1 Peatland types influence the inhibitory effects of a humic substance analog on methane
2 production

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Abstract: Methane (CH₄) production is often impeded in many northern peatland soils, although competitive inorganic terminal electron acceptors (TEAs) used in anaerobic respiration are usually present in low concentrations in these soils. Recent studies suggest that humic substances in wetland soils can be utilized as organic TEAs for anaerobic respiration and may directly inhibit CH₄ production. Here we utilize the humic analog anthraquinone-2, 6-disulfonate (AQDS) to explore the importance of humic substances, and their effects on the temperature sensitivity of anaerobic decomposition, in two peatland soils. In a bog peat, AQDS was not initially utilized as a TEA, but greatly inhibited the fermentative production of acetate, carbon dioxide (CO₂), and hydrogen (H₂), as well as CH₄ production. When added together with glucose, AQDS was partially reduced after a lag period of 5 to 10 days. In contrast, no inhibitory effect of AQDS on fermentation was found in a fen peat and AQDS was readily reduced. The addition of glucose and AQDS to both bog and fen peats caused complicated temporal dynamics in the temperature sensitivity of CH₄ production, reflecting temporal changes in the temperature responses of other carbon processes with effects on methanogenesis. Our results, for the first time, show that the inhibitory effects of a humic analog on CH₄ production depend on peatland type, acting primarily as a toxic compound in bog soils and as a competitive TEA in fen soils. The results also suggest that the high concentrations of humic substances in northern peatlands may greatly influence the effect of climate change on soil carbon cycling in these ecosystems.

Keywords: Anaerobic decomposition, Methane production, Humic substances, Anthraquinone-2, 6-disulfonate (AQDS), Temperature sensitivity, Peatland

1. Introduction

Wetlands store globally significant amounts of soil organic carbon (SOC) (Maltby and Immirzi, 1993), which may decompose to either carbon dioxide (CO₂) or methane (CH₄) via anaerobic metabolism. Given that on a mass basis CH₄ has a global warming potential 28 to 34 times greater than CO₂ over 100 years (Myhre et al., 2013), the ratio of CO₂:CH₄ produced during anaerobic C decomposition may have substantial impacts on the Earth's future climate. It is therefore essential to understand the fundamental controls over organic C mineralization to CO₂ and CH₄ in these systems.

Rates of anaerobic C mineralization and the ratio of its end products, CO₂ and CH₄, are the result of a suite of complicated interactions among multiple microbial functional groups (Bridgham et al., 2013; Megonigal et al., 2004). In the absence of oxygen, organic polymers are firstly hydrolyzed and degraded to alcohols, short-chain fatty acids, CO₂, and dihydrogen (H₂) during primary fermentation. Syntrophic bacteria convert the alcohols and short-chain fatty acids to acetate, H₂, and CO₂. Subsequently, acetate and H₂ are utilized for microbial respiration and methanogenesis. In general, microbes will preferentially use a variety of thermodynamically favorable terminal electron acceptors (TEAs), such as nitrate (NO₃⁻), iron (Fe(III)), manganese (Mn(III, IV)), and sulfate (SO₄²⁻), for respiration before CH₄ production becomes important, which results in a higher ratio of CO₂:CH₄ production. The relative importance of TEAs for anaerobic respiration will depend on the initial pool size of TEAs and the rate of electron supply from fermentation, which in turn depends on the availability of labile organic compounds and the temperature dependence of anaerobic respiration. After these TEAs have been depleted, methanogens use either acetate (acetoclastic methanogenesis) or CO₂/H₂ (hydrogenotrophic methanogenesis) to produce CH₄, resulting in an approximately equal

molar production of CO₂ and CH₄ (Conrad, 1999). CO₂ acts as a TEA in hydrogenotrophic methanogenesis, but here we more narrowly use the term TEAs to refer to those compounds that accept electrons in CO₂-generating respiratory reactions.

Despite northern peatlands generally having low concentrations of inorganic TEAs (Keller and Bridgham, 2007; Vile et al., 2003a), their ratio of CO₂:CH₄ production is often much greater than 1 (Duddleston et al., 2002; Hines et al., 2001; Keller and Bridgham, 2007; Ye et al., 2012). Moreover, the production ratio of CO₂:CH₄ can vary by several orders of magnitude among different types of peatlands suggesting distinctive pathways and controls of anaerobic decomposition (Bridgham et al., 1998; Hines et al., 2008; Ye et al., 2012). To date it is not clear what ultimately limits CH₄ production and causes the large variations of CO₂:CH₄ production in northern peatlands (Bridgham et al., 2013), but there is a growing consensus that these patterns cannot be explained by respiration associated with inorganic TEAs.

Humic substances have been hypothesized to play multiple roles in anaerobic C cycling beyond their effect as organic electron donors for decomposition. Humic substances are traditionally thought to be a unique, heterogeneous class of macromolecules, yet recent research suggests that they are collections of relatively small molecules derived from biological materials (Lehmann et al., 2008; Piccolo, 2002; Sutton and Sposito, 2005). Irrespective of the exact chemical nature of humic substances, aromatic substances have been shown to occur at high concentrations in peatlands (Collins and Kuehl, 2001; Tfaily et al., 2013). It is well recognized that humic substances can act as organic TEAs (Cervantes et al., 2000; Keller et al., 2009; Klupfel et al., 2014; Lovley et al., 1996). Galand et al. (2010) hypothesized that the unequal production of CO₂ and CH₄ in peat soils results from the reduction of humic substances as TEAs and that this process is more significant in bogs than in rich fens (Galand et al., 2010). Keller

and Takagi (2013) verified in a bog soil that organic TEAs could explain a significant fraction of the CO_2 produced during anaerobic respiration and that CH_4 was not produced until the electron-accepting capacity of the organic TEAs was exhausted. Recent research has shown that humic substances are able to oxidize sulfur species, promoting sulfate reduction and contributing to high $\text{CO}_2:\text{CH}_4$ production ratios (Heitmann and Blodau, 2006; Minderlein and Blodau, 2010). Humic substances can also promote iron reduction in wetland sediments by serving as electron shuttles (Roden et al., 2010). The use of humic substances as TEAs in the process of anaerobic CH_4 oxidation in peatland soils has also been hypothesized (Gupta et al., 2013).

It is generally believed that quinone moieties contained in humic substances are important electron-accepting groups (Aeschbacher et al., 2009; Ratasuk and Nanny, 2007; Scott et al., 1998). Quinone moieties are aromatic molecules with an even number $-\text{C}(=\text{O})$ groups, which upon reduction are converted into phenolic groups (Cory and McKnight, 2005). Humic respiration has been frequently investigated with a functional analog, anthraquinone-2, 6-disulfonate (AQDS) in many systems (Cervantes et al., 2000; Keller et al., 2009; Lovley et al., 1996). AQDS reduction (i.e., quinone respiration) to anthrahydroquinone-2,6-disulfonate (AHQDS) is thermodynamically more favorable than methanogenesis, which should lead to an increase in $\text{CO}_2:\text{CH}_4$ production ratio in soils where AQDS-like humics are being utilized for microbial respiration (Cervantes et al., 2000). Keller et al. (2009) demonstrated that additions of AQDS to wetland soils resulted in decreased CH_4 production and increased ratios of $\text{CO}_2:\text{CH}_4$, although this pattern was confounded by changes in pH. Amendment of AQDS to Arctic peat soils also stimulated iron reduction and resulted in higher production ratios of $\text{CO}_2:\text{CH}_4$ (Lipson et al., 2010).

The large variety of aromatic compounds in peatlands (Tfaily et al., 2013) may also have direct inhibitory effects on various microbial groups due to their high concentrations of phenolic and quinone moieties. For example, the addition of a “humic”-rich peat extract was found to be inhibitory to CO₂ production, sulfate reduction, and methanogenesis, but not to acetogenesis in a bog soil (Minderlein and Blodau, 2010). Phenols suppress C mineralization by inhibiting microorganisms, binding proteins and polysaccharides, and inactivating enzymes (Freeman et al., 2012; Harborne, 1997). These compounds are oxidized by phenol oxidase (often back into quinone moieties), but the activity of these enzymes is constrained by the low oxygen availability, low pH, and low temperature common to many peatlands (Freeman et al., 2012; Limpens et al., 2008). These factors along with vegetation with high foliar phenolic concentrations (Bragazza et al., 2013) often cause very high soluble phenol concentrations in the porewater of many peatlands. Moreover, *Sphagnum* mosses, a dominant component of the plant community in many peatlands, contain high concentrations of unique polyphenolic compounds that have long been known to have antibiotic properties (McClymont et al., 2011; van Breemen, 1995; Verhoeven and Toth, 1995). Thus, quinone compounds have important roles in soils as TEAs and in their reduced state as potential inhibitory compounds. Quinone compounds also have strong antibiotic effects in their oxidized state (Monks et al., 1992). For example, Cervantes et al. (2000) suggested that AQDS may have a direct toxic effect on methanogens in some sediment.

While it is apparent that humic substances can potentially influence anaerobic C mineralization in multiple ways, untangling these multiple roles in peatland decomposition remains a challenge. We have recently observed different rates of CO₂ and CH₄ production in soils from six peatland types across a hydrogeomorphic landscape gradient, ranging from

ombrotrophic bogs (i.e., atmospheric inputs only) to minerotrophic rich fens (i.e., with surface- or groundwater inputs) even when incubated at common pHs (Ye et al., 2012). All of the peats contained minimal concentrations of inorganic TEAs, yet none of them exhibited the equal molar production of CO₂ and CH₄ expected under methanogenic conditions during a 43-day incubation. Bog peats exhibited particularly high CO₂:CH₄ production ratios (Ye et al., 2012). We hypothesized that humic substances in these peats were particularly inhibitory to methanogens.

Temperature is another important control over anaerobic respiration, CO₂:CH₄ production ratios, and CH₄ emissions; however, it is unclear to what extent the multiple roles of humic substances affect these temperature relationships. Reported apparent Q₁₀s for CH₄ production vary greatly in wetland soils, ranging from 1.3 to 28 (Segers, 1998). Better defining the temperature response of overall anaerobic C cycling and CH₄ production was recently identified as a major impediment to modeling CH₄ emissions from wetlands in response to climate change (Bridgman et al., 2013). van Hulzen et al. (1999) suggested while the processes controlling CH₄ production (i.e., TEA reduction, fermentation reactions, and methanogenesis) all have intrinsic Q₁₀s of ~ 2, typical for microbial processes, their complex temporal dynamics could give very high apparent Q₁₀s for CH₄ production. In direct opposition to this hypothesis, a meta-analysis recently showed that CH₄ production from pure cultures of methanogens had similar Q₁₀s to CH₄ production in sediments and CH₄ emissions (Yvon-Durocher et al., 2014). Additionally based upon net emission data, this study suggested that the temperature response for CH₄ production was substantially greater than that for CO₂ production at an ecosystem level. Thus, the mechanisms controlling the temperature dependence of anaerobic respiration is an important topic, and the role of humic substances in this regard remains largely unstudied.

In the present study, we used the humic analog AQDS to examine 1) inhibitory effects of humic substances on CH₄ production in bog and rich fen soils, 2) whether the inhibitory effects differs in the two soils, and 3) how the inhibitory effects, in combination with substrate availability, influence the temperature responses of anaerobic decomposition, including methanogenesis.

2. Methods and materials

2.1. Site description

We collected soil samples from 0 to 15 cm below the water table from a bog and a rich fen in the Upper Peninsula of Michigan, USA in June 2011. The bog (N46°6'6", W88°16'25") had a soil pH of 3.7 and a peat depth of ~3.8 m with an average water table of -27 cm during the growing season (water tables were measured in hollows). It is dominated by > 90% of cover of *Sphagnum* spp. mosses with stunted (< 1-m height) ericaceous shrubs such as leatherleaf (*Chamaedaphne calyculata* (L) Moench), small cranberry (*Vaccinium oxycoccos* L.), and bog Labrador tea (*Rhododendron groenlandicum* Oeder), and scattered low-stature black spruce (*Picea mariana* (Mill.) Britton, Sterns & Poggen). The rich fen (N46°13'27", W89°29'53") had a soil pH of 5.9 and a peat depth of ~ 6.4 m with consistent standing water. It is dominated by upright sedge (*Carex stricta* Lam.) tussocks with leatherleaf also present on the tussocks.

Previous studies have extensively examined the anaerobic C cycling dynamics of peatlands in this area, including the relative predominance of methanogenic pathways, importance of homoacetogenesis, effect of pH, substrates dynamics, and use of humic substances as a terminal electron acceptor (Keller and Takagi, 2013; Ye et al., 2012; Ye et al., 2014), which

provide a context for the current study. These sites were previously described as “Bog 1” and “Rich Fen” in Ye et al. (2012).

2.2. Sample preparation

In June 2011, four soil cores were randomly extracted from hollows in each site with PVC pipes (10-cm diameter) to a depth of 15 cm below the water table (-14 cm) at the bog site or 15 cm below the soil surface at the rich fen site (water table + 15 cm). In situ soil temperatures at the time and depth of sampling were 5.1 °C in the bog and 10.1 °C in the fen, and the mean annual temperature of both sites is approximately 5 °C. Upon extraction, cores were intermediately capped after filling with porewater to prevent oxidation of the peat and transported on ice to our laboratory at the University of Oregon. The cores were stored at 4 °C and used within a week after collection.

Peat was processed in a glove box (Coy Laboratory Products Inc., Grass Lake, MI, USA) filled with 98% of N₂ and ~2% H₂ gas. Following the removal of green vegetation, large roots, and woody material, cores from the same site were combined and homogenized in a food processor with degassed deionized water at a peat:water ratio of 5:19 by mass. A subsample was collected and dried at 60 °C for 3 days to determine the moisture content. Subsamples of ~24 mL of the homogenized peat slurries were transferred to 125-mL serum bottles, which were capped with butyl septa and incubated at room temperature (22 ± 1 °C) in the dark for 15 days to reduce any electron acceptors that were initially present.

2.3. Laboratory experiment

After pre-incubation, each sample was amended with one of the following treatments: 1) control (as water), 2) 1.4 mM glucose, 3) 10.2 mM AQDS or 4) 1.4 mM glucose plus 10.2 mM AQDS. The purpose of the glucose was to act as a ready source of electrons for AQDS reduction to decouple this from the lability of the two different peats. Glucose was added at a rate not larger than the CO₂ production from these soils (Ye et al., 2012), while AQDS was added as suggested by documented literature (Cervantes et al., 2000). All treatments were added as 11 mL of degassed solutions by direct injection in the anaerobic glove box and were mixed well with peat slurries by gently shaking, followed by bubbling the slurries with oxygen-free N₂ gas for 10 minutes. Parallel samples were incubated at 7°C, 15°C, and 25°C in the dark. Four replicates of each treatment incubated at each temperature were destructively sampled (see below) on the 2nd, 5th, 10th, 15th, 30th, and 45th day of incubation.

2.4. CO₂, CH₄, and H₂ measurement

Bottles were shaken gently to release trapped gas bubbles before sampling. Headspace concentrations of CO₂ and CH₄ were analyzed by gas chromatography using a flame ionization detector equipped with a methanizer (SRI Instruments, Torrance, CA, USA). Headspace H₂ concentrations were measured with a Peak Performer gas chromatograph with a reducing compound photometer (Peak Laboratories, Mountain View, CA, USA). Total CO₂ and CH₄ production rates were calculated from the cumulative production in both gas and liquid phases up to a sampling point divided by the time of incubation, adjusting for solubility, headspace pressure, temperature, and pH (Stumm and Morgan, 1995).

2.5. Water chemistry

Following gas measurement, 10 mL of water was collected from each sample and centrifuged at 5,000 rpm for 5 minutes in the glove box. Aliquots of the water sample were used for quantifications of reduced AQDS (AHQDS) and glucose, while the remaining sample was frozen at -20°C for acetate analysis. Reduced AQDS was determined as described by Cervantes et al. (2000) with slight modifications. In brief, 1 mL of water from the incubation was mixed well with 2 mL of degassed 60 mM bicarbonate buffer, pH 6.7, in a 5 mL cuvette, followed by measurements of the absorbance at 450 nm with a spectrophotometer (Genesys 5, Thermo Scientific, Waltham, MA, USA). Stock AHQDS standards were obtained by chemically reducing AQDS with dithionite, while working standards were prepared by serially diluting the stocks with degassed water. All procedures were performed anaerobically in the glove box. Glucose concentration was determined colorimetrically (Fournier, 2001), and acetate was analyzed with a Dionex DX500 ion chromatography system equipped with a HC-75 (H⁺) column (Hamilton Company USA, Reno, NV, USA) and a Dionex AD20 absorbance detector (Dionex Corporation, Bannockburn, Illinois, USA). pH was measured at each sampling point, and did not differ between treatments within a peat type (data not shown).

2.6. Temperature sensitivity of CO₂ and CH₄ production and AQDS reduction

Temperature sensitivity was described by activation energy (E_a), based on the Arrhenius equation:

$$y = A * e^{-E_a/RT}$$

where y is the production rate at various temperatures (7, 15, and 25°C), A is a constant, R is the universal gas constant (8.314 J mol⁻¹ K⁻¹), and T is the absolute temperature (K). E_a was estimated as the slope of the linear regression of the natural logarithm of y and -1/RT. The

production rates were calculated from cumulative production of CO₂ and CH₄, or the cumulative reduction of AQDS (measured as the production of AHQDS), up to a sampling point divided by the incubation time and the dry weight of the soil, and expressed as $\mu\text{m production g}^{-1} \text{ soil d}^{-1}$. Data generally fit the Arrhenius equation excellently ($R^2 > 0.96$) except in cases where inhibition or depletion of glucose over time was evident (see Results).

2.7. Statistical analyses

Results were analyzed with the MIXED procedure of SAS 9.1 (SAS Institute). Tukey's test was conducted to determine significant pairwise differences at $\alpha = 0.05$. The data were tested for normality and log-transformed if the transform resulted in significant improvements in the overall distribution. Regressions to estimate E_a and its standard error were done in Microsoft Office Excel 2010 (Redmond, WA). For cumulative production of the 45 day incubation, differences in E_a relative to the control treatment were done with the "slopestest" function (<http://www.real-statistics.com>) in Excel.

3. Results

3.1. AQDS reduction

Background AHQDS concentrations in treatments without AQDS amendment were minimal (consistently $< 0.1 \text{ mM}$) (Fig. 1), suggesting that measured increases in AHQDS accurately approximated AQDS reduction. AHQDS concentrations were only slightly greater in the bog peat in the AQDS treatment at all temperatures, and in the glucose + AQDS treatment at 7°C (0.15 to 0.21 mM), and did not increase through time (Fig. 1a, b, c). AHQDS production

increased in the glucose + AQDS treatment after a lag period of 10 days at 15°C and of 5 days at 25°C (Fig. 1b, c). In the rich fen peat, AHQDS concentrations in peats amended with AQDS, with or without glucose, increased from day 2 to the last day of the experiment and were highest at 25°C (Fig. 1d, e, f). Regardless of temperature and time, AHQDS concentrations were generally higher in the rich fen peat when both glucose and AQDS were added than when AQDS was added alone.

3.2. Glucose concentration

Glucose concentrations were generally higher in the control than in the AQDS treatment in both the bog and fen peat, although the difference was not always significant (Fig. 2). In bog peat, adding only glucose increased its concentration in the early stages of the experiment relative to the control, but the difference diminished as the experiment continued and disappeared on day 30 at 7°C, on day 10 at 15°C, and on day 5 at 25°C (Fig. 2a, b, c). However in the glucose + AQDS treatment, the difference persisted through the entire incubation at 7°C and disappeared only at day 30 at 15°C and 25°C (Fig. 2a, b, c). In contrast, added glucose (with or without AQDS) was rapidly consumed in the rich fen peat such that its concentration was not different from the control by day 2 at 25°C and by day 5 at 7°C and 15°C (Fig. 2d, e, f). Glucose concentrations in rich fen peat in the glucose + AQDS treatment were less than the control after day 5 regardless of the temperature, although the difference was not always significant (Fig. 2d, e, f).

3.3. Acetate concentration

In the bog peat, acetate concentrations in the control increased constantly from day 2 to 45 and were generally greater at higher temperatures (Fig. 3a, b, c). Adding glucose greatly

promoted acetate accumulation, but adding glucose in combination with AQDS caused a lag period in acetate accumulation of 30 days at 7°C, 10 days at 15°C, and 5 days at 25°C. On days 30 and 45, acetate concentrations were similar in the glucose and glucose + AQDS treatments at 15°C and 25°C. In both the bog and fen peats, acetate was not detected at all temperatures during the entire course of the experiment when AQDS was added alone (Fig. 3). In the rich fen peat, acetate concentrations were consistently low ($< 12 \mu\text{M}$) in the control treatment, which was generally lower than the glucose and glucose + AQDS treatments (Fig. 3d, e, f). However, the difference disappeared by day 30 at 15°C and by day 10 at 25°C. Acetate concentrations were initially lower in the glucose + AQDS treatment than in the glucose treatment, but the difference was not significant by day 45 at 7°C, day 30 at 15°C, and day 10 at 25°C.

3.4. H_2 Partial pressure

In the bog peat, higher partial pressures of H_2 were generally observed at higher temperatures in the control (Fig. 4a, b, c). Addition of glucose increased H_2 partial pressures at all temperatures, with a maximum on day 30 at 7°C, on day 15 at 15°C, and on day 10 at 25°C. In contrast, addition of glucose with AQDS did not cause significant increases in H_2 at any temperature, except at 15°C after a 10-day lag period. H_2 partial pressures in both the bog and rich fen peats amended with AQDS alone were consistently $< 2 \text{ Pa}$ regardless of the temperature (Fig. 4). In the rich fen peat, addition of glucose increased H_2 production at all temperatures, with a maximal value on day 10 at 7°C and on day 5 at 15°C and 25°C (Fig. 4d, e, f). The H_2 partial pressures decreased at all temperatures after their peak and were all $< 2 \text{ Pa}$ at end of the experiment. H_2 partial pressures in the glucose + AQDS treatment were consistently very low.

3.5. CO_2 production rates

CO₂ production in both the bog and rich fen controls increased with temperature (Fig. 5). In the bog peat, addition of glucose caused a substantial increase in CO₂ production at all temperatures. In contrast, addition of AQDS generally caused lower CO₂ production relative to the control after day 2 at all temperatures, though the difference was not always significant. The glucose + AQDS treatment occasionally caused a small increase in CO₂ production at 15°C and 25°C. In rich fen peat, addition of glucose increased CO₂ production at all temperatures (Fig. 5d, e, f). Addition of glucose and AQDS caused even a larger increase in CO₂ production initially, though this stimulatory effect decreased through time. Addition of AQDS alone had no effect on CO₂ production, except for a small stimulatory effect on day 2 at 25°C.

3.6. CH₄ production rates

CH₄ production increased with temperature in both the bog and rich fen peats (Fig. 6). In the bog peat, addition of glucose did not result in significantly higher CH₄ production, except on day 45 at 7°C, day 5 at 15°C, and days 5 and 30 at 25°C (Fig. 6a, b, c). Amendment with AQDS, with or without glucose, decreased the rates of CH₄ production after day 5 at 7°C and after day 2 at both 15°C and 25°C. In contrast to the bog peat, addition of glucose in the rich fen peat increased CH₄ production rates at all temperatures during the entire course of the experiment (Fig. 6d, e, f). Addition of AQDS decreased CH₄ production after day 2 at all temperatures. However, there was no difference between the glucose + AQDS treatment and the control.

3.7. E_a for CO₂ and CH₄ production and AQDS reduction

The E_a for cumulative CO₂ production over the 45 day incubation in bog peat was similar in the control, AQDS, and AQDS + glucose treatments (Q₁₀ 1.8 to 2.6), but was lowest in the

glucose treatment (Table 1). However, the poor fit of the Arrhenius equation in the glucose treatment precluded a statistically significant difference between the glucose and control E_a 's. The Arrhenius equation fit poorly in the glucose treatment because of its complicated temporal dynamics in temperature sensitivity (Fig. 7a), with the E_a peaking at day 5 and decreasing to close to 0 by day 30. In contrast, the glucose + AQDS treatment had an increasing E_a through the first 15 days of the incubation and then leveled off, but it decreased somewhat on day 45. The E_a in the rich fen peat was similar in the control, glucose, and AQDS treatment (Table 1, Q_{10} 1.8 to 2.3), and was lowest in the glucose +AQDS treatment (Table 1, $Q_{10} = 1.5$).

In the bog peat, addition of glucose did not affect the E_a of cumulative CH_4 production relative to the control ($Q_{10} \sim 2.4$), but addition of AQDS with or without glucose eliminated the temperature response because of its inhibitory effect (Table 1). In the fen peat, addition of glucose did not change the E_a relative to the control, but addition of AQDS caused a marginally significant increase in the E_a of cumulative CH_4 production, which however was not observed when the AQDS was added with glucose (Q_{10} 1.8 to 2.7). Similar to CO_2 production, cumulative CH_4 production masked complicated temporal changes in the temperature sensitivity of methanogenesis (Fig. 7b, e). The E_a increased from days 2 to 10 and levelled off thereafter in the bog peat in a parallel manner in the control and glucose treatments, but there was a flat response except for one anomalous value on day 10 when AQDS was added with or without glucose (Fig. 7b). In this anomalous case, a small increase was observed at higher temperature in a flux that was close to the limit of detection. In the rich fen peat, the E_a of CH_4 production in the control and glucose treatments was generally similar except for the sharp drop in E_a from days 2 to 5 in the glucose treatment (Fig. 7e). Addition of AQDS decreased the E_a through day 15, but this effect was ameliorated by the addition of glucose with AQDS by day 10, and by day

30 in the AQDS only treatment. On the last day of the experiment, the E_a in the AQDS treatment was higher than the control.

When AQDS was added alone to bog peat, there was almost no temperature response in cumulative AQDS reduction (Table 1), because of low reduction rates at all temperatures (Fig. 1a, b). However, cumulative AQDS reduction showed a substantial temperature response in the AQDS + glucose treatment. The lag period in this treatment before substantial AQDS reduction (Fig. 1a, b, c) was evident also in the temporal dynamics of the E_a for AQDS reduction, with the E_a increasing steady after day 10 to a E_a of 88 by day 45 (Fig. 7C). In contrast in the fen peat, the temperature response of AQDS reduction was higher in the AQDS only treatment than in the AQDS + glucose treatment (Table 1). These two treatments also had very different temporal responses with the E_a increasing in the AQDS only treatment and decreasing in the AQDS + glucose treatment to day 10 and remaining relatively steady after that (Fig. 7f).

4. Discussion

Northern peatlands contain low concentrations of inorganic TEAs, and their reduction is generally not the major pathway for C mineralization (Keller and Bridgham, 2007; Vile et al., 2003b; Ye et al., 2012). Humic substances can be utilized as organic TEAs by humic-reducing microbes in many systems (Cervantes et al., 2000; Keller et al., 2009; Lovley et al., 1996), and it has recently been shown that the reduction of both dissolved and solid phase humic substances can account for a significant fraction of CO_2 production in a bog soil (Keller and Takagi, 2013). In the present study, all peat samples were collected below the water table and processed anaerobically and we assume that after the 15-day pre-incubation period, reduction of

endogenous inorganic and organic TEAs was likely minimal, as supported by measurements under similar laboratory conditions in Ye et al. (2012). Thus, in our experimental protocol, added AQDS was likely the most abundant TEA that could be used for respiration which allowed us to explore the role of this humic analog as both an organic TEA and a potentially inhibitory compound.

4.1. Anaerobic decomposition

There was no evidence of substantial AQDS reduction in the bog peat when it was added alone (Fig. 1a, b, c). Instead AQDS had a severe inhibitory effect on fermentation and respiration reactions (summarized in Table 2), with acetate concentration below detection (Fig. 3a, b, c), very low H_2 partial pressures (Fig. 4a, b, c), and suppressed rates of CO_2 production (Fig. 5a, b, c). As expected, we observed increased fermentative production of acetate, H_2 , and CO_2 when glucose was added alone to bog peat. However, the stimulatory effect of added glucose on fermentation had a lag period that was temperature-dependent when added in combination with AQDS. Major decreases in glucose were seen on day 45 at 7°C and day 10 at 15°C and 25°C (Fig. 2a, b, c). At the two warmer temperatures, this was coincident with increases in acetate concentration and H_2 partial pressure, and generally increased CO_2 production. This enhanced anaerobic activity was accompanied by the reduction of AQDS (Fig. 1b, c), indicating that it was acting as a TEA. Thus, it was only with a substantial input of labile C and a lag period that the microbial community in the bog peat was able to use AQDS as a TEA in respiration, and the dominant effect of AQDS was inhibitory.

In contrast, AQDS stimulated both fermentation and respiratory activity in the fen peat through its role as a TEA, as demonstrated in both the AQDS and AQDS + glucose treatments

by sustained production of AHQDS (Fig. 1d, e, f), rapid initial production of acetate (Fig. 3d, e, f), and an increase in CO₂ production (Fig. 5d, e, f) at all temperatures (summarized in Table 2). Glucose was rapidly consumed in the fen peat with or without AQDS addition (Fig. 2d, e, f), indicating high fermentation potential compared to the bog peat, and a lack of an inhibitory effect of AQDS in this peat.

Our results are intriguing when compared to those of Keller and Takagi (2013), who conclusively demonstrated that fully oxidized humic substances from the same bog as used in the present study acted as TEAs and accounted for a significant fraction of anaerobic CO₂ production until the humic substances were fully reduced. In their study, the solid phase peat was responsible for the vast majority of the reductive capacity. However, AQDS applied in our study is a surrogate for dissolved-phase quinone substances that may provide range of toxic effects (Monks et al., 1992; O'Brien, 1991; Shyu et al., 2002). The amount of our AQDS addition was similar to other studies that have examined its electron-accepting capabilities (e.g. Cervantes et al., 2000; Keller et al., 2009; Lovley et al., 1996) and was biologically reasonable considering the observed reductive capacity of the fen peats (up to 100% reduction of the added AQDS). Since AQDS addition caused no change in pH, its inhibitory effect in the bog peat in the present study was not due to an increase in acidity. Furthermore, addition of a “humic”-rich peat extract was found to be inhibitory to CO₂ production, sulfate reduction, and methanogenesis, but not to acetogenesis in a bog soil (Minderlein and Blodau, 2010). Thus, it appears that both AQDS and humic substances can act as TEAs or be inhibitory for anaerobic microbial metabolism under different circumstances. We hypothesize that solid phase humics act primarily as TEAs and solution-phase humics can act as inhibitory substances in certain environments (e.g., bogs).

4.2. Methanogenesis

As expected, CH₄ production was stimulated by glucose amendment regardless of the peat type and temperature (Fig. 6). Apparently, fermentation of glucose provided acetate (Fig. 3) and H₂ (Fig. 4) to methanogens resulting in an increase in CH₄ production. In contrast, AQDS amendment significantly decreased CH₄ production in both peats (Fig. 6) but due to different effects of the substance (Table 2). In the fen peat, AQDS acted as a TEA, and addition of glucose fully compensated for the reduction in CH₄ production by AQDS (Fig. 6d, e, f), providing strong evidence that the effect of AQDS on CH₄ production was primarily through substrate competition. In contrast, in the bog peat CH₄ production was not recovered in the glucose + AQDS treatment (Fig. 6a, b, c), despite high concentrations of acetate as a result of glucose fermentation after the lag period discussed above (Fig. 3a, b, c). We have previously shown that acetoclastic methanogenesis dominates in this bog soil (Ye et al., 2012), suggesting that AQDS suppressed CH₄ production in this bog soil through a direct inhibitory effect even in the presence of high concentrations of the dominant methanogenic substrate. Our results are in agreement with other studies that have found that AQDS can have an inhibitory effect on methanogenesis (Cervantes et al., 2000; Keller et al., 2009).

CH₄ production in the controls was up to 47-times lower in the bog peat compared to the rich fen peat (Fig. 6). Lower CH₄ production in bog soils compared to soils of other peatland types has been widely observed, indicating that methanogenesis in bog peats may be intrinsically inhibited (Bridgham et al., 2013). The low soil pH of these systems has often been implicated as a reason for this inhibition (Brauer et al., 2004; Dunfield et al., 1993; Valentine et al., 1994). However, in a pH manipulation experiment with soils from six peatlands across the ombrotrophic-minerotrophic gradient, we found that while pH was an important control over

CH₄ production, this rate remained low in bog peat even after prolonged incubation at circumneutral pH and with substantial acetate accumulation (Ye et al., 2012).

In a review on this subject, Bridgham et al. (2013) hypothesized that bogs contain high concentrations of aromatic compounds that are particularly inhibitory to methanogens. Bog porewater has very high concentrations of aromatic compounds compared to fen porewater (Tfaily et al., 2013). *Sphagnum* mosses have high concentrations of phenolic substances that are inhibitory to microbial activity (McClymont et al., 2011; Williams et al., 1998), and Hines et al. (2008) found that CH₄ production in peat soils was highly negatively correlated with the proportional cover of *Sphagnum* mosses. Our results are in agreement with Hines et al. (2008) in that plant cover was dominated by *Sphagnum* spp. in the bog and by vascular plants in the rich fen. Our experimental results with the quinone analog AQDS support the hypothesis that the inherently low CH₄ production in bogs is attributable to the toxic effect of dissolved aromatic substances. Quinones can be toxic through the formation of semiquinone radicals (O'Brien 1991) or through the formation of polyphenols. Our study provides an important additional perspective to the 'enzyme-latch' hypothesis that peatlands accumulate C because of the low activity of phenol oxidase under anaerobic conditions and the resultant accumulation of inhibitory phenolic compounds, which would include the phenolic moieties of humic substances that are formed through the reduction of quinone moieties (Bragazza et al., 2013; Freeman et al., 2012; Freeman et al., 2001; Limpens et al., 2008). Under aerobic conditions, polyphenol oxidase can in turn convert at least some polyphenols back into quinones, so the biogeochemistry of quinone and polyphenol moieties is intimately linked through redox reactions. This important body of research has to date not addressed the effects of aromatic substances on methanogenesis to our knowledge. While we are unaware of any studies that have examined

phenol oxidase activity across the ombrotrophic-minerotrophic gradient, it is likely that quinone-like humic substances have a broad inhibitory effect on anaerobic C mineralization in an ombrotrophic bog, with a particularly strong inhibitory effect on methanogenesis, whereas these substances largely act as organic TEAs in a minerotrophic fen. Additional research is needed to verify our findings in other peatlands, to identify the source of the inhibitory substances in bogs (e.g., are they derived primarily from *Sphagnum* spp. mosses?), and to relate these findings to the enzyme-latch hypothesis.

4.3. Temperature sensitivity of CH₄ production

As described in the introduction, the role of humic substances in controlling the temperature dependence of CH₄ and CO₂ production is largely unknown. Our anaerobic handling of samples and 15 day pre-incubation should have reduced any TEAs in the samples, and thus we isolated the temperature response of AQDS, as a humic substance analog, with and without a ready source of electrons caused by the addition of glucose in the two peats, and its effect on the E_a of methanogenesis. In the bog peat, addition of AQDS caused almost complete inhibition of CH₄ production (Fig. 6 a, b, c) and thus no obvious temperature response was observed (Table 1). In contrast, the addition of AQDS to the fen peat, where it acted as a TEA, increased the E_a of cumulative CH₄ produced. van Hulsen et al. (1999) suggested the presence of TEAs would increase the temperature sensitivity for CH₄ production, which provides a relatively straight forward explanation of the temperature response for cumulative CH₄ production in the fen peat. However, the complex temporal dynamics of temperature effects on CH₄ production (Fig. 7b, e) suggests a more complicated set of factors controlling the apparent temperature sensitivity of methanogenesis. For example, the temporal dynamics of E_a in the control and glucose treatments in both peats generally mirrored each other but changed greatly

through time, especially in the bog peat, for reasons that are not clear. Similarly quizzical, the negative effect of AQDS on the E_a for CH_4 production in the fen peat was ameliorated by day 10 in the glucose + AQDS treatment and by day 30 in the AQDS treatment (Fig. 7e), although the majority of the 11.25 mM of added AQDS was not reduced by the end of the experiment (Fig. 1).

These complicated temporal dynamics in the temperature sensitivity of CH_4 production likely reflect temporal changes in the temperature responses of other microbial groups with effects on methanogens. For example, the initial decrease in the E_a for AQDS reduction (Fig. 7f) and CO_2 production (Fig. 7d) in the AQDS + glucose treatment in the fen peat was likely due to rapid consumption of the added glucose in that treatment (Fig. 2d, e, f). In contrast, the increase in the E_a in AQDS reduction (Fig. 7f) and CO_2 production (Fig. 7d) in the AQDS treatment in the fen peat from day 2 to 5 was likely because of a small stimulatory effect of AQDS on mineralization of native peat on day 2 (Fig. 5d, e, f). The E_a in AQDS reduction and CO_2 production in the AQDS + glucose treatment in the bog peat (Fig. 7a, c) increased substantially as it began to be used as a TEA (Fig. 1a, b, c), but it likely continued to strongly direct inhibit methanogenesis also (Fig. 1a, b, c). The addition of glucose increased the E_a of CO_2 production by day 2 in the fen peat (Fig. 7d), and by day 5 in the bog peat (Fig. 7a), but in both cases the E_a thereafter tended to be lower than the control, with the temperature effect almost disappearing from this treatment in the last half of the incubation in the bog peat (Fig. 7a). These complicated temporal dynamic are likely due to differences the temporal dynamics of glucose consumption (Fig. 2) and acetate pooling (Fig. 3) among treatments.

There is an increasing acceptance in the terrestrial ecosystem literature that while the intrinsic temperature response of most microbially mediated reactions is ~ 2 , the apparent

temperature response of soil respiration can be quite variable because of the effects of temperature on interacting processes (Conant et al., 2011; Schmidt et al., 2011). Our results strongly suggest that this is also the case in peatlands where we demonstrated complicated temperature sensitivity of anaerobic C cycling and CH₄ production under carefully controlled conditions of substrate and TEA availability. Our results also extend the work of van Hulzen et al. (1999) by demonstrating inhibitory quinone moiety effects on the temperature sensitivity of many anaerobic processes in bog soils. Our results and the studies cited above also contradict the recent meta-analysis by Yvon-Durocher et al. (2014), where they showed that the temperature dependence of CH₄ production did not vary across studies in pure cultures of methanogens, slurries, and field fluxes. Meta-analyses can be relatively blunt instruments compared to mechanistic studies focused on the temperature dependence of multiple, interacting processes, but at a minimum, the contradictory results call for future research on this important topic.

5. Summary

We demonstrated that the humic analog, AQDS, has broad-scale inhibitory effects on anaerobic C cycling in a bog soil, with methanogenesis being particularly sensitive, and it was only when glucose was added and a lag period that any reduction of AQDS was observed. In contrast, AQDS acted as an organic TEA in a rich fen soil. It is apparent that a humic analog can suppress CH₄ production in different types of peatlands through different pathways by acting as either an inhibitory substance or a TEA, but the circumstances that determine when one or the other effect is dominant are unclear at this point. We hypothesize that solid phase humics act primarily as TEAs and solution-phase humics can act as inhibitory substances in certain environments (e.g., bogs). We further suggest that the enzyme-latch hypothesis that has

structured much current thinking about why peatlands accumulated C needs to be expanded to incorporate the effects of phenolic substances on anaerobic C cycling.

The addition of glucose and AQDS caused complicated temporal dynamics in terms of the apparent temperature sensitivity of various anaerobic C cycling processes. Under natural conditions the availability of TEAs and available substrates will vary dramatically in space and time in peatlands, and we suggest that these interactions will make modeling the temperature response of CH₄ production in peatlands particularly challenging. Our research adds to the growing body of literature in terrestrial soils that climate effects on soil C cycling will be mediated through complicated, interactive ecosystem responses.

Acknowledgements. We thank James Ziemer and Yvonne Ziemer for access to their private field site and the University of Notre Dame Environmental Research Center for access to field sites and laboratory facilities. This work was supported by NSF grant DEB-0816575.

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 698

699 **Figure Legends:**

700 Fig. 1. AHQDS concentrations of peat slurries from a bog and a rich fen with different
701 treatments. a-c, bog peats incubated at 7°C, 15°C, and 25°C, respectively; d-f, rich fen peats
702 incubated at 7°C, 15°C, and 25°C, respectively. Bars indicate mean $\pm$ 1 standard error. Note
703 differences in scales between the bog and rich fen soils.

704 Fig. 2. Glucose concentrations of peat slurries from a bog and a rich fen with different
705 treatments. a-c, bog peats incubated at 7°C, 15°C, and 25°C, respectively; d-f, rich fen peats
706 incubated at 7°C, 15°C, and 25°C, respectively. Bars indicate mean $\pm$ 1 standard error.

707 Fig. 3. Acetate concentrations of peat slurries from a bog and a rich fen with different treatments.
708 a-c, bog peats incubated at 7°C, 15°C, and 25°C, respectively; d-f, rich fen peats incubated at
709 7°C, 15°C, and 25°C, respectively. Bars indicate mean $\pm$ 1 standard error.

710 Fig. 4. H₂ partial pressures in response to different amendments in peat slurries from a bog and a
711 rich fen. a-c, bog peats incubated at 7°C, 15°C, and 25°C, respectively; d-f, rich fen peats
712 incubated at 7°C, 15°C, and 25°C, respectively. Bars indicate mean $\pm$ 1 standard error. Note
713 differences in scales between the bog and rich fen soils.

714 Fig. 5. Cumulative CO₂ production rates in response to different amendments in peat slurries
715 from a bog and a rich fen. a-c, bog peats incubated at 7°C, 15°C, and 25°C, respectively; d-f,
716 rich fen peats incubated at 7°C, 15°C, and 25°C, respectively. Bars indicate mean $\pm$ 1 standard
717 error.

718 Fig. 6. Cumulative CH₄ production rates in response to different amendments in peat slurries
719 from a bog and a rich fen. a-c, bog peats incubated at 7°C, 15°C, and 25°C, respectively; d-f,

720 rich fen peats incubated at 7°C, 15°C, and 25°C, respectively. Bars indicate mean $\pm$ 1 standard
721 error. Note differences in scales between the bog and rich fen soils.

722 Fig. 7. Activation energy (E_a) from 7-25°C for cumulative CO₂ and CH₄ production and AQDS
723 reduction in peat slurries from a bog and a rich fen. a-c, CO₂, CH₄, and AHQDS in bog peats,
724 respectively; d-f, CO₂, CH₄, and AHQDS in fen peats, respectively.

1 Table 1. Activation energy values and standard errors (KJ mol⁻¹) from 7-25°C for cumulative
2 production of CH₄, CO₂, and AHQDS reduction in peats from a bog and a rich fen with different
3 treatments. Treatments in bold are different than the control at p < 0.05 and in italics are
4 different than the control at 0.10 > p > 0.05 for CH₄ and CO₂. For AHQDS, only the AQDS and
5 Glucose + AQDS treatments are compared.

	CH ₄	CO ₂	AHQDS
<u>Bog</u>			
Control	60 (13)	71 (12)	N.A.
Glucose	61 (15)	3 (5)	N.A.
AQDS	4 (6)	70 (19)	10 (6)
Glucose + AQDS	-2 (7)	73 (32)	88 (14)
<u>Rich Fen</u>			
Control	46 (7)	49 (9)	N.A.
Glucose	55 (10)	51 (13)	N.A.
AQDS	77 (10)	60 (7)	46 (7)
Glucose + AQDS	60 (8)	29 (4)	20 (4)

6 Note: N.A., not available.

7

Table 2. Summary of significant effects of temperature (T), glucose (Glu), and AQDS addition and their ecological interpretation. ↑ = stimulatory effect, ↓ inhibitory effect.

Response Variable	Bog	Rich Fen
AQDS reduction	<p>↑ T, ↑ Glu + AQDS</p> <p>Warm T and Glu addition necessary to overcome AQDS inhibition</p>	<p>↑AQDS, ↑ T, ↑ Glu + AQDS</p> <p>Rapid use of AQDS as TEA with addition of electron donor Glu</p>
Glucose consumption	<p>↑ T, ↑Glu, ↓ AQDS</p> <p>AQDS reduced stimulatory effect of warm T and Glu addition</p>	<p>↑ T, ↑ Glu, ↑AQDS</p> <p>Rapid regardless of treatment, but enhanced by AQDS</p>
Acetate concentration & H ₂ partial pressure	<p>↑ Glu, ↓ AQDS, T x Glu x AQDS</p> <p>Warm T reduced inhibition lag from AQDS</p>	<p>↑ Glu, ↓ AQDS</p> <p>Glu enhanced production, AQDS enhanced respiratory consumption</p>
CO ₂ production	<p>↑ T, ↑ Glu, ↓ AQDS</p> <p>Warm T and Glu increased respiration, AQDS caused large reduction</p>	<p>↑ T, ↑ Glu, ↑AQDS</p> <p>Respiration increased with warmer T, substrate addition of Glu, and AQDS as TEA</p>
CH ₄ Production	<p>↑ T, ↑ Glu, ↓ AQDS</p> <p>Warm T and Glu increased methanogenesis, AQDS caused large reduction through direct inhibition</p>	<p>↑ T, ↑ Glu, ↓ AQDS</p> <p>Warm T and Glu increased methanogenesis, AQDS caused large reduction through competitive inhibition</p>

Figure

Fig. 1.

Fig. 2.

Fig. 3.

Fig.4.

Fig.5.

Fig.6.