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Solid-phase organic matter reduction regulates anaerobic decomposition in bog soil

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Abstract. Peatlands store globally significant amounts of carbon and are important sources of the greenhouse gas methane (CH₄) to the atmosphere. However, for reasons which are not well understood, many peatland soils produce smaller amounts of CH₄ than theoretically predicted, and carbon dioxide (CO₂) produced during anaerobic decomposition in peatland soils cannot be accounted for by commonly measured microbial processes. Here we show that the reduction of solid-phase organic matter (i.e., humic substances) suppresses CH₄ production in a bog soil and can be responsible for 33–61% of the total carbon mineralization in this soil. These results demonstrate that the reduction of organic matter is a key component of anaerobic decomposition in peatlands, and is at least partially responsible for their low CH₄ production. Thus, organic matter reduction may be a key regulator of how peatlands respond to ongoing global change.

Key words: bog; humic substances; methane; organic matter reduction; peatlands; wetlands.

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INTRODUCTION

Peatland ecosystems store approximately one-third of the terrestrial soil carbon and release globally significant amounts of the greenhouse gas methane (CH₄) to the atmosphere (Jobbágy and Jackson 2000, Bridgham et al. 2006, Denman et al. 2007). Over geologic time, CH₄ flux from peatlands has been an important component of global climate forcing (Loulouergue et al. 2011), and it is crucial to understand the mechanistic controls of peatland carbon cycling to predict how these ecosystems will respond to and affect ongoing global change (Bridgham et al. 1995, Limpens et al. 2008). Like other wetlands, peatlands are characterized by flooded or saturated soils where oxygen is not readily available as a terminal electron acceptor (TEA) for microbial decomposition. Under these anaerobic conditions, decomposition is mediated by a complex suite of microbial processes (Megonigal et al. 2004). Initially, complex organic molecules are degraded through a series of successive fermentation reactions that ultimately generate low molecular weight alcohols, fatty acids (including acetate), and dihydrogen (H₂). These fermentation products serve as substrates for anaerobic respiration using a variety of alternative TEAs in place of oxygen to mineralize organic carbon to carbon dioxide (CO₂). Available inorganic TEAs are generally consumed sequentially based on thermodynamic favorability in the following order: NO₃⁻ (denitrification), Mn(IV, III) (manganese reduction), Fe(III) (iron reduction) and SO₄²⁻ (sulfate reduction). Only after these competitively superior TEAs have been consumed is CH₄ produced through the splitting of acetate and/or the reduction of CO₂ by H₂ in acetoclastic
and hydrogenotrophic methanogenesis, respectively (Megonigal et al. 2004).

The ratio of the gaseous end products of anaerobic decomposition—CO₂:CH₄—is useful for exploring the relative importance of CO₂-producing TEA processes in comparison to methanogenesis. This ratio also has great significance for global change given that the global warming potential of CH₄ is 25-times that of CO₂ (Forster et al. 2007). Under purely methanogenic conditions, i.e., when more competitively favorable TEAs have been exhausted, a CO₂:CH₄ ratio of 1:1 is predicted (Conrad 1999). The use of more favorable TEAs in anaerobic decomposition generates CO₂ while competitively suppressing CH₄ production, resulting in higher CO₂:CH₄ ratios. Many wetland soils yield high CO₂:CH₄ ratios during anaerobic incubations (van Hulzen et al. 1999), and this pattern is particularly pronounced in many peat soils despite low availability of inorganic TEAs (Updegraff et al. 1995, Bridgham et al. 1998, Blodau 2002). Efforts to account for the excess CO₂ (i.e., the CO₂ above the 1:1 CO₂:CH₄ ratio) in peatlands frequently reveal that this CO₂ cannot be explained by measured rates of known inorganic TEA reduction processes (Keller and Bridgham 2007).

One hypothesized mechanism for this unexplained CO₂ is the use of organic matter as a TEA. In this process, organic matter serves as both the electron donor and the electron acceptor for microbial respiration. The use of organic TEAs was first reported by Lovley et al. (1996) who demonstrated that microbes were capable of respiring humic acids and humic analogues in pure culture. It is worth noting that recent research has challenged the existence of humic substances as discrete, chemically complex molecules in soils, suggesting instead that humics are “supramolecules” composed of small, identifiable biopolymers held together by hydrophobic interactions and hydrogen bonds (Sutton and Sposito 2005, Kelleher and Simpson 2006). While the dissolved humic pool is relatively small, the much larger pool of solid-phase, soil-associated wetland organic matter has also been shown to serve as a TEA (Roden et al. 2010), and humic acids extracted from many wetland soils increased the CO₂:CH₄ ratio in anaerobic incubations (Keller et al. 2009). While a role for organic matter reduction in wetland carbon cycling has gained support, it is unknown to what extent anaerobic decomposition and CH₄ production are affected by this microbial process.

We hypothesized that electron transfer to solid-phase organic matter explains the excess production of CO₂ over CH₄ during anaerobic incubations of soil from an ombrotrophic (not connected to surface and ground water) bog characterized by a high CO₂:CH₄ production ratio. We tested this hypothesis by comparing the number of electrons transferred from the soil to a ferric iron solution (i.e., the electron shutting capacity (ESC)) (Roden et al. 2010) as well as CO₂ and CH₄ production in soils which were either chemically reduced (CR) to exhaust the available humic TEA pool or biologically reduced (BR) by the active microbial community over a 6-week anaerobic laboratory incubation.

**MATERIALS AND METHODS**

**Soil collection and processing**

Soil was collected on 15 August 2009 from an ombrotrophic bog in the Upper Peninsula of Michigan (46°6.100 N, 88°16.417 W). The pH of the surface soil at the site is ~3.7. Typical of other bogs in the region, the vegetation is dominated by >90% cover by Sphagnum spp. mosses with stunted ericaceous shrubs including leatherleaf
(Chamaedaphne calyculata (L) Moench), small cranberry (Vaccinium oxycoccos L.), and bog Labrador tea (Rhododendron groenlandicum Oeder) as well as scattered low-stature black spruce (Picea mariana (Mill.) Britton, Sterns & Poggen) (Ye et al. 2012). Five soil cores were collected from below the water table, which averaged 34 cm below the soil surface at the time of sampling, and were frozen prior to being shipped to Chapman University.

We removed large roots and woody material from the peat using forceps in the ambient atmosphere, and 80 g of root-free, field-moist soil from each of the 5 samples was composited (400 g total) and homogenized in a food processor. Homogenized peat was spread as a thin layer on a tray and allowed to oxidize for 24 h at 4°C. Oxidized peat was re-homogenized in the food processor and 4.0 g (± 0.2 g) aliquots were added to 160 mL serum bottles. Slurries were created by adding 20 mL of de-ionized water. Percent moisture was measured on additional subsamples of peat by drying to a constant mass at 60°C.

Soil incubation

Half of the slurries were chemically reduced (CR) by adding 10 palladium-coated alumina pellets, capping with grey butyl septa, evacuating the headspace for 1 min, and replacing the headspace with 100 mL of 99.99% GC grade H₂ (Roden et al. 2010). The remaining biologically reduced (BR) slurries were handled similarly except that 10 3-mm glass beads were added instead of palladium pellets. Slurries were shaken in the dark at 150 rpm for 6 days. After 2 days on the shaker, the pH of each slurry was measured in an anaerobic chamber (Coy Laboratory Products, Grass Lake, Michigan, USA) filled with <2% of H₂ and a balance of N₂. Previous research has demonstrated that manipulation of humic chemistry influences pH (Keller et al. 2009), which is known to be an important control of peatland carbon mineralization (Ye et al. 2012). To remove this potential confounding effect, the pH of all CR samples was adjusted with 0.5 M HCl to match the pH in the BR soils on day 2 of the chemical reduction phase. After re-capping, all slurries were evacuated for 1 min and 100 mL of H₂ was added to the serum bottles which were returned to the shaker table. At the end of the 6-day chemical reduction phase, the pH of the CR soils was adjusted a second time in the anaerobic chamber as described above. Slurry pH did not differ between treatments at the start of the incubation, but was allowed to change over the course of the experiment (Appendix: Fig. A1). Following the pH adjustment after the 6-day chemical reduction phase, all palladium and glass pellets were removed from the slurries in the anaerobic chamber. All serum bottles were re-capped and the headspace was flushed with N₂ for 15 min to ensure anaerobic conditions. Slurries were incubated in the dark at 20°C for 6 weeks.

CO₂ and CH₄ production

On days 2, 7, 14, 21, 28, 35 and 42, CO₂ and CH₄ were measured in the headspace of 4 slurries from each treatment using a gas chromatograph equipped with a flame ionization detector and an in-line methanizer (SRI 8610C, SRI Instruments, Torrance, CA). Total CO₂ and CH₄ production were calculated after correcting for solubility and pH (Eaton et al. 1995, Drever 1997) and were expressed per gram dry weight of peat. The headspace of the remaining slurries were flushed with N₂ for 15 minutes on days 7, 14, 21, 28, 35, and 42 and rates of CO₂ and CH₄ production were calculated as the accumulation of gases between sampling points.

Electron shuttling capacity

Samples used to measure CO₂ and CH₄ production on each sampling date were sacrificed to measure the electron shuttling capacity (ESC) of dissolved and solid-phase organic matter (Roden et al. 2010). In the anaerobic chamber, the soil slurries were transferred to 50-mL centrifuge tubes which were capped and centrifuged at 4100 rpm for 5 min. The supernatant was decanted and filtered through a pre-leached Whatman GF/F filter. We added 0.25 mL of the filtrate to 1.0 mL of 5 mM ferric iron complexed with nitriloacetic acid (Fe(III)-NTA) and vortexed for 1 min. A 0.25 mL subsample was added to 2 mL of buffered ferrozine solution (0.1% ferrozine in HEPES buffer, pH = 7.0) and the absorbance at 562 nm (Lovley and Phillips 1986) was immediately measured to quantify Fe(II) produced as a result of electron shuttling.
from dissolved organic matter to Fe(III)-NTA. ESC of dissolved humics (μmol e⁻ equivalent mL⁻¹) was calculated as the amount of Fe(II) produced after correcting for Fe(II) produced by a deionized water blank.

Solid-phase ESC was determined by adding 15 mL of 5 mM Fe(III)-NTA to the soil sample remaining after decanting the supernatant. The centrifuge tubes were briefly shaken to re-suspend the soil and centrifuged for a second time at 4100 rpm for 5 min. A 0.25 mL subsample of the resulting supernatant was added to 2 mL of buffered ferrozine solution and the absorbance at 562 nm was immediately measured to quantify Fe(II) produced as a result of electron shuttling from the soil slurry to Fe(III)-NTA. The remaining supernatant was decanted and the mass of dry soil that reacted with Fe(III)-NTA was calculated by drying the soil to a constant mass at 60°C. ESC of solid-phase organic matter (μmol e⁻ equivalent gdw⁻¹) was calculated as the amount of Fe(II) produced by this reaction. The resulting value was corrected for Fe(II) produced by porewater present in the reaction with Fe(III)-NTA based on the percent moisture of the soil sample used in the extraction.

Statistical analyses
Linear regressions were performed using PASW Statistics 18 (SPSS 2009).

RESULTS
At the start of the incubation, the ESC of the CR soil was 26.85 ± 0.32 μmol e⁻ equivalents gdw⁻¹ (mean ± SE) compared to 1.48 ± 0.14 μmol e⁻ equivalents gdw⁻¹ in the BR soil (Fig. 1A). This demonstrates that our chemical reduction protocol effectively reduced this soil and that 94% of the redox-active organic matter pool was available for microbial reduction in the BR soil. Thus, comparing CR and BR soils during the subsequent anaerobic incubation (see below) allowed us to isolate the effect of oxidized redox-active moieties in the soil organic matter on organic matter mineralization. In both treatments, the majority of ESC was explained by solid-phase rather than dissolved organic matter (Appendix: Fig. A2). Over the course of the incubation, ESC of the BR soil increased, presumably as a result of the transfer of electrons from anaerobic microbial respiration to solid-phase organic matter serving as a TEA, reaching levels comparable to the CR soils after 4 weeks (Fig. 1A).

In the same samples used to measure ESC, rates of CH₄ production were suppressed in the BR soil compared to the CR soil through week 3 of the incubation (Fig. 1B). Following the complete reduction of the solid-phase organic matter in the BR soil (as indicated by the increased ESC, Fig. 1A), rates of CH₄ production increased, and surpassed rates in the CR soil, which produced CH₄ over the course of the entire incubation (Fig. 1B). During the initial 4 weeks of the incubation, CO₂ production was higher in the BR soil than in the CR soil, but rates
Fig. 1. (A) Solid-phase electron shuttling capacity (ESC), (B) CH$_4$ production, and (C) CO$_2$ production over a 6-week anaerobic incubation at 20°C. Bog soil used in this incubation was either biologically reduced (BR, open symbols) or chemically reduced (CR, closed symbols). Error bars represent SEM (N = 4).

Rates of CO$_2$ production in both treatments decreased over the course of the experiment (Fig. 1C) as is common in anaerobic incubations of peatland soils where labile carbon available for microbial decomposition becomes limiting through time (Updegraff et al. 1995).
DISCUSSION

The microbial reduction of solid-phase organic matter is an important carbon mineralization pathway in this bog soil. ESC in the BR treatment increased over the course of this incubation (Fig. 1A), demonstrating that the active microbial community was using solid-phase organic matter as a TEA (Roden et al. 2010). During the first 3 weeks of this experiment, when there was evidence of organic matter reduction, there was no measurable CH4 production in the BR treatment (Fig. 1B). We attribute this suppression to the thermodynamic favorability of humic reduction compared to methanogenesis, as has been shown for AQDS (Cervantes et al. 2000) as well as other humic acids and model quinones (Aeschbacher et al. 2011). Excess CO2 produced by the BR treatment over the same time period was likely generated as a respiratory end product of microbial reduction of solid-phase TEAs, including humic substances. This hypothesis is supported by a strong relationship ($P = 0.003; r^2 = 0.91$) between the additional CO2 at each time point during the incubation (i.e., BR CO2 - CR CO2) and the change in ESC in the BR soil over the same time periods (Fig. 2).

It is worth noting that inorganic TEA dynamics likely also differed between our CR and BR treatments; however, there are multiple lines of evidence to suggest that our results are best explained by the reduction of solid-phase organic TEAs, and not inorganic TEA reduction. The availability of dissolved inorganic TEAs (e.g., SO4$^{2-}$ and NO3$^{-}$) in the BR treatment compared to the CR treatment likely contributed minimal amounts of the excess CO2 production observed in the BR soil (Fig. 1C). While we do not have direct measurements of inorganic TEAs in this experiment, previous work with soil collected from the same peatland during the same sampling effort showed that SO4$^{2-}$ concentrations were <20 μM and NO3$^{-}$ was not detected after 2 days of an anaerobic incubation (“Bog 1” in Ye et al. [2012]). This is consistent with other work quantifying pathways of anaerobic carbon mineralization in peatland soils that reveals low availability of these inorganic TEAs (e.g., Keller and Bridgham 2007). Our data do not allow us to determine if the reduction of Fe(III) was done directly by iron reducing microbes or indirectly through electron shuttling from reduced humic substances (Lovley et al. 1996, Roden et al. 2010). While iron reduction could have directly contributed to CO2 production in the BR soil, this excess CO2 would have been limited to the initial stages of the incubation when Fe(III) was available.

Further, our solid-phase ESC measurements are based on Fe(II) produced following short-term exposure to an Fe(III)-NTA solution and thus likely do not capture solid-phase iron dynamics. There are 2 mechanisms by which the microbial reduction of solid-phase Fe(III) could have contributed to our measurements of solid-phase ESC. First, reduced Fe(II) could have been measured directly in our assay. We feel that
this possibility is unlikely due to the non-acidic nature of the Fe(III)-NTA solution (pH = 7.7) used in our ESC assay which was unlikely to effectively extract solid-phase iron from our soil. Second, reduced soil-associated Fe(II) could have transferred electrons to the Fe(III)-NTA solution. The transfer of electrons between different iron molecules is conceptually possible given the range of iron chemistry in soil systems, but we are unaware of similar explanations in peatland soils. Thus, it seems most likely that the excess CO₂ production as well as the ESC measured in Fig. 2 are the result of the reduction of solid-phase organic TEAs and not the reduction of solid-phase Fe(III).

Assuming that excess CO₂ produced in the BR soil compared to CR soil represents humic-derived CO₂, we can calculate, for the first time in any ecosystem, the percentage of total anaerobic carbon mineralization explained by microbial reduction of organic matter (i.e., humic-derived CO₂/(total CO₂ + CH₄)). This percentage was 33, 61, and 49% during the first, second and third weeks of the incubation, respectively. This humic-derived CO₂ could result directly from the reduction of humic substances by microbes or indirectly from sulfate reduction driven by the abiotic reoxidation of reduced sulfur species by oxidized humics (Heitmann et al. 2007). The anaerobic oxidation of CH₄ using humics as a terminal electron acceptor is also conceptually possible, although there is currently limited empirical evidence of this process (Smemo and Yavitt 2011). Regardless of the specific mechanism, these results suggest that solid-phase organic matter reduction can be the dominant microbial process in peatlands and may explain a large fraction of previously ‘unexplained’ CO₂ produced during anaerobic incubations (Keller and Bridgham 2007). The role of this process in situ, and in particular the possibility to re-oxidize reduced humics below the water-table level, remain largely unexplored.

We have demonstrated that solid-phase organic matter reduction is important; however, this process cannot fully explain the high CO₂:CH₄ ratios seen in these soils. The CO₂:CH₄ ratios in the CR soil ranged from 75:1 to above 300:1 over the course of the incubation, and ratios in the BR soil after week 3 ranged from 28:1 to 70:1. In both cases, this excess CO₂ did not result from the reduction of TEAs which were not available in these soils. The high CO₂:CH₄ ratios may be due to CO₂ produced directly by fermentation (Vile et
al. 2003b, Galand et al. 2010). Presumably, CO₂ produced by fermentation would coincide with an accumulation of fermentation intermediates (e.g., acetate) as has been observed in other peatland systems (Hines et al. 2008). Ye et al. (2012) demonstrated that acetate did accumulate in soil collected from the same site (“Bog 1” in Ye et al. [2012]) when incubated at near in situ pH and concluded that fermentation dominated anaerobic decomposition at this site. Future work investigating the importance of fermentation and acetate dynamics in peatland soils is necessary to further explore these dynamics in the context of anaerobic carbon mineralization.

It is also possible that an inhibition of CH₄ production that cannot be explained by competitive suppression by more favorable TEAs was occurring in these soils. There is growing evidence, for example, for a direct toxic effect of humic substances on microbial processes (Cervantes et al. 2000), with methanogens potentially being particularly sensitive to this effect in bog soils. For example, the addition of humic-rich dissolved organic matter to a bog soil reduced methanogenesis and sulfate reduction but not the accumulation of acetate (Minderlein and Blodau 2010). We have also found that additions of the humic analog AQDS more strongly inhibit CH₄ production than CO₂ production in the same bog soil used for this study (R. Ye and S. Bridgham, unpublished manuscript). In addition to their role as organic TEAs, the direct toxicity of humic substances may be another important regulator of peatland carbon cycling. We also cannot rule out the possibility that O₂ toxicity to a slow growing methanogen community inherently limited CH₄ production in the BR samples (presumably O₂ would have been consumed in the CR soils following chemical reduction).

While the role of organic matter as a TEA has been previously shown (Lovley et al. 1996, Heitmann et al. 2007, Keller et al. 2009, Roden et al. 2010), we have demonstrated for the first time that the reduction of solid-phase soil organic matter can dominate anaerobic carbon mineralization by producing substantial amounts of CO₂ while competitively suppressing CH₄ production in a peatland soil (Fig. 1). These results suggest that the reduction of organic matter plays an important role in regulating carbon storage and greenhouse gas dynamics in peatland ecosystems. Biogeochemical models of peatland carbon cycling do not currently include the dynamics of humic substances, although these substances may place a fundamental limitation on the ability of peatlands to respond to future warming in terms of increased CH₄ fluxes.

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LITERATURE CITED


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Fig. A1. Slurry pH during the chemical reduction phase (negative time values) and during a 6-week anaerobic laboratory incubation at 20°C. Bog soil used in this incubation was either biologically reduced (BR, open symbols) or chemically reduced (CR, closed symbols). pH of the chemically reduced soil was adjusted on day 2 and day 6 of the 6-day chemical reduction phase using 0.5M HCl. Error bars represent SEM (N = 4).

Fig. A2. Contributions of solid-phase (white bars) and dissolved (gray bars) electron shuttling capacity (ESC) to total ESC in soil slurries during a 6-week anaerobic laboratory incubation at 20°C. ESC values are corrected for the total mass of dry soil and total volume of porewater in serum bottles. Bog soil used in this incubation was either biologically reduced (hatched bars) or chemically reduced (open bars). Error bars are omitted for clarity.
Fig. A3. (A) 0.5M HCl-extractable iron and (B) dissolved iron as Fe(II) (circles) and total iron (squares) during a 6-week anaerobic laboratory incubation at 20°C. Bog soil used in this incubation was either biologically reduced (BR, open symbols) or chemically reduced (CR, closed symbols). Error bars represent SEM (N = 4 for day 3 samples and N = 2 for all remaining sampling dates).