

**Factors controlling the relative abundances of anaerobic conditions in upland soils.**

by

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## Abstract

Anaerobic microenvironments, or microsites, are important regulators of C cycling in upland soils, but environmental factors that control their abundance are not well known. To help fill this knowledge gap, we examined variation in the redox state of upland soils at seven locations across the Kansas precipitation gradient. At each location, we used a Gidding's probe to collect samples from three depth intervals (0-5, 5-15, and 15-30cm) at three land use types (native prairie, restored prairie, and agricultural). We measured 0.5 M HCl extractable Fe(II) and Fe(III), total organic carbon (TOC), pH, and particle size distribution, and used Fe(II) as our measure of the abundance of anaerobic microsites. We assessed the statistical significance of relationships between our parameters using Spearman's rank correlation tests ( $p < 0.05$ ).

Results show that Fe(II) content was positively correlated with TOC overall ( $r = 0.52$ ,  $p = 2.99E-7$ ), consistent with greater TOC storage in soils with more abundant anaerobic microsites. Fe(II) ( $r = 0.51$ ,  $p = 4.849E-7$ ) and TOC ( $r = 0.25$ ,  $p = 0.019$ ) were both positively correlated with average annual precipitation. Increasing precipitation has the potential to increase anaerobic microsite abundance through two mechanisms: by decreasing O<sub>2</sub> transport within soil and by increasing organic C inputs. A positive correlation of clay abundance and Fe(II) ( $r = 0.31$ ,  $p = 0.003$ ) but not TOC in our dataset is consistent with the former mechanism. However, it is possible that both mechanisms contribute. Analysis of our results by land-use category shows that relationship between Fe(II) and TOC and their relationships with precipitation were significant for samples from agricultural and restored prairie fields but not native prairie fields.. Taken together, these findings provide evidence that precipitation and land use influence soil redox and its relationship to carbon storage in upland soils. We believe that our results show the

need for more studies to examine the relationships between soil redox parameters, TOC, and precipitation data at different time scales to aid in refining soil carbon models.

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## **Dedication**

To my friends and family and everyone who has helped me get here I could not have done it without you. professor Kirk you really believed in me over these last two years and I cannot stress enough how grateful I am for that confidence; it has helped me get a lot done. I'll take with me the skills and beers that I've learned here. To Bre and Javille, y'all were pretty incredible to work alongside and I'm very glad I got to meet you. To my thesis committee, thank you for the thoughts and guidance on this project, it was truly invaluable. Lastly to the K state community as a whole, I'm glad that people in the Midwest turned out to be just as nice as everyone says they are.



# Chapter 1 - Introduction

Soil contains the largest terrestrial pool of organic C, making it important to understand factors that affect soil C cycling. One of the primary controls on C cycling is the redox state of soil, which strongly affects organic carbon mineralization (i.e., oxidation to inorganic C) (Brewer et al., 2018; Keiluweit et al., 2017; Liang et al., 2017; Phillips & Nickerson, 2015; Schimel, 2018). Until recently, anoxic microenvironments, termed anaerobic microsites, were thought to be insignificant in upland soils (Brewer et al., 2018; Keiluweit et al., 2017). However, recent studies have shown that they play an important role in organic C stabilization by decreasing C mineralization rates (Keiluweit et al., 2016, 2017). Improving our understanding of factors that influence anaerobic microsite abundance will help inform models of soil C cycling in the future as especially considering the implications of climate change.

Anaerobic microsite abundance has been linked with higher soil clay content and decreased oxygen concentrations in soils (Brewer et al., 2018; Keiluweit et al., 2016, 2018). The interiors of soil peds and aggregates can become anaerobic if local respiratory demand for oxygen surpasses its supply (Keiluweit et al., 2016; Sexstone et al., 1985b). Soil oxygen transport is highly controlled by soil structure, texture, porosity, and moisture content (Keiluweit et al., 2016; Kristensen et al., 2010; Neira et al., 2015). Therefore, factors that affect these variables can have the potential to impact the abundance of anaerobic microsites.

These observed relationships suggest that the abundance of anaerobic microsites can be affected by the amount of precipitation. Elevated soil moisture negatively impacts soil oxygen diffusion primarily through decreased occupying what would otherwise be open pore space (Neira et al., 2015). Additionally, soil pore structure and aggregation are dramatically influenced by plant and soil microbe abundances, composition, productivity, byproducts, and more (Cotrufo

et al., 2013; Neira et al., 2015; Sexstone et al., 1985a; Johan Six et al., 2002). Plants are capable of altering soil structure and porosity both physically with their roots and chemically through various exudates that can aggregate soil particles (Lynch & Bragg, 1985; Neira et al., 2015). Similarly, a variety of soil microbes can also both physically and chemically bind soil particles together into aggregates, thus influencing soil structure and porosity (Lynch & Bragg, 1985). The availability of water, which is inherently tied to precipitation, in soils has demonstrated effects on the productivity and composition of both plant and soil microbial communities (Schimel, 2018; Wu et al., 2011). Increased precipitation is generally associated with higher primary productivity for plants and soil microbial communities become stressed in drier conditions (Deng et al., 2017; Schimel, 2018; Wu et al., 2011). These findings suggest that anaerobic microsite abundance increases with precipitation.

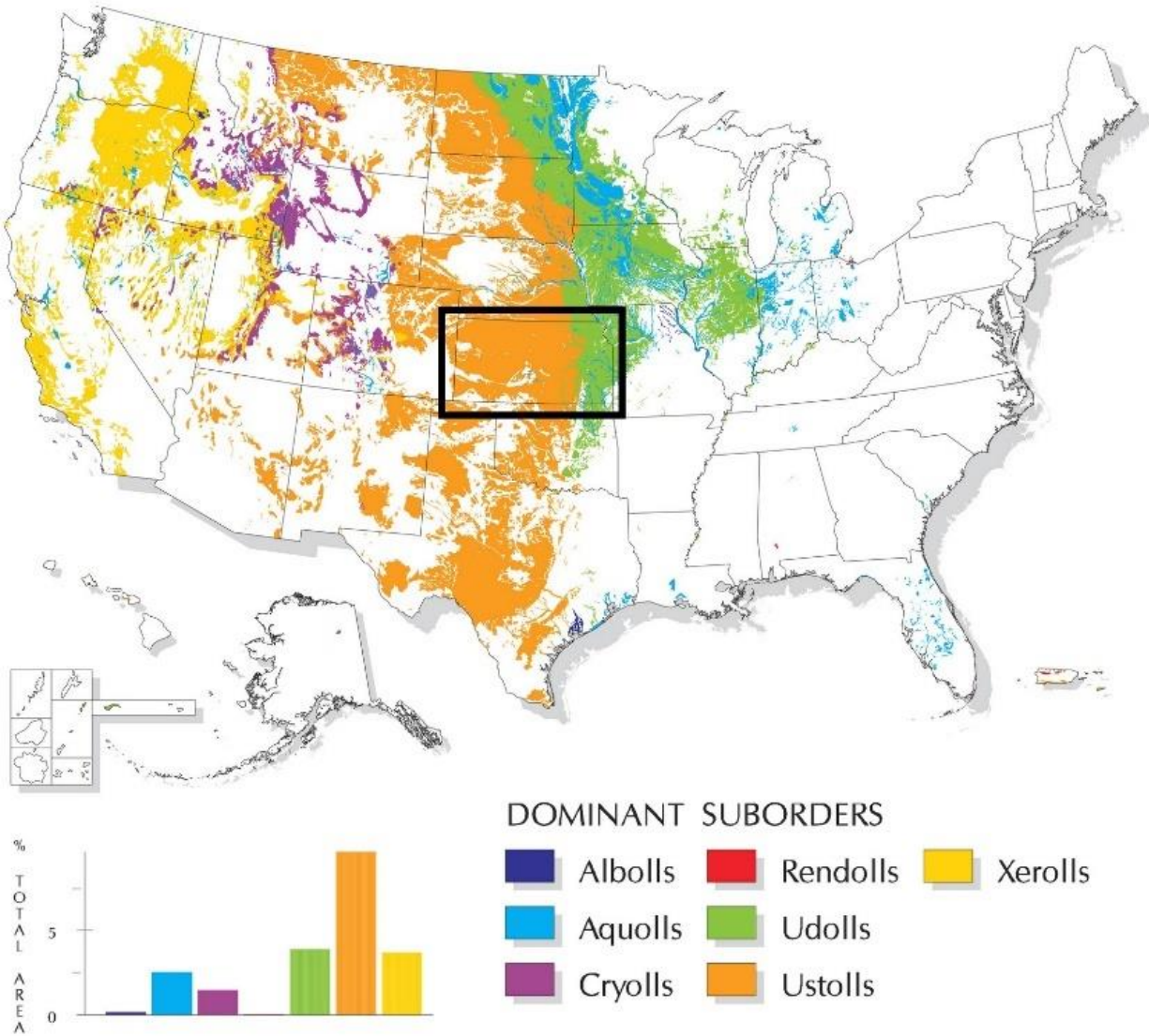
Similarly, land use also has the potential to affect the abundance of anaerobic microsites and merits study. Agricultural practices such as tillage break up soil aggregates, in the process reducing the quantity and quality of soil roots and microbial populations (Blanco-Canqui & Lal, 2004). These effects in turn have implications for soil oxygen transport and thus anaerobic microsites. Soil anaerobic microsites that may have existed in soil would likely be profoundly disturbed by tillage, likely resulting in an overall decrease in their overall abundance. Lending further support to this idea is the fact that conversion of natural soil ecosystems to agricultural use is traditionally associated with GHG emissions and net losses of organic carbon from soils (Jenny, 1930; Lal, 2002; Levine et al., 2011; Tubiello et al., 2015; Whisler et al., 2016). As for clay, Keiluweit et al., (2018) found the abundance of anaerobic microsites to be strongly correlated with clay content. What remains unclear is how precipitation, through various possible

indirect mechanisms, together with land use, impact anoxic microsite abundance on a broad spatial scale in upland soils.

In order to learn more about controls on anaerobic microsite abundance and its impact on soil carbon storage on a broad special scale, this study asks the question: how do the abundances of anaerobic microsities and organic carbon content of upland soils vary with land use across the Kansas precipitation gradient? We hypothesize that (1) the abundance of anaerobic microsities will be positively corelated with soil TOC content, consistent with previous studies that have found regulation of C cycling by anaerobic microsities (Keiluweit et al., 2016, 2017). Additionally, (2) the abundance of anaerobic microsities and soil TOC content will both be positively correlated with precipitation, and lastly (3) the native and restored sites will have higher TOC content and abundances of anaerobic microsities.

To test these hypotheses, we have sampled and analyzed soils from three land-use types across Kansas' precipitation gradient: native prairie, restored prairie, and agricultural land. This natural laboratory is ideal for our study as Kansas is predicted to become both warmer and more arid in the future (EPA, 2016), possesses a large precipitation gradient (Fig. 2), and has a relatively uniform soil type (Fig. 1). The breadth and density of our sampling over Kansas' precipitation gradient has allowed us to produce a novel dataset to examine our hypotheses.

# MOLLISOLS



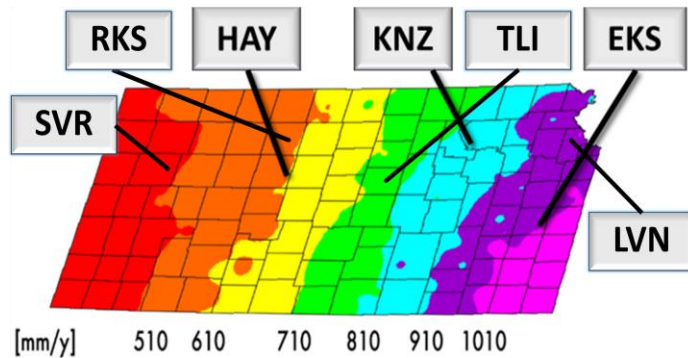
**Figure 1: Map of Mollisols in the United States with Kansas highlighted with a black box (Mollisols Map | NRCS Soils, n.d.).**

## **Chapter 2 - Methods**

### **2.1 Study Site Selection**

We collected samples from seven sites across the Kansas precipitation gradient (Fig. 2). Study sites selection criteria included the availability of desired land use types and location on the precipitation gradient. Each research site had plots of all three land use types present within close proximity of each other. Plots were regarded as being native following assessment in which the plant community present and current land management practices were deemed to be adequate for a native prairie. Restored plots were selected in essentially the same manner, the only difference being that they were previously agricultural fields. Agricultural plots were plots that were currently being farmed. Soils at the selected research sites are all silt to silt-clay loam soils with characteristics of Mollisols. USDA descriptions of the soil series for each of the research locations can be found in the appendix (Appendix A).

Precipitation and temperature both increase eastward across the study area (Fig. 2). From east to west across the study area, 30-year normal precipitation increases from 476 to 1040 mm/y while temperature increases from 11.6 to 13.3°C based on data from PRISM climate group at Oregon State University. Thus, precipitation gradient is much larger than the temperature gradient. Due to the relatively small change in temperature compared to the change in precipitation, we focus on precipitation in this study.

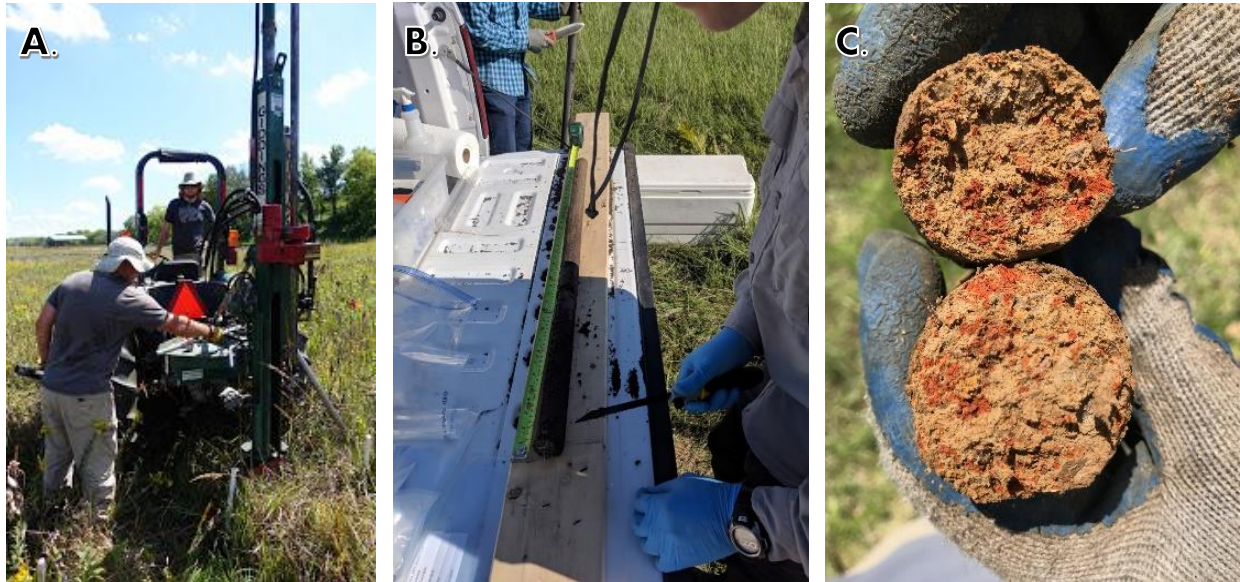


**Figure 2: (Left) Map of Kansas with 30-year average annual precipitation bands shown in color and sampling locations marked. (Right)**

## 2.2 Sample Collection

At each research site, native prairie restored prairie, and active agriculture plots were identified and sampled. Samples were collected using a Gidding’s soil probe (Figure 3) and were split into three depth-intervals for analysis: 0-5cm, 5-15cm, and 15-30cm, with each plot being cored several times. At each plot, one 30cm core from each land-use type was designated as our “redox” sample for Fe, C, N, and particle size analysis. These redox samples were immediately sealed in BD Gaspak EZ pouches with oxygen consuming packets in the field and kept at 4°C until being stored in a -80°C freezer.

Sampling took place during two separate time intervals in 2018 and 2019 with two separate sampling strategies. Each land use plot at the HAY, KNZ and EKS research site has four plots. In 2018, one redox sample core was taken from each plot, yielding four redox sample soil cores per land use type at these three locations (12 samples). During the 2019 sampling, each land use type had only one redox sample taken from it (3 samples). Soil pH was measured from soils collected alongside the redox samples, but not from the actual redox samples themselves. These collected samples were stored at 4°C, homogenized, and analyzed as soon as possible.



**Figure 3: A: Giddings probe used for collecting soil cores. B: Soil core extracted by Giddings probe. C: a cross sectional view of a collected soil core.**

### **2.3 Chemical Analysis**

We measured soil pH twice, once in deionized water and then again in 0.1 N  $\text{CaCl}_2$  suspensions following agitation for 20 minutes. The suspensions consisted of 2 g of fresh soil and 10 mL of solution. The suspensions were agitated on an orbital shaker and then measured for pH using a single junction general-purpose pH electrode connected to a PC-450 meter (Oakton).

We used ferrous iron ( $\text{Fe(II)}$ ) as a measure of abundance of anaerobic microsites in our samples, consistent with Keiluweit et al. (2017). Ferrous iron can be produced by biotic and abiotic reactions but can be oxidized when exposed to oxygen or nitrate (Bethke et al., 2011). Thus, ferrous iron can be used as a tracer for anoxic conditions (Heron et al., 1994; Keiluweit et al., 2017). To analyze the Fe content of our samples, we used the ferrozine method, which has shown to be effective for sediments, to extract Fe from our samples (Heron et al., 1994; Stookey, 1970). Step by step procedures of how  $\text{Fe(II)}$ ,  $\text{Fe(III)}$  and  $\text{Fe(tot)}$  concentrations were obtained

using this methodology can be found in Appendix C and Appendix D. The samples used for our Fe analysis came from the anaerobic pouches that were kept at -80°C. During processing in the lab, samples were additionally handled inside of an anaerobic chamber to avoid exposure to oxygen. Our Fe data comes from the analysis of these samples. It is important to note that since we used 0.5M HCl to extract Fe, the Fe values in this study are not total Fe values but 0.5M HCl extractable Fe values. Measured Fe concentrations were corrected for soil moisture to be on a per-dry-kg of soil basis. All samples were weighed, left to air dry for 3 days and then weighed again to calculate their moisture content. The moisture content was then used to correct the Fe measurement to be

For TOC and TN analysis, one gram of sample was taken from each soil sample, air dried, sieved (>2mm), and then ground into fine powder with a mortar and pestle before being analyzed at Kansas State University Soil Testing Laboratory. The lab used a LECO TruSpec CN Carbon/Nitrogen combustion analyzer for the measurements. The soil laboratory reports, the standard deviation for this instrument and the above method as 0.06 with 2.18% Rel. Std. Dev. for soil carbon and 0.006 with 3.35% Rel. Std. Dev. for soil nitrogen.

## **2.4 Particle Size Analysis**

We analyzed the particle size of our samples in order to see if there were any significant correlations between the clay, silt, and or sand content with TOC or our Fe redox parameters. About 4.5 grams of each sample was weighed out in 30mL Falcon tubes to which 4mL of 30% hydrogen peroxide was added. After the samples had stopped visibly reacting with the H<sub>2</sub>O<sub>2</sub>, the tubes were filled with water up to 25ml and left for 24hrs to react. After 24 hours the samples were then centrifuged and rinsed with DI water several times to remove any excess H<sub>2</sub>O<sub>2</sub>.



Particle size analysis was done with a Malvern Mastersizer 3000 laser diffraction particle size analyzer with a Hydro EV wet dispersion accessory. The USDA particle size classification scheme states that particles from 0.05-2mm are considered sand, 0.05-0.002mm as silt, and >0.002mm as clay. To account for the underestimation of clay content by laser diffraction, however, we adjusted the clay boundary to 0.008mm. This adjustment helps to compensate for the tendency of laser methods to underestimate clay content consistent with the observations of (Konert & Vandenberghe, 1997).

## **2.5 Statistical Analysis**

To test the significant of relationships between our parameters, we used Spearman's rho rank correlation, a non-parametric analysis for the monotonic relationship between two variables, in GraphPad Prism 8. There were two main reasons that we chose this methodology: first, our data failed the Kolmogorov-Smirnov, Shapiro-Wilk, D'Agostino & Pearson, and Anderson-Darling tests for gaussian distribution. To assess the impact of land-use we separated the samples out by land-use and compared the results of our analysis, spearman's rho correlation, among the different land use types.

We assessed statistical significance by using an alpha value of 0.05 in line with conventional practice. In line with the approaches of (Chapelle et al., 2013; Kirk et al., 2016) we have not included correlation analysis of parameters from data sets of less than 20 samples. This was done in order to help limit the possibility of random samples and outliers impacting our overall conclusions. Consequently, we do not explore correlations between our parameters separated out by depth and land-use simultaneously, as this created data sets with only 9

samples. However, we do examine variation with depth and land use separately, as these datasets had 29 samples per depth and 27 samples per land use.

## Chapter 3 - Results

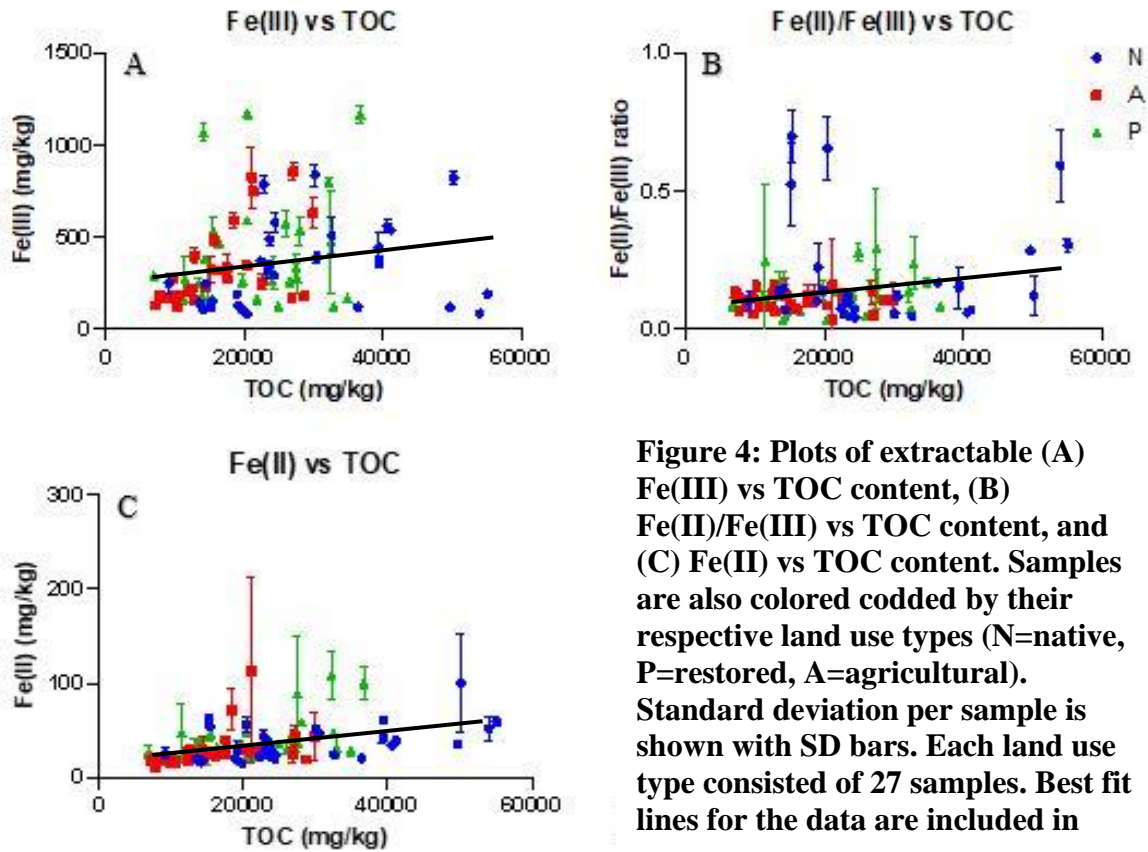
### 3.1 Variation in TOC with Redox

TOC concentrations varied greatly, ranging from 6900-55100mg/kg and decreased with depth in the soil profile (Appendix B). Extractable Fe, Fe(tot), and Fe(III) concentrations ranged from 7.92-187.3mg/kg, 120.8-1266mg/kg, and 85.95-1085mg/kg respectively, also decreasing with depth (Appendix B). Table 1 shows a general summary of the redox, particle size and pH analysis when analyzed collectively versus the TOC content of the samples. Overall, besides total nitrogen content, Extractable Fe had the strongest correlation with TOC out of all of our parameters ( $r=0.52$ ,  $p=3.00E-7$ ) (Fig. 4, Table 1, Appendix B). Fe(tot) and Fe(III) were also correlated with TOC content but the strength of these correlations were notable weaker than that with Extractable Fe (Table 1). The ratio of Extractable Fe to Fe(III) was not significantly correlated with TOC content overall or in any of the depth intervals. These correlations for the samples overall, varied significantly when samples were analyzed by their respective depth intervals.

parameter	$n^1$	range	Mean	SD <sup>2</sup>	$r^3$	$P$
pH (0.01 M CaCl <sub>2</sub> )	63	3.83	6.57	1.11	-0.163	0.202
Clay( %)	87	35.97	26.32	7.85	-0.036	0.739
Silt (%)	87	22.60	52.96	4.87	0.136	0.208
Sand (%)	87	41.78	20.72	8.08	-0.076	0.484
Fe(II) (mg/kg)	87	179.4	36.80	26.70	0.517	3.00E-07
Fe(tot) (mg/kg)	87	1145	394.90	262.00	0.342	0.001
Fe(III) (mg/kg)	87	1085	357.90	248.90	0.315	0.003
Fe(II)/Fe(III)	87	1.01	0.15	0.16	0.135	0.214
TOC (mg/kg)	87	48200	22177	10710	N/A	N/A

**Table 1: Summary of particle size and geochemical data for all samples with results of statistical analysis. (1) number of samples, (2) Standard deviation, (3) Spearman's r and P values indicate the given parameters correlation with TOC.**

Only extractable Fe ( $r=0.42$ ,  $p=0.023$ ) was significantly correlated with TOC content in the 0-5cm samples (Table 2). In the 5-15cm samples extractable Fe(tot), Fe(III), and Fe(II), in order of strength, were significantly correlated with TOC content. Only Fe(tot) and Fe(III) were significantly correlated with TOC content in the 15-30cm samples. The significant and strength of the correlations between Fe(III), Fe(tot), and TOC, generally increased with depth, but decreased for Fe(II).



**Figure 4: Plots of extractable (A) Fe(III) vs TOC content, (B) Fe(II)/Fe(III) vs TOC content, and (C) Fe(II) vs TOC content. Samples are also colored coded by their respective land use types (N=native, P=restored, A=agricultural). Standard deviation per sample is shown with SD bars. Each land use type consisted of 27 samples. Best fit lines for the data are included in**

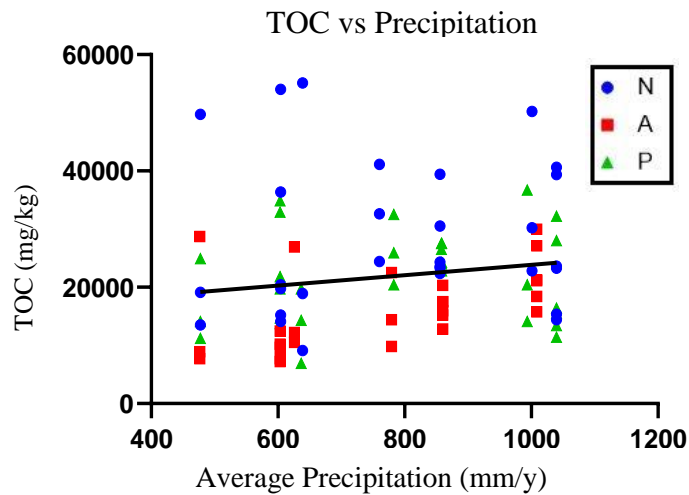
r values	5cm		15cm		30cm		All	
	r	p	r	p	r	p	r	p
<b>N=29</b>								
<b>Fe(II) (mg/kg)</b>	0.420	0.0231	0.380	0.0423	0.3564	0.0577	0.5167	2.99545 E-07
<b>Fe(tot) (mg/kg)</b>	0.102	0.6004	0.464	0.0113	0.5922	0.0007	0.3424	0.0012
<b>Fe(III) (mg/kg)</b>	0.092	0.6346	0.466	0.0108	0.5286	0.0032	0.3152	0.0029
<b>Fe(II)/Fe(III) ratio</b>	0.226	0.2376	-0.256	0.1793	-0.1030	0.5951	0.1345	0.2142

**Table 2: Results of spearman’s rho correlation analysis between extractable Fe parameters and TOC content, separated by depth intervals. The results of the Spearman’s rho analysis for all the samples are provided in the “All” column for context. Each depth interval consisted of 29 samples with 87 samples overall.**

### 3.2 TOC and Soil Redox Variation with Precipitation

TOC content was positively correlated with precipitation overall ( $r=0.25$ ,  $p=0.019$ ) (Fig. 5, Table 3). Nevertheless, the highest TOC values were observed towards the more arid end of the precipitation gradient (Fig. 5). Additionally, in spite of the overall trend, when the samples were analyzed by depth, TOC was only significantly correlated with precipitation in the 5-15cm and 15-30cm samples (Table 3). For soil redox, extractable Fe, Fe(tot), and Fe(III) were all significantly correlated with precipitation overall, Fe(tot) having the strongest correlation with precipitation followed by Fe(III) and Fe(II) (Table 4, Fig. 6). Unlike with TOC, the highest Fe(II) and Fe(III) concentrations were found on the wetter side of the precipitation gradient (Fig. 6). Lastly, the ratio of extractable Fe(II) to Fe(III) was negatively correlated with precipitation overall.

Analyzing the samples separated by depth interval revealed that the overall correlations previously described did not hold true for all the separate depth intervals. The correlation between extractable Fe(II) and precipitation was significant in all three depth intervals, with the 0-5cm samples having the strongest correlation followed by the 15-30cm and 5-15cm samples (Appendix B). Fe(tot) and Fe(III) were significantly correlated with precipitation in the 5-15cm and 15-30cm samples, with the 5-15cm samples having the stronger correlations (Appendix B). Finally, the ratio of extractable Fe(II) to Fe(III) was negatively correlated with precipitation in the 0-5cm ( $r=-0.54$ ,  $p=0.002$ ) and 5-15cm ( $r=-0.59$ ,  $p=0.001$ ).



**Figure 5: Plot of TOC vs precipitation for all samples separated by land use type. (N= native, P=restored, A=agricultural). Each land use type consisted of 27 samples. Best fit line for all samples pooled together included in black.**

TOC vs precipitation	All land-use types	
N=29	r	p
0-5cm	0.094	0.629
5-15cm	0.428	0.021
15-30cm	0.419	0.024
Overall	0.250	0.019

**Table 3: Results of spearman rho analysis of TOC content vs precipitation separated by depth. Each depth interval consisted of 29 samples with 87 samples overall.**

Fe vs precipitation	All land-use types	
N=87	r	p
Fe(II) (mg/kg)	0.509	4.849E-07
Fe(tot) (mg/kg)	0.765	5.980E-18
Fe(III) (mg/kg)	0.746	1.154E-16
Fe(II)/Fe(III)	-0.325	0.0021

**Table 4: Spearman's rho analysis of the all Fe parameters vs precipitation. Results are from all samples pooled together (87 samples).**

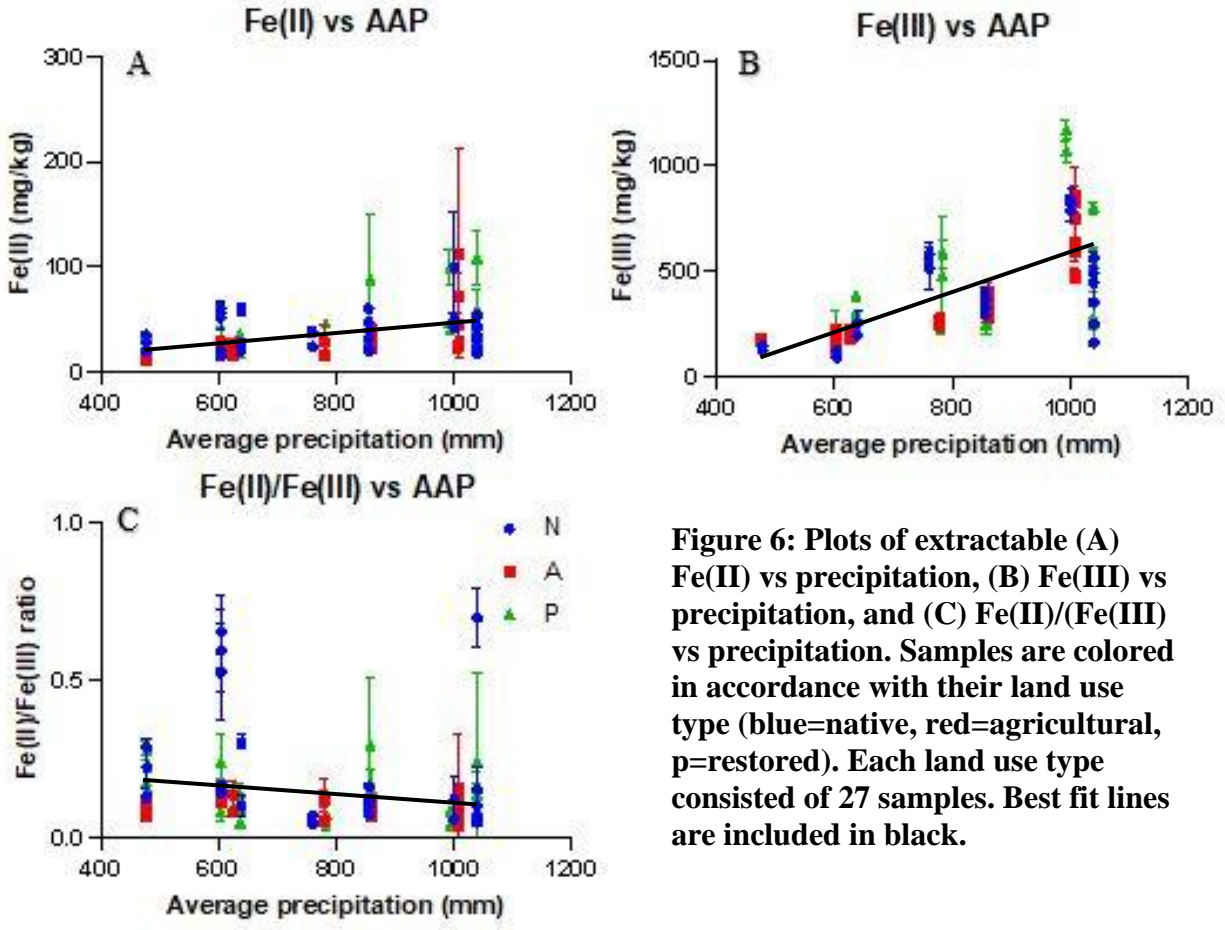


Figure 6: Plots of extractable (A) Fe(II) vs precipitation, (B) Fe(III) vs precipitation, and (C) Fe(II)/(Fe(III)) vs precipitation. Samples are colored in accordance with their land use type (blue= native, red= agricultural, p= restored). Each land use type consisted of 27 samples. Best fit lines are included in black.

### 3.3 Variation with Land use

#### 3.3.1 Variation in TOC and soil redox with Land Use

The native samples had the highest TOC content of the three land use types, ranging from 9100-55100mg/kg with an average concentration of 28219mg/kg. The restored samples followed the native samples, ranging from 6900-36700mg/kg with an average of 21615mg/kg. Lastly, the agricultural samples had the lowest TOC concentrations, ranging between 7200-29950mg/kg with an average of 16087mg/kg. The means, ranges, and standard deviations, of the redox parameters for the three land use types are listed in Table 5. The restored samples had the highest



average extractable Fe(II), Fe(tot), and Fe(III) concentrations followed by the native and then agricultural samples. On the other hand, the native samples had the highest average Fe(II) to Fe(III) ratios, then the restored and agricultural samples.

Restored (N=27)	Fe(II)	Fe(tot)	Fe(III)	Fe(II)/Fe(III)
Minimum	17.59	133.8	116.1	0.03515
Maximum	187.3	1266	1171	1.04
Range	169.7	1133	1055	1.005
Mean	43.26	444.1	400.8	0.1595
Std. Deviation	35.6	314.5	298.7	0.1797
Native (N=27)				
Minimum	7.92	120.8	85.95	0.04261
Maximum	100.2	923.9	838.3	0.6987
Range	92.24	803.1	752.3	0.656
Mean	36.54	373.6	336.5	0.186
Std. Deviation	19.47	230.5	223.9	0.1855
Agricultural (N=27)				
Minimum	10.64	142.3	122.8	0.03593
Maximum	113.1	938.7	856.1	0.1649
Range	102.4	796.4	733.3	0.129
Mean	29.91	363.8	333.9	0.1025
Std. Deviation	20.61	230.5	215.3	0.03375

**Table 5: Mean, minimum, maximum, standard deviation, and range values for the Fe parameters separated by land use type. Each land use type consisted of 27 samples.**

### 3.3.2 Variation in Soil Redox, TOC and Precipitation with Land Use

The correlations that existed for our samples when analyzed as a whole, varied when the samples were analyzed by land use type. Table 6 shows the variation in the correlations between our redox parameters and TOC content for the three land use types. Additionally, Figure 5 shows how the TOC content of the different land use types varied with precipitation. The correlations between extractable Fe(II), Fe(III), and Fe(tot) and TOC were the strongest in the agricultural samples (Table 6). Extractable Fe(II) and TOC had a stronger relationship in the restored

samples than the native samples, but Fe(III) and Fe(tot) correlations with TOC were stronger in the native samples.

Redox vs TOC		N		A		P	
		r	p	r	p	r	p
Overall	Fe(II)	0.418	0.022	0.669	1.351E-04	0.453	0.012
	Fe(tot)	0.390	0.033	0.624	0.001	0.209	0.268
	Fe(III)	0.373	0.042	0.606	0.001	0.181	0.337
	Fe(II)/Fe(III)	-0.031	0.871	-0.023	0.911	0.247	0.188

**Table 6: Spearman’s rho analysis of the all the Fe parameters vs TOC. Spearman’s r and p values are grouped within columns for land use. Results are from analysis of all the samples in that respective land use type. N=native, A=agricultural, P=restored. Each land use type consisted of 27 samples.**

The correlation between TOC content and precipitation also varied between the land use types. Despite the overall trend, there was no correlation between TOC and precipitation in the native ( $r=0.14$ ,  $p=0.447$ ) or restored ( $r=0.101$ ,  $p=0.596$ ) samples. TOC was however still positively correlated with precipitation in the agricultural ( $r=0.63$ ,  $p=4.816E-4$ ) samples.

As with TOC, the relationships between our redox parameters and precipitation also varied by land use type. Table 7 lists how the relationships between our redox parameters and precipitation were different for the respective land use types. The agricultural samples had the strongest correlation between extractable Fe(II) and precipitation ( $r=0.70$ ,  $p=4.58E-05$ ) followed by the restored samples ( $r=0.64$ ,  $p=2.0E-4$ ), and no significant relationship between extractable Fe(II) and precipitation in the native samples. Extractable Fe(tot) and Fe(III) were significantly correlated with precipitation in all three land use types with the agricultural samples again having the strongest correlations with precipitation.

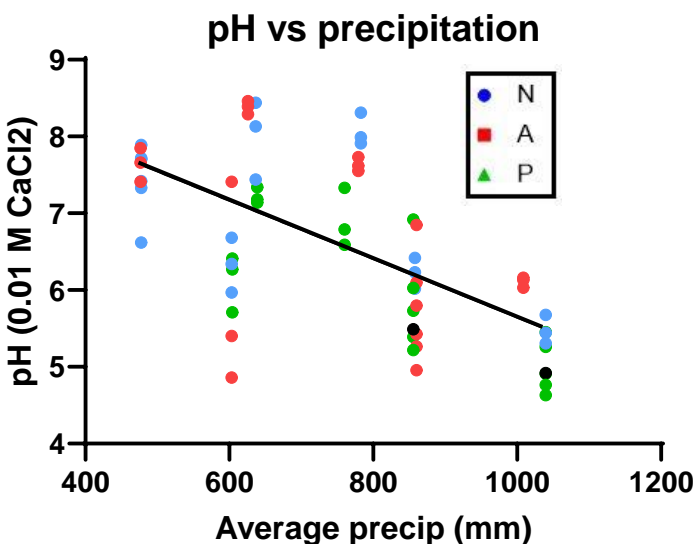
Redox vs precipitation		N		A		P	
		r	p	r	p	r	p
<i>Overall</i>	<b>Fe(II)</b>	0.154	0.416	0.701	4.58E-05	0.637	1.550E-4
	<b>Fe(tot)</b>	0.691	2.380E-05	0.935	1.010E-12	0.714	9.550E-6
	<b>Fe(III)</b>	0.685	3.0E-05	0.929	2.740E-12	0.706	1.298E-5
	<b>Fe(II)/Fe(III)</b>	-0.490	0.006	-0.293	0.138	-0.266	0.157

**Table 7: Spearman’s rho analysis of the all the Fe parameters vs precipitation. Spearman’s r and p values are grouped within columns for land use. Results are from analysis of all the samples in that respective land use type. (N=ative, P=restored, A=agricultural). Each land use type consisted of 27 samples.**

### 3.4 pH

We focus on the 0.01M CaCl<sub>2</sub> pH readings because this solution accounts for the presence of soluble ions that influence pH readings (Minasny et al., 2011). The average pH for our samples was 6.57 with a range of 3.83 and standard deviation of 1.11. Precipitation and pH were negatively correlated ( $r=-0.60$ ,  $p=1.726E-7$ ), meaning soils became more acidic with higher precipitation (Fig. 7). Overall, pH was neither correlated with TOC content of any of the Fe

redox parameters.



**Figure 7: Plot of pH values vs precipitation. Data points are color coded to their respective land use types.(N=ative, P=restored, A=agricultural). Each land use type consisted of 21 samples. Best fit line for data is included in black.**

The pH of our samples did not change significant with depth ( $r=0.007$ ,  $p=0.954$ ) and pH was negatively correlated with precipitation in every depth interval. In terms of variance by land use, the N samples were the most acidic (mean=6.116, SD=0.9363) followed by the A samples (mean=6.731, SD=1.192) and then P samples (mean=6.979, SD=1.068). Additionally, pH was not significantly correlated with either TOC content or and redox parameters in any of the land-use types. The relationship between pH and TOC was only significant in the 15-30cm samples with a negative correlation ( $r=-0.52$ ,  $p=0.015$ ). Additionally, for the relationship between pH and soil redox, pH was only significantly correlated with extractable Fe(II) ( $r=-0.45$ ,  $p=0.040$ ) and the ratio of extractable Fe(II) to Fe(III) ( $r=-0.44$ ,  $p=0.047$ ) in the 15-30cm samples.

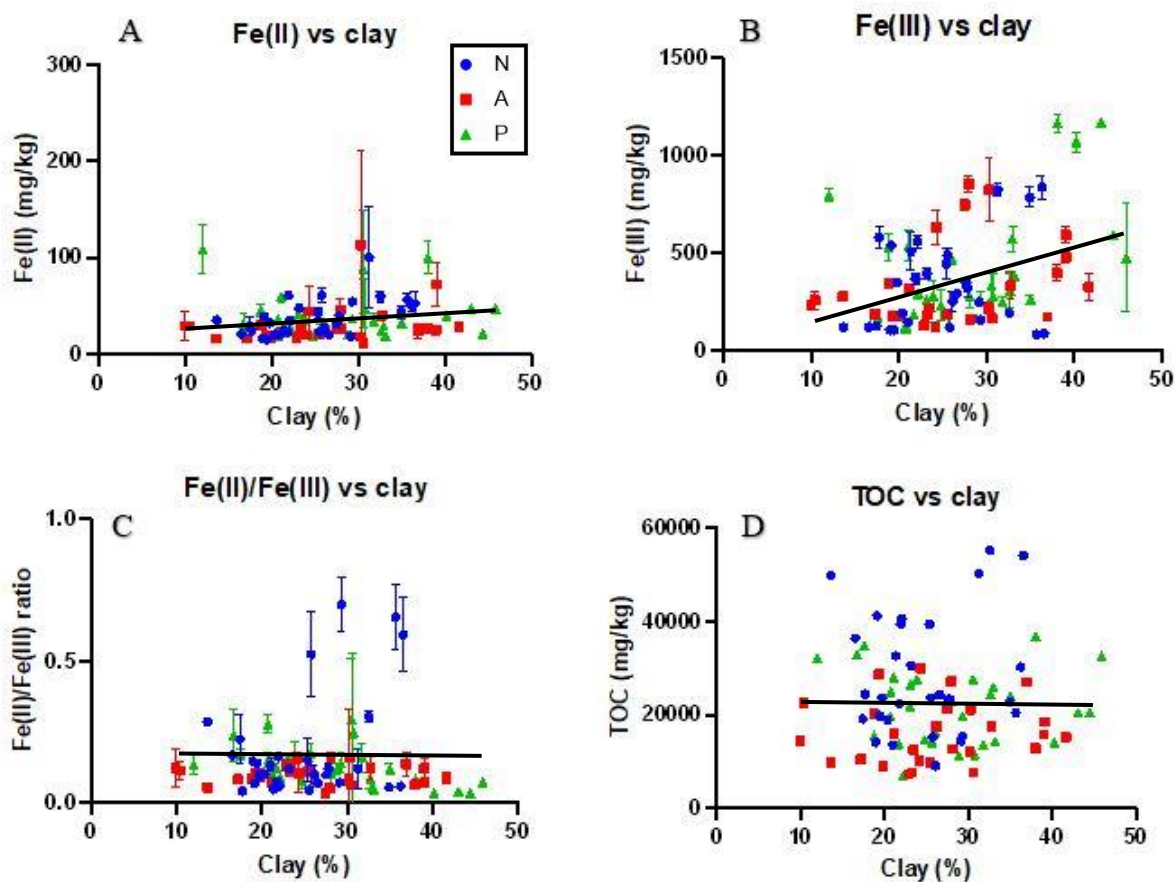
### **3.5 Particle Size**

Table 8 shows how clay, silt, and sand content varied across all the samples. Samples were generally mostly comprised of silt, then clay and sand. Overall, TOC was not significantly correlated with clay (Fig. 8), silt, or sand. When land-use was accounted for, TOC was still not significantly correlated with particle size at all. Despite the lack of any correlation between TOC and particle size within the land use types or overall, there were two significant relationship when depth was accounted for. In the 5-15cm samples, TOC was negatively correlated with sand content ( $r=-0.42$ ,  $p=0.024$ ) and positively correlated with silt ( $r=0.37$ ,  $p=0.046$ ). No other significant correlations between TOC and particle size existed in our samples.

N=87	Range	Mean	SD <sup>2</sup>	r <sup>3</sup>	P
Clay(%)	35.97	26.32	7.85	-0.036	0.739
Silt(%)	22.60	52.96	4.87	0.136	0.208
Sand(%)	41.78	20.72	8.08	-0.076	0.484

**Table 8: Summary of particle size data for all samples with results of statistical analysis. (1) number of samples, (2) Standard deviation, (3) Spearman's r and P values indicate the given parameters correlation with TOC.**

As for the relationship between soil redox and particle size, clay content was positively correlated with extractable Fe(II), Fe(III), and Fe(tot) overall (Fig. 8, Table 9). In the 5-15cm and 15-30cm samples, clay content was also positively correlated with Fe(tot) and Fe(III). The only significant correlation that silt had with soil redox parameters was with the ratio of extractable Fe(II) to Fe(III) in the 15-30cm samples (Table 9). Lastly, sand content was negatively and significantly correlated with extractable Fe(II), Fe(tot), and Fe(III) content in all of the depth intervals but had no significant correlation with the ratio of extractable Fe(II) to Fe(III) at any depth.



**Figure 8: Plots of clay content vs extractable (A) Fe(II), (B) Fe(III), (C) Fe(II)/Fe(III), and (D) TOC content for the three land use types. (N=native, P=restored, A=agricultural). Each land use type consisted of 27 samples. Best fit lines for data are included in black.**

N=87	Clay		Silt		Sand	
	r	p	r	p	r	p
Fe(II)	0.313	0.003	0.178	0.099	-0.410	8.181E-05
Fe(tot)	0.352	0.001	0.132	0.222	-0.454	1.008E-05
Fe(III)	0.329	0.002	0.126	0.243	-0.429	3.364E-05
Fe(II)/Fe(III)	-0.135	0.212	0.117	0.279	0.113	0.297

**Table 9: Spearman rho analysis results of particles size vs extractable Fe parameters. Results are from all depth intervals pooled together, 87 samples total used for analysis.**

### 3.6 Variation with Time

The 2018 sampling protocol involved taking four replicates from each land use type at the three research sites: HAY, KNZ, and EKS. Figure 9 shows soil redox parameters, TOC, and particle size variability amongst these replicates in the 2018 samples and the variability between the 2019 and 2018 samples. There were no consistent trends in the listed parameters amongst the different land use types in either the 2018 or 2019 samples. For example, TOC was not always higher in the N samples than the P or A samples between the three research sites. Some of the parameters are tightly constrained for certain research sites such as extractable Fe(II) and Fe(III) at Hays but then show considerably higher variability at KNZ and EKS (Fig. 9). The variability between the 2018 and 2019 samples is generally small. The 2019 data, when plotted on top of the 2018 data, generally overlaps well, indicating that in most cases, with a few exceptions, variation was not significant (Fig. 9).

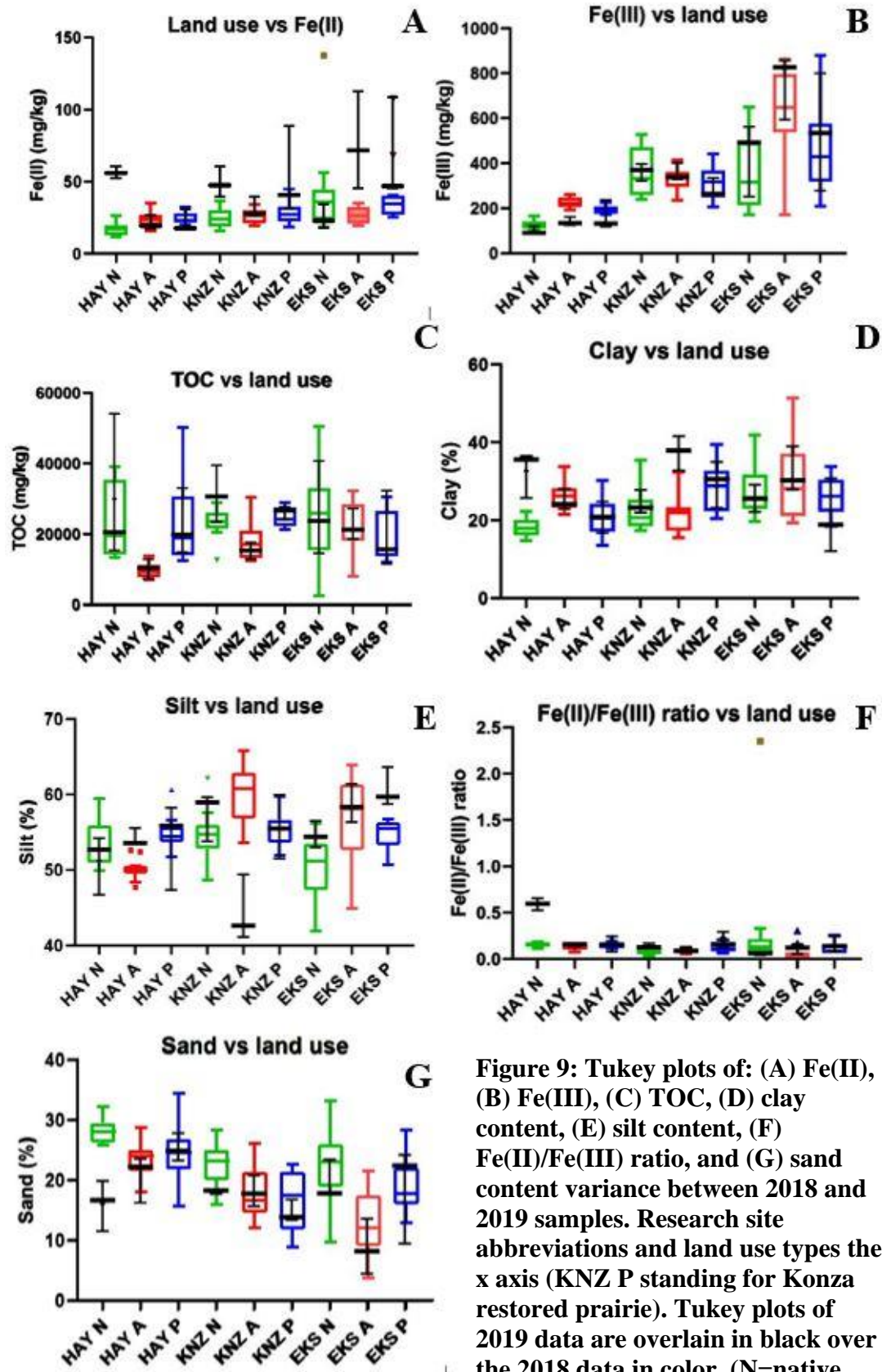


Figure 9: Tukey plots of: (A) Fe(II), (B) Fe(III), (C) TOC, (D) clay content, (E) silt content, (F) Fe(II)/Fe(III) ratio, and (G) sand content variance between 2018 and 2019 samples. Research site abbreviations and land use types the x axis (KNZ P standing for Konza restored prairie). Tukey plots of 2019 data are overlain in black over the 2018 data in color. (N= native, P=restored, A=agricultural).



## **Chapter 4 - Discussion**

We investigated (1) the relationships between the relative abundance of soil redox (anaerobic microsites) and TOC, (2) the relationships between both soil redox and TOC with precipitation, and (3) the effect of land use on these relationships. In the following subsections, we explore mechanisms behind the correlations observed in our data and we reflect on their possible implications.

We hypothesized that (1) native and restored soils would have a greater abundance of anaerobic microsites than the agricultural soils, (2) anaerobic microsites and TOC would be positively correlated with AAP, and (3) TOC would be positively correlated with the abundance of anaerobic microsites. In analyzing all of our samples, all three of our original hypotheses proved correct. However, when we parsed out of samples by depth or LU, our hypotheses were only valid for certain depth intervals and certain land use types, indicating that land use and depth also influence the relationships between our measured parameters.

### **4.1 Correlation Between Redox Parameters and TOC**

The strong correlation seen between extractable Fe(II) and TOC ( $r=0.52$ ,  $p=2.99e-7$ ) in our data is consistent with higher abundances of anaerobic microsites being associated with increased soil TOC content. Along with the correlation between extractable Fe(II) and precipitation, our findings imply that changes in precipitation may lead to a decrease in anaerobic microsite abundance and in turn decreased TOC storage. Also implied is that significant mechanism of the loss of anaerobic microsites would be decreased soil moisture, as a result of decreased precipitation, which would increase soil oxygen transport (Kristensen et al., 2010; Neira et al., 2015).

Greater concentrations of oxygen in previously anaerobic soil fractions would make greater fraction of TOC within these previously anaerobic environments, more susceptible to biotic and abiotic degradation (Boyd, 1995; Cheng et al., 2006; Y. Luo & Zhou, 2006; Phillips & Nickerson, 2015), likely resulting in losses of soil TOC. Consistent with this, decreases in soil moisture have been put forward as a mechanism of soil carbon losses from wetlands. This has been attributed to both increased rates of mineralization and increased susceptibility of soil organic matter to mineralization (Arnold et al., 2015; Chen et al., 2018). Moreover, warming related soil C losses are greater in dryer tundra ecosystems than in wetter tundra ecosystems, (McGuire et al., 2009; Oberbauer et al., 2007). Lastly, previous studies in the Central/Great plains area in the United States have found positive correlations between precipitation and TOC in upland soils (Burke et al., 1989a; Honeycutt et al., 1990; Klopfenstein et al., 2015). These findings from wetland, tundra, and upland ecosystems are consistent with our proposed mechanism for soil anaerobic microsites and TOC content loss through decreased precipitation.

Extractable Fe(II) content was significantly correlated with clay content ( $r=0.31$ ,  $p=0.003$ ) which fits well with previous findings of anaerobic microsite abundance being positively associated with clay content in upland soils (Keiluweit et al., 2018). The results of (Keiluweit et al., 2018) imply that elevated clay content is a driver of increased anaerobic pore volume in upland soils through limiting oxygen diffusion into soil microaggregates. Greater abundances of clay are associated with more microaggregates existing in soil profiles. The higher water-retention and tortuosity of these aggregates constrain oxygen diffusion, in turn leading to the formation of greater abundances of anaerobic microsites (Keiluweit et al., 2018). Consistent with our work, this study also used extractable Fe(II) as one of its measures of anaerobic respiration, lending further support to our conclusions.

TOC results positively correlate with Fe(III) ( $r=0.32$ ,  $p=0.003$ ) and Fe(tot) ( $r=0.34$ ,  $p=0.001$ ). Fe can be found in largely four different pools in soil environments speciated as Fe(II) or Fe(III). These pools are: primary and secondary minerals, (hydro)oxides, as soluble and exchangeable Fe, and lastly Fe associated with organic matter (Colombo et al., 2013; Lindsay, 1988). In (hydro)oxides, Fe (III) can readily complex with and in sometimes “protect” said organic matter from mineralization (Kleber et al., 2015; Lehmann & Kleber, 2015). We suggest that this mineral protection of organic compounds is the main reason behind the significant association seen between Fe(III) and Fe(tot) and TOC in our samples.

### **4.3 Influence of Precipitation**

Extractable Fe(II) was significantly correlated with increasing average annual precipitation ( $r=0.51$ ,  $p=4.85E-7$ ). Our suggested mechanism for this correlation the lower rates of oxygen diffusion in soils with higher moisture content (Kristensen et al., 2010; Neira et al., 2015). Upland soils in regions of higher average precipitation would have higher average soil moisture levels than in more arid conditions. As a higher proportion of soil pore spaces become filled with water, it accordingly become more difficult for oxygen to diffuse through that soil (Kristensen et al., 2010; Neira et al., 2015).

As we did not measure actual total concentrations of Fe(II), Fe(III) or for Fe(tot), the significant correlations between extractable Fe(tot), Fe(III) and precipitation are reflective of precipitation being linked with a larger reactive amount of Fe in our samples. While we do not know how actual total Fe chemistry was effected by precipitation, we argue our results clearly show that precipitation effects the speciation of extant soil Fe and thus the abundances of extractable Fe(II) and Fe(III). The negative correlation between the ratio of extractable Fe(II) to

Fe(III) and precipitation suggest that as precipitation increases, a smaller fraction of the total iron will be speciated as Fe(II). Our results suggest that as precipitation increases the pool of extractable Fe(III) increases at a greater rate than extractable Fe(II). We argue that the negative correlation between the ratio of extractable Fe(II) to Fe(III) and precipitation is a function of the increase in total extractable Fe simply “outpacing” the increase in extractable Fe(II).

The overall significant correlation between TOC and precipitation ( $r=0.25$ ,  $p=0.02$ ) agrees with previous work (Brye et al., 2004; Brye & Kucharik, 2003; Burke et al., 1989b; Z. Luo et al., 2017). One of the main mechanisms we credit this correlation with is increased primary productivity of the vegetation at the sites with higher precipitation (Deng et al., 2017). It is well known that local vegetation inputs significant amounts of organic C into soil (Sokol & Bradford, 2019). It then follows that higher productivity by local plants would be correlated with greater inputs of C into soil, resulting in the positive correlation between TOC and precipitation seen in our samples.

### **4.3 Influence of Land Use**

The native samples containing the highest TOC concentrations and the agricultural samples containing the lowest concentrations fits well with the established literature. Numerous studies have concluded that conversion of native soil ecosystems normally results in losses of TOC (Burke et al., 1989b; Lal, 2004; Levine et al., 2011; Z. Luo et al., 2017; Tubiello et al., 2015). Tillage, fertilization, and other agricultural practices significantly disrupt native soil biogeochemistry, often resulting in significant losses of soil C (Balesdent et al., 1990; J. Six et al., 1999). That the restored samples had more TOC than the agricultural samples also fits well with previous studies, as it has been shown that it is possible to increase soil TOC levels by

restoration of agricultural plots to native vegetation and ecosystem functioning (Braman, 2012; Cahill et al., 2009). However, as we do not have any data for the restored plots prior to restoration, we are largely unable to comment on the effects of restoration here.

The restored sites had the highest average extractable Fe(II), Fe(III), and Fe(tot) content. As mentioned, the possible reasons behind this are many and the difficulties in picking a single explanation stem from reasons explained in our uncertainty section. The native samples had higher average concentrations of all the Fe parameters than the agricultural samples. We had originally hypothesized that the native samples would have the highest extractable Fe(II) content due to our hypothesized link between TOC and extractable Fe(II). We put forward that the main reason for the lower general concentrations of Fe in the agricultural samples is uptake by crops. Fe is an essential nutrient for plants and the repeated removal of biomass from agricultural plots over time would lower soil Fe concentrations unless they were later amended (Kim & Guerinot, 2007; Morrissey & Guerinot, 2009).

While TOC was generally correlated with precipitation ( $r=0.25$ ,  $p=0.019$ ) in the native samples this correlation was not significant. This lack of a significant correlation in the native samples was surprising given the body of research on upland soils where TOC and precipitation have been positively correlated (Brye et al., 2004; Jenny, 1930). We suggest local factors such as primary plant productivity and the soil microbial community had a stronger influence on TOC content than precipitation. The influence of a local factor or factors, was further supported by the fact that the highest TOC values were found on the drier side of the precipitation gradient (Fig 5.). Another possible reason for the lack of a significant correlation between TOC and precipitation in the native samples comes from a long-term experiment that was conducted at KNZ. Wilcox et al., (2016) artificially increased precipitation over a 25 year period on an

annually burned plot at KNZ but found no change in the soil C pool, suggesting that native upland soil C pools may be somewhat stable in the face of shifting precipitation. Finally, the native sites are all not managed the same way. Some of the sites, such as KNZ, are annually burned while others harvested for hay. Both processes would influence the TOC content of the native sites through altering C inputs from local vegetation into the soil and could be part of the reason for the lack of a significant correlation.

While there is a wealth of research concerned with the response of native upland soil ecosystem TOC stocks to changes in precipitation, there is a dearth of research on the same topic in agricultural soils, leaving gaps as far as plausible explanations for the correlation in our data between precipitation ( $r=0.63$ ,  $p=4.816e-4$ ) and TOC. The strong correlation between the abundances of anaerobic microsites (extractable Fe(II)) and TOC in our agricultural samples, is our main mechanistic justification for our noted positive TOC-precipitation correlations. While unfortunate, this does identify a potentially important knowledge gap: what are the mechanistic responses of agricultural soil to altered precipitation regimes and are they significant enough to be seen through agriculture related interference?

## **4.4 Sources of Uncertainty**

While we are confident in the conclusions that we have come to on our data, we acknowledge that there are some important sources of uncertainty. One of these primary sources is our use of the ferrozine method to assess the iron content of our soil samples. Our procedure uses 0.5 N HCl to extract the iron from the soil samples. Research has shown that the fraction of Fe extracted by 0.5 N HCl is not all of the iron present in soils, specifically Fe contained in silicate minerals will not all dissolve (Anastácio et al., 2008). Consequently, it is important to

acknowledge that our study does not consider the entire soil Fe budget that may be available for soil redox reactions and C cycling. As Fe redox reactions can be important parts of soil C cycling processes (Adhikari et al., 2019; Fimmen et al., 2008; Jacobs et al., 2002; Weber et al., 2006), this is an important limitation to acknowledge.

Other research has also revealed that at high concentrations (1mM Fe(III) and above) waiting longer periods of time for color to develop, while using the Ferrozine method, can cause extractable Fe(II) concentrations to be overestimated due to interference from dissolved Fe(III) (Im et al., 2013). We tested this on a subset of three of our samples, waiting for first 2.5, then 5, then 18 hours, measuring the extractable Fe(II) concentrations at each time stamp. We found that our extractable Fe(II) concentration did in fact “drift” with passing time. We estimated based on the slopes of the lines formed by measuring this time series data that our extractable Fe(II) may have been overestimated by as much as 15%. While this does skew the accuracy of our measurements, we do not believe it ultimately undermines our conclusions. The extractable Fe(II) concentrations in our samples ranged from 7.9mg/kg to 187.3mg/kg, or an increase of over 2270%. Furthermore, the standard deviation of our extractable Fe(II) concentrations was 26.7mg/kg with a coefficient of variation of 72.56%. We are confident that this range and variability in our measured concentrations is enough to compensate for this overestimation of extractable Fe(II).

Another important source of uncertainty was the age and history of our restored (P) sites. The restoration sites across our research area varied drastically in their age. In addition to not knowing the time of restoration, we also do not have data on the land management history. Time since restoration, method of restoration, and management since restoration would all be extremely important factors that would drastically affect the biogeochemistry of these plots.

Consequently, we were generally reticent to make any significant claims about the relevance of our P soils analysis.

## 4.5 Conclusion

In our study we examined the correlations between soil redox and TOC content, TOC and precipitation, and soil redox and precipitation. The strong correlation between extractable Fe(II) and TOC content provides evidence for the protection of soil TOC by anaerobic microsites and further underscores their importance in line with recent research. While the impact of these anaerobic microsites had been established in laboratory studies, broad-scale field research examining the relationship between anaerobic microsites and soil TOC are currently sparse. A substantial implication of this study, supported by the strong links between extractable Fe(II), TOC, and precipitation, is that as climate change causes some upland soil ecosystems become more arid, the abundance of anaerobic microsites, and consequently TOC content, may accordingly decrease.

Considering the effect of land use, we suggest that the different correlations of our redox parameters, TOC, and precipitation among the different land use types show the significant impact of land-use on soil redox and TOC biogeochemistry. More specifically, native upland soil TOC stocks and abundances of anaerobic microsites appear to be less sensitive to changes precipitation than in agricultural soils. However, more long-term studies will be needed to support this claim over time as well as to help uncover the mechanisms responsible. Questions remain about the extent to which the abundance of anaerobic microsites will change with precipitation patterns as well as the time scales over which a significant response would occur. More mechanistic studies looking into upland soil redox dynamics involving the analysis of



additional redox parameters such as Eh, Mn speciation, and direct measurements of oxygen concentrations would aid in resolving these questions.

## Chapter 5 - References

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## Appendix A - USDA soil series designations for sampling sites

Appendix A1: USDA soil series designations for the sampling sites.

EKS Native	Kenoma-Olpe silt loam complex
EKS Agriculture	Woodson silt loam
EKS Restored	Kenoma silt loam
KNZ Native	Reading silt loam
KNZ Agriculture	Reading silt loam
KNZ Restored	Reading silt loam
HAY Native	Harney silt loam
HAY Agriculture	Harney silt loam
HAY Restored	Armo loam
TLI Native	Hord silt loam
TLI Agriculture	Hord silt loam
TLI Restored	McCook silt loam
LVN Native	Sharpsburg silty clay loam
LVN Restored	Martin silty clay loam
RKS Native	Harney silt loam
RKS Agriculture	Harney silt loam
RKS Restored	Harney silt loam
SVR Native	Ulysses silt loam
SVR Agriculture	Colby silt loam
SVR Restored	Ulysses silt loam

**Appendix B - Parameter abundance data and varied Spearman's  
rho analysis**

<b>TOC (mg/kg)</b>		<b>N</b>	<b>A</b>	<b>P</b>
<b>5cm</b>	<i>minimum</i>	23550	12450	19700
	<i>maximum</i>	55100	29950	36700
	<i>mean</i>	42940	22011	29588
<b>15cm</b>	<i>minimum</i>	18900	8900	6900
	<i>maximum</i>	32600	21250	25900
	<i>mean</i>	24070	14319	18755
<b>30cm</b>	<i>minimum</i>	9100	7200	11200
	<i>maximum</i>	24400	18400	27500
	<i>mean</i>	17648	11933	16503
	<b><i>Overall mean per Land-use</i></b>	<b>28219</b>	<b>16087</b>	<b>21615</b>

**Appendix B1: Minimum, maximum and mean TOC concentrations of the different land use types, with overall means of each land-use included as well. N= Native, P= Restored, A=agricultural**

Appendix B2: (Bottom) p values from Spearman's rho analysis of all the parameters against each other. (Top)  
r values from Spearman's rho analysis of all the parameters against each.

r values	Bottom cm	Latitude	Longitude	0.01 M Ca	pH (D)	average Tempage	precip	OC (mg/kg)	N (mg/kg)	Clay	Silt	Sand	G Fe2+	mg Fe	Total r3	Fe(III)	mgG Fe(II)/Fe
Bottom cm	1	0	0.007484	0.016037	0	-0.62198	-0.63312	0.233756	-0.07119	-0.16761	-0.40473	-0.06979	-0.05634	-0.2901			
Latitude	0	1	-0.07247	0.3391	0.118219	-0.46627	-0.24002	0.065217	0.006094	0.151308	-0.11655	-0.09107	-0.04277	0.050792	0.066966	-0.14835	
Longitude	0	-0.07247	1	-0.60512	-0.60619	0.669644	0.960093	0.276126	0.167852	0.4067	0.233568	-0.53975	0.512394	0.82669	0.813211	-0.40434	
pH (0.01 M	0.007484	0.3391	-0.60512	1	0.747447	-0.35373	-0.60271	-0.16296	-0.17538	-0.04064	-0.48415	-0.23139	-0.24283	0.26296	-0.154317	-0.0337	
pH (D)	0.016037	0.118219	-0.60619	0.747447	1	-0.45551	-0.59312	-0.10573	-0.09732	-0.05345	-0.59312	-0.10573	-0.09732	-0.09732	-0.16346	-0.10963	-0.30508
Average Te	0	-0.46627	0.669644	-0.35373	-0.45551	1	0.74156	0.295494	0.212112	0.067476	0.074045	-0.16901	0.323805	0.644832	0.626664	-0.30737	
Average pr	0	-0.24002	0.960093	-0.60271	-0.59312	0.74156	1	0.250434	0.15313	0.332871	0.26425	-0.46937	0.508831	0.765425	0.745834	-0.32478	
TOC (mg/k	-0.62198	0.065217	0.276126	-0.16296	-0.10573	0.295494	0.250434	1	0.952755	-0.03621	0.136234	-0.0761	0.516734	0.342404	0.315192	0.134503	
N (mg/kg)	-0.63312	0.006094	0.167852	-0.17538	-0.09732	0.212112	0.15313	0.952755	1	-0.08273	0.158994	-0.02888	0.497643	0.188471	0.157198	0.26926	
Clay	0.233756	0.151308	0.4067	-0.04064	-0.05345	0.067476	0.332871	-0.03621	-0.08273	1	-0.21887	-0.8217	0.312571	0.351564	0.328581	-0.13522	
Silt	-0.07119	-0.11655	0.233568	-0.48415	-0.23139	0.074045	0.26425	0.136234	0.158994	-0.21887	1	-0.29992	0.178074	0.132282	0.126486	0.117328	
Sand	-0.16761	-0.09107	-0.53975	0.233253	0.16346	-0.16901	-0.46937	-0.0761	-0.02888	-0.8217	-0.29992	1	-0.40955	-0.4539	-0.42913	0.112972	
AVG Fe2+	-0.40473	-0.04277	0.512394	-0.24283	-0.10963	0.323805	0.508831	0.516734	0.497643	0.312571	0.178074	-0.40955	1	0.494822	0.425463	0.331875	
AVG Fe Tc	-0.06979	0.050792	0.82669	-0.15549	-0.30508	0.644832	0.765425	0.342404	0.188471	0.351564	0.132282	-0.4539	0.494822	1	0.99373	-0.55284	
AVG Fe(III)	-0.05634	0.066966	0.813211	-0.14317	-0.29992	0.626664	0.745834	0.315192	0.157198	0.328581	0.126486	-0.42913	0.425463	0.99373	1	-0.61734	
AVG Fe(II)	-0.2901	-0.14835	-0.40434	-0.0337	0.157317	-0.30737	-0.32478	0.134503	0.26926	-0.13522	0.117328	0.112972	0.331875	-0.55284	-0.61734	1	

vs precipitation		All LU together	
		r	p
5cm	Fe(II) (mg/kg)	0.5947	0.0231
	Fe(tot) (mg/kg)	0.8474	0.6004
	Fe(III) (mg/kg)	0.8479	0.6346
	Fe(II)/Fe(III)	-0.5411	0.2376
15cm	Fe(II) (mg/kg)	0.5576	0.0017
	Fe(tot) (mg/kg)	0.8114	9.3200e-8
	Fe(III) (mg/kg)	0.8091	1.0764e-7
	Fe(II)/Fe(III)	-0.5925	0.0007
30cm	Fe(II) (mg/kg)	0.5734	0.0011
	Fe(tot) (mg/kg)	0.6340	0.0002
	Fe(III) (mg/kg)	0.5492	0.0020
	Fe(II)/Fe(III)	0.0002	0.9990
All depths	<b>Fe(II) (mg/kg)</b>	<b>0.5088</b>	<b>4.8487E-07</b>
	<b>Fe(tot) (mg/kg)</b>	<b>0.7654</b>	<b>5.9796E-18</b>
	<b>Fe(III) (mg/kg)</b>	<b>0.7458</b>	<b>1.1535E-16</b>
	<b>Fe(II)/Fe(III)</b>	<b>-0.3248</b>	<b>0.0021</b>

**Appendix B3: Spearman's rho analysis of the all of the Fe parameters vs precipitation. Results are broken apart by depth in the rows and the results within the All depths section of the table are from analysis of all the samples.**

		0-5cm			5-15cm			15-30cm		
		Clay	Silt	Sand	Clay	Silt	Sand	Clay	Silt	Sand
<b>r</b>	<b>Fe(II)</b>	0.419	0.182	-0.564	0.466	0.108	-0.560	0.545	0.341	-0.599
	<b>Fe(tot)</b>	0.235	0.310	-0.399	0.407	0.128	-0.489	0.670	-0.133	-0.590
	<b>Fe(III)</b>	0.223	0.301	-0.372	0.389	0.125	-0.469	0.640	-0.164	-0.558
	<b>Fe(II)/ Fe(III)</b>	0.071	-0.161	-0.023	-0.236	0.083	0.218	-0.264	0.524	0.066
<b>p</b>	<b>Fe(II)</b>	0.024	0.344	0.001	0.011	0.578	0.002	0.002	0.070	0.001
	<b>Fe(tot)</b>	0.219	0.102	0.032	0.028	0.510	0.007	7.025E-5	0.492	0.001
	<b>Fe(III)</b>	0.246	0.112	0.047	0.037	0.519	0.010	1.855E-4	0.395	0.002
	<b>Fe(II)/ Fe(III)</b>	0.713	0.403	0.904	0.217	0.670	0.257	0.167	0.004	0.736

**Appendix B4: Results of Spearman rho analysis of particle size vs Fe parameters are displayed in this table separated by sample depth interval. Results highlighted in red are statistically insignificant.**



Redox vs TOC		N		A		P	
		r	p	r	p	r	p
5cm	Fe(II)	0.624	0.060	0.167	0.678	0.442	0.051
	Fe(tot)	-0.067	0.865	0.383	0.313	0.210	0.375
	Fe(III)	-0.067	0.865	0.383	0.313	0.146	0.539
	Fe(II)/Fe(III)	0.467	0.179	-0.583	0.108	0.302	0.196
15cm	Fe(II)	0.406	0.247	0.733	0.031	0.018	0.973
	Fe(tot)	0.818	0.006	0.917	0.001	0.139	0.707
	Fe(III)	0.806	0.007	0.917	0.001	0.139	0.707
	Fe(II)/Fe(III)	-0.612	0.067	-0.300	0.437	-0.188	0.607
30cm	Fe(II)	-0.079	0.838	0.767	0.021	0.055	0.892
	Fe(tot)	0.685	0.035	0.900	0.002	0.576	0.088
	Fe(III)	0.648	0.049	0.867	0.005	0.527	0.123
	Fe(II)/Fe(III)	-0.479	0.166	0.067	0.880	-0.212	0.560
Overall	Fe(II)	0.418	0.022	0.669	1.351E-04	0.453	0.012
	Fe(tot)	0.390	0.033	0.624	0.001	0.209	0.268
	Fe(III)	0.373	0.042	0.606	0.001	0.181	0.337
	Fe(II)/Fe(III)	-0.031	0.871	-0.023	0.911	0.247	0.188

**Appendix B5: Spearman's rho analysis of the all the Fe parameters vs TOC. Spearman's r and p values are grouped within columns for land use. Results are broken apart by depth in the rows and the results within the Overall depths section of the table are from analysis of all the samples in that respective land use type. N= Native, P= Restored, A=agricultural**

Vs precipitation		N		A		P	
		r	p	r	p	r	p
5cm	Fe(II)	0.190	0.597	0.937	0.001	0.707	0.027
	Fe(tot)	0.826	0.005	0.886	0.030	0.848	0.003
	Fe(III)	0.826	0.005	0.886	0.030	0.896	0.001
	Fe(II)/Fe(III)	-0.716	0.025	-0.641	0.069	-0.762	0.014
15cm	Fe(II)	0.147	0.685	0.928	0.015	0.575	0.003
	Fe(tot)	0.697	0.030	0.726	2.205e-4	0.630	0.020
	Fe(III)	0.697	0.030	0.726	2.205e-4	0.630	0.020
	Fe(II)/Fe(III)	-0.758	0.014	0.101	0.537	-0.661	0.016
30cm	Fe(II)	0.135	0.711	0.447	0.768	0.557	0.016
	Fe(tot)	0.508	0.137	0.751	0.937	0.460	0.161
	Fe(III)	0.489	0.154	0.785	0.937	0.404	0.185
	Fe(II)/Fe(III)	-0.251	0.481	-0.144	0.042	0.122	0.483
<b>Overall</b>	<b>Fe(II)</b>	<b>0.154</b>	<b>0.416</b>	<b>0.701</b>	<b>4.58E-05</b>	<b>0.6367</b>	<b>2.000E-4</b>
	<b>Fe(tot)</b>	<b>0.691</b>	<b>2.380E-05</b>	<b>0.935</b>	<b>1.010E-12</b>	<b>0.714</b>	<b>9.550E-6</b>
	<b>Fe(III)</b>	<b>0.685</b>	<b>3E-05</b>	<b>0.929</b>	<b>2.740E-12</b>	<b>0.706</b>	<b>1.300E-5</b>
	<b>Fe(II)/Fe(III)</b>	<b>-0.490</b>	<b>0.006</b>	<b>-0.293</b>	<b>0.138</b>	<b>-0.266</b>	<b>0.155</b>

**Appendix B6: Spearman's rho analysis of the all the Fe parameters vs precipitation. Spearman's r and p values are grouped within columns for land use. Results are broken apart by depth in the rows and the results within the Overall depths section of the table are from analysis of all of the samples in that respective land use type. N= Native, P= Restored, A=agricultural**

Redox parameters		N	A	P
5cm	Fe(II) (mg/kg)	47.44	31.76	48.25
	Fe(tot) (mg/kg)	408.7	383.1	459.2
	Fe(III) (mg/kg)	361.3	351.3	411
	Fe(II)/Fe(III)	0.2027	0.1142	0.1776
15cm	Fe(II) (mg/kg)	31.77	32.64	30.24
	Fe(tot) (mg/kg)	378.6	383.4	431
	Fe(III) (mg/kg)	346.8	350.7	400.7
	Fe(II)/Fe(III)	0.1578	0.1037	0.1033
30cm	Fe(II) (mg/kg)	30.4	25.34	33.28
	Fe(tot) (mg/kg)	333.5	325	413.7
	Fe(III) (mg/kg)	301.5	299.7	380.4
	Fe(II)/Fe(III)	0.1976	0.08962	0.1233
All depths	Fe(II) (mg/kg)	36.54	29.91	43.26
	Fe(tot) (mg/kg)	373.6	363.8	444.1
	Fe(III) (mg/kg)	336.5	333.9	400.8
	Fe(II)/Fe(III)	0.186	0.1025	0.1595

**Appendix B7: Average values for the Fe parameters measured separated by land use type and depth. Averaged values for all the depth intervals of each land use are presented in the bottom section of the table. N= Native, P= Restored, A=agricultural**

## Appendix C - Fe extraction procedure

### 0.5 N HCl Extraction procedure

Analysis of iron extracted with 0.5 N HCl can provide a rapid measure of the redox state of a sediment sample (Heron et al., 1994). In our lab experiments, it can also help us estimate the abundance of sorbed ferrous iron+FeS+FeCO<sub>3</sub>.

#### Procedure

1. Inside an anaerobic chamber, add 0.1 to 1 g of sediment (or 1 mL of well-mixed bioreactor slurry) to 10 mL of 0.5 M HCL in 15 mL centrifuge tube. Cap the tube and place it on a shaker for 1 h. The 0.5 M HCL does not need to be anoxic.
2. Remove the vial from the shaker and either let it sit for 1-2 h to allow solids to settle. Alternatively, centrifuge the tube for a short time at a rate that won't damage the tube.
3. Measure Fe(II) using the strongly-buffered ferrozine approach.
4. Measure Fe(III)+Fe(II) using the total Fe approach. Note – for bioreactor experiments, we typically skip this step because we are primarily interested in Fe(II). Ask if unsure.

#### Calculation

Calculate Fe(II) concentration in the sample using a calibration regression and the dilution factor for the ferrozine analysis – this is the concentration of Fe in your extract solution.

Then calculate the amount of Fe(II) in your sample based on the proportion of sample to 0.5 N HCl in the extraction. Note that for a bioreactor slurry, you also need to subtract the concentration of Fe(II) in the aqueous portion of the sample. We want to know how much Fe(II) is present in the sediment.

If you want to evaluate Fe(III) concentration, then you need to subtract Fe(II) from the Fe concentration measured by the total Fe approach:

$$\text{Fe(III)} = \text{Fe(tot)} - \text{Fe(II)}$$

Then you can calculate the redox ratio (Fe(II)/Fe(III)).

#### **Reference**

Heron G, Crouzet C, Bourg ACM, Christensen TH (1994) Speciation of Fe(II) and Fe(III) in contaminated aquifer sediments using chemical-extraction techniques. *Environ. Sci. Technol.*, 28, 1698-1705.

## Appendix D - Ferrozine protocol

### Analyzing Ferrous Iron [Fe(II)] using the Ferrozine Method

Ferrozine (the disodium salt of 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine) reacts with ferrous iron to form a stable magenta complex that is soluble in water. The visible absorption spectrum of the ferrous-ferrozine complex has a single sharp peak with maximum absorbance at 562 nm. The complex will form in solution between pH 4 and 9. References – Stookey (1970) and Gibbs (1976).

**Standard approach** – Use this approach to analyze the Fe(II) concentration of water samples.

#### Reagents

- Ferrozine solution – add per liter 1 g of ferrozine and 11 g of HEPES (46 mM HEPES). Adjust the pH to 7.0 and store at 4°C. Note – it makes a difference if the HEPES is HEPES acid [238.302 g/mol] or HEPES sodium salt (HEPES-Na [260.284 g/mol]). If it is the sodium salt, add 12 g.
- Ferrous iron standards – add 0.70213 g of ferrous ammonium sulfate hexahydrate ( $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ ; FW 392.14) to 100 mL of DI (confirm). This should have 1000 mg/L ferrous iron. Make dilutions from this solution to obtain standards ranging in concentration from as low as 0.1 mg/L to as high as 10 mg/L. All standards, including the 1000 mg/L starting solution, need to be made fresh immediately before analysis. The ferrous iron begins to oxidize immediately.
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#### Procedure

1. Add 2.5 mL of ferrozine reagent to a test tube.
2. Add 1 mL of each sample or standard. Mix and observe the color. The solution will become purple-ish in color if ferrous iron is present. A deep blue/purple indicates that too much ferrous iron is present. If this is the case, dilute the sample (e.g., add 0.1 mL of sample + 0.9 mL of DI for 1:10 dilution).
3. Set wavelength on spectrophotometer to 562 nm.
4. Normalize absorbance on the spectrophotometer to absorbance of a blank sample consisting of 2.5 mL of ferrozine solution + 1 mL of DI water. Pour the blank sample into a cuvette, place the cuvette in the spectrophotometer, close the lid, and press “Measure Blank”.
5. Measure and record the absorbance for each sample/standard. To accomplish this task, you simply add the sample/standard to a cuvette, place it in the spectrophotometer, close the lid, and read the absorbance value on the display.

6. Perform a linear regression on the absorbance and concentration data from the standards. Use this regression line and the absorbance of the samples to calculate sample concentrations.

**Extra-buffering approach** – use this method to analyze the Fe(II) content of samples from 0.5N HCl extractions.

#### Reagents

- Ferrozine solution –Because the formation of the ferrous-ferrozine complex is pH dependent, some buffering needs to be present to ensure consistency between samples and standards. For this purpose, the standard ferrozine solution contains 46 mM HEPES. For samples from 0.5 N HCl extractions, however, more buffering is needed. Make the ferrozine solution as described above but with 1 M HEPES. Per L of solution: 238.3 g of HEPES, 1 g of ferrozine. Adjust the pH to 7.0 and store at 4°C.
- Ferrous iron standards – Make the standards as described above, except make the dilutions with 0.5 N HCl.
- 0.5 N HCl – Partially fill a 1 L volumetric flask with DI water. Inside a fume hood, slowly add 41.7 mL of concentrated HCl (12 N) to the flask, and then fill the flask to the mark with DI.

#### Procedure

1. Add 2.5 mL of ferrozine reagent to a test tube.
2. Add 1 mL of sample/standard and wait for color development. Wait 1 hour and start analyzing samples. You need to be consistent between samples and standards because absorbance changes over time. Therefore, I suggest you work only with small batches of samples (e.g., <20). It may be necessary to dilute the sample (e.g., add 0.1 mL of sample + 0.9 mL of 0.5 N HCl for 1:10 dilution).
3. Set wavelength on spectrophotometer to 562 nm.
4. Normalize absorbance on the spectrophotometer to absorbance of a blank sample consisting of 2.5 mL of ferrozine solution + 1 mL of 0.5 N HCl. Pour the blank sample into a cuvette, place the cuvette in the spectrophotometer, close the lid, and press “Measure Blank”.
5. Measure and record the absorbance for each sample/standard. To accomplish this task, you simply add the sample/standard to a cuvette, place it in the spectrophotometer, close the lid, and read the absorbance value on the display.
6. Perform a linear regression on the absorbance and concentration data from the standards. Use this regression line and the absorbance of the samples to calculate sample concentrations.

## Total Fe approach – for analysis of Fe(II)+Fe(III)

This ferrozine method can only measure the concentration of ferrous iron [Fe(II)]. It is insensitive to ferric iron [Fe(III)]. In order to use the ferrozine method to measure the total concentration of iron in a sample that contains both ferric and ferrous iron, therefore, the ferric iron must first be reduced to ferrous iron.

### Reagents

- 0.5 N HCl – Partially fill a 1 L volumetric flask with DI water. Inside a fume hood, slowly add 41.7 mL of concentrated HCl (12 N) to the flask, and then fill the flask to the mark with DI.
- 6 M hydroxylamine hydrochloride ( $\text{NH}_2\text{OH}\cdot\text{HCl}$ ; FW = 69.49 g/mol). Do not make a large volume of this reagent. Just make slightly more than you need. It should be used soon after it is made.

### Procedure

1. Add 1 mL of acidified sample (e.g., sample from 0.5 N HCl extraction, acidified water sample, etc.) to a 15 mL centrifuge tube.
2. Add 0.5 mL of hydroxylamine solution.
3. Add 8.5 mL of 0.5 N HCl. Be careful when pipetting acid. You do not want to draw the solution too rapidly into the pipet. If that happens, acid may splash inside the pipet and damage seals.
4. Cap and vortex the tube and then allow it to incubate overnight.
5. The next day, measure iron concentration in the sample using the ferrozine method - Extra-Buffering Approach outlined above. Note that this digestion dilutes the sample 10-fold.

### **References**

- Gibbs, C.R., 1976, Characterization and application of ferrozine iron reagent as a ferrous iron indicator: *Analytical Chemistry*, v. 48, p. 1197-1201.
- Stookey, L.L., 1970, Ferrozine - a new spectrophotometric reagent for iron: *Analytical Chemistry*, v. 42, p. 779-781.