

7-3-2017

Hydrogenation of Organic Matter as a Terminal Electron Sink Sustains High CO₂:CH₄ Production Ratios During Anaerobic Decomposition

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Recommended Citation

Wilson, R.M., Tfaily, M.M., Rich, V.I., Keller, J.K., Bridgham, S.D., Medvedeff Zalman, C., Meredith, L., Hanson, P.J., Hines, M., Pfeifer-Meister, L., Saleska, S.R., Crill, P., Cooper, W.T., Chanton, J.P., Kostka, J.E., Hydrogenation of Organic Matter as a Terminal Electron Sink Sustains High CO₂:CH₄ Production Ratios During Anaerobic Decomposition, *Organic Geochemistry* (2017), doi: <http://dx.doi.org/10.1016/j.orggeochem.2017.06.011>

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Accepted Manuscript

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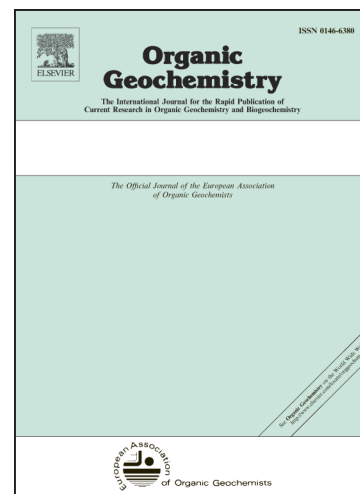
PII: S0146-6380(16)30208-X
DOI: <http://dx.doi.org/10.1016/j.orggeochem.2017.06.011>
Reference: OG 3567

To appear in: *Organic Geochemistry*

Received Date: 6 October 2016
Revised Date: 19 June 2017
Accepted Date: 27 June 2017

Please cite this article as: Wilson, R.M., Tfaily, M.M., Rich, V.I., Keller, J.K., Bridgham, S.D., Medvedeff Zalman, C., Meredith, L., Hanson, P.J., Hines, M., Pfeifer-Meister, L., Saleska, S.R., Crill, P., Cooper, W.T., Chanton, J.P., Kostka, J.E., Hydrogenation of Organic Matter as a Terminal Electron Sink Sustains High CO₂:CH₄ Production Ratios During Anaerobic Decomposition, *Organic Geochemistry* (2017), doi: <http://dx.doi.org/10.1016/j.orggeochem.2017.06.011>

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Title: Hydrogenation of Organic Matter as a Terminal Electron Sink Sustains High CO₂:CH₄ Production Ratios During Anaerobic Decomposition

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Abstract:

Once inorganic electron acceptors are depleted, organic matter in anoxic environments decomposes by hydrolysis, fermentation, and methanogenesis, requiring syntrophic interactions between microorganisms to achieve energetic favorability. In this classic anaerobic food chain, methanogenesis represents the terminal electron accepting (TEA) process, ultimately producing equimolar CO₂ and CH₄ for each molecule of organic matter degraded. However, CO₂:CH₄ production in *Sphagnum*-derived, mineral-poor, cellulosic peat often substantially exceeds this 1:1 ratio, even in the absence of measureable inorganic TEAs. Since the oxidation state of C in both cellulose-derived organic matter and acetate is 0, and CO₂ has an oxidation state of +4, if CH₄ (oxidation state -4) is not produced in equal ratio, then some other compound(s) must balance CO₂ production by receiving 4 electrons. Here we present evidence for ubiquitous hydrogenation of diverse unsaturated compounds that appear to serve as organic TEAs in peat, thereby providing the necessary electron balance to sustain CO₂:CH₄ >1. While organic electron acceptors have previously been proposed to drive microbial respiration of organic matter through the reversible reduction of quinone moieties, the hydrogenation mechanism that we propose, by contrast, reduces C-C double bonds in organic matter thereby serving as 1) a terminal electron sink, 2) a mechanism for degrading complex unsaturated organic molecules, 3) a potential mechanism to regenerate electron-accepting quinones, and, in some cases, 4) a means to alleviate the toxicity of unsaturated aromatic acids. This mechanism for CO₂ generation without concomitant CH₄ production has the potential to regulate the global warming potential of peatlands by elevating CO₂:CH₄ production ratios.

Keywords: anaerobic methanogenesis; C cycle; greenhouse gas; terminal electron acceptor; peatland; microbial respiration

Abbreviations

DOM—dissolved organic matter

FTICRMS—Fourier transform ion cyclotron resonance mass spectrometry

KMD—Kendrick mass defect

TEA—terminal electron acceptor

VFA—volatile fatty acid

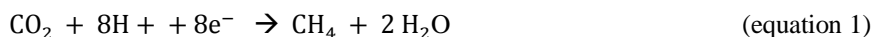
Funding: this work was supported by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research [grants: DE-AC05-00OR22725, DE-SC0004632, DESC0010580, DE-SC0012088 and DE-SC0014416].

1. Introduction

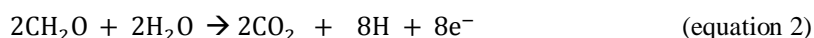
Over the past ten thousand years boreal peatlands have accumulated approximately 500 Gt of carbon (C), an amount equal to half of the atmospheric C reservoir (Yu 2012). This vast store of soil C could substantially impact the global C cycle if peatlands shift from net C sinks to net C sources. Peatlands act as C sinks by sequestering CO₂ as organic C in deep anoxic peat (i.e., the catotelm). In the catotelm, cold temperatures, water-logged conditions and the resultant lack of O₂ all contribute to slow decomposition rates (Clymo, 1987). If these slow decomposition rates are the result of kinetic control—and not due to some inherent recalcitrance of the organic compounds themselves—then increased warming under changing climate conditions could stimulate the conversion of this organic matter to the respiration products CO₂ and methane (CH₄), which could then be released back to the atmosphere creating a positive feedback to future warming. Since the sustained-flux global warming potential of CH₄ is 45-times greater than that of CO₂ on a 100 y timescale (Neubauer and Megonigal 2015), the magnitude of this potential feedback will ultimately depend on the balance between net CO₂ and CH₄ emissions (Frolking et al. 2011).

Sphagnum-derived ombrotrophic peatland soils are low in mineral content and comprised mostly of cellulosic organic matter (Clymo 1987; Conrad 1999; Corbett et al. 2013). In the absence of a substantial mineral component, the traditional inorganic terminal electron acceptors (TEAs) for organic matter degradation are rapidly depleted or non-existent. For example, iron reduction and denitrification have been shown to be inconsequential in at least some ombrotrophic peat systems (Keller and Bridgham 2007) and nitrate, nitrite, and sulfate concentrations may be low or undetectable (Corbett et al. 2013; Hodgkins et al. unpublished). The majority of C stored in peatlands resides below the water table in the catotelm, where organic matter decomposition is dominated by fermentation reactions, syntrophy, and methanogenesis (Drake et al. 2009; Bridgham et al. 2013). These low-energy yielding reactions are only marginally thermodynamically favorable and depend on a delicately balanced syntrophic microbial consortium to break down organic molecules into CO₂ and molecular hydrogen (H₂) (Conrad 1999; Burdige 2006). The build-up of H₂ in peat porewater resulting from this metabolic decomposition, however, inhibits the very fermentation processes that produce it (e.g. Lovley and Klug 1982). Thus, syntrophic reactions require a coupling reaction that consumes H₂ in order to maintain electron flow and make the sum of the reactions energy-yielding (Conrad 1999).

Hydrogenotrophic methanogenesis converts CO₂ and H₂ into CH₄ thereby reducing H₂ concentrations and yielding sufficient energy to make the overall syntrophic reactions thermodynamically favorable (Conrad 1999; Whiticar 1999):



In this reaction, the oxidation state of C goes from +4 to -4 thereby serving as an electron sink. This reaction is not a net sink for CO₂, as 2CO₂ are generated from 0-valent organic matter to produce the hydrogen in the first place, as shown below (with sufficient reductant produced to then convert one CO₂ to CH₄ by Eq. (1)):



The classic paradigm of cellulosic organic matter decomposition under anaerobic conditions assumes that, following the depletion of inorganic TEAs, hydrogenotrophic methanogenesis is the final H₂ sink (Conrad 1999).

While acetotrophic methanogenesis is also an important CH₄ production pathway in peatlands, acetoclasty produces equimolar CO₂:CH₄ from 0-valent acetate, and therefore does not present an electron imbalance.

Therefore, considering the breakdown of the model organic molecule as glucose (the monomer of which cellulose is constructed), electron balance would lead one to expect equimolar production of CO₂ and CH₄ as the net result in methanogenic cellulosic environments (Tarvin and Buswell 1934; Barker 1936; Schink 1997; Conrad 1999; Yavitt and Seidman-Zager 2006; Corbett et al. 2013).



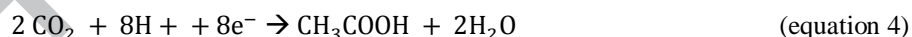
The overall C oxidation state in glucose is 0, and Eq. (3) produces 3 CO₂ in which the C has an oxidation state of +4, and 3 CH₄ where the oxidation state of C is -4 thereby preserving electron balance. When CO₂ is produced without concomitant CH₄ production, those 4 electrons must be transported to another sink. In mineral-rich settings, inorganic TEAs commonly serve that function allowing CO₂:CH₄ > 1, while many organic-rich anaerobic environments which are low in inorganic TEAs (such as lake sediments and landfills) conform to the predicted 1:1 CO₂:CH₄ production ratio (Lovley and Klug 1982; Tchobanoglous and Kreith 2002; Hines et al. 2008; Conrad et al. 2011). However, mineral-poor cellulosic peatlands—i.e. those dominated by *Sphagnum*—pose an exception (Yavitt and Seidmann-Zager, 2006; Hines et al. 2008; Nilsson and Öhlquist 2009). Despite a dearth of measurable inorganic TEAs in peat (e.g. Bridgham and Keller 2007; Corbett et al. 2013), measurements of CO₂:CH₄ production ratios in numerous field and incubation studies conducted across many different peatland sites have failed to demonstrate equimolar CO₂ and CH₄ production from deep (i.e., permanently saturated and anoxic) catotelm peat (Table 1; Valentine et al. 1994; Romanowicz et al. 1995; Updegraff et al. 1996; van Hulzen et al. 1999; Chasar et al. 2000a, b; Keller and Bridgham 2007; Wright et al. 2011; Corbett et al. 2013; McCalley et al. 2014; Hodgkins et al. 2014, 2015). CO₂ production is much greater than expected in most cases resulting in a CO₂:CH₄ production ratio much greater than 1.

While *Sphagnum*-derived peat is mineral-poor and composed primarily of cellulose (Clymo 1987; Corbett et al., 2013), a large fraction of the organic matter is comprised of aromatic and lignin-like molecules. Could the decomposition of such aromatic compounds account for the apparent CO₂:CH₄ imbalance? The earliest studies suggested that lignin was resistant to decomposition in the absence of O₂ (Hackett et al. 1977; Zeikus et al. 1982; Odier and Monties 1983), but later work reported that limited anaerobic degradation of lignin is possible under some conditions—particularly in the presence of sulfate or nitrate as electron acceptors (Kaiser and Hanselmann, 1982; Benner et al. 1984; Colberg and Young 1985). However, very little was known about the mechanism of anaerobic decomposition of lignin-like compounds until recent studies demonstrated that the major lignin-derived aromatic compounds—syringate and vanillate—also produce equimolar CO₂:CH₄ under TEA-limited, methanogenic conditions (Kato et al. 2015). Thus, similar to cellulose, anaerobic decomposition of lignin-like aromatic compounds is expected to result in a CO₂:CH₄ near 1, and therefore the polyphenol content of peat cannot account for the observed non-stoichiometric production ratios.

Organic matter itself serves as an electron acceptor for microbial respiration in a variety of aquatic environments including peatlands, but the exact mechanism or extent to which this occurs is poorly understood (Lovley et al. 1996; Coates et al. 2002; Kappler et al. 2004; Keller et al. 2009; Roden et al. 2010; Smemo and Yavitt

2011; Keller and Takagi 2013; Tfaily et al., 2013, 2014). Addition of humic acids to peat incubations drastically reduces methane production (Blodau and Deppe 2012). Both dissolved and solid-phase humic substances appear to have the ability to accept electrons, which has been proposed to account for elevated CO₂:CH₄ ratios (Scott et al., 1998; Heitmann and Blodau, 2006; Heitmann et al., 2007; Keller and Takagi, 2013). Quinone moieties are thought to act as the electron-accepting sites (Lovley et al., 1996; Kappler et al., 2004; Keller et al., 2009; Roden et al., 2010; Keller and Takagi 2013; Ye et al., 2016). However, quinone reduction is a reversible reaction similar to inorganic TEAs that requires re-oxidation to sustain high CO₂ production rates, thus, this mechanism cannot fully explain persistently high CO₂:CH₄ production ratios in permanently saturated catotelm peat (Keller et al., 2009; Bridgham et al. 2013; Keller and Takagi 2013) and long-term, closed-system incubations (Bridgham et al. 2013) where no mechanism for reoxidation exists. Similarly, anaerobic oxidation of CH₄ may occur to some extent at these sites and could result in CO₂:CH₄ > 1 (Smemo and Yavitt 2011; Gupta et al. 2012), but again an electron sink is required to accomplish this oxidation so we are left with the same problem—where are the electrons transferred? Further, stable C isotopic evidence does not support the amount of oxidation necessary to explain the observed excess CO₂ in all sites (e.g. Corbett et al. 2013; Hodgkins et al. 2014).

Another mechanism that may explain an elevated CO₂:CH₄ ratio is fermentation. However, CO₂ is not the only product of fermentation and we must account for the production of volatile fatty acids (e.g. acetate, butyrate, and propionate) and sometimes hydrogen resulting from fermentation if we are to accept this explanation. Acetate accumulation signals the inhibition of acetoclastic methane production which is associated with higher than expected CO₂:CH₄ ratios (Dudleston et al 2002; Hines et al., 2008), but acetate is not an electron sink since both acetate and glucose have an oxidation state of 0 and therefore acetate accumulation does not account for the electron imbalance imposed by high CO₂ production. Homoacetogenesis, i.e. acetate formation via the Acetyl-CoA pathway, is a H₂ sink in anoxic peat that could help draw down the H₂ produced during fermentation. However, this reaction also consumes CO₂ (Ye et al. 2014):



which would result in lower CO₂:CH₄ ratios than expected, not higher, and again acetate and glucose have the same oxidation state, so homoacetogenesis does not serve as a terminal electron sink. In addition, recent studies show that homoacetogenesis is outcompeted by other anaerobic processes in boreal and Arctic peatlands (Tveit et al., 2015; Schmidt et al., 2016).

Animal rumen are similar in many ways to peat soils. They are C-rich, devoid of inorganic electron acceptors, and the end products of fermentation (volatile fatty acids, CO₂, and CH₄) are slow to be removed suggesting that the ruminal system may be subject to similar thermodynamic limitation (Ungerfeld and Kohn 2006). In rumen, fermentation can be manipulated to increase propionate production. Since propionate has an oxidation state of -2, there is no production of H₂ and CH₄ production is suppressed relative to fermentative acetate production thereby promoting elevated CO₂:CH₄ ratios (Mackie et al. 1984; Callaway and Martin 1996; Nagaraja et al. 2007). However, one difference from peatlands, is that, in rumen, the propionate (and acetate) can be absorbed by the ‘host’ animal for use in glandular metabolism. Thus propionate is removed from the rumen and does not accumulate. No such mechanism exists in peat, thus to sustain CO₂ production by propionate production, propionate

must either (1) accumulate in the peat, (2) be interconverted with succinate, or (3) be broken down into acetate with H_2 as a byproduct. We do not always observe the build-up of propionate (or succinate) in peatlands where $CO_2:CH_4$ ratios are high (Hodgkins et al. 2014; McCalley et al. 2014; Wilson et al. unpublished), thus neither propionate nor succinate can be the final electron acceptor in these systems and interconversion with acetate without subsequent CH_4 production would lead to excess H_2 .

Studies of the rumen system also point to another pathway in which organic matter may serve as an electron acceptor, namely the hydrogenation of aromatic double bonds. Biohydrogenation of double bonds is an important process in the rumen of cattle and sheep that is linked to a shunting of electrons away from methanogenesis (Rosenfeld and Tove, 1971; Kemp et al, 1975; Jenkins et al., 2007; McKain et al., 2010; Hristov et al., 2013) thereby suppressing CH_4 production and sustaining elevated $CO_2:CH_4$ ratios (Nagaraja et al. 1997). Following ingestion, lipids are considerably altered in the rumen, and metabolomics analyses provide evidence that biohydrogenation reactions abound. For example, while the fatty acid profiles of lipids in cattle diet mostly contain unsaturated fatty acids, the lipids that exit the rumen are mostly saturated fatty acids (Jenkins et al. 2007). The rumen is a strictly anaerobic environment and hydrogenation of unsaturated hydrocarbons is thought to provide a selective advantage for microbial groups in the absence of inorganic electron acceptors (Kemp et al. 1975; McKain et al., 2010). Kemp et al. (1975) state that it is possible “that some anaerobes can use unsaturated fatty acids as a hydrogen sink, thus gaining a slight advantage over organisms not able to hydrogenate”. Any rumen microorganisms would also benefit from biohydrogenation through a reduction in toxicity, since unsaturated fatty acids are considered toxic and the reduction of their double bonds is thought to relieve their toxicity. Additionally, Hughes and Tove (1980) have suggested that the hydrogenation of double bonds in the rumen may regenerate quinones, which could provide a mechanism for sustaining CO_2 production by ultimately receiving the electrons that are passed to quinone moieties thereby reoxidizing the quinones and allowing them to accept more electrons.

Studies of enrichment cultures and model anaerobes also point to organic electron accepting processes and the hydrogenation of double bonds. A number of bacterial cultures isolated from the rumen have been demonstrated to be capable of reducing aromatic acids, a process which involves hydrogenation (Krumholz and Bryant, 1986; Imkamp and Mueller, 2002). The metabolism of benzoyl-CoA, a key intermediate in nearly all known pathways for the microbial degradation of aromatic compounds, involves hydrogenation during the initial ring reduction (Fuchs et al., 2011; Meckenstock and Mouttaki, 2011; Porter and Young, 2014). Ring cleavage by addition of H_2 is a nearly ubiquitous first step in the degradation of aromatic hydrocarbons under anaerobic conditions (Porter and Young 2014; Stenson et al. 2003). In a number of anaerobes (e.g., *Wolinella succinogenes*), fumarate respiration is coupled to hydrogen oxidation (i.e., H_2 serves as the electron donor), and energy is produced through oxidative phosphorylation by hydrogenation of a double bond in fumarate (Kroger et al., 2002).

Microbial growth could elevate the $CO_2:CH_4$ ratio as microbial biomass is largely composed of compounds that are reduced relative to cellulose (Hungate et al., 1961; Nilsson and Öquist 2009). This is considered to be an important mechanism maintaining non-stoichiometric CH_4 production in rumen (Hungate et al. 1961). While peatlands share many similarities with anaerobic digestion in rumen, there yet remain some important differences. The rumen acts more like a (near) constant addition flow through reactor as fresh readily fermentated carbohydrates

are added to the milieu at least daily, volatile acid fermentation products are absorbed by the ruminant cells for glandular metabolism thereby removing them from the system (Nagaraja et al. 1997) and microbial cells and other products are lost from the gut. In both rumen and peatlands, the turnover of microbial biomass releases the reduced compounds (e.g. lipids and proteins) back into the DOM pool. Decomposition of proteins and lipids which generally have a lower oxidation state than carbohydrate, would shift production towards more CH_4 than CO_2 (Nilsson and Öquist 2009). Thus the influence of microbial growth on elevating $\text{CO}_2:\text{CH}_4$ ratios is likely much stronger in rumen than in peatlands which are comparatively more stable and presumably microbial biomass is closer to a dynamic equilibrium.

The goal of this study was to examine the potential for biohydrogenation of organic matter to serve as an electron sink capable of sustaining CO_2 production in peatlands without concomitant CH_4 production. To accomplish this goal we used Fourier transform ion cyclotron resonance mass spectrometry (FTICRMS) followed by Kendrick mass transform analysis to investigate molecular changes in the dissolved organic matter (DOM) of two northern peatlands. The Kendrick mass (i.e., Kendrick 1963) transform analysis identifies gain or loss of specific chemical moieties (e.g. CH_2 , H_2 , CH_2O , etc) via mass differences, and was used to infer potential microbial degradation pathways by which compounds were consumed in these samples. We examined peat chemistry from two boreal peatlands (with different cryogenic histories) at multiple depths in the field and over time in incubations, in tandem with measurements of $\text{CO}_2:\text{CH}_4$ production ratios.

2. Material and methods

For field comparisons, porewater was collected from the S1 bog, a *Sphagnum*-dominated site at northern Minnesota peatland in the Marcell Experimental Forest (47.51911°N, 93.45964°W, USA) using permanently-installed piezometers, which provided profiles from the watertable surface to 200 cm depth. Porewater was also collected from an additional *Sphagnum*-dominated site within Stordalen Mire (68.350°N, 19.033°E, Sweden), a palsa mire underlain by thawing permafrost, using perforated stainless steel tubes that were inserted into the peat to the desired depths (13 cm and 32 cm). Porewater was immediately filtered to 0.7 μm in the field at both sites using Whatman glass fiber filters then stored in polycarbonate vials and frozen for shipment. Volatile fatty acid (VFA) analysis was conducted following the methods of Hines et al. (2008). Acetate, propionate, and butyrate concentrations in filtered pore water were determined using a Dionex ICS-2100 Reagent-Free ion chromatograph (Thermo Scientific) fitted with an AS11-HC column, EGC III Potassium Hydroxide eluent generator, and ASRS 300 suppressor. Standards were prepared using sodium acetate, propionic acid, and sodium butyrate, respectively. Samples were neutralized using 0.1 M sodium hydroxide prior to analysis and run in duplicate (relative standard deviation < 5%) using a hydroxide gradient from 1 mM to 50 mM for optimal peak separation. Level of detection for VFA analysis was 0.1 mg/L.

Anaerobic incubation studies were conducted on peat from 8-18 cm deep collected from Stordalen Mire following the procedures of Hoddgkins et al. (2014, 2015). This depth was chosen as the shallowest permanently inundated depth at that site. The peat was gently homogenized by gloved hands using a pulling-shredding motion to adequately homogenize the sample while maintaining the integrity of the peat. Peat was aliquoted into six 125 mL

serum vials (40 g each) to which we added 40 mL of degassed deionized water and then capped. The vials were flushed with N₂ gas and shaken gently until CO₂ concentrations in the headspace were below 0.1% as measured by flame ionization gas chromatography with use of a methanizer. The sealed vials were then stored in the dark at ambient room temperature (18-22°C) for a 25-day pre-incubation period to equilibrate the samples and consume any oxygen that was inadvertently introduced during the setup and reduce any inorganic TEAs that might have been present (e.g. Hodgkins et al. 2014, 2015). At the end of this pre-incubation period, vials were flushed with N₂ and shaken again, until a final CO₂ concentration <0.1% and CH₄ concentration <0.5% was attained in the headspace. At this point, two vials were sacrificed for the ‘day 0’ porewater sampling. Porewater was removed and filtered to 0.2 µm to remove cells. The remaining vials were stored undisturbed in a dark, room-temperature location for 50 days. Periodically, 50-150 µL of the headspace was removed to monitor the concentration and isotopic ratio (δ¹³C‰) of the produced CO₂ and CH₄ by isotope ratio mass spectrometry. At the end of 50 days, two additional replicates were sacrificed for the ‘day 50’ porewater analysis. Again the porewater was filtered to 0.2 µm then frozen.

Thawed porewater samples were mixed with 2 parts methanol for direct injection on a 12T Bruker Solarix Fourier transform ion cyclotron ratio mass spectrometer (FTICRMS) at the Environmental Molecular Sciences Laboratory, a national user facility (Richland, WA, USA). A standard Bruker ESI source was used to generate negatively charged molecular ions. Samples were then introduced to the ESI source equipped with a fused silica tube (200 µm i.d) through a syringe pump at a flow rate of 3.0 µL/min. Experimental conditions were as follows: needle voltage, +4.4 kV; Q1 set to 150 m/z; and the heated resistively coated glass capillary operated at 180°C. Ninety-six individual scans were averaged for each sample and internally calibrated using organic matter homologous series separated by 14 Da (–CH₂ groups). The mass measurement accuracy was typically less than 1 ppm for singly charged ions across a broad m/z range (i.e. 200, <m/z <1200). Chemical formulas were assigned using an in-house built software program following the Compound Identification Algorithm, described by Kujawinski and Behn (2006) and modified by Minor and Stephens (2008). Chemical formulas were assigned based on the following criteria: S/N >7, and mass measurement error <1 ppm, taking into consideration the presence of C, H, O, N, S and P and excluding other elements. All observed ions in the spectra were singly charged as confirmed by the 1.0034 Da spacing found between isotopic forms of the same molecule (between ¹²C_n and ¹²C_{n-1}¹³C₁). In terms of overall coverage, we were able to assign >85% of the peaks observed in each spectrum. Duplicate injections were run for the Sweden field samples with 80% of peaks replicated in both analysis runs. Methanol was also analyzed as a blank. Forty peaks appeared in the methanol and were subtracted from the samples. Only peaks appearing in all instrument and method replicates for each sample were considered in further analyses.

Peaks were divided into two categories: ‘consumed’ and potential ‘end products’. For the field data, compounds that appeared in shallower samples but were missing from deeper samples (up to 200 cm and 32 cm for Minnesota and Sweden respectively) were considered to have been “consumed” while all compounds present in the deep samples were considered potential “end products”, regardless of their appearance elsewhere. The vertical progression of peat decomposition has been confirmed in these (Tfaily et al., 2014; Hodgkins et al., 2014, 2015) and other peatland ecosystems (Tfaily et al. 2013). Similarly in the incubations, peaks that were detected at the

beginning of the incubation (day 0) but disappeared by the end of the incubation (day 50) were considered to have been “consumed” during the course of the incubations. Compounds that were present at day 50 were considered potential “end products” regardless of their appearance elsewhere. The total number of peaks detected in each sample as well as the number of consumed compounds for each depth in the field or sampling time in the incubations are provided in SI Table 1.

Kendrick mass analysis involves mathematically transforming the data to alternate mass scales through the use of conversion factors that set the mass of a desired chemical moiety to an integer atomic mass value (sensu Kendrick 1963). The Kendrick mass defect (KMD) is defined as the difference between the resulting exact and nominal masses. In the newly calculated masses, peaks that have identical KMD have molecular formulae that differ by integer multiples of the chemical moiety (transform) investigated. The Kendrick mass (KM) of a peak is determined by: $KM = \text{observed mass} \times \frac{F_{\text{nominal_mass}}}{F_{\text{exact_mass}}}$ where F is the transform of interest. We applied Kendrick mass transforms to the samples based on a list of 162 transforms assembled from the literature (SI Table 2) that represented potential microbial decomposition strategies. To further constrain our data we also calculated z^* scores for each peak (sensu Hsu et al. 1992). This score, which is calculated as the modulus of the division of the peak nominal mass by $F_{\text{nominal_mass}}$, ensures that compounds with the same z^* score have molecular weight differences consistent with the transform of interest and reduces the number of false KMD matches due to measurement errors (Hsu et al. 1992; Stenson et al. 2003). The KMDs of ‘consumed’ peaks were compared to ‘end product’ peaks to identify potential pathways by which the consumed peaks may have been degraded. When a consumed peak and an end product peak had KMD values within 0.000001 mass units and an identical z^* score, those peaks were counted as a transform pair and it was assumed that the consumed peak was degraded to the end product peak by the relevant transform. For each of the 162 transforms that we investigated, we counted the total number of such transform pairs in each sample and present them as the fraction of the total number of consumed peaks present in the samples (Fig. 2). It is important to note that a single peak can be involved in multiple transforms, from which we infer that there are different pathways by which it can be degraded. Thus, the sum of the transform counts could be somewhat greater than the sum of consumed compounds. By mass comparison, we noted whether the consumed compound was transformed by addition or removal of the relevant chemical moiety.

3. Results

In our incubations of Stordalen Mire peat, CO₂ and CH₄ concentrations increased linearly throughout the incubation period (Fig. 1a). The CO₂:CH₄ ratio in the incubations remained fairly stable from day 8 through day 50, approaching a value of ~1.5 (Fig. 1b), these results are in agreement with observations made by Hodgkins et al., (2014) at this same Stordalen Mire site. For comparison, previous incubations of peat from S1 bog yielded CO₂:CH₄ ratios that varied from 2 to 100, and increased as a function of depth (Tfaily et al., 2014).

In the Stordalen Mire bog field samples, the concentrations of VFA’s that serve as electron sinks—butyrate and propionate—were maintained at very low levels (<10 μM and <0.1 μM respectively measured from 12 cm to 70 cm deep) (Hodgkins et al., 2014; McCalley et al., 2014). Electron harboring VFA concentrations at S1 bog in Minnesota are similarly low—propionate averaged 2.3 ± 7.5 μM and butyrate averaged 0.4 ± 1.3 μM.

The Kendrick mass (i.e., Kendrick 1963) transform analysis identifies gain or loss of specific chemical moieties (e.g. CH_2 , H_2 , CH_2O , etc) via mass differences, and was used to infer potential microbial degradation pathways by which compounds were consumed in these samples. This is an environmental analogue to “traditional” small-molecule metabolomics, in which small molecule metabolites are used to identify cellular processes. Environmental metabolomics differs in its complexity and molecule size; it is not a set of well-defined chemical fingerprints, but rather the entire DOM pool. Microbial processes are encoded in the changes in DOM pool composition (e.g. over time) rather than appearance/disappearance of specific metabolites.

The most commonly occurring transform in the field samples from both peatlands was the addition of 2 hydrogens. We will write this transform ambiguously as $+2[\text{H}]$ recognizing that when H_2 is not the direct electron donor, nevertheless, metabolic hydrogen is in equilibrium with aqueous H_2 (Ungerfeld and Kohn 2006). In either case, transform results indicate that hydrogenation was an important step in the transformation of DOM in these systems (Fig. 2) accounting for ~3.5-5% of the transforms observed in field samples. Similarly, $+2[\text{H}]$ was the second-most commonly occurring transform in the incubations, accounting for ~2% of the total number of transforms. The most commonly occurring transform in the incubations was CH_4 exchange for O_2 , which could represent the accumulation of multiple smaller transforms and closely matches reactions for degrading lignin in which $2[\text{H}]$'s are removed from side chains, followed by demethylation, and aromatic ring cleavage by addition of O_2 (e.g., Stenson et al. 2003). Thus hydrogenation and dehydrogenation appear to be simultaneously occurring reactions important in the degradation of organic matter.

FT-ICR MS analysis allows inferences about some of the chemical characteristics of the compounds undergoing these transforms to be examined. A diversity of consumed compounds across both field and incubation data were linked by the hydrogenation transform (Fig. 3). In total, we identified 216 unique organic compounds that were transformed by the addition of $2[\text{H}]$ (given that the later incubation time points show addition relative to earlier time points, and that deeper catotelm samples show addition relative to shallower ones). These compounds spanned most of the overall DOM pool's O/C vs H/C space, and represented a range of degree of oxidation, double bond equivalences, and heteroatom (N,S,P) contents (Fig. 3). While in general higher molecular weight, heteroatom-substituted compounds appear less susceptible to the $+2[\text{H}]$ consumption mechanism, overall, the potential substrates of the $+2[\text{H}]$ transform appeared numerous and chemically diverse.

4. Discussion

Our results suggest that, as in the rumen, hydrogenation of unsaturated organic matter in peatlands not only serves as a requisite for the degradation of these compounds, but also as a terminal electron sink, sustaining fermentation without subsequent CH_4 generation and possibly reducing H_2 production, thereby explaining the non-stoichiometric production of CO_2 relative to CH_4 . The parallels between the rumen and peatlands are striking. In the rumen, the addition of tannins and saponins have been specifically used to inhibit the production of CH_4 (Hristov et al. 2014). In peatlands, when dissolved humic acids are added to incubations, methanogenesis is similarly curtailed (Blodau and Deppe, 2012).

Hydrogenation of C-C double bonds is involved in the degradation of complex unsaturated organic molecules and may be a strategy to reduce the toxicity of aromatic acids released during the decomposition of plant organic matter (Rosenfeld and Tove 1971; Chesson et al. 1982). *Sphagnum*-dominated peatlands, as well as other anaerobic ecosystems, contain an abundance of plant-derived aromatic acids (e.g., cinnamic, vanillic, syringic, caffeic, ferulic acid) (Williams et al., 1998). In fact, the highly unsaturated sphagnic acid (p-hydroxy-B-[carboxymethyl]-cinnamic acid) is among the most abundant degradation products of *Sphagnum* litter and is thought to inhibit decomposition in *Sphagnum*-dominated bogs (Rudolph and Samland, 1985; Williams et al., 1998). Because hydrogenation of C-C double-bonds also represents an electron sink, this mechanism could be important both for alleviating toxicity and as an electron sink to sustain microbial respiration of cellulosic material. Addition of 2[H] across a C-C double bond changes the C oxidation state by -1, i.e., the reduction of the double bond is electron accepting. Thus, reduction of unsaturated organic compounds acts as an electron and putative molecular hydrogen sink allowing CO₂ production without subsequent CH₄ generation. However, because metabolic hydrogen is in equilibrium with H_{2(aq)} (Ungerfeld and Kohn 2006), it may be that the biohydrogenation of double bonds serves as an alternative electron sink bypassing proton reduction which simply results in suppressing the formation of H₂.

The first phase of decomposition in anaerobic environments where inorganic TEAs other than CO₂ are not available has been dubbed the “acid phase”, in which fermentation produces fatty acids, alcohols, CO₂ and H₂ (Conrad 1999; Tchobanoglous and Kreith 2002). The predominant fermentation intermediates in peatland soils all represent potential electron sinks (with the exception of acetate as discussed in the introduction) (Tveit et al., 2015; Schmidt et al., 2015; 2016). Syntrophic bacteria then further degrade the alcohols and fatty acids to acetate or formate, CO₂ and H₂. In the commercial production of volatile fatty acids (VFAs) in anaerobic digesters, conditions are manipulated to maintain the system in the acid production phase. This is sometimes accomplished by addition of fresh organic matter substrate (e.g., Pervin et al. 2013; Vanwonterghem et al. 2015), which serves to overcome the thermodynamic constraints to H₂ production by increasing the concentration of the reactants (Ungerfeld and Kohn 2006) thereby driving the reaction towards VFA + H₂ production (Burdige 2006). In anaerobic incubations and in peatlands where fresh organic matter enters the anaerobic zone slowly or not at all, the acid production phase occurs very early in diagenesis. Then, if H₂ concentrations build up faster than organic matter is added, VFA concentrations increase, and an H₂ sink is required to allow syntrophic reactions to continue (Conrad 1999; Tchobanoglous and Kreith 2002). For example, at Stordalen Mire, the concentrations of VFAs build up only in the early-stage permafrost thaw habitats, but in the bog habitat examined in this study—which is a more mature thaw feature (Hodgkins et al., 2014; McCalley et al., 2014) — fermentation products such as acetate are maintained at very low levels (<10 μM and <0.1 μM respectively measured from 12 cm to 70 cm deep) or are below detection (butyrate and propionate). Concentrations of VFA's with reduced oxidation states at the Minnesota bog site are similarly low—propionate averaged 2.3 ± 7.5 μM and butyrate averaged 0.4 ± 1.3 μM—indicating that at the sites investigated in this study, fermentation is coupled to VFA consuming reactions which require concomitant H₂-consuming reactions to be thermodynamically favorable.

Much of the anaerobic food chain and electron flow pathways in peatlands remain as a “black box”, with the majority of studies of fermentation and syntrophy conducted in the laboratory (Schmidt et al., 2016). However, a

growing body of microbiological evidence from the laboratory and field corroborates our environmental metabolomics results pointing to unsaturated organic compounds as an electron sink and possibly an H₂ sink. Nearly all microbial groups detected in abundance in freshwater wetland soils under anaerobic conditions have been shown to be capable of the fermentation or respiration of aromatic compounds (Schink and Stams, 2006; Sieber et al., 2012). Acetogens and other syntrophs, sulfate-reducers, and metal-reducers were all shown to respire organic electron acceptors such as fumarate in pure culture (Sieber et al., 2012). Fumarate reduction involves hydrogenation of a double bond to produce succinate (Kroger et al., 2002). Of these groups, classic syntrophs (*Syntrophococcus*, *Clostridium*) and acetogens (*Acetobacterium*) are capable of the fermentation or respiration of aromatic acids as the sole electron acceptor (Bache and Pfennig, 1981; Tschech and Pfennig, 1984; Krumholz and Bryant, 1986; Imkamp and Muller, 2002).

Microbiological studies from the S1 bog in Minnesota and from Stordalen Mire in Sweden corroborate environmental metabolomics results from the field. The most abundant microbial taxa detected in the anaerobic catotelm of the S1 bog are groups which are capable of utilizing organic electron acceptors, particularly fumarate (Lin et al., 2014a,b; 2015). A combination of amplicon sequencing and metagenomics data showed that novel members of the Thaumarchaeota, which are among the most abundant taxa in the S1 catotelm, are syntrophic and capable of fumarate respiration (Lin et al., 2014a; 2015). In that study, members of the Syntrophobacterales and Geobacter groups increased in the catotelm of S1 bog where our transform data indicate hydrogenation of organic matter is important. These groups which mediate fermentation, syntrophy, and utilize fumarate as the sole electron acceptor by direct interspecies electron transfer (Sieber et al., 2012; Wang et al., 2016) are also prevalent at Stordalen Mire (Mondav and Woodcroft et al. 2014, supplemental data). Furthermore, Tfaily et al. (2015) identified a unique fluorescent chromophore in pore waters from the nearby Glacial Lake Agassiz peatlands in Minnesota that appeared to be a microbial by-product originating from humic matter, consistent with the microbial transformation of unsaturated compounds. Increasing alkylation of dissolved organic matter with increasing depth was also observed in the Glacial Lake Agassiz peatlands which is consistent with our hypothesis of increasing hydrogenation of organic matter (Tfaily et al., 2013).

Mechanistically, the FTICRMS results only show that an addition of 2 hydrogens is occurring; we cannot be certain that molecular hydrogen, H₂, is being consumed in these reactions. Tracer studies in the rumen with labeled H in water, glucose, formate and succinate have indicated the H from these organic molecules does not come from molecular H₂ added across the double bond to saturate it, but rather the H that is added comes from water which acts as a shuttle accepting and donating hydrogens. These studies indicate the addition of a proton and a hydride ion with NADH serving as an electron donor (Rosenfeld and Tove 1971; Jenkins et al. 2007). These authors clearly state, however, that their results do not “preclude the direct reduction of a carrier by an organic substrate if the hydrogen undergoes rapid exchange with water”. Similarly in hydrogenotrophic methanogenesis (Eq. (1)), during the hydrogenation of CO₂ to form CH₄—which is often written as a reduction of H₂: CO₂ + 4H₂ → CH₄ + 2H₂O—the H is apparently actually derived from or is at least in isotopic equilibrium with water (Whiticar et al., 1986; Waldron et al., 1999; Whiticar 1999; Chanton et al., 2006). Thus it remains unclear in all of these

transformations whether H_2 itself is reduced during the hydrogenation step or whether the actual hydrogen comes from another source (e.g., water) with organic matter supplying the electrons.

The diversity of the hydrogenation process—where H_2 is used as the electron carrier—and its widespread distribution is supported by the recent discovery of hydrogenase genes in 51 bacterial and archaeal phyla that are ubiquitous in aquatic and terrestrial habitats (Greening et al. 2015). Hydrogenases actively catalyze H_2/H_2O exchange in addition to catalyzing reversible reactions with H_2 as reactant or product (Vogt et al., 2008). Greening et al. (2015) note that H_2 was the first electron donor for life, and that the great diversification of hydrogenases may have allowed the growth and survival of microbiota across many ecosystems. Thus it seems plausible that H_2 could be the electron carrier for the hydrogenation mechanism identified in this study. In any case, metabolic hydrogen is in equilibrium with $H_{2(aq)}$ and thus the implications for the thermodynamics of the system are the same whether H_2 is the direct participant or not (Ungerfeld and Kohn 2006).

As described above, organic matter has previously been proposed as a potential electron acceptor for microbial respiration in a variety of aquatic environments including peatlands (Lovley et al. 1996; Coates et al. 2002; Kappler et al. 2004; Keller et al. 2009; Roden et al. 2010; Smemo and Yavitt 2011; Keller and Takagi 2013). While previous evidence pointed to the potential for quinone moieties to serve as the active electron accepting site (Lovley et al., 1996; Kappler et al., 2004; Keller et al., 2009; Roden et al., 2010; Blodau and Deppe 2012; Keller and Takagi 2013) quinone reduction is a reversible reaction that requires reoxidation to sustain high CO_2 production rates. The prevalence of the $+2[H]$ reaction in both the field and incubation samples, and the diversity of substrates consumed by this mechanism (Fig. 3), supports the idea that reduction of C-C double bonds can serve as an abundant hydrogen sink and TEA. Unlike other TEAs which are quickly depleted in the shallow subsurface, this electron sink may indeed be quite large, less easily depleted, and therefore able to sustain prolonged secondary fermentation associated with the breakdown of the large peatland organic matter pool, leading to sustained high CO_2 production. The hydrogenation reaction may additionally serve to reoxidize quinones or other TEAs replenishing their electron accepting capabilities (Nilsson and Öquist 2009). Furthermore, observations of modern C fueling CO_2 and CH_4 production in deep peat (Chanton et al., 2008; Corbett et al., 2013; Wilson et al. 2016) suggest that downward advection of DOM through the peat column may refresh organic C stores enhancing their ability to act as a large electron sink in the catotelm.

Because CH_4 has a sustained flux global warming potential approximately 45 times that of CO_2 on the 100-y timescale (Neubauer and Megonigal 2015), the ratio of $CO_2:CH_4$ emissions from peatlands has far-reaching implications for understanding the role that these systems play in future climate change. As peatlands thaw, mechanisms, such as the one proposed in this paper which have the potential to control $CO_2:CH_4$ emission ratios, will become increasingly pertinent to global climate modeling efforts.

This proposed mechanism does not preclude the involvement of quinone moieties as intermediate electron acceptors in catotelm peat, however. In fact, it is entirely possible that quinone reduction could be a mechanism of electron transport and would be consistent with previous evidence of quinone reduction in such systems (Roden et al. 2010; Keller and Takagi 2013, etc). We suggest that quinone reduction does not represent a terminal electron accepting step, however. As indicated by Hughes and Tove (1980) reduced quinones (quinols) could be regenerated

by reducing C-C double bonds in organic matter, thereby themselves being re-oxidized and becoming available for further electron shuttling capabilities (Keller and Bridgham, 2007; Keller et al., 2009). The hydrogenation step we propose could be a mechanism for reoxidizing quinones or small quantities of other TEAs thereby sustaining their electron accepting capabilities (e.g. Nilsson and Öquist 2009).

Because the potential pool of unsaturated C compounds is large, such quinone recycling has the potential to be a significant control on the ratio of CO₂ to CH₄ produced during anaerobic decomposition in peatlands and appears to be rapidly renewed with downward vertical advection of surface DOM. The hydrogenation mechanism proposed in this study therefore links microbial respiration with the reduction of large (150-700 Da) ubiquitous unsaturated organic compounds and has the potential to control long-term CO₂:CH₄ emission dynamics from peatlands by promoting CO₂ over CH₄ production while serving as an electron sink.

Further support that the proposed hydrogenation mechanism can be used to explain the CO₂:CH₄ imbalance could be garnered from a quantitative mass balance. Unfortunately, FTICRMS is currently not a quantitative technique thus such a mass balance is currently unavailable. Future work should focus on improving the quantification of the hydrogenation transform to determine whether or how much of the CO₂:CH₄ imbalance could potentially be explained by this mechanism.

Thus far we have limited our investigation to the dissolved organic matter pool, however, the solid phase peat itself represents an even larger reservoir of organic C that may also have the potential to act as an electron sink (Roden et al., 2010; Keller and Takagi, 2013). Application of the FTICRMS technique to strictly solid phase samples is still under development and quantification of this and the DOM electron sinks will, in part, require identification of those compounds that are most susceptible to +2[H] addition. However, results of our previous analyses of these and other peatland sites over more than a decade have indicated that CO₂ and CH₄ production in these systems are more strongly influenced by DOM than solid phase organic C dynamics (Wilson et al., 2016; Tfaily et al. 2014; Corbett et al. 2013, Chanton et al. 2008; Chasar et al. 2000a) thus we feel that our focus on the DOM in this study is highly relevant to understanding the CO₂:CH₄ production dynamics.

It is possible that fluctuating water table depths could transiently expose peat within the mesotelm to O₂ changing redox conditions and reoxidizing TEAs that were previously reduced, recycling them for further oxidation and sustaining microbial respiration (Bridgham et al. 2013). Such a mechanism could allow sulfate or iron reduction in spite of very low measured concentrations of these potential TEAs by rapid turnover (Keller and Bridgham 2007). However, the field samples collected from Stordalen Mire were taken from below the mesotelm of peat that is permanently inundated and the closed incubations were highly anaerobic with little chance of changing redox conditions. Thus reoxidation events are unlikely to be influencing our present results and explaining the elevated CO₂:CH₄ observed in these samples.

FTICRMS environmental metabolomics data in this study combined with the metagenomics results together suggest a new view of peatland anaerobic decomposition, in which organic electron acceptors, that potentially scavenge H₂ and maintain electron flow in boreal peatlands, regulate the CO₂:CH₄ production ratio in these systems (e.g., Blodau and Deppe 2012). This mechanism challenges the conventional understanding of controls on CO₂:CH₄ production in TEA-depleted environments by explaining the sustained breakdown of organic

matter to CO₂ without concomitant CH₄ production. This work, thus, provides an important step in understanding the greenhouse gas balance of these systems and the microbial and thermodynamic determinants of feedbacks that may play an important role in future climate change.

Data Availability Data are available publicly from the Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, U.S. Department of Energy, Oak Ridge, TN U.S.A. at: *DOI pending*.

The authors declare that they have no conflict of interest.

Acknowledgements

This material is based upon work supported by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research. Oak Ridge National Laboratory is managed by UT-Battelle, LLC, for the U.S. Department of Energy under contract DE-AC05-00OR22725. Work in Sweden was funded by the US Department of Energy Office of Biological and Environmental Research under the Genomic Science program (Awards DE-SC0004632 and DESC0010580). Work in Minnesota was supported by the Terrestrial Ecosystem Science (TES) Program, under U.S. Department of Energy contracts # DE-SC0012088 and DE-SC0014416. We'd like to thank the Environmental Molecular Sciences Laboratory at Pacific Northwest National Laboratory (Richland, WA) for access to their 12T FTICRMS and for support to MM Tfaily. We thank the Abisko Scientific Research Station for sampling infrastructure and Kelsey Crossen for assistance with the microbial data from Sweden. SR Saleska and VI Rich received support through the Ecosystem Genomics Initiative, by the University of Arizona Technology and Research Initiative Fund, through the Water, Environmental and Energy Solutions Initiative.

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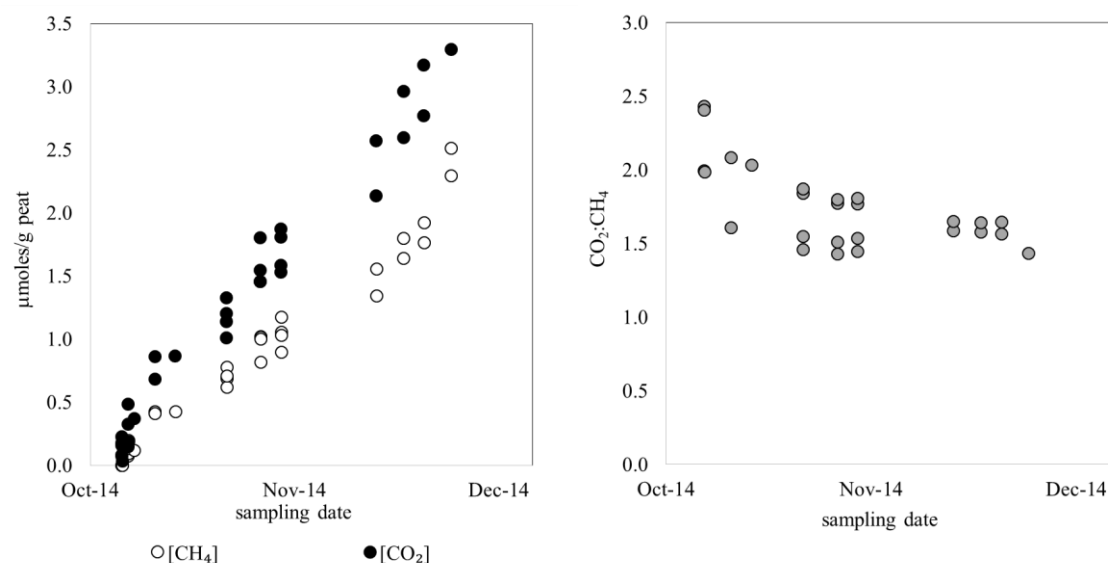


Fig. 1: CO_2 and CH_4 production (a) and $\text{CO}_2:\text{CH}_4$ production ratios (b) in the incubation experiment over time. Incubation days start counting following the pre-incubation period (day 0). Symbols indicate individual replicates measured over time. The number of replicates decreases over time as incubation vials were sacrificed for destructive sampling described in section 2. Materials and methods.

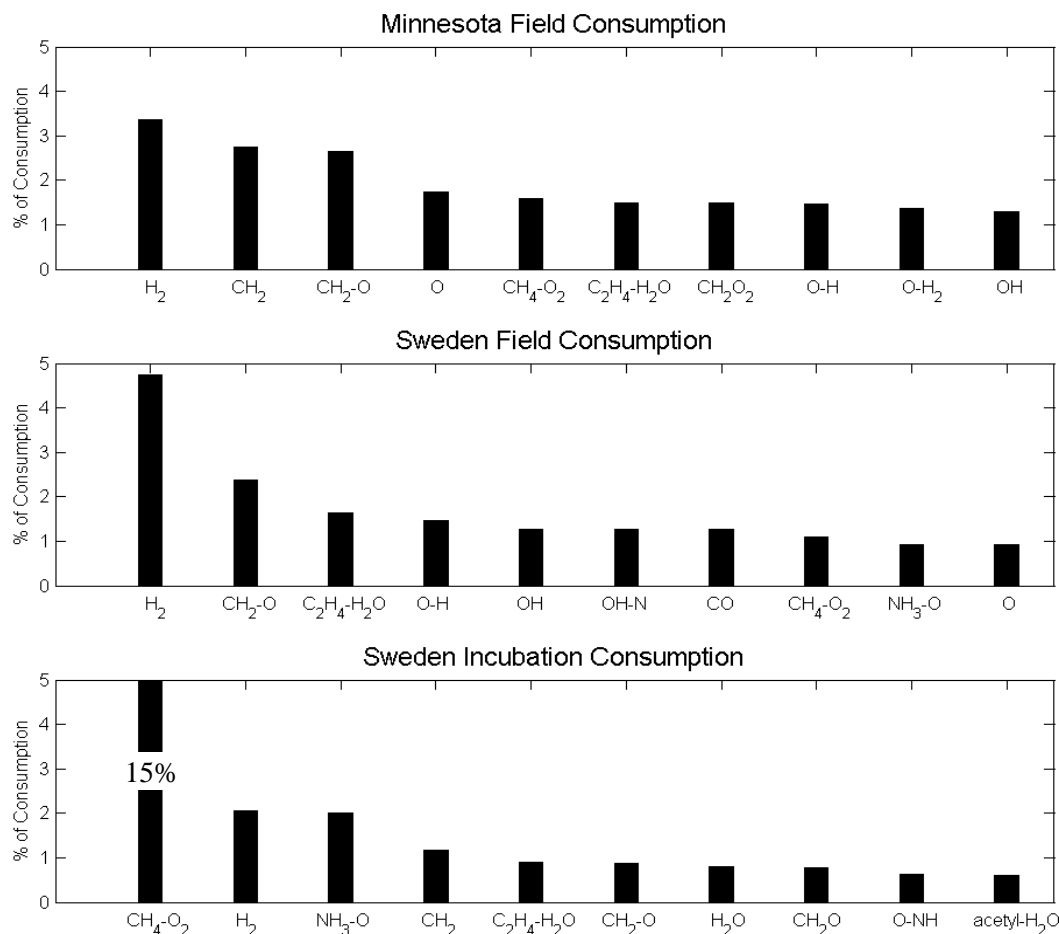


Fig. 2 Transform results in the field based on differences between shallow and deep samples for the Minnesota (a) and Sweden (b) peatlands. Panel (c) presents transform results for the incubations based on differences between day 0 and day 50 of the incubations. Transforms are ordered by frequency for each site. The dash (-) indicates exchange, for example CH₂-O indicates a net exchange of CH₂ for O in the molecule. Only the top ten most frequent transforms are shown. Note axes are truncated to 5% for comparison purposes, however, in the incubations graph CH₄-O₂ accounted for 15% (off scale) of total consumption. This transform is thought to represent the accumulation of -2[H], -CH₂, and +O₂.

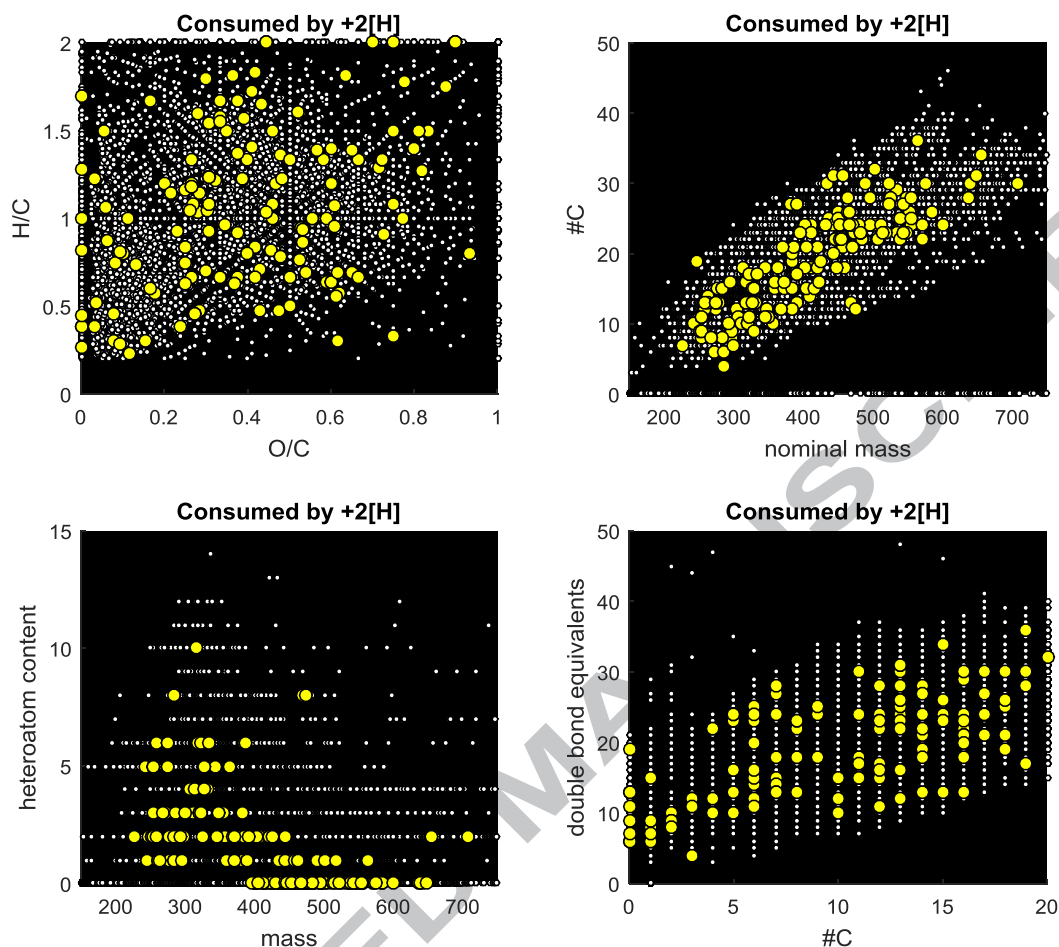


Fig. 3: Characteristics of all consumed compounds (incubations and both field sites combined) highlighting those compounds that are linked to +2[H] addition. In all panels, the yellow symbols indicate compounds that are consumed via addition of +2[H] and the grey symbols represent the molecular characteristics of all other compounds consumed by mechanisms other than +2[H]. Panel (A) presents the van Krevelen diagram, Panel (B) presents the number of C atoms (#C) vs. nominal mass, Panel (C) presents the total number of heteroatoms (N,S,P) vs. nominal mass, and Panel (D) presents the number of C atoms vs. double bond equivalents (DBE). No systematic differences between the chemical characteristics of +2[H] compounds (yellow) and those of compounds consumed by other mechanisms (grey) either in field sites or incubations was observed other than that there are fewer +2[H] additions at higher nominal mass and heteroatom content.

Table 1: CO₂:CH₄ Ratios reported from field and incubation studies. Peat type are indicated by the abbreviations CP = collapsed palsa feature, B = Sphagnum-dominated bogs, and F = sedge-dominated Fen sites, the site investigated by Wright et al. (2011) was a *Myrica-Cyrilla* bog-plain, indicated with an asterisk (*).

Reference	CO ₂ :CH ₄	Type of Study	Peat Type	Duration/depth
Hodgkins et al. (2014)	1.3 to 5	Incubation	CP, B, F	50 days
Hodgkins et al. (2015)	5 to 10	Field	CP, F, B	0-22 cm
McCalley et al. (2014)	1.2 to 5	Field	F, B	fluxes
Tfaily et al. (2014)	2 to 100	Incubation	B	75 days
Corbett et al. (2012)	6 to 15	Incubation	F,B	250-300 days
Chasar et al. (2000a,b)	4.6 to 12.8	Field	F,B	0-100 cm
Romanowicz et al. (1995)	0.6 to 1.3	Field	F,B	0 -250 cm
Valentine et al. (1994)	10 to 20	Incubations	F,B	28-42 days
Keller and Bridgham (2007)	1.9 to 17	Incubation	F,B	3-4 days
Wright et al. (2011)	10 to >20	Field	B*	< 200 cm
Updegraff et al. (1996)	3.8 to 882.6	Incubation	B	560 days
van Hulzen, et al. (1999)	1.7 to 10	Incubation	F	100 days

Figure Legends:

Fig. 1: CO₂ and CH₄ production (a) and CO₂:CH₄ production ratios (b) in the incubation experiment over time. Incubation days start counting following the pre-incubation period (day 0). Symbols indicate individual replicates measured over time. The number of replicates decreases over time as incubation vials were sacrificed for destructive sampling described in section 2. Materials and methods.

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Highlights

- Organic matter hydrogenation provides an electron sink during anaerobic decomposition
- This mechanism sustains CO₂ production and suppresses methanogenesis
- Thus this mechanism controls climate feedbacks by altering CO₂:CH₄ production ratios

ACCEPTED MANUSCRIPT