**HATCH Startup: *In situ* soil pH measurements for improved microbial community characterization in Wisconsin's soils**

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## Introduction

Soil pH is often referred to as "the master variable" (Rengel, 2003). It regulates nutrient availability and biogeochemical cycles, affects fundamental metabolic processes in plants and microbes, and is the strongest predictor of soil microbial community composition across biomes (Lauber *et al.*, 2008; Fierer and Jackson, 2006). Because microbes control so many soil processes that are critical to agriculture – nutrient cycling, organic matter decomposition, mineral weathering – understanding how pH affects these microbes is essential for effective management. Understanding the effects of pH on soil microbes and their functions is particularly important in the state of Wisconsin, which has a wide range of soil pHs, from ~4.5 to ~7.5. However, to date, the methods used to characterize soil pH have largely been designed to reflect nutrient availability for plants, rather than the ambient pH that a given microbial consortium actually experiences in the soil. While this has worked well for plants, in order to link soil pH to the microbial processes that we care about, we need to establish effective methods of measuring pH under conditions that are more relevant to microbes. We will consider approaches not yet widely applied to soils: using microelectrodes and adapting ratiometric fluorescence analysis to soils, in order to determine the pH of <1 mL volumes of extracted soil solution under environmentally realistic water contents. **We propose to (1) apply and test microelectrode and fluorescence-based approaches for lab measurement of soil pH under more realistic soil conditions, and (2) determine which measurement approaches best allow us to predict soil microbial community composition, using soils from across UW-Madison agricultural field stations and from a single-site long term pH manipulation trial.**

## Background and justification

*Soil pH controls key soil functions*

Soil pH is a critical parameter for soil management, because it affects so many important processes and properties, including nutrient cycling, greenhouse gas emissions, and soil microbial community composition. It is well known as a key control on nutrient availability (Figure 1). For example, mineral P availability is highly dependent on pH, forming precipitates with Al, Fe, and Mn at low pH and with Ca and Mg at high pH. Soil microbes are responsible for numerous nutrient cycling processes in soil, including N, P, and S mineralization, $N_2$ fixation, nitrification, and denitrification, and are strongly affected by pH. pH is also an important determinant of soil greenhouse gas emissions. For example, soil microbial activity is often highest around neutral pH values, resulting in greater decomposition and higher $CO_2$ emissions (Rousk *et al.*, 2009). Methane emissions (and uptake) are also often largely controlled by soil microbial activities, and are strongly affected by soil pH and soil redox conditions (Jugsujinda *et al.*, 1996). Additionally, in another study, although total bacterial denitrification genes (*nos* and *nir*)

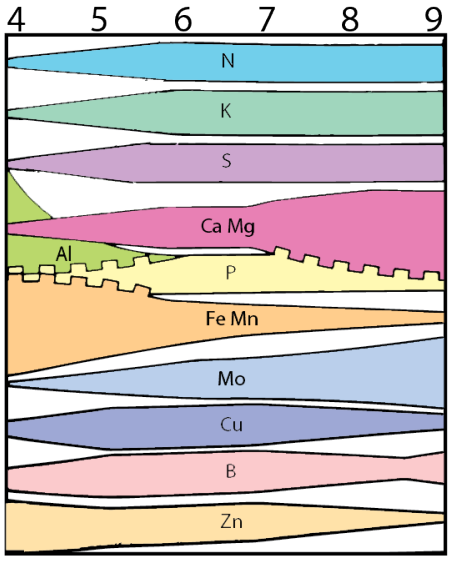


Figure 1. Relative nutrient availability varies with pH (Modified from Brady and Weil, 2008). Band thickness indicates availability, with thinner bands representing lower relative availability.

increased with higher soil pH, the relative emissions of the potent greenhouse gas $N_2O$ were greatest at lower pHs, which Samad *et al*. (2016) attributed to changes in the soil bacterial community. Because any effects of pH on soil microbial communities will often have additional, indirect effects on nutrient availability and greenhouse gas emissions, understanding bacterial response to soil pH is essential for robust soil management.

*Soil pH structures soil microbial communities*

Soil bacteria rely on intra-extra-cellular pH gradients to drive their core metabolism. This phenomenon, known as proton motive force, enables cells to make energy from soil organic matter and to carry out basic life processes (Krulwich *et al*., 2011; Mitchell, 1966). While bacteria exist that can function across an extreme range of pH values, the optimum pH ranges for many soil bacteria are narrow and soil-specific (Fernández-Calviño and Bååth, 2010; Thomas and Booth, 1992). Because of this, and because it affects so many other key parameters, soil pH consistently proves to be one of the best predictors of soil microbial community composition or activities (Bååth and Arnebrant, 1994; Bååth and Anderson, 2003; Pietri and Brookes, 2008; Pietri and Brookes, 2009; Shen *et al*., 2013; Seuradge *et al*., 2017). For example, Fierer and Jackson (2006) examined the diversity of soil bacteria in samples from across North and South America, and found that pH was a much better predictor of diversity than mean annual temperature, latitude, or potential evapotranspiration. In a single-site study, Rousk *et al*. (2010) used a long-term liming experiment at the Rothamsted research station in the UK to compare bacterial and fungal communities across a pH gradient of 4.0-8.3. They found that community composition was strongly correlated with pH for both bacteria and fungi and, using high-throughput sequencing, were able to determine specific phyla or sub-groups that responded consistently to pH. For example, *Acidobacteria* as a phylum were more abundant at lower pHs, but some subgroups within that phylum increased with increasing pH. In a similar study, Bartram *et al*. (2013) found that bacterial communities in soils were strongly affected by soil pH, using a long-term pH manipulation trial (pH range 4.5-7.5) at the Craibstone research farm in Scotland. We see a similar trend again in our ongoing study of fire effects on soil microbial communities in boreal Canada, where microbial communities clearly cluster by pH (Figure 2).

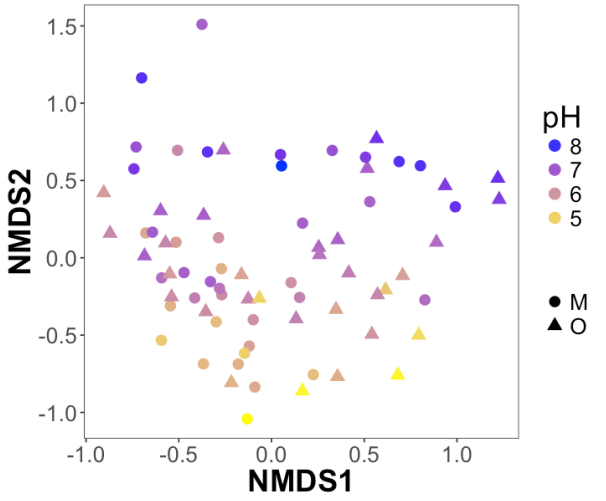


Figure 2. NMDS of Bray distances between soil bacterial communities (16S) across boreal forest sites in mineral (circles) and organic (triangles) horizons, coloured by pH (light yellow = more acidic, dark blue = more basic) (k=2, stress=0.156).

Soil pH is consistently a strong predictor of soil microbial community composition and activity, but the conditions under which we usually measure it are inconsistent with the conditions under which microbes interact with their environments. Supplementing standard pH measurement with methods of pH determination that better reflect the microbial environment may improve our ability to understand core soil functions that occur at microbially-relevant scales.

*Current methods of measuring soil pH: mechanisms and limitations*

Soil pH is most often measured with a glass electrode placed in a paste or supernatant of equal masses of water and soil and allowed to settle (Kellogg Soil Survey Laboratory, 2014; Thomas, 1996). This low-resolution standard soil pH metric is currently in wide use, and provides a single value for what is likely a highly heterogeneous and dynamic soil acidity profile that controls bacterial community dynamics (Matthiesen, 2004). While taking the pH of the supernatant of a settled soil slurry is largely sufficient for the characterization of soil responses to acidity at the root- and plot-scale, it may not represent the pH of soil solution in non-saturated soils under typical field conditions, which are the conditions in which soil bacterial communities develop and grow. For example, changes in soil salinity or the amount of dissolved $CO_2$ from the air can significantly change measured pH (Miller and Kissel, 2010; Sasowsky and Dalton, 2005). One common and simple adjustment for such limitations is to measure pH in a dilute salt solution (*e.g.,* 0.01 M $CaCl_2$), which better represents the soil solution, and can shift reported pH by more than 1 unit (Miller and Kissel, 2010). While this is a useful step toward more realistic pH determinations, the specific conditions of each soil will vary, and it would clearly be best if we could simply measure the pH of the actual soil solution – to determine the "*in situ* pH".

When using a standard glass electrode to measure pH (Conkling and Blanchar, 1986), a charge from two electrodes causes a hydrogen ion potential across the surface of a hydrated glass bulb separating the test solution and reference solution. A meter detects and registers the potential as a pH value after calibration with known buffered solutions. A microelectrode works similarly to the glass electrode, but the reference electrode and the tip of the electrode are miniaturized to allow the detection of pH of much smaller volumes, down to 15 µL or smaller (Dennis *et al.*, 2009). However, some uncertainties of measuring soil pH are unchanged at any scale – while these methods have miniaturized the glass electrode, they still rely on the same charged thin-surface electric potentiometry method. This means they may suffer from the "suspension effect" (Feldman, 1956), whereby the pH of the supernatant of a settled soil slurry is different from the soil. Ion-sensitive field-effect transistors (ISFET), however, use new ion sensor technology with applications to measuring soil pH (Artigas *et al*., 2001; Rossel and Walters, 2004). Briefly, a series of layers are deposited on a silicon chip, with a final layer that has a selective affinity for $H^+$ ions, and results in an electrical signal that is converted directly to a pH measurement. ISFET probes are more durable than glass electrodes, measure $[H^+]$ directly rather than via a difference in ion potential, take faster measurements, and can be stored dry. In addition, ISFET electrodes can be used by applying a drop of solution to the electrode's surface, allowing for the measurement of extremely small volumes. We will consider both a glass microelectrode (from our lab, for volumes as low as 45 µL) and also an ISFET electrode (to be purchased).

A second approach to pH measurement is based on chemical dye indicators. These dyes change conformation under different pH conditions, reflecting light differently. This includes traditional dyes for estimation of pH in the field (Kellogg Soil Survey Laboratory, 2014), as well as newer fluorescent dyes and polymers for in-lab determination of pH. The ratiometric fluorescent polymer method takes the ratio of two emission peaks that respond differently to pH, rather than just the absolute value of a single peak. This method uses a standard microplate reader, such as the BioTek Synergy 2 in our lab, with relatively high throughput (Zhang *et al*., 2016). We will investigate a series of different pH-sensitive dyes, including 8-hydroxy-1,3,6-pyrenetrisulfonic acid trisodium salt (HPTS) (Zhu *et al*., 2005), to characterize the pH of soil solution extracts.

HPTS and other fluorophores are widely used in microbiology for their high sensitivity to pH, and recent studies have shown HPTS is relatively non-reactive and may be suitable for direct soil solution analysis (Pedersen *et al*., 2015; Santner *et al*., 2015). However, these approaches have not yet been optimized for application in soil solutions. Possible challenges will include accounting for potential interference from autofluorescence of materials in the soil solution, sensitivity of the indicators to dissolved ions other than $H^+$ (which will vary from one soil to the next), and choosing an indicator or set of indicators that spans a soil-appropriate range of pHs.

*Relationship to national and state research priorities*
This proposed research is directly responsive to national and state-level research priorities. While we recognize that land managers who rely on soil pH measurements may not be likely to switch to a microelectrode or fluorescence-based pH measurement system any time soon, we expect that our research will be directly useful in two ways. First, we will aim to relate *in situ* pH to standard pH measurements using soil properties including electrical conductivity, texture, moisture contents, and organic matter contents, allowing users to convert a standard pH measurement into a predicted *in situ* pH value. Second, our findings will improve our understanding of the fundamental processes carried out by soil microbes, and the effects of pH on these processes and communities.

At the national level, we are addressing priority 4 ("Sustainable use of natural resources - USDA supports research to improve soil, air and water resources while supporting agricultural and forest production on working lands."). Specifically, we are directly addressing priority area 4 in that soil pH is a key variable that controls a wide range of fundamental soil properties and processes, from nutrient availability to soil microbial community composition. Many current land management recommendations are based on soil pH, and if we can develop an improved method of assessing pH, we may also improve these recommendations. Furthermore, better understanding the mechanisms through which pH affects soil microbes and the many nutrient cycling processes that they control will also improve soil resources and production on working lands.

At the state level, we are addressing Wisconsin priority 5 ("Sustainable agricultural and forestry production and processing systems that provides improved food safety and security, environmental protection, economically viable communities, protection of public goods, and human well-being. This need requires an understanding of basic life processes and model plant/animal systems in order to manage biotic systems for human use."). Specifically, we will improve our understanding of the basic life processes that drive soil systems by improving our understanding of soil pH and its effects on soil microbes and the processes that they control. This understanding will help inform land management decisions. Additionally, we will be carrying out our research on a range of soils from across the state of Wisconsin.

**Objectives and hypotheses**

Objective 1. Test and compare three alternative methods for measuring pH under microbially-relevant, unsaturated soil conditions ("*in situ* pH") and relate these to standard pH measurements and other soil properties.

Hypothesis 1. For all three methods, *in situ* pH as measured under conditions that are more representative of the soil environment (maintaining soil atmosphere and soil moisture contents) will differ substantially from standard pH measurements, and will vary depending on soil properties, including texture and organic matter (OM). Specifically, fine-textured (clayey) soils will diverge more from standard pH measurements than coarse-textured (sandy) soils; soils with high OM will diverge more from standard pH measurements than soils with low OM. Additionally, for the ISFET electrode, we anticipate less error due to interference from variations in the concentration of ions other than $H^+$ because, unlike the glass microelectrode and ratiometric fluorescence methods, the ISFET electrode directly measures $[H^+]$.

Objective 2. Determine whether or under what conditions *in situ* pH better predicts soil microbial community composition than standard pH, using a trans-Wisconsin sampling campaign and a long-term soil pH manipulation experiment. We will use the pH measurement techniques investigated in Objective 1 and high-throughput soil microbial community sequencing to address this objective.
Hypothesis 2. Soil microbial community composition will be strongly correlated with all pH measurement approaches, but it will be better predicted by *in situ* pH than by standard pH measurements.

**Methodological approach**

*Sample collection*
We will investigate the relationship between *in situ* soil pH, soil properties, and soil microbial communities using two sample sets: (A) We will sample soils from University of Wisconsin Agricultural Research Stations from across the state, taking advantage of the wide range of pHs naturally found in WI. (B) We will sample soils from a long-term pH manipulation trial at the Spooner Agricultural Research Station, which will allow us to investigate soil pH while controlling for factors such as soil mineralogy and texture. Soils will be collected during the spring and summer of 2018 by the Ph.D. student.

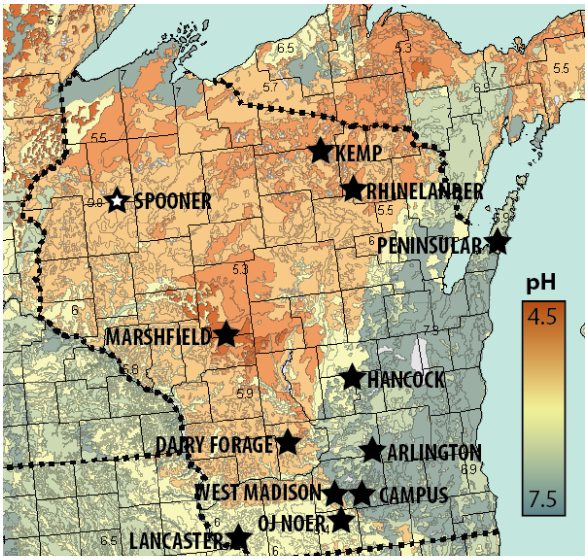

Figure 3. pH map of the soils of WI. Black stars indicate UW-Madison agricultural research stations, and the white star indicates Spooner station, where the pH manipulation trial is maintained. (BONAP, 2008.)

*Sites and sampling design*
The pH in WI soils ranges from ~4.5 to ~7.5, which is relatively wide, and comparable to that considered in other similar studies of pH effects on soil microbial communities (Bartram *et al.*, 2013; Rousk *et al.*, 2010). The original sites of the UW-Madison Agricultural Research Stations were chosen to represent a broad range of agricultural systems and natural landscapes across the state of WI. We will take advantage of the broad range of soil types covered by the research stations, using them to collect soils that represent a range of pHs, OM contents, and textures. Using the Web Soil Survey and Agricultural Research Station records, we will aim to select sites with all 12 combinations of low (4.5-5.5), medium (5.5-6.5) and high (6.5-7.5) pHs, lower (<2%) and higher (>2%) OM, and fine- and coarse-textured soil, with three sites representing each of

these combinations, and sampling two depths (0-5 cm and 5-30 cm, focusing on the Ap or A horizons). This will result in 72 total samples from Agricultural Research Stations. At each site, we will obtain vegetation cover, management history, relief, and climatic data.

In order to test the effects of pH while keeping many other soil properties constant, we will also target a long-term pH manipulation trial at Spooner Agricultural Research Station (see letter of collaboration from Philip Holman, Superintendent of Spooner ARS). This trial has been established since 1991, with 5 lime-adjusted pH targets of 4.7, 5.2, 5.7, 6.2, 6.7, and 4 field replications of each pH target, and planting history (corn and soy since 2002), yields, and soil property data are available. The experimental area is 23 m x 15-18 m. We will take three samples from the centre of each of the 20 field-replicated plots, at two depths within the Ap horizon, for a total of 120 samples.

For each sample from the multi-farm and the pH manipulation trial, soils will be immediately sieved <2 mm, and sub-sampled for soil microbial community analyses. Microbial samples will be kept frozen on dry ice and preserved in a -80°C freezer as soon as possible. If dry ice is not available in the field, microbial samples will have DMSO-EDTA-salt solution added to help preserve them while they are kept on ice in coolers (Tatangelo *et al.*, 2014). The remaining soil samples will be sealed in plastic bags and kept on ice or in a refrigerator until returned to the lab, where they will be stored at -4°C until analysis.

*Sample analysis – soil properties*
Soil samples will be air-dried for lab analyses, with a sub-sample dried at 105 °C to determine moisture content. We will take *in situ* and standard pH measurements in our lab using the methods developed and described below. In addition, for each sample, we will analyze standard soil properties, including texture (using the particle size analyzer in the Department of Soil Science), organic and inorganic C contents and organic N contents (in Dr. Randall Jackson's lab at UW-Madison), total elemental analysis (using the XRF in the Department of Soil Science), electrical conductivity (in our lab), microbial biomass (in our lab, with DOC measured in the Jackson lab), and mineral N (at the UW Soil and Forage Analysis Lab). We will also make a rough estimate of soil C mineralizability by incubating dried soils under a standardized water holding capacity over the course of one week in Mason jars multiplexed to a Picarro CRDS $CO_2$ analyzer autosampler currently being developed in our lab.

*Sample analysis – soil bacterial communities*
DNA will be extracted from frozen soil samples using the MoBio/QIAGEN PowerSoil® DNA DNEasy Isolation Kit. Extracted DNA will be quantified with PicoGreen and used for amplicon metagenomics. We will use the 16S rRNA gene as a marker for the bacterial/archeal community. After PCR amplification of the 16S region with barcoded primers, samples will be normalized using Sequal plates, pooled, purified with a Wizard gel cleanup system, and sequenced on an Illumina MiSeq platform (2x250 paired ends) at the UW-Madison Biotechnology Centre. Sample processing will be performed using a combination of QIIME2 (Caporaso *et al.*, 2010), mother (Schloss *et al.*, 2009), and UPARSE (Edgar, 2013) pipelines, using similar approaches to our past work (Whitman *et al.*, 2016).

*Sample analysis – soil pH by traditional and alternative methods*
For standard pH measurements, we will follow the Soil Science Society of America methods (Thomas, 1996). Briefly, soils will be mixed in a 1:1 soil:solvent ratio, using water and 0.01M $CaCl_2$ as solvents, stirred for 10 min and allowed to settle for 10 min, after which the solution supernatant will be measured with a standard pH probe.

We will also assess microelectrode- and fluorescence-based methods of measurement of *in situ* pH. We will optimize these methods in the lab, with the overarching aim of obtaining and maintaining small volumes of soil solution under conditions that are as representative of the soil environment as possible. As with standard pH measurement methods, we will bring the soils to a consistent moisture level. However, unlike standard pH measurement, this target moisture level will be chosen to be more representative of standard soil conditions, rather than total saturation, and we will characterize the effect of a range of moisture levels during optimization experiments. We will extract small volumes of soil solution via centrifugation of unsaturated soils under a range of ambient moisture conditions. Samples will be protected from changes in dissolved $CO_2$ by containing the samples in sealed tubes with custom valve ports through which microelectrodes can be inserted to test solution pH directly or through which small solution volumes can be extracted via a syringe and transferred for measurement under controlled conditions. The three instruments and protocols we will use to measure *in situ* pH of the extracted soil solutions are (1) a glass microelectrode, (2) an ISFET electrode, and (3) ratiometric fluorescent polymers analyzed by a microplate reader.

For the fluorescence-based methods, we will adapt for soils methods similar to those Zhang *et al*. (2016) used for microbial cultures. Briefly, we will aliquot small volumes of soil solution into a microplate, adding the fluorescent dye indicator, and measuring the fluorescence ratios on our lab's microplate reader. We will research and investigate the wide range of possible fluorescent indicators, including HPTS. We will aim to select an indicator or set of indicators that has low sensitivity to other dissolved ions, is sufficiently sensitive across a soil-appropriate range of pH values, and is photo-stable. One possible challenge for this approach is that soils contain an unpredictable mixture of organic and inorganic substances, which can contribute an unknown level of autofluorescence, contaminating fluorescent dye-based measurements. Using ratiometric fluorescence methods (taking the ratio of two emission peaks that respond differently to pH, rather than just the absolute value of a single peak), combined with a no-dye control, may help limit these confounding factors. We will also investigate whether filtering the soil solution helps control for this potential issue. Because there are many possible indicators available (Santner *et al*., 2015), we expect it is likely that we will be able to choose a fluorescent dye or combination of dyes that work well for a wide range of soils.

*Objective 1 statistical analyses – linking in situ soil pH, standard soil pH and soil properties*
Our goal for objective 1 is to determine the relationship between various *in situ* pH measurements and standard soil pH, and to determine whether differences between these are related to fundamental soil properties. To do this, we will use standard multivariate linear models, testing each parameter for significance, similar to the approaches used by Miller and Kissel for standard pH (Miller and Kissel, 2010). This will allow us to determine how the three *in situ* methods deviate from standard soil pH. In addition, we will determine whether using other soil properties as model factors help improve predictions of *in situ* pH.

*Objective 2 statistical analyses – linking soil pH and microbial communities.*
Our objective is to determine whether or under what conditions *in situ* pH better predicts soil microbial community composition than standard pH. To do this, we will use a number of approaches. First, we will use a permutational multivariate ANOVA (Anderson, 2001) using the Adonis function in the R package vegan (Oksanen *et al.*), to determine whether pH is a significant predictor of soil community composition for bacteria, archaea and fungi. While we expect that pH will correlate significantly with soil microbial community composition no matter what the measurement technique, we will compare the adjusted $R^2$ values associated with *in situ* pH *vs*. standard pH measurements to determine which pH measurement approach is a better predictor of soil microbial community composition. However, this approach only considers the community as the whole. To determine which specific microbes are positively or negatively associated with pH, we will use a differential abundance approach, where we determine how the relative abundance of each microbe changes with a change in pH, using the R package DESeq2 (Love *et al.*, 2014).

*Next steps, future research, and dissemination*
Although we know pH is a key determinant of soil microbial communities, there are many mechanisms through which this may occur, including direct impacts (metabolic stress from high or low hydrogen ion concentrations in the soil solution) and indirect impacts (such as changing nutrient availability), the relative importances of which are not currently well-characterized. Further research will be needed to experimentally identify the specific mechanisms by which soil pH influences the growth and development of soil microbial communities, beyond correlation-based analyses. While the research proposed here will not directly address these questions, the Ph.D. student will investigate them in their broader dissertation, and the results from this proposed research will help form the foundation for their hypotheses and experiments. Most importantly, determining *in situ* soil pH will help highlight possible mechanisms by which pH influences soil microbial communities, and vice versa.

Our future research will also delve into the issues of scale. Bacterial cells are often only 1-2 μm wide, and, if immobile, most of their interactions with their surroundings are limited to a range of about 20 μm (Raynaud and Nunan, 2014). However, pH can vary over very short gradients in the soil – *e.g.*, in the ~2 mm zone of soil affected by plant roots (the "rhizosphere"), soil pH can vary by >1 unit (Rudolph *et al.*, 2013). Despite this micro-to milli-scale heterogeneity in soil microbial communities and in pH, we usually sample soils at scales that are orders of magnitude greater than the scales at which this heterogeneity exists. Our long-term goal is to develop an understanding of micro-scale location-specific differences in soil pH, and the implications for soil microbial communities and their activities. In order to do this, though, the first step is to optimize the measurement of pH at very small scales, which is what we propose to do here.

Results from the work will be published in peer-reviewed journals and on open science pre-print servers and presented at scientific conferences and farmer field days.

**Management plan**
Dr. Thea Whitman will be the sole PI on this project and will oversee all work described within this proposal.

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