Humic acids as electron acceptors in wetland decomposition

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A B S T R A C T

Decomposition of organic matter in inundated wetland soils requires a number of interdependent microbial processes that ultimately generate CO\textsubscript{2} and CH\textsubscript{4}. Largely as the result of anaerobic decomposition, wetland soils store globally significant amounts of organic carbon and are currently net sources of CH\textsubscript{4} to the atmosphere. Given the importance of wetlands in the global carbon cycle, it is important to understand controls on anaerobic decomposition in order to predict feedbacks between wetland soils and global climate change. One perplexing pattern observed in many wetland soils is the high proportion of CO\textsubscript{2} resulting from anaerobic decomposition that cannot be explained by any measured pathway of microbial respiration. Recent studies have hypothesized that humic substances, and in particular solid-phase humic substances in wetland soils, can support anaerobic microbial respiration by acting as organic electron acceptors. Humic substances may thus account for much of the currently unexplained CO\textsubscript{2} measured during decomposition in wetland soils. Here we demonstrate that humic acids extracted from a variety of wetland soils act as either electron donors or electron acceptors and alter the ratio of CO\textsubscript{2}:CH\textsubscript{4} produced during anaerobic laboratory incubations. Our results suggest that soil-derived humic substances may play an important, and currently unexplored, role in anaerobic decomposition in wetland soils.

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1. Introduction

Microbial decomposition in wetland ecosystems is fundamentally different than in upland systems. In most upland soils, organic carbon can be completely mineralized to carbon dioxide (CO\textsubscript{2}) by a single microorganism using oxygen (O\textsubscript{2}) as a terminal electron acceptor (TEA). In contrast, anaerobic decomposition in inundated wetland soils requires many interdependent microbial processes and can generate both CO\textsubscript{2} and methane (CH\textsubscript{4}) as end products of mineralization (Megonigal et al., 2004). Anaerobic decomposition is less thermodynamically favorable than aerobic decomposition, and this limitation has resulted in the storage of ~500 Pg of carbon (19% of the terrestrial soil carbon pool) in wetland soils worldwide (Bridgham et al., 2006). Further, wetlands are currently responsible for between 15% and 40% of the global CH\textsubscript{4} flux (Denman et al., 2007). Wetlands could release additional CO\textsubscript{2} or CH\textsubscript{4} if carbon mineralization in wetland soils is stimulated by ongoing climate change, augmenting anthropogenic emissions of these important greenhouse gases (Gorham, 1995; Bridgham et al., 1995). Thus, understanding the factors that control anaerobic carbon decomposition in wetland soils has important implications for understanding the global carbon cycle.

In wetland soils, organic molecules are initially degraded by a series of hydrolysis and fermentation reactions that generate successively lower molecular weight products. Ultimately, the end products of these fermentation reactions are acetate, hydrogen (H\textsubscript{2}) and CO\textsubscript{2}, which are subsequently used as substrates for microbial respiration. In the absence of O\textsubscript{2}, microbes preferentially reduce a variety of alternative TEAs for respiration. The primary inorganic TEAs are, in order of decreasing thermodynamic yield: NO\textsubscript{3}\textsuperscript{−} (denitrification), Fe(III) (iron reduction), Mn(III, IV) (manganese reduction) and SO\textsubscript{4}\textsuperscript{2−} (sulfate reduction). Reduction of these inorganic TEAs is coupled to oxidation of organic matter to CO\textsubscript{2} and competitively suppresses CH\textsubscript{4} production. Once more favorable TEAs have been depleted, methanogens produce CH\textsubscript{4} either by splitting acetate to CO\textsubscript{2} and CH\textsubscript{4} (acetoclastic methanogenesis) or reducing CO\textsubscript{2} in a reaction coupled to H\textsubscript{2} oxidation (hydrogenotrophic methanogenesis).

The ratio of the two terminal products of anaerobic decomposition – CO\textsubscript{2} and CH\textsubscript{4} – provides useful information on the dominant
processes contributing to anaerobic decomposition. Under methanogenic conditions (i.e., once more favorable TEAs have been consumed), CO₂ and CH₄ are produced in equal amounts resulting in a 1:1 ratio of CO₂:CH₄ (Conrad, 1999). However, the ratio of CO₂:CH₄ produced during anaerobic incubations of wetland soils is often >1 (van Hulzen et al., 1999), suggesting reduction of non-methanogenic TEAs is important in these systems. Many freshwater peatland soils have high CO₂:CH₄ ratios (e.g., Bridgham et al., 1998; Blodau, 2002; Yavitt and Seidman-Zager, 2006) despite low availability of inorganic TEAs (i.e., NO₃, Fe(III), Mn(III, IV), and SO₄²⁻). In a particularly striking example, Updegraff et al. (1995) observed a CO₂:CH₄ ratio of 883 after an 80-week anaerobic incubation of bog soil. A number of detailed process measurements in peatland soils demonstrate that most of the CO₂ produced during anaerobic incubations cannot be explained by microbial reduction of inorganic TEAs (summarized in Keller and Bridgham, 2007). Similarly, 80% of CO₂ produced in anaerobic incubations of an organic brackish marsh soil could not be accounted for by measured rates of sulfate reduction, iron reduction or methanogenesis (Neubauer et al., 2005).

What accounts for the ‘unexplained’ CO₂ produced during anaerobic decomposition in some wetland soils? It has been suggested that CO₂ produced directly during fermentation contributes to the high CO₂:CH₄ ratio in peatland soils (Vile et al., 2003). Many fermentation reactions do produce CO₂ in the processing of organic molecules (e.g., Schink, 1997); however, Yavitt and Seidman-Zager (2006) suggested that the amount of CO₂ produced during these reactions is typically smaller than the amount of H₂ produced. Thus, any CO₂ produced by fermentation reactions should be more than balanced by CH₄ produced by hydrogenotrophic methanogenesis, producing a CO₂:CH₄ ratio closer to the theoretical value of 1.

A number of researchers have hypothesized the utilization of humic substances as organic TEAs to explain the high CO₂:CH₄ ratio observed in many wetland soils (Segers, 1998; Neubauer et al., 2005; Heitmann et al., 2007; Keller and Bridgham, 2007). Lovley et al. (1996) first demonstrated that bacteria can use humic substances as organic electron acceptors, and bacteria capable of reducing the humic substance analog arthaquinone-2,6-disulphonate (AQDS) have been isolated from natural wetland environments (Coates et al., 1998). From a thermodynamic perspective, reduction of AQDS is more favorable than methanogenesis, which should lead to an increase in the CO₂:CH₄ ratio in soils where AQDS-like humics are being used for microbial respiration; however, direct inhibition of methanogenesis by AQDS-like humics is also possible (Cervantes et al., 2000). Recent research in a Canadian peatland demonstrated that dissolved organic matter is an important electron acceptor, contributing directly (through humic reduction) or indirectly (by regenerating oxidized sulfur species for sulfate reduction) to high CO₂:CH₄ ratios (Heitmann and Blodau, 2006; Heitmann et al., 2007). Although the total electron accepting capacity of dissolved humic substances was relatively small, it was hypothesized that the much larger pool of humic-like organic matter in the solid phase of wetland soils could be used in a similar manner (Heitmann and Blodau, 2006). Scott et al. (1998) also demonstrated that the electron accepting capacity of humic substances extracted from soils was much greater than dissolved humic substances across a number of aquatic systems.

Here we explore how humic substances extracted from wetland soils alter the ratio of CO₂:CH₄ produced during anaerobic incubations. In particular we hypothesize that if humic substances are used as terminal electron acceptors they will increase CO₂ production at the expense of CH₄ production, resulting in an increase in the ratio of CO₂:CH₄ produced.

2. Materials and methods

2.1. Soil collection

Soils were collected beneath plant species that represented a range of tissue chemistry in areas where each species was locally dominant. Soils from unvegetated patches were also collected. All soils were collected in wetlands of the upper Chesapeake Bay (Table 1). Pahokee peat reference soil from the International Humic Substances Society (IHSS) was also included in this experiment. Either one or two samples were collected from each sampling site (Table 1).

2.2. Humic acid extraction and yield

Humic acids were serially extracted from 2.5 g of field-moist soil using a modification of the IHSS protocol (Posner, 1966). An initial 1 M HCl pretreatment was used (pH = 1.5) to remove metals and fulvic acids. Samples were then serially extracted with 0.5 M Na₂CO₃:NaHCO₃ (2:1); 0.1 M Na₄P₂O₇; 0.5 M NaOH; and finally 1 M HCl pretreatment was used (pH = 1.5) to remove metals and fulvic acids. Thus, any reduction of humic substances as organic electron acceptors, and bacteria capable of oxidizing some humic substances. Thus, any reduction of humic substances is also possible (Cervantes et al., 2000). Recent research in a Canadian peatland demonstrated that dissolved organic matter is an important electron acceptor, contributing directly (through humic reduction) or indirectly (by regenerating oxidized sulfur species for sulfate reduction) to high CO₂:CH₄ ratios (Heitmann and Blodau, 2006; Heitmann et al., 2007). Although the total electron accepting capacity of dissolved humic substances was relatively small, it was hypothesized that the much larger pool of humic-like organic matter in the solid phase of wetland soils could be used in a similar manner (Heitmann and Blodau, 2006). Scott et al. (1998) also demonstrated that the electron accepting capacity of humic substances extracted from soils was much greater than dissolved humic substances across a number of aquatic systems.

Table 1

<table>
<thead>
<tr>
<th>Collection site</th>
<th>Plant species</th>
<th>n*</th>
<th>Sample code</th>
</tr>
</thead>
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<tr>
<td>Battle Creek Cypress Swamp</td>
<td>Taxodium distichum</td>
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<td>BCC-TD</td>
</tr>
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<td>Blackwater National Wildlife Refuge</td>
<td>Morella sp.</td>
<td>1</td>
<td>BWR-MC</td>
</tr>
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<td>(Cambridge, MD)</td>
<td>Schoenoplectus americana</td>
<td>2</td>
<td>BWR-SC</td>
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<td>Hoopers Island Fire Station</td>
<td>Phragmites australis</td>
<td>2</td>
<td>HIF-PA 1</td>
</tr>
<tr>
<td>(Fishing Creek, MD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jug Bay Wetland Sanctuary</td>
<td>Nuphar advena</td>
<td>2</td>
<td>JUG-SC</td>
</tr>
<tr>
<td>(Lothian, MD)</td>
<td>P australis</td>
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<td>JUG-PA</td>
</tr>
<tr>
<td></td>
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<td>JUG-QL</td>
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<td></td>
<td>Unvegetated</td>
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<td>JUG-UV</td>
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<tr>
<td>Muddy Creek (Edgewater, MD)</td>
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<td>1</td>
<td>MUC-BA</td>
</tr>
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<td>International Humic Substance Society</td>
<td>Pahokee peat reference soil</td>
<td>1</td>
<td>PEAT 1</td>
</tr>
<tr>
<td></td>
<td>Pahokee peat reference soil</td>
<td>1</td>
<td>PEAT 2</td>
</tr>
</tbody>
</table>

* Number of replicate extracts from each soil.
Research Center where live roots were removed by hand in an anaerobic chamber with a headspace of ~98% nitrogen and <2% hydrogen (Coy Laboratory Products, Grass Lake, Michigan). Root-free soil from all cores was mixed in a Mason jar which was tightly capped in the anaerobic chamber. The Mason jar was covered with aluminum foil and the headspace was flushed with nitrogen for 30 min. The soil was pre-incubated at room temperature to allow metagenomic conditions to develop prior to treatments, and lasted until headspace concentrations of CO₂ and CH₄ were equal (i.e., CO₂:CH₄ ratio = 1).

On 15 January 2008, the Mason jar was returned to the anaerobic chamber (headspace of ~99% nitrogen and <1% hydrogen) and opened. The soil was once again mixed by hand. Subsequently, 5 g of field-moist soil was added to 100 ml serum bottles and slurried with 10 ml of one of the following treatments (n = 4): control (deionized water); 10 mM organic carbon (as 35,000–50,000 Da dextran); 10 mM FeCl₃; 10 mM Fe₂(SO₄)₃; 1 mM AQDS; or 10 mM AQDS. Dextran is a polyglucose and was used as a fermentable organic carbon source (i.e., a known electron donor, but not an electron acceptor). FeCl₃ and Fe₂(SO₄)₃ were added as known inorganic TEAs. AQDS has been used as a homologue for quinone moieties in natural humic substances (e.g., Cervantes et al., 2000), and was added as a known organic TEA. Freeze-dried humic acids from each of the four serial extracts were quantitatively transferred from scintillation vials with two successive rinses with deionized water (10 ml total volume), and used to create a soil slurry with 5 g of field-moist soil.

The different soil types resulted in different yields of humic acids and the distribution among the four serial extracts all differed dramatically. For these incubations, we combined the humic acids from all serial extract fractions and did not correct the mass of the incubated soils for mass of extracted humic acids added, which varied among the different wetland soil types. Thus, we amended 5 g (wet weight) of a single soil type with the total extractable humic acid pool from 2.5 g (wet weight) of a variety of soils. The yield of humic acids from the Pahokee peat reference soil was considerably greater than other soil samples. For the first replicate (‘PEAT 1’), four successive washes with distilled water (20 ml total volume) were used to transfer the humic acids from the 0.5 M Na₂CO₃:NaHCO₃ (2:1) extract to the incubation bottles. For the second replicate (‘PEAT 2’), 6 successive washes with distilled water (30 ml total volume) were used to transfer the humic acids from each of the four serial extracts to the incubation bottles. Even with these additional washes, visual inspection suggested that there was not a quantitative transfer of Pahokee peat humic acids and that the quantities added to the incubations were lower than the total yield.

Serum bottles were removed from the anaerobic chamber, capped with gray butyl septa and flushed with N₂ for 30 min. Bottles were covered with aluminum foil and incubated in the dark at 20 °C. Headspace concentrations of CO₂ and CH₄ were measured on days 1, 2, 3, 4 and 7. Headspace pressure was maintained by replacing sampled volume with N₂. CO₂ was measured on a LiCor LI-7000 and CH₄ was measured on Shimadzu GC-14A equipped with a flame ionization detector. Dissolved CH₄ and CO₂ were calculated using Henry’s Law, adjusting for solubility, temperature and pH (Clesceri et al., 1989; Drever, 1997). Headspace concentrations of CO₂ and CH₄ were corrected for headspace pressure measured at the end of the incubation using an Omega HHP 520 pressure meter (Omega Engineering, Stanford, Connecticut). Following the incubation, pH was measured on the soil slurries and all samples were dried at 60 °C for 1 week. Values from the first sampling point were set to zero and the subsequent production of CO₂ and CH₄ was expressed as μmol C g⁻¹w⁻¹. Rates of CO₂ and CH₄ production were generally linear over the course of the incubation; however, we focus on the total CO₂ and CH₄ at the end of the incubation to allow for comparisons among all treatments regardless of the temporal patterns of gas production.

3. Results and discussion

The addition of humic acids extracted from a variety of wetland soils altered the relative production of CO₂ and CH₄, resulting in a change in the CO₂:CH₄ ratio at the end of a 7 d anaerobic incubation (Fig. 1). This suggests that solid-phase humic acids in wetland soils can play a significant role in anaerobic decomposition, with important implications for wetland carbon cycling.

Untreated control soil was methanogenic with a CO₂:CH₄ ratio of 0.7 ± 0.04 (mean ± 1 SE) at the end of the 7 d incubation (Fig. 1). This ratio is below the predicted theoretical CO₂:CH₄ ratio of 1. One explanation for this lower than expected ratio is a stimulation of hydrogenotrophic methanogenesis, which consumes CO₂, due to the presence of excess H₂ in the headspace of the incubations. H₂ is a reactant for removing O₂ in the anaerobic chamber. Despite flushing with N₂ prior to starting the incubation, excess H₂ was possibly present at the start of the incubation. However, we cannot rule out the possibility that this ratio was a result of uptake of CO₂ through other processes (e.g., homoacetogenesis) or simply a reflection of non-equilibrium conditions (Conrad, 1999). Regardless, the CO₂:CH₄ ratio of <1 demonstrates that anaerobic

![Figure 1](https://example.com/figure1.png)

Figure 1. (a) Percent increase in CO₂ and CH₄ production relative to the control treatment at the end of a 7 d anaerobic incubation. Negative values represent a decrease in CH₄ concentrations. (b) Ratio of CO₂ and CH₄ concentration in the same samples. Data are means ± 1 SE in cases where multiple replicates were available. Codes for extracted soil humic acids are described in Table 1.
decomposition in the control treatment was dominated by methanogenesis.

The addition of dextran, a fermentable carbon source (i.e., an electron donor), stimulated both \( \text{CO}_2 \) and \( \text{CH}_4 \) production as predicted for a methanogenic system. The final \( \text{CO}_2: \text{CH}_4 \) ratio of 1.2 ± 0.04 (mean ± 1 SE), was close to the theoretical ratio of 1 predicted for methanogenic conditions (Fig. 1). The slightly elevated ratio could be the result of excess \( \text{CO}_2 \) released during fermentation of dextran; although this would not be expected if excess \( \text{H}_2 \) was present in the incubation headspace leading to a stimulation of hydrogenotrophic methanogenesis (Yavitt and Seidman-Zager, 2006). A second possibility is that slow growth of methanogens able to respire fermentation end products delayed \( \text{CH}_4 \) production compared to \( \text{CO}_2 \) production (i.e. the system was not at steady state). Our experimental design does not allow us to explore the dynamics of fermentation intermediate and end products. In contrast, the addition of known organic (AQDS) or inorganic (FeCl or Fe\((\text{SO}_4)\)) TEAs stimulated \( \text{CO}_2 \) production while inhibiting \( \text{CH}_4 \) production, resulting in final \( \text{CO}_2: \text{CH}_4 \) ratios ranging from 1.4 ± 0.04 for the 1 mM AQDS treatment to 64.2 ± 3.67 for the Fe\((\text{SO}_4)\) treatment (Fig. 1). These patterns are presumably the result of a shift in the dominant pathway of anaerobic decomposition from methanogenesis to the utilization of more thermodynamically favorable TEAs.

The comparison of \( \text{CO}_2 \) and \( \text{CH}_4 \) dynamics in incubations amended with humic acids to the patterns produced by known electron donors and acceptors provides valuable insights into the role humic substances may play in anaerobic decomposition. In some cases, the net effect of humic acid addition was an electron donor-like response in which both \( \text{CO}_2 \) and \( \text{CH}_4 \) production were stimulated (Fig. 1A), resulting in a final \( \text{CO}_2: \text{CH}_4 \) ratio near 1 (Fig. 1B). In other cases, there was an electron acceptor-like response in which \( \text{CO}_2 \) production increased while \( \text{CH}_4 \) production decreased, resulting in increased \( \text{CO}_2: \text{CH}_4 \) ratios. A number of humic acids had a final \( \text{CO}_2: \text{CH}_4 \) ratio > 2, suggesting that humic acids from some wetland soils may strongly limit the production of \( \text{CH}_4 \) by serving as thermodynamically favorable organic electron acceptors. We hypothesize that the observed range in \( \text{CO}_2: \text{CH}_4 \) ratios is a result of differences in the chemical makeup of humic substances extracted from different wetland soils; however, we also discuss the potential importance of humic yield and humic-induced changes in pH in this experiment.

When all four serial extracts were combined, the total yield of humic substances was generally less than 0.10 g. Although there was a positive relationship between the final \( \text{CO}_2: \text{CH}_4 \) ratio and the mass of humic acids added to the incubations, the relationship was weak and explained only 8% of the variation (Fig. 2). This suggests that humic chemistry – and not simply humic quantity – determines the extent to which humic substances may act as terminal electron acceptors. The humics extracted from Pahokee peat soil mines the extent to which humic substances may act as terminal electron acceptors. The humics extracted from Pahokee peat soil

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A potentially confounding artifact in our experimental design was soil pH. Soils with higher \( \text{CO}_2: \text{CH}_4 \) ratios consistently had a lower pH at the end of the 7 d incubation (Fig. 3A). This pattern generally reflected a negative relationship between the final pH and the final concentration of \( \text{CO}_2 \) as well as a positive relationship between the final pH and the final concentration of \( \text{CH}_4 \) (Fig. 3B). It is unlikely that \( \text{CO}_2 \) production by non-methanogenic respiration was stimulated by more acidic conditions, and we interpret the relationship between pH and \( \text{CO}_2: \text{CH}_4 \) to be a consequence of well-known changes in carbonate chemistry that occur as \( \text{CO}_2 \)...
accumulates over the course of the incubations (Stumm and Morgan, 1995). There were a few exceptions to this pattern in which samples with low pH had relatively low rates of CO₂ production. In these cases, the amendments were the two known inorganic TEA treatments and the treatments that were amended with comparatively high quantities of Pahokee Peat humic acids (Fig. 3B). We interpret the high CO₂:CH₄ ratios in these four treatments to be primarily the result of direct pH effects from adding humic acids or inorganic TEAs. As we did not measure the initial pH of the incubations, we cannot fully evaluate to what extent differences in final pH were caused by the initial addition of humic acids or by changes in carbonate chemistry.

Our results demonstrate that soil-derived humic acids can serve as either electron donors or electron acceptors during anaerobic decomposition in a wetland soil. Although largely descriptive, this raises the intriguing possibility that solid-phase humic substances may inhibit CH₄ production by serving as thermodynamically favorable organic TEAs. The extent to which this process contributes to decomposition in natural wetlands needs to be further explored, particularly in highly organic peatland soils where humic substances are found in high concentrations.

We suggest four future directions that will likely prove fruitful in understanding how humic substances influence microbial decomposition in wetland soils. (1) The confounding effect of pH must be removed to demonstrate conclusively that humic substances are acting as TEAs rather than influencing microbial decomposition solely through changes in pH. (2) Comparisons between the CO₂:CH₄ ratios observed in incubations with highly modified extracted soil humic substances and in situ CO₂:CH₄ production potentials from the source soils would put the laboratory incubations into a stronger ecological context. (3) There is evidence that the abiotic reduction of soil-derived humics (i.e., chemical reducing capacity) is related to the microbial reduction of these compounds (Pereytazhko and Sposito, 2006). As we did not measure abiotic electron accepting capacity of humics extracted in this experiment, it remains to be seen if this property is related to the reduction of humic substances by the diverse, natural wetland microbial community in our experiment. The measurement of the abiotic electron accepting capacity of soil-derived humic substances over longer-term incubations may also demonstrate that this pool is being reduced in the course of anaerobic decomposition. (4) It appears that humic chemistry and not simply humic quantity is important when considering the role of humic reduction in wetland soils. However, it remains to be seen which functional groups are responsible for the range of electron accepting capacities observed in humic substances from wetland soils. Quinone moieties are generally considered important in humic redox chemistry (Scott et al., 1998), but other functional groups have also been identified (Stryk and Sposito, 2001; Ratusak and Nanny, 2007). Relationships between the functional composition of humic substances and the electron accepting capacity of those substances would allow for mechanistic hypotheses about where and when humic reduction is likely to be important in natural wetlands, and how this process may respond to ongoing global change.

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