Wetland methane emissions altered by vegetation disturbance: an interaction between stem clipping and nutrient enrichment

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Abstract

Plant-mediated transport is the dominant means of methane release from vegetated wetlands. Whether aboveground plant disturbances affect the emission of methane is largely unknown. We tested the effects of stem clipping on methane emissions from freshwater wetland mesocosms vegetated with *Sagittaria lancifolia*, *Panicum hemitomon*, *Echinochloa walteri*, or *Eleocharis macrostachya* under different nutrient regimes. Mesocosms vegetated with *E. macrostachya* showed an effect of treatments on CH₄ emission which was elevated in high nutrient level treatment and suppressed in mid nutrient level treatment as compared to controls when the plant was clipped to 3 cm above water line. *S. lancifolia* and *P. hemitomon* showed a similar pattern, and *E. walteri* showed no response to treatments. Mean CH₄ emission significantly differed between species, being up to 56% higher in mesocosms vegetated with *P. hemitomon*. Treatments where plants were clipped below the water line had low initial emission rates, but these increased after three days. Sediment samples were analyzed for patterns in the microbial functional genes *mcrA* (methanogens) and *pmoA* (methanotrophs), but no changes in microbial communities in response to treatments were observed. Changes in methane emissions due to plant disturbances in wetlands warrant further investigation, but this study demonstrates that the available nutrient pool must also be considered. Additionally, initial responses to treatments were observed, but these responses tended to dissipate after three days, emphasizing the importance of monitoring CH₄ emissions over time rather than only observing initial responses. Our results suggest that nutrient enrichment alone can suppress CH₄ emission, however interactions with plant disturbances can be of equal importance.

Keywords: Methane, Plant Disturbance, Wetlands, Methanogens, Methanotrophs, DGGE

1. Introduction

Wetland ecosystems are the largest natural source of methane (CH₄) emissions, representing 20 – 40% of global emissions (Kirschke et al., 2013), even as wetlands constitute only 5-8 % of worldwide land area (Lehner and Döll, 2004). CH₄ concentrations in the atmosphere have exhibited an increasing trend since 1750 (Ciais et al., 2013), but there is a large amount of interannual variability in global emissions with an estimated 50 – 70% of the global interannual variability attributed to wetland CH₄ emissions (Bousquet et al., 2006; Chen and Prinn, 2006). Wetland vegetation is known to influence the production and consumption of CH₄ (Carmichael et al., 2014), modulating CH₄ fluxes to the atmosphere directly and indirectly (Schimel, 1995; Chanton et al., 2008). Given the influence of vegetation over CH₄ consumption, production, and emission, it is likely that disturbances to wetland vegetation can affect CH₄ processes (King et al., 1998; Ding et al., 2005; Schultz et al., 2011), and may play a role in the observed variability in wetland CH₄ emissions.

Transmission of sediment produced CH₄ through vegetation to the atmosphere is particularly important in wetlands; up to 90% of total CH₄ emissions from vegetated wetlands may result from emergent aquatic vegetative transport (Sebacher et al., 1985; Van der Nat and Middelburg, 1998; Bergstrom et al., 2007). Gas transport through wetland vegetation could potentially be altered by herbivores feeding on the stems of these plants (Bodelier et al., 2006; Dingemans et al., 2011; Petruzzella et al., 2015). The wetlands in southeastern Louisiana support many species of waterfowl and mammals (Nyman et al., 2013), but the effects of aboveground herbivory on the release of CH₄ from these wetlands are unknown. Extrapolations from previous herbivory or clipping studies provide little guidance due to the lack of studies utilizing plant species common to the region. Previous studies examining CH₄ emission in relation to herbivory

or stem clipping have also maintained constant nutrient conditions (Kelker and Chanton, 1997; Greenup et al., 2000; Ding et al., 2005), whereas it is quite common for southeastern Louisiana wetlands to vary widely in nutrient content due to surrounding land use, river diversion control structures, wastewater treatment, and management (Brantley et al., 2008, Hyfield et al., 2008). In this study, we examined the effects of plant stem clipping on CH₄ emission via three levels of clipping on four different aquatic plants common to freshwater marshes of southeastern Louisiana (*Sagittaria lancifolia*, *Panicum hemitomon*, *Echinochloa walteri*, and *Eleocharis macrostachya*) and under three levels of nutrient enrichment. Sediment samples from each treatment were analyzed for methanogen and methanotroph diversity using denaturing gradient gel electrophoresis (DGGE) of genes linked to methane cycling (*mcrA*, *pmoA*) to determine if treatments also led to structural changes in the methane-active microbial community. By measuring CH₄ emission and methane-related microbial diversity, we sought to determine if plant disturbances similar to herbivory altered plant mediated CH₄ emission, and whether any observed effects could be related to the community of methanogenic and methanotrophic microorganisms.

2. Methods

This experiment was conducted in wetland mesocosms (18 total) that were constructed from galvanized steel farm watering troughs (1.8 X 0.6 X 0.3 m, 280 L; Nasco, Fort Atkinson, WI), and were located outdoors on the Louisiana State University campus, Baton Rouge, LA. Each mesocosm was filled with high clay content fill dirt to approximately one-half of total depth. The upper sediment material consisted of surface sediment collected from a freshwater marsh at the confluence of Bayou Verret and Lake Cataouatche in southeastern Louisiana, and was added to a depth of approximately 20 cm. Each mesocosm was then subdivided into four 0.6

X 0.3 m sections using flat sheets of PVC plastic (Professional Plastics Inc., Fullerton, CA) pushed into the sediment to limit belowground competition between plants. Each subdivision within each trough was then vegetated with one of four plant species: *Sagittaria lancifolia*, *Panicum hemitomon*, *Eleocharis macrostachya*, or *Echinochloa walteri*, and two small remaining sections on each end of the trough were left unplanted. *S. lancifolia* was collected from the lower Atchafalaya delta (29°24'49.49 N, 91°19'37.45 W) and Bayou Verret, LA (29°52'32.54 N, 90°14'42.68 W). *P. hemitomon* was collected from a site just south of Raceland, LA, along Highway 182 West (29°40'37.76 N, 90°39'26.99 W). *E. macrostachya* was collected from the Farr Park Equestrian Center, Baton Rouge, LA (30°22'57.50 N, 91°12'39.35 W). All field-collected plants were transplanted into mesocosms on the day of collection. *E. walteri* was not collected, but was grown from seed outdoors at the Louisiana State University greenhouse facility, in a mixture of high clay fill dirt, potting soil, and vermiculite. Plants were randomly assigned to a subdivision within each trough and each subdivision received enough plant material to fill the area. Each trough was filled with water to the top of the trough and water levels were monitored daily.

The experimental design consisted of three levels of each of two treatments: nutrient enrichment (at zero, mid, and high nutrient addition), and clipping (at no clipping, clipping to 3 cm above the water line, and clipping to below the water line). Nutrient enrichment consisted of powdered 20-10-5 planting tablets (20% total N consisting of 4% ammoniacal N, 4.65% water soluble organic N, and 11.35% water insoluble N, 10% available phosphate, 5% soluble potash; Forestry Suppliers Inc., Jackson, MS) that were wrapped in a filter and enclosed in a 30 ml narrow mouthed sample bottle (Fisher Scientific Inc., Pittsburgh, PA), which contained 20 holes of approximately 0.15 cm diameter. Bottles were pushed into the sediment of each tank receiving

nutrient enrichment. This method of nutrient enrichment was used to promote slow release over the course of the experiment (Williams and Ruckelshaus, 1993; Silliman and Zieman, 2001). The high nutrient addition was set to 20 g N m^{-2} , just above the yearly loading rate recommended for rice fertilization in Louisiana (Saichuck et al., 2011). The mid-level nutrient treatment was set at half of this amount for a total concentration of 10 g N m^{-2} , which is near the N-levels in marshes receiving diverted Mississippi river water (Hyfield et al., 2008). For troughs receiving no nutrient enrichment, sand was used in place of the powdered planting tablet to control for any disturbance effects. Nutrient enrichment began six weeks before sampling. Clipping was carried out just prior to sampling and consisted of clipping all of the plants in a particular mesocosm down to either 3 cm above the water, or to below the water line. Controls were not clipped. Each combination of nutrient amendment (zero, mid, high) and clipping (no, clipped to 3 cm above the water, clipped below water line) was carried out in two replicate mesocosms with 4 differing plant species (4 x 3 x 3 x 2 design).

Methane emissions were measured immediately following clipping of the stem. Sampling took place over three separate sampling events, each lasting three days, with six mesocosms sampled during each event. For each event and after methane measurements, the clipped plant material was collected, dried (70°C , 48 h), and weighed to determine biomass. Plants clipped to 3 cm above the water were fully harvested to the top of the sediment after gas sampling on day 3, dried, and weighed. The roots of each plant within the confines of the base chamber were subsequently removed and washed clean of sediment and debris. Roots were then dried to determine belowground biomass. Sediment samples for mass measurements were weighed, dried (70°C , 48 h), and reweighed to determine moisture content (Carter and Gregorich, 2007). Dry sediment was then ashed (500°C , 2 h) and reweighed to determine organic matter content as ash

free dry mass (% C) (Carter and Gregorich, 2007). Other environmental variables measured included stem number, water temperature and depth, pH, and percent cover and dieback were assessed.

2.1. Methane Sampling

Methane sampling followed the common protocol of Lindau et al., (1991) for closed chamber measurements. Methane emissions were measured immediately after applying clipping treatments and again after three days, in order to detect responses other than the immediate effect of clipping. Clear Plexiglas chambers (30 X 30 X 30 cm), as described by Lindau et al. (1991), were installed into each subdivision of six randomly selected mesocosms three days prior to sampling, and this process was repeated 3 times, once per week for three weeks, until all 18 mesocosms were sampled. Chambers were installed to 10 cm of sediment depth. At the beginning of each sampling event, plants were clipped to the appropriate treatment level, and gas chamber tops added and sealed to the base units. A time-zero gas sample was taken and internal chamber temperature recorded. Each chamber top consisted of a sampling port (rubber septum), battery operated 12 v fan, pressure control tube, and a thermometer. The pressure control consisted of 1 m of plastic tubing (1.5 mm I.D.) that maintained equilibrium gas pressure between the outside and inside of chambers. Prior to sampling, Vacutainers were placed on a high-vacuum preparation line to remove any residual gases and then re-sealed with silicone rubber. For each headspace sample, 15 ml was removed from the flux chambers with a syringe and injected into a 10 ml evacuated gas Vacutainer. A slight over pressure of headspace gas was injected into each Vacutainer to prevent atmospheric contamination (Lindau et al., 1991). After collecting each sample, 100% silicone rubber sealant was spread over the puncture in the rubber septum. Headspace samples were collected via the rubber septum sampling port with a gas tight syringe

and stainless steel hypodermic needle. As well as the time zero gas sample, headspace gas samples were withdrawn after 1 h in order to calculate emission rates (IAEA, 1992). After the last sampling of a chamber, internal chamber temperature was recorded again, and the chamber tops removed. Water level inside chambers and headspace height was measured for each sampling. Prior to data collection, gas samples were collected from an unclipped plant at time 0, 15, 30, and 60 min to determine the linear rate of CH₄ increase and to set the duration of incubation time. All gas samples were analyzed on Shimadzu 14-A gas chromatograph (Shimadzu Scientific Instruments, Inc., Columbia, MD) fitted with a flame ionization detector within 24 h of collection and 1 ml of sample was injected for analysis.

2.2. Amplification and DGGE Analysis of Methane-Active Microorganisms

To investigate the methane-active sediment microbial community, sediment was collected from each mesocosm 1 day and 3 days after CH₄ sampling. Sediment was collected from a depth of 5-10 cm (staying within the rhizosphere to observe plant effects) with a metal scoop, transferred to 50 ml centrifuge tubes and returned to a lab freezer. Day 3 samples were primarily used for community analyses as the time between treatment and sampling would to allow for potential growth of microbial communities, however, a selection of day 1 samples were analyzed for comparison. DNA was extracted using a PowerSoil DNA kit (MoBio, San Diego, CA) following the manufacturers recommended protocol for wet soil samples. The presence of community DNA was confirmed by electrophoresis in agarose gels. PCR amplification of functional genes was performed in a Bio-Rad MyCycler (BioRad Inc., Hercules, CA) thermal cycler in 50 µL reaction volumes containing 2 µL of sample and 48 µL of a reaction mixture described by Jackson et al. (2001) and consisting of 0.4 mM each PCR primer, 0.2 mM of each dioxynucleotide triphosphate, 1.0 U of *Taq* polymerase, and a buffer consisting of 500 mM

KCl, 100 mM Tris-HCl, with 15 mM Mg²⁺. Two sets of primers were used to target the functional *mcrA* and *pmoA* genes of methanogens and methanotrophs, respectively (Table 1). For methanogens, a portion of the *mcrA* gene was amplified following the protocol of Luton et al. (2002), where denaturation, annealing, and extension were carried out at 95, 55, and 72 °C, respectively, along with the ramp in temperature between annealing and extension slowed to 0.1 °C s⁻¹ for the first five cycles of the 40-cycle program to allow for mismatched primer extension (Luton et al., 2002). A 40 bp GC clamp was added to the 5' end of the *mcrA* forward primer to allow for analysis of amplification products by DGGE (Muyzer et al., 1993). For methanotrophs, a portion of the *pmoA* gene was amplified following a modified protocol of Tuomivirta et al. (2009). The amplification consisted of an initial denaturation at 95 °C for 3 min, followed by 42 cycles of denaturation at 95 °C for 30 s, a touchdown annealing step from 66 °C to 55 °C for 45 s, and extension at 75 °C for 30 s, and ending with final extension at 72 °C for 10 min. As with the *mcrA* primers, a 40 bp GC clamp was added to the 5' of one *pmoA* primer to allow for DGGE analysis. Presence of amplification products of the correct size was confirmed by electrophoresis in agarose gels.

Samples showing positive amplification results for *mcrA* or *pmoA* gene fragments were analyzed using DGGE. For the *mcrA* gene, the amplified products were electrophoresed through a 20 to 40% urea-formamide gradient (Mosoni et al., 2011) in 8% acrylamide gels at 60 °C and 200 V for 5 h in a DGGE-2001 system (C.B.S. Scientific Co., Del Mar, CA). For the *pmoA* gene, a denaturing gradient of 35 to 70% was used, with electrophoresis running at 155 V for 9 h at 60 °C (conditions based on a modified protocol of Jaatinen et al., 2005). Following electrophoresis, gels were stained with SYBR Green (BioRad Inc., Hercules, CA) and banding patterns examined under ultraviolet transillumination.

2.3. Statistical Analysis

Data were tested to examine an effect of nutrient enrichment and/or clipping on CH₄ emissions. Statistical analysis of the split-plot design was performed in R version 2.15.2 (<http://www.r-project.org>) using the nlme package for linear and nonlinear mixed effects models. The experiment was analyzed with mesocosm as the whole-plot factor and plant species as the split-plot to examine an effect of treatments on CH₄ emission ($n = 145$). The assumption of normality was checked using plots of residuals versus predicted values and QQ plots of residuals, and tested via Shapiro-Wilks test. MANOVA was used to determine differences among species and troughs based on measured covariates and Pearson correlations were examined. DGGE banding patterns were analyzed based upon mobility, or movement of bands through the gel. Bands with relative intensities that accounted for >1% of the total intensity within a particular sample were regarded as positives, and the presence/absence of specific DGGE bands used to create binary data for each sample. Binary data was used to generate a Jaccard dissimilarity matrix between samples for both *mcrA* and *pmoA* genes, and these matrices used to ordinate samples through nonmetric multidimensional scaling (NMDS) to assess community similarity across different treatments.

3. Results

Total above and belowground biomass within each subdivision differed significantly ($p < 0.01$) among plant species and aboveground biomass was correlated with belowground biomass ($r = 0.71$) (Table 2). Mean sediment % C for all treatments was 14.84 ± 1.04 % C and was weakly correlated to percent moisture of the sediment ($r = 0.27$). Species were not significantly ($p > 0.05$) different in terms of % C or pH (Table 3). CH₄ emission was weakly

positively correlated to aboveground biomass ($r = 0.46$), stem height ($r = 0.32$), belowground biomass ($r = 0.27$), and percent cover ($r = 0.36$), and showed a weak negative correlation with pH ($r = -0.25$).

A significant ($p < 0.05$) three-way interaction between plant species, nutrient addition, and clipping level was detected via a mixed effects model. Mesocosms vegetated with *E. macrostachya* showed a significant ($p < 0.05$) difference in CH₄ emissions based on nutrient treatment level when clipped to 3 cm above the water on day 1, where the high nutrient treatment showed elevated emissions as compared to the treatment with no nutrient addition, and the mid-level nutrient treatment showed suppressed emissions rates as compared to the treatment with no nutrient addition (Figure 1). This same pattern is also seen in *S. lancifolia* on day 1 (Figure 1). For *P. hemitomon*, emissions did not show a difference between treatments with no nutrient enrichment and mid-level nutrient enrichment when clipped to 3 cm above the water (Figure 1). Also for *P. hemitomon*, on both day 1 and 3, treatments receiving no clipping and mid-level nutrient enrichment were significantly ($p < 0.05$) suppressed in terms of CH₄ emission as compared to the treatments of high level, and no nutrient addition (Figure 1). Mesocosms vegetated with *E. walteri* showed no response to treatments (Figure 1). Mean CH₄ emission significantly ($p < 0.05$) differed among plant species, being up to 56% higher from mesocosm subdivisions vegetated with *P. hemitomon* as compared to other species.

Time since clipping was also a factor affecting CH₄ emission where a significant interaction ($p < 0.05$) was detected with time since clipping and clipping level. When plants were not clipped, or clipped to 3 cm above the water, CH₄ emissions were not different between day 1 and day 3 (Figure 2). However, when plants were clipped below the water line, emission rates after 3 days were elevated significantly ($p < 0.05$) (Figure 2). Sediment samples from *E. walteri*

(representing no treatment effect), as well as *S. lancifolia* and *P. hemitomon* (representing a treatment effect) were analyzed for methanogen and methanotroph microbial community structure. A total of 39 distinct bands were detected in DGGE gels of the *pmoA* gene, but only three distinct bands for the *mcrA* gene. The mean ($\pm$ SE) number of *pmoA* bands present each sample were 9 ± 1.9 , 7 ± 1.1 , and 12 ± 1.7 for *S. lancifolia*, *P. hemitomon*, and *E. walteri* respectively, which did not vary significantly ($p > 0.05$) between treatments, plant species, or sampling day. NMDS ordinations of *pmoA* gene structure in each treatment showed no clear grouping of samples based on either treatment type or vegetation (Figure 3). However, there may have been a weak suggestion of high nutrient treatments tending to group more tightly than other treatment levels along the second axis (Figure 3). As bands for the *mcrA* gene showed little to no diversity, NMDS analysis was not performed for the *mcrA* gene.

4. Discussion

Our experiment supports the hypothesis that the interacting effects of plant disturbance and nutrient enrichment can affect plant mediated CH₄ emissions, but also shows that CH₄ emission responses are plant species specific, possibly related to species differences in lacunar gas exchange processes. It is likely that the stimulatory or inhibitory effects of nutrient addition on CH₄ emission seen in this study depend on the gas transport capacity of the specific plant species and their contributions to belowground substrate materials for microbial production or oxidation of CH₄. Previous experiments investigating CH₄ flux after plant clipping have yielded variable findings, where CH₄ emissions have been found to increase after clipping above the water level in *Carex lasiocarpa*, *Carex meyeriana*, and *Deyeuxia angustifolia* (Ding et al., 2005), *Carex aquatilis*, *Carex rostrata* (Kelker and Chanton, 1997), and *Eleocharis equisetoides* (Petruzzella et al., 2015), but to be unchanged for *Eriophorum vaginatum* (Greenup et al., 2000).

Experiments examining the effects of live herbivory on CH₄ emissions have shown both decreases and increases in CH₄ emission (Bodelier et al., 2006; Dingemans et al., 2011), potentially because of differences in foraging behavior. Animals that forage by rooting into the sediment could increase sediment O₂ concentrations which would select for alternative modes of respiration other than methanogenesis and support aerobic methanotrophic bacteria that oxidize CH₄. When animals graze on the shoots of emergent macrophytes above the water line, transport of CH₄ to the atmosphere could be altered by removal of the shoots and changes in the internal pressurization of the plant. When plants are grazed, or clipped below the water line, our results suggest that CH₄ emissions will increase over time, likely due to less O₂ transport to belowground organs by plants, as well as increasing reducing conditions due to belowground dieback of plant roots.

The different clipping treatments used in this study simulate different grazing approaches, and although our data are not directly comparable to previous studies because of the nutrient level interaction (nutrient level was not manipulated in other herbivory, or clipping experiments), similar responses were observed. Under high nutrient enrichment, clipping *S. lancifolia* to 3 cm above the water increased the emission of CH₄, the same outcome seen in other studies involving plant disturbances (e.g., Kelker and Chanton, 1997; Ding et al., 2005; Dingemans et al., 2011; Petruzzella et al., 2015). However, this was only an initial response observed in the day 1 samples, and after three days CH₄ emissions from the different *S. lancifolia* treatments were similar, indicating a temporary response. This finding emphasizes the importance of monitoring CH₄ emissions over a period of time rather than observing only initial responses.

Comparisons of CH₄ flux across differing studies can be difficult due to the inherent variability of CH₄ emission rates, often arising from large diel and seasonal fluctuations (Batjes

and Bridges, 1994) as well as the composition of the plant community (Ding et al., 2005; Kao-Kniffin et al., 2010; Schultz et al., 2011). Mean CH₄ emission rates from *S. lancifolia* over the course of the experiment were comparable to that observed by Alford et al. (1997) from the Mississippi River Deltaic Plain, but 39.5% lower than that observed in a Louisiana freshwater marsh dominated by *S. lancifolia* (Crozier and DeLaune, 1996). It is likely that we observed lower CH₄ concentrations than Crozier and DeLaune (1996) because they measured CH₄ from field collected soil cores in the laboratory, as oppose to *in situ* measurements made in mesocosms as described here. With regards to the other plant species tested, to our knowledge CH₄ emissions from monospecific or dominant stands of *P. hemitomon*, *E. walteri*, and *E. macrostachya* have not been previously reported.

The nutrient content of sediments and soils influences CH₄ production and consumption, although experimental effects of nutrient addition on CH₄ emissions vary with the type of nutrient enrichment, the quantity applied, and the method of application (Lindau, 1994). The effects of nutrients on CH₄ dynamics *in situ* are poorly understood, however the addition of compounds containing nitrate or sulfate should cause metabolic competition that is unfavorable to methanogens, thereby decreasing CH₄ production and emission (Jugsujinda et al., 1995; Conrad, 1996). Indeed, nitrogen addition has been shown to inhibit CH₄ production, and combined nitrogen and phosphorus to inhibit CH₄ production to an even larger extent than nitrogen addition alone due to increased substrate competition with denitrifying bacteria (Kim et al., 2015). Our results support a reduction in CH₄ emission only at the mid-level nutrient enrichment treatments, and only significantly in *P. hemitomon* when no clipping treatment was applied. When plants were clipped to 3 cm above the water, mid-level nutrient enrichment still led to a reduction in emissions, however treatments that received a high-level of nutrient

enrichment exhibited a significant increase in emissions from *E. macrostachya* and *S. lancifolia* on day 1. These results suggest that while nutrient enrichment alone can suppress CH₄ emissions, interactions with plant disturbances can alter CH₄ emission even further and warrants further study.

Nitrogen fertilizers have been shown to inhibit CH₄ oxidation (Crill et al., 1994; Alam and Jia, 2012) and to increase methanotrophic activity (Bodelier et al., 2000a, b; Kruger and Frenzel, 2003). In our study, DGGE of methanotrophic genes (*pmoA*) did not show any shifts in methanotroph community structure with our nutrient treatments, or in response to clipping, although there was a suggestion of increased *pmoA* diversity at higher nutrient levels for *S. lancifolia* and *P. hemitomon*. Both type I and II methanotrophs require ammonium (Bodelier et al., 2000a), and increased nitrogen availability may select for type I methane oxidizers (Hanson and Hanson, 1996; Bodelier, 2011), potentially explaining the weak suggestion of increased *pmoA* diversity at higher nutrient levels for sediments vegetated with *S. lancifolia* and *P. hemitomon*.

Changes in aboveground vegetation in response to nutrient enrichment likely influence herbivore grazing preferences, where wetland vegetation with a higher nutrient content may be preferred by herbivores (Ialeggio and Nyman, 2014). Our results would indicate that wetlands under a high nutrient load and with grazing pressures by herbivores may emit more CH₄ to the atmosphere than a wetland under moderate, or very little nutrient load with the same grazing pressure. Future studies could further elucidate the plausibility of this under field conditions, for example by utilizing wastewater treatment wetlands receiving high nutrient loads as field sites to compare to wetlands not receiving wastewater. Additionally, further research into modifications of methanotrophic and methanogenic communities due to aboveground disturbances to plants *in*

situ may reveal relationships missed with our artificial mesocosm study. Changes in CH₄ cycling due to nutrient enrichment is especially interesting as changes in the N-cycle can potentially lead to increased nitrification, and subsequently denitrification, a more thermodynamically favorable process than methanogenesis, potentially reducing CH₄ emission and increasing nitrous oxide (N₂O) emissions. Interactions between these possible alterations in N-cycling and plant disturbance warrant further investigation. Even as CH₄ responses to disturbance and nutrient addition are likely plant species specific, further study into the interaction between these variables and CH₄ emission could lead to changes in wetland management decisions in a changing climate where the goal is to reduce wetland CH₄ emissions.

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Figure Legends

Figure 1. Methane emission ($\text{CH}_4 \text{ kg ha}^{-1} \text{ d}^{-1}$, $\pm \text{SE}$) from wetland sediments in mesocosms vegetated with *Eleocharis macrostachya*, *Echinochloa walteri*, *Panicum hemitomon*, or *Sagittaria lancifolia* at three levels of nutrient enrichment (0, 10, 20 g N m^{-2}) and clipping treatments (no clipping, clipped to 3 cm above water line, clipped below water line). Emissions

were measured immediately following the herbivory treatment (day 1) and after three days (day 3) and values are means ($\pm$ SE). * denotes significant difference between day 1 and day 3 samples.

Figure 2. Methane emissions ($\text{CH}_4 \text{ kg ha}^{-1} \text{ d}^{-1}$, $\pm$ SE) from vegetated wetland sediments for a linear mixed effects model showing a significant ($p < 0.05$) interaction between time since clipping (immediate, day 3) and clipping level (no clipping, clipped to 3 cm above water line, clipped below water line). Open circles represent mean ($\pm$ SE) day 1 samplings and closed circles represent mean ($\pm$ SE) day 3 samplings.

Figure 3. NMDS plot for *pmoA* gene based on a binary data sheet consisting of presence/absence of DGGE bands for each mesocosm treatment vegetated with *S. lancifolia*, *P. hemitomom*, or *E. walteri* from day 3 samples. Triangles are *S. lancifolia*, circles are *P. hemitomom*, and squares are *E. walteri*. Black shapes are treatments that received no nutrient addition, gray shapes are treatments that received 10 g N m^{-2} , and white shapes are treatments that received 20 g N m^{-2} .

Primer	Gene	Sequence (5'-3')	Reference
<i>mcrA</i> f	<i>mcrA</i>	GGTGGTGTMGGATTCACACARTAYGC WACAGC	Luton et al., (2002); Mosoni et al., (2011)
<i>mcrA</i> r	<i>mcrA</i>	TTCATTGCRTAGTTWGGRTAGTT	Luton et al., (2002); Mosoni et al., (2011)
A189 f	<i>pmoA</i>	GGNGACTGGGACTTCTGG	Holmes et al., (1995); Tuomivirta et al., (2009)
A621 r	<i>pmoA</i>	CGCTCGACCATGCGGAT	Tuomivirta et al., (2009)
GC Clamp		CCCCCCCCCCCCCGCCCCCGCCCCCGCCCCCGCCGCCC	

Table 1. Properties of PCR primers used to amplify the *mcrA* and *pmoA* region in this study.

Species	Nutrient Treatment (g N m ⁻²)	Aboveground Biomass (g)	Belowground Biomass (g)
<i>E. macrostachya</i>	0	29.47 ±4.7	42.09 ±9.3
	10	40.74 ±3.2	42.54 ±5.7
	20	32.44 ±3.2	44.32 ±5.5
<i>E. walteri</i>	0	3.65 ±2.1	15.86 ±2.3
	10	3.56 ±1.1	20.78 ±6.0
	20	4.43 ±0.7	12.72 ±3.9
<i>P. hemitomon</i>	0	63.18 ±8.8	194.68 ±52.4
	10	61.57 ±7.2	154.03 ±20.4
	20	75.07 ±5.7	209.63 ±30.4
<i>S. lancifolia</i>	0	46.68 ±7.0	269.35 ±45.0
	10	59.56 ±8.5	252.93 ±27.2
	20	50.73 ±9.5	165.78 ±36.3

495

496 **Table 2.** Above and belowground biomass for *Eleocharis macrostachya*, *Echinochloa walteri*,
497 *Panicum hemitomon*, and *Sagittaria lancifolia* at three levels of nutrient enrichment (0, 10, 20 g
498 N m⁻²). Values are means ±SE (*n*=6 for each species at each nutrient treatment level).

Species	Sediment AFDM (g)	Water Temperature (°C)	pH	Water Depth (cm)	Stem Number	Cover (%)	Dieback (%)	Sediment Moisture (%)
<i>E. macrostachya</i>	14.59 ±2.67	31.67 ±0.84	7.80 ±0.11	7.22 ±0.47	221.94 ±23.69	27.50 ±2.56	6.39 ±2.81	66.57 ±2.89
<i>E. walteri</i>	17.99 ±1.59	32.0 ±0.90	7.93 ±0.13	6.11 ±0.43	24.50 ±2.96	7.72 ±1.01	86.11 ±1.88	75.41 ±1.98
<i>P. hemitomon</i>	14.95 ±2.57	31.44 ±0.73	7.76 ±0.12	6.14 ±0.37	91.06 ±9.53	39.47 ±4.08	0.94 ±0.33	71.62 ±1.40
<i>S. lancifolia</i>	11.84 ±0.92	31.61 ±0.85	7.79 ±0.13	5.25 ±0.36	34.50 ±7.60	26.11 ±1.64	4.33 ±1.06	69.81 ±1.97

500

501 **Table 3.** Environmental characteristics for mesocosms vegetated with *Eleocharis macrostachya*, *Echinochloa walteri*, *Panicum*
502 *hemitomon*, or *Sagittaria lancifolia*. Values are means ±SE (*n*=18 for each species).

Figure 2

Figure 3