1 **Accumulation of soil carbon drives denitrification potential and**
2 **lab-incubated gas production along a chronosequence of salt marsh**
3 **development**

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Abstract: We measured sediment organic carbon and nitrogen accumulation, and rates of denitrification enzyme activity and greenhouse gas (CO₂, CH₄, N₂O) production from slurries of sediments of a mudflat that formed in 2002, a young (8-year-old) natural *Spartina alterniflora* salt marsh that developed on part of the mudflat, and four mature (> 200 years old) salt marshes in southeastern Georgia to examine microbial processes related to carbon (C) and nitrogen (N) cycling during succession from mudflat to mature marsh. Soil organic C and N and C: N ratio (0-30 cm) increased across the chronosequence from mudflat (791 ± 35 g C/m², 125 ± 17 g N/m²) to young marsh (2520 ± 131g C/m², 190 ± 10 g N/m²) to mature marshes (5827 ± 250 g C/m², 372 ± 20 g N/m²). After 8 years of colonization by *S. alterniflora*, sediment organic carbon increased 3.2 times, and nitrogen increased 1.5 times relative to the mudflat. The high rate of organic C and N accumulation based on time series measurements (188 g C/m²/yr, 7.8 g N/m²/yr) and feldspar marker layers (359 g C/m²/yr, 26.2 g N/m²/yr) was attributed to high accretion (3 cm/yr) in this low elevation (0.18 m NAVD88) emerging marsh. Carbon dioxide production increased with increasing sediment organic C from mudflat to mature marshes. Un-amended denitrification enzyme activity, measured in slurry incubation, ranged from an average of 0.020 ± 0.005 µg g⁻¹ hr⁻¹ in the mature marshes to 0.094 ± 0.03 µg g⁻¹ hr⁻¹ in the young marsh. We also measured denitrification potential in slurry incubations amended with C (glucose), N (nitrate), and C+N to assess the potential for substrate limitations. Denitrification potential in the mudflat did not show strong nutrient limitation. In the young marsh, denitrification potential was C-limited, and in the mature marsh, it was co-limited by C and N. In July samples, CO₂ production showed a statistically significant increase with age from the mudflat to the mature marshes. However, in both months, CO₂ production efficiency (expressed on a per g C basis) was significantly higher in the mudflat sediment slurries than in the young marsh and mature marsh samples. *Spartina* colonization of mudflats and the subsequent accumulation of organic matter are key to

enriching sediment organic C and N pools that control microbial heterotrophy, particularly denitrification and CO₂ production, that play an important role in marsh C and N cycling.

Keywords: denitrification potential, greenhouse gas production, soil carbon, organic matter accumulation, salt marsh, ecosystem development, USA, Georgia, Sapelo Island

Introduction

Salt marshes are the predominant intertidal habitats along temperate zone coastlines. These wetlands play an important role in sustaining biodiversity, providing habitat for finfish, shellfish, and water birds, sequestering carbon, and purifying water (Zedler and Kercher, 2005). Young ecosystems generally are characterized by relatively simple community structure and open nutrient cycles, whereas mature ecosystems are characterized by much greater complexity and more efficient internal nutrient cycles (Odum, 1969; Childers et al., 1993). Although there is a wealth of information regarding the development of community structure and nutrient cycling of created and restored salt marshes, there is limited data on ecosystem development of natural salt marshes. However, there have been some observations of newly formed natural marshes, including newly formed *Phragmites australis* salt marshes in the Yellow River Delta (Gao et al., 2012, Bai et al., 2012, and Ye et al., 2014), an emerging Mississippi River delta marsh (Henry and Twilley, 2013), young barrier island *Spartina alterniflora* marshes in Virginia (Tyler and Zieman, 1999, Tyler et al., 2003), and a previous study of young *Spartina alterniflora* marshes in coastal Georgia (Krull and Craft, 2009). Developmental trajectories in a created young marsh may not necessarily reflect those in a naturally developed young marsh due to inherent differences in hydrology, parent material, nutrient and sediment supply, and other key drivers of ecosystem development.

During their initial stages of development, tidal marshes and other young ecosystems are characterized by small pools of organic matter and nitrogen (Odum, 1969; Krull and Craft, 2009, Henry and Twilley, 2013) that may limit the development of microbial processes that

drive carbon (C) and nitrogen (N) cycling. These processes are important for supporting heterotrophic food webs and contributing to water quality improvement via denitrification (Day et al., 1989, Groffman et al., 2006). However, young ecosystems, including tidal marshes, exhibit higher rates of net primary production than mature ecosystems (Craft et al., 1999). Even though they contain less sediment organic C and N than mature marshes, young natural marshes accumulate C and N at rates 5 to 7 times greater than accumulation rates in mature marshes (Krull and Craft, 2009; Henry and Twilley, 2013).

Soil microbial processes are critical to the development and maintenance of C and N biogeochemical cycles, as well as important ecosystem functions associated with these cycles, such as denitrification and C sequestration. Yet, little is known about how quickly these functions change or what limits their development during succession. Few studies have investigated how denitrification potential and microbial production of CO₂, CH₄, and N₂O (greenhouse gases) change during salt marsh ecosystem development. Coastal ecosystems are often N-limited, and denitrification is one of the pathways by which reactive nitrogen is removed from the water column. Denitrification requires labile C to transform NO₃⁻ to N₂, and studies of created salt marshes indicate that denitrification is lower in created marshes compared to natural marshes due to low carbon availability (Broome and Craft, 2009). N₂O, a greenhouse gas with 265 times the global warming potential of CO₂ (Myhre et al., 2013), is a product of incomplete denitrification. The production of N₂O has been observed to increase with available C substrate (Bergstrom et al., 1994; Weier et al., 1993), with the C: N ratio of organic material (Huang et al., 2004), and with available nitrate (Moseman-Valtierra et al., 2011). Although the mechanism is not well understood, these studies suggest there could be substantial changes in denitrification rates and N₂O production as ecosystems age and accumulate C.

Due to concerns about global climate change, there is growing emphasis on the

regulation of greenhouse gas (GHG) production. Wetlands can be sources or sinks of greenhouse gases such as CO₂, CH₄, and N₂O (Moseman-Valtierra et al., 2011). Since carbon is a key driver of CO₂ and CH₄ production from sediments, production of these greenhouse gases could be expected to change as C accumulates in marsh sediments. Studies of created marshes show that overall soil organic matter mineralization increases with age as soil organic C accumulates (Cornell et al., 2007). Similarly, additions of nitrate can increase N₂O production from a tidal salt marsh, enough to change it from a sink to a source of N₂O (Moseman-Valtierra et al., 2011). These results suggest that fluxes of GHGs from young wetlands can be expected to increase over time as organic matter and nutrients accumulate.

To provide a better understanding of the development of microbially-mediated soil biogeochemical processes in newly developed salt marsh ecosystems, we measured denitrification potential in sediment slurries along a chronosequence from a mudflat to a young (8-year-old) salt marsh to mature (>200 years old) marshes. We used data which Krull and Craft (2009) collected at the young marsh in 2006, hereafter referred to as the 3-year-old marsh, to provide an additional time series measurement. We hypothesize that sediment organic matter limits microbial processes, thus as a mudflat develops to a marsh and accumulates sediment organic C and N, denitrification should increase. We directly assessed carbon and nitrogen limitation of denitrification potential by amending sediments with NO₃⁻ and labile C (glucose). We also measured greenhouse gas production (CO₂, CH₄, N₂O) in additional sediment slurries from the same sites, with a similar hypothesis that as organic matter accumulates, greenhouse gas production will increase. We also measured rates of sediment organic C and N accumulation using time series measurements of sediment C standing stocks (0-30 cm) and feldspar marker layers.

Methods

Site Description

A mudflat formed at the mouth of the Altamaha River in 2002 and was partially colonized by *Spartina alterniflora* in 2003 (Krull and Craft, 2009). Krull and Craft (2009) sampled the sediment of the unvegetated mudflat and the portion of the mudflat now colonized by the young (3-year-old) marsh in 2006. Since then, the island has continued to recruit vegetation

Figure 1. Location of sampling site in Sapelo Island, Georgia. Mudflat and young marsh: GCE9; mature marshes: GCE3, GCE6, GCE9 and GCE10.

and build large expanses of mudflat and marsh. We sampled the mudflat, the young (8-year-old) marsh and four mature marshes at Sapelo Island, Georgia in 2011. The four mature marshes are part of the Georgia Coastal Ecosystems Long Term Ecological Project (GCE3, GCE6, GCE9 and GCE10; Figure 1). Based on historical data, the mature marshes are greater than 200 years old. All marshes are dominated by *S. alterniflora* and experience tidal inundation of 2.3 m (see Craft 2007 for a detailed site description). The salinity of tidal flood water at the mudflat, young marsh, and mature marsh (GCE9; Figure 1) near the mouth of the Altamaha River ranged from 15 to 20, while the salinity of the other mature salt marshes (GCE 3, 6, and 10) further from the Altamaha River was higher, 32-37, during the study period (2011).

Sediment Carbon and Nitrogen Accumulation

To estimate sediment C and N pools and accumulation during ecosystem development, five cores of young (8-year-old) marsh sediment (8.5 cm diameter by 30 cm deep) were randomly collected in May of 2011 and divided into 0-10 and 10-30 cm depths, dried at 70°C, weighed, ground, and sieved through a 2 mm mesh screen (Craft et al., 2003). For consistency across years, the field sampling methods used were the same as those used by Krull and Craft (2009). Bulk density was calculated from the dry weight per unit volume for each depth increment. Organic C and N were measured using a Perkin-Elmer 2400 CHN analyzer (Perkin-Elmer, Norwalk, Connecticut). Subsamples were tested for carbonates by adding one drop of dilute (0.1 mol L⁻¹) HCl and observing whether effervescence occurred. Samples containing carbonates were treated with 0.1 mol L⁻¹ HCl prior to CHN analysis. Bulk density and C and N concentrations were weighted by depth (1x for 0-10 cm, 2x for 10-30 cm) and mean values were calculated. Nutrient pools were calculated from the mean values as follows:

Nutrient Pool (g m⁻²) = bulk density (g cm⁻³) · C, N(g/g) · 10,000 cm² m⁻² · 30 cm depth
(Krull and Craft, 2009).

Accumulation rates of organic C and N of the young marsh were calculated as:

Accumulation Rate = C, N (g/m^2 , 2011, this study) – C, N (g/m^2 , 2006, Krull and Craft, 2009) / 5 years, where Krull and Craft (2009) measured C and N in the same marsh five years earlier (2006), using the same sampling and analytical protocols.

We also measured organic C and N accumulation of the young (8-year-old) marsh using the bulk soils data (bulk density, percent organic C) and feldspar marker layers placed on the sediment surface of the young marsh. Three feldspar marker layers (0.25 m^2) were placed in the young marsh on March 14, 2011. Plots were sampled three times, May 20, July 26, and December 19, 2011 by taking three small (3 cm diameter) cores in each plot. On each sampling date, accretion rates were determined by measuring the thickness (mm) of fresh sediment atop the marker layer. Accretion data were normalized and expressed on an annual basis. Organic C and N accumulation were calculated using the feldspar marker data, sediment bulk density, and percent organic C and N in the 0-10 cm sampling depth.

Sediment Denitrification Potential

An additional five replicate sediment cores (8.5 cm diameter by 10 cm deep) each were randomly collected from the mudflat, young (8-year-old) marsh, and mature marshes in May and July in 2011. Surface water from each site was collected at the same time for use in the incubations. Sediment and water samples were placed on ice and transported to the laboratory at Indiana University Bloomington for analysis.

We measured denitrification potential in sediment slurry incubations using the chloramphenicol-amended acetylene inhibition technique (Wall et al., 2005). Field moist sediment was homogenized and sieved through a 2-mm mesh, and 25 grams of sediment were placed into 125 mL Wheaton bottles fitted with gray butyl septa. Sediments were treated with 0.2 mM chloramphenicol to inhibit new enzyme production. To each incubation we added 50

184 mL site water containing 0.2 mM chloramphenicol (to inhibit *de novo* enzyme production) and
185 amended with either glucose (18 mM), potassium nitrate (12 mM NO₃⁻), both glucose and
186 potassium nitrate, or no amendment (un-amended). These concentrations of glucose and
187 potassium nitrate were used because previous work indicated that they were sufficient to
188 eliminate C and N limitations (Marton et al., 2012). The nitrate concentrations used were an
189 order of magnitude higher than the nitrate concentrations typically found in water of the
190 Altamaha River (5-40 µM). Incubations were maintained at 25°C (room temperature) in shaded
191 conditions. Five samples, one from each core, were incubated for each treatment (un-amended,
192 C, N, C+N). Incubations under light conditions may favor some uptake of NO₃⁻ by
193 photosynthetic organisms such as benthic algae. However, this is not likely given the short
194 duration (1 hour), low ambient light level, and ample nitrate (12 mM) added to our incubations.

195 The headspace of the incubation bottles were flushed with ultra-high purity N₂, then
196 injected with 5 mL acetylene (C₂H₂) to block the conversion of nitrous oxide to N₂. At 10
197 minute intervals over a one hour period (n=6), we shook the bottles to equilibrate gas between
198 the water and headspace and drew a 5 mL sample with a gas-tight syringe, which was injected
199 into pre-evacuated 2 mL glass vials equipped with gray butyl septa (see Marton et al., 2012).
200 Samples were analyzed for N₂O on a gas chromatograph equipped with a ⁶³Ni electron capture
201 detector (SRI Instruments, Torrance, CA, USA).

202 N₂O accumulated at each sampling time was corrected for dilution caused by multiple
203 sample collections and the concentration was regressed against time. The denitrification rate
204 was calculated using the slopes of the regressions. Only regressions based on at least four
205 (usually six) samples with an r² of 0.80 or higher were used. Rates were expressed on a dry
206 weight basis after drying a 5 g field moist subsample to a constant weight at 70°C.

Greenhouse Gas Production

For measurement of greenhouse gas (GHG) production rates, 25 g of field moist sediment with the roots removed, collected using the coring method described above, were placed into 125 mL bottles as described above. Fifty mL site water was added to the incubation bottles and they were flushed with ultra-high purity N₂. Incubations were maintained at 25°C and a 5 mL gas sample was drawn every half hour over 6 hours as described above. Five mL of ultra-high purity N₂ was added to each bottle after each sampling period to maintain constant pressure.

Samples were analyzed for N₂O as described above. CH₄ was analyzed using a flame ionization detector and CO₂ was analyzed using a flame ionization detector after passing through a methanizer (SRI Instruments, Torrance, CA, USA). GHG production rates were calculated as described above for N₂O. We calculated areal rates of greenhouse gas production by multiplying our soil mass-based measurements (expressed as $\mu\text{g g}^{-1} \text{hr}^{-1}$) by bulk density (g/cm^3) and sample depth (10 cm) to express our measurements as $\mu\text{g m}^{-2} \text{hr}^{-1}$. Since heterotrophic microbial activity is driven by carbon availability, we calculated greenhouse gas production efficiency rates for CO₂, N₂O, and CH₄ by expressing our results on a per g organic carbon basis.

Statistical Analysis

We used data from Krull and Craft (2009) (the mudflat, 3-year-old marsh, and mature [200-year-old] marshes) and this study (8-year-old marsh) to construct a two-way analysis of variance (ANOVA) based on age (0-mudflat, 3 years, 8 years, and mature) and depth (0-10, 10-30 cm) to test for differences in bulk soil properties (bulk density, percent C and N) and C and N pools. We used data published by Krull and Craft (2009) for the unvegetated mudflat, young (3-year-old marsh), and mature marshes to compare with our newly collected data from the now 8-year-old marsh.

As there was a significant interaction between site age and depth, one-way ANOVAs were run for the 0-10 cm and 10-30 cm depths. Means were compared using the Ryan-Einot-Gabriel-Welsch multiple range test (SAS, 2011). All tests of significance were conducted at $\alpha = 0.05$.

We used a three-way ANOVA (SPSS Statistics, IBM Corp., 2013) for denitrification potential, with age (mudflat, young marsh, and mature marsh), sample month (May and July), and amendment type (un-amended, N-amended, C-amended, and C+N-amended) as fixed factors. Sample month was not significant ($p=0.513$), so we subsequently used a two-way ANOVA with age and amendment type as fixed factors. We also used one-way ANOVAs to test for differences among amendment types within a given age. Means were compared using Tukey's honestly significant difference test (IBM Corp., 2013).

We constructed a two-way ANOVA for each greenhouse gas (CO_2 , CH_4 , and N_2O) with sample month (May and July) and age (mudflat, young marsh, and mature marsh) as fixed factors. As there was a significant interaction between sample month and age, one-way ANOVAs to compare GHG emission by age were run for May and July data separately. Means were compared using Tukey's honestly significant difference test (IBM Corp., 2013). Welch's robust test of equality of means and the Games-Howell post hoc test were used when Levene's test of homogeneity of variances was significant ($p < 0.10$). A two-sample t-test was also run to compare average GHG production by sample month.

To investigate the effects of sediment organic carbon (SOC) on GHG production and denitrification potential, we ran two-way correlations between the average SOC and each GHG and the un-amended potential denitrification rate for each site (mudflat, young marsh, and mature marshes at sites GCE3, GCE6, and GCE9) in IBM SPSS Statistics (IBM Corp., 2013). We report Spearman's ρ and the p value for each correlation.

Results

Sediment Carbon and Nitrogen Accumulation

Marsh age and sampling depth had significant effects on tidal marsh sediment properties. Bulk density decreased with marsh age and, after 8 years, it was comparable to mature marshes (Table 1). Sediment organic C and N increased with marsh age. Percent organic C and N were greater in the 8-year-old marsh than in the 3-year-old marsh but less than in mature marshes, especially in the 10-30 cm depth (Table 1). Sediment C:N ratios also increased with marsh age, and the C:N ratio of the 8-year-old marsh did not differ from that of mature marshes at either the 0-10 cm or the 10-30 cm depth (Table 1).

Sediment organic C pools in the 8-year-old marsh averaged 936 g/m² in the 0-10 cm depth and 1584 g/m² in the 10-30 cm depth (Table 1). Surface sediment C and N pools did not differ from measurements made 5 years earlier. However, subsurface C pools more than doubled, from 774 g/m² to 1584 g/m², during that time period, and subsurface N pools increased by 34%, from 87 g/m² to 117 g/m². Still, C and N pools in the 8-year-old marsh were considerably less than pools measured in mature marshes (Table 1). Rates of C and N accumulation (0-30 cm) based on differences in C and N pools measured after 3 and 8 years were 188 g C/m²/yr and 7.8 g N/m²/yr, with most of the accumulation (162 g C/m²/yr, 6 g N/m²/yr) occurring in the 10-30 cm depth.

Measurements of sediment accretion atop feldspar marker layers yielded accretion rates of 3.5 ± 0.4 cm/yr. Based on this rate and the mean bulk density and percent organic C in the surface (0-10 cm) depth, the young marsh accumulated 359 g C/m²/yr and 26.2 g N/m²/yr (Table 2). These values are more than double the rate of C accumulation estimated by comparing sediment C pools in 2011 with measurements made five years earlier in 2006. For N accumulation, the differences were even greater, 26 g N/m²/yr based on feldspar marker layer versus 6 g N/m²/yr based on the change (increase) in sediment N pools.

Table 1. Bulk density, percent organic C, total N, and organic C and N pools in mudflat, 3-year-old marsh, 8-year-old marsh, and mature marshes (>200 years old). Means within a given depth (0-10, 10-30 cm) with the same letter are not significantly different (p=0.05) according to the Ryan-Einot-Gabriel-Welsch multiple range test. Mudflat (n=5 cores), 3-year-old marsh (n=5) and mature marshes (n=5) data are from Krull and Craft (2009).

	Bulk density (g/cm ³)	Organic C (%)	Nitrogen (%)	C:N ratio (mol)	C Pool (g/m ²)	N Pool (g/m ²)
0-10 cm:						
Mudflat:	1.21±0.02 a	0.20±0.02 a	0.03±0.01 a	9±2.2 a	244± 21 a	36± 5 a
3-year-old marsh:	0.58±0.03 b	1.41±0.11 a	0.11±0.01 b	15±0.4 b	807± 58 b	64± 5 b
8-year-old marsh:	0.25±0.03 c	4.10±0.40 b	0.30±0.02 c	15±0.6 b	936± 45 b	73± 4 b
Mature marshes:	0.35±0.03 d	5.97±1.19 c	0.40±0.07 c	18±1.6 b	1918±238 c	128±12 c
10-30 cm:						
Mudflat:	1.16±0.02 a	0.24±0.02 a	0.04±0.01 a	7±0.3 a	547± 48 a	89± 5 a
3-year-old marsh:	0.86±0.03 b	0.46±0.06 a	0.05±0.01 a	10±0.5 b	774±100 a	87± 5 a
8-year-old marsh:	0.42±0.03 c	1.90±0.20 b	0.14±0.01 b	16±0.3 c	1584±109 b	117± 4 b
Mature marshes:	0.38±0.04 c	5.36±0.49 c	0.33±0.03 c	19±1.8 c	3909±212 c	244±13 c

Table 2. Sediment accretion based on feldspar marker layers (n=3) and organic C and N accumulation in the 8-year-old marsh. C and N accumulation were calculated using the mean bulk density (0.25 g/cm³), and organic C (4.1%) and nitrogen (0.30%) contents in the 0-10 cm depth of the 8-year-old marsh (see Table 1).

	Accretion Rate (mm/yr)	Organic C (g/m ² /yr)	Nitrogen (g/m ² /yr)
Plot: A	32	328	24
B	43	441	32.2
C	30	307	22.5
Mean:	35±4	359±42	26.2±3.0

Sediment Denitrification Potential

Differences in denitrification potential were apparent among sediment slurries from the mudflat and the young and mature marshes. Un-amended denitrification potential was significantly greater in the young marsh ($0.094 \pm 0.03 \mu\text{g g}^{-1}\text{hr}^{-1}$) than in the mudflat ($0.052 \pm 0.02 \mu\text{g g}^{-1}\text{hr}^{-1}$) and the mature marsh ($0.020 \pm 0.005 \mu\text{g g}^{-1}\text{hr}^{-1}$; Figure 2). Denitrification potential was not significantly different between sampling months (data not shown). The addition of C and C+N promoted denitrification potential of young marsh and mature marsh sediment slurries, while N additions did not significantly increase denitrification potential compared to un-amended sediment slurries for any marsh age (Figure 2). In the young marsh, additions of C and C+N significantly increased denitrification potential compared to un-amended sediment slurries, and potential denitrification was not significantly different between the C and C+N treatments (Figure 2). In sediment slurries from the mature marshes, C additions significantly increased denitrification potential compared to un-amended sediment slurries, but the C+N treatment resulted in significantly higher denitrification potential than C alone (Figure 2). The denitrification potential of mudflat sediment slurries was not significantly increased by any of the amendments.

When all treatments were pooled, the young marsh sediment slurries had significantly higher denitrification potential than either the mudflat or mature marsh sediment slurries (Figure 2). Denitrification potential in mudflat and mature marsh sediment slurries were not significantly different from one another.

Greenhouse Gas Production

CO₂ production by the mudflat and marsh sediments was generally 3-4 orders of magnitude greater than N₂O and CH₄ production (Figure 3). Production of CO₂ from incubations exhibited fairly consistent trends across months. In May, CO₂ production was

significantly greater in mature marshes than in the young marsh and mudflat ($p<0.0005$; Figure 3A). In July, CO_2 production was greatest in the mature marshes, intermediate in the young marsh, and lowest in the mudflat ($p<0.0005$; Figure 3B). Average CO_2 production across all age groups was significantly greater in May (60.79 ± 7.96) than in July (13.66 ± 1.59 ; $p<0.0005$).

Figure 2. Denitrification potential in mudflat, young marsh, and mature marsh sediment slurries. The lowercase letters represent the differences between un-amended, carbon-amended, nitrogen-amended, and nitrogen + carbon-amended denitrification potential within the same marsh age, while uppercase letters represent the differences between age groups (all treatments averaged). Means labeled by the same letters are not significantly different according to Tukey's honestly significant difference test ($p>0.05$).

Figure 3. GHG production in mudflat, young marsh, and mature marsh sediment slurries. A May, B July. Means labeled by the same letters are not significantly different according to Tukey's honestly significant difference test ($p > 0.05$).

Trends in CH₄ and N₂O were less evident. There was no significant difference in CH₄ production among sites in either May or July (Figure 3). N₂O production differed by age in May, when the young marsh produced significantly more N₂O than the mature marshes ($p = 0.049$; Figure 3A). However, in July, N₂O production was not significantly different

between the mudflat, young marsh, and mature marsh samples. Average N_2O production across age groups was not significantly different between May and July. Greenhouse gas (CO_2 , CH_4 , and N_2O) production efficiency rates expressed per gram carbon were significantly greater in the mudflat than in the young marsh and mature marshes ($p < 0.05$) (Figure 4), and generally were higher in May than in July.

Figure 4. GHG production efficiency (per g C) in mudflat, young marsh, and mature marsh sediment slurries. A May, B July. Means labeled by the same letters are not significantly

different according to Tukey's honestly significant difference test ($p>0.05$).

CO₂ production from the mudflat and both ages of marsh sediments was positively correlated with sediment organic C in July (Spearman's $\rho=0.900$, $p=0.019$), but not in May. CH₄ production in May was also positively correlated with sediment organic C (Spearman's $\rho=0.900$, $p=0.019$), while N₂O production and un-amended denitrification were not significantly correlated with sediment organic C in either month ($p>0.05$).

Discussion

Sediment Carbon and Nitrogen Accumulation

Sediment organic C, organic N, and C: N ratios increased with the age of the salt marsh. The trend was consistent with many studies of natural and restored salt marshes (Tyler and Zieman, 1999, Craft et al., 2003; Krull and Craft, 2009; Henry and Twilley, 2013). After 3 years of colonization, the young *Spartina alterniflora* marsh (1581 g C/m², 0-30 cm) contained double the sediment organic carbon present in the mudflat (791 g C/m²). After 8 years, sediment C density increased to 2520 g/m² (0-30 cm), an increase of nearly 60% during the 5-year period.

Sediment organic nitrogen increased less than carbon. After 3 years, the marsh contained 1.2 times (151 g/m², 0-30 cm) the N measured in the mudflat (125 g N/m², 0-30 cm), or an additional 26 g/m². Between three and eight years, sediment N exhibited a 26% increase, to 190 g/m². Surface (0-10 cm) sediment C: N ratios (15) did not differ between the 3-year-old marsh and the 8-year-old marsh. However, in subsurface (10-30 cm) sediments, it increased from 10 to 16, indicating preferential enrichment of C relative to N during the 5-year period. Decomposition of litter and roots could contribute to the preferential enrichment of C over N. Zhao and colleagues (2015) showed that over a 200-day period, up to 70% of the original nitrogen in *Phragmites australis* litter was lost, while only 40% or less of the carbon was lost.

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376 If most of the C and N lost from the leaf litter does not remain in the sediment, the increased
377 C: N ratio of the leaf litter would explain why sediment C: N ratio increased over time.

378 Nitrogen enrichment of the sediment also is likely linked to greater N fixation in young
379 marshes. In Virginia, Tyler et al. (2003) reported rates of N fixation in a 7-year-old marsh (18
380 g/m²/yr) that were 2-3 times greater compared to a 15-year-old (9 g/m²/yr) and a 150-year-old
381 natural marsh (6 g/m²/yr). The authors suggested that N fixation could be an important source
382 of N for primary producers during marsh ecosystem development.

383 Measurement of organic C and N accumulation in the young marsh using feldspar
384 marker layers yielded rates (369 g C/m²/yr, 26 g N/m²/yr) that were more than double those
385 of the time series measurements described above. Sediment accretion atop marker layers in
386 the young marsh was more than 3 cm/yr, well above accretion on feldspar plots placed in
387 mature marshes (0.2-0.3 cm/yr) during the same period (C. Craft, unpublished data). The high
388 rate of accretion in the young marsh is attributed to its low position in the tidal frame (0.18 m
389 NAVD88) relative to the mature marshes (0.72 to 0.87 NAVD88).

390 Our two methods of measuring C and N accumulation likely bracket lower and upper
391 estimates of accumulation. The time series approach represents the low-end estimate since it
392 does not account for the high rate of sediment accretion in the young marsh. On the other
393 hand, accumulation measured using feldspar marker layers represents the high-end estimate
394 since marker layers tend to over-estimate accretion as compared to longer-term measurements
395 such as ¹³⁷Cs and ²¹⁰Pb (Craft, 2007).

396 Henry and Twilley (2013) compared sediment organic matter, N, and P along a
397 chronosequence (10, 23, and 35 years) of tidal freshwater wetlands of the Wax Lake delta,
398 Louisiana. Sediment organic matter, N, and P concentration (0-4 cm) increased with wetland
399 age at rates much greater than observed in our study. For example, organic matter increased
400 from 2.8% at the 10-year-old site to 18% at the 35-year-old site, with the intermediate-aged

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401 site containing about 10% organic matter. In contrast to our study, where we observed an
402 increase in sediment C: N ratios along the chronosequence, the C: N ratios in Wax Lake delta
403 sediments did not vary along the chronosequence. The higher rate of organic matter
404 enrichment measured by Henry and Twilley (2013) can be attributed to a combination of
405 factors, including (1) the shallow depth of sampling (0-4 cm), where sediment organic matter
406 preferentially accumulates initially, (2) inclusion of root and rhizomes in the sample, and (3)
407 the predominantly freshwater inputs to the Wax Lake delta. Our Georgia study sites are
408 brackish to saline with the mature marshes having about 12% organic matter and 0.40% N at
409 a depth of 0-10 cm, as compared to 18% organic matter and 0.56% N in sediments of the Wax
410 Lake delta.

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411 When compared with constructed marshes in Virginia and North Carolina (Craft et al.,
412 2003; Krull and Craft, 2009), the young marsh in this study contained nearly twice the C and
413 N pools of constructed marshes of similar age at both 3 and 8 years. After 8 years, our marsh
414 contained comparable carbon and nitrogen pools to those of 26- to 28-year-old constructed
415 *Spartina alterniflora* marshes in North Carolina with similar tidal range but higher salinity
416 (Craft et al., 2003; Krull and Craft, 2009). Though these results could indicate that naturally
417 formed young salt marshes develop at a faster pace than constructed marshes of the same age,
418 it is more likely that geomorphic and climatological factors (Moreno-Mateos et al., 2012),
419 especially freshwater inputs from the Altamaha River, drive the observed differences between
420 young, constructed marshes in North Carolina and young, naturally-formed marshes in
421 Georgia. It is well known that constructed and restored marshes are not equivalent to mature
422 natural marshes that often are >200 years old. Our data suggest that further comparison
423 between natural and restored marshes of similar age, geographic location, and geomorphic
424 position (riverine versus estuarine marshes) could improve our understanding of how
425 ecosystem development of restored systems is different from that of natural systems.

Sediment Denitrification Potential

Denitrification potential was significantly greater in sediment slurries from the young marsh than in those from the mudflat and mature marshes (Figure 2). We hypothesize that the greater abundance of live roots and labile organic C in young marsh sediments support the higher denitrification potential in these habitats versus the mature marshes. Craft et al. (2003) reported that, even though young (constructed) *Spartina* marshes contain less sediment organic C than mature natural marshes, the quality of accumulating organic matter was higher based on lower lignin: N ratios in macro-organic matter. Similarly, the mudflat samples contained less sediment organic C than mature marshes, but the C produced by the resident benthic microalgae is likely of higher quality due to its high N content and lack of lignin (Giblin et al., 1997). These results point to a need for understanding the quality as well as quantity of C in wetland sediments as a controlling factor for the development of microbial functions like denitrification.

Un-amended denitrification potential in sediment slurries from the mature marshes in this study ($0.020 \pm 0.005 \mu\text{g g}^{-1} \text{hr}^{-1}$) was similar to measurements of un-amended denitrification potential reported by Craft et al. (2009) in mature marshes ($0.021 \mu\text{g N}_2\text{O g}^{-1} \text{sediment hr}^{-1}$) of the Altamaha River using the same methods. Un-amended denitrification potential in sediment slurries from the mudflat ($0.052 \pm 0.017 \mu\text{g g}^{-1} \text{hr}^{-1}$) and young marsh ($0.094 \pm 0.027 \mu\text{g g}^{-1} \text{hr}^{-1}$) were of a similar magnitude, but were higher than the rate observed by Craft et al. (2009).

Addition of N did not significantly increase denitrification potential of sediment slurries from mudflat, young marsh, or mature marsh sites (Figure 2). This result was unexpected, given nitrate's importance in denitrification (Woodward et al., 2009). Denitrification potential in the +N incubations was likely limited by low availability of labile C (Woodward et al., 2009, Fellows et al., 2011). In addition, salt marshes tend to lose equal amounts of nitrate to

N₂ and NH₄, so it is likely that a significant portion of the added nitrate in the +N treatment was converted to ammonium (Joye and Hollibaugh, 1995, Giblin et al., 2010).

Additions of C and C+N increased the denitrification potential of sediment slurries from the young and mature marshes, and C+N additions significantly increased denitrification potential compared to C additions alone in the mature marsh sediment slurries. These results suggest that the young marsh is C-limited, while the mature marsh is co-limited by C and N. Fellows et al. (2011) also found C-limitations, rather than N-limitations, for denitrification potential in slurries of floodplain soils. In sediment slurries from the mudflat, potential denitrification exhibited no increase when C, N, and C+N were added. Denitrification potential in the mudflat sediment slurries may have been limited by a lack of sufficient enzymes due to the low *in situ* concentrations of organic C and N (Table 1).

Anaerobic estuarine mudflat and marsh sediments are often low in nitrate (NO₃; Koike and Sorensen, 1988). However, the lack of response of young and mature marsh sediment slurries to nitrate additions and the strong response to labile C additions (glucose) suggests that the availability of labile C (rather than NO₃⁻) limits denitrification potential in these sediments. Studies have shown that labile carbohydrates can limit denitrification even if NO₃⁻ is present (Thomas et al., 2012). In marsh sediments, C exists as relatively recalcitrant compounds (Craft et al., 2003). This recalcitrant C mineralizes slowly under the anaerobic conditions present in marsh sediments (Gale and Gilmour, 1988), which explains why labile C additions in this study significantly increased denitrification potential in sediment slurries from young and mature marshes compared to un-amended sediment slurries (Figure 2).

Salinity and nitrogen availability can both affect denitrification potential. Microbial reduction of seawater sulfate to sulfides inhibits the activity of the reductase enzymes involved in the final steps of denitrification (Joye and Hollibaugh, 1995, Joye and Anderson, 2008), resulting in a decrease in denitrification as salinity increases along estuarine salinity gradients

(Rysgaard et al., 1999, Giblin et al., 2010). Salinity can also affect denitrification by inhibiting nitrification, the conversion of ammonia to nitrate, which is coupled with denitrification in low nitrate environments. Mudflat and marsh sites (young marsh, mature marsh at site 9) on the Altamaha River are exposed to higher nitrate-N concentrations (0.1-18 μ M) than the high salinity mature marshes (sites 3, 6, 10; 0.1-1.3 μ M), but concentrations overall are low. Because nitrification decreases with salinity (Rysgaard et al., 1999, Noe et al., 2013), we might expect low salinity sites to exhibit higher denitrification potential than the high salinity mature marshes. However, we observed no difference in denitrification potential between the low salinity mature marsh (site 9) and the high salinity marshes (sites 3, 6, 10). These findings further suggest that differences in potential denitrification among sites are driven by C and not N availability, and that salinity is not a major driver of denitrification potential in these systems.

In this study, sediment pH (measured using a 1:1 ratio of field moist sediment and distilled water) was 8.0 in the mudflat, 7.4 in the young marsh, and 7.1-7.6 in the mature marshes. Though we did see differences in pH among study sites, studies of denitrification across a range of soil pH (3-10) suggest that factors other than acidity, such as O₂ availability, nitrogen loading, and organic carbon, control denitrification potential (Waring and Gilliam, 1983, Groffman, 1994, Bai et al., 2014), indicating that pH was not a major factor determining denitrification potential in this study.

Acetylene-inhibited laboratory incubations in which no nutrients are provided may underestimate field denitrification rates, as nitrification is also inhibited and may cause artificial nitrate limitations (Seitzinger et al., 1993, Wall et al., 2005). However, Bernot et al. (2003) found the acetylene-inhibited, chloramphenicol-amended method to be comparable to other methods for estimating denitrification when incubated for short time periods (i.e., less than 6 hours). In addition, Groffman and Tiedje (1989) found denitrification potential to be a

good predictor of annual denitrification N loss. Despite the limitations of laboratory incubations, denitrification potential provides valuable insight into differences in denitrification rates between systems (Wall et al., 2005).

Greenhouse Gas Production

We observed increasing production of CO₂ from the mudflat to the young marsh and mature marshes (Figure 3). We speculate that the increase in CO₂ production during ecosystem development can be attributed to the observed increase in sediment organic C (Table 1) that serves as an energy source for heterotrophic microorganisms (Burden et al., 2013). When expressed on an area basis, the production of CO₂ ranged from 9.6 g m⁻² d⁻¹ in the young marsh to 41.3 g m⁻² d⁻¹ in the mature marsh. Howarth (1984) reported chamber-based fluxes of CO₂ between 9 g m⁻² d⁻¹ and 20 g m⁻² d⁻¹ in salt marshes of the eastern seaboard of the United States. Magenheimer et al. (1996) documented that mean sediment CO₂ flux using chambers in the Bay of Fundy (New Brunswick, Canada) ranged from 0.3 g m⁻² d⁻¹ to 3.7 g m⁻² d⁻¹, and was greatest in the middle marsh compared to upland edge and low salt marshes. The higher rate of CO₂ production measured in our study may be attributable to artifacts associated with bottle incubations of sediment slurries (Robertson et al., 1999), which limit scaling from bottle to the field.

Trends in N₂O production with ecosystem development were less evident. In May, N₂O production was greater in the young marsh than in the mature marsh while, in July, there was no difference among sites (Figure 3). Cheng et al. (2007) reported that the *in situ* flux of N₂O ranged from 0.1 to 0.4 mg m⁻² d⁻¹ in *Spartina alterniflora* salt marsh in the Yangtze River estuary. The production of N₂O in this study was 1-2 orders of magnitude higher, ranging from 4.1 mg m⁻² d⁻¹ to 12.0 mg m⁻² d⁻¹ from the mature marshes to the mudflat, again likely reflecting the difference between measurements using slurry incubations versus *in situ*

measurements.

Methane production was very low and there was no trend in CH₄ production with ecosystem development in either month (Figure 3). It is well known that the presence of sulfate in seawater suppresses methanogenesis by providing substrate for a more efficient metabolic pathway, sulfate reduction, which produces CO₂ as an end product of organic matter mineralization (Poffenbarger et al., 2011). In our study, the production of methane ranged from 0.2 mg m⁻² d⁻¹ in the young marsh to 0.5 mg m⁻² d⁻¹ in the mature marsh (0.055 and 0.166 g m⁻² yr⁻¹, respectively). Our production rates from laboratory incubations of sediment slurries were lower than even the minimum reported ecosystem flux of 0.2 g m⁻² yr⁻¹ reported for 10 polyhaline marshes (salinities >18) in a review by Poffenbarger et al. (2011), and the 3.8 to 27 mg m⁻² d⁻¹ in *Spartina alterniflora* salt marsh in the Yangtze River estuary reported by Cheng et al. (2007). Our observation of lower methane production in comparison to the cited ecosystem flux measurements may be attributed to the limitations imposed by laboratory incubations of sediment slurries. Labile carbon limitation is also possible in incubated sediments as there is no introduction of new carbon from photosynthetic organisms, and methanogens utilize only a small subset of carbon forms which can become rapidly depleted in incubations.

Carbon quality is also important for the control of metabolic pathways. Although total CO₂ and N₂O production were lower in the mudflat sediments than in young and mature marsh sediments, when expressed on a per gram of carbon basis, production efficiency of these GHGs was significantly greater in mudflat than in marsh sediments (Figure 4). We attribute greater greenhouse gas production efficiencies to the greater proportion of labile C from sources such as algae in the mudflat, as opposed to more recalcitrant macrophyte biomass in mature marshes (Craft et al., 2003).

In conclusion, sediment C and N accumulate along a chronosequence from mudflat to

marsh, reaching 43% of C and 51% of N pools of mature marshes in only 8 years of ecosystem development. CO₂ production from sediment slurries increased along the gradient of ecosystem development from mudflat to mature marsh and was positively related to sediment organic C (Spearman's $\rho=0.900$) across all sites in July, but not in May. Production efficiency of greenhouse gases on a per gram sediment carbon basis decreased with site age (Figure 4) suggesting that, while sediment C increases with time, organic matter quality decreases. Methane and N₂O production and denitrification did not show strong trends with age. Carbon and N limitations of denitrification potential also varied with age, displaying evidence of initial carbon limitation in young marshes with low sediment C and switching to co-limitation by C and N as the ecosystems age and recalcitrant carbon accumulates. Our results indicate that young marshes and mature marshes function differently with respect to C and N cycling. Carbon sequestration is rapid at first and declines with age. Carbon pools increase with time, leading to greater heterotrophic CO₂ production and alleviating C limitation of microbial processes such as denitrification.

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