Electron Acceptors in the Arctic: The Roles of Iron, Humic Acids, and Organic Chlorine in Anaerobic Respiration

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by

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Chair

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2016
DEDICATION

This dissertation is dedicated to my dad, the first true scientist I ever met

- and -

to my mom, who showed me how to revel in the natural world.
EPIGRAPH

Life is nothing but an electron looking for a place to rest.

-Albert Szent-Györgyi
# TABLE OF CONTENTS

Signature Page ........................................................................................................ iii

Dedication .................................................................................................................. iv

Epigraph .................................................................................................................... v

Table of Contents ...................................................................................................... vi

List of Figures ............................................................................................................ ix

List of Tables ............................................................................................................. xi

Acknowledgements .................................................................................................. xii

Vita ............................................................................................................................... xiv

Abstract of the Dissertation ....................................................................................... xv

Chapter 1: Introduction .............................................................................................. 1

References .................................................................................................................. 5

Chapter 2: Electrochemical and spectroscopic properties of humic acids from soils of
the Arctic Coastal Plain ............................................................................................... 7

Abstract ..................................................................................................................... 7

Introduction ............................................................................................................... 7

Materials and Methods ............................................................................................. 10

Site Description ......................................................................................................... 10

Soluble Iron Measurements .................................................................................... 10

Soil Sampling ............................................................................................................ 11

Humic Acid Extraction ............................................................................................ 11

Inductively Coupled Plasma Optical Emission Spectrometry ............................... 12

Potentiometric Redox Titrations ............................................................................. 13

Cyclic Voltammetry ................................................................................................. 15
Results ................................................................................................................................. 16
Soluble Iron .......................................................................................................................... 16
Humic Acid Yield .................................................................................................. 17
Metal Content of Humic Acids ............................................................................................ 17
Electron Accepting Capacity of Humic Acids ................................................................. 18
Spatial Variations in Humic Acid Electron Accepting Capacity .............................. 19
Discussion ............................................................................................................................ 20
Acknowledgments .................................................................................................................. 23
References .............................................................................................................................. 24
Figures and Tables ................................................................................................................... 28

Chapter 3: Role of iron as mediator and structural component in microbial humic
reduction ................................................................................................................................ 36
Abstract ................................................................................................................................. 36
Introduction ............................................................................................................................. 37
Materials and Methods ............................................................................................................. 40
Site Description ....................................................................................................................... 40
Field Experiment ....................................................................................................................... 44
Humic Acid Extraction ............................................................................................................. 44
Laboratory Incubation ............................................................................................................. 44
Sequential Iron Extraction ...................................................................................................... 47
Inductively Coupled Plasma Optical Emission Spectrometry............................................. 47
pH-Dependence of Humic Extract ORP .............................................................................. 48
Results .................................................................................................................................... 49
Humic Acid Addition Oxidizes Soil Iron Pools ................................................................. 49
Microbial Reduction of Soluble Humic Acids ................................................................. 51
Iron Content of Soils ............................................................................................................... 51
Iron Content of Humic Acids ............................................................................................... 52
pH-Dependence of Humic Extract ORP .............................................................................. 53
Discussion ............................................................................................................................... 54
Interactions Between Terrestrial Humic Acids and Iron ..................................................... 54
Iron as a Structural Component of Arctic Humic Acids ..................................................... 56
LIST OF FIGURES

Figure 2.1: Fe(II)/total iron ratio of soluble iron in soil pore water over the field season of 2010 ................................................................. 28

Figure 2.2: Fe(II)/total iron ratio of soluble iron in soil pore water over the field season of 2011 ................................................................. 29

Figure 2.3: Concentrations of aluminum (Al), calcium (Ca), iron (Fe), magnesium (Mg), and manganese (Mn) in extracted soil humic acids by inductively coupled plasma optical emission spectrometry ................................................................. 30

Figure 2.4: Potentiometric redox titrations of humic acid extracts by basin age ........ 31

Figure 2.5: Compiled cyclic voltammetry scans of representative humic acid extracts from each age drained thermokarst lake basin superimposed on a blank buffer scan ........................................................................................................... 32

Figure 2.6: Voltage potentials used in discriminant function analysis to bin humic acid sample cyclic voltammetry scans by basin age ................................................................. 33

Figure 2.7: Integral averages of cyclic voltammetry curves by basin age ................. 34

Figure 3.1: Fe(II)/total Fe in ancient basin during soil amendment experiment ...... 64

Figure 3.2: Total soluble iron in ancient basin during soil amendment experiment .... 64

Figure 3.3: Fe(II)/total Fe in medium basin during soil amendment experiment ....... 65

Figure 3.4: Total soluble iron in medium basin during soil amendment experiment... 65

Figure 3.5: Bacterial concentrations from colony counts of humic acid treatment..... 66

Figure 3.6: Total iron concentrations in vials with insoluble humic acids over the incubation ........................................................................................................... 67

Figure 3.7: Concentration of Fe(II) in vials with insoluble humic acids over the incubation ......................................................................................... 68

Figure 3.8: Endpoint redox potentials of insoluble humic acids from incubations..... 69

Figure 3.9: Sequential iron extractions ......................................................................... 70
Figure 3.10: Relationship between Eh and pH of dissolved humic acids ....................... 71

Figure 4.1: Soil pore water chloride concentrations over the 2010 growing season.. 105

Figure 4.2: Analysis of soil chlorine using TSX-Cl................................................. 106

Figure 4.3: Cl-XANES on soil and vegetation samples .............................................. 107

Figure 4.4: Chloride concentration over time in autoclaved and non-autoclaved soils provided with dichloroethylene (DCE) and tetrachloroethylene (PCE) ............ 108

Figure 4.5: Relative abundance of *Dehalococcoides* 16S rRNA sequences by depth in areas of high and low topography ................................................................. 109
LIST OF TABLES

Table 2.1: Humic acid yield and electron accepting capacity (EAC) by basin age ..... 35

Table 2.2: Statistics for discriminant function analysis (DFA) of cyclic voltammograms .................................................................................................................. 35

Table 4.1: Numbers of sequences matching chlorine cycling genes and organisms in metagenomes ........................................................................................................................................ 110

Supplemental Table 4.1: Phylogenetic assignment of sequence matches to Haloperoxidases ........................................................................................................................................ 111

Supplemental Table 4.2: Phylogenetic assignment of sequence matches to Halogenases ........................................................................................................................................ 112

Supplemental Table 4.3: Phylogenetic assignment of sequence matches to Reductive Dehalogenases ........................................................................................................................................ 112

Supplemental Table 4.4: Phylogenetic assignment of sequence matches to Haloacid Dehalogenases ........................................................................................................................................ 113
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PUBLICATIONS


ABSTRACT OF THE DISSERTATION

Electron Acceptors in the Arctic: The Roles of Iron, Humic Acids, and Organic Chlorine in Anaerobic Respiration

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Professor David Lipson, Chair

Bacteria and archaea have evolved the ability to respire using diverse compounds to produce energy. The use of alternative electron acceptors for anaerobic respiration is critical in environments where oxygen is limited or absent – such as soils of the Arctic Coastal Plain. Continuous permafrost below the active layer of soil restricts drainage, creating anoxic conditions. Thus, anaerobic respiration dominates all but the top few centimeters of soil.

Climate change effects acutely impact the Arctic, and the potential for positive feedback from soil respiration is substantial. Biogeochemical cycling in this environment warrants further study, particularly concerning anaerobic electron
acceptors which contribute to CO₂ fluxes and can compete with methanogenesis, further impacting greenhouse gas emissions.

The objective of this dissertation is to explore metabolism of unusual electron acceptors in Arctic tundra soils, focusing on the importance of humic substances, their interactions with iron, and the role of organohalide respiration in the Arctic carbon cycle. The research contained within unites microbiological techniques, soil chemistry methods, and innovative interdisciplinary tools to study these compounds from a variety of vantage points.

Soil extract measurements, potentiometric redox titrations, and cyclic voltammetry revealed that metabolism involving humic acids in this environment may contribute nearly 400 moles of electrons per square meter of soil (e⁻/m²), accounting for over 10% of ecosystem respiration. Performing a field-based soil amendment experiment and laboratory incubations validated that reduction of large, insoluble humic acids can be facilitated by way of soluble iron intermediates, iron is an electron-accepting moiety in humic acids, and iron-reducing bacteria liberate complexed iron from the structure of humic acids. Chlorinated organic compound cycling in tundra soils was studied with field measurements, biological exploration using laboratory incubations and metagenomics, and chemical investigation using elegant tools including Oxidative Combustion Microcoulometry and X-ray absorption near edge structure (XANES). These corroborating methods demonstrated chlorinated organic compounds are widespread, dynamic, and used for anaerobic respiration by diverse microorganisms in essentially pristine Arctic soils. The work contained within this
dissertation provides fresh insight into vital yet understudied Arctic soil microbial
anaerobic processes which have broader impacts on Arctic carbon cycling and climate
change feedback mechanisms.
CHAPTER 1

Introduction

Arctic soil microbiology

Soil microorganisms of the Arctic Coastal Plain are adapted to harsh, cold, and predominantly anaerobic conditions. Here, deep continuous permafrost is found beneath the active layer of soil. The active layer undergoes a yearly freeze-thaw cycle, and soils are generally waterlogged as the permafrost restricts drainage. In the absence of the electron acceptor, oxygen, microbes must use alternative sources to produce energy. In soils of the Arctic Coastal Plain, many of the compounds – such as nitrate, manganese, and sulfate – reduced commonly in other anoxic systems are also limited (Lipson et al., 2010). Thus, these microorganisms must reduce other compounds to carry out respiration.

The landscape of Northern Alaska’s Arctic Coastal Plain is dominated by thermokarst lakes and drained thaw (or thermokarst) lake basins (DTLB) which contain unique soil characteristics (Hinkel et al., 2005). DTLB eventually form when lakes inevitably drain due to erosion or other natural processes; over time both the organic layer depth and soil organic carbon content increase, generating an age gradient system useful for basin characterization (Hinkel et al., 2003). Basin ages are assigned based on the number of years since the basin formed and the resulting soil and organic layer characteristics: basins formed less than 50 years before present
(y.b.p.) are classified as young, 50-300 y.b.p. are medium aged, 300-2000 y.b.p. are old, and basins between 2000 and 5500 years old are termed ancient (Hinkel et al., 2003). These landscape features provide a chronosequence useful for studying soil and organic matter development.

Arctic soils are of interest for their high sequestered carbon content and potential for positive biosphere-climate feedbacks. Processes involving the release of soil carbon as carbon dioxide and methane gas are of particular interest, as climate change is occurring more rapidly in the Arctic than anywhere else on Earth (IPCC, 2014). The active layer of soil, which thaws and refreezes annually, is affected by warming trends and may grow to encompass a larger portion of the soil profile as well as take longer to refreeze each year – effects which increase the potential amount of carbon lost during microbial activity including anaerobic respiration (Hinzman et al., 2005). Anaerobic respiration employing electron acceptors such as iron, humic acids, and potentially chlorinated organics, can compete with methanogenesis, resulting in the production of carbon dioxide instead of the more potent greenhouse gas methane (Blodau and Deppe, 2012; Bond and Lovley, 2002; Miller, 2015).

Iron is particularly available in this environment, with microbes reducing the ferric form Fe(III) to the ferrous form Fe(II) in anaerobic respiration (Lipson et al., 2013). The terrain is dominated by mosses, sedges, and grasses which break down and decompose to form complex organic compounds, including humic acids (Zona et al., 2009). Humic acids are a main component of organic matter, and are chemically reactive yet resist degradation in the soil (McKnight and Aiken, 1998). These
heterogeneous compounds play a role in anaerobic respiration, accepting electrons as well as transferring them to insoluble iron particles to facilitate dissimilatory iron reduction (Lovley et al., 1996). Another metabolically active class of compounds is chlorinated organic compounds (Cl$_{org}$), which are prevalent throughout the landscape and can be reduced and dechlorinated in organohalide respiration (Mohn and Tiedje, 1992). My dissertation affirms that these largely-unexplored electron acceptors are involved in dynamic metabolic cycles that have important implications for the Arctic ecosystem.

In Chapter 2, a combination of measurements and chemistry techniques reveal the remarkable electrochemical potential of humic acids in these Arctic soils and their significant role in soil respiration. The assemblage of humic acids in these soils matures over time both in quantity and chemical complexity. Over a chronosequence of soil development, humic acid molecules transform with time into compounds with increased electron accepting capacity. Using an approach which combines a field amendment experiment with controlled laboratory incubations in Chapter 3, I show that the redox cycles of humic acids and iron in the Arctic ecosystem are coupled in a previously unrecognized way. While supporting previously described electron shuttling in which microbes use humic acids as intermediates to reduce insoluble iron (Lovley et al., 1996), these experiments demonstrate that the reverse process can occur – microorganisms can use dissolved iron to pass electrons to insoluble humic compounds. In addition, iron atoms serve as moieties inside the humic complex, contributing to the redox abilities of humic compounds. Complex and diverse, humic
acids are useful to Arctic anaerobic microorganisms in sometimes independent, and
often complementary, processes. Chapter 4 presents the first study investigating
biological cycling of chlorinated organic compounds in non-contaminated Arctic soils.
Using field experiments, laboratory incubations, and specialized biological and
chemical methods, I discovered that chlorinated organic compounds are ubiquitous
throughout the coastal tundra, and I found chlorinated organics are used for anaerobic
respiration by diverse microbes in essentially pristine Arctic soils. My work sheds
light on the role of these elusive and complex molecules in the biogeochemistry of the
Arctic ecosystem.
References


Abstract

Terrestrial humic acids from Alaskan tundra soils were analyzed for electron accepting capacity and chemical attributes to quantify their involvement in microbial anaerobic respiration in this environment. Electron accepting capacity of humic acids varied with soil basin age as a result of organic matter content and soil development. Electrochemical techniques of potentiometric redox titrations and cyclic voltammetry revealed that metabolism involving humic acids contributes up to 4 moles of electrons per square meter of soil ($e^-/m^2$), accounting for up to 8.3% of ecosystem respiration. The humic acid pool in the Arctic Coastal Plain is a substantial electron acceptor for soil anaerobic respiration pathways, contributing to ecosystem respiration and soil carbon release.

Introduction

Humic acids are the high-molecular-mass, alkaline-soluble constituent of humic substances (HS). HS are an assortment of complex, heterogeneous compounds derived from decaying organic matter (OM) in soils, and are therefore abundant in
soils with high OM (Janos, 2003; Orlov, 1985; Stevenson, 1994). An early study of humic acid electron accepting capacity showed that the redox potential of humic acids became more negative with increased humification, or humic development (Visser, 1964). Despite the chemical capacity to accept electrons, humic substances were generally viewed as static, largely inactive compounds in soils until more direct evidence determined that HS could be used to accept electrons from soil microorganisms (Lovley et al., 1996). Investigations into the mechanism of microbial humic acid reduction asserted that quinone groups are the primary functional groups of humic acids responsible for accepting electrons (Scott et al., 1998). However, humic acid structure includes bound elements, such as metals. In a study of metal complexed within the structure of humic acids, the specific temperature required for iron removal suggested that the complexed iron plays an explicit role in the redox capability of humics (Jansen et al., 1996). Humic acids with high ash and mineral content had a greater capacity to transfer electrons to oxidants, suggesting that complexed iron within humic acid structure is significantly involved (Struyk and Sposito, 2001).

Electron transfer between iron and humic substances has been well established (Helburn and MacCarthy, 1994; Lovley et al., 1998; Roden et al., 2010). Soil dissolved organic matter containing humic acids accepted electrons from dissolved iron and reduced H₂S to thiosulfate in a northern peatland, participating in anaerobic heterotrophic respiration (Heitmann et al., 2007). In Arctic peat soils, the addition of a humic acid analog stimulated soil respiration (Lipsonet et al., 2010), and coupling
between iron reduction and humic acids is suggested in Arctic soils where humic acids are prevalent and iron reduction is a major metabolic pathway (Lipson et al., 2013b). Humic acid reduction may be important in the anoxic soils of the ACP, where concentrations of many common electron acceptors such as nitrate, nitrite, and sulfate are low (Lipson et al., 2010, 2013b). Humic acids and other dissolved organic compounds competitively suppressed methane release when supplemented to soils, demonstrating further impact of humic acids on carbon flux (Blodau and Deppe, 2012; Heitmann et al., 2007; Miller et al., 2015). Interactions between humic acids and soil respiration and methanogenesis have the potential to influence climate change feedbacks in the Arctic, a system well-known for its large reserves of soil carbon and OM (Tarnocai et al., 2009).

Humic acids play a role in terrestrial anaerobic respiration (Peretyazho and Sposito 2006), but the extent to which these complex and variable compounds contribute to respiration in soils of the Arctic Coastal Plain (ACP) is unknown. Large-scale spatial heterogeneity complicates estimates of the influence of terrestrial humic substance metabolism to regional and ecosystem respiration. A mosaic of differently-aged lake basins formed after thermokarst lake drainage covers over 50% of the ACP landscape, leading to different soils at widely varying humification stages (Hinkel et al., 2005). Thus, the aim of this study was to determine the concentrations of humic acids in different representative soils of the ACP and define the electron accepting capacity (EAC) of humic acids from this environment, for use in estimates of the impact of humic acid metabolism on anaerobic respiration in Arctic tundra soils.
Materials and Methods

Site Description: Study sites are located on the ACP, near Barrow, Alaska. The active layer of soil averages 36 cm in depth and thaws and refreezes yearly with seasonal changes; beneath this active layer is deep continuous permafrost (Hinkel and Nelson, 2003; Nelson et al., 1998). ACP soils are often anoxic below 5 cm depth, and oxygen is generally depleted within 2 cm of the height of the water table (Lipson et al., 2010). Nearly half of the ACP near Barrow contains drained thermokarst lake basins (DTLB) (Hinkel et al., 2003). Thermokarst lakes inevitably drain due to soil erosion, leaving behind basins which undergo senescence and revegetation. Basin classification is based on the age of the basin following this drainage event and provides an excellent chronological sequence to study soil OM development. The established system classifies DTLB in four age categories: young, medium, old, and ancient. Young basins are those which drained in the last fifty years and exhibit the least organic matter development, while medium basins drained between 50 and 300 years ago and tend to be variable; old basins show a deepening organic layer, having been developing for 300-2200 years, and the most developed of the basins are the ancient basins which drained between 2200 and 5000 years before present (Hinkel et al., 2003).

Soluble Iron Measurements: Soil pore water dissolved iron pools were measured over the active seasons of 2010 and 2011. Soil pore water samples were
collected using soil water suction microlysimeters (Rhizon, Eijkelkamp) from June through September of 2010, and June through August of 2011 (Lipson et al., 2013b). Seven to fifteen spatial replicates of each basin age were collected. Iron was stabilized by adding HCl to samples to reach a final concentration 20 mM HCl. Samples were stored in 4°C until measurement. The 1,10-phenanthroline colorometric method was used to measure total soluble iron and Fe(II) (Analytical Methods Committee, 1978). Measurements were performed in in 96-well plates, using a Spectra MAX 190 (Molecular Devices Corp.) at 510 nm after 15-30 minutes. Values were analyzed with SOFTmax PRO 4.0 software (Life Sciences Edition by Molecular Devices Corp).

**Soil Sampling:** Soil samples were collected in June and August of 2011 from DTLB near Barrow, Alaska. Samples were removed from the ground using a portable electric drill fitted with a masonry hole bit of 3 cm diameter if frozen, or a Russian peat corer (Rickly Hydrological Company, Columbus, Ohio) if thawed (Lipson et al., 2013b). Soil cores averaged 24 cm deep. Four to five spatial replicate samples were collected in each individual basin. Samples were transported to the lab and frozen at −40°C. To transfer samples to SDSU for further work, samples were shipped frozen overnight by commercial courier.

**Humic Acid Extraction:** Frozen soil core samples were cut using a band saw vertically along the profile, including the entire depth of soil collected. Samples were
processed to extract HA as follows. Approximately 10 g wet soil was placed into 30 mL of N₂-bubbled 0.1 M NaOH/0.1 M Na₄P₂O₇ solution in 50 mL polypropylene tubes under an N₂ headspace; samples in solution were shaken overnight to begin extraction (Agnelli et al., 2000; Janos, 2003). Tubes were centrifuged at 10,000 g for 6 min, followed by acidification of the supernatant with concentrated HCl to pH 1 to separate humic acids from fulvic acids. Upon acidification, the humic acid portion precipitated out and was pelleted by centrifugation (10,000 g, 6 min). Pellets were rinsed with diH₂O, and shaken 2 days with O₂-purged 0.3 M HF/0.1 M HCl to remove impurities such as clay, silica, aluminum, and iron (Andjelkovic et al., 2006; Velthorst et al., 1999). Humic acid extracts were again pelleted by centrifugation and rinsed with 1 mM HCl and again with diH₂O before resuspension in O₂-purged 0.1 M, pH 7 KH₂PO₄. To preserve the redox state of humic extracts, all extraction steps were carried out using solutions bubbled with N₂ gas to remove oxygen, and headspaces were exchanged with N₂ gas. Yields of humic acids were compared using Analysis of Variance (ANOVA). Humic acid content by volume of soil was calculated from bulk density values of these soils.

*Inductively Coupled Plasma Optical Emission Spectrometry:* Inductively coupled plasma optical emission spectrometry (ICP-OES) was used to quantify tightly bound iron and other trace metals remaining in humic acids after extraction (Perkin Elmer DV4300 ICP-OES). Four replicates from each age basin were measured (16
total). Dry humic acid extracts were combusted in ceramic crucibles (CoorsTek, CO) and fully dissolved in 10% nitric acid. This solution was diluted with ultrapure water to a final concentration of 2% nitric acid according to manufacturer recommendations. Differences in metal concentration between humic acids from different basin ages was examined by ANOVA.

*Potentiometric Redox Titrations*: Potentiometric redox titrations were performed on humic acid samples to quantify the EAC of these substances. Humic acid extracts were investigated by basin age; four spatial replicate extracts from a single basin were combined in equal volumes to normalize for landscape variation. As described below, titrations started with reduced humic acids and quantified the electrons donated to an oxidant over the course of the experiment. In this way, potentiometric redox titrations provided the electron donating capacity of the humic acids tested, assumed to be equivalent to their EAC as determined in previous studies of this region (Lipson et al., 2013b).

Humic acids were chemically reduced by incubation with \( \text{H}_2 \) gas and Palladium catalyst (10% on activated charcoal, 2.5 mg/10 mL) for at least 72 hours; such chemical reduction produces humic acids with comparable electron donating capacity to microbially-reduced humic acids (Peretyazhko and Sposito, 2006; Struyk and Sposito, 2001; Visser, 1964). The Ag/AgCl ORP electrode used contained 4 M KCl gel (Thermo Orion probe #9179BNMD). Voltage potential was corrected from
Ag/AgCl to Standard Hydrogen Electrode (SHE) according to factors supplied by the manufacturer (+219 mV at 22°C). Accurate electrode function was tested before each titration with fresh saturated solutions of quinhydrone in buffers of pH 4 and pH 7.

Potentiometric redox titrations of reduced humic acid extracts involved potassium ferricyanide (K₃Fe(CN)₆, 5 mM) as the oxidant. Potassium ferricyanide was added incrementally to the apparatus containing 5 mL of solution with reduced humic acid extracts in pH 7, 100 mM KH₂PO₄. Nitrogen gas flowed through inlet and outlet ports on the cap to preserve the anaerobic atmosphere. After each addition of oxidant, the electrode was allowed to equilibrate for 10 min before recording the potential (Struyk and Sposito, 2001).

Midpoint and endpoint redox potentials were estimated from each titration curve (Struyk and Sposito, 2001). The midpoint potential was estimated for each sample using the inflection point of the curve. The endpoint potential is the point on the graph of a potentiometric redox titration curve where the slope changes from steeply increasing potential to a plateau, and refers to the potential at which no more electrons can be released by the volume of the compound under analysis. Thus, at this point, the substance being oxidized is considered fully oxidized; using the volume of the humic acids in the vial and the volume of oxidant added, the total number of electrons released can be determined. Each molecule of potassium ferricyanide can accept one electron from the reduced humic acids, reducing potassium ferricyanide to potassium ferrocyanide (Kolthoff and Tomsicek, 1935). The electron accepting
capacity (EAC) of humic acids measured in titrations, as determined by endpoint potential, was extrapolated to the landscape level using the yield of humic acids from soils, bulk density, and the depth to which these samples were collected (24 cm). Therefore, these extrapolations are conservative as the EAC of humic acids from the bottom of the active layer is not included. Landscape EAC values for humic acids were related to ecosystem respiration calculations from 2009 (Zona et al., 2009) by dividing the landscape EAC of humic acids by the number of electrons used to oxidize the CO$_2$ molecule, and comparing these values to the corrected CO$_2$ daily flux in moles e$^-$/m$^2$/year for the length of the active season.

*Cyclic Voltammetry:* Cyclic voltammetry (CV) was employed to analyze electron accepting features of humic acids and detect such differences between samples. Potassium phosphate (pH 7, 100 mM) was used as the buffer in which humic acid samples were dissolved; a mix of 4 mL sample in buffer and 1 mL NaNO$_3$ as supporting electrolyte were combined to fill the electrochemical cell. Once assembled, the solution was bubbled with N$_2$ gas for five to ten minutes to purge the cell of oxygen. This was followed by a gentle flow of N$_2$ to the headspace of the cell during all scans to isolate the environment from the oxidizing effect of O$_2$. Potential was applied from -0.81 V to 0.8 V and back (relative to Ag/AgCl) using a 2 mm gold working electrode. The resulting current was measured and recorded. The reference electrode was Ag/AgCl, and the counter electrode was platinum wire.
Sample currents were plotted against the potential in mV (Ag/AgCl) in cyclic voltammograms. Curves of the reducing direction (positive to negative voltage) from each sample were integrated, and the integral of the corresponding blank was subtracted to obtain a normalized integral value. The upper portion (0.4 – 0.8 V Ag/AgCl) of voltage was omitted from integration due to anomalous nature of the samples at high potential. Cyclic voltammograms from four spatial replicates from each age basin and blank samples of buffer were analyzed using discriminant function analysis (DFA) to determine qualitative differences on humic acids by basin age. CV data were averaged into 50 mV bins, resulting in 54 variables used for the DFA analysis in the range of -0.81 to 0.8 V Ag/AgCl. Cross-validation was performed, in which one sample was omitted at a time in analysis runs to determine if the omitted sample could be correctly classified using only the other replicates. To calculate the intensity of the CV signal, integrals of curves from each experimental scan were evaluated. The integral of the buffer blank was subtracted from sample integrals.

**Results**

*Soluble Iron:* Soils of the Arctic Coastal Plain are unique for their high levels of soluble iron and insoluble humic substances, and the redox state of soluble iron indicates the redox state of the humic acids (Lipson et al., 2010, 2013b). Thus, measurements of Fe(II) relative to total soluble iron during the active seasons of 2010 and 2011 reveal the fluctuations of the redox states of both the dissolved iron pool and
the humic acid pool. The ratio of Fe(II)/total iron describes the redox state of the soluble iron pool; the higher this value, the more Fe(II) is present in the iron pool (more reduced), and if the iron pool is more oxidized, the value will be lower.

The soluble iron pools in all basin ages exhibited similar trends. In June of both years, the iron pool was fairly oxidized. Over the course of the season, the iron pool became more reduced with time (Figures 2.1, 2.2). From the end of sampling in 2010 to the beginning of sampling in 2011, an unknown mechanism caused the iron pool to become re-oxidized in all soils. As the redox state of iron and humic acid pools are related, the reset of redox state observed in soil pore water dissolved iron is presumed to reflect a re-oxidation of the humic acid pool.

**Humic Acid Yield:** Yields of humic acids from soils range from 4.9 to 100.6 mg HA/ g dry soil. The average yield for humic acids by basin age is shown in Table 2.1. Yields of humic acids from medium aged basins were significantly lower than in other basin ages (P=0.01). Humic acids are also presented as proportion of soil organic matter (Table 2.1).

**Metal Content of Humic Acids:** Iron was found at higher concentrations than many other ions such as Ca, Mg, and Mn, in humic acid extracts, indicating that iron has a structural role in humic acids (Figure 2.3). There was no significant difference
between age basins for iron content of humic acids. Average concentrations of iron across the landscape was 3984 ppm by dry mass of humic acids.

Concentration of aluminum varied between humic acids from different basin ages with the highest in old basin humic acids (old= 6888 ppm, average= 2740 ppm, P=0.01). The presence of Al in humic acid extracts indicates metal binding, but Al bound in organic matter is always in the same oxidation state and does not contribute to electron exchange. Concentrations of calcium, magnesium, and manganese were low in all samples (Averages: Ca= 132 ppm, Mg= 204 ppm, Mn= 7 ppm). Molybdenum was below detection limits.

Electron Accepting Capacity of Humic Acids: Starting potentials of reduced humic acids from three aged basins (medium, old, ancient) were in the range of −4.9 to −46.1 mV SHE; however, the young basin humic acids started at +126.4 mV SHE (Figure 2.4). This titration was repeated on the same young humic acid extracts, freshly assembled and reduced for the repeat, to verify that this difference was actual and not an artifact; the repeat starting potential was +119.2 mV SHE.

Average EAC by gram of dry humic acid extract and the calculated prospective contribution of humic acid EAC in one square meter of tundra soil increased with soil basin age (Table 2.1). Humic acids account for an increasing portion of ecosystem respiration as basins increase in age. Reduction of humic acids in less developed soils accounts for 0.37% (young) and 0.53% (medium) of ecosystem
respiration, while humic acid reduction in more developed soils produce approximately 2.32% (old) and 8.33% (ancient) of ecosystem respiration (Table 2.1).

_Spatial Variations in Humic Acid Electron Accepting Capacity:_ Analysis of humic acids using cyclic voltammetry provides similar data to potentiometric redox titrations. As titrations were performed on pooled replicates for logistical reasons, CV scans on multiple samples allow for an assessment of variability in samples within basins. Features and peaks on voltammograms display qualitative differences between samples, while integrals of curves are quantitative.

Representative cyclic voltammetry scans, or voltammograms, show visual differences between age basins of soil (Figure 2.5). DFA on the entire scanned range of -0.81 to 0.8 mV (Ag/AgCl) binned scans into correct age categories (or blank) with 95.5% accuracy (Table 2.2). Cross-validation classified scans into proper category with 86.4% accuracy, indicating that the features and intensities of voltammetry curves represent differences in humic acids based on basin age. Using a restricted range of -0.4 to 0.4 mV, DFA grouped samples and blanks with 94.4% accuracy (Table 2.2).

DFA omitting blank buffer scans classified humic acids by basin age with 90.9% accuracy using the entire scan range (-0.81 to 0.8 mV), and 83.3% accuracy using only the restricted range (-0.4 to 0.4 mV). Wilk's lambda was low for all DFA (0.006-0.071, Figure 2.2); thus, most of the variation between samples is accounted for
by the grouping variable (basin). Chi-squared tests on the significance of Wilk’s lambda produced low P values (<0.001 for all). For visual representation of major contributing differences between samples, significant bands used to distinguish scans of humic acids in the restricted range of voltage potentials are designated on cyclic voltammograms in Figure 2.6.

Integrals of curves from each experimental scan, normalized by subtracting the integral of the blank, indicate the intensity of signal as current is proportional to the concentration of electroactive species in a sample. Due to the low yield of humic extracts from medium aged basin soils, concentrations comparable to those used in CV for the three other age classes were not attainable. Thus, medium aged basin humic extracts were removed from statistical analysis on integrals of CV curves. Integrals shows a significant increasing trend in signal intensity with basin age (P=0.002), suggesting development of humic acids over time corresponding to improved EAC of the compounds (Figure 2.7).

Discussion

Release of stored soil carbon as greenhouse gases from Arctic regions is important to understand and predict as climate change continues. I have shown that humic acid reduction contributes to ecosystem respiration in soils of the ACP. Arctic soils are particularly impacted by effects of climate change and have extraordinary potential for substantial climate feedbacks, as competing soil processes contribute to
the release of soil carbon as methane from methanogenesis or carbon dioxide from microbial respiration. (Schuur and Bockheim, 2008). Humic acids represent a missing link in models of Arctic carbon flux and ecosystem respiration; my results show that the impact of humic acid reduction to Arctic soil respiration can be predicted from organic matter content and soil basin age, allowing for extrapolation to large scales.

The proportion of humic acids in soils varies by environment. In soils of the Arctic Coastal Plain, where carbon and organic matter concentrations are high in both the active layer and stored deep within permafrost (Tarnocai et al., 2009), I show that humic acids are prevalent, making up between 9.8 and 15.8% of SOM (Table 2.1). In contrast, humic acids make up between 2 - 10% of soil organic matter (SOM) in a Spanish bog, about 40% of SOM in high organic matter Brazilian histosols, and from 50% up to 100% of SOM in alpine soils, with more found in cultivated soils than undisturbed soils (Bongiovanni and Lobartini, 2006; Gondar et al., 2005; Kumada et al., 2012; Valladares et al., 2007).

I have shown that the humic acid pool matures into an assemblage of molecules with more electroactive properties as Arctic tundra soils become more humified and decomposed (Figure 2.4, 2.7, Table 2.1). Cyclic voltammetry scans and potentiometric redox titrations show a corresponding increase in redox activity of humic acids along the age gradient. Thus, humic acid molecules are more active in ecosystem respiration in older DTLB, a metric which can be used to improve estimates of humic acid contribution in ecosystem models.
Along with improved redox activity of humic acids in more decomposed soils, microorganisms in ACP soils may be more dependent on respiration involving these compounds in older basins. As DTLB age and the organic layer containing humic substances expands, the mineral-rich layer tends to recede. I predict this phenomenon begins a phase shift in which the bacterial communities previously relying more heavily on direct iron reduction become more dependent on humic acids to fulfill their energetic needs. Like Fe(III), which becomes re-available for reduction each year, the redox state of humic acids appears to cycle completely every year based on the redox state of the soluble iron pool (Lipson et al., 2013b). The underlying mechanism for reoxidation is unclear but may be attributed to a low water table late in the year, penetration of oxygen-rich snow melt early in the active season, or oxygen gas entering through soil cracks in the fall as observed with CO₂ and methane release (Mastepanov et al., 2008).

I conclude that approximately 8.3% of total ecosystem respiration in ancient basins is accounted for by humic acid reduction, representing a substantial source of energy for the Arctic microbial community. As roughly 50% of ER is attributed to vegetation, the reduction of humic acids in ancient basins may be the origin of 16.6% of total microbial ER. My work illuminates the metabolic importance of these heterogeneous and complex molecules to the biogeochemistry of the Arctic ecosystem and future climate change models.
Acknowledgements

I would like to thank Lisa Thurn for technical support with ICP-OES, and Ukpeagvik Iñupiat Corporation (UIC/UMIAQ) for assistance in Barrow, Alaska. This work was supported in part by NSF grants 0808604 and 1204263. The authors have no conflicts of interest to declare.

Chapter 2, in part, is in preparation for journal submission. Jaime Zlamal, Gregory Kalyuzhny, Dominic Goria, and David Lipson; 2016. The dissertation author was the primary investigator and author of this paper.
References


Figure 2.1: Fe(II)/total iron ratio of soluble iron in soil pore water over the field season of 2010.
Figure 2.2: Fe(II)/total iron ratio of soluble iron in soil pore water over the field season of 2011.
Figure 2.3: Concentrations of aluminum (Al), calcium (Ca), iron (Fe), magnesium (Mg), and manganese (Mn) in extracted soil humic acids by inductively coupled plasma optical emission spectrometry.
Figure 2.4: Potentiometric redox titrations of humic acid extracts by basin age.
Figure 2.5: Compiled cyclic voltammetry scans of representative humic acid extracts from each age drained thermokarst lake basin superimposed on a blank buffer scan.
Figure 2.6: Voltage potentials used in discriminant function analysis to bin humic acid sample cyclic voltammetry scans by basin age. Arrows on representative cyclic voltammetry scans point to most statistically important potentials for differentiating scans by basin, not including the blank. This figure magnifies the restricted range of −0.4 V to 0.4 V.
Figure 2.7: Integral averages of cyclic voltammetry curves by basin age. Integration was performed on the reducing direction of the scan from -0.81 V to 0.4 V. The low yield of humic extracts from medium aged basin soils precluded inclusion in analysis of integrals.
Table 2.1: Humic acid yield and electron accepting capacity (EAC) by basin age. Abbreviations: y.b.p.: years before present, HA: humic acid, cc: cubic centimeter, OM: organic matter, SHE: standard hydrogen electrode, eq: equivalents (moles of electrons), ER: ecosystem respiration.

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Medium</th>
<th>Old</th>
<th>Ancient</th>
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<tr>
<td>Fiducial Age (y.b.p.)</td>
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<td>50-300</td>
<td>300-2000</td>
<td>2000-5500</td>
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<td>HA yield (mg/g soil)</td>
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<td>28.1</td>
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<td>HA % of total OM</td>
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<td>EAC (µeq e⁻/g HA)</td>
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<td>190</td>
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<tr>
<td>Landscape EAC (e⁻/m²)</td>
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<td>Contribution to ER (% of ER)</td>
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<td>0.53</td>
<td>2.32</td>
<td>8.33</td>
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</table>

Table 2.2: Statistics for discriminant function analysis (DFA) of cyclic voltammograms. Bands used in DFA to distinguish samples by basin age are reported as midpoints of 50 mV wide bands.

<table>
<thead>
<tr>
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<th>Full Range (-.8 to 0.8 V)</th>
<th>Restricted Range (-.4 to 0.4 V)</th>
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<tr>
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</tr>
<tr>
<td>% Classified</td>
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<tr>
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<td>90.9</td>
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<td>22</td>
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<tr>
<td>No. Functions</td>
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<td>4</td>
</tr>
<tr>
<td>Wilk's Lambda</td>
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</tr>
<tr>
<td>Chi-square</td>
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<td>63.353</td>
</tr>
<tr>
<td>P</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Oxidizing Curve Bands (V)</td>
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<td></td>
<td>0.409</td>
<td>0.409</td>
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<tr>
<td></td>
<td>0.587</td>
<td></td>
</tr>
<tr>
<td>Reducing Curve Bands (V)</td>
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<tr>
<td></td>
<td>0.418</td>
<td>-0.350</td>
</tr>
<tr>
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</table>
CHAPTER 3

Role of iron as mediator and structural component in microbial humic reduction

Abstract

Terrestrial humic acids interact with iron in anoxic soils of the Arctic Coastal Plain. Iron and humic acids are in high concentration in Arctic Coastal Plain soils, and the goal of my investigation was to determine the extent of interactions between humic acids and iron. The first experiment amended soils with humic acids to describe the effect on soluble iron pools. The iron pool in soil that contained developed organic matter was initially oxidized by the addition of humic acids, and then reduced by the microbial community, demonstrating that soluble iron served as an electron shuttle in the microbial reduction of humic acids. An incubation experiment tested the effect of an iron reducing bacterial culture on insoluble humic acids extracted from tundra soil. The iron pool increased in treatments containing humic acids, suggesting the bacteria removed complexed iron from the humic acids. Measurements of redox potential on humic acids of pH 6, 7, and 8 displayed that iron is an active electron accepting moiety in the structure of humic acids from Arctic soils. Therefore, iron is an essential part of humic acid structure and metabolism in the Arctic Coastal Plain.
Introduction

Humic substances (HS), which are complex and heterogeneous compounds formed from the decomposition of living organisms, are found ubiquitously in soils (Orlov, 1985; Stevenson, 1994). These compounds serve as terminal electron acceptors in anaerobic respiration (Visser, 1964). Humus, the organic matter assemblage containing humic substances, such as humic acids, increases the rate of reduction of nitrobenzenes, establishing the hypothesis that HS might act as electron shuttles (Dunnivant et al., 1992). Transfer of electrons from humic substances to ferric iron (Fe(III)) or other elements has been confirmed (Benz et al. 1998; Coates et al., 1998; Lovley et al., 1996, 1998; Osterberg and Shirshova, 1997; Scott et al., 1998), and soluble humic acids are used as electron shuttles to insoluble iron forms during microbial dissimilatory iron reduction (Lovley et al., 1996, 1998). Thus, humic substances participate in redox reactions in soils both directly and indirectly as an electron shuttle.

In addition to humic substance interactions with iron in soils, it appears that iron is an important component of humic acids in some environments. The structure of humic acids is that of an organic polymer, principally composed of aromatic compounds (Griffith and Schnitzer, 1989). Quinones are assumed to be the major electron-accepting functional groups of humic acids (Paul et al., 2006; Roden et al. 2010; Scott et al. 1998). However, structural analyses of humic acids show multiple metal binding sites (Jansen et al., 1996a). Complexed metal within humic acid
structure aids in attachment to soil minerals (Jansen et al., 1996b), and there may be several more purposes of these metal groups. While investigating the transfer of electrons from humic acids to strong oxidants, Struyk and Sposito (2001) proposed that abiotic transfer of electrons from humic acids may involve complexed Fe(III) moieties. *Geobacter metallireducens* reduced humic acid-bound Fe(III); however, the inability of this reduced iron to subsequently release electrons led to the conclusion that complexed iron is not an important electron-accepting group in humic acids, and the assertion of quinone groups dominating this role in humic acids persisted (Lovley and Blunt-Harris, 1999). An evaluation of the chemical properties of humic acids determined that a variety of functional groups with different redox potentials contributed to the electron accepting capacity of humic acids (Peretyazhko and Sposito, 2006). I propose iron constitutes one of the major electron-accepting functional groups of humic acids.

Addition of the humic analog anthraquinone-2,6-disulfonate (AQDS) stimulated soil respiration in the Arctic, and humic substance reduction contributed notably to ecosystem respiration (Lipson et al., 2010, 2013; Chapter 2). In peatland systems, soil dissolved organic matter is a fundamental electron acceptor (Heitmann et al., 2007). Electron exchange between iron and soluble humic acids has been well demonstrated, and the redox state of the dissolved iron pool is indicative of the redox state of humic acids in Arctic soils (Helburn and MacCarthy, 1994; Lipson et al., 2013; Roden et al., 2010). Direct addition of chelated Fe(III) to Arctic soils increased respiration and decreased soil oxidative-reductive potential, indicating that the
chelated iron facilitated transfer of electrons to another pool of electron acceptors, the humic acids (Lipson et al., 2010).

In contrast to soluble humic acids, the role of solid-phase humic substances is only recently becoming recognized. Solid-phase humic acids shuttled electrons from iron-reducing bacteria to iron oxide surfaces in a wetland ecosystem (Roden et al., 2010). The soil system of the Arctic Coastal Plain near Barrow, Alaska exhibits high concentrations of soluble and organic-bound iron and large pools of insoluble humic substances (Lipson et al., 2010). Iron reduction is a major respiratory pathway in ACP soils (Lipson et al., 2010, 2013), and with high levels of both iron and humic acids, interactions between the compounds may contribute to respiration and carbon cycling in the Arctic ecosystem. Electron flow between soluble iron and insoluble humic acids therefore requires further examination. I present a field study and supporting incubation data indicating transfer of electrons from soluble reduced iron to insoluble humic acids reversing the roles of these compounds as demonstrated in iron reduction (Lovley et al., 1996, 1998). The incubation experiment validates microbial reduction of insoluble humic acids and removal of complexed iron from their structure. The inclusion of iron as a component and functional group of terrestrial Arctic humic acids was confirmed by complementary measurements of soil iron composition, humic acid iron content, and humic acid pH-dependent redox potential.
Materials and Methods

Site Description: My study site is located near the town of Barrow, Alaska on the North Slope of the Arctic Coastal Plain (ACP). Here, deep continuous permafrost lies beneath an active layer of tundra soil characterized by a seasonal freeze-thaw cycle (Hinkel et al., 2005). Experiments were conducted in a medium (71.254° N, -156.564° W) and ancient (71.248° N, -156.583° W) aged drained thermokarst lake basin (DTLB) (Sturtevant and Oechel, 2013). DTLB are a dominant landscape feature of the ACP, present on nearly half of the terrain near Barrow (Hinkel et al., 2003). These basins form from the erosion and drainage of thermokarst lakes, after which they undergo revegetation, forming a chronosequence of soil development. Ages of these DTLB are classified into four age categories: young, medium, old, and ancient. These ages are based on the amount of time since drainage of the thermokarst lake (Hinkel and Nelson, 2003). Basins used in the field experiment included a medium aged basin, which drained between 50 and 300 years before present, and an ancient aged basin, which drained between 2000 and 5000 years ago (Hinkel et al., 2003). In contrast, all four age classes of DTLB soils were used for iron extractions and redox measurements on humic acids.

Field Experiment: The field component was performed to test the hypothesis that soluble Fe(II) transfers electrons to oxidized humic acids, making the iron available for further microbial reduction. Supplementary humic acids were added to
ACP soils, and soluble soil iron was monitored over time. This experiment was executed during the active season of 2013 between July 18 and July 31. Soils were isolated using circular PVC collars of 20 cm depth and 11.4 cm inner diameter. Collars were pushed 15 cm deep into the soil, leaving 5 cm of each collar remaining above the soil. Installation of collars was aided by the use of a knife to cut the soil and provide a trench. To control for landscape variation, each soil within a collar receiving treatment was paired with a collared soil receiving an equivalent volume of water as control. Four of these groupings were set up in a medium (M sentinel) and an ancient (A sentinel) basin, with paired collars located within 1 m of each other and at least 15 m from other clusters. After insertion, collars and soils were undisturbed for 5 days to allow the soil to equilibrate and recover from effects of soil manipulation.

Soils within collars were injected with 60 mL ultrapure water containing 20 g/L concentration of Humic Acids from Sigma Aldrich, or with 60 mL ultrapure water as experimental control. Potentiometric redox titrations (Lipson et al., 2013) performed on these commercial Sigma Aldrich humic acids (data not shown) demonstrated that these humic acids exhibit redox properties comparable to humic acids extracted from these Arctic soils. Commercially available humic acids were selected for repeatability and consistency. Humic acid treatment of experimental soil plots was designed to achieve approximate final concentrations of 1 g/L in water saturating the soil contained within the collar. Assuming an electron accepting capacity of these humic acids of 0.33 meq/g from potentiometric redox titrations (unpublished data), the final concentration of 1 g/L humic acid to soil water would
provide sufficient electron accepting capacity to fully oxidize 330 µmoles of iron. The value was chosen from measurements of soluble iron in basin soils nearby which had an average concentration of 300 µM soluble Fe in basin soil pore water (Lipson et al., 2013).

Humic acid solution or water was injected with a syringe and long steel needle starting at 15 cm depth, applying even pressure as the needle was withdrawn up the soil profile to distribute 20 mL of liquid per 5 cm increment of soil. Soil water suction microlysimeters were installed at a depth of 0-10 cm (Rhizon, Eijkelkamp). Soil pore water was sampled regularly by connecting vacutainers to installed lysimeters (Becton Dickinson). Sampling occurred just before injection with treatment, and at 3, 24, 48, 72, 96, 120, and 168 hrs after treatment. An additional time point was sampled from the ancient basin 24 hr before addition of treatment. Water samples were acidified to pH 2 with 20-40 µL of 1 N H₂SO₄ to stabilize the Fe(II)/Fe(III) ratio before measurement.

The ratio of Fe(II)/total Fe is a useful metric to describe the redox state of the soluble iron pool (Lipson et al., 2013). The closer this value is to 1, the more reduced the iron pool; in reverse, if the pool is more oxidized, the value will be closer to 0. Total soluble iron and Fe(II) were measured using the 1,10-phenanthroline colorimetric method in 96-well plates, using a Spectra MAX 190 (Molecular Devices Corp.) at 510 nm after 15-30 minutes (Analytical Methods Committee, 1978). Values were analyzed with SOFTmax PRO 4.0 software (Life Sciences Edition by Molecular
Devices Corp). Total iron concentrations and Fe(II)/total Fe values were analyzed by time series analysis of covariance (ANCOVA) including cluster to control for landscape variation.

**Humic Acid Extraction:** Humic acids for use in the laboratory incubation experiment were extracted from soil samples collected from DTLB of all age classes in June 2011. Soil sample collection is described in Chapter 2. Extractions began with a 10:1 ratio of 0.1 M NaOH/0.1 M Na₄P₂O₇ solution to soil, shaken overnight (Agnelli et al., 2000; Janos, 2003). This mixture was centrifuged at 10,000 g for 6 min, and the supernatant containing fulvic and humic acids was acidified to pH 1 with HCl. The humic acid portion was precipitated out and collected by centrifugation (10,000 g, 6 min), rinsed with diH₂O, and was shaken 2 days with 0.3 M HF/0.1 M HCl to remove clay particles, silica, aluminum, and iron (Andjelkovic et al., 2006; Velthorst et al., 1999). Humic acid extracts were rinsed with 1 mM HCl and again with diH₂O before removing as much water as possible via centrifugation (Agnelli et al., 2000). To ensure oxidation of the humic acids, all steps were carried out with exposure to the air, and final extracts were stirred over two days with Parafilm M® (Beemis, Neenah, WI) to avoid drying out while allowing contact with oxygen.

Humic acids for Inductively Coupled Plasma Optical Emission Spectrometry and redox measurements were extracted as described in Chapter 2. There are two differences between extraction of humic acids for these measurements and the
extraction procedure described above for laboratory incubations. First, all extraction steps were carried out under N₂ gas to avoid oxidation and preserve the natural redox state for samples used in measurements. Conversely, humic acid extracts were provided as an electron acceptor for reduction in the laboratory incubation; thus, an oxidized state was preferred, and extraction was performed in oxic conditions. Second, humic acid extraction for measurements involved 16 samples (4 basin ages, 4 replicates) to examine differences by age class and increase statistical power for analysis. Subsamples of two soil samples (Young 16, Medium 1) were extracted omitting the HCl/HF rinse step to compare iron content in humic acids extracted with and without HF acid wash. In contrast, multiple soil samples from all four basin ages were combined in the humic acid extraction for laboratory incubations to provide cultures with a more homogeneous representation of ACP humic acids.

**Laboratory Incubation:** The laboratory incubation experiment involved an iron-reducing bacterial culture isolated from ACP soils and previously grown in two media containing either soluble iron or a soluble humic acid analog. The ability of this bacterium to grow by reducing field-sourced solid-phase humic acids was tested.

Media used for these incubations included 10 mM pH 5 sodium lactate as a carbon source, 1 mM NH₄Cl, 100 µM KH₂PO₄, 100 µM MgSO₄, 100 µM CaCl₂, and the following trace elements: 1.6 µM CoCl₂, 1.4 µM MnCl₂, 1 µM ZnCl₂, 2 µM H₃BO₃, 320 nM Na₂MoO₄, 200 nM NiCl₂, and 200 nM CuCl₂. Individual vials were
filled with 10 mL of oxygen-purged media. Cysteine was added to each vial (1 mM final concentration) to act as an oxygen scavenger.

To test for microbial effects, 8 of the vials started with $1.7 \times 10^3$ cfu/mL iron-reducing bacteria provided with 100 mg (dry mass) of insoluble extracted humic acids as electron acceptor, while another 8 vials had bacteria but no humic acids. Uninoculated controls of both treatments were also employed: four vials contained 100 mg humic acid extract and four did not (24 total).

The iron reducing culture used in laboratory experiments was isolated from a young basin (Y1) soil sampled in the field season of 2010 and grown in anaerobic conditions with media containing 50 mM AQDS, a humic acid analog, as the sole electron acceptor and 3.33 mM glucose or 120 mg/L xylan and carboxymethyl cellulose as carbon sources. The 16S rRNA gene from the isolate was sequenced. From results of the NCBI BLASTN platform, the isolate is a *Pseudomonas* species, and closely matches GenBank: EF515554.1 from a microbial fuel cell and EU978842.1, a sequence from a glacier ice metagenome. The isolate was subcultured in anaerobic vials with media containing 20 mM iron pyrophosphate as sole electron acceptor prior to inoculating the vials in this experiment. Initial concentration of inoculum was $1.7 \times 10^3$ cfu/mL, as determined by plate counts.

Glass 10 mL Wheaton serum vials (Cat: 223686) were fitted with chlorobutyl rubber stoppers (Cat: W224100-173). Aluminum seals (Cat: 224178-01) were crimped onto the lids to secure the septa and avoid pressure-related issues. All media were
purged of oxygen using N\(_2\) gas; after filling vials, headspaces were exchanged with N\(_2\) gas. The addition of cysteine aided in the elimination of residual oxygen. Although these isolates are facultative anaerobes and can grow using oxygen, the experimental design required oxygen to be unavailable as an electron acceptor.

Vials were sampled over the course of the experiment using a syringe and needle to maintain the anaerobic environment inside the vials. Vials were vortexed for 30 s and let to rest for 10 min prior to sampling. Liquid from the buffer was withdrawn and split for colony count and iron testing. Samples were diluted and plated on Tryptic Soy Agar (TSA) plates to calculate bacterial concentration and incubated at 22° C until colonies were visible. For iron measurement, each sample was combined with HCl (final concentration 20 mM) to stabilize Fe(II) and Fe(III) forms before storing in 4° C until measurement involving the 1,10-phenanthroline colorimetric method described above.

At the end of the incubation experiment, the oxidation-reduction potential (ORP) of humic acids in each vial was tested. The probe used was an Ag/AgCl ORP electrode containing 4 M KCl gel (Thermo Orion probe #9179BNMD). Voltage potential was corrected from Ag/AgCl to Standard Hydrogen Electrode (SHE) according to manufacturer instructions (+219 mV at 22°C). Electrode function was verified before measurement with freshly saturated solutions of quinhydrone in buffers of pH 4 and pH 7.
Preparation of humic acids for ORP measurement are as follows: Humic acid samples were quickly removed from vials and placed in a conical tube, headspaces were replaced with N₂ gas, samples were centrifuged (5 min, 11,000 g) to pellet humic acids, supernatants were removed, and humic acids were rinsed and resuspended in oxygen-purged pH 8, 0.1 M KH₂PO₄. The probe was fitted to a conical tube cap and attached to the tube enclosing the humic acid sample. Nitrogen gas flowed through inlet and outlet ports on the cap to preserve the anaerobic atmosphere during ORP measurement. The probe was permitted to equilibrate for 10 min before recording the value.

**Sequential Iron Extraction:** Sequential iron extraction was performed on soils collected in June 2011 from basins of each age class to quantify the amount of soil iron bound in organic compounds (3 young, medium, and ancient; 4 old basins; 4 replicates per basin). Extraction followed methods described by Poulton and Canfield (2005) with the exception of adding the nitric acid extraction step from Claff et al. (2010). This nitric acid step was critical in extracting organic bound iron from the soil.

**Inductively Coupled Plasma Optical Emission Spectrometry:** Inductively coupled plasma optical emission spectrometry (ICP-OES), performed in Chapter 2, quantified iron in humic acid extracts from all basin ages (Chapter 2). Humic acids were extracted from subsamples of two soils, omitting the HCl/HF rinse step to
compare iron values before and after acid wash (Young 16, Medium 1). The HCl/HF rinse step is used in humic acid extraction to eliminate silica, metals such as Fe and Al, and clay. Iron concentrations in humic extracts as determined by ICP-OES were compared to soil organic bound iron values from sequential iron extraction to calculate the proportion of organic bound soil iron which is complexed within humic acids.

**pH-Dependence of Humic Extract ORP:** A separate experiment measured ORP of oxidized humic acids extracted from soils of all four age classes (8 basins total) dissolved in buffer of three pH values to calculate the relationship of reduction potential (Eh) and soil pH. The change in Eh by pH gives clues to electron accepting moieties of the humic acid structure (Osterberg and Shirshova, 1997; Struyk and Sposito, 2001). Subsamples of each humic acid sample were dissolved in pH 6, pH 7, and pH 8 KH$_2$PO$_4$ (0.1 M). ORP of the dissolved humic acids was measured using the redox probe as above, and corrected to SHE. A linear regression was performed on all data points to determine the relationship between the Eh and pH of the humic acids. The slope of the regression was compared to a theoretical value for one proton per electron (−59.16 mV/pH unit) derived from the Nernst equation.
Results

*Humic Acid Addition Oxidizes Soil Iron Pools:* The addition of humic acids affected the redox state and total concentration of the soluble iron pool in Ancient basin soils. Initial Fe(II)/total ratios in the ancient basin soils were higher in experimental plots than control plots, but this difference was not significant (Figure 3.1). The Fe(II)/total ratio dropped within 24 hours of addition of humic acids, indicating oxidation of the soluble iron pool; soluble Fe(III) then became reduced at a slower rate over the following 7 days. These results show that electrons were transferred from the reduced soil iron pool to the added humic acids. This electron transfer reset the redox potential of the iron pool which then trended again toward reduction for the remainder of the time course.

The treatment effect of humic acid addition was significant in collar pairs 1-3 (F = 11.363, dfe = 47, P = 0.002). Cluster 4 was removed from the analysis of the redox state of iron in this basin because total Fe levels were very low, and Fe(II) levels were below detection limits (however, inclusion of these data in the time series analysis did not change the significance). Both treated and control soils exhibited a trend toward iron reduction throughout the remainder of the experiment, as expected from previous soil iron redox measurements in the ACP (F = 3.529, dfe = 47, P = 0.067) (Lipson et al., 2013; Chapter 2). The effect of treatment on Fe(II)/total ratio was significant when control values were subtracted from paired treatment values to correct data for spatial variability and means of pre-treatment values were compared to
means of post-treatment values ($F = 33.303$, dfe = 2, $P = 0.029$). Post-treatment mean values of treated soils were significantly more oxidized than control soil post-treatment mean values ($F = 255.047$, dfe = 2, $P = 0.004$). Landscape variation between paired collars produced high standard error bars (Figure 3.1), but all statistical analyses accounted for this spatial variability.

Total soluble iron was comparable in the ancient basin soils preceding amendment, and total soluble iron concentrations remained close to starting values in control soils throughout the study (Figure 3.2). Humic acid-treated soils exhibited an increase in total iron after amendment, and values remained higher than controls. Treatment effects were significant ($F = 53.737$, dfe = 63, $P < 0.001$), cluster effects from spatial variability were significant ($F = 21.542$, dfe = 63, $P < 0.001$), and effects of time were significant ($F = 4.593$, dfe = 63, $P = 0.036$). The impact of humic acid treatment on total iron in the ancient basin was also significant ($F = 11.363$, dfe = 6, $P = 0.014$) when means of post-treatment time points were compared between treatment and controls.

The ratio of Fe(II)/total Fe in the medium aged basin was comparable between treated and control soils, with values increasing toward reduction over time and exhibiting similar fluctuations (Figure 3.3). Humic acid addition did not show an effect. Total soluble iron concentration in humic acid-amended soils in the medium basin was higher than controls both at the beginning and during the course of the
experiment (Figure 3.4). Concentrations of soluble iron in the medium aged basin soils were 2 to 4 times higher than in the ancient basin.

**Microbial Reduction of Soluble Humic Acids:** Bacterial growth over time occurred in inoculated treatments, while uninoculated control vials remained uncontaminated. Growth curves for humic acid treatment vials are displayed in Figure 3.5. Total iron and ferrous iron (Fe(II)) increased over time in all vials supplemented with insoluble humic acids (Figures 3.6, 3.7). The slope of the increase in both total iron and Fe(II) over time is significantly higher in inoculated vials than controls (Total Iron: P = 0.04, Fe(II): P = 0.027). The significant increase in soluble iron exhibited in vials with iron reducing bacteria indicates that the microbes liberated complexed iron from the insoluble humic acids. In comparison, iron concentrations in all vials lacking humic acids remained below detection limits throughout the incubation.

Endpoint humic acid ORP measurements were lower for replicates treated with iron reducing bacteria than for uninoculated controls (P = 0.0047, t-test, equal variances, Figure 3.8). The lower ORP of inoculated replicates in contrast to control vials demonstrated microbial reduction of the humic acids.

**Iron Content of Soils:** Soils in the ACP have high levels of iron found in many forms including free iron adsorbed on the soil matrix, poorly crystalline
oxyhydroxides, crystalline minerals, and organic-bound iron, some of which includes humic acid-bound iron as revealed by sequential iron extraction (Figure 3.9). The organic bound iron pool is removed in the nitric acid fraction (HNO₃, Figure 3.9). The nitric acid fraction can also contain pyrite; however, based on the average oxidation state of S in ACP soils, there is no evidence for pyrite (Ted K. Raab, personal communication). These data are presented to determine the organically bound iron in soils in relation to the iron complexed in humic acids. Organic bound iron in ACP soils ranges from 8.2 to 42.2 µmoles Fe/cm³ with an average of 19.3 µmoles Fe/cm³. This represents an average of 17.5% of total soil iron (7.4 to 38.0% range).

Iron Content of Humic Acids: General ICP-OES results of humic samples are presented in Chapter 2. Average iron content of humic acids was 3984 ppm by dry mass, with no significant difference between age basins. The contribution of iron to humic acid electron accepting capacity (EAC) was calculated using the concentration of iron in humic acid extracts and the EAC of humic acids determined in Chapter 2.

Iron was higher in the two humic acid extracts processed without the HCl/HF rinse step than in the replicates processed with HF: young 16 contained 10226 ppm Fe without the rinse, and 4809 ppm Fe after HF rinse, medium 2 contained 17932 ppm Fe without the rinse, and 3576 ppm Fe after rinsing with HF (ppm Fe by dry mass humic acid). These differences indicate that incubating with HF removes a portion of the iron
from humic acids; not all humic acid-associated iron is removed by HF, supporting that the remaining iron is tightly bound in the structure of humic acids.

**pH-Dependence of Humic Extract ORP:** The ORP of humic acids decreases with an increase in pH (Figure 3.10). Minor differences existed between the ORP of different samples at any one pH, but the slopes of each basin are not statistically different. A linear regression on redox potential (SHE) by pH (Eh/pH) provided a slope of `43.3 ± 3.6 mV/pH unit (R^2 = 0.931).

Protons are consumed from organic functional groups such as quinones and phenolics when they are reduced, while complexed Fe(III) moieties gain electrons without consuming protons. The slope of a linear regression on Eh/pH data quantifies this phenomenon. The theoretical slope of `59.16 mV/pH unit signifies consumption of one proton per electron. The difference between the theoretical and experimental slopes indicates that fewer than one proton are consumed per electron gained in reduction of ACP humic acids, revealing that quinone and phenolic compounds are not the only electron accepting functional groups on these compounds. If bound in the structure of humic acids, Fe(III) would accept an electron without consuming protons. Thus, the difference between slopes signifies that complexed iron constitutes up to 26.6 ± 0.9% of the electron accepting moieties of humic acids. Using values for ecosystem respiration from Chapter 2, up to 2.7% of total Arctic ecosystem respiration is contributed by iron functional groups in the structure of humic acids.
Discussion

*Interactions Between Terrestrial Humic Acids and Iron*: Addition of humic acids to Arctic soil triggered an increase in ferric iron as a proportion of the total soluble iron pool in the Ancient basin. This change in the ratio of reduced to oxidized soluble iron indicates that electrons from ferrous iron in the soil were transferred to the supplemented humic acids, thus oxidizing the soluble iron pool. Following the initial oxidation event, the iron pool in amended soils showed a gradual subsequent trend toward reduction, as microorganisms in these soils utilized the refreshed pool of oxidized iron for respiration. These two observations support the model that microorganisms can use soluble iron to transport electrons to solid humic acids, a novel link connecting two concepts: microorganisms reduce soluble Fe(III) in anaerobic respiration, and Fe(II) donates electrons to insoluble humic acids.

Laboratory incubations of iron-reducing bacteria with insoluble humic acids demonstrated reduction of the humic acids and liberation of iron from their molecular structure. The humic acids remained in a predominantly insoluble state in this experiment, yet the bacteria reduced them as shown by ORP measurements (Figure 3.8). Reduction of large, insoluble compounds often requires a mediator (Lovley et al., 1998). I propose that these microorganisms solubilized iron from the humic acids for use as an electron shuttle to reduce the insoluble humic acids, reversing the roles of iron and humic acids described in previous studies (Lovley et al., 1996, 1998). The mechanism for removing the complexed iron is unclear, but may have been
accomplished through production of siderophores which are common in ACP soils (Lipson et al., 2013). An alternative explanation is that electrons may be conducted to Fe(III)-rich reaction centers of humic acids where they reduce Fe(III) to the more soluble Fe(II) form which diffuses more easily. While vials containing bacteria exhibited a steeper increase in total iron, the iron in uninoculated control vials also increased, suggesting that the lactate provided as a carbon source caused dissolution of some humic-bound iron after incubation for 6 weeks.

An increase in total soluble iron was observed in the ancient basin following humic acid addition. Two possible explanations exist for this observation. Soluble humic acids have been implicated as electron shuttles used in microbial reduction of extracellular insoluble iron; thus, the addition of humic acids to ACP soils may have stimulated the release of previously recalcitrant solid-phase iron (Lovley et al., 1996). In contrast, the commercially-available humic acids (Sigma-Aldrich) used for soil amendments contain iron much like humic acids sourced from ACP soils (0.187 mmol/g) (Benz et al., 1998). Similar to laboratory incubations, microorganisms in the soil may have liberated the iron from supplemented humic acids, increasing the soluble iron pool.

Unlike observations from the ancient basin amendments, humic acid addition to medium basin soils did not significantly affect the soluble iron pool. Concentrations of total dissolved iron were substantially higher in the medium aged basin than the ancient basin, in agreement with previous observations (Lipson et al., 2013). The high
soluble iron content of medium aged basins can be explained by their flat topography and especially wet, anoxic soil conditions despite lower overall mineral content (Lipson et al., 2013). The same volume of humic acids was added to each plot, regardless of basin age. Effects of humic acid addition on the redox state of dissolved iron are expected to be more pronounced in lower iron concentrations than higher concentrations due to the capacity of a given volume of supplemental humic acids to accept a finite number of electrons. Therefore, in a larger total soluble iron pool, a relatively smaller share of the iron would be impacted than in a smaller iron pool provided with the same volume of oxidizing humic acid, explaining why neither total soluble iron nor the redox state of the iron in the medium aged basin showed measurable difference between treatment and control soils.

Iron as a Structural Component of Arctic Humic Acids: ICP-OES measurements and the results of the Eh/pH experiment produce striking estimates of the role of iron as a structural component and electron acceptor in humic acids. ICP-OES measurements revealed the presence of iron in humic acid extracts from Arctic soils of varying age class, and the iron in humic acids account for approximately 27.4% of the EAC of humic acids. Concentrations of iron in humic acids measured with ICP-OES were lower in HF-rinsed samples than in samples extracted with the rinse step omitted, showing that HF is useful to remove some iron from humic acids. However, even after this step, iron persisted in the extracts, demonstrating the iron is
complexed within the humic acid structure. Despite avoiding removal by HF, iron was released from insoluble humic acid extracts by iron-reducing bacteria in laboratory incubations (Figure 3.6), supporting that the iron in these extracts was tightly bound in their structure.

Soils of the ACP contain various forms of iron, including organic-bound iron (Figure 3.9). Iron bound to organic compounds varies in stability; a portion can be removed by acid washes (HCl, HF), while some requires microbe-mediated chelation. The contribution of humic acid-complexed iron to total organic-bound iron in ACP soils was calculated. Iron associated with humic acids accounts for 1 to 19% of total organic-bound iron in ACP soils, differing by basin age (3% young, 1% medium, 9% old, 19% ancient). These values increase when iron concentrations are used from humic acids extracted without HF (12% young, 9% medium), signifying that iron loosely-associated with humic acids accounts for a larger portion of soil organic iron. Organically-complexed iron is a common and important feature in soils and aquatic environments (Sjöstedt et al., 2013; Sundman et al., 2014; Yu et al., 2015), and has been implicated in the preservation of organic matter (Lalonde et al., 2012; Wagai et al., 2014). The ability for bacteria to remove tightly bound iron from OM has consequences for the long-term stability of soil carbon.

A structural component in all humic acids extracted from ACP soils, iron complexed in humic acids must serve a purpose. ORP measurements on humic acids dissolved in solutions of increasing pH determined the extent to which this complexed
iron serves as an electron accepting moiety in humic acids. My Eh/pH data produce a slope of \(-43.3 \pm 3.6 \text{ mV/pH}\), similar to others (\(-42\) and \(-44 \text{ mV/pH}\), Osterberg and Shirshova), indicating that the remaining share (26.6 \pm 0.9\%) of electron accepting groups of humic acids do so independently of pH (Osterberg and Shirshova, 1997; Struyk and Sposito, 2001). Electron accepting capacity estimates from ICP and Eh/pH data agree (27.4\% and 26.6\% of humic acid EAC). I propose that organic functional groups such as phenolics and quinones are not the only major functional groups responsible for accepting electrons in microbial humic acid reduction; complexed iron constitutes more than a quarter of the electron accepting moieties of humic acids, and reduction of humic acid-complexed iron is responsible for up to 2.7\% of total ecosystem respiration in the Arctic.

My results confirm that iron interacts with humic acids in cooperating ways. Soluble iron acts as an electron shuttle to mediate the reduction of insoluble humic acids, and complexed iron in humic acids can be solubilized by bacteria for direct reduction or use as a mediator. Iron also serves a structural component and a major redox active functional group of humic acids.
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Chapter 3, in part, is in preparation for journal submission. Jaime Zlamal and David Lipson; 2016. The dissertation author was the primary investigator and author of this paper.
References


Figures

Figure 3.1: Fe(II)/total Fe in ancient basin during soil amendment experiment. (0: oxidized, 1: reduced).

Figure 3.2: Total soluble iron in ancient basin during soil amendment experiment.
Figure 3.3: Fe(II)/total Fe in medium basin during soil amendment experiment. (0: oxidized, 1: reduced).

Figure 3.4: Total soluble iron in medium basin during soil amendment experiment.
Figure 3.5: Bacterial concentrations from colony counts of humic acid treatment.
Figure 3.6: Total iron concentrations in vials with insoluble humic acids over the incubation.
Figure 3.7: Concentration of Fe(II) in vials with insoluble humic acids over the incubation.
Figure 3.8: Endpoint redox potentials of insoluble humic acids from incubations.
Figure 3.9: Sequential iron extractions.
Figure 3.10: Relationship between Eh and pH of dissolved humic acids.
CHAPTER 4

Biological chlorine cycling in the Arctic Coastal Plain

Abstract

The present research explores the largely unstudied chlorine cycle in pristine Arctic tundra soils of the North Slope near Barrow, Alaska. Microbial communities in these soils are adapted to the predominantly anaerobic conditions and are capable of utilizing a surprising diversity of metabolic pathways. I used pore water chloride measurements, total organic and inorganic chlorine measurements, Cl-XANES, laboratory incubation techniques, and metagenomic analyses to uncover chlorine cycling in Arctic tundra soils along a chronosequence of soil development. Concentrations of soil organic chlorinated compounds (Cl$_{org}$) were correlated with organic matter content. The concentration and chemical diversity of Cl$_{org}$ increased with soil development, Cl$_{org}$ in younger soils resembled that of vegetation, and older soils had more complex and variable Cl$_{org}$ XANES signatures. Metagenomic analyses revealed numerous genes for both synthesis (haloperoxidases) and breakdown (reductive dehalogenases, halo-acid dehalogenases) of Cl$_{org}$ in Arctic tundra soils, originating from diverse microbial genomes representing 11 phyla of both Bacteria and Archaea. Many genome sequences with close similarity to known organohalide respirers (e.g. *Dehalococcoides*) were identified. Furthermore, laboratory incubations demonstrated microbial organohalide respiration *in vitro*. This study provides multiple
lines of evidence for the existence of an intricate and dynamic chlorine cycle in a pristine Arctic tundra ecosystem.

**Introduction**

Biogeochemical cycling of naturally occurring chlorinated organic compounds (Cl$_{\text{org}}$) has received increased attention in recent scientific literature (Öberg, 2002; Biester et al., 2006; Svensson et al., 2007; Leri and Myneni, 2010; Van den Hoof and Thiry, 2012; Bastviken et al., 2013). Chloride (Cl$^-$) has generally been considered inert in ecosystems, and therefore often used as a conservative tracer in hydrological studies (Leibundgut et al., 2009). Microbial chlorine metabolism has consequently been studied mainly in the context of contamination from chlorinated compounds used in industry (Asplund and Grimvall, 1991; Holliger et al., 1997; Öberg, 2002; Hiraishi, 2008; Futagami et al., 2013; Lohner and Spormann, 2013; Leys et al., 2015), and radioactive $^{36}$Cl released following decommissioning of nuclear reactors (Van den Hoof and Thiry, 2012; Bastviken et al., 2013). Globally, soil chlorine cycling also has significance for the destructive impact of methyl halides and volatile halogenated organic compounds (VHOC) on the ozone layer (Keppler et al., 2000). Sources, sinks, and concentrations of VHOC have been studied in Arctic environments (Rhew et al., 2007; Teh et al., 2009; Wetzel, 2015). However, there have been no studies of internal chlorine cycling in non-contaminated Arctic soils.
Many organisms produce Cl$_{org}$, including fungi, lichen, bacteria, terrestrial and marine plants and invertebrates, and even higher animals such as frogs and mammals (Gribble, 1998; Gribble, 2003; Peng et al., 2005). Halogenases and haloperoxidases are enzymes catalyzing the chlorination of organic compounds (Niedan et al., 2000; van Péé and Unversucht, 2003; van Péé, 2012; Bengtson et al., 2013). Some halogenated compounds produced by plants/algae are powerful insecticides, such as Telfairine produced by the red algae *Plocamium telfairiae*, and many bacterially-produced antibiotics (such as vancomycin) contain chlorine (Gribble, 1998). In addition to synthesis of antibiotics, bacteria may non-specifically halogenate organic compounds as a form of competitive antagonism or as a defense against reactive oxygen species (Bengtson et al., 2009; Bengtson et al., 2013). Abiotic processes, such as volcano eruptions and forest fires, produce Cl$_{org}$, and Fe(III) can catalyze the abiotic formation of organohalogens (Gribble, 1998; Keppler et al., 2000; Comba et al., 2015).

Bacterial species, such as those in the *Dehalococcoides* genus, respire using organic chlorinated compounds as the terminal electron acceptor in a process known as organohalide respiration (also referred to as dehalorespiration or halorespiration) (Mohn and Tiedje, 1992; Rupakula et al., 2013). This process results in the liberation of chloride ions from Cl$_{org}$ (Holliger et al., 1993). A variety of dehalogenase enzymes exist and catalyze slightly different reactions depending on substrate specificity, utilizing one of several of mechanisms to cleave the bond between the carbon and
halogen atom (Kurihara et al., 2000; Tang and Edwards, 2013; Wagner et al., 2013; Bommer et al., 2014).

In wet tundra soils of the Arctic Coastal Plain, continuous permafrost blocks drainage, and the dominant moss communities on the surface hold soil water (Brown et al., 1967). The microbial communities in these waterlogged soils host a diverse range of anaerobic pathways (Lipson et al., 2013a; Lipson et al., 2015; Tveit et al., 2013). Permafrost-affected soils support one of the largest pools of carbon on earth (Hinkel et al., 2003; Schuur et al., 2008; Tarnocai et al., 2009; Hultman et al., 2015), and are especially vulnerable to climate change (IPCC 2014).

Our study site is located near Barrow, Alaska on the North Slope of the Arctic Coastal Plain. Much of the landscape is comprised of thermokarst lakes which drain and become revegetated slowly over the course of a roughly 5500 year cycle (Hinkel et al., 2003; Sturtevant and Oechel, 2013; Bockheim et al., 2004). Drained thermokarst lake basins (DTLB; sensu Grosse et al., 2013) accumulate organic matter and develop topography due to formation of ice-wedge polygons over time (Hinkel et al., 2003; Bockheim et al., 2004). Geomorphic stages are defined as young, medium, old, and ancient, which drained <50 y.b.p. (years before present), 50-300 y.b.p., 300-2000 y.b.p., and 2000-5500 y.b.p., respectively (Hinkel et al., 2003). DTLB provide a convenient system to study soil properties, such as chlorine or iron pools, in different stages of soil development (Bockheim et al., 2004; Hinkel et al., 2005; Lipson et al., 2013b).
Here, I present a study using several different approaches that reveal a dynamic and complex chlorine cycle in Arctic tundra soils. I present DNA sequence data with close matches to organisms that participate in chlorine cycling pathways and many genes for these pathways are identified from the metagenomes. I provide qualitative and quantitative measurements of inorganic and organic chlorinated compounds in soils within and among variations in landscape and depth. I show that organohalide respiration occurs in these soils using laboratory incubations.

**Experimental Procedures**

*Site Description:* Soil and soil pore water samples were collected from DTLB near Barrow, Alaska located on the North Slope of the Arctic Coastal Plain. Basins were situated near 71.24°N 156.48°W (Sturtevant and Oechel, 2013). Sampling occurred over the active seasons (summer) of 2010 through 2013. These tundra soils are characterized by a seasonal freeze-thaw cycle following active layer atop deep continuous permafrost.

*Soil Sampling:* Soil samples were collected in June and August of 2011 from replicate basins of all age classes surrounding Barrow, Alaska. Samples of 3 cm diameter and 30 cm length were removed using coring drill bits and extenders on a handheld power drill. Deep, 7.5 cm diameter cores were extracted to a depth of 40 cm using a SIPRE corer (Nb-steel drill bit) and small gas-powered engine (Miller et al.,
Samples from July 2013 were collected from Young 1, Medium Sentinel, Old 1, and Ancient Sentinel basins (as described in Sturtevant and Oechel, 2013) using a long serrated knife. The entire thawed soil profile was collected up to the depth of the frozen layer (approximately 20 cm). Samples were immediately returned to the lab and frozen at -40°C. Samples were shipped frozen overnight to labs in California by commercial courier.

**Soil Solution Chloride Measurements:** Soil pore water samples were collected from a depth of 0 - 10 cm using installed soil water suction microlysimeters (Rhizon, Eijkelkamp) from late June through October 2010 (Lipson et al., 2013b). Seven to ten spatial replicate samples were collected from a representative basin of each age class. Chloride content was measured using a colorimetric assay (Merchant, 2009). Plates were read by a Spectra MAX 190 (Molecular Devices Corp.) at 480nm and analyzed using SOFTmax PRO 4.0 software (Life Sciences Edition by Molecular Devices Corp).

**Total Organic Halides (TOX):** Soil samples were analyzed for Total Organic Halides (TOX), using chloride-titration. One set was used to study spatial variability of organic and total chlorine among and within basins; the soils comprised of segments spanning from the soil surface to 15 cm deep with three spatial replicates collected along a 30 m transect across each of three distinct basins for each age class.
The other set was used to analyze patterns in TOX by depth. Depth profiles were created from SIPRE cores using the following approximate depth increments: 0-5 cm, 10-15 cm, 20-25 cm, and 30-35 cm. Soils were prepared by drying overnight in a 65°C drying oven and homogenizing using a clean mortar and pestle. Between 10 - 60 mg of homogenized samples were weighed and combined with 100-150 mg tungsten powder (100 mesh, Santa Cruz Biotechnology) as a combustion accelerant before being placed into a clean, new, ceramic sample boat (COSA Xentaur; Yaphank, NY) and introduced by the Automatic Boat Controller to a Mitsubishi Chemical TOX-100 Chlorine analyzer set up for TSX-Cl analysis by pyrohydrolysis (Svensson et al., 2007). The gas (Matheson Inc.) profile was argon (carrier) and purified oxygen (for combustion). TOX-100 operating software (ver. 5.1.0.0) was used to analyze the resulting Cl\(^-\) containing hydrolysate by coulometry. The method detection limit by coulometry for this equipment was < 100 ppb Cl.

Inorganic chloride measurements were made on subsamples of the same homogenized soil samples used for TSX-Cl analyses. Each sample was shaken for 2 nights with a 1:10 or 1:20 ratio of dry soil to acidified potassium nitrate solution (0.2 M KNO\(_3\), 0.02 M HNO\(_3\)) to extract soluble chloride (Svensson et al., 2007). These supernatants were analyzed using the colorimetric chloride assay described above (Merchant, 2009). The chloride content of the nitrate extraction solution (“extraction blank”) was measured to correct for inadvertent addition of Cl\(^-\), but was not detectable. To increase sensitivity of the assay, the ratio of sample to working reagent was modified, as the original method was optimized to be linear over a higher
concentration of chloride (5 mM). Instead, 100 µL of sample was combined with 50 µL of working reagent, considerably lowering the background. Inorganic chloride concentration results were subtracted from total chlorine concentration values to determine the soil organic chlorinated component. These results were compared to inorganic-Cl leached extracts run on the TSX-Cl to verify the accuracy of each method.

_Cl-XANES:_ Samples 5-10 cm in length were obtained from soil monoliths about 20 cm deep collected in August 2013, separating shallow surface soil from deeper soil near the thaw depth boundary at the time of collection. Soils were cut with a band saw and dried fully in a 65°C drying oven. Vegetation samples were as follows: _Arctophila fulva_ (grass, Ancient Sentinel basin), _Carex aquatilis_ (sedge, Medium Sentinel basin), _Sphagnum_ (moss, Medium BE basin). Humic extract samples from a young and an ancient basin were also used in this study (Lipson et al., 2013b). Briefly, humic substances from 5-10 g of wet, frozen soil were extracted overnight in N₂-bubbled 0.1 M NaOH/0.1 M Na₃P₂O₇, precipitated by acidification with HCl to pH 1, rinsed with 0.001 M HCl, and finally redissolved in N₂-bubbled 50 mM NaHCO₃ (Lipson et al., 2013b). These extracts were precipitated in H₂SO₄ and rinsed with dilute H₂SO₄ before being dried at 65°C. Dried samples were ground to a fine paste with a clean, ethanol-rinsed mortar and pestle before mounting onto carbon tape. Polyethylene glycol was used as a binder for some soil samples.
K-edge chlorine XANES was performed at energies of 2800-2860 eV to elucidate the chemical forms of chlorine found in Arctic samples. XANES spectra were collected at Beamline 9-BM-C at the Advanced Photon Source (Argonne National Laboratory; Lemont, IL) in partial fluorescence mode in an experimental arrangement essentially as described in Bolin (2010) and consisted of a Si(111)-monochromator, with focusing achieved using a Rhodium-coated toroidal mirror. Harmonics were rejected through a flat, Rhodium-coated mirror; this provided a maximum energy resolution of 0.1-0.2 eV at 2.8 keV. Qualitative exploration of chlorinated compounds was achieved by linear combination fitting of normalized soil/plant spectra to a standard library collected under the identical beamline conditions as the soil spectra (Manceau et al., 2012). Standards dispersed in boron nitride mulls (BN₃ to minimize Cl over-absorption) served as Cl-peak energy standards by which we compared whole soils. Vulcan Carbon XC72 was used for immobilizing organic solvents.

ATHENA software was used to analyze and compare the spectra (Ravel and Newville, 2005). All sample spectra were normalized after pre-edge and post-edge corrections. The relative contributions of known chlorine-containing compounds to each spectrum was analyzed using Linear Combination Fitting (LCF) (Manceau et al., 2012). Standards were chosen to span the canonical oxidation states of Cl, and those used to fit spectra included NaCl, KCl, CaCl₂, monochlorodimedone, sucralose, polyvinylchloride, trichloroethylene, chrome azurol-S, and chloroacetic acid. LCF analysis produced weights attributed to each standard, and these weights were used in
a Principal Component Analysis (PCA) to qualitatively compare the overall similarity of each sample.

**Organohalide Respiration in Laboratory Incubations:** About 1 g of soil from 15 cm depth profiles collected from a medium aged basin in July 2013 was added to 25 mL of sterile, oxygen free, 10 mM pH 5.5 sodium acetate buffer containing 900 µM tetrachloroethylene (PCE), 1.8 mM dichloroethylene (DCE), and 0.1 mg/L thiamine (Holliger et al., 1998) in sterile 50 mL Wheaton crimp top glass vials fitted with butyl rubber septa. Septa were replaced and secured with metal clamps, and needles were used to exchange vial headspaces with N\textsubscript{2} gas. Treatments included a matrix of the following: with and without vitamin B\textsubscript{12} (0.5 mg/L) (Bommer et al., 2014; Yan et al., 2013), with and without carbon dioxide gas (sufficient to replace N\textsubscript{2} headspace) (Stevens and Tiedje, 1988; Nonaka et al., 2006), and with and without hydrogen gas as an electron donor (10 cc / vial) (Aulenta et al., 2008; Futagami et al., 2008). A subset of vials was autoclaved as a control for abiotic chlorine liberation; thus, this treatment received vitamin B\textsubscript{12}, hydrogen and carbon dioxide gases to provide the best environmental conditions for reductive dechlorination to test the effect of soil sterilization. The five different biotic treatments were as follows: (1) +B\textsubscript{12}, +H\textsubscript{2}, +CO\textsubscript{2}; (2) +H\textsubscript{2}, +CO\textsubscript{2}; (3) +B\textsubscript{12}, +H\textsubscript{2}; (4) +B\textsubscript{12}, +CO\textsubscript{2}; (5) +CO\textsubscript{2}. These vials were incubated at 10°C for the duration of the experiment, and buffer samples were extracted with a syringe regularly to test for soluble chloride concentration while maintaining the internal atmosphere. The colorimetric chloride assay described above
was used to measure liberated chloride (Merchant, 2009). The sample to working reagent ratio for these measurements was modified to 2:1 as discussed earlier. Concentrations were normalized to µmole Cl⁻ per gram of soil. Following chloride measurement and analysis, all biotic treatment results were combined as none of the specific treatments had a significant effect.

**Metagenomics and Bioinformatics:** A total of eight metagenomes were created using two depths from each of four age classes; each metagenome library included combined DNA from three spatial replicate samples taken from different locations along a 30 m transect to make each metagenome more representative of spatial variability. Shallow (5-6 cm depth) and deep (15-16 cm depth) soils were analyzed from the following basins: Young 1, Medium Sentinel, Old 1, and Ancient Sentinel (8 classes in total).

Samples were thawed and processed (~1 g) using MO BIO PowerSoil® DNA isolation kit (MO BIO Cat# 12888-100). DNA was quantified using Quant-iT pico green dsDNA assay kit (Life Technologies, Cat# P11496). Environmental DNA samples (500 ng) were sheared using a Covaris focused-ultrasonicator M220 with a target fragment size of 500 bp. Shotgun libraries were prepared using Roche rapid library Lib-L, Multiple-Prep (MV) preparation methods and multiplex identifier (MID) adaptors. Libraries were sequenced on a Roche 454 Life Sciences GS Junior platform at San Diego State University.
MIDs, reads with mean quality scores less than 20, reads less than 60 bp, duplicates, and reads with more than 1% ambiguous bases were removed. Read ends with quality scores less than 20 were trimmed from left and right using PRINSEQ v0.20.4 (Schmieder and Edwards, 2011). Processed sequencing reads were uploaded to MG-RAST (Meyer et al., 2008) and are publicly available (Project ID 7998, MG-RAST ID numbers 4554152.3-4554159.3).

I re-analyzed our previously published metagenomes from a medium aged basin as detailed by Lipson and others (Lipson et al., 2013a). These four metagenomes are from four depths (0-10 cm, 10-20 cm, 20-30 cm, and 30-40 cm); each metagenome explored pooled soils from four spatially replicated soil cores (GenBank SRA accession number SRP020650). The four previously published metagenomes had larger coverage than the eight described in the current study; however, for consistency all twelve were generated using the same sequencing platform and analyzed using MG-RAST with hit comparisons to SEED and GenBank databases.

Sequencing of 16S rRNA: Pyrosequencing was performed on the 16S rRNA gene from old and ancient basin soils collected in June 2011 (Lipson et al., 2015). Four depths were studied from high/dry and low/wet topographical features (rims and centers of low-centered ice wedge polygons). Sample processing, sequencing and core amplicon data analysis were performed by the Earth Microbiome Project (www.earthmicrobiome.org), and all amplicon and meta-data made public through the
Results

Soil Chlorine Content: The chloride levels in medium aged basin soil pore water samples were higher than in the other three basin ages (Figure 4.1). These medium basin chloride levels displayed a clear increase in the early part of the season and remained higher than the other basins throughout the season. A linear regression on the data using date as a continuous value found the medium and ancient basin chloride concentrations increased significantly over the season (medium basin: slope = 14.676 µM/day, R² = 0.168, P = 0.008; ancient basin: slope = 5.229 µM/day, R² = 0.181, P = 0.006). The high slope displayed in the medium basin was driven by the initial increase early in the season, after which the concentrations remained relatively constant (Figure 4.1). However, a quadratic fit was only marginally better than the linear fit (P = 0.074). The chloride concentration in soils of young and old basins did not exhibit significant changes over the growing season, and the linear slopes of these data are not statistically different than zero (Young basin: slope = 0.673 µM/day, R² = 0.003, P = 0.754; Old basin: slope = 0.471 µM/day, R² = 0.002, P = 0.793).
Total Soil Organic Chlorine: Total chlorine levels ranged from 69.9 ppm to 2000 ppm Cl (Figure 4.2 A). Concentrations of organic chlorinated compounds ranged from around 20% to 80% of total soil chlorine, with most values between 50-70% (Figure 4.2 B). The proportion of soil chlorine bound in organic compounds increased with basin age. With basin age coded as a categorical variable, this trend was marginally significant (P = 0.067). However, Cl$_{org}$ as percent of total chlorine increased significantly with log-transformed age inferred by the mean of each basin fiducial age category (P = 0.029) (Hinkel et al., 2003). Total soil chlorine did not increase significantly with age (Figure 4.2 A), yet when the two outliers from the medium basins were omitted from the analysis, this trend became significant (P = 0.024, age as categorical; P = 0.01, log-transformed mean fiducial age).

The relationship between soil organic matter content and Cl$_{org}$ is shown in Figure 4.2 C, including samples from among the depth profile and spatial replicates. An analysis of covariance (ANCOVA) was used to determine the interactive effects of organic matter and basin age on soil chlorine. After correcting for the natural increase in soil organic matter with basin age, more organic chlorine was observed in older basins. Certainly, soil organic chlorine was highly dependent upon soil organic matter content (P< 0.001), and the slope varied with basin age (Figure 4.2 C). Soil Cl$_{org}$ in old and ancient basins exhibited a steeper increase than young and medium basin soils (basin * organic matter interaction P = 0.013). This analysis indicates higher chlorine content in soil organic matter of old and ancient basins than in young and medium aged basins.
Soil Chlorine Structure: The energy of the edge (white line) feature of XANES spectra reflects the oxidation state of the chlorine in the soil, and is used to determine whether the compound signature is more organic or inorganic (Leri et al., 2006; Leri et al., 2007; Leri and Myneni, 2010). XANES peaks occur at lower energies for organic chlorinated compounds than inorganic salts. The pre-edge features give information on the chemical bonding environment of the chlorine atoms. The medium and young basin soil spectra resembled each other more closely than other spectra (Figure 4.3 A). Spectral edges of the young and medium soils occurred at higher energy (shifted right) than other samples and pre-edge features, pointing to increased inorganic chlorine concentrations as a proportion of total Cl. Qualitatively, the spectra of the medium and young soils displayed a likeness to spectra of vegetation, which old and ancient soils did not share (Figure 4.3 A). The edge features on spectra from old and ancient basin soils shifted to lower energies, indicating higher organic contribution to these samples compared with young and medium soils. Furthermore, spectra from these older soils were more variable and did not resemble each other or the spectra of vegetation.

These relationships were borne out in the PCA (Figure 4.3 B). Young and medium soil samples clustered with vegetation samples (moss, sedge, and grass), demonstrating the similarity in chlorine signature among these samples. Old and ancient soil spectra were dispersed on the graph, indicating diversification in soil chlorine signatures as soils develop. Likewise, the spectra from humic substances (relatively complex and old compounds) extracted from both young and ancient aged basins were distinct from the spectra of both soils and vegetation.
**Organohalide Respiration in Laboratory Incubations:** In each of the five biotic treatments a gradual increase in free soluble chloride appeared over the course of the incubation (Figure 4.4). The combined biotic treatments fit a linear trend with a slope of $0.0015 \mu$M Cl/g soil/hr and an $R^2$ value of 0.5614. Conversely, autoclaving the soils in the abiotic control treatment appears to have liberated chloride initially, after which there was no significant increase in chloride (non-significant trend line shown for clarity, Figure 4.4). The first measurement occurred prior to autoclaving the vials; all other measurements were taken after this step. A Mann-Whitney U test, performed on the slopes of individual flasks from this experiment, determined the slopes were significantly different between autoclaved and non-autoclaved treatments ($P = 0.002$).

**Metagenomics and 16S rRNA:** Metagenomic data revealed the widespread presence of various genes for enzymes integral to biological chlorine cycling in different depths of Arctic soils of all age classes. Haloperoxidases, important in the generation of chlorinated organics, were found in all but one soil surveyed. Halogenases, which catalyze the halogenation of organic compounds, were found in young and medium aged basins, primarily at the shallower depths of the soil profile. Reductive dehalogenases, responsible for transferring electrons to $\text{Cl}_{org}$ in the final step of organohalide respiration (Holliger et al., 1998), were found in young and medium soils; however, haloacid dehalogenases responsible for liberating halogens from 2-halo carboxylic acids (Goldman, 1968) were found throughout the landscape everywhere except the shallow ancient soil. These genes originated from an
unexpectedly diverse set of genera of bacteria and archaea (Supplementary Tables 4.1-4.4). Detected in all surveyed soils, was the canonical organohalide respiring genus *Dehalococcoides*. In addition, numerous genomic DNA hits to the well-studied dechlorinating genus *Anaeromyxobacter* and the perchlorate-reducing genus *Dechloromonas* were found in all soils analyzed (Table 4.1).

Data from our 16S rRNA gene survey showed higher numbers of *Dehalococcoides* in lower topography, and the peak for abundance appeared at the intermediate depth of 15 cm below the surface (Figure 4.5). The proportion of *Dehalococcoides* sequences varied with topography and depth (P = 0.012 and P = 0.011, respectively, R^2 = 0.485, log-transformed frequency data). Both bacterial and archaeal genera known for their capacity to dechlorinate halogenated organic compounds occur in these soils (the number of sequences detected, out of 2,027,920 total, is shown in parentheses): *Delftia* (9) (Zhang et al., 2010), *Desulfo bacterium* (52) (Egli et al., 1987), *Desulfomonile* (16) (Louie and Mohn, 1999), *Desulfovibrio* (36) (Sun et al., 2000), *Methanobacterium* (10649) (Egli et al., 1987), and *Methanosarcina* (2135) (Holliger et al., 1992). Notably, four genera found throughout these soils are capable of both dechlorination and dissimilatory iron reduction, another critical component of Arctic soil metabolism: *Anaeromyxobacter* (199) (He and Sanford, 2002; He and Sanford, 2003), *Desulfitobacterium* (1) (Niggemeyer et al., 2001, Nonaka et al., 2006; Villemur et al., 2006), *Desulfiuromonas* (67) (Löffler et al., 2000; Sung et al., 2003), and *Geobacter* (33602) (Coates et al., 2001; Sung et al., 2006).
Additionally, 59 sequenced matching the perchlorate-reducer containing genus *Dechloromonas* were found throughout the samples (Achenbach et al., 2001).

**Discussion**

Substantial concentrations of chlorinated organic compounds and the presence of chlorine cycling genes in soils of all basin ages indicate widespread chlorine cycling activity in the Arctic ecosystem, driven by remarkably diverse microbial communities. My results are similar to observations made of soils from a boreal forest catchment in southeast Sweden, in which soil organic matter content was positively correlated with Cl$_{\text{org}}$ (Svensson et al., 2007). The Arctic soil Cl$_{\text{org}}$ values range from 18.1 ppm to 1016 ppm, with an average of 244.8 ppm. These concentrations are higher compared to a boreal forest soil where Cl$_{\text{org}}$ ranged from 26 ppm to 178 ppm, the disparity expected to arise from the higher level of organic matter in Arctic peat soils compared to forest soils, as well as the natural enrichment of halogens in peat soils (Biester et al., 2006). However, the Cl$_{\text{org}}$ to inorganic chlorine (Cl$_{\text{in}}$) ratio was very similar to the boreal forest, which measured about two-thirds of the soil chlorine in an organically bound state (Svensson et al., 2007). The change in slope for Cl$_{\text{org}}$ concentration between the age classes indicates organic chlorinated compounds accumulate in the organic matter as the Arctic soils age.

The similarity of young and medium soil XANES spectra to those of vegetation signifies mosses and other plants contribute heavily to the initial
chlorinated compounds that develop in the early stages of these soils. A development of soil Cl$_{\text{org}}$ with time is indicated by the shift in spectra toward a more organic signature seen as soils age, and by the increased variability in spectra of older soils and humic substances which do not resemble each other or the vegetation (Figure 4.3 A-B). The TOX data agree with XANES trends, as more Cl$_{\text{org}}$ is seen in older and ancient basins, even when corrected for the natural accumulation of organic matter with time (Figure 4.2 B-C). Taken together, the trend shown by both TOX and XANES data suggests a net increase in quantity and complexity of halogenated organic compounds with time. It follows that, while vegetation is a key input of soil Cl$_{\text{org}}$, chlorinated organic compounds in these soils continue to change into more complex structures with increased functional group diversity as a result of soil development (Fahimi et al., 2003; Hinkel et al., 2003). Considering humic substances are likely the most developed and aged portion of the soil Cl$_{\text{org}}$, the divergence of humic sample spectra relative to the spectra of soils and vegetation further indicates the chlorine signature becomes more rich and diverse over time (Figure 4.3 B).

The soil landscape around Barrow, as a whole, becomes less anoxic with development (Lipson et al., 2013b). Anoxic conditions support anaerobic processes, such as organohalide respiration, while halogenating reactions are favored in the presence of oxygen (Bengtson et al., 2013). An increase in aerobic conditions as soils age leads to accelerated Cl$_{\text{org}}$ accumulation rates in older soils. Corroborating metagenomic analyses support the activity of Cl$_{\text{org}}$ synthesis pathways as exhibited by numerous genetic matches to haloperoxidases and halogenases (Table 4.1). Young and
medium basin chlorine metabolism may be dominated by organohalide respiration or reductive dehalogenation, slowing the accumulation of Cl_{org}, while synthesis pathways (e.g., haloperoxidases, which require peroxide) may dominate in the more aerated soils of older basins. Alternatively, the rate of Cl_{org} generation may be slightly faster than the rate of degradation at all stages of soil development; simply leading to accumulation of Cl_{org} in older soils.

The *in situ* increase observed in the medium aged basin soil chloride levels indicates net dechlorination occurred over the season (Figure 4.1). This medium basin increase cannot be attributed to accumulation of salts due to evaporation, as the other basins examined were subjected to the same conditions and did not produce similar trends. Given the anaerobic nature of ACP soils, the most likely explanation for this trend is organohalide respiration, a pathway validated for all soil depths and basin ages analyzed by genetic surveys. Metagenomic data showed that populations capable of organohalide respiration were present, revealed by the presence of reductive dehalogenase genes and organohalide respirers in all soils investigated (Table 4.1). Further dehalogenating capabilities are supported by prevalent haloacid dehalogenases and 16S rRNA matches to genera of numerous Cl_{org} degraders.

Chlorine cycling is a dynamic process in these Arctic soils. The young and old basins harbor organisms and enzymes significant in chlorine cycling pathways but do not show variation in chloride concentration, suggesting that Cl_{org} generation and degradation is tightly coupled. A majority of the studies on organohalide respiration have centered on model organisms such as *Dehalococcoides*, but this Arctic
environment with few major anthropogenic sources of Cl\textsubscript{org} demonstrates that there is a diverse chlorine-cycling microbial community awaiting characterization. While the range of organisms participating in the natural chlorine cycle is vast as revealed by numerous reports on unexpected chlorine cycling microbes, the limited annotation of genomes restricts the ability to use genetic insight alone to determine the full diversity of organisms participating in the chlorine cycle in any new environment (Holliger et al., 1992; Louie and Mohn, 1999; Nonaka et al., 2006; Sung et al., 2006; Zhang et al., 2010). For this reason, many genera with strains known to participate in Cl\textsubscript{org} metabolism were listed as potentially involved in the Arctic chlorine cycle (Table 4.1). Discovering chlorine cycling genes in 11 phyla of such a variety of organisms from cyanobacteria to Archaea was one of the more surprising aspects of the present research (Supplementary Tables 4.1-4.4).

The peak abundance of *Dehalococcoides* 16S rRNA genes at intermediate depths (Figure 4.5) likely signifies a sort of “Goldilocks” necessity of these organisms to both be in anaerobic conditions (not too shallow) as well as close to their substrate (not too deep). As organic matter is the best predictor of Cl\textsubscript{org}, these organisms follow the pattern of their substrate, decreasing as organic matter declines with depth (Lipson et al., 2013a). The metabolic process of organohalide respiration may be further constrained by pH and available energy (Paul and Smolders, 2014). While Fe(III) and humic substances are important electron acceptors for anaerobic respiration in soils of the Arctic Coastal Plain (Lipson et al., 2010; Lipson et al., 2013a; Lipson et al., 2013b) and can compete with other anaerobic process such as methanogenesis (Miller
et al., 2015), organohalide respiration can coexist with high levels of these electron acceptors (Aulenta et al., 2007; Azizian et al., 2010). Furthermore, Fe(III) and humic substances react to create Cl_{org} (Keppler et al., 2000; Fahimi et al., 2003), potentially increasing the opportunity for chlorine cycling in these soils. Although the redox potential of Cl_{org} is difficult to gauge due to its complex and varied chemical composition (Gribble, 2003), the coexistence of organohalide respiration with Fe(III) reduction suggests the energy potentials may overlap (Azizian et al., 2010; Shani et al., 2013).

Chloride liberation in the biotic treatments of the laboratory incubation confirmed the organohalide respiring capability of these Arctic soils in vitro. Interestingly, the process of autoclaving soils used as controls in the laboratory incubation resulted in a large release of chloride, possibly caused by the sudden release of chloride from the cytoplasm of soil microbes (Figure 4.4). This spike shows the soil was severely disturbed by the process of autoclaving, which affected both the chloride pool and the microbial community of the soil. Chloride data from all vials demonstrated noise; larger deviations observed in biotic samples may point to the expected dynamic nature of multiple chlorine metabolism pathways occurring in these vials during the investigational incubation. As the process of organohalide respiration is sensitive to certain soil conditions, the high iron content and low pH of these soils may have had an inhibitory effect, complicating the data obtained in these incubations (Paul and Smolders, 2014). Chlorine tracing experiments using labeled $^{37}$Cl or $^{36}$Cl
would allow for more sensitive observation of chlorine cycling (Bastviken et al., 2013).

This is the first study demonstrating that an active chlorine cycle exists in soils of the Arctic Coastal Plain. Naturally occurring Cl_{org} is present throughout the tundra soils investigated, and more organic chlorinated compounds accumulate as soils age. Microorganisms distributed among all soils sampled partake in the chlorine cycle, with a wide diversity of bacteria performing organohalide respiration in uncontaminated soils. As the Arctic continues to be a major focus of investigations of climate change and atmospheric impacts of VHOC, an increased understanding of the natural chlorine cycle in this ecosystem will prove indispensable.
Acknowledgements

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Chapter 4, in part, is under review in Environmental Microbiology. Jaime Zlamal, Ted K. Raab, Mark Little, and David Lipson; 2016. The dissertation author was the primary investigator and author of this paper.
References


Figures and Tables

Figure 4.1: Soil pore water chloride concentrations over the 2010 growing season.
Figure 4.2: Analysis of soil chlorine using TSX-Cl. (A) Total soil chlorine by basin age; (B) Cl$_{org}$ as percent of total chlorine; (C) The relationship between Cl$_{org}$ and soil organic matter by basin age. Regression lines are shown for young and medium (YM) versus old and ancient (OA) soils.
Figure 4.3: Cl-XANES on soil and vegetation samples. (A) Cl-XANES spectra from representative basins and vegetation, with normalized absorption plotted as function of x-ray energy (eV); (B) A principal component analysis (PCA) of the linear combination fitting results for experimental XANES spectra using weights of each standard
Figure 4.4: Chloride concentration over time in autoclaved and non-autoclaved soils provided with dichloroethylene (DCE) and tetrachloroethylene (PCE). Regressions lines for the two treatments are shown.
Figure 4.5: Relative abundance of *Dehalococcoides* 16S rRNA sequences by depth in areas of high and low topography.
Table 4.1: Numbers of sequences matching chlorine cycling genes and organisms in metagenomes. Analysis performed using the PATRIC database and MG-RAST. Abbreviations are as follows: HPO: Haloperoxidase; H: Halogenase; HADH: Haloacid Dehalogenase; RDH: Reductive Dehalogenase. Functional genes, *Anaeromyxobacter*, and *Dechloromonas* are from the SEED database matches. *Dehalococcoides* hits are from GenBank matches.

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Supplemental Table 4.1: Phylogenetic assignment of sequence matches to Haloperoxidases.

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Supplemental Table 4.2: Phylogenetic assignment of sequence matches to Halogenases.

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Supplemental Table 4.3: Phylogenetic assignment of sequence matches to Reductive Dehalogenases.

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Supplemental Table 4.4: Phylogenetic assignment of sequence matches to Haloacid Dehalogenases.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Matches</th>
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<td>Solibacter</td>
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<td>Opitutus</td>
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<tr>
<td>Clostridium</td>
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<td>Pelobacter</td>
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</tr>
<tr>
<td>Conexibacter</td>
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<tr>
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<td>Roseiflexus</td>
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<td>Azorhizobium</td>
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<td>Janthinobacterium</td>
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<td>Ralstonia</td>
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<tr>
<td>Sulfitobacter</td>
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</tr>
<tr>
<td>Syntrophobacter</td>
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<tr>
<td>Thermomonospora</td>
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<td>Bacillus</td>
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<td>Bordetella</td>
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<td>Desulfitobacterium</td>
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<td>Flavobacteriales</td>
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<td>Geobacter</td>
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<td>Haemophilus</td>
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<td>Hyphomonas</td>
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<td>Janibacter</td>
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<td>Kribbella</td>
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<td>Treponema</td>
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<td>Total Sequence Matches</td>
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CHAPTER 5

Conclusions

Summary

The objective of my dissertation was to explore anaerobic metabolism involving understudied organic terminal electron acceptors found abundantly in soils of the Arctic Coastal Plain. I conclude that humic acids and organic chlorinated compounds are important for anaerobic respiration in these tundra soils. Chapter 2 provides electrochemical evidence that anaerobic respiration using humic acids contributes to ecosystem respiration in anaerobic tundra soils, and humic acid compounds increase in electron accepting capacity along a chronosequence of soil development. In Chapter 3, links between humic acids and soluble iron are identified, demonstrating that soluble iron serves as a mediator to microbial humic acid reduction in Arctic soils, and that iron plays a role as an electron-accepting moiety complexed within the structure of humic acids. Chapter 4 explores the use of chlorinated organic compounds as an electron acceptor by soil microorganisms and provides molecular data demonstrating that the Arctic chlorine cycle is biologically active. This final chapter proposes the incorporation of these results into future investigations on metabolism and climate models.
Future Directions

Future work into humic acid and chlorinated organic compound metabolism should seek to compose a fuller picture of their cycles. Determining the structure of common humic acids and chlorinated organics will allow for directed studies on metabolism of these compounds. Organisms participating in these pathways should be isolated from various environments and defined.

Reduction-oxidation couples are commonly classified by redox potential, and at present, the thermodynamic favorability of both humic acids and chlorinated organic compounds as electron acceptors remains unresolved. Arctic pools of humic acids and chlorinated organics are complex and heterogeneous. Functional groups active in electron exchange are diverse in these compounds and differ in chemical structure between molecules. A fuller characterization of the redox properties of humic acids and chlorinated organic compounds would help predict whether reduction of these terminal electron acceptors competes for resources with important soil processes such as methanogenesis.

Anaerobic respiration involving humic acids and chlorinated organic compounds was my focus. Processes forming these organic electron acceptors in Arctic soils require further study, as do the organisms responsible. The oxidation state of humic acids from the Arctic Coastal Plain seems to reset every year, being fully oxidized and primed for reduction at the beginning of the active season. The mechanisms responsible for this pattern are unclear. My dissertation lays the
foundation for study of anaerobic respiration involving humic acids and organic chlorinated compounds in Arctic soil. Increased knowledge of humic acids and chlorinated organics and their cycles will help determine their full impact on the Arctic and other ecosystems where they are prevalent.

**Conclusion**

Arctic soils are important reservoirs of soil carbon and therefore have the potential to heavily influence climate change feedback through soil respiration processes. My dissertation has demonstrated the significance of anaerobic respiration involving the organic terminal electron acceptors of humic acids and chlorinated organic compounds in soils of the Arctic Coastal Plain.

Climate models are incomplete without incorporating the effects of metabolic pathways involving humic acids and organic chlorinated compounds in Arctic soils. If these essential cycles are left out of the equation, methane release estimates will be exaggerated as respiration involving these compounds competes with methanogenesis. Likewise, CO$_2$ fluxes will be underestimated without integrating these commonly ignored anaerobic electron acceptors which contribute to CO$_2$ release. Predicting landscape and seasonal patterns of greenhouse gas fluxes and their responses to climate change requires an understanding of the environmental controls over humic acid and chlorinated organic compound reduction. This dissertation contributes novel insight into these critical soil microbial processes. I determined that Arctic soil humic
acids develop in complexity over time, and their reduction contributes more to ecosystem respiration in older soils. The dynamic biological chlorine cycling processes discovered in my study were not expected to exist in Arctic soils with such little human influence. I show evidence that the chlorine cycle is more active in unpolluted soils than previously thought; therefore, Cl\textsuperscript{−} is not an appropriately inert tracer for hydrological studies. The greater understanding of the development and electron accepting capacity of terminal electron acceptors from my dissertation is a large step into determining respiratory processes impacting Arctic soil organic matter and carbon cycling, and therefore, predicting climate change feedback processes worldwide.