Q., Li, Y. 2015. Archaeal communities associated with roots of the
536 common reed (*Phragmites*) in Beijing Cuihu Wetland. World Journal of Microbiology and
537 Biotechnology 31, 823-832.
538 López, D., Fuenzalida, D., Vera, I., Rojas, K., Vidal, G. 2015. Relationship between the
539 removal of organic matter and the production of methane in subsurface flow constructed
540 wetlands designed for wastewater treatment. Ecological Engineering 83, 296-304.
541 Lv, X., Yu, J., Fu, Y., Ma, B., Qu, F., Ning, K., Wu, H. 2014. A meta-analysis of the bacterial
542 and archaeal diversity observed in wetland soils. The Scientific World Journal, 2014.
543 Mander, Ü., Dotro, G., Ebie, Y., Towprayoon, S., Chiemchaisri, C., Nogueira, S., Jamsranjav,
544 K., Truu, J., Tournebize, Mitsch, W. 2014. Greenhouse gas emissions in constructed
545 wetlands for wasteweater treatment: review. Ecological Engineering 66, 19-35
546 Maucieri, C., Barbera, A.C., Vymazal, J., Borin. M. 2017 A review on the main affecting
547 factors of greenhouse gases emission in constructed wetlands. Agricultural and Forest
548 Meteorology 236, 175-193.
549 Mburu, N., Tebitendwa, S., Rousseau, D., Van Bruggen, J., Lens, P. 2012. Performance
550 evaluation of horizontal subsurface flow–constructed wetlands for the treatment of domestic
551 wastewater in the tropics. Journal of Environmental Engineering 139, 358-367.

552 Muyzer, G., De Waal, E., Uitterlinden, A. 1993. Profiling of complex microbial populations by
553 denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified
554 genes coding for 16S rRNA. Applied and environmental microbiology 59, 695-700
555 Niu, C., He, Z., Ge, Y., Chang, J., Lu, Z. 2015. Effect of plant species richness on methane
556 fluxes and associated microbial processes in wetland microcosms. Ecological Engineering
557 84, 250-259.
558 Pedescoll, A., Corzo, A., Álvarez, E., García, J., Puigagut, J. 2011. The effect of primary
559 treatment and flow regime on clogging development in horizontal subsurface flow
560 constructed wetlands: an experimental evaluation. Water Research 45, 3579-3589.
561 Raskin, L., Stromley, J., Rittmann, B., Stahl, D. 1994. Group-specific 16S rRNA hybridization
562 probes to describe natural communities of methanogens. Applied and Environmental
563 Microbiology 60, 1232-1240.
564 Ruiz, I., Díaz, M. A., Crujeiras, B., García, J., Soto, M. 2010. Solids hydrolysis and
565 accumulation in a hybrid anaerobic digester-constructed wetlands system. Ecological
566 Engineering 36, 1007-1016.
567 Sidrach-Cardona, R., Martínez, O. S., Garrido, L., Bécares, E. 2015. Molecular characterization
568 of microbial communities in constructed wetlands. In Nova Science Publishers, Inc.
569 Song, M., Shin, S., Hwang, S. 2010. Methanogenic population dynamics assessed by real-time
570 quantitative PCR in sludge granule in upflow anaerobic sludge blanket treating swine
571 wastewater. Bioresource technology 101, S23-S28.
572 Soto, M., Méndez, R., Lema, J. M. 1993. Methanogenic and non-methanogenic activity tests.
573 Theoretical basis and experimental set up. Water Research 27, 1361-1376.
574 Sun, C. L., Brauer, S. L., Cadillo-Quiroz, H., Zinder, S. H., Yavitt, J. B. 2012. Seasonal changes
575 in methanogenesis and methanogenic community in three peatlands, NewYork State.
576 Microbiology of wetlands, 134 pp.

577 Tai, P., Li, P., Sun, T., He, Y, Zhou, Q., Gong, Z., Motoyuki, M., Inamori, Y. 2002. Green
578 house gas emissions from a constructed wetland for municipal sewage treatment. Journal of
579 Environmental Sciences 14, 27-33.
580 Tanner, C., Adams, D., Downes, M. 1997. Methane emissions from constructed wetlands
581 treating agricultural wastewaters. Journal of environmental quality 26, 1056-1062.
582 Tran, N., Ngo, H., Urase, T., Gin, K. 2015. A critical review on characterization strategies of
583 organic matter for wastewater and water treatment processes. Bioresource technology 193,
584 523-533.
585 Trang, N., Konnerup, D., Schierup, H., Chiem, N., Brix, H. 2010. Kinetics of pollutant removal
586 from domestic wastewater in a tropical horizontal subsurface flow constructed wetland
587 system: effects of hydraulic loading rate. Ecological Engineering 36, 527-535.
588 Traversi, D., Villa, S., Acri, M., Pietrangeli, B., Degan, R., & Gilli, G. (2011). The role of
589 different methanogen groups evaluated by Real-Time qPCR as high-efficiency bioindicators
590 of wet anaerobic co-digestion of organic waste. *AMB express*, *1*(1), 28.
591 United States Environmental Protection Agency (USEPA) 2000. Manual: Constructed Wetlands
592 Treatment of Municipal Wastewaters. Cincinnati, Ohio, USA, 166 pp.
593 Valdebenito-Rolack, E., Araya, T., Abarzúa, L. Ruiz-Tagle, N., Sossa, K., Aroca, G., Urrutia,
594 H. 2011. Thiosulphate oxidation by *Thiobacillus thioparus* and *Halothiobacillus*
595 *neapolitanus* strains isoplated from petrochemical industry. Electronic Journal of
596 Biotechnology 14, 1262-1268.
597 Vera, I., García, J., Sáez, K., Moragas, L., Vidal, G. 2011. Performance evaluation of eight
598 years experience of constructed wetland systems in Catalonia as alternative treatment for
599 small communities. Ecological engineering 37, 364-371.
600 Vymazal, J. 2005. Horizontal sub-surface flow and hybrid constructed wetlands systems for
601 wastewater treatment. Ecological engineering, 25, 478-490.

602 Vymazal, J., Kröpfelová, L. 2011. A three-stage experimental constructed wetland for treatment
603 of domestic sewage: first 2 years of operation. Ecological Engineering 37, 90-98.
604 Wang, J., Song, X., Wang, Y., Bai, J., Li, M., Dong, G., Yan, D. 2017. Bioenergy generation
605 and rhizodegradation as affected by microbial community distribution in a coupled
606 constructed wetland-microbial fuel cell system associated with three macrophytes. Science of
607 The Total Environment 607, 53-62.
608 Wang, Q., Xie, H., Ngo, H. H., Guo, W., Zhang, J., Liu, C., Zhao, C. 2016. Microbial
609 abundance and community in subsurface flow constructed wetland microcosms: role of plant
610 presence. Environmental Science and Pollution Research 23, 4036-4045.
611 Wang, W., Xie, L., Luo, G., Zhou, Q., Angelidaki, I. 2013a. Performance and microbial
612 community analysis of the anaerobic reactor with coke oven gas biomethanation and in situ
613 biogas upgrading. Bioresource technology 146, 234-239.
614 Wang, Y., Yang, H., Ye, C., Chen, X., Xie, B., Huang, C., Xu, M. 2013b. Effects of plant
615 species on soil microbial processes and CH_4 emission from constructed wetlands.
616 Environmental pollution 174, 273-278.
617 Watanabe, T., Kimura, M., Asakawa, S. 2006. Community structure of methanogenic archaea in
618 paddy field soil under double cropping (rice–wheat). Soil Biology and Biochemistry 38,
619 1264-1274.
620 Winter, K., Goetz, D. 2003. The impact of sewage composition on the soil clogging phenomena
621 of vertical flow constructed wetlands. Water science and technology 48, 9-14.
622 Yavitt, J., Yashiro, E., Cadillo-Quiroz, H., Zinder, S. 2012. Methanogen diversity and
623 community composition in peatlands of the central to northern Appalachian Mountain
624 region, North America. Biogeochemistry 109, 117-131.

Zhang, D., Zhu, W., Tang, C., Suo, Y., Gao, L., Yuan, X., .Cui, Z. 2012. Bioreactor performance and methanogenic population dynamics in a low-temperature (5–18° C) anaerobic fixed-bed reactor. Bioresource technology 104, 136-143.

Zhang, J., Yang, Y., Zhao, L., Li, Y., Xie, S., Liu, Y. 2015. Distribution of sediment bacterial and archaeal communities in plateau freshwater lakes. Applied microbiology and biotechnology 99, 3291-3302.

Zhang, K., Liu, Y., Chen, Q., Luo, H., Zhu, Z., Chen, W., Mo, Y. 2018. Effect of submerged plant species on CH_4 flux and methanogenic community dynamics in a full-scale constructed wetland. Ecological Engineering 115, 96-104.

Table Captions

Table 1. Characteristics of HSSF units.

Table 2. Seasonal physicochemical characterization of the influent and effluent from HSSFs.

Table 3. Seasonal specific methanogenic activity, potential methane production and bacterial and archaeal quantification of HSSFs.

Table 4. Bacteria found in biomass from HSSFs by DGGE−sequencing technique.

Table 5. Archaea found in biomass from HSSF by DGGE−sequencing technique.

Characteristics	Unit	Value	
Support medium			
Type	-	Gravel	
Size	mm	19−25	
Porosity	-	0.4	
Geometric			
Surface area	m^2	4.5	
Length/width relation	-	2	
HSSF average height	m	0.57	
Water table height	m	0.4	
Bottom slope	m/m	0.05	
Total volume	m^3	1.8	
Active volume	m^3	0.73	
Control parameters			
Hydraulic loading rate	mm/d	Cold season	30.86 ± 10.68
		Warm season	27.47 ± 4,85
Hydraulic retention time	d	Cold season	6.10 ± 1.30
		Warm season	5.86 ± 2.13
Organic loading rate	$gBOD_5/m^2 \cdot d$	Cold season	4.64 ± 1.30
		Warm season	4.74 ± 1.01
Operational conditions			
Temperature (Constructed Wetland)	ºC	Cold season	12.07 ± 1.80
		Warm season	20.30 ± 3.27
ORP	mV	Cold season	-226.8 ± 50.4
		Warm season	-259.8 ± 53.3
Dissolved Oxygen	mg/L	Cold season	0.47 ± 0.25
		Warm season	0.43 ± 0.39
Rainfall	L/m^2 d	Cold season	3.10 ± 1.01
		Warm season	0.60 ± 0.22

ORP: Oxidation Reduction Potential; HSSF: horizontal subsurface flow constructed wetland; BOD_5: biological oxygen demand; cold season: average of fall/winter; warm season: average of spring/summer

Table 1

Season	Parameter	Concentration (mg/L)						Removal (%)			
		Influent		Effluent				HSSF-Phr		HSSF-Sch	
				HSSF-Phr		HSSF-Sch					
		Average	Range	Average	Range	Average	Range	Average	Range	Average	Range
Cold season	BOD_5	205.8	102.0-372.0	71.9	23.5-213.0	69.2	22.5-198.0	64.7	42.7-87.6	67.1	46.8-87.9
	COD	286.2	131.6-464.5	102	33.5-216.1	99.1	35.3-189.8	60.7	43.2-87.5	61.7	43.2-87.5
	TSS	273.4	100.0-526.0	14.2	5.2-27.5	14.7	7.0-33.5	89.3	72.3-99.4	88.5	67.4-99.1
	VSS	262.9	33.3-770.0	12.7	2.4-27.5	16.5	4.2-33.0	92.7	72.8-99.3	92.8	74.2-98.9
Warm season	BOD_5	164.4	48.0-264.0	76.8	21.0-162.0	74.7	22.5-165.0	53.0	22.8-71.1	54.6	27.4-68.7
	COD	269.1	115.2-425.0	123.2	67.1-266.6	143.4	55.3-271.1	56.7	17.1-78.2	50.5	17.1-78.2
	TSS	262.2	125.0-480.0	26.1	12.5-25.0	41.7	7.5-142.5	88.0	75.6-97.7	84.5	64.0-98.4
	VSS	198.6	102.0-365.0	17.9	6.0-37.5	26.1	7.5-77.5	86.4	64.3-98.0	86.0	64.7-96.5

HSSF-Phr: units planted with *Phragmites australis*; HSSF-Sch: units planted with *Schoenoplectus californicus*; BOD_5: biological oxygen demand; COD: chemical oxygen demand; TSS: total suspended Solids; VSS: volatile suspended solids; n=15 for all parameters of warm season, and n=26 for all parameters of cold season; cold season: average of fall/winter; warm season: average of spring/summer

Table 2

Seasons	HSSF	SMA_m ($gCOD_{CH4}/gVSS \cdot d$)	Attached biomass ($g\ VSS/m^2$)	Potential methane Production ($mg\ CH_4/m^2 \cdot d$)	Number of copies (Nº of copies/mg VSS)	
					Bacteria	Archaea
Cold season	HSSF-Phr	0.02 ± 0.01	84.05 ± 36.07	1434 ± 283	5.57E+06	2.38E+04
	HSSF-Sch	0.01 ± 0.00	80.76 ± 69.65	1799 ± 447	5.80E+06	4.19E+04
Warm season	HSSF-Phr	0.07 ± 0.03	171.04 ± 72.88	1485 ± 86	4.81E+06	5.47E+04
	HSSF-Sch	0.06 ± 0.01	274.98 ± 63.13	3075 ± 1149	9.26E+06	8.26E+04

HSSF-Phr: units planted with *Phragmites australis*; HSSF-Sch: units planted with *Schoenoplectus californicus*; SMA_m; Specific methanogenic $activity_{maximum}$; COD: chemical oxygen demand; VSS: volatile suspended solids; cold season: average of fall/winter; warm season: average of spring/summer. n:3

Table 3

Sample	Sample source	Related clone	Id (%)	Isolation source	Taxonomic group	Closest culturable match	Accesion number
Bacteria							
1	Phr- cold season	*Uncultured bacterium clone*	97	Sewadge sludge	Firmicutes	*Clostridium sp.*	KF765651
2	Phr- cold season	*Uncultured bacterium clone GDIC2IK01D8TXV*	97	Methanogenic Enrichments	Bacteroidetes	*Bacteroidales bacterium*	JF600605
3	Phr- cold season	*Uncultured bacterium clone N1_3_1934*	96	Anaerobic sludge digester	Bacteroidetes	*Antarcticimonas flava*	JQ121396
4	Phr- cold season	*Uncultured bacterium clone RDX-5CFa3*	100	RDX contaminated groundwater	Firmicutes	*Clostridium sp.*	JX470466
5	Phr- cold season	*Uncultured bacterium clone A46*	100	Activated sludge	Firmicutes	*Clostridium sp.*	KP238600
6	Phr- cold season	*Uncultured bacterium clone KS-150*	98	Proglacial soil	Firmicutes	*Clostridium sp.*	EU809898
7	Phr- cold season	*Uncultured Nitrosomonadaceae bacterium*	91	Upland river	Proteobacteria	*Herbaspirillum chlorophenolicum*	AF540017
8	Phr- cold season	*Clostridium sp.*	99	TCE dechlorination consortium	Firmicutes	*Clostridium sp..*	AB596885
9	Sch-warm season	*Uncultured denitrifying clone BC84*	91	Biofilm anaerobic reactor	Proteobacteria	*Alcaligenes sp.*	JN125790
10	Sch- warm season	*Uncultured bacterium clone T1S106D04*	92	Freshwater lake	Proteobacteria	*β-proteobacteria Iso10-11*	KC624081
11	Sch- warm season	*Uncultured γ-proteobacterium clone HPC1173*	91	Marine sediment	Proteobacteria	*γ-Proteobacteria HPC1173*	EF503561
12	Sch- warm season	*Uncultured bacterium clone GDIC2IK01DPDLA*	99	Methanogenic enrichment	Bacteroidetes	*Bacteroides sp.*	JF674519

Phr: units planted with *Phragmites australis*; Sch: units planted with *Schoenoplectus californicus*; Id: identity; cold season: average of fall/winter; warm season: average of spring/summer.

Table 4

Sample	Sample source	Related clone	Id (%)	Isolation source	Taxonomic group	Closest culturable match	Accesion number
Archaea							
13	Phr- cold season	*Uncultured archaeon clone UAFB*	96	Anaerobic sludge digester	Methanosarcinales	*Methanosarcina Mazei*	KJ476545
14	Phr- cold season	*Uncultured archaeon clone B102P50*	100	Paddy soil	Methanosarcinales	*Methanosaeta concilii*	KP327890
15	Phr- cold season	*Uncultured Methanosarcinales*	100	Groundwater	Methanosarcinales	*Methanosaeta concilii*	LN796162
16	Phr- cold season	*Uncultured Methanosaeta Clone*	100	UASB granular sludge	Methanosarcinales	*Methanosaeta concilii*	KP343675
17	Phr- cold season	*Uncultured archaea*	80	Anaerobic farm reactor	Methanomassiliicoccales	*Methanomassiliicoccus intestinalis*	KT252415
18	Phr- cold season	*Uncultured archaea*	96	Rice paddy soil	Methanosarcinales	*Methanosaeta concilii*	AB650608
19	Phr- cold season	*Uncultured archaeon clone B102P50*	100	Paddy soil	Methanosarcinales	*Methanosaeta concilii*	KP327890
20	Phr- cold season	*Uncultured archaea clone SGA35G*	99	Anaerobic digester	Methanosarcinales	*Methanosaeta concilii*	GU389089
21	Phr- cold season	*Uncultured archaeon clone E126P700*	100	Paddy soil	Methanosarcinales	*Methanosarcina mazei*	KP328045
22	Phr- cold season	*Uncultured archaeon clone AR_44*	100	Rice paddy soil	Methanosarcinales	*Methanosaeta concilii*	KP327890
23	Sch- cold season	*Uncultured euryarchaeote clone S2C05344af*	100	Cold freshwater spring	Methanosarcinales	*Methanosaeta concilii*	KJ566501
24	Phr- cold season	*Uncultured archaeon clone REG3547*	100	Wetland soil	Methanosarcinales	*Methanosaeta concilii*	KJ645022

Phr: units planted with *Phragmites australis*; Sch: units planted with *Schoenoplectus californicus*; Id: identity; cold season: average of fall/winter; warm season: average of spring/summer.

Table 5