# The Acidity and Proticity of Soil Microhabitats

by

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## 0.1 Abstract

Soil scientists, microbiologists, and biogeochemists will encounter values of "soil pH" throughout the scientific literature and various public databases, and this dissertation reviews and advances the metrology of soil acidity as represented by soil pH. Typical levels of soil moisture and carbon dioxide are experimentally integrated into the determination of soil pH, revealing a mismatch of aqueous chemical models with *in situ* soil solution. Standard and non-standard values of soil pH are used to predict soil microbial communities, establishing that the novel method is more optimal than the standard method in use today. Soil is demonstrated to poorly meet the assumptions required to apply pH as a metric of soil acidity, and a new metric—"proticity"—is formalized and proposed with potential to replace pH altogether. *In situ* soil solution and proticity then inform the redesign of diffusion isolation-cultivation devices, or "iplates", that cultivate rare soil bacteria and improve the functionality, customization, and affordability of existing "ichip" technology.

## 0.2 Acknowledgements

The following people have made seminal or supportive contributions to the content and qualility of this work: Michael Piotrowski, Linda Graham, Mattie Urrutia, Alfred Hartemink, the Soil Ecology Laboratory, and the faculty and staff of the Departments of Soil Science, Botany, and Bacteriology.

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*This work is dedicated to my*

*curious students,*

*clever colleageus,*

*and caring family.*

*Thank you.*

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*"Lux lucet in tenebris quamvis nihil obscurius luce. [Light shines in darkness, however, nothing darkens the light.]"*

Freiherr Christian Johann Dietrich Theodor von Grotthuss (1785-1822)

---

*"It is indispensable also to study, alongside matter and energy, the manifestation of time in living processes."*

V. I. Vernadsky (1863-1945)

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*"Life on Earth is more like a verb. It repairs, maintains, re-creates, and outdoes itself."*

Lynn Margulis, *What Is Life?* (2000, 14)

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# 1 Introduction

## 1.1 Overview

This dissertation describes and interprets the history, measurement, significance, and future of the property called "soil pH" in the fields of biogeochemistry, soil science, and microbial ecology. At the outset of my doctoral research in May of 2016, I received the very open-ended task to investigate "soil microhabitats" in some novel and impactful way. Soil microbial ecology today is a maturing science, undergoing a kind of *Bildungsroman*, and over the first two years of my doctoral program, I designed my primary research project to ease our collective growing pains where I was able to do so. From coursework and independent study, there surfaced many important hypotheses, generalized theories, and mental models that exhibited a great need for further investigation, and ultimately the metrological foundations of soil acidity became the primary subject of the experimental designs, the talks I delivered at various conferences, and my subsequent research.

I will explain in this brief introductory chapter why the metric called "soil pH" so powerfully caught my interest as a scientist and educator, and I will present the findings of my investigations in the remaining chapters of this dissertation. Chapters 2 and 3 are a two-part development and measurement of standard and non-standard soil pH values in relation to the nutrient profiles of Wisconsin's agricultural research stations and the bacterial ecology of the soils of Wisconsin. Chapter 4 presents a new metric named "proticity", which utilizes the new chemical model of temporal bond state dynamics, with prospects of replacing the pH scale. Chapter 5 presents a diffusion microchamber device named the "Iplate" that allows investigators to customize the recent but underutilized "Ichip" invention for culturing uncultivated soil microbes. Chapter 6 is the final chapter providing several personal anecdotes and concluding remarks, and Chapter 7 is not a chapter $perse$ but several appendices where one can find direct quotes from the last century of scientific literature, access to my retranslation of the seminal and foundational paper by Debye and Hückel (1923) on their seminal theory of electrolyte solutions, which is the basis of "pH" itself, and a brief guide to that publications's mathematics and interpretation.

## 1.2 Motivation of Research

Soils offer a pleasing but capricious inscrutibility to investigators who are fortunate enough to take an interest, and, upon joining such investigators, I naturally became fascinated with the embedded uncertainties, complexities, and relationships between "pH", soil, biogeochemistry, and bacterial ecology. Whether by fate or fluke, soil science and bacteriology both treat pH as a "master variable". pH is noticeably causal or correlative with many other factors in most systems of study. Soil pH in soil science strongly indicates the surface chemistry of soil particles, which over time impacts the chemical mechanisms of pedogenesis, stability of inorganic and organic carbon and nitrogen in soil, nutrient availabilities for uptake by plant roots and microbial cells, and the rates of mineral weathering throughout Earth's history. Soil pH participates in many "pedotransfer functions", the useful collinear relationships where one property can be used as a rough but sufficiently predictive proxy for many other soil properties. Likewise, with direct relation to soil pH, microbiologists have intensely researched the internal pH and the external pH of cells. The gradient that is formed bewteen internal and external acidity drives the fundamental bioenergetics of bacterial growth, movement, and thresholds of tolerance to various microenvironments including cultivation growth media, freshwater, and even the human body.

However, it remains unknown whether values of soil pH represent the external acidity of a microbe's immediate surroundings. An unknown or unverified mechanism warrants the discernment of both causation from correlation and fact from fiction regarding the influence of pH on and between soil and microbial physiology. The need for a new paradigm is found not solely in experimental analyses of leading models but also in the fundamental dimensional analysis of leading models' variables themselves. I believe that very few paths of investigation hold as much promise as the endeavor of measuring acidity, whereby we may one day move beyond the "pH scale" altogether to advance the knowledge and abilities of biogeochemists, agronomists, and soil microbiologists, with great possibilities for use by physicians and physical chemists.

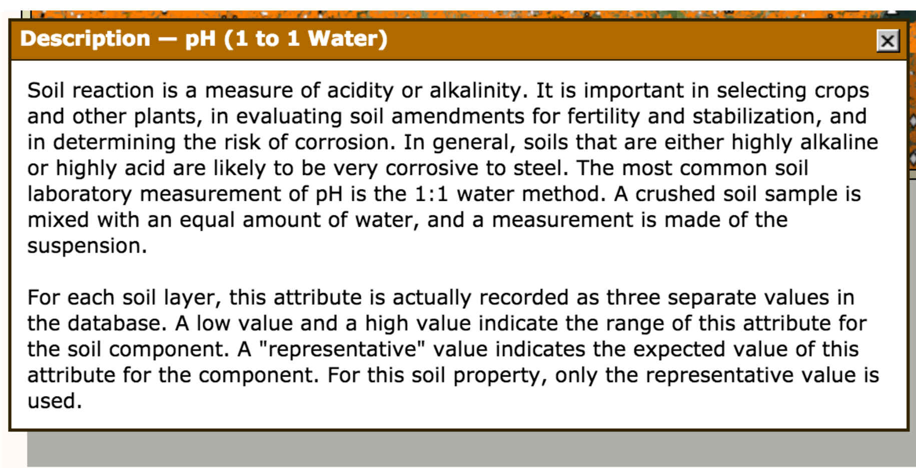
To initiate this critical analysis of "soil pH", I propose that the physical sciences have several fundamental principles that both guide and concretize the truth and knowledge we seek, and I also propose the disclaimer that these principles may be phrased in a multitude of formats and iterations:

1. Only comparisons of physical quantities (PQ), i.e. data with both a numerical value (NV) and a calibrated and dimensionally homogeneous unit of measurement (UM), are used to characterize a system or organism of interest, or, more succinctly, $\{PQ\}_i = (NV)_i[UM]_i$, where $i$ signifies each datum.
2. It is necessary, if an investigator wishes to determine a causal link between the status or behavior of an organism of interest and a certain physical quantity, to match or surpass the capabilities possessed by that organism to detect that physical quantity.
3. Neither a single physical quantity nor single resulting model can fully characterize an organism of interest or the organism's habitat, yet we try anyway (and try again and again, of course, until satisfied).

### 1.3 Topic of Research

Soil pH is the predominant representation of soil acidity today, and Figure 1.1 shows a description from the Web Soil Survey (WSS) of the common standard soil pH method that has populated the WSS database describing soils across the United States. Similar methods have produced the soil pH values used to describe soils across the rest of the world. However, (1) other metrics exist to represent different aspects of soil acidity, such as exchangeable acidity and aluminum, (2) the pH scale defined by Bates and Guggenheim (1960) excludes soils of moisture content below field capacity, and (3) the standard soil pH method may not represent *in situ* soil acidity because the pH of the supernatant of a slurry is unlikely to correspond fully with the acidity of thin hydrofilms ($< 10$ [$\mu$m]) coating unsaturated soil particles.

Conceptually, despite great progress in disparate fields, there remains a mechanistic gap between soil pH and soil microbial communities (Figure 1.2), which prevents a clear distinction between correlation and causation between soil acidity and the microbial life in soils. This mechanistic gap entails many research questions: How do solvated protons behave when they exist in soils, which range from being completely saturated to nearly completely dry? Do substances with acidic effects when added to water behave identically in soil moisture? Can soil pH values be modified using other chemical data to represent what soil bacteria and other microorganisms experience *in situ*? What excactly is "pH" if "hydrogen ions" do not exist in water? Is it true that pH $= -\log[\text{H}^+]$, or that pH $= -\log[\text{H}_3\text{O}^+]$, if these species are autoionizing with the solvent (water) as well as the


**Description — pH (1 to 1 Water)**

Soil reaction is a measure of acidity or alkalinity. It is important in selecting crops and other plants, in evaluating soil amendments for fertility and stabilization, and in determining the risk of corrosion. In general, soils that are either highly alkaline or highly acid are likely to be very corrosive to steel. The most common soil laboratory measurement of pH is the 1:1 water method. A crushed soil sample is mixed with an equal amount of water, and a measurement is made of the suspension.

For each soil layer, this attribute is actually recorded as three separate values in the database. A low value and a high value indicate the range of this attribute for the soil component. A "representative" value indicates the expected value of this attribute for the component. For this soil property, only the representative value is used.

Figure 1.1: Web Soil Survey description of standard soil pH methodology ("pH 1 to 1 Water").

hydrated particle surfaces? If not, is there a better metric for acidity other than "pH", "acids", and "bases"? Is the movement and transfer of protons and their respective charge perhaps the *lingua franca* by which "soil microhabitats" mutually interact with their "soil microinhabitants"?

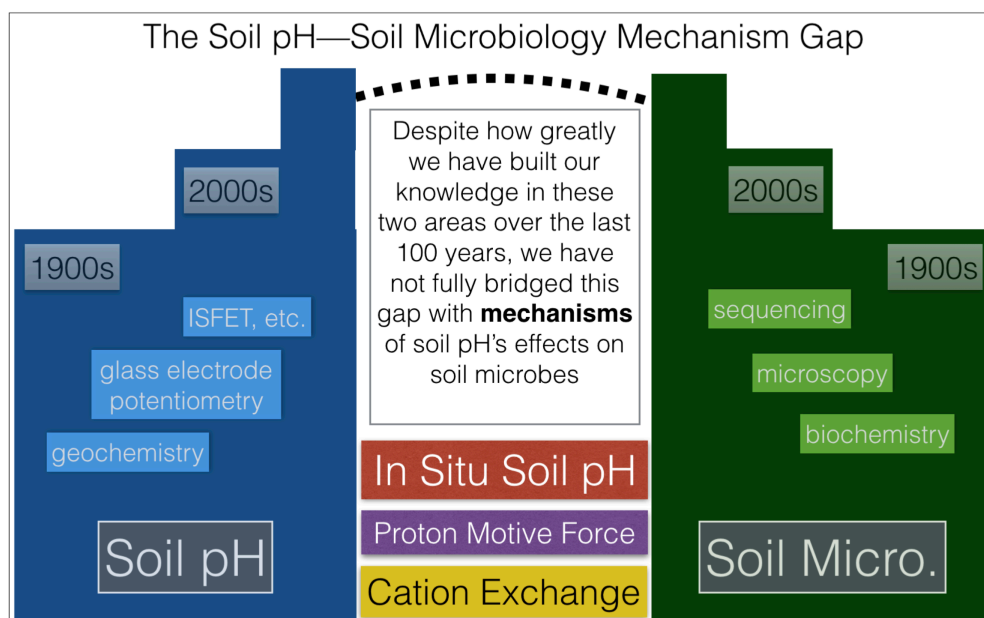



Figure 1.2: A conceptual diagram of the existing mechanistic gap between soil pH and soil microbial communities (January 2018).

To answer these questions, I developed an overarching model of proton flow in soil microhabitats (Figure 1.3). From the various mechanisms and systems within soil microhabitats, acidity emerges and ostensibly confers the soil pH values found in agricultural databases. Soil acidity is a property

that governs the reactivity of both biotic and abiotic entities in dynamic, and there is untapped potential to incorporate a temporal dimension such that we may measure "solvated proton flow" throughout the environment.

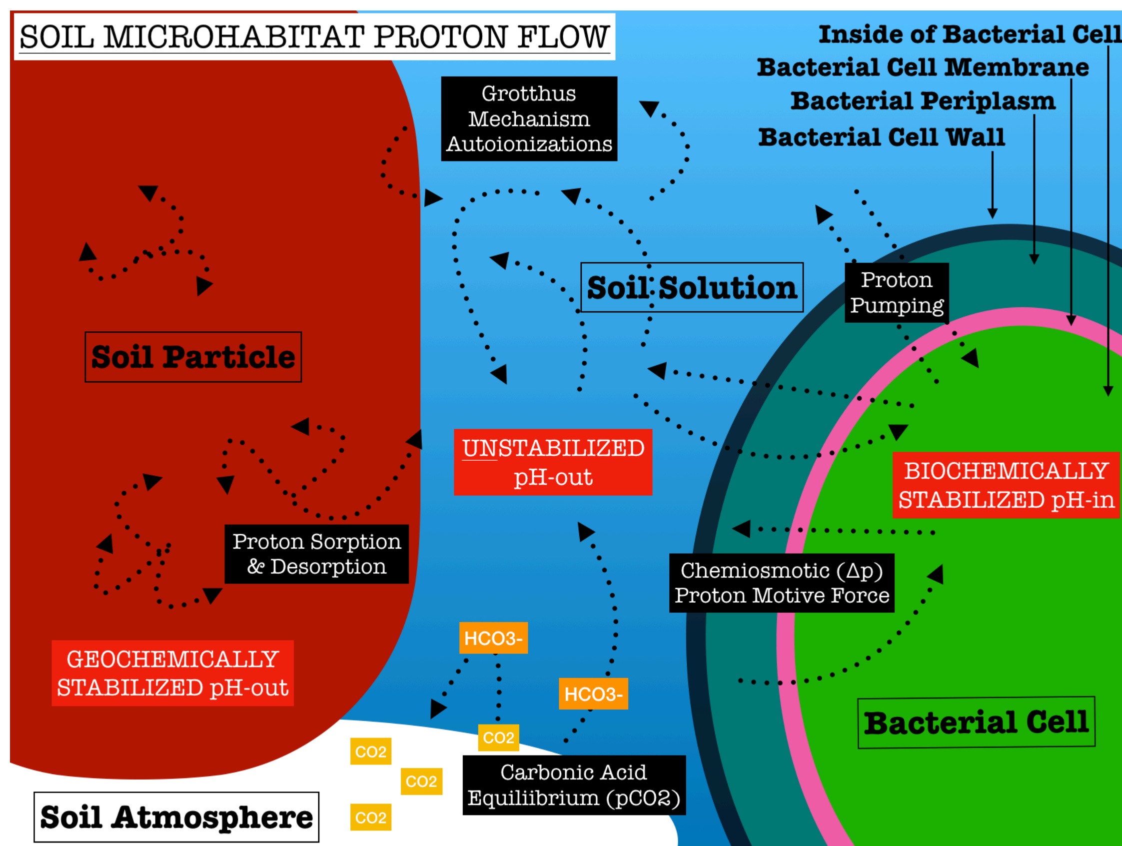



Figure 1.3: A conceptual model of mechanisms from which proton flow (proticity) emerges in soil microhabitats (January 2018).

## 1.4 Research Challenges

A model is only as applicable as its assumption allow. Soil pH operates under aqueous chemical models, but soils at a typical moisture content are not aqueous solutions at all. I will use two examples to briefly introduce the metrological problems resulting from this discrepancy. First, nearly every chemist throughout the 20th century has admitted that single-ion activities are not measurable, and values of pH are estimates of the single-ion activity of protons in water strictly when sufficiently dilute, i.e. of sufficiently low ionic strength. This dilute solution assumption necessarily results from using the theory of Debye & Hückel (1923), who greatly simplified a key equation in their work (replacing equations $10$ with $10'$) by assuming a sufficiently large

distance between ions: "The further one moves away from the specified ion, the smaller will be the potential $\psi$ and for larger distance the sufficient approximation $\mathfrak{Sin}\frac{\epsilon\psi}{kT}$ can replace $\frac{\epsilon\psi}{kT}$." Ion exchange and the hydrofilms adsorbed to particles of unsaturated soils clearly violate this assumption. Debye & Hückel used ionic strength to set the upper threshold for "dilute solutions", which has been reiterated dozens of times as $0.1$ moles per liter since 1923 (see Appendix A). In brief, unsaturated soil is not a dilute aqueous solution.

A second example of the metrological challenges of this research is the estimation of ionic strength in soils. Nocco, Ruark, and Kucharik (2019) has estimated "apparent" ionic strength but do not use actual values of electrical conducitivity (EC) from a conductivity meter immersed in an aqueous solution but instead the "apparent soil conductivity" ($EC_a$) from a Veris Soil EC 3100 (Veris Technologies in Salina, KS). This instrument does not measure the electrical conductivity of a solution but instead the conductivity through a volume of soil matrix. Soils are composed of highly electrically resistant materials, notably silica, which cannot be fairly compared to an aqueous solution. Therefore, the interconversion of ionic strength $(I_s)$ and EC using the formula described by Alva, Sumner, and Miller (1991), $I_s = (1.6 * 10^{-2}) * \text{EC[dS/m]}$, can only be made possible when used for soil solution extracts or the dilute supernatants of soil slurries. $EC_a$ will be low, of course, owing to highly resistant materials of soils, not owing to low ionic strength by dilution of a solution. Therefore a low $EC_a$ reading neither signifies nor pertains to the ionic strength of a soil to determine if pH, which requires $I_s$ less than than $0.1$[M], may apply. The observation that the ionic strength of unsaturated soil must be enormously greater than $0.1$[M] is obvious: the water composing soil moisture is so concentrated that it is bound to the soil and will not exit except by centrifugation or evaporation (i.e. adding energy). Bound water existing in many thin hydrofilms in soils is therefore incommensurable with liquid water in a beaker or tube, and the measurement of pH requires by its own definition the latter aqueous system and excludes the former soil system.

Addressing the use of strictly aqueous models to soil systems—and a great number more—to measure the acidity of soils and other systems offers abundant opportunities with a very lively future for collaborations, inventions, discussions, and investigations yet to perform. Overall, my goal is to discern exactly whether soil pH is a satisfactory or unsatisfactory representation of the

acidity of soil microhabitats and provide recommendations on the topic to other scientists and researchers of soil biogeochemistry.

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# 2 Standard and Non-Standard Measurement of Acidity in Wisconsin's Soils. I. Is 'Soil pH' a Dilution or a Delusion?

## 2.1 Introduction

### 2.1.1 A brief history of soil pH measurements: Challenges and limitations

Since its inception over a century ago (Myers 2010; Shoemaker, McLean, and Pratt 1961; Woodruff 1948; Olsen 1921), "pH" became the most common measurement of acidity collected, and "soil pH" in turn became the most common measurement of the acidity of soils (Totsche et al. 2010; Miller and Kissel 2010). Although the specific protocols for quantifying soil acidity differ in different regions—and even in different laboratories—these protocols universally operate as follows: the suspension of a mass of soil in at least an equal mass of an aqueous dilute electrolyte solution yields a settled sediment equilibrated with a supernatant analyte, and the acidity of this supernatant is measured by colorimetric dye or by inserting a glass pH probe after calibration to traceable standard pH solutions (Burt and Staff 2014). This standard method, although effective for maximizing crop production, was not designed to represent or estimate *in situ* soil conditions (Matthiesen 2004; Elberling and Matthiesen 2007; Samaranayake and Sastry 2010). Furthermore, according to the metrological literature and guidelines for the commensurability of acidity measurements, "pH" itself is inapplicable to brines, saline solutions, and the tightly bound hydrofilms of soils, because the pH standard solutions are incommensurable to concentrated solutions. For over a century, the notion of "pH" itself was considered a property strictly pertaining to dilute aqueous solutions—precisely the phase that soil is not.

The techniques for measuring "pH" are by definition applied to solutions of ionic strength ($I_s$) greater than $0.1$ molar, whereby the measurement error is maintained below $5\%$ and where

$$I_s = \frac{1}{2}\sum(c_i z_i^2) \qquad (2.1)$$

estimates charge density of electrostatically charged ions or substances added to an aqueous solution (Lewis and Randall 1921) using the concentration ($c_i$) of each ion ($i$) and the charge of each ion ($z_i$). The upper theshold of $0.1$ [M] is also called the "the dilute solution assumption" (Dobrovolskii et al. 2018, 87; Covert and Hore 2016, 235–38; Levie 2014, 615, 2010; Spitzer and Pratt 2011, 75; Wright 2007, 382; Baucke 2002, 774; Sparks 1998, 112; Anderegg and Kholeif 1994, 1521; Galster 1991, 16; Butler 1998, 462–63; Volk and Rozen 1977; p. 1569; Pourbaix

1974, 14; Sena 1972, Appendix 3; Ashcraft 1957, 3, 1947, 29; Feldman 1956, 1865; MacInnes 1939, 148; Debye and Hückel 1923, 197), and it is a fundamental limitation of the measurement of pH. Given this limitation, I ask what exactly is the pH—much less the "*in situ* pH"—of a soil, which is a complex substance that is more accurately categorized as a semi-solid colloidal matrix than a dilute aqueous solution? In other words, is "soil pH" a *dilution* or a *delusion*?

Prior to the development of quantitative measurements of acidity, soil acidity was characterized by taste and smell, often reported by the purely qualitative scale of "sourness". As described by Chang (2012), the qualitative metrics for acidity have also been measured by litmus paper as well as an analyte's corrosive effects on skin, textiles, and metals. Evidence of ionization of solutes in water emerged from the works of Michael Faraday, John Dalton, Jacobus Henricus van 't Hoff, and their contemporaries during the 19th century, and, by 1887, Svante Arrhenius and Wilhelm Ostwald performed the first electrochemical measurement of "hydrogen ions". This work informed the development by Walther Nernst in 1889 of his equation for membrane potential, which Max Cremer (father of chemist Erika Cremer, a pioneer in gas chromatography) used to detect the unusual selectivity of glass membranes to the activity of acids and bases. Søren Sørensen (1909) at Carlsberg Laboratory (now Carlsberg Research Center), which was the research division of the Carlsberg brewery, coined the term "pH" in 1909 to spare himself and his assistantcs from writing in decimal format the extremely small values in units of molarity (F. Sgambato et al. 2011a, 151). The measurement of "soil pH" began shortly after by one of his assistants, Carsten Olsen.

Sørensen's notation, widely evangelized by Michaelis (1926, 22), set off a flurry of inventions and models of measuring acidity in aqueous solutions, which led Lawrence Joseph Henderson to develop his equation demonstrating the relationship of acids, bases, and buffers. The Henderson equation was written in "hydrogen ion concentration", and only later in 1917, well after the shorthand of "pH" was developed, did Karl Albert Hasselbalch suggest keeping all values in logarithmic form, yielding the Henderson-Hasselbalch equation in use today.[1] "pH" has since been defined the same way in the hundreds of publications discussing acid-base chemistry, geochemistry, biochemistry, and medical physiology published in the interim century (F. Sgambato et al. 2011a; Levie 2014). The definition has slightly varied but is best summarized as $\text{pH} = -\log[\text{H}^+]$. Upon

[1] The Henderson-Hasselbalch equation is not a physical law but an approximation, as described by De Levie (2002).

learning of the problems associated with this definition, however, which I describe in more detail below, many authors cleverly hedge their version of the definition with special stipulations, such as $[H^+] = [H_3O^+]$ or $[H^+] = c(H^+) = a(H^+)$, where "$H^+$" *operationally* signifies all cations of autoionizing water, $c$ signifies "concentration", and $a$ signifies "activity."[2]

Levie (2014), Spitzer and Pratt (2011), and Bak (1974) have provided further historical details and insights regarding events surrounding the development of Sørensen's pH scale, the similar definitions by Brönsted (1923) ("electric charge type") and Lowry (1923) ("the uniqueness of hydrogen"), the complementary definition of acidity by Lewis (1923, 142), the highly precise potentiometer of Thackray and Myers (2000, 124–42), and the many other innovations that ultimately led to the definition we use today by Bates and Guggenheim (1960). Luder and Zuffanti (1961), Peel (1971), and Drago (1974) presented improvements and alternatives, whereby some models show greater effectiveness and accuracy, but the Bates-Guggenheim convention still predominates today despite the overwheliming evidence that the bare proton connoted by the term "hydrogen ion", as it was originally conceived, indeed does not exist in water (Hynes 1999; Wolke et al. 2016).

Emil Truog (1914), working at the University of Wisconsin, performed one of the first studies to measure soil acidity, noting that the elevated carbonic acid in soils caused measurements following the preparation of soil samples from pits and cores to be highly error-prone. He used a small field kit to create a soil slurry, similar to the standard protocol used today (Burt and Staff 2014) but heated to degas the carbon dioxide, and he measured the supernatant's acidity using litmus paper. Truog also performed various titrations using hydrogen sulfide and zinc sulfide in the laboratory, but the new method was intended for convenience and speed in the field for use by farmers and agronomists, with little quantitative accuracy for the characterization of *in situ* soil acidity. Wherry (1920) attempted to quantify soil acidity as well, but he admitted he was highly limited by the physical chemical models available to him at the time, raising more questions than he was capable of answering.

The first controlled experiment incorporating the factor of "soil pH" was published in 1921 by

[2]Thermodynamic activity was originally defined by Lewis (1907, 262–63) specifically with units moles per liter (molarity). However, the International Union of Pure and Applied Chemistry (IUPAC), reporting in their "Green Book" (Renner 2007, 70–71), that thermodynamic activity is unitless by way of dividing the concentration by an arbitrary "standard state" concentration. It remains unclear why the IUPAC would make this change to Lewis's original definition of activity.

one of Sørensen's assistants, Carsten Olsen (Olsen 1921; Sharp and Hoagland 1916). He found certain crop plants exhibited a preference for neutral or slightly acidic soils, as measured by growth rate and size, with clear soil pH optima. However, a little-known paper by a well-known scientist of the time, Danish chemist Niels J. Bejrrum, appeared two years prior to Olsen's publication, which greatly informed Olsen's methodology and initial interpretation as to how soil pH might govern various plant growth rates. Bjerrum and Gjaldbæk (1919, 13–14) iterated the problem of the dilute solution assumption when applying acidity measurement to soils, which was extremely prescient: "[It] must be mentioned that [the hydrogen ion concentration] is only exactly valid for very dilute solutions, since only then can one set the activities of the ions equal to their concentrations... In very dilute solutions the activity coefficients are equal to 1 and the same expression results for $a_{H^+}$ and $C_{H^+}$. In concentrated solutions the activity coefficients are not equal to 1 and the apparent hydrogen ion activity and hydrogen ion concentration are then different."

The metrology of pH advanced greatly from 1920 to the present (Buck et al. 2002; Spitzer and Werner 2002; Myers 2010), but the standard soil pH method did not. However, it is apparent that many of the same problems for soil scientists and biogeochemists of the early 20th century, namely Bjerrum and Truog but also their many contemporaries (Duncan A MacInnes 1915; Fisher 1922; Pierre 1925), plague soil scientists and biogeochemists today. To summarize, (1) soil solutions, as found in nature, generally have ionic strength greater than $0.1$[M], which places them outside the bounds within which standard pH measurements are applicable; (2) neither "hydrogen ions", as originally conceived, nor hydronium exist in the same solvated state as other ions in solution, because hydrogen ions are highly autoionizing in addition to being solvated; and (3) even if we were able to measure the single-ion activity of hydronium ions, in concentrated solutions such as soil solution, their activity will not be equavalent to their respective concentrations as is required to measure pH. For these reasons, "soil pH" is worth critical reanalysis, update, or even replacement.

#### 2.1.2 Representing field conditions in the laboratory for soil pH measurements

In addition to the fundamental limitations of pH as currently measured in soils, we are also faced with the dilemma that the conditions under which we measure soil standard pH as described above

rarely represent those found in soils and are continuously changing on a daily and seasonal basis. Unfortunately, non-standard protocols and analytical procedures are sparse and somewhat scattered in the literature, and few methodological studies have addressed the underlying metrological challenge of "soil pH" itself. On the one hand, the use of non-standard methods of measuring soil acidity risks violating the commensurability of an investigator's pH values to the standard soil pH values found in large databases (Minasny et al. 2011). On the other hand, the large diversity and variability through time of soil environments warrants diversification and customization of methods as well as the subsequent interpretation of the values that novel or adapted methods yield. For example, a saturated peatland may require neither drying nor addition of solution but simply gentle centrifugation and analysis of the supernatant with a glass pH probe. On the opposite extreme, a study of saline desert soils inhabited by plants having halotolerant root physiology would require the addition of a solution, most likely in excess of the typical $1:1$ ratio by mass, to create solution extract dilute enough for pH measurement. In many regions of Earth, the soil solution is frozen for a large period of the year, whereby the solid phase of solution is intractably shifted away from standard state.

Furthermore, how can one reconcile the differences of the chemistry of soil as it exists in the field and soil as it exists as an analyte in the laboratory? We can juxtapose the laboratory and the field to conclude, generally, the chemical properties of solutions in the controlled conditions of the laboratory ("*ex situ*"), further altered with the addition of solutions and processing of extracts, are highly incommensurable with the same chemical properties of undisturbed solutions in the field ("*in situ*").

The atmospheres of soils often have much higher partial pressures of carbon dioxide, and these partial pressures change with depth (Vernadsky 1913; Cary and Holder 1982; Belnap, Hawkes, and Firestone 2003; Jury and Horton 2004, 215; Oh, Kim, and Richter Jr 2005) reaching maxima estimated to be approximately $4\%$ to $6\%$ at depths at or below $2$ [m] and minima of atmospheric carbon dioxide levels ($400$ [ppm] or $0.04\%$) at depths of $< 5$ cm, with a vertical distribution skewed around $2\%$ $CO_2$. Under these conservative thresholds of typical mineral soils, which are customary of agricultural lands, one may then deem $0.04\%$ "very low $CO_2$", $5.0\%$ "very high $CO_2$" and $2\%$ "typical $CO_2$". Laboratory atmospheres would therefore accurately be considered

low $CO_2$, though this varies greatly with the presence of investigators indoors, and from this low and variable level naturally follows several important questions for the field of soil biogeochemistry. If a soil sample collected from a soil profile at $1$ [m] is moved to the laboratory for measurement of acidity or other chemical characteristic, this would mean the *in situ* $CO_2$ levels were likely high $CO_2$ levels, whereas the *ex situ* measurement occurred in a very low $CO_2$ atmosphere. Can the discrepancy of $CO_2$ levels between soil and laboratory be reversed to approximate *in situ* acidity of a soil profile using *ex situ* measurements? Can we also control soil moisture, which has been shown to influence the atmosphere of soil as microorganisms respire $CO_2$ (Keith, Jacobsen, and Raison 1997, 136), when measuring soil pH?

Soils span a wide range of texture, mineralogy, and organic matter, with subsequent diversity of physical chemistry and hydrostatics, all of which contribute to acidity as it is measured by soil pH. Figures presented by Jackson (1958) (Figure 2.1) and Mubarak and Olsen (1976) (Figure 2.2) illustrated the variety and extremes of the effects of dilution on values of soil pH of the same sample, whereby non-standard soil pH values yielded from pastes or slurries of higher or lower saturation than the $1:1$ water:soil ratio can be quite different. Overall, as soil moisture decreases from a slurry to a paste, clayey (argillaceous) soils generally rise in pH, while sandy (arenaceous) soils generally drop in pH as soil moisture decreases. Although very few replications of these studies have been conducted to certify there is indeed a stark divergence of acidity of clays and sands in a predictable pattern across all soils, two hypotheses exist for such a trend. First, the smallest clay particles may cause the soil solution to become decreasingly acidic as a result of alkaline buffering by the high surface area of the reactive silica of clays (Papirer 2000, 301), despite the fact that the percent organic matter is often much higher in clayey soils (Rasmussen et al. 2018). Conversely, sandy soils would be unbuffered and therefore their organic matter, despite often being lower in percentage of soil mass than clayey soils, would cause the soil solution to become more acidic when the soil moisture is lowered and therefore acids less diluted. Second, the same smallest clay particles may simply cause the soil solution to appear to decrease in acidity. The charge of these clay particles in the supernatant may cause an artificial potential across the liquid junction of the glass probe's salt bridge (Oman et al. 2007), whereas a sandy soil, lacking such interfering charged clay particles, would cause little error of this kind.

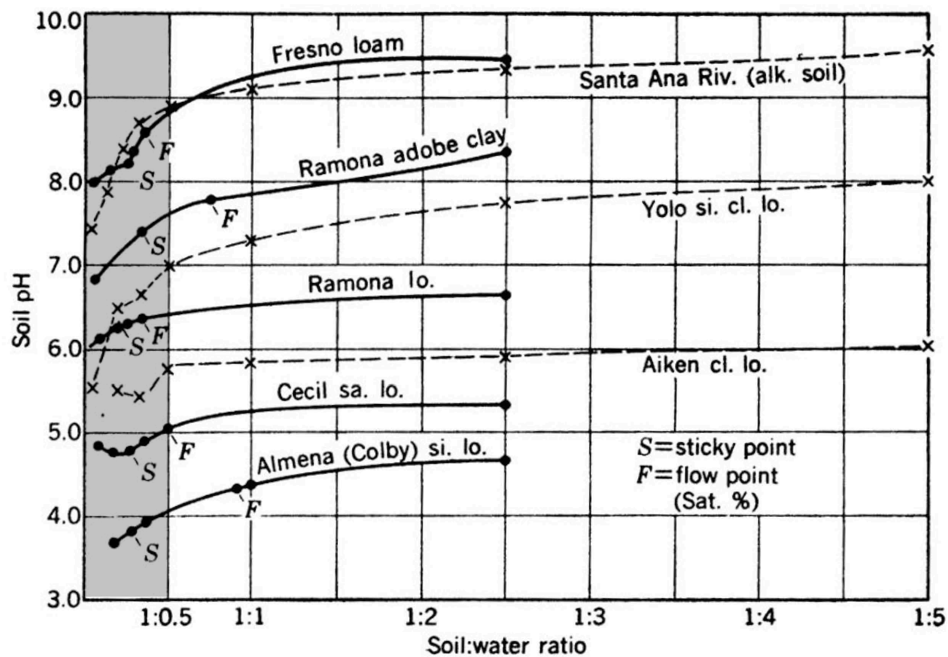



Figure 2.1: The slurry-to-paste dilution pH differentiation trend, adapted from Jackson (1958), page 43: solid lines from Chapman, Axley, and Curtis (1941) and dashed lines from Huberty and Haas (1940). The moisture levels considered typical and representative of *in situ* conditions better than standard soil pH (1 : 1 water:soil by mass) in this study are greyed. (I have received permission to use this figure with minor modification by the publisher and copyright owner.)

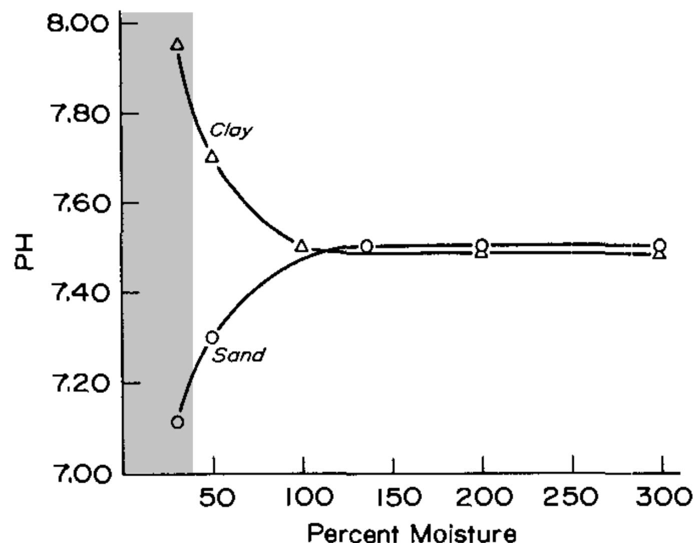



Figure 2.2: The slurry-to-paste dilution pH differentiation trend, adapted from Mubarak and Olsen (1976), page 882: "Clay" signifies a clay soil and "sand" signifies a fine sandy loam. The moisture levels considered typical and representative of *in situ* conditions better than standard soil pH (1 : 1 water:soil by mass) in this study are greyed. (I have received permission to use this figure with minor modification by the publisher and copyright owner.)

A combination of these two effects and additional temporal effects and reactions in this complex mixture are also possible, which cannot be tracked with as much rigor or closeness in the field as is made possible in the laboratory.

In order to reconcile the differences between field conditions and laboratory measurements, two approaches present themselves: (1) perform direct measurements in the field while minimizing the marring of the original conditions of soil profiles and functionality of instruments, or (2) simulate the original conditions of intact soils during the analysis of soil samples that have been collected from the field and brought to the laboratory. Both of these approaches have complementary advantages and disadvantages, but both approaches are also a significant departure from traditional methods listed throughout such tomes as the Soil Sciencie Society of America's publication *Methods of Soil Analysis* (Jacob, Clarke, and Dick 2002). The existing evidence of the divergence of soil pH values with the decrease of soil moisture and increase of carbon dioxide (detailed below) suggests that it would be valuable to develop the in-lab approach. While *in situ* measurements offer prospective data of the current chemical conditions of soils, *ex situ* chemical analysis in simulated microenvironments enable experimental data to predict the reaction of soil solution to changes to soil conditions such as those predicted by climate change models (Shukla et al. 2019; Handelsman and Liautaud 2016). We could use such experiments to perform chemical analysis at elevated carbon dioxide levels to represent thawing permafrost (Song et al. 2020; Huissteden 2020, 85) and both extremes of moisture in samples from soil undergoing drought (Kopittke, Tietema, and Verstraten 2012) or flooding (Unger, Kennedy, and Muzika 2009).

As elaborated above, the alteration of soil samples between the field and laboratory may yield misleading values of soil pH. Miniaturization and simulation of soil conditions during chemical analysis of such samples may provide sufficient reversal or correction of such disturbances. The tools and equipment common to a molecular microbiology laboratory offer investigators the ability to miniaturize the soil-water suspension. The combination of miniaturization to sample more extensively, simulation in the laboratory to measure *in situ* soil pH, and modeling of temporal variability would provide an ideal characterization of soil for use by both agronomists and microbial ecologists. Whereas the standard soil pH protocol necessitates a single standardized ratio of soil to solution, usually $1:1$ or between $1:1$ and $1:5$ (Miller and Kissel 2010), control of this

ratio combined with extraction via centrifugation offers investigators more freedom to approach the typical moisture conditions of their study systems. Below I present soil pH measurements using non-standard methodology, specifically the removal of charged particles from the analyte, miniaturization of analyte to allow for finer-scale measurements, and simulation of soil conditions during pH measurement.

## 2.2 Methods

In order to explore pH measurements across a wide range of soil properties, I assembled two sets of study sites: the first from a 25-year soil pH manipulation trial at UW-Madison's Spooner Research Station, and the second drawn from soils collected from across the state of Wisconsin. For each of these datasets, I applied a miniaturized, centrifuge-based soil solution extraction, manipulating water:soil ratios and atmospheric $CO_2$ levels during measurement, and using a microprobe to measure pH. The methods of this study, described in greater detail below, measured soil pH values using the following factors to generate a multifactorial analysis:

$$\{\text{Research Station}\} * \{\text{Site (i.e. Pit)}\} * \{\text{Soil Horizon}\} * \{\text{Water:Soil Ratio}\} * \{\text{CO}_2\ \text{Level}\}$$

### 2.2.1 Sample Collection and Storage

Sampling of soils was completed at the Spooner Research Station (Spooner soils), with sample ID codes for each sample formatted as

Sample Number—Site Letter with Pit Number—Core Number—Upper Depth (cm)—Lower Depth (cm)

and soils from across Wisconsin (Wisconsin soils) were given sample ID codes formatted for each sample as

Sample Number – Site Letter with Pit Number – Upper Depth (cm) – Lower Depth (cm)

For example, a sample of ID `034-H2-0-30` signifies "sample 34 is from Hancock Agricultural Research Station's pit number 2 between depths $0$ [cm] and $30$ [cm]." Where noted, "Topsoil" signifies any combination of A horizons, and "Subsoil" signifies all horizons beneath the A horizon (E, B, etc.).

The first set (Spooner soils) was collected on November 3rd, 2017, from an ongoing soil pH multifactorial experiment at the Spooner Agricultural Research Station begun in 1994. Four

replicates of standard field plots have been maintained at target soil pH values of $4.7$, $5.2$, $5.7$, $6.2$, and $6.7$, but the measured soil pH values varied slighly from the target soil pH values. The standard soil pH values were measured and reported independently in this study's multifactorial ($1 : 1$ water-to-soil ratios at laboratory carbon dioxide levels). Three $1$-inch diameter cores to $20$ [cm] depth were randomly sampled by random number generator using the lengh of the long retangular plots, avoiding the plot edges by $5$ [ft]. All soil samples were placed in sterile bags and transported within 24 hours of collection to the Department of Soil Science at the University of Wisconsin-Madison and placed in a refrigerator ($4$ [$^{\circ}$C]). Within two days of arrival, each bag of soil was homogenized, subsampled, and stored at $-80^{\circ}$C.

The second set (Wisconsin soils) were selected using the legacy chemical and physical data, which were retrieved on August 7th, 2018, from Web Soil Survey. A depth of $0$ [cm] to $50$ [cm] was selected for the following parameters: calcium carbonate, cation exchange capacity at pH 7 (CEC-7), electrical conductivity (EC), gypsum, soil pH, sodium adsorption ratio, available water capacity and supply, bulk density at $\frac{1}{3}$ bar, liquid limit, percent organic matter, percent clay, percent sand, percent silt, and saturated hydraulic conductivity ($K_{sat}$), parent material, and representative slope. These features were used to select the widest variety possible across Wisconsin, namely a wide breadth of texture, organic matter content, and soil pH values. The following sites were selected from the many University of Wisconsin Argicultural Research Stations (ARS) to yield the Wisconsin soils, with ID letter and soil pH values according to WSS listed in parentheses: Kemp (K, $5.40$), Rhinelander (R, $5.50$), Marshfield (M, $5.65$), Spooner (S or Sp, $5.80$), Hancock (H, $6.20$), Arlington (A, $6.50$), Lancaster (L, $6.60$), West-Madison (W, $6.70$), and Peninsular (P, $7.20$). Supplemental Figure 8 shows a map of the locations of these sites along the north-south standard soil pH gradient of Wisconsin, and Supplemental Figure 2.9 lists the latitude and longitude of each site.

The Wisconsin soils were collected from each of the two or three most common soil series of each agricultural research station listed above, between August and September, 2018, where a pit was dug to $> 50$ [cm] depth and several kilograms of soil were gathered from each horizon evenly spanning the upper to the lower boundary. These boundaries were easily visible.

#### 2.2.2 Agronomic Soil Chemical Analyses

All samples of the Wisconsin set were homogenized, subsampled, and submitted to the University of Wisconsin Soil and Forage Laboratory where the sampels were dried and seived to conduct the following analyses: Routine Tests (pH using 1:1 water, P using Bray No 1 extraction test, K also using Bray No 1 extraction test, and OM using loss on ignition), Cation Exchange Capacity (summation, including calcium and magnesium extracted using ammonium acetate), and total nitrogen and organic carbon (dry combustion). See "North Central Regional Research Publication No. 221 (Revised 2015) Recommended Chemical Soil Test Procedures for the North Central Region" for specific protocols. The Spooner Agricultural Research Station performed chemical analysis of the long-term experimental soil pH plots in 2017, which determined the organic matter at that time was $2.15\%$ $(\pm 0.24)$, phosphorus level was $33$ $(\pm 6)$ [ppm], and potassium level was $93$ $(\pm 25)$ [ppm] (personal correspondence with Superintendent Phil Holman).

#### 2.2.3 Soil Solution Extraction

Soil solution was extracted using the following protocol, resembling earlier recommendations (Gillman and GP 1976; Wolt 1994, 95–120), to gather supernatants of extracts:

1. Label and weigh empty tubes and record the tubes' masses.
2. Add $1.0$ mL to $1.3$ mL packed undried soil, and record the mass of soil added.
3. Use the soil mass to calculate the volume of $0.01$ [M] KCl solution (specific mass approximately equal to water, or $1.0$g mL$^{-1}$) to the target water:soil ratio (1:1, 1:2, 1:3, or 1:4).
4. Vortex tubes and let rest $40$ minutes to $1$ hour.
5. Centrifuge for at least $60$ seconds at $8000$[rpm] ($4651$[$\times$g] if $g = (1.118 * 10^{-5})RS^2$, where $R \equiv$ radius of the rotor's center to tube and $S \equiv$ rotations per minute).
6. Pipet from each tube $100$[$\mu$L] of supernatant into a $0.5$ mL tube.
7. Freeze all aliquots for later thawing and measurements. The probe, owing to the sorption of solution to its surface, will remove approximately $5$[$\mu$L] per measurement, and the error during this study was $< \%15$ while performing $\leq 3$ repeated measurements of the same extracts in different simulated conditions.
8. Dry the original soil remaining in the $1.5$ mL tubes, then record the dry mass value. These

values enable the calculation of original gravimetric water content, which may slightly differ from the target water:soil ratio because undried soils have variable soil water content.

I used microcentrifuges with rotors capable of spinning $1.5$ mL tubes at $> 10,000$ rpm and glass microprobes capable of measuring at least $100[\mu$L$]$ of analyte. I was consistently able to yield at least $100[\mu$L$]$ from $1.5$ mL of soil. Minimum volumes of added solution for sufficient yield were tested by extracting via centrifugation fixed volumes of solution added to an especially dry soil, the Ap horizon of Plano silt loam that was dried and stored for $1$ year. The addition of a weak electrolyte ($0.01$ [M] KCl or $CaCl_2$) minimizes the liquid junction potential of glass probe pH acidimetry (Duncan A. MacInnes 1915; Bates 1973, 31–58; Kadis and Leito 2010; Libohova et al. 2014), and throughout this protocol I added aliquots of $0.01$ [M] KCl. This solution produces highly dilute spectator ions without acid-base reactivity that cannot increase ionic strength past the threshold beyond which pH is applicable while minimizing liquid junction potentials. The pH for each solution was measured under ambient ($0.04\%$ or $400$ [ppm]) and high ($2\%$) carbon dioxide (chamber conditions described in detail below) with a pH microprobe (probe details also described in detail below).

#### 2.2.4 Simulation of Soil Atmospheric Carbon Dioxide

Using a vinyl anaerobic airlock chamber (Coy Laboratory Products, Inc., Grass Lake, Michigan), I adjusted the soil atmosphere simulation chamber to $2.0 \pm 0.2\%$ for a "high carbon dioxide" level. The "glove box" is an ideal chamber to elevate partial pressures (see Figure 2.3 and Supplemental Figure 2.10) to resemble soil macropores, in accordance with Henry's law, $K_H = a_i/P_i$, where $i$ is the index of each gaseous species, $K_H$ signfies Henry's constant that is approximately $3.4 * 10^{-4}$ $[\frac{\text{mol}}{\text{m}^3\text{Pa}}]$ at $T^{\ominus}$ for carbon dioxide in water (Sander 2015, 4488), $a_i$ (unitless) signifies the thermodynamic aqueous activity benchmarked to the standard state, and $P_i$ [Pa] signifies the partial pressure. Using the equilibrium constants of the sequential reactions between $CO_2$(g) as $H_2CO_2$(aq) in water, (Strawn, Bohn, and O'Connor 2020, 90–97) describes the pH of a solution in equilibrium with calcite as a function of partial pressure of carbon dioxide, with the combination of acid dissocation constants of the various species of carbonate:

$$\text{pH} = 5.93 - \frac{2}{3}\log P_{\text{CO}_2}. \tag{2.2}$$

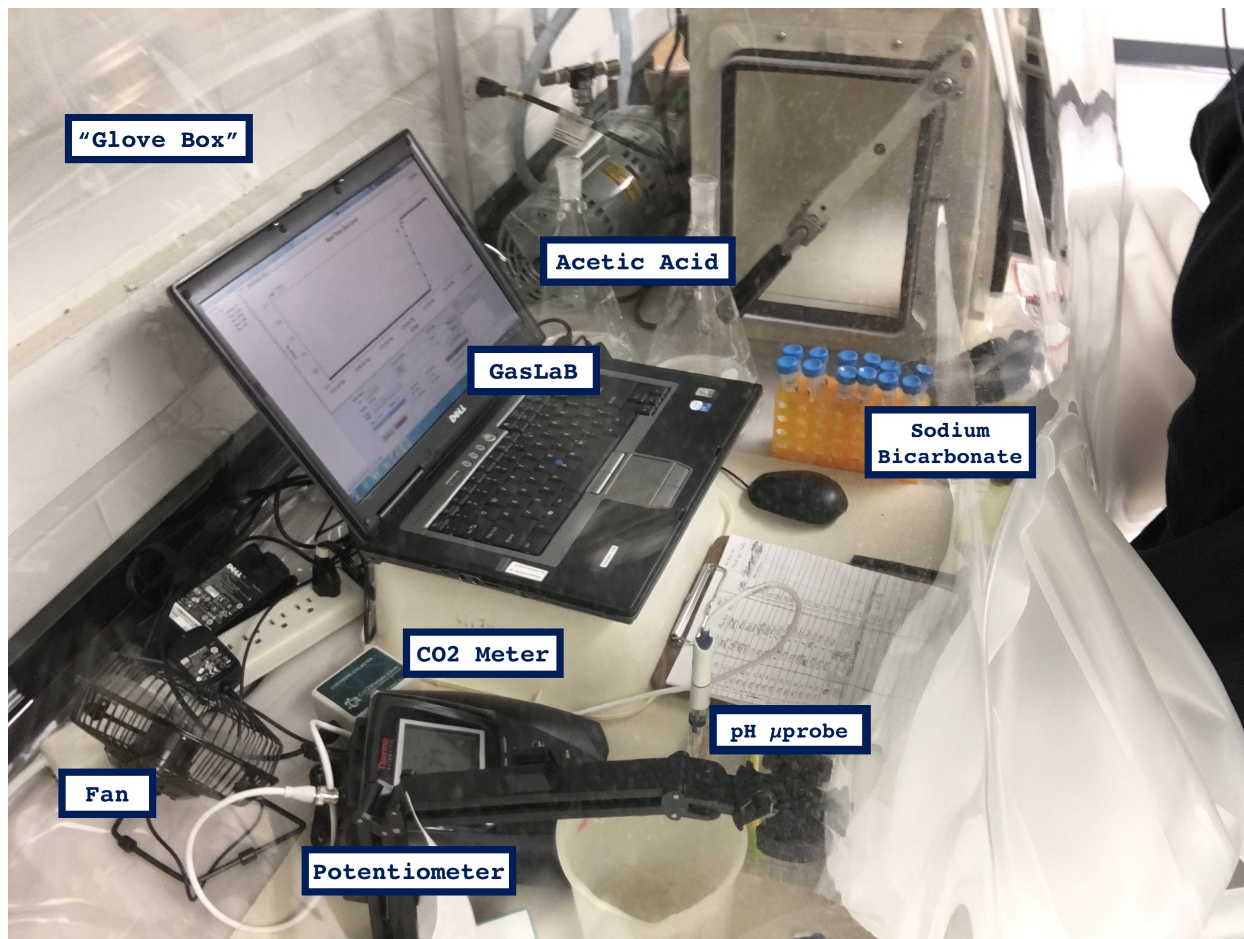



Figure 2.3: "Simulated soil pH" experimental rig, equipment, and reagents for acidimetry under elevated carbon dioxide resembling a typical *in situ* soil atmosphere.

This model predicts that any increase in carbon dioxide will decrease pH, but I will reiterate the authors' caveat: "Several simplifying assumptions were required to solve the carbonate system equations that may not be possible or appropriate in other aqueous equilibrium problems. Additionally, the assumption that activity and concentrations are equal (ideal solution) is fine for showing trends, but activity corrections can cause significant changes in the predicted pH or concentrations of the species." According to the Bjerrum curves that relate the concentrations of carbonic acid to mono- and di-proticic carbonate (Andersen 2002), elevated carbon dioxide partial pressures should also increase acidity.

The addition of $100$ [g] of sodium bicarbonate (baking soda) to excess $5\%$ acetic acid (vinegar) elevated the carbon dioxide inside the approximately $1.0$ m$^3$ chamber from $0.04\%$ to $3.0\%$, and the addition of more sodium bicarbonate was adjusted in the chamber up to $5.0\%$. However, beyond this quantity, the chamber began to pressurize beyond atmopsheric pressure ($1.013$ bar), which artificially carbonated solutions inside, so I maintained carbon dioxide levels below this threshold with a combination of bicarbonate reaction and venting. A USB CO2 Probe Data Logger (CO2Meter.com, K-30 Probe, CM-0040) with a measurement range of $0\%$ ($0$ [ppm]) to $30\%$ ($300,000$ [ppm]), with a maximum error of $3\%$ of the quantity measured, was used to measure the carbon dioxide inside the soil atmosphere simulation chamber. This gas probe was calibrated with a single point to the outdoor atmosphere (approximately $400$ [ppm]), then installed inside the chamber with a small fan that gently circulated the internal atmosphere during operation. Readings were logged using the GasLab software (v. 2.2.1.36).

#### 2.2.5 Measurement of pH with a Microprobe

Westcott (1978, 1–16) and Yu (1992, 207–11) have provided detailed overviews of the inference of stoichiometric concentration and thermodynamic activity of species of hydrogen ions from potentiometric measurements. The "suspension effect" has also long been observed (Jenny et al. 1950; Gorham and others 1960; Ponnamperuma, Martinez, and Loy 1966; Oman et al. 2007), which is the apparent decrease in pH when a pH probe is moved between the supernatant and sediment of a settled suspension. However, competing evidences for the effect being error or other explanations have left the problem somewhat unresolved (Feldman 1956; Fornasier, Fornasier, and Di Marco 2018). This study has avoided the suspension effect by first removing or minimizing

density of soil particles from solution extracts via centrifugation, and although the standard soil pH values were measured in a slurry, their values are of the supernatant and not the sediment. Centrifugation was also utilized to miniaturize the creation of soil slurries at varying soil water content (also calculated interchangeably as "water-to-soil ratio") in $1.5$ mL tubes.

pH was measured using an InLab Micro pH glass microelectrode (Mettler-Toledo, Material No. 51343160) that was optimized for application to microplates, small test tubes, and micro-scale samples. The electrode's shaft length was $60$[mm] and diameter of $3$[mm] with a built-in ARGENTHAL reference system of $3$[mol/L] KCl reference electrolyte. The probe was stored in either $3.0$ [M] KCl saturated electrolyte solution or InLab storage solution (Material No. 30111142). The probes's glass was made from U Glass with a membrane resistance of $< 600$[Mohm] and was connected to an Orion Star A215 pH/Conductivity Benchtop Multiparameter Meter. The pH probe connected to the potentiometer was calibrated using NIST-traceable standards for pH $4$, $7$, and $10$, then recalibrated when probe drift is detected with a retest of a standard solution every two hours. Mettler-Toledo reported the error of the probe to be $\pm 0.25$ pH units ($0.78$ stoichiometric), but our measurement error rarely exceeded $\pm 0.15$ pH units.

To monitor the quality of measurements throughout the analysis at elevated carbon dioxide, the pH of identical volumes of several controls were taken alongside the soil extract, including $0.01$[M] CaCl, $5\%$ $(0.833[\text{M}])$ acetic acid, and deionized water. These values deviated $< \pm 0.12$ pH units during each incubation across the entire experiment.

#### 2.2.6 Statistical Analysis

To determine which soil properties were the most influential predictors of different measurements of soil pH, linear models correlating soil chemical measurements and all values of pH were analyzed using Bayesian information criterion (BIC), or

$$k \log(n) - 2 \log(L(\hat{\theta}), \tag{2.3}$$

where $n$ is the sample size or number of observations, $k$ is the number of parameters the model estimates (degrees of freedom), $\theta$ is the set of all parameters, and $L(\hat{\theta})$ is the maximum likelihood of the model tested using the input data (Kass and Wasserman 1995). These calculations were performed using the `regsubsets` function from the R package `leaps` (Lumley and Miller

2020). Interpretation of these plots involves assessing which factors, when added to the model as represented by darkened rectangles that together create a heatmap, produced the most negative BIC, where more negative BIC values indicate better models. The group of most negative BIC values are the "BIC dropoff" region of the plot, which offers an assortment of models most likely to be predictive of the input factor. I have calculated BIC for models of the factors composing the soil nutrient profiles predicting each of the the four soil pH values generated in the extremes of this study's multifactorial: carbon dioxide ($400$ [ppm] and $2\%$) and soil water content (1-to-1 and 1-to-4 water-to-soil ratio by mass).

## 2.3 Results

### 2.3.1 Non-Standard Soil pH Values at Four Levels of Soil Moisture

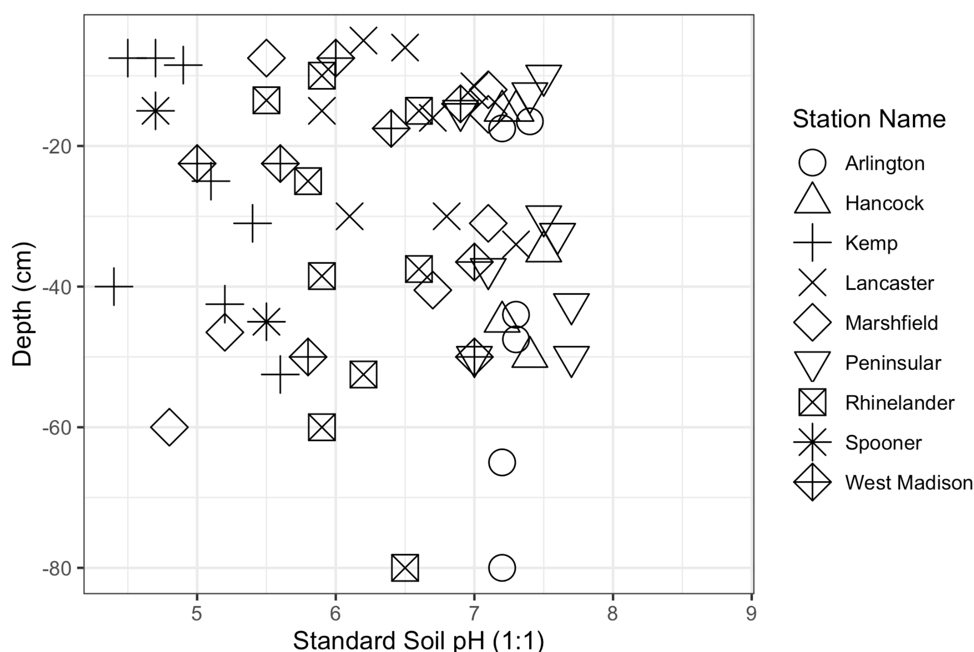



Figure 2.4: Soil pH values of all samples as a function of depth measured at Wisconsin agricultural field stations. The combination of pedogenesis and glaciation of the state of Wisconsin has caused this region to exhibit a gradient of soil acidity, whereby the soils of the south are more basic and the soils of the north are more acidic. See also Supplemental Figure 2.8 depicting the relative locations in Wisconsin of these stations.

Soil samples from multiple depths and from across Wisconsin's natural north-south soil pH gradient yielded a wide range ($4.4$ to $7.8$) of standard soil pH values (Figure 2.4), providing us a sufficient range and variety of samples to test the measurement and interpretation of non-standard soil pH methods using simulated water content and carbon dioxide levels. The replication of re-

search reviewed by Jackson (1958, 43) similarly showed that the values of soil pH of topsoils (triangles in Figure 2.5) were more responsive to moisture level than the values of soil pH of subsoils (circles in Figure 2.5). Soil pH both increased and decreased in stages within and between soil horizons unpredictably, where both the variance of magnitude and variability of sign ("zig-zagging") increased while lowering the soil moisture before extraction of soil solution. It remains unclear whether clayey soils tended to be more alkaline at the lowest moisture level while sandy soils tended to be more acidic at the lowest moisture level. Among the Spooner soils, the more alkaline soil pH values $> 6.5$ increased approximately $0.2$ and up to $1.0$ when soil moisture was lowered from the soil slurry of a $1:1$ water:soil ratio by mass, whereas the soils of soil pH $< 6.5$ changed little but decreased ("dipped") at a $3:1$ soil:water ratio, and nearly all soils increased in soil pH by appoximately $0.15$ when reaching $20\%$ soil moisture. Comparisons of soil pH measured at $1:1$ water:soil ratio to all other water:soil ratios at ambient and elevated ($2\%$) carbon dioxide levels can be viewed in Supplemental Figures 2.11-14. These relationships raise many questions as to which soil acidity metric is most useful to agronomists, land managers, and soil microbiologists, respectively.

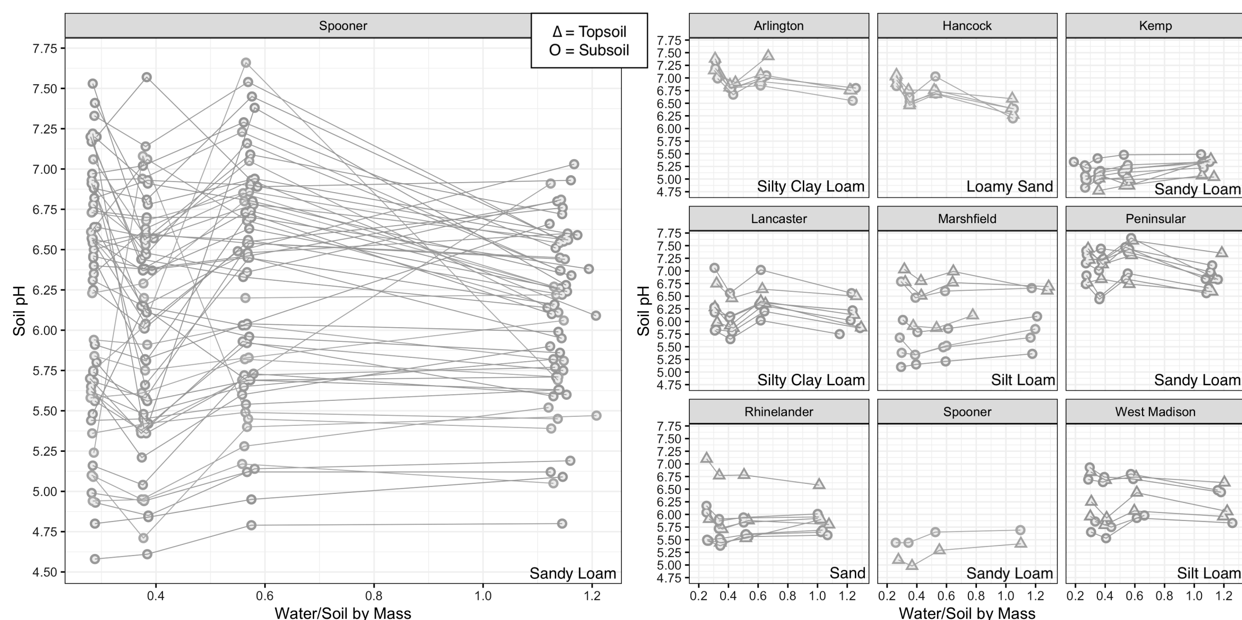



Figure 2.5: Soil pH values of the set of soils from across Wisconsin's natural soil pH gradient (rightward plots) and the set of soils from an experimental soil pH gradient in Spooner (leftward plot), WI, as a function of water-to-soil ratio, replicating the research reviewed by Jackson (1958), page 43, presented in the introduction. Each point represents a single pH measurement.

### 2.3.2 Non-Standard Soil pH Values at Two Levels of Carbon Dioxide

Soil pH values measured at different water-to-soil ratios and different levels of carbon dioxide were also different, having magnitudes and signs dependent on the interaction of moisture and the dissolved gases of the soil atmosphere simulation chamber. Exponentiation of the soil pH values signifies activity of hydrogen ions ($a_{\mathrm{H}^+}$), which adopts the units of moles per liter to represent effective concentration when the activity coefficient of hydrogen ions (i.e. hydronium and related cationic species of solvated protons) is $1.0$, and the exponentiated value enabled a better fit of linear regression for all treatments.

If all conditions were to follow Henry's law and Bjerrum's plot, one would have expected all solutions to have been acidified under elevated carbon dioxide, but this was not the case—in fact, this trend was inverted among the soil solutions extracted from the lowest-moisture soil slurries (Figure 2.6). The addition of carbon dioxide caused the most concentrated soil solution extracts to increase by up to half in acidity in stoichiometric units, the change in number density of acidic species per volume. Only solution extracts prepared according to the standard ($1:1$) ratio exhibited the trend of acidification at elevated carbon dioxide.

### 2.3.3 Relating Soil Properties to Non-Standard Soil pH Values

The factors significantly correlated with standard and simulated soil pH values fall into the broad categories of textural (sand, silt, and clay content), chemical (SOM, C, N, P, K), and exchangeable (CEC and exchangeable acidity), each of which have a unique influence on soil chemistry (Volk and Jackson 1963; Parfitt, Percival, and Van Der Lee 1995). The most consistently influential correlates in the soil nutrient profiles for soil pH values were the exchangeable factors and the Bray-extracted phosphorus (Figure 2.7). The decrease of water content from a water:soil ratio of 1:1 to 1:4 generally caused the influence of textural factors to decrease and chemical factors to increase. Calcium was too low in all soil samples to be influential in any model. However, the calcium content, which is a spectator ion in acid-base chemical equilibria, that was reported in these data may not signify calcium carbonate, which is a buffer, and residual calcium carbonate may have remained reactive in soil samples. The BIC plots between carbon dioxide level exhibited little difference.

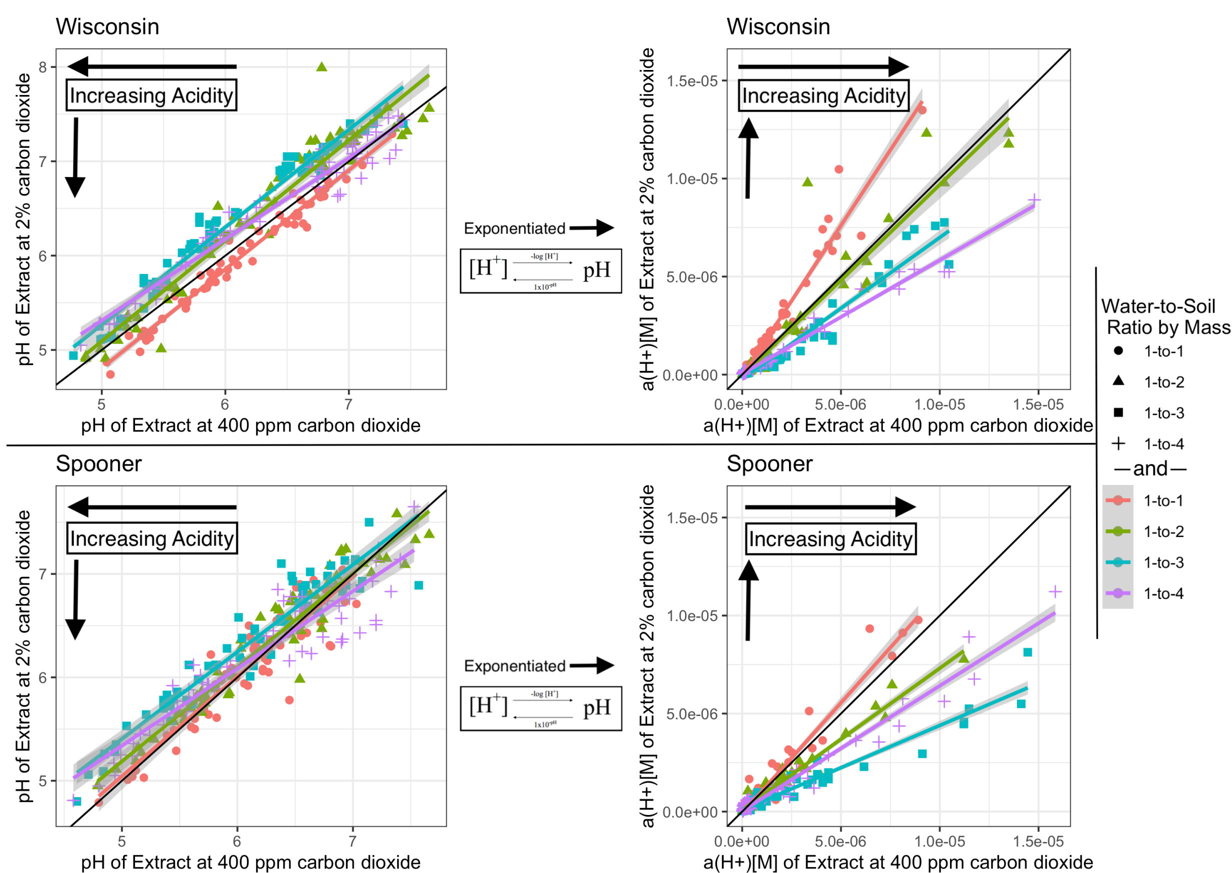



Figure 2.6: Soil pH and a($H^+$) values, which are interchangeable according to the definition of pH scale, measured at ambient or low (400 [ppm]) and high (2%) carbon dioxide levels and soil water content at four levels from the natural Wisconsin soil acidity gradient and experimental Spooner soil acidity gradients. Grey regions surrounding linear regression lines are standard error, and the solid black line signifies $y = x$. Points have been given redundant labels by color and shape, both signifying water:soil ratio, to aid the disentanglement of complex interactions.

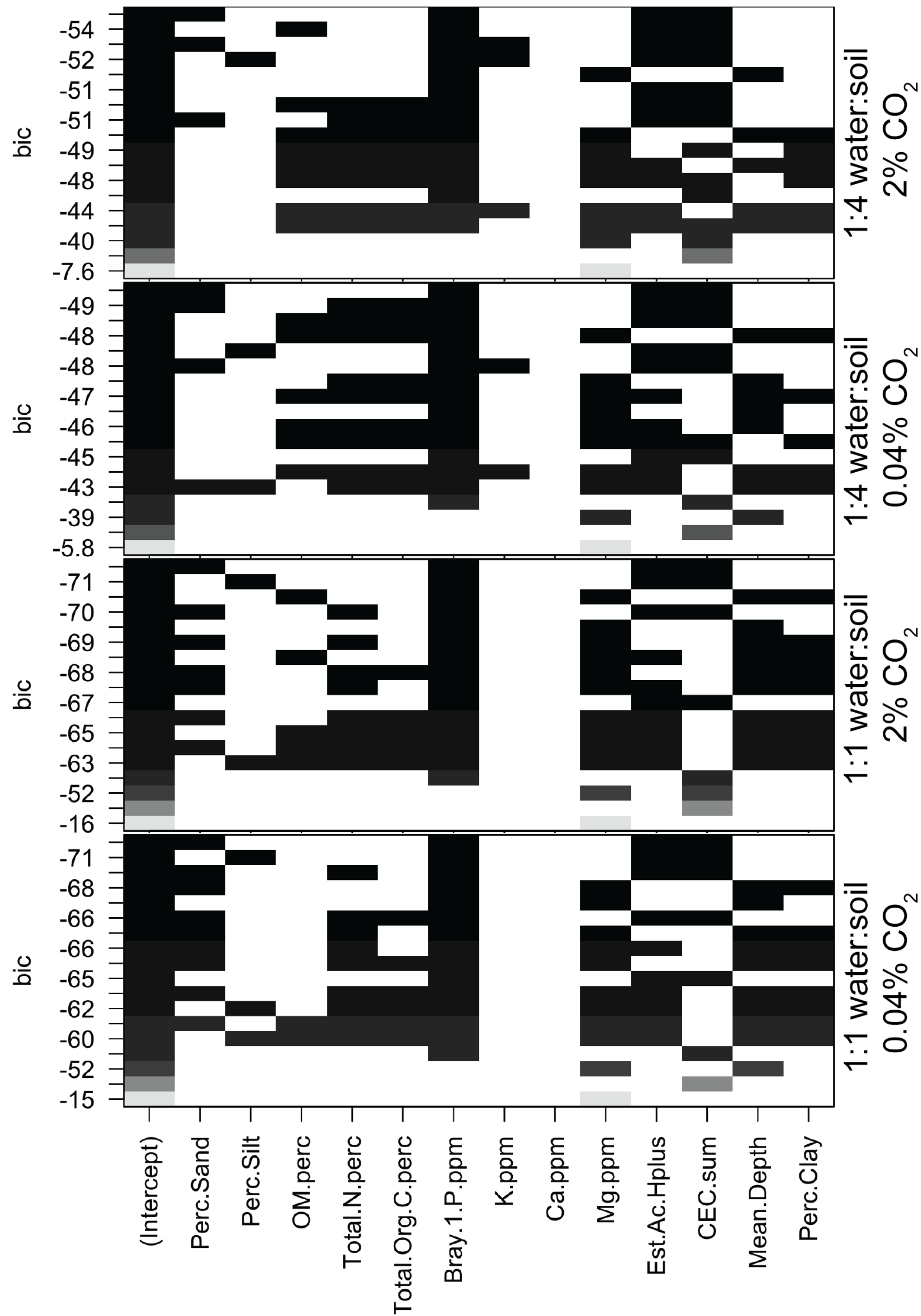



Figure 2.7: Bayesian information criterion (BIC) plot for soil properties as possible correlates of soil pH as determined by a ratio of water-to-soil of 1-to-1 compared to 1-to-4 and approximately 400 [ppm] compared to 2% carbon dioxide. Vertical axes are discrete and not continuous, where each value represents the ranked BIC value of the model using the input factors indicated by blocks.

## 2.4 Discussion

### 2.4.1 Measurements under simulated in situ conditions differed from standard pH measurements

I have measured soil pH values while simulating both typical soil moisture levels and carbon dioxide levels common to soils collected from across the natural acidity gradient of Wisconsin and across a long-term experimental soil acidity gradient in Spooner, Wisconsin. The combined methods of extraction via centrifugation and miniaturization of analyte proved both possible and essential to more accurately characterize the *in situ* acidity of soil microhabitats. Standard soil pH values were flawed predictors of simulated soil pH values, a confounding characteristic of soil biogeochemistry first predicted by Bjerrum and Gjaldbæk (1919), reviewed by Jackson (1958, 43), proposed again by Kilian (1961) and Mubarak and Olsen (1976, 882), and then further confirmed in the present study. Here I discuss two "inversions" observed, both of which suggest that it is best to measure properties of intact microhabitats—not settled slurries in beakers—because it is in intact microhabitats where the majority of Earth's soil microorganisms carry out their unique strategies to live and successfully reproduce.

The first inversion is one of textural composition and chemical composition. Models, when ranked by their ability to predict soil pH from the agricultural nutrient profiles and *vice versa*, showed that standard soil pH values of dilute slurries were largely determined by the textural characteristics of the soils, whereas pH values of soil solutions extracts from suspensions of very low water-to-soil ratios were more greatly influenced by the chemical characteristics of the soils. It is possible that some clays ascended into the supernatant in the moments from their removal from the centrifuge to the pipetting of $100[\mu\text{L}]$ of analyte for pH measurement, and that clay in suspension caused sufficient effect either on the solution or directly on the microprobe to influence the pH value. Overall, the "zig-zagging" appearance of the curves representing the intermediate conditions from standard to *in situ* soil pH values, where soil moisture was lowered from a slurry ($1:1$ water:soil by mass) to a more typical soil moisture content ($1:4$ water:soil by mass), may be a result of a "competition" between the various acidic and basic buffers present in soil solutions. Multiprotic acids, multiprotic bases, and the liquid junction potential together may compete for dominance in their influence on the solutions' acidities, causing the "zig-zagging" of pH values observed as soil solution extracts grew increasingly concentrated. For example, the inital increase in carbonate

may have caused the pH to rise but later become overwhelmed as the concentration of the acids of the soil organic matter in solution was further increased, much in the same manner as a titration curve will reach an infelction point. I have observed that carbon dioxide may dissolve as carbonic acid but ultimately buffers the existing acidity in solutions that are already highly acidic, rather than further acidifying the solution as proposed elsewhere (Strawn, Bohn, and O'Connor 2020, 90–97).

### 2.4.2 Carbon dioxide effects on pH

The second inversion observed in this study occurred as carbon dioxide was reintroduced to soil solution extracts. Carbon dioxide, when added back into soil solution extracted from soils at moisture levels more typical of the field, appeared to solvate and react with an alkalifying effect instead of the acidifying effect as predicted by Henry's law (Figure 2.6). However, few (if any) aqueous electrolyte solution models are available to predict acid-base potentials or equilibria in the hydrofilms of soil particle surfaces. Degassing carbon dioxide has been shown to raise pH by $0.25$ to $0.40$ but unpredictably lower the pH of some soils by approximately $0.1$, as demonstrated by the work of Dahlgren, Percival, and Parfitt (1997), using a similar methodology to this study, concluding that "failure to recognize this artifact could seriously affect the interpretation of data resulting from collection and analysis of soil solutions extracted by centrifugation." Experimental work by Mubarak and Olsen (1976, 881) showed that, using standard $1:1$ soil slurries, "the loss of $CO_2$ from the soil samples caused the pH to increase from $0$-$0.3$ pH units. In other words, an error of as much as $+0.3$ pH units can occur simply by allowing loss of $CO_2$ from the sample by equilibration with the atmosphere." Kaupenjohann and David (1996) found that degassing carbon dioxide raised soil pH values by as much as $0.5$, but these experiments were conducted using contained bottles, which may not correspond to experiments using soils exposed to the large reservoir of carbon dioxide in the open atmosphere. The degassing of the intact soil atmosphere during sampling from the field and dilution of collected soil to create slurries in the laboratory very likely cause meaningful deviations from in situ conditions. Whereas standard soil pH values appear erratic, non-standard soil pH values may both more accurately and more precisely represent *in situ* soil acidity.

### 2.4.3 Soil moisture effects on pH

Although carbon dioxide played a large role in the determination of soil pH under simulated soil atmosphere, the water content of the analyte was much more influential than carbon dioxide when correlating soil pH with the nutrient profiles of Wisconsin's soils. I have reproduced here much of the conclusions of Chapman, Axley, and Curtis (1941, 200), namely that soils having a moisture content above approximately $30\%$ gravimetric soil water content, traditionally called the "sticky point", exhibit a more consistent soil pH value approaching neutral with further dilution, whereas in soils of lower moisture content (i.e. most soils), these pH values will diverge in a variable fashion. Highly diluted solutions resemble the highly dilute solutions to which aqueous models will apply well, but soils at typical soil moisture levels must be considered highly concentrated solutions, perhaps even colloidal gel-sol matrices. Therefore a soil slurry does not represent well the soils from which the slurry was created, because the soil intractably violates the dilute solution assumption. Without meeting this key assumption, I cannot accurately apply—without extreme caution—most aqueous models, such as Debye-Hückel theory, Sørensen's acidity function ("pH"), Michaelis-Mentin enzyme dynamics, and mean ionic activity (at unity). Generally, I recommend seeking alternative measurements for the circumstances where microbial or biogeochemical models require pH values. Only highly saturated soils, those that would not require the addition of solution to produce a supernatant for analysis, would enable commensurability of soil pH to *in situ* pH. Drained mineral soils, such as Earth's non-rice farmlands, and the sediments of brackish regions, such as the coasts of all oceans and saline seas, have an ionic strength surpassing that which permit standard applications of pH measurements altogether.

### 2.4.4 Limitations when measuring the pH of concentrated solutions

Several guides exist for the measurement of pH of concentrated solutions (e.g. Thermo Fisher Scientific Application Note 009, 2014) and invariably provide cautionary notes for the interpretation of the pH values of such solutions: "ion mobility decreases in the high ionic strength samples and the activity differs from the concentration... High ionic strength solutions change the liquid junction potential. This may lead to bias and considerable time may be required to establish a stable reading." ("Measuring pH of Concentrated Samples" 2014, 1) However, such guidance offers little by way of illustration of solving the underlying metrological problem of the highly

narrow thresholds of applicability of pH to such systems as soils. Solution extracts from soils of typical moisture constitute "highly concentrated solutions" owing to their greater density of ions, biomolecules, and organic matter, in addition to clays, the smallest of which are chemically and catalytically reactive. Pourbaix (1974, 14) stipulates, for the entirety of the Eh-pH diagrams listed in his seminal *Atlas of Electrochemical Equilibria in Aqueous Solutions*, that "only when a dissolved species is greatly predominant, in the case of dilute solutions at least, can one assume, as has been done in this *Atlas*, that the activities are virtually the same as the molarities." Pourbaix's strictly chemical approach to Eh-pH modeling was inspired by Becking, Kaplan, and Moore (1960, 245), who was more transparent about the errors and limitations of these models to dilute solutions: "In the account of the natural environment that follows, there is little attempt to interpret the readings. The literature on the subject is full of examples of too much interpretation with too little factual basis, so we have been content to record the actual variation possible in any one environment and relate it to the microflora." What may seem to be a damning admission is rather an acknowledgement that "pH" and "Eh" are semi-qantitative notions, and violation of its assumptions merits transparent recognition.

Investigators will need to break the mold and step outside standard methods to meet the specific needs of their soils and systems of interest, and this study proposes that all investigators are sufficiently equipped to make appropriate modifications to standardized protocols and reagents. We agree with Rengel (2003, 166) that the measurements of soil acidity used in models of soil acidification "can, in principle, operate with pH determined by any method. However, there must be consistency between all parts of the model, including pH buffer capacity calculations. Thus, it is a user decision." Such user-specific decisions carry the added responsiblity to adapt user-specific interpretations of soil pH values (or values of other metrics of acidity) across and between studies, not only for the benefit of agronomy and biogeochemistry but also, for example, protistology (Dupont et al. 2016; Oliverio et al. 2020), botany (Wherry 1927; Lapp and Wherry 1951; Gough et al. 2000; Isermann 2005), and amphibian zoology (Frisbie and Wyman 1992; Mushinsky 1975). There remain many unexplored avenues to improve global models incorporating soil pH (Slessarev et al. 2016), aided in large part by non-standard soil pH metrology that approximates *in situ* acidity of soil microhabitats. For example, most soils collected at field capacity would not require

the addition of excess analytical solution to extract soil solution via centrifugation (Wolt 1994, 104; Geibe et al. 2006), and the pH of these extracts would greatly complement or even replace standards soil pH values.

#### 2.4.5 Suggestions for future studies and additional implications of pH measurement methods

Further studies measuring the acidity of soil microhabitats are necessary, be it using an entirely new metric of acidity, an extension of the standard soil pH method, such as planar optodes (Blossfeld et al. 2010), or, as presented and utilized in this study, the miniaturization and simulation of key soil conditions in the laboratory to produce measurements of non-standard soil pH values. Simulation of soil atmosphere may improve our understanding of the acidity that soil bacteria experience in their microhabitats, paired with a finer-scale and more frequent series of data points to surmount failures to detect short-range spatiotemporal variation (Hartemink et al. 2017). Using a miniaturized and subsequently more high-throughput method of measuring the pH of many small soil extracts, periodic pH readings throughout a study now become more feasible.

A composite of non-standard soil pH values, made possible using miniaturization and extraction by centrifugation of soil extracts, could take the place of a single standard soil pH value when characterizing the dynamic acidity of each soil horizon or soil profile, a *proviso* originally proposed by Niels Bjerrum (1919, 4), as translated by J. K. Rundo at the Atmoic Energy Research Establishment (Harwell Laboratory, Oxfordshire, 1956):

> "One can naturally agree to carry out the determination of the degree of acidity of a sample of soil by treating the soil with water in a carefully defined manner and determining the hydrogen ion exponent in this aqueous solution. In this manner one would probably succeed in getting a well defined characteristic measure for the degree of acidity of a sample of compact soil, but this measure will not give an expression for all the hydrogen ion exponents which the moisture in the sample of soil in question will have in the course of the year with changing climatic and biological conditions, and therefore it does not provide us with an exhaustive specification of the state of acidity of the soil in question. In what follows when we speak of the reaction of a sample of

> compact soil we are thinking of the hydrogen ion exponent in an aqueous extract of soil obtained in a suitable manner . . . In most cases there will be no difficulty in practice in measuring the reaction, but we must not think that by determining the reaction we can characterise the acidic or basic properties of the earth in an exhaustive manner. As well as the reaction itself we need to know the strength with which the earth maintains its reaction on addition of acids or bases."

Our findings here mirror Bjerrum's cautionary words, questioning the meaning and utility of standard soil pH values when these values are (1) single points in time and space and (2) heavily reliant upon the dilute solution assumption. Pourbaix diagrams, reduction potential half-reactions, and the law of mass action, with particular regard to the application of acid dissociation constants to soil microhabitats, will therefore require more careful interpretation or elimination when applied to soils. First, the exponentiation of potentiometric acidity "pH" to stoichiometric acidity "$a_{H^+}$" clarifies the correlation of soil acidity to nutrient profiles and the unusual carbonate reaction whereby the acidifying effect of carbonate in dilute acidic solutions inverts to alkalify concentrated acidic soil solutions. The concept of $a_{H^+}$ is inadmissable in biogeochemical models as reported by "pH", because (1) $H^+$ is not a static ion but the lyonium or autioinization with the aqueous solvent and (2) the definition of the logarithmic function is violated when logarithmically transforming a unitful physical quantity (Molyneux 1991; Boggs 1958). The definition of "activity" as the observed concentration divided by "standard concentration" does not obviate or solve this problem, because the calculation does not "cancel units" to force the value to be dimensionless, but instead creates a percentage. Percentages may only be considered unitless if the percentage itself is ignored, but in no case are percentages dimensionless. Second, perhaps "soil pH" is not the acidity of a soil but is more accurately a soil's acidifying or akalifying effect on a target solution of known initial acidity and buffering capacity. This new (or perhaps old) interpretation of soil pH would be more apt, and the interpretation inverts the current attribution of soil pH as a property of soil itself, such that the physical quantity one would call "*in situ* soil pH" would be deprecated to the status of oxymoron.

The metrological and chemical tests and analyses above warrant a reassessment of the interpretation of soil pH within biogeochemistry, the microbiology of soils and sediments, the chemistry

of unsaturated soils, and even astiobiology (Kounaves et al. 2010; Stroble, McElhoney, and Kounaves 2013). Soil pH will always remain useful to agronomists and farmers as well as the improvement of soil DNA extraction protocols, but only through extensive customization does soil pH reliably represent the acidity experienced by soil particles or microorganisms. Soil acidity by any measurement will remain invaluable to agricultural needs, such as determining liming requirements, irrigation, and fertilization regimens, with many new opportunities for using soil acidity to investigate soil microbial communities, as I do in the second part (Chapter 3) of this work.

## 2.5 Supplemental Materials

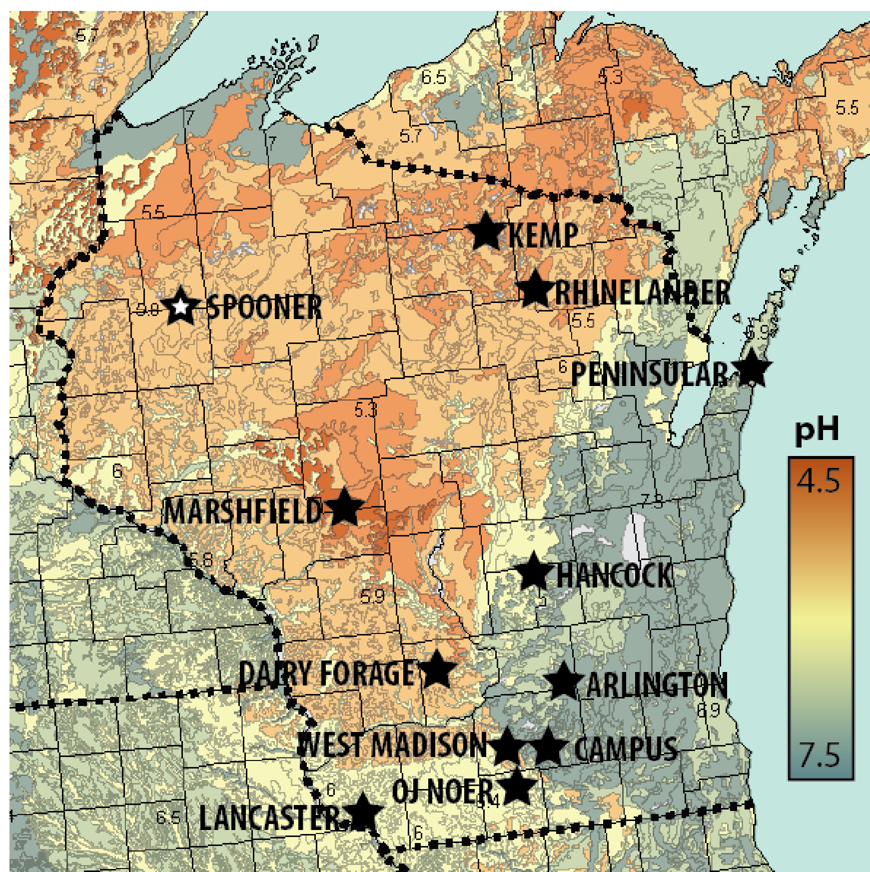



Figure 2.8: Map of field locations in Wisconsin.

| Pit.ID | Research.Station | Latitude | Longitude | Soil.Series | Soil.pH..WSS. |
|---|---|---|---|---|---|
| K1 | Kemp | 45.84073 | -89.67555 | Sayner loamy sand, 15 to 45 percent slopes | 5.4 |
| K3 | Kemp | 45.83834 | -89.67427 | Sayner loamy sand, 15 to 45 percent slopes | 5.4 |
| K4 | Kemp | 45.85040 | -89.65060 | Vilas loamy sand, 6 to 15 percent slopes | 5.5 |
| R1 | Rhinelander | 45.66480 | -89.26794 | Vilas loamy sand, 0 to 6 percent slopes | 5.5 |
| R2 | Rhinelander | 45.65433 | -89.26533 | Vilas loamy sand, 0 to 6 percent slopes | 5.5 |
| R3 | Rhinelander | 45.66651 | -89.21747 | Padus-Pence sandy loams, 0 to 6 percent slopes | 5.4 |
| M1 | Marshfield | 44.76046 | -90.09719 | Withee silt loam, 0 to 3 percent slopes | 5.6 |
| M2 | Marshfield | 44.76225 | -90.09930 | Loyal silt loam, 1 to 6 percent slopes | 5.7 |
| M3 | Marshfield | 44.76370 | -90.11234 | Marshfield silt loam, 0 to 2 percent slopes | 5.1 |
| Sp | Spooner | 45.82540 | -91.86877 | Mahtomedi-Cress complex, 2 to 6 percent slopes | 5.8 |
| H1 | Hancock | 44.12066 | -89.53984 | Sparta loamy sand, 0 to 2 percent slopes | 6.2 |
| H2 | Hancock | 44.11900 | -89.54606 | Plainfield sand, 0 to 2 percent slopes | 5.3 |
| A249 | Arlington | 43.30450 | -89.36342 | Channahon silt loam, 12 to 30 percent slopes, eroded | 7.3 |
| A341 | Arlington | 43.30205 | -89.35450 | Saybrook silt loam, 6 to 12 percent slopes, eroded | 6.5 |
| L2 | Lancaster | 42.83506 | -90.79082 | Fayette silt loam, uplands, 6 to 10 percent slopes, moderately eroded | 6.0 |
| L3 | Lancaster | 42.82901 | -90.79458 | Palsgrove silt loam, 6 to 12 percent slopes, moderately eroded | 6.4 |
| L4 | Lancaster | 42.84232 | -90.79415 | Dubuque soils, deep, 10 to 15 percent slopes, moderately eroded | 6.2 |
| W4 | West Madison | 43.05465 | -89.53524 | Griswold loam, 12 to 20 percent slopes, eroded | 6.9 |
| W5 | West Madison | 43.06537 | -89.54614 | Plano silt loam, gravelly substratum, 2 to 6 percent slopes | 6.6 |
| W7 | West Madison | 43.07023 | -89.54216 | Dresden silt loam, 6 to 12 percent slopes, eroded | 6.6 |
| P1 | Peninsular | 44.87988 | -87.33316 | Onaway-Ossineke fine sandy loams, moraine, 1 to 6 percent slopes | 6.6 |
| P2 | Peninsular | 44.88135 | -87.33140 | Longrie Loam, 2 to 6 percent slopes | 6.7 |
| P4 | Peninsular | 44.88060 | -87.32387 | Summerville loam, 0 to 2 percent slopes | 7.3 |

Figure 2.9: Table of latitude, longitude, soil series, and soil pH of field sites according to the Web Soil Survey database.

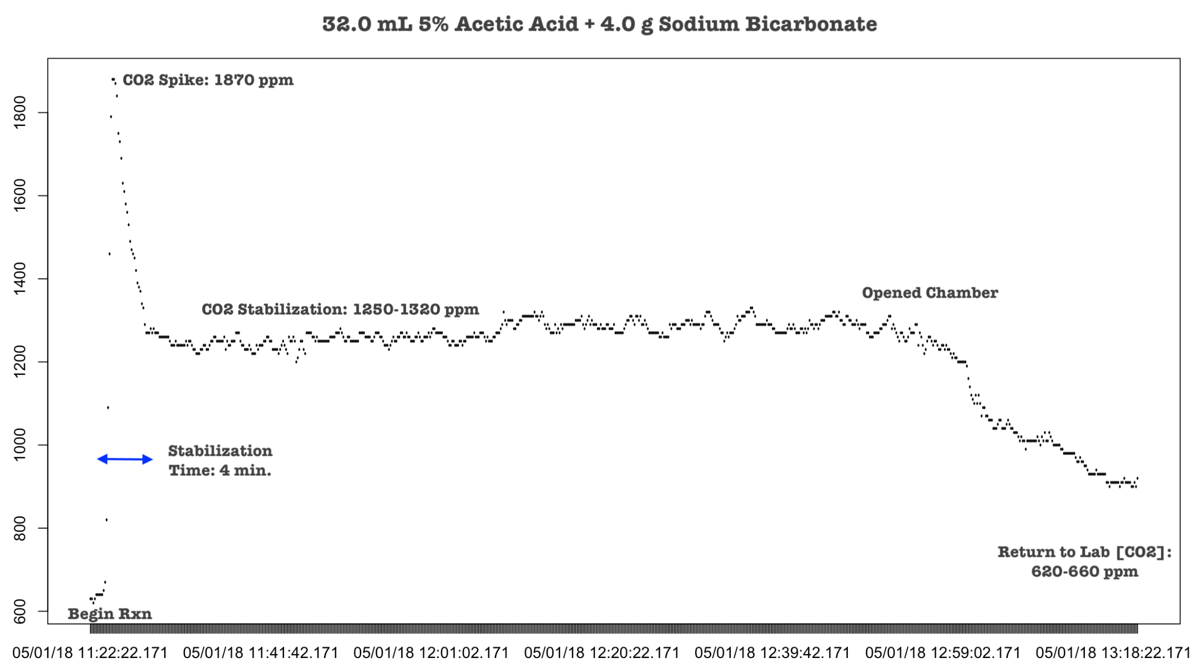



Figure 2.10: Spike, stabilization, and persistence periods of an elevated carbon dioxide chamber ("glove box") creating a partially simulated soil atmosphere, using an effervescent reaction of excess 5% acetic acid with sodium chloride. This test shows that this chamber maintains a stable elevated carbon dioxide atmosphere for $> 10$ hours, which enables the elevation of carbon dioxide up to 5% mol/mol ($50,000$ [ppm]) to simulate soil atmospheric conditions.

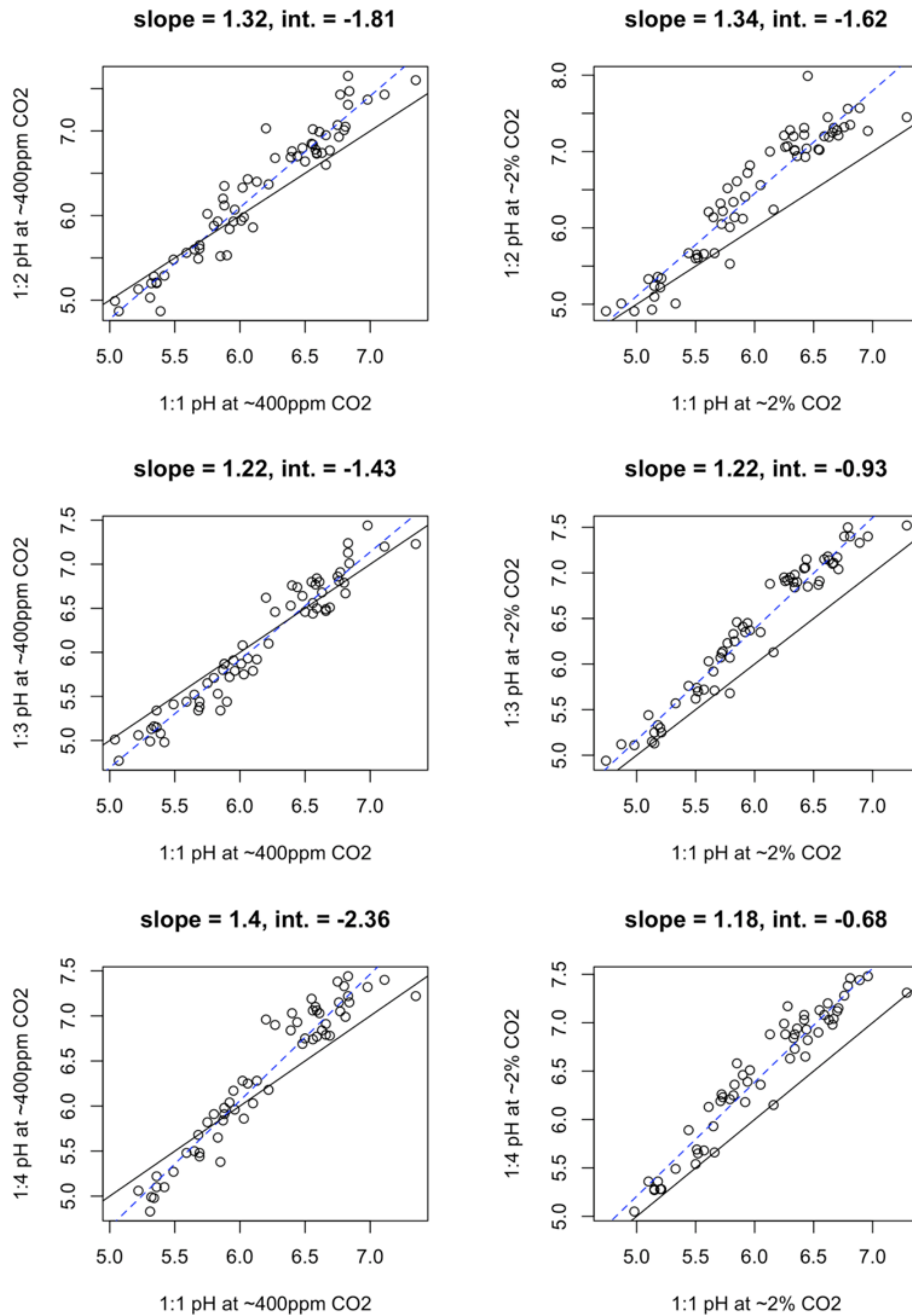



Figure 2.11: Standard soil pH ($1 : 1$ water:soil ratio) of Wisconsin soils compared to three other ratios ($2 : 1$, $3 : 1$, $4 : 1$) at two levels of carbon dioxide (400 [ppm] and 2%).

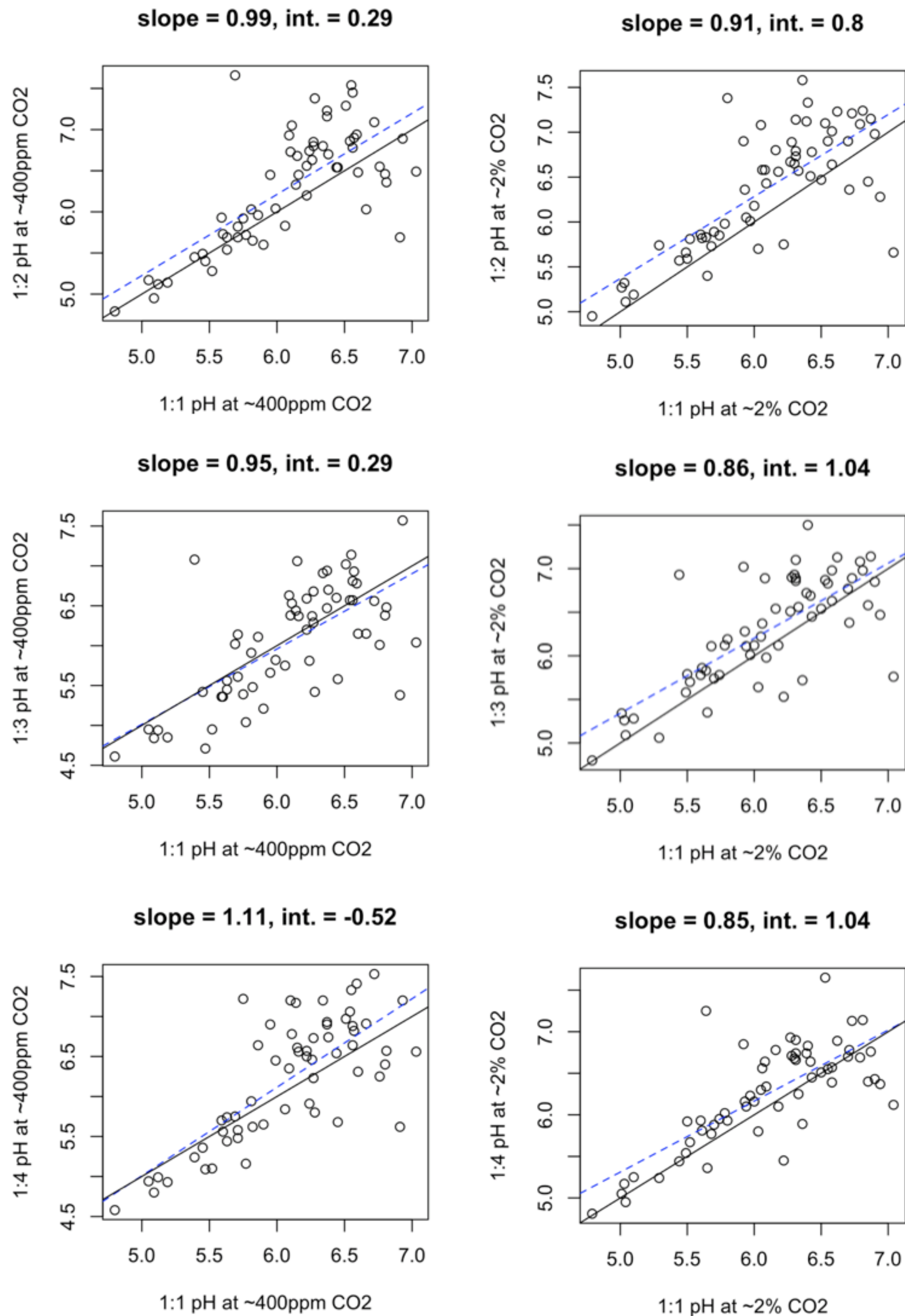



Figure 2.12: Standard soil pH (1 : 1 water:soil ratio) of Spooner soils compared to three other ratios (2 : 1, 3 : 1, 4 : 1) at two levels of carbon dioxide (400 [ppm] and 2%).

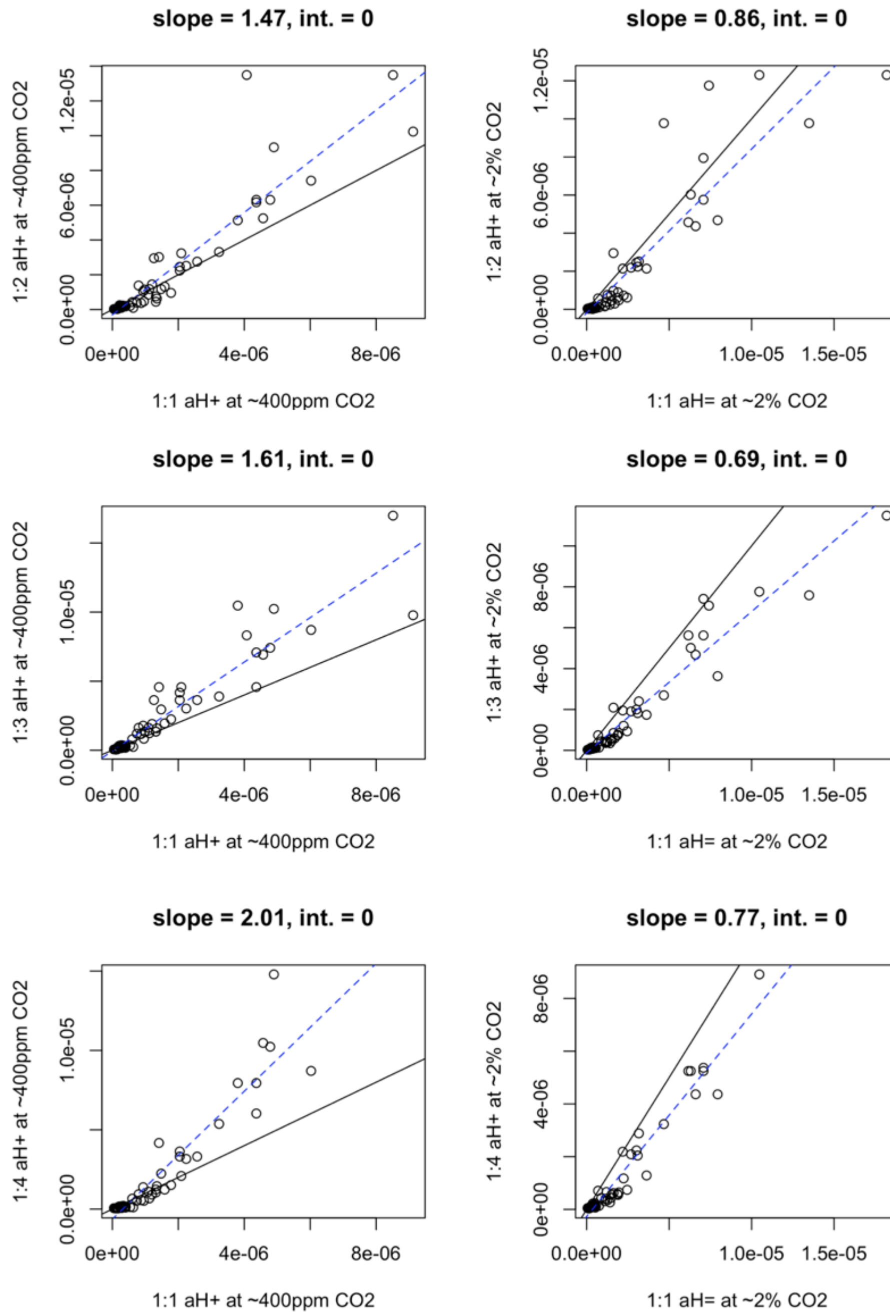



Figure 2.13: Soil $a_{H^+}$ (1 : 1 water:soil ratio) of Wisconsin soils compared to three other ratios (2 : 1, 3 : 1, 4 : 1) at two levels of carbon dioxide (400 [ppm] and 2%).

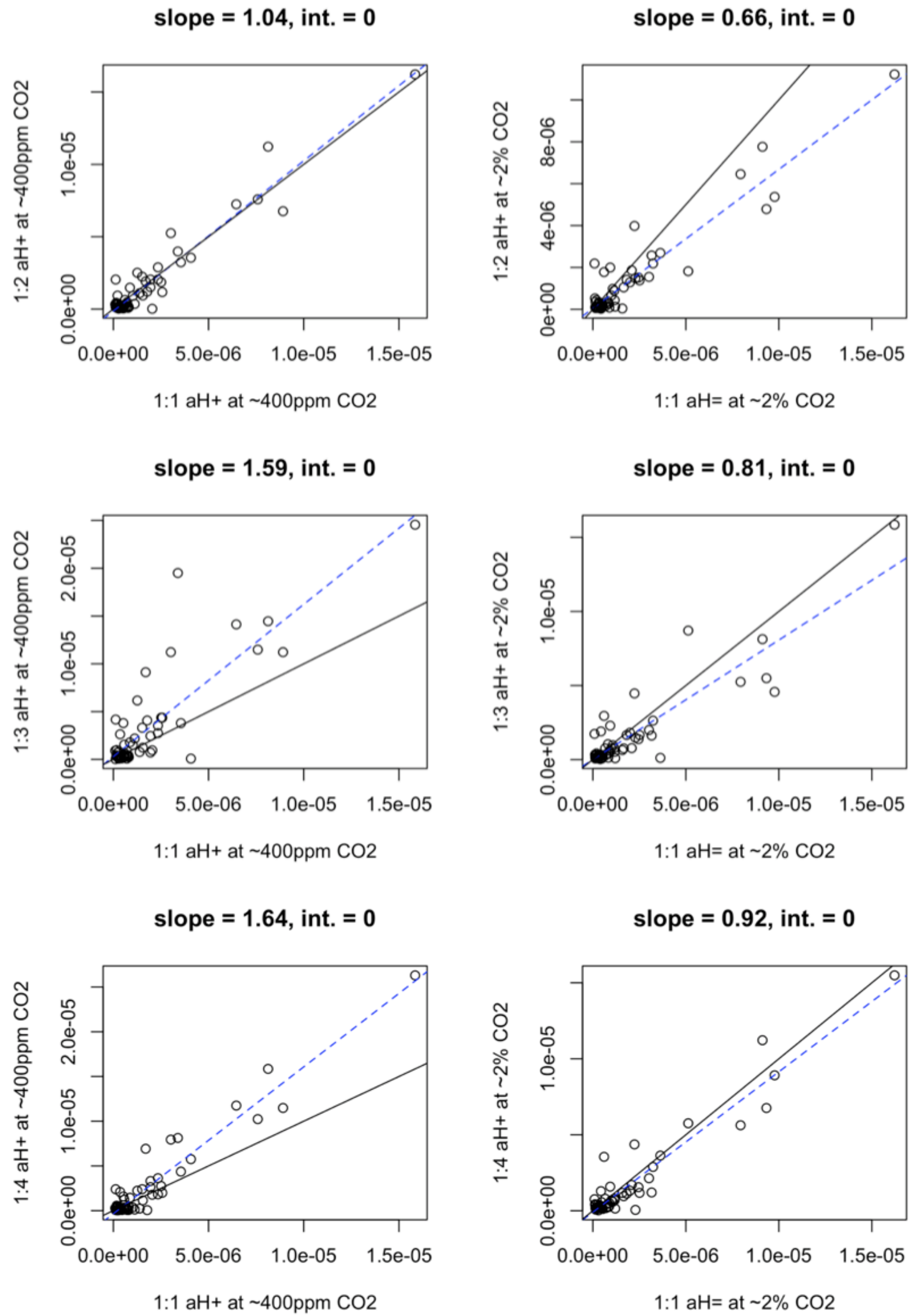



Figure 2.14: Soil $a_{H^+}$ ($1:1$ water:soil ratio) of Spooner soils compared to three other ratios ($2:1$, $3:1$, $4:1$) at two levels of carbon dioxide (400 [ppm] and 2%).

# 3 Standard and Non-Standard Measurement of Acidity in Wisconsin's Soils. II. Do Bacterial Communities Correspond with Soil pH or Not?

## 3.1 Introduction

Standard soil pH measurements have guided land management and biogeochemical research (Libohova et al. 2012; Miller and Kissel 2010), and today these values are also one of the best predictors of many characteristics of soil microbial communities (Wakelin et al. 2016; Shen et al. 2013). Historically, soil pH has aided agronomists in maximizing crop yield from acid-affected soils, but recently the standard method of measurement of soil acidity strongly correlates with bacterial community diversity and composition in soils (Tripathi et al. 2012; Lauber et al. 2009, 5114; Fierer and Jackson 2006, 627; Bååth and Anderson 2003, 958–59). This correlation remains poorly understood, though soil microbial ecologists have found "soil pH" to be one of the greatest correlates of soil microbial community composition and diversity as determined by both culturing (Small 1954, 212) and molecular methods (Vos et al. 2013; Lauber et al. 2008; Tecon and Or 2017; Rousk et al. 2010), generally showing greater diversity in more neutral soils. Both the acidity of soils and the acidicification of soil are of great interest to sustainable crop production and biogeochemical research, but non-standard soil pH values may offer both microbiologists and agonomists improved—or at least more diverse—metrics to monitor and improve soil health worldwide (Meena 2019, 113–59).

The pH of soil microhabitats are highly relevant to crops but equally relevant to the microbial life of soils, many of which directly depend on their microenvironments to provide growth factors via ion-exchange and protonmotive force to generate adenosine triphosphate (ATP) to remain competitive for limited nutrients. However, precise mechanistic theories for the responsiveness of bacteria to their microenvironments remain diverse, roughly falling into three broad categories. First, research such as Carson et al. (2009) propose that the availability of nutrients may be the limiting factors of soil bacterial growth. Second, research such as Ranjard and Richaume (2001) propose that the conditions of soils are highly selective and stressful, whereby the gene-dependent ability of bacteria to resist fluctuating environmental conditions generally limits growth. Third, research such as Schimel (2018) propose that the abiotic factors and biotic factors are

not only partial contributors to determining soil microbial community composition and size but also interact. Soil pH also affects our ability to assess microbial communities through molecular methods, namely DNA extraction, PFLA extraction, and others, most of which report substances whose chemical behaviors are highly dependent on pH and ionic strength (Young et al. 2014; Naidu et al. 1994; Barrow 1984; Kerndorff and Schnitzer 1980; Kirk et al. 2004, 171).

As described above, microbial community composition is regularly strongly correlated with soil pH. However, the conditions under which we measure pH rarely resemble the soil conditions that microbes experience. Referring to the most comprehensive metrological literature of the acidity of aqueous solutions (Buck et al. 2002), a soil's atmosphere regularly exhibits a much higher $CO_2$ partial pressure than in the laboratory (Dahlgren, Percival, and Parfitt 1997; Ponnamperuma, Martinez, and Loy 1966), while a soil's moisture is generally much lower than that of a soil slurry in which we measure pH. Furthermore, these conditions are known to affect our quantification of pH (as investigated in Chapter 2). Thus, we might wonder whether simulating conditions in the laboratory to generate pH values with which we may begin to predict the *in situ* acidity of soils in which soil microorganisms grow, live, and reproduce would lead to even better correlations with characteristics of microbial communities.

In this study, we explore the hypothesis that using the simulation of soil conditions (elevated carbon dioxide and typical soil water content) to generate non-standard soil pH values will predict soil microbial community diversity and composition better than the soil pH values resulting from the standard protocol. There exist large opprotunities to improve the standard soil pH protocol, which historically has been a good preliminary but limited measurement of the acidity of soil microhabitats, and by using methods for measuring soil acidity that simulate the *in situ* conditions of soils, we may improve the predictive models of the ecology of soil bacteria.

## 3.2 Methods

### 3.2.1 Standard and Non-Standard Soil pH Values

Our objective in this study was to determine whether soil pH measurements under conditions designed to better reflect *in situ* soil conditions were better predictors of bacterial community composition. For the purposes of this study, "standard soil pH" is defined as the pH value

measured at ambient (approximately $400$ [ppm]) carbon dioxide and $1:1$ water:soil ratio, and "simulated soil pH" is defined as the pH value measured at elevated (approximately $2\%$) carbon dioxide and $4:1$ water:soil ratio. These soil pH values were determined and described in the metrological study conducted as part of Chapter 2 (Part I of this two-part work), both for soils across the natural soil pH gradient of Wisconsin's agricultural research stations and for soils across the long-term (1994 to today) experimental soil pH gradient of the Spooner Agricultural Research Station.

Because soil pH can potentially interact with the chemicals used for extracting DNA, we also investigated the predictive value of the pH of solutions of the first two lysate steps of the DNA extraction protocol (see supplementary information for details).

#### 3.2.2 Soil DNA Extraction and 16S Amplification

Soils collected as described in Chapter 2 were subsampled and stored at -80°C until DNA extraction. Total genomic DNA was extracted using the PowerLyzer PowerSoil DNA Isolation Kit (Catalog No. 12888, Qiagen, Germantown, MD, USA). All DNA was stored at or below -20°C from the date of extraction throughout stages of sequencing. 16S rRNA genes of extracted DNA were amplified using polymerase chain reaction (PCR) to yield three replicate reactions per sample. Variable region V4 of the 16S rRNA gene was targeted using forward primer 515F and reverse primer 806R with modification by Walters et al. (2016), which increased degeneracy of bases that have caused detection bias among some bacterial clades. Primers also had barcodes and Illumina sequencing adapters added, following Kozich et al. (2013). The following reagents were added to each PCR reaction: (1) $12.5[\mu$L] Q5 Hot Start High-Fidelity 2X Master mix (New England BioLabs INC., Ipswich, MA), (2) $1.25[\mu$L] 515f forward primer ($10$[mM]), (3) $1.25[\mu$L] 806r reverse primer ($10$[mM]), (4) $1[\mu$L] DNA extract, and (5) $7.75[\mu$L] PCR-grade water. The plate was sealed and gently vortexed and briefly centrifuged to ensure all liquids were well mixed. The plate was then run on an Eppendorf Mastercycler nexus gradient (Hamburg, Germany) thermal cycler using the following parameters: $98[^{\circ}$C] for 2 minutes + ($98[^{\circ}$C] for $30$ seconds + $58[^{\circ}$C] for 15 seconds + $72[^{\circ}$C] for $10$ seconds) x $(30+72)$ [$^{\circ}$C] for 2 minutes and 4 [$^{\circ}$C] hold.

Successful amplification was verified via gel electrophoresis. TAE buffer and agarose were mixed

and microwaved for one minute until dissolved. Invitrogen SYBR safe DNA gel stain was added to the mixtures once the beaker was cool enough to touch. The mixture was poured into gel mold (with well plate mold in) and set. The exact gel composition was 2.00 [g] agarose + $200$[mL] 1xTAE + $20$[$\mu$L] SYBR Safe stain. Each well was loaded with $1$[$\mu$L] gel loading dye, purple ($6$X) (New England BioLabs Inc., Ipswich, MA) and $5$[$\mu$L] DNA. Loading buffer was pipetted onto fresh Parafilm paper. DNA was added to buffer and pipetted up and down to mix then was loaded into the well. 2-Log DNA Ladder (0.1-10.0 kb) at a 1:6 dilution (New England BioLabs INC., Ipswich, MA) and loading buffer were added to end wells. The gel was placed into the Axygen Horizontal Gel Box, 20 [cm] (Corning, NY). 1X TAE buffer was poured over the gel filling the end wells and just barely covering the gel. The gel was run for one hour at $115$ $[V]$ using the Thermo EC135-90 (Holbrook, NY). A photo was taken when the gel was finished running and wells were checked visually for successful amplification.

The SequalPrep Normalization Plate Kit (Invitrogen Corporation, Thermo Fisher Scientific, Waltham, MA, USA) was used to purify amplicons and normal PCR products using a limited binding capacity solid phase in each well, which after elution yields a similar number of amplicons per sample. The PCR triplicates were combined into one sample on a new PCR plate. $25$[$\mu$L] of the pooled PCR product and $25$[$\mu$L] of the SequalPrep Normalization Binding Buffer were transferred to the SequalPrep Normalization Plate and mixed by pipetting. The plate was incubated at room temperature for one hour. The liquid was aspirated from the wells. Then $50$[$\mu$L] of the SequalPrep Normalization Wash Buffer was added to each sample well and mixed by pipetting. The SequalPrep Normalization Wash Buffer was then aspirated for each well. $20$[$\mu$L] of the SequalPrep Normalization Elution Buffer was added to each sample well and mixed by pipetting. The plate was incubated at room temperature for 5 minutes. Eluted DNA was then pooled into a single tube per sample and stored at $4$[$^{\circ}$C].

The Wizard SV Gel and PCR Clean-Up System A9282 (Promega, Madison, WI) was used to extract and purify the combined PCR product library according to manufacturer's instructions except for the following two deviations: (1) the SV Minicolumn incubation and centrifugation (steps 5.A.2-5.A.3) steps were repeated twice for each sample, and (2) nuclease-free water application was divided into $30$[$\mu$L] and $20$[$\mu$L] increments with the incubation step and centrifuge

step after each addition (step 5.A.6). DNA was concentrated using a SpeedVac Vacuum Concentrator System (Thermo Fisher Scientific, Waltham, MA, USA) before and after using the Wizard SV Gel and PCR Clean-Up to meet the requirements of $15[\text{ng}/\mu\text{L}]$.

#### 3.2.3 Microbial Community Analysis

Prosser (2015) and Morton et al. (2019) have described the various limitations of inferring the microbial load of taxa detected using amplicon sequencing, so I have utilized 16S amplicon sequencing for the sole purpose of "fingerprinting" microbial communities such that I may measure relative differences between soil microbial communities from different sites and conditions. Paired-end, 250 base pair sequencing was performed using the Illumina MiSeq sequencer (2 x 250 bp). I quality-filtered and trimmed (truncation length 235 bp for forward and 144 bp for reverse reads, left trim of 5 bp for forward and reverse reads with other default settings), learned errors (using all sequences), dereplicated, determined operational taxonomic units (OTUs) (default settings), and removed chimeras using dada2 (McLaren, Willis, and Callahan 2019) as implemented in R, run on the UW-Madison Center for High-Throughput Computing cluster.

The operational taxonomic units (OTUs) were filtered for those identified as bacterial in origin and for each sample were normalized by relative abundance. These reads were merged with both soil chemical data and all soil pH values to be analyzed using the R packages `phyloseq` (McMurdie and Holmes 2013) and `vegan` (Dixon 2003). The permutational multivariate analysis of variance (PERMANOVA), which is a permutational semiparametric dissimilarity measure described by Anderson (2014), was used to assess the influence of environmental factors on community compositions. The Shannon index (Shannon 1998) and Simpson index (Simpson 1949) of diversity, each of which incorporate both relative abundance and evenness across OTUs in a community, were calculated to compare bacterial diversity between treatments. Non-metric multidimensional scaling (NMDS) was used to cluster microbial communities (Agarwal et al. 2007) with a matrix of Bray–Curtis dissimilarity distances (Bray and Curtis 1957). All reads have been deposited at the National Center for Biotechnology Information Short Reads Archive under BioProject ID PRJNA643927, and code for all analyses can be found at `https://github.com/michaeljbraus/usda-wisconsin-soil-ph`.

### 3.3 Results

The Spooner soils clustered well according to standard soil pH values (PERMANOVA, $R^2 = 0.1341$, p$= 0.001$; Figure 3.1) whereas the Wisconsin soils clustered less (PERMANOVA, $R^2 = 0.0864$, p$= 0.001$; Figure 3.1). Other factors besides soil pH also contributed to the microbial community dissimilarity found among the Wisconsin soils but to a lesser degrees (percent clay, $R^2 = 0.0546$; pH of DNA extraction lysate after adding C1, $R^2 = 0.0481$; pH of DNA extraction lysate after adding C2, $R^2 = 0.0447$; percent organic matter, $R^2 = 0.0553$; total nitrogen, $R^2 = 0.0571$; and depth, $R^2 = 0.0675$). The diversity of soil microbial communities plotted as a function of pH estimated as observed taxa, Shannon index, and Simpson index (Figure 3.2) did not exhibit clear trends with pH in either of the sample datasets, or with different methods of measuring soil pH.

Soil pH and $a_{H^+}$ values explained between $7\%$ and $16\%$ of bacterial composition (Figure 3.3), and $a_{H^+}$ was a poorer predictor than pH. For the Spooner soils, soil pH measured at $1:3$ water:soil ratio and elevated carbon dioxide explained the most $(0.16)$ of bacterial composition, but this improvement did not persist with decreasing moisture, as soil pH measured at $1:4$ water:soil ratio was a poorer predictor $(0.15)$. Decreasing moisture in pH measurements did not improve the predictive value for the Wisconsin soils ($R^2 = 0.08 \pm 0.005$ throughout; Figure 3.3). Carbon dioxide levels showed little influence on the predictive power of any measurement of soil acidity for both Spooner and WI datasets.

### 3.4 Discussion

In this study, I explored the possibility that soil pH is a key predictor of soil microbial community differences, diversity, and composition among bacteria. I hypothesized that soil pH values taken during the simulation of soil conditions, namely elevated carbon dioxide and typical water:soil ratios, more closely represent *in situ* conditions of microhabitats and therefore would predict bacterial communities better than standard soil pH values. I tested soil pH because it is a key predictor of soil microbial community differences, diversity, and composition, among bacteria (Lim, Choi, and Son 2017, 7; Saeki and Kunito 2010, 189). Bacterial communities of soils from the Spooner study were predicted best by the pH of soil solution prepared at a moisture

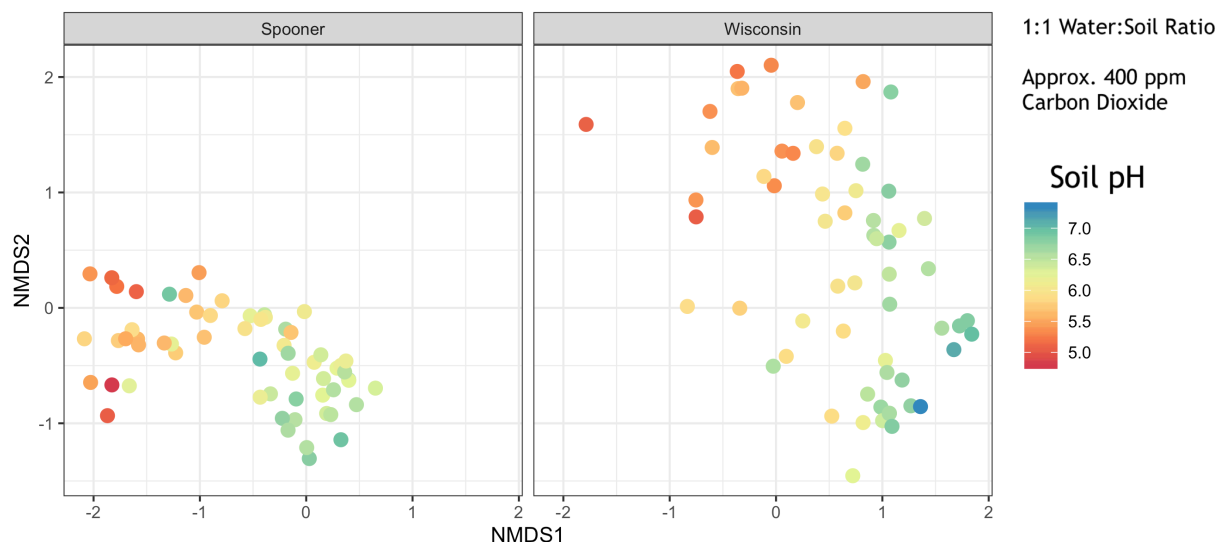



Figure 3.1: Non-metric multidimensional scaling plots of Bray-Curtis dissimilarities for soil microbial communities from 16S amplicon analysis of two sets of samples (Wisconsin soils and Spooner soils) (k=3, stress = 0.112). Points are coloured by standard soil pH.

level of approximately field capacity, but the prediction of soil bacterial communities among soils from across Wisconsin's agricultural research stations was unchanged by simulation of *in situ* physicochemical conditions. Soil acidity measurements taken at ambient carbon dioxide and reported as pH were the best predictors of soil microbial community composition in this study. However, this was only the case when the water-to-soil ratio was lowered to 1-to-3, and this was only true among the soils of a long-term experimental soil pH gradient and not among samples collected from across Wisconsin's natural soil pH gradient. Interestingly, when the water content was lowered further in the Spooner samples, the predictive power of pH values decreased instead of increasing further.

While the Wisconsin soils were very diverse in texture, organic matter, and region across the state, the soils from Spooner were highly similar in all characteristics except soil pH. The long-term experimental plots in Spooner have been amended with lime to raise the soil pH and sulfur to lower the soil pH of the soil of that region (Mahtomedi loamy sand). The excess unreacted amendment may have been a residue in the samples, whose suspension during preparation for analysis would have dissolved and reacted to affect the analyte. The Wisconsin soils were not amended with the same quantity of lime or sulfur, and therefore their solution extracts may not have changed with the decrease of water-to-soil ratio. The diversity observed in these soils were

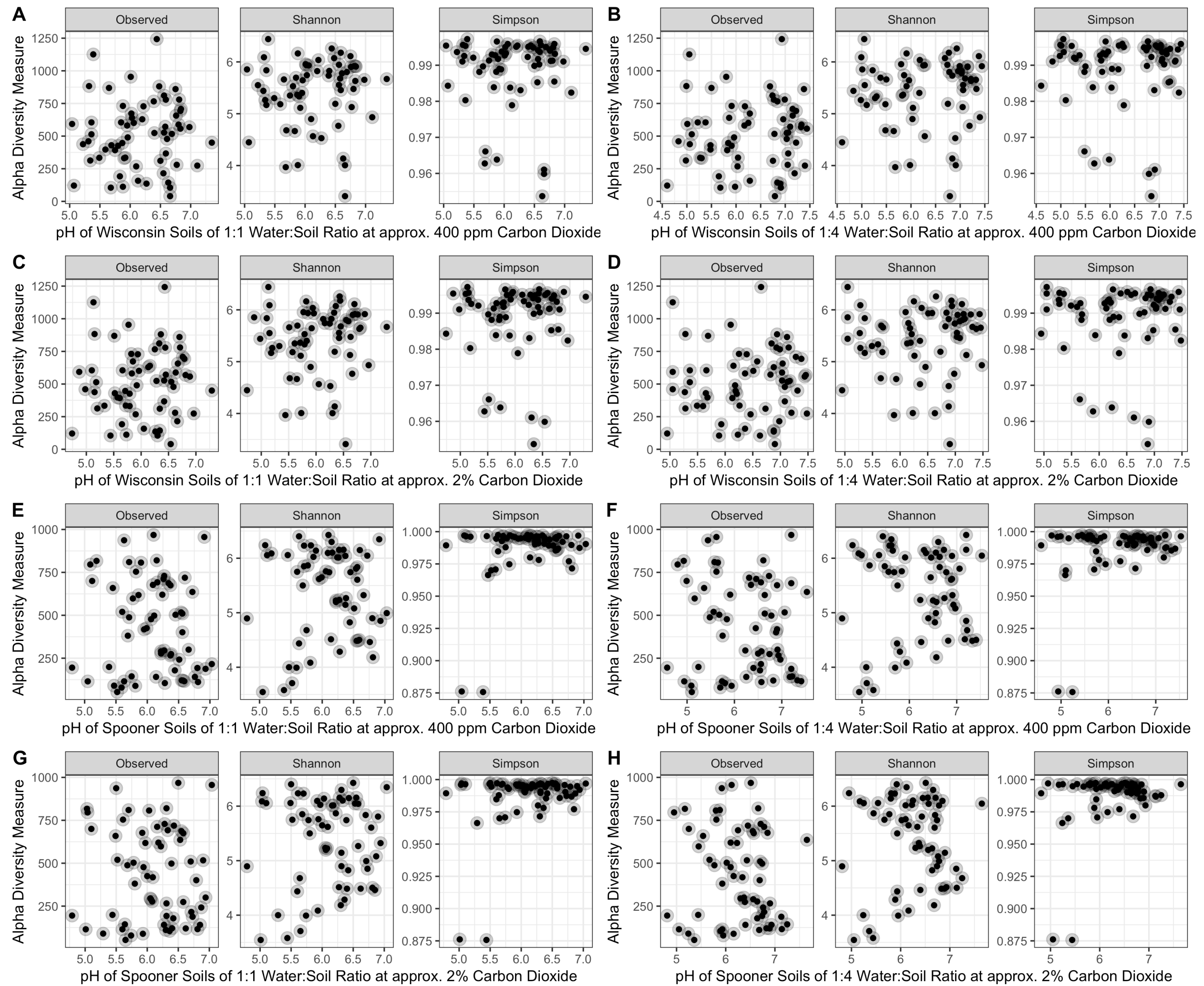



Figure 3.2: Diversity of soil bacterial communities plotted iteratively as a function of the measured pH of extracts taken from soils at two moisture levels (1 : 1 (A, C, E, G) and 1 : 4 water:soil ratios (B, D, F, H)) and two $CO_2$ levels (~400[ppm] (A, B, E, F) and 2% (C, D, G, H)) and two sets of samples (Wisconsin soils (A-D) and Spooner soils (E-F)), using the metrics of observed taxa, Shannon index, and Simpson index.

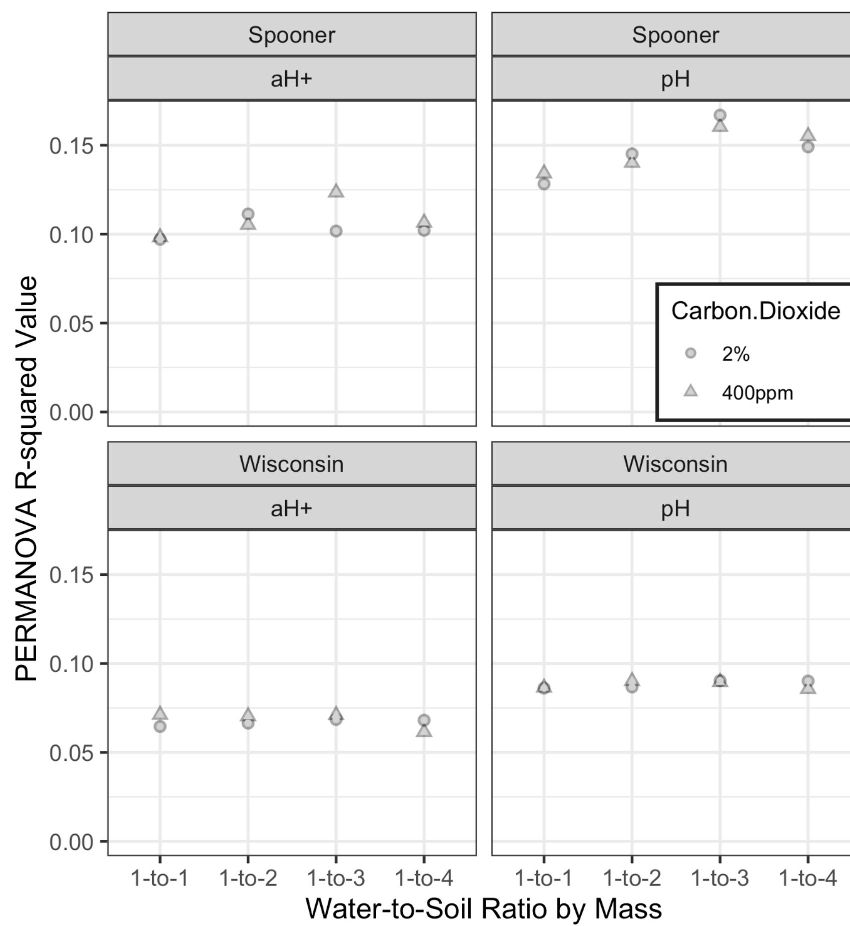



Figure 3.3: $R^2$ values yielded from a PERMANOVA analysis of all soil pH values and $a_{H^+}$ values as factors predicting bacterial community composition as determined by 16S amplicons for the Wisconsin soils set and Spooner soils set analyzed in this investigation.

also largely unresponsive to soil pH of the range of this study, approximately $4.5$ to $8.0$, even with amendment, which suggests that the bacterial diversity of soils only decrease among the most extremely acidic or basic soils (Bahram et al. 2018). Fortunately, our findings suggested that the DNA extraction itself is unlikely to cause artifactual patterns within community composition data, despite the occurrence of "pH swings" during the first two lysis steps (Supplemental Figure 3.4).

One may reformulate soil pH measurement recommendations for the improved use of such values in microbial ecology, possibly viewing the elevated concentration of solutes and carbonate in the analytes of these sites as a means of both heightening the detection of important acids and bases found in typical soil solution by the glass probe as well as improving the representation of *in situ* conditions of soil microhabitats (Sumner 1994). However, the further concentration of analyte beyond a 4-to-1 water-to-soil ratio may have caused the analyte to begin interfering with the functioning of the glass probe, which only functions in error of $< 5\%$ in analytes of ionic strength $< 0.01$ moles per liter (Dobrovolskii et al. 2018, 87). Measurement of acidity with a probe without a liquid junction would allow us to determine whether (1) the effect is an error preventing further predictive power in lowering the soil moisture levels and/or (2) the 1-to-3 water-to-soil ratio at ambient carbon dioxide levels is a more optimal protocol for predicting the microbial communities in experimentally controlled soil acidity experiments. Additionally, the benefits of this approach will depend on the specific dataset. If other soil properties are the primary underlying drivers of microbial community composition, then adjusting pH measurement techniques may only marginally improve its predictive value.

Poole (1999, 252) presented a discussion of whether bacteria respond to pH because the production of ATP relies upon the maintenance of acidity gradients between the interior of bacteria and their exterior. While this may be correct, our ability to quantify and understand this effect is constrained by methodological issues with pH quantification (as discussed in detail in Chapter 4). Not only is the ionic strength of the interior of bacteria several times larger than the upper threshold of ionic strength to which pH may be applied, the ionic strength of the exterior environment is also potentially very large as well as fluctuating throughout the usual cycles of most soils' cycles of wetting and evapotranspiration. Fenchel et al. (2012, 115) has offered an alternate

or complementary explanation for the strong effects of pH on soil microbes, that the pH of soil solution determines the charge topology of sources of carbon, nitrogen, and other growth factors that enable bacterial growth and reproduction. For example, the response of lignocellulose to enzymatic attack has been shown to change greatly when the pH of its solution is shifted $> 1.0$ units. pH shifts of a similar magnitude were observed in this study after the simulation of typical moisture levels and elevated carbon dioxide, but changes to the predictive value of our pH estimates for soil microbial communities were generally slight and limited to the highly controlled experimental soil pH gradient. For these reasons, I recommend seeking an alternative metric of soil acidity before further attempts to measure "*in situ* soil pH", despite its superior ability to predict soil microbial communities.

The greater predictive power of pH over hydrogen ion activity would appear to indicate that pH is a better metric. However, when samples collected at periodic intervals across the colog scale of pH are exponentiated to give $a_{H^+}$, they result in a skewed distribution. (This can be seen in Figure 2.6 above.) While this observation may seem obvious, it asks us to reconsider the fundamental assumptions underpinning our predictive models. As employed in this paper, the PERMANOVA is essentially testing whether there is a linear response of microbial community dissimilarities to pH or $H^+$. Our implied expectation of a linear response through this model suggests that we expect that the mechanisms driving this response should affect microbes in a steadily increasing manner along the pH gradient. However, what if the microbial response is driven proportionally to the activity of $H^+$, rather than as its cologarithm? In future studies, it would be interesting to design sampling schemes such that roughly even intervals of activity are gathered, producing an even distribution from low to high acidity in molar units. A more fundamental inquiry, beyond the statistical vagary of the transformation of data during the fitting of models, is to what extent pH is an electrical phenomenon or a chemical phenomenon. On the one hand, pH is a scale converted from the millivoltage of a glass probe using the Nernstian slope, so we may treat the acidity of soil solution as a field of electrostatic charge. On the other hand, pH is currently defined as representative of "hydrogen ions", which are chemical entities virtualy absent from all substances on Earth,[3], so we may or may not be able to consider the acidity of soil solution as a

[3] Hydrogen ions (both positive and negative valencies) are abundant in outer space as the ionized hydrogen ejecta from stars, or "solar wind" (Lühr et al. 2018), but hydrogen ions bind too strongly to other substances on Earth or its atmosphere to exist in any measurable amount.

concentration of particular ions. This is an open research question in biogeochemistry.

## 3.5 Supplemental Materials

### 3.5.1 Supplemental Note: "pH Swings" of Soil DNA Lysate During Extraction

The pH values of miniaturized analytes of the first two steps of a standard soil DNA extraction protocol were measured. Two sets of DNA extraction kits with bead-beating tubes and solutions C1 and C2, which are identical to the solutions and materials used in the PowerLyzer PowerSoil DNA Isolation Kit used for 16S amplicon sequencing in this study, were used to generate lysates of the first two steps of the soil DNA extraction. Excess addition of C1 and C2 solutions allowed for the removal of small aliquots of solution without disrupting the chemical events and buffers of the first steps of DNA extraction. $100[\mu L]$ was removed from the lysate after the addition and bead-beating with solution C1, and another $100[\mu L]$ was removed from the lysate after the addition of solution C2. These pH values ("after C1" and "after C2") were compared to the standard soil pH values (i.e. $1:1$ water:soil ratio at ambient carbon dioxide levels).

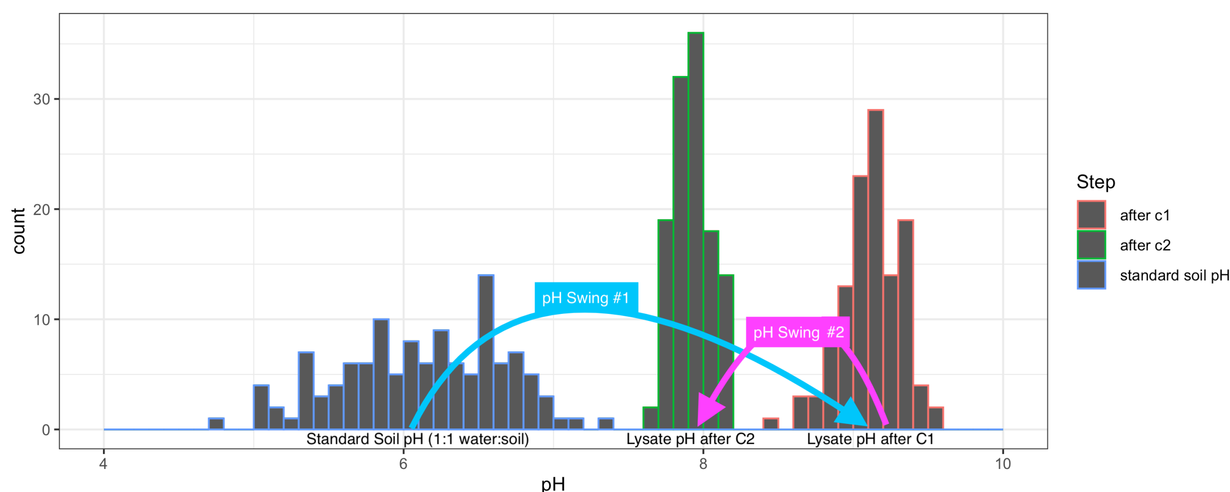



Figure 3.4: Histograms of standard soil pH and the ph of the lysate supernatants after treatment with buffers "C1" and "C2", respectively, of the first two steps ("pH swings") of the soil DNA extraction protocol. The first pH swings to $> 9$ from the the more variable and acidic standard soil pH values (1:1 water:soil), then the second pH swings down to approximately 8, narrowing the range of pH values as the DNA extraction progresses. The acidic soils ($< 5.5$) were nearly $100\times$ more acidic than the neutral-to-basic soils ($> 7$) according to their standard soil pH measurement, but the DNA extraction kit treated these soils with an identical alkaline buffer in the first step.

# 4 Acidity as Proticity in Soils, Blood, and Brines with Theory and Evidence for Temporal Bond State Dynamics

## 4.1 Introduction

While reviewing a topical subset of older literature and research of the influential agricultural and biogeochemical measurement called "soil pH", as well as "pH" itself, I often wondered why some terms and theories become more widespread than others. Are scientific theories entrained within the utterances of their terminology, or perhaps *vice versa*? For example, with a brilliantly flowing string of coherent coinings, as if mining a shallow vein of precious metals from the scientific landscape of his time, Michael Faraday instantly canonized the terms "ion", "electrolyte", "anode", and "cathode" in a single publication (Faraday 1834). A century later, Niels Bjerrum, an equally brilliant and prolific electrochemist, also coined a term, "lyonium", to name the unique chemical entity of autoionizing chemical species (Bjerrum 1949, 230). However, despite Faraday's and Bjerrum's virtually equal aptitude, scientific prestige, and public influence in their regions, Bjerrum's term failed to become as widely-adopted as Faraday's terms. Perhaps Faraday's terms gained an advantage by predating Bjerrum's term, perhaps Bjerrum's terminology were built upon Faraday's and therefore sequential, or perhaps the language in which Faraday published (English) was more widely read than the language in which Bjerrum published (Danish). However, I also wondered whether the behaviors of materials are stable and virtually identical and uniform, free of all human influence.

It would appear that the theories and terminology change but physical reality stays the same. A substance will dissolve or react at a certain rate, regardless of whether the scientist speaks English or Danish, for example. Likewise, how a proton behaves in water or any other medium will not respond to the investigator's thoughts and beliefs on the matter, but, for better or worse, such thoughts and beliefs are the fundamental substance of the scientific record. Keeping this firmly in mind, one will observe that the concept represented by the term "pH" has many uses today, but even a cursory review of pH values—and the pH scale as a summary metric of acidity—quickly reveals key discrepancies between "pH" as a mental model and "acidity" as a physical phenomenon. In this monograph, I will explore acidity, beginning by reviewing the metrological history of "pH" and subsequently commenting on its pedagogy today in the form of a somewhat lighthearted

essay. I then more seriously formalize a novel metric of "proticity", which describes acidity in terms of bond state dynamics, which may enable one to abandon the metric of "pH" altogether. Finally, to substantiate the largely theoretical considerations of these prior sections, I provide the methods and results of a simple but profound experiment that demonstrates acidity as proticity.

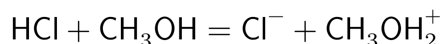
## 4.2 History

History has much more to inform us on the subject of acidity, and theories of acidity are more nuanced than what the introductory blurbs found in chemistry textbooks today would suggest. Niels Bjerrum (1879-1958), whose works and accomplishments have been surveyed by Kauffman (1980b, 1980a), canonized the Brønsted model of acidity, named after Johannes Nicolaus Brønsted (1879-1947), which defines acidity by the transfer of protons. Although Luder and Zuffanti (1961) and Drago (1974) attempted to revivify the electronic theory of acidity, proposed by Lewis and Randall (1921), the Brønsted model has persisted as the dominant paradigm for acids and bases. The "lyonium" molecule, however, defined by Bjerrum (1949, 230), is a separate category of covalently yet dynamically solvated ions, most notably the suite of cations of water (i.e. Eigen cations and Zundel cations) that emerge as protons transfer rapidly or "flit", a behavior that stands apart from the static solvated ions (i.e. calcium, sodium, chloride, etc.). I will quote Bjerrum directly:

> My early work with aquo salts and their hydrolysis, and with the constitution of the amino acids, made it especially easy for me to see the usefulness of Brønsted's extension of the acid concept. And I believe that I may take credit for having seen, even earlier than the originator, the applicability of the new use of the word to the hydrogen ion in aqueous solution. By that time it had become a commonly accepted idea that the hydrogen ion, $H^+$, is not found free in its solutions, but is always present in solvated form, for example, as $H_3O^+$ (hydroxonium ion) in aqueous solution. This idea, which can be traced back to the work of Franklin, Goldschmidt, and Fitzgerald and Lapworth received strong support shortly before 1923 in a paper by Fajans. In my work on polybasic acids I start out from the supposition that the hydrogen ion exists in aqueous solution as hydroxonium ion, and in agreement with Bronsted's concept, I regard the hydroxonium ion as a tribasic acid and discuss the values of its three dissociation

constants.

While the hydrogen ion is present in water as $H_3O^+$ (hydroxonium ion), in methyl alcohol it is present as $CH_3OH^+$ (methyloxonium ion), in liquid ammonia as $NH_4^+$ (ammonium ion), and so on. Chemists speaking of hydrogen ions in daily conversation practically always have in mind these solvated hydrogen ions. This is a somewhat dangerous use of words, — especially dangerous when it is necessary to distinguish between free and solvated hydrogen ions. If it is decided to call the free hydrogen ion a hydrogen kernel or proton, the term "hydrogen ion" can still be used for the solvated hydrogen kernel. But I regard it as distinctly more practical to introduce a special common name for these solvated hydrogen ions, and I propose to call them "lyonium" ions (the Greek word "lyo," to dissolve, is used in the prefix of the terms "lyophilic" and "lyophobic" colloids). When an acid ionizes in a medium, its hydrogen ion is given to the solvent with formation of a lyonium ion. For example,



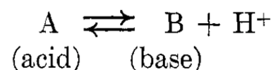
$$HCl + CH_3OH = Cl^- + CH_3OH_2^+$$

In this reaction the original acid (hydrogen chloride) disappears, but there is formed in its stead a new acid, i.e., the lyonium ion (in our example, the methyloxonium ion). In solutions of strongly dissociated acids we have in fact no longer the original acid, but only the lyonium-ion acid.

With regard to distinguishing bases from the basic species formed from the solvent reacting with acidic species, Bjerrum (1949, 237) continues, admitting "after long hesitation I have finally come to the conclusion that it is best to try to push through the use of the term 'base' for the extended concept, and introduce the new name 'lyate' for the acidates of the solvent." It must be noted here that Brønsted originally proposed that the donation of any solute or chemical leaving group with a *net positive charge* will acidify a solution, not the transfer of hydrogen *per se*, reaching the obvious conclusion that hydrogen has the largest positive charge density of all substances after shedding its electron (i.e. a proton). The summary of Brønsted's true paradigm, possibly misinterpreted or oversimplified today, is as follows (Bronsted 1926, 785):

**Summary**

1. The definition of acids and bases by means of the scheme:



$$A \rightleftarrows B + H^+$$
(acid) (base)

involves the admission of ions as well as electrically neutral molecules as acids and bases.

2. The positive electric aquo ions, for instance $Al(H_2O)_6^{+++}$, are acids. The acid property is the more pronounced, the higher the positive charge. The corresponding hydroxions are bases.

3. In correspondence with the theory of Kossel the charge of the central atom of a molecular configuration plays a very great part in imparting to it acid and basic properties.

4. The acidity and basicity of the various molecules formed by the elements in any group of the periodic table show a graduate change in correspondance with the statements in 1., 2. and 3.

5. The electrostatic effect tends to make acids with two or more positive charges more strongly ionizing in solvents of low dielectric constant than in water.

6. The free $H^+$-ion does not exist. Ionization of acids and bases takes place as a double acid-basic equilibrium involving the solvent molecules. The ionization tendency is measured by the product of an acid and basic constant. This accounts for the slight ionization in pure solvents as HCl or $H_2O$ and the strong ionization in mixture: HCl + $H_2O$.

7. Absolute comparison of the strengths of two acids or two bases is impossible because it requires the absence of any solvent medium.

*Copenhagen,*
*Denmark,*
*Dec. 11, 1925.*

The 1920's was indeed a seminal decade in the history of aqueous chemistry. Much of electrochemistry, acid-base chemistry, and many other scientific fields were inspired by Debye and Hückel (1923), and I have created a new translation with updated language and clear typesetting of that publication entitled "*Zur Theorie der Elektrolyte. I. Gefrierpunktserniedrigung und verwandte Erscheinungen*" in the journal *Physikalische Zeitschrift* (see Appendix C). The Debye-Hückel theory is highly relevant to the history of pH, and the reader will not have failed to notice the extremely long list of references that followed the key claim of the second chapter of this dissertation, and a direct quote of each has been provided in Appendix A:

Specifically, these techniques for measuring "pH" are incapable of being applied (with-

out measurement errors above $5\%$) to solutions of ionic strength ($I_s$) greater than $0.1$ molar, where $I_s = \frac{1}{2}\sum(c_i z_i^2)$ estimates charge density added to an aqueous solution (Lewis and Randall 1921) using the concentration ($c_i$) of each ion ($i$) and the charge of each ion ($z_i$). The upper theshold of $0.1$ [M] is also called the "the dilute solution assumption" (Dobrovolskii et al. 2018, 87; Covert and Hore 2016, 235–38; Levie 2014, 615, 2010; Spitzer and Pratt 2011, 75; Wright 2007, 382; Baucke 2002, 774; Sparks 1998, 112; Anderegg and Kholeif 1994, 1521; Galster 1991, 16; Butler 1998, 462–63; Volk and Rozen 1977; p. 1569; Pourbaix 1974, 14; Sena 1972, Appendix 3; Ashcraft 1957, 3, 1947, 29; Feldman 1956, 1865, 1956, 1865; MacInnes 1939, 148; Debye and Hückel 1923, 197), and it is a fundamental limitation of the measurement of pH.

Reading these works, one will find that the literature strongly suggests there is a problem today in the field of acid-base chemistry, summarized by the words of Roger G. Bates himself (Ashcraft 1957, 3), who was one of the pair of chemists after whom the Bates-Guggenheim convention (Bates and Guggenheim 1960) was named that exactly defines "pH" as we use it today:

> These fundamental applications of measured pH values are permissible for many rather dilute aqueous systems. However, there is always the temptation to extend the interpretation beyond the limited range of its validity, to systems that are not dilute or not aqueous. It must always be borne in mind that the experimental (that is, operational) pH value in such media as $50$ per cent alcohol, molasses, and glacial acetic acid cannot be interpreted in terms of hydrogen ion concentration or the conventional hydrogen ion activity.

Bates, among dozens of his colleagues, cautioned future scientists to utilize the concept and measurement of "pH" within the bounds of its applicability, which relies on the Debye-Hückel theory of *dilute* electrolyte solutions. However, it is clear that these thresholds are nearly ubiquitously violated. Consider seawater, which has an ionic strength ($I_s$) of $0.70$ [M] (Kennish 1989, 60), making this a solution that lies far outside of the upper threshold of ionic strength. The pH values calculated from millivoltage measurements of a glass probe in seawater are therefore highly incommensurable with pH values calculated from millivoltage measurements of a glass probe in the familiar traceable pH standard solutions (typically 4, 7, and 10). Another example would be

soil organic matter (SOM), the brownish surfaces coating soil particles that give them their dark color, an amalgam of weak acids and bases that are glommed to particle surfaces. SOM has a high density of charged particles and very low water content, and these substances are then further concentrated during evaporatranspiration, which generates an intensely strong ionic atmosphere (i.e. brine) in addition to the particles' silica surface charge itself. The combination of inter-ionic interactions and subsequent liquid junction potential on a glass pH probe are enormous and possibly immeasurable with the instruments at hand. However, although the ionic strength among substances whose acidity influences life on Earth, especially blood ($I_s > 0.6$ M) and seawater ($I_s > 0.7$ M), it is important to note that ionic strength is by convention a "fudge factor" and not a real theory or law of the electrochemical concentration or behavior of electrolyte solutions (Darvell and Leung 1991). Additionally, it is likely that non-ions are just as influential to ionic strength, but there have been few efforts to include these substances in the calculations of ionic strengths, warranting reinvention of ionic strength alongside a reinvention of acidity such that soils and other typical environmental systems may be accurately studied.

From the original theory of Brønsted regarding acidity as positive charge density, we may use Bjerrum's "lyonium" to begin to describe the autoionization that produces this acidity to reformulate acidity itself. What if we considering water a reactant with other reactants in the mixture, with no "solvent" or "solutes" whatsoever? New questions emerge: What if the autioinized species of protonated and deprotonated water were to be considered "mono-, di-, and tri-protic oxygen", whereby the substances with which these multiprotic oxygen interact have equally varying numbers or protons, what we might call "proticity"? If the addition of only a small volume of acid to a solution can cause it to become a strong acid, then, if one's skin is splashed with this acid, for example, does the actual substance causing the acidification of the solution or the acidified solvent itself (i.e. the water) cause the resulting burn? If I don a swimsuit and jump in one end of a long swimming pool while a very large beaker of concentrated HCl is added to the other end of the pool, will I feel the acidity on my skin before, at the same moment, or after the chloride diffuses to reach me? Would not the net charge travel as current through the mildly conductive solvent and reach far more rapidly than even on of the diffusing chloride ions? Such questions—be they for research or teaching—require an new and more apt mental model to embrace physical

reality.

### 4.3 Pedagogy

Chemical educators, with regard to the subject of acidity, have received valid criticism in the past several decades, but I will argue that their troubles are no fault of their own. Although many educators have used playing cards (Zhang 2017) and other games and techniques to communicate the concepts, most students still develop a distaste for chemistry—namely electrochemistry—reporting that the ideas are an affront to their common sense. The willingness of the students to pay their valuable attention is really the proximal problem and not the ultimate problem to address, and games and motivational preambles only become effective when the ultimate problem is first made apparent. The ultimate problem of teaching about acidity lies within the metrology and dimensional analysis of acidity itself. Cartrette and Mayo (2011), Hawkes (1992), and many others outside of the literature have illustrated the dilemma—or perhaps trilemma—that a single chemical phenomenon (acidity) has been permitted to be explained by three unique models: Arrhenius acids as dissociated solutes, Brønsted acids as proton-donors, and Lewis acids as electron-acceptors. Each theory even has respectively unique notations. Can scientists and educators "stress one model over the other" while permitting the three models to co-exist?

Cooper, Kouyoumdjian, and Underwood (2016, 1704) summarized the most common and most logical solution to this problem, which is to adopt the Lewis acid theory. They write, "One further reason to support earlier emphasis on the Lewis acid—base model is that it provides an entreé [sic] into reasoning about chemical reactions from a mechanistic view by helping students think about how reactions occur. If students think about acid—base reactions as proton transfers, without considering how the transfer happens, they will be less likely to make the connection between acid—base reactions and (for example) nucleophilic substitutions. The move to Lewis acid—base theory means that students will be introduced to mechanistic reasoning." Cartrette and Mayo (2011) also make a similar recommendation that the Lewis acid model confuses students less than other models of acidity, although "failing to confuse students" is less of an endorsement than would be "successfully educating students". Indeed, Luder and Zuffanti (1961) has shown that acidity as solely an elecronic theory is evidently simpler to comprehend, more interoperable between systems, and also more rigorously supported than Brønsted's model alone. Referring to

any chemistry textbook published in the last 40 years, however, one will see the three models of acidity listed with the Brønsted acid model upstaging the others.

The non-competetive co-existence of models will have a detrimental effect on scientific progress and science education, postponing rather than accepting the requisite rigor of testing and retesting until we may rank the theories or one day announce a single victor. The problem of "stressing one model over the others" is that this approach is identical to ignoring all models but one, which would act as the antithesis of science as the scientific method initially assumes all hypotheses are plausible at once with the assumption that only one will survive rigorous testing. Under the implicit assumption that all theories are worthy of refutation *and worthy of study whether correct or incorrect*, I suggest we finish listing all possible models of acidity then attempt to disprove all but one. In this way, we may find that the three extant models of acidity could be abandoned for a newer consilient model of acidity. As an example of initiating this approach, it is obvious that Brønsted acids and Lewis acids are, colloquially speaking, two sides of the same coin, as expressed by Luder (1952): "If one follows Lewis, it is unnecessary to make any distinction between 'Lewis acidity' and 'Brønsted acidity.' On the contrary, when applied to hydrogen acids, the two theories are absolutely identical."

It is entirely probable that separate models of acidity are unnecessary, because they are all extensions of the law of mass action (Lund 1965; Guldberg and Waage 1899). However, the behavior of protons is "nonconformist", as Lowry (1923), Bronsted (1926), Drago (1974), Ernsberger (1983), and Knauth and Di Vona (2012) have all suggested, meaning that ions resulting from protonation are different, atypical, more dynamic. Nonconformity is the common thread throughout the history of acidity, and therefore the model of acidity, in turn, must not conform either. What if "acidity" is not a phenomenon resulting from a concentration (or acitivity) of "acids" and "bases" at all but instead purely a reactivity and autoionization via protonation over time in response to net charge densities? What if the proton were treated as an entity that ineluctably moves and bonds in response to fields of charge density, which is present in all protic substances (Biswas and Voth 2017), and the notions of "pH" and "dissociation" were abandoned for positive charge transfer?

I believe that most the questions above are scientific questions wrapped in a pedagogical questions,

so one must answer both questions to answer either. $H_2O$ is oxygen bonded to two hydrogen, and this is the physical model of water. We may more accurately call this molecule diprotic oxygen. Other terms for the presence of hydrogen as a chemical group on a molecule are "saturated", "hydrogenated", and sometimes "reduced", but the nature of water can be very concisely described by its composition: one oxygen and approximately two hydrogen undergoing bonding dynamics in a cloud of electron orbitals. To add a proton and subtract a proton is to respectively increase or decrease its affinity for protonation and ability to protonate a nearby molecule as its charge ranges from negative to neutral to positive. The transfer of protons does not require any neologism, because it is already most succinctly called "protonation" or "proton transfer".

One will rarely hear objections to the statements above, but this is precisely the place where the competing models of acidity diverge. A notional model might contradict the simple accounting of atoms by evoking an entity called "water", giving it the role of "the solvent" in which "solutes" are "solvated by the solvent". Diprotic oxygen, being a particle, has a finiteness, whereas "the solvent", being a notion, does not. Likewise, a particle such as a sodium ion has a finite charge field around a real nucleus with mass, whereas "a solute" has neither charge nor mass explicitly. Why would this distinction become a problem for researchers and educators? Because the schism of notional and physical models is a source of confusion but not a source of knowledge. For example, Dr. Todd Silverstein has maintained a discussion on this subject of what constitutes an entity in chemistry, emphasizing the theories of acidity. Silverstein, from his works spanning almost two decades from (2000, 849–50) to (2014, 608–10), appraised the "quarterback" model, which supposes that molecules with acid-base properties are like football players that toss a ball—the proton—from one to the other in aqueous solutions. While the mental image is pleasing and familiar, many will begin to wonder how an hydroxide ion would ever form. "pH is supposed to be complemented by pOH, right?" a student might ask. "How do the football players lose a football when they are not carrying a football?" Silverstein's football model embodies the problem I have proposed above, that it is the educators who are succesful while the theories they have been given to use and teach are lacking in common sense.

Let us try to use a mental model that assumes there is no such thing as a solvent at all but instead many diprotic oxygen, and that proton transfer is the rule instead of the exception. *Jugglers* might

provide a much better mental model, and, though the analogy remains highly limited, we can abandon the ill-conceived "balls" for jugglers' preferred term for what they creatively manipulate, "props". Consider a room crowded with jugglers, most of whom have two props. Some have one prop, grasping around to fill their empty hand, and some have three props, juggling so furiously they sometimes toss a prop to another juggler. All of these individuals begin passing the props in a semi-periodic manner because, growing somewhat tired, they try to exert as little effort as possible. Moreover, when one of the jugglers has three props, a small crowd forms to watch with keen attention, but at any moment the juggler with three props may toss one of his or her props to a member of the little audience, who then immediately becomes the focus of the group's attention.

We may now recapitulate: the juggler is oxygen, their attention is the cloud of electrons, and the evanescent crowds are the clustering and reclustering of water as it autoionizes. The props are the protons, and the minimization of effort represents the energy of the system. Although still highly limited in scope, this "jugglers model of the proticity of water" is a much more more accurate mental model for discussing protonation dynamics and describing acidity, and the model is both coherent and evokes entities instead of notions.

Essentially, if we are going to present several theories of acidity as truthful for students and as testable for researchers, we cannot allow multiple theories to coexist forever. In other words, if we are going to call chemical entities "species", we would be wise to allow these species to evolve. For example, the notion of "chemical activity" (Burgot 2017) has many key problems. Activity has been represented as $a_{\mathsf{x}} = c_{\mathsf{x}}\gamma_{\mathsf{x}}$, but it has also been presented by the IUPAC as

$$a_{\mathsf{x}} = \frac{c_{\mathsf{x}}}{c_{\mathsf{x}}^{\ominus}}\gamma_{\mathsf{x}} \ , \tag{4.1}$$

which suggests that activity is dimensionless. This is not true, because (1) activity was defined as explicitly having units moles per liter (Lewis 1907, 262–63) and (2) the primary purpose of dividing the measured concentration ($c_{\mathsf{x}}$) by a standard concentration ($c_{\mathsf{x}}^{\ominus}$), which is inexplicably set as $1[\mathsf{M}]$ for solutes but not for solvents, is to ensure that the quantities involved cause the equilibrium constant to be a "pure number" for logarithmic transformation to calculate pKA. Consider a reaction where the reactants are A and B, and the products are C and D, all of

stoichiometries of $1$:

$$K = \frac{a_{\mathsf{C}} a_{\mathsf{D}}}{a_{\mathsf{A}} a_{\mathsf{B}}} \tag{4.2}$$

It is unclear whether $K_a$ is indeed $K$ as it is written above, or whether it is

$$K_a = K * 55.5[\mathsf{M}]\ \mathsf{H_2O} = \frac{a_{\mathsf{A}^-} a_{\mathsf{H_3O^+}}}{a_{\mathsf{AH}}} \tag{4.3}$$

as written in some (but not all) chemistry textbooks. If activity were truly dimensionless, it would be impossible to interpolate a concentration of the system, so it can be seen that these values are not pure numbers but percentages. This model does not adapt to the situation wherebe a reactant is the same as the solvent, which is exactly the case of autoionization of water to produce the acidity we wish to understand and measure. Additionally, the activity of water in the acid-base reaction is $55.5[\mathsf{M}]$ when water is the solvent, and this will remain this magnitude whether it is dimensionless or not. This is very important to clarify, because if the activity of water is incorporated into $K_a$ and then logarithmically transformed to become pKa, the current definitions by the IUPAC of acid dissociation and pH would render them inapplicable to all but the most limited systems (e.g. pasta water). To remedy the problem, one would need to exponentiate pKa, incorporate again the concentration of water, then recalculate Ka, but this calculation is rarely performed as aqueous chemistry adopts the dilute solution assumption.

What if one is not investigating dilute solutions (i.e. $I_s > 0.1[\mathsf{M}]$)? Ultimately, acid dissocitation has omitted the necessary factors of solvation, as well as time, when applied the acid-base chemistry of non-standard and non-ideal solutions throughout the environment. The formula

$$\mathsf{AH} + \mathsf{H_2O} \leftrightharpoons \mathsf{A}^- + \mathsf{H_2OH}^+ \tag{4.4}$$

results in a more realistic view of the equilibrium reached by deprotonation of an acid with the dominant proton-acceptor, $H_2O$, and the generalized equation for acid dissociation would therefore be

$$K_a = \frac{[\mathsf{A}^-]\gamma_{\mathsf{A}^-}[\mathsf{H_2OH}^+]\gamma_{\mathsf{H_2OH}^+}}{[\mathsf{AH}]\gamma_{\mathsf{AH}}[\mathsf{H_2O}]\gamma_{\mathsf{H_2O}}}. \tag{4.5}$$

This equation now includes $H_2O$ as a reactant and not "the solvent", enabling the lowering of the concentration of $H_2O$ as solutions (or, of notable interest here, soils) evaporate water. The addition of solvation shells to each ion would provide additional information to how much water becomes structured in response to the ions' increasing charge density, and these solvation shells

can only be represented when we discontinue the practice of ignoring the concentration of water in the equation for Ka. More troublesome still is that, when the concentration of water is multiplied by the acid dissociation constant, a logarithm function cannot be applied to a unitful physical quantity (Boggs 1958). "What is the logarithm of 10 apples?" is the old question, and we may ask here, "What is the logarithm of $55.5$ moles of water?" The exclusion of a key reactant such as $H_2O$ from this calculation is a remarkable omission.

Pending adoption of a new notation or the transition from "pH" to something actually sensible, I will recommend for the sake of teaching and immediate practicality we revivify the representation of acidity as "hydrogen ions", perhaps reported as an index rather than a physical quantity, for such fields as medicine and soil science (F. Sgambato et al. (2011a); F. Sgambato et al. (2011b); and personal correspondence). The Henderson equation originally used the raw concentration of hydrogen ions, and the Henderson-Hasselbalch equation later used pH so researchers need not constantly refer to tiresome log tables. All pH values can be readily expressed in nanomolar, sparing investigators instead the equally tiresome cologarithmic values, which a computer or calculator can handle today. For example, when one expresses human blood pH values in "nanomoles of hydrogen ions" or more aptly "nanomoles of protons" as the scale of acidity, the healthy range of $7.45$ to $7.35$ on the pH scale is almost exactly $35$[nM] to $45$[nM], respectively (see Figure 4.1). The numerical continuity of $35$'s and $45$'s present in both scales is a convenient coincidence, albeit inverted, and the nanomoles of protons scale makes the physical quantity of acidity both unitful in terms of accounting for active protons as well as much more intuitive as the increase in numerical value connotes a linear (not negative logarithmic) increase in acidity. This would therefore be applicable to soil biogeochemistry as well as microbial physiology and medical physiology, and the notation of suffixes for magnitudes of molarity (e.g. "pico-", "milli-", etc.) already exists to allow a seamless transition from the unnecessary (and somewhat incorrect) "colog of unitless activity" to the coherent (although still lacking physical representation) of units of molarity. This reversion to "hydrogen ions" or "active protons" will at the very least lessen the suffering of students asked to think in cologs, and considering protons as charged and mobile entities will also unburden us of narrow thresholds of applicability, which has been the case when using "pH" since its inception.

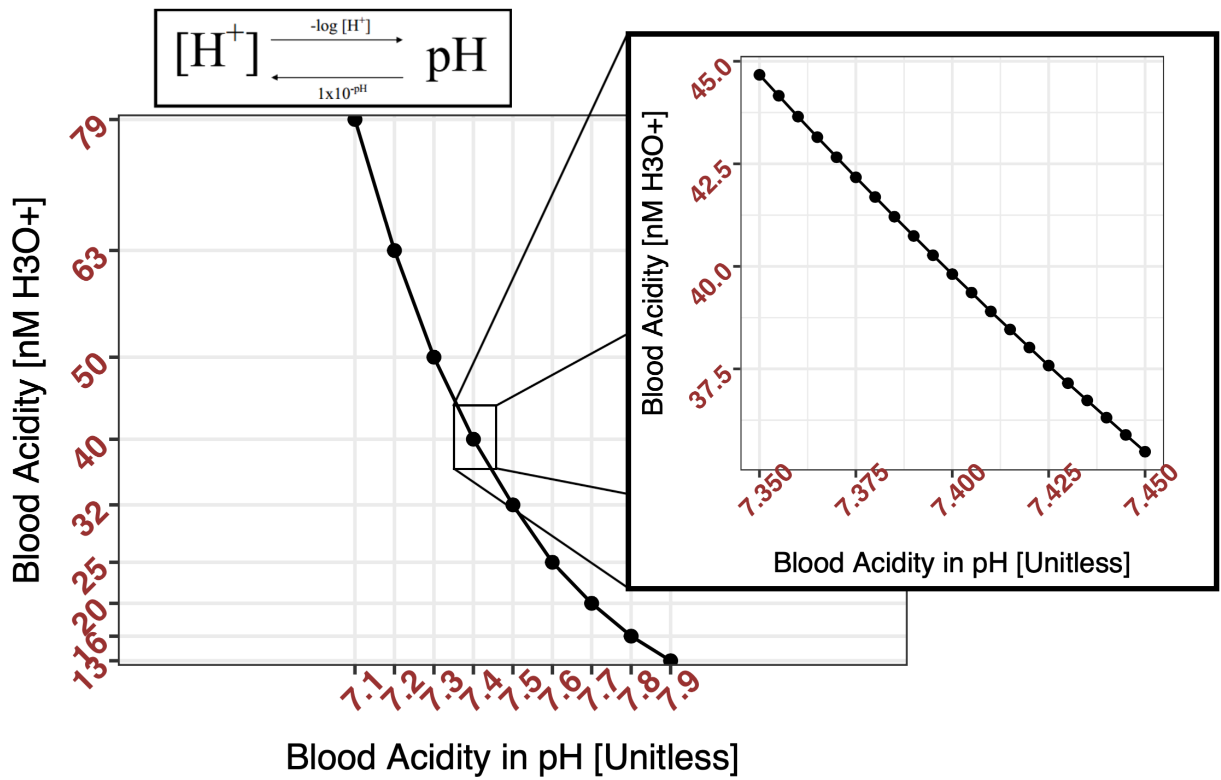



Figure 4.1: Interchangeable conversion between Henderson-like metric of "hydrogen ion concentration" and Henderson-Hasselbalch-like metric of "pH" for the healthy range of human blood acidity. "Hydrogen ions" no dot exist in blood—or any other solutions—but provide a more intuitive metric in nanomolar than the negative base-10 logarithm.

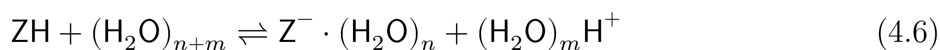
## 4.4 Metrology

The bonding dynamics of protic substances can been referred to as "proticity" (Garland 1978) to describe the states and rates of protonations per unit time per unit volume. "Temporal bond state theory" is a framework for measuring and communicating the patterns by which bond states emerge over time, referring to any substances undergoing any type of bonding together. This theory assumes that the mass and charge topology of molecules solely determine their collective behavior, be it in isolation or in a mixture. The proton carries a positive charge, and the electron carries a negative charge, of course, such that every nucleus has an inherent positive charge density and a variable negative charge density acquired as electron orbitals. In other words, postive charge density flows as positive current (proticity), and negative charge density flows as negative current (electricity), interacting over time via charge-carriers. We may alternatively call this "charge entrainment".

To conceptualize reactivity within a volume, we may merge the parametric 3-dimensional cartesian space used in calculus with the spatiotemporal mental model of the voxel used in biogeochemistry, medicine, and pedology. This entails a space with a dimensionality mimicking reality well, and it is in that space we may allow reactions of all kinds to play out. The 3-D coordinate system arranges points ($P_i$) on three axes ($x$, $y$, and $z$) stemming from a single origin, and each point is given a position where $P_i = (x_i, y_i, z_i)$. The distances ($d$) between points can be calculated by $d(P_1, P_2) = \sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2 + (z_2 - z_1)^2}$, and the function of lines and curves within the 3-D coordinate system can be parameterized by time or any other factor. Existing concepts used in geography (especially pedology) offer us an intuitive terminology to complement these mathematics. An object of a single dimension is considered a "point". An object of two dimensions is considered a "pixel", which is a portmanteau of "picture" and "element". An object of three dimensions is considered a "voxel", which is a portmanteau of "volume" and "element". We can see a pattern here, such that a "fluxel" would be the subsequent four-dimensional element undergoing a flux, and with this entity we now have both a mathematical and conceptual substructure in which to build and communicate chemical reactivity. There is no requirement to use the awkward term "fluxel", of course, but the fluxel simply represents a very useful concept for precisely measuring reactivity (e.g. autoionization, colloids, etc.) and reporting

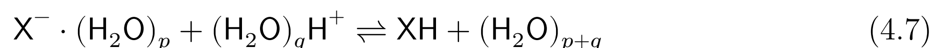
reactivity and formation/deformation rates to other investigators.

Each bond can be represented by a bond state from the point of view of one molecule, and each bond state may exist with a finite frequency and duration over the lifetime of the molecule, exhibiting a pattern of bond shifts as it accepts and donates a target chemical group (e.g. protonation, methylation, phosphorylation, etc.). The mean bond state describes the average bond state (or "level", meaning the number of such bonds) of a single molecule over a long period of bonding pattern or of many molecules of the same species and bonding pattern at once. "Bulk proticity" is the rate of protonations per second per unit volume, or the set of protic bond states per fluxel. Consider a protonated substance (Z) undergoing a proton transfer with autoionizing diprotic oxygen to produce an anion with a solvation shell and lyonium of triprotic oxygen. This reaction would have the formula



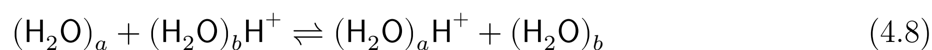
$$\mathrm{ZH} + (\mathrm{H_2O})_{n+m} \rightleftharpoons \mathrm{Z}^- \cdot (\mathrm{H_2O})_n + (\mathrm{H_2O})_m\mathrm{H}^+ \quad (4.6)$$

where $n$ and $m$ are the number of diprotic oxygen per solvation cluster, and the small dot signifies weak, or ionic, bonding. Also consider a deprotonated substance ($X^-$) undergoing a proton transfer with autoionizing diprotic oxygen to produce an anion with a solvation shell and lyonium of monoprotic oxygen. This reaction has the formula



$$\mathrm{X}^- \cdot (\mathrm{H_2O})_p + (\mathrm{H_2O})_q\mathrm{H}^+ \rightleftharpoons \mathrm{XH} + (\mathrm{H_2O})_{p+q} \quad (4.7)$$

where $p$ and $q$ are the number of diprotic oxygen per solvation cluster. We would not apply the Guldberg-Waage mass action or acid dissociation equation but instead apply a bonding regime to all species of each reaction, then assemble the reactions just as they would assemble when reacting together in the same solution. When the concentration of X or Z is high, their bonding would grow large enough to enter one another's set of bond states, encompassing the phenomenon traditionally called "ion pairing". Likewise, reactions of the kind whose reactants and products are identical, as in the case of



$$(\mathrm{H_2O})_a + (\mathrm{H_2O})_b\mathrm{H}^+ \rightleftharpoons (\mathrm{H_2O})_a\mathrm{H}^+ + (\mathrm{H_2O})_b \quad (4.8)$$

where $a = b$ and even some of the water of cluster $a$ is shared by cluster $b$, are also accounted for.

Referring to Figure 4.2, I will illustrate three commonplace examples to demonstrate temporal bond state dynamics. First, diprotic oxygen carries a dipole but no net charge. When we consider the bonding of protons with diprotic oxygen from the oxygen's point of view, the most accurate model of this species will form two patterns: (1) small, smooth oscillation as a product of hydrogen-bonding and (2) large, marked oscillations up and down from shifts in bond state as it is protonated and deprotonated over time. After a long period—or considering diprotic oxygen gaining protons to become triprotic or losing a proton to become monoprotic—the mean of the overall distribution will be approximately $2$ protons bonded to an oxygen atom. Please note that, much as an average of two different numbers represents these numbers but actually is neither, so too we must consider the mean bond state of diprotic oxygen to be non-integer despite each bond state being an integer, owing to the highly charge-dense protons creating strong covalent bonds.

Second, a bare ion, such as the univalent sodium, of course does not exist as a bare atom. Instead, when introduced to many water molecules, the exposed electron orbitals of the oxygen atoms cluster around the sodium to form a cluster. In fact, neither a bare ion nor a static solvated ion exist, because water ebbs and flows to and from the hydration shell, forming and deforming around the sodium point charge. Although a sodium ion may have in one instant $20$ water molecules clustering around it, one weakly bonded water molecule may fall away ($-1$ bond shift) and be replaced by another ($+1$ bond shift). This will be far slower than the proton bonding described above, but no less significant to the behavior of solutions upon addition of substances with ionic bonding dynamics. The mean bond state approximates the average number of water molecules clustering around the atom over a long period or considering many such molecules.

Third, and most important to this work, is proton transfer bonding dynamics. As noted above, a proton without an electron is a powerfully postively-charged atom such that it transfers extremely rapidly and strongly. The third plot in Figure 4.2 depicts theoretical bond state shifts when we take the point of view of the proton. Usually a proton is bonded to water to form any protic state of oxygen, but during a proton transfer this proton shifts down one bond state to $0$ for an instant and then returns to $1$. One will note the very steep slopes of protonation bond shifts. Although highly theoretical, the mean bond state of hydrogen is very likely almost its entire lifetime spent

in a single bond state of 1, only taking brief energetic moments to transfer to any willing molecule. As such, hydronium and even bare protons do indeed exist in solution, but they are staggeringly brief, hence the aptness of the term "flitting" for this phenomenon.

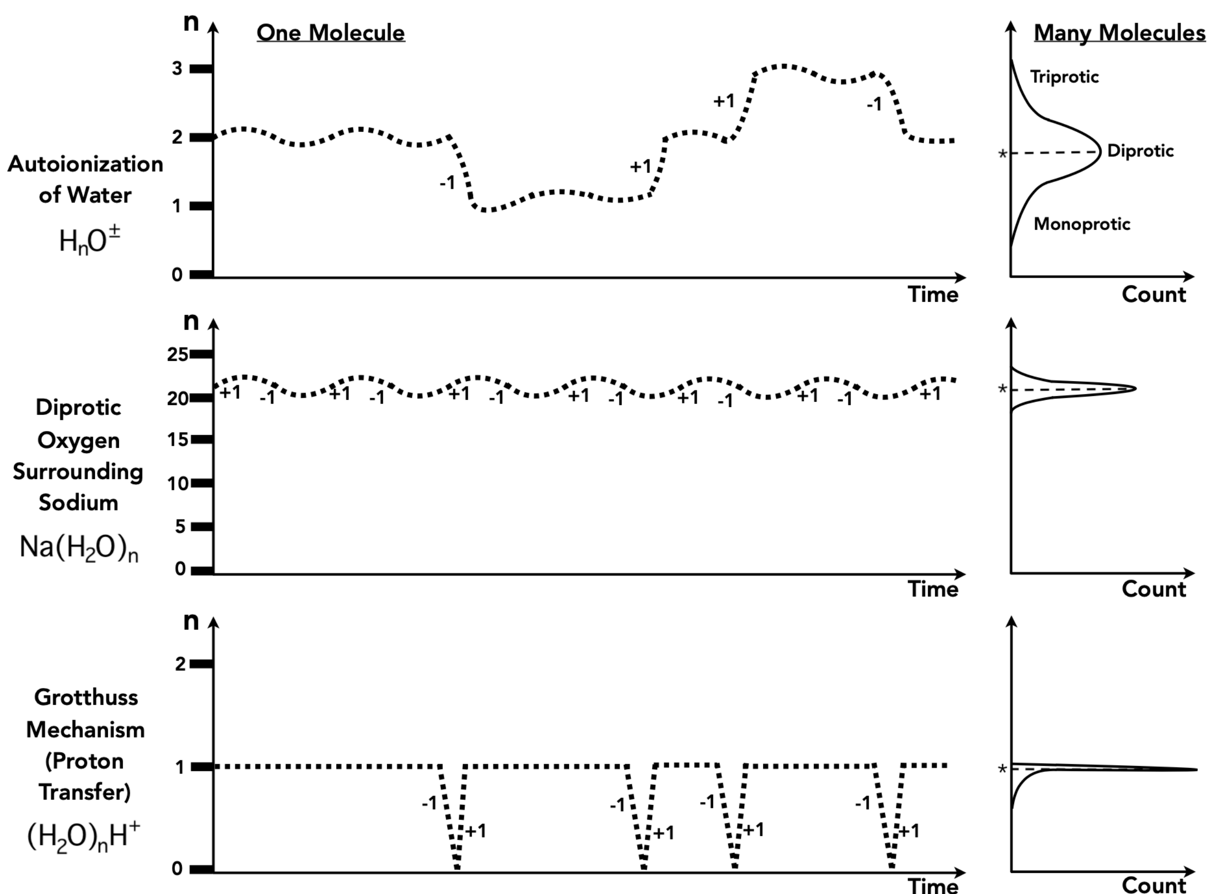



Figure 4.2: Theoretical temporal bond state curves connoting, from top to bottom, protonation and deprotonation (i.e. autoionization) of water, hydration shell of a sodium ion, and the Grotthuss mechanism as a proton is transferred, each with histograms representing mean bond state of many such curves occurring within a volume. The y-axes represent bond states in terms of number of bonds with the target chemical group, the leftmost x-axes represent time, and the rightmost x-axes of histograms represent frequency, i.e. "counts". An asterisk (*) indicates the theoretical mean bond state of many molecules of the species of interest.

A generalized bonding regime is composed of the time a particle exists in each of its finite bond states throughout its lifetime. Considering that the substance we have given the overly vague term "water" is not an entity but is in fact composed of many polar multiprotic oxygen, we would expect the set of multiprotic molelcules ($X$) to have a real mean protic state ($\mu$), and deviation ($\sigma$) over time as a result of protonation and deprotonation, creating either the familiar bell-shaped

distribution, X $\sim N(\mu, \sigma)$, or any other function that describes its shape. There exist neither "solvent" nor "solutes" in a bonding regime, only the reactive entities and particles, and therefore the mean protic bond state of the oxygen in a solution would represent the acidity of the solution. An increase in the mean protic bond state of the oxygen, e.g. from $2.01$ to $2.03$, would signify that the triprotic bond state has increased in frequency over time. Additionally, the bulk proticity of the fluxel in units of protonations per second per liter will change as substances are added to the aqueous solution, where "acids" are simply molecules that are more often deprotonated and "bases" are simply molecules that are more often protonated. Every molecule would then have a lifetime in the solution composed of dynamic protic bond states as they interact, which togehter emerge as acidity. Protonation events are extremely rapid (nearly vertical slope of bond transitions), whereas ionic bonding is extremely sluggish (nearly horizontal slope of bond transitions), as depicted by the theoretical bond state curves in Figure 4.2.

Acidity has traditionally been a measurement following the question, "*How many* hydronium are there per liter?" despite hydronium only being an intermediate of many rapidly forming and deforming cations of water undergoing autoionization. Considering instead temporal bond states instead, we may now ask the question, "*How long*, as a percentage of its lifetime, is the average molecule in triprotic state?" Therefore, neutrality could be more accurately represented as the maximum bulk proticity, or protonations per second per liter, in a system (see Figure 4.3). This concept would better explain why the neutral point of "pH" changes with temperature, as higher heat content will cause more and even faster protonations per second per liter. This would also better explain why life on Earth tends to regulate its acidity, be it called "neutral", "having a pH of $7.0$", or "maximum bulk proticity" that signifies the highest rate of protic bonding at the lowest energy state.

Proticity is measurable using a glass probe to test the theories above. The glass probe's surface that interacts with the internal and extrenal solutions are partially hydrated, forming a silica gel that undergoes competetive cation exchange (Eisenman 1967, 177–78) of protons with all other cations in solutions. The internal solution of the probe does not change, but the extrenal analyte solution will change. The protonation of the outer surface of the bulb reaches a stable potential measured in millivoltage of the potentiometer in one solution, then rises or falls when the probe

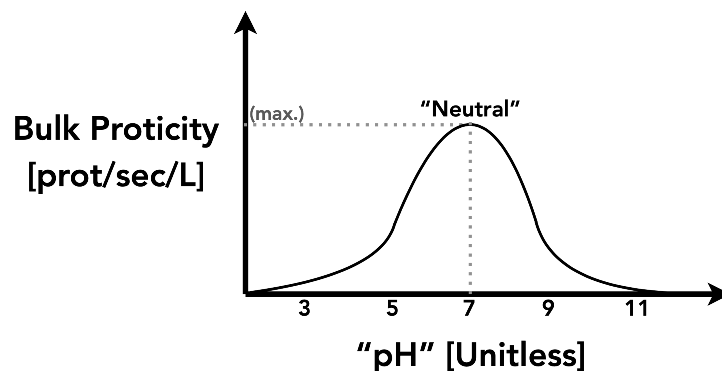



Figure 4.3: The representation of "neutrality" as maximum bulk proticity. Both highly acidic and highly basic solutions have decreased rates of protonation and deprotonation per second per unit volume.

is moved to a new analyte. The change in potential is often measured by the Nernst equation to determine the change in acidity, but rarely is the *rate of change in potential* measured as well. The ionic strength loses its influence because it can be made equivalent between the solutions without interfering with the measurement from the glass probe, which bypasses the dilute solution assumption of Debye-Hückel theory.

The curve observed during operation of a glass probe (Figure 4.4) strongly resembles the rightmost half of the familiar logistic function:

$$N = \frac{K}{1 + be^{-rt}} \ ; \ b = \frac{K - N_0}{N_0} - 1 \tag{4.9}$$

The maximum value here is instead the final potential at the final time, the inflection point is the initial potential at the initial time, and steepness ($r$) of the curve the indicator of proticity, which slows as the surface reaches the equilibriated protic bond state. This model then requires a real-time reading from initial time ($t_i$) to final time ($t_f$) of potential ($\phi$) plotted over time in seconds.

Acidity measurements are therefore captured by the relative potential and change through time in bulk proticity,

$$\alpha = t_f - t_i \ \ ; \ \ \beta = \phi_f - \phi_i \ , \tag{4.10}$$

and therefore

$$f(\phi, t, r) = \frac{\phi_f - \phi_i}{1 + be^{-r(t_f - t_i)}}. \tag{4.11}$$

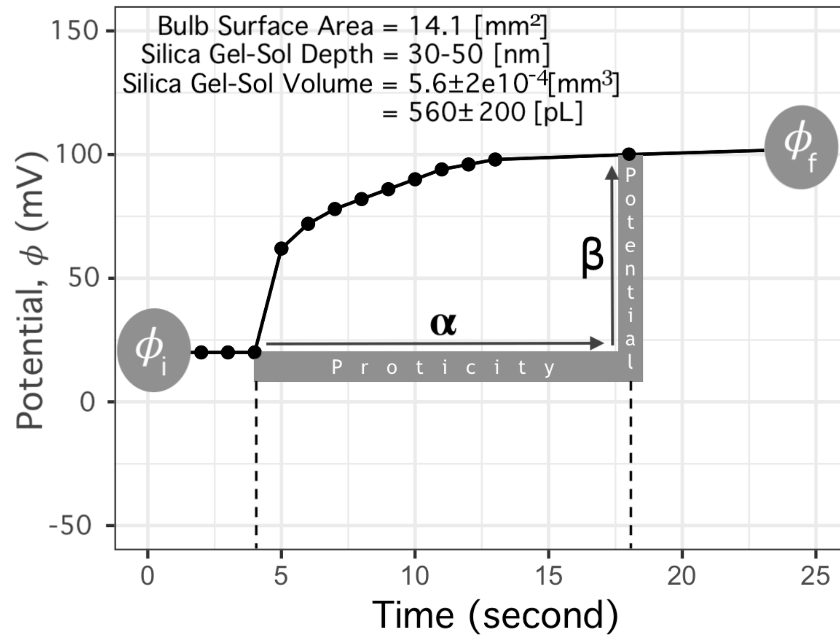



Figure 4.4: Real-time reading of a potentiometer connected to a glass probe inserted in the supernatant of a soil suspension, like all pH measurements, requires roughly 30 seconds to one minute to "saturate" and "stabilize", which represents the rate at which and the extent to which the hydrated silica surface of the glass probe's acidity-sensitive bulb has protonated. Although rarely recorded, this time to stabilization represents the proticity of the solution, which represents the acidity.

In finding the derivative of the model above, we may also incorporate Michealis-Mentin kinetics,

$$r_{\text{prot}} = \frac{V_m C_{\text{prot}}}{K_{\text{prot}} + C_{\text{prot}}} \tag{4.12}$$

where $r_{\text{prot}}$ is the protonation rate, but $C_{\text{prot}}$ would signify the concentration of protonated sites, $V_m$ the maximum rate of protonation in the system, and $K_{\text{prot}}$ the concentration where the reaction rate is half its maximum ($V_M/2$).

The time it takes to protonate via cation exchange of the glass bulb's surface, whose density of protonation sites per silica gel can be measured using gel depth with density and surface area of the bulb (see Figure 4.4), represents the proticity of the solution in contact with the bulb. If the two solutions have equivalent ionic strength, the glass probe will detect the different charge density caused by the substance of interest in that particular mixture. For example, a glass probe will equilibrate to an initial potential in any solution then, upon the addition of some substance whose influence on the acidity of the solution we wish to measure, will shift to a final potential. From the shape of the curve of this shift we may interpolate proticity, and because proticity represents

real particles undergoing bonding and not the purely notional theory of "pH shifting up or down", we may infer both the energy and mass action that have occurred. Measuring acidity in this way would be of particular interest to soil chemists because soils contain very complex surfaces of silica gels that closely resemble the surfaces of glass probe bulbs. Furthermore, there are many opportunities to explore the coating of glass probes with organic films—or even embedding these coatings with bacterial cells themselves—to further resemble the skins of organic matter that typically coat soil particles.

The theory of acidity as proticity in response to the currents of net positive charge density and negative charge density therefore explains why NaCl does not acidify a solution while HCl strongly acidifies a solution. The positive charge density of the briefly flitting $H^+$ as it enters the matrix of protic oxygen is greater than the negative charge denstity of $Cl^-$, whereas the positive charge density of $Na^+$ and the negative charge density of $Cl^-$ are nearly equivalent. The difference in final charge densities causes the solution to exhibit acidity (higher positive charge density) or alkalinity (lower positive charge density). Proticity solely relies upon the mass and charge densities of the substances of interest themselves, without reliance on operational notions of "pH" or even "standard states". Proticity also creates opportunities to explain the behaviors of ammonia, nitrate, phosphate, surgars, cellulose, alcohols, and any species whose protonations and deprotonations over time determine their behaviors, and temporal bond state theory offers opportunities for understanding methylation, phosphorylation, beta-keto acid decarboxylation, micelles, and other chemical groups or aggregates. We may also consider a bacterial cell or mitochondria as a fluxel and perhaps ask, "What is the ratio of protonations to phosphorylations per second per femtoliter of this cell?" and then "zoom in" to the ATP synthase or "zoom out" to the entire cell, reporting these values at any and all scales. In the same manner, we may ask, "What is the rate of cation exchange of protons in place of aluminum on the silt particles of this vertisol?" and then "zoom in" to the silica surface and "zoom out" to the whole profile, reporting these values with greater commensurability than "soil pH" would allow. It is no coincidence that the term "lyotropic series", which describes the affinity of different ions for particle surfaces, has the same root as Bjerrum's term "lyonium".

## 4.5 Experimentation

Consider again the "jugglers model" of acidity presented above, which I have formalized as "proticity". What would happen if a truckload of props (protons) were dumped into one side of a large group of these jugglers (multiprotic oxygen), most of whom have only two props, minding their own business? The new props will enter one side of the group, causing a flurry of (re)activity, but the transfer of props from each newly overwhelmed juggler to their hapless neighbor across the room will be much faster than the slow migration of the newly introduced props themselves. Even the jugglers at the farthest opposite end of the room may acquire three props in a matter of minutes, but the new props will not diffuse rapidly at all.

Using this mental model to return to reality once more, I suggest that by the combination of electric current and protic current, acidity itself would travel many times faster than diffusion of an acidic substance itself. By using an experiment similar to that of Theodor von Grotthuss (1806), one can in fact view in real time how the proticity of aqueous solutions enables the positive charge density of an acidic solution to conduct through a solution.

### 4.5.1 Methods

I used a flame to shape an unusued borosilicate tube of $25$[cm] total length, $6$[mm] inner diameter, $9$[mm] outer diameter, and $8.91$[mL] volume as measured gravimetrically, into a u-tube (called a "G-tube" as it resembles the same tube used by Grotthuss (1806)). The G-tube was primed with $0.01$[M] KCl and thymol blue, which is an acid-sensitive dye that changes color according to the pH scale from blue to yellow at $8.0$, from yellow to borwn at $3.0$, and from brown to crimson at $1.2$. The two probes (Mettler-Toledo InLab Micro) were "co-equilibrated" by connecting their bulbs with a short length of tube that was filled with $3.0$ [M] KCl and allowed to rest until their respective potentiometric readings were within $15$ mV of one another and stable (see Figure 4.5) as the internal solutions reached neutrality with one another across the liquid junctions (glass frits) of both probes.

I arranged the experiment (Figure 4.6) to capture via camera the change in color of the thymol blue and overlayed these images with the simultaneous measurements of real-time potential from the two microprobes. These probes were calibrated to the pH scale using NIST traceable standards $4$,

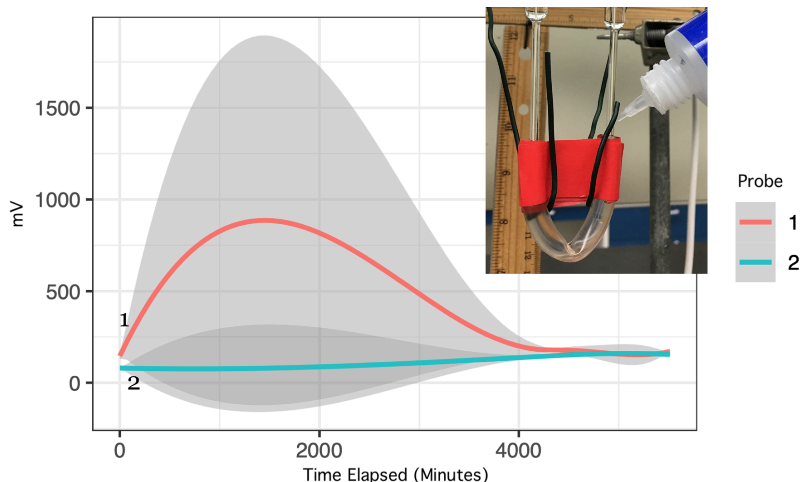



Figure 4.5: Co-equilibration of two glass microprobes in 3.0 M KCl.

7, and 10 prior to beginning the experiment, but millivotages were also reported. The experiment consisted of inserting each probe into its respective end of the G-tube, adding $400[\mu\text{L}]$ of $0.50$ [M] HCl to the side where probe 1 was situated, and recording images and potential readings for at least $60$ minutes.

The slow migration of particles through a solution is ostensibly governed by Fick's law,

$$J = -D\frac{d\varphi}{dx}, \tag{4.13}$$

which is a relation of diffusion flux ($J$), the diffisuion coefficient ($D$), concentration of the substance ($\varphi$), and position ($x$). Fick's second law predicts diffusion with respect to time ($t$):

$$\frac{\partial\varphi}{\partial t} = D\frac{\partial^2\varphi}{\partial x^2}, \tag{4.14}$$

and the simplified equation for time of diffusion is

$$t \approx \frac{x^2}{2D}. \tag{4.15}$$

$D_{HCl}$ varies in its estimation because $D_{\text{H}^+}$ is often reported as $9.31[10^{-9}\text{m}^2\text{s}^{-1}]$ and $D_{\text{Cl}^-}$ is often reported as $2.03[10^{-9}\text{m}^2\text{s}^{-1}]$. Lobo, Helena, and Teixeira (1979) have estimated the diffusion coefficient of HCl at $298[\text{K}]$ and $0.1[\text{M}]$ to be $3.017[10^{-5}\text{cm}^2\text{s}^{-1}]$, and at $0.01[\text{M}]$ to be $3.165[10^{-5}\text{cm}^2\text{s}^{-1}]$. This latter estimate is $50\%$ higher than the other estimate, but this could be the weighted mean of $D_{\text{H}^+}$ and $D_{\text{Cl}^-}$ or an estimate at a much lower concentration of HCl. (Note: $[10^{-5}\text{cm}^2\text{s}^{-1}]$ is the same unit as $[10^{-9}\text{m}^2\text{s}^{-1}]$.)

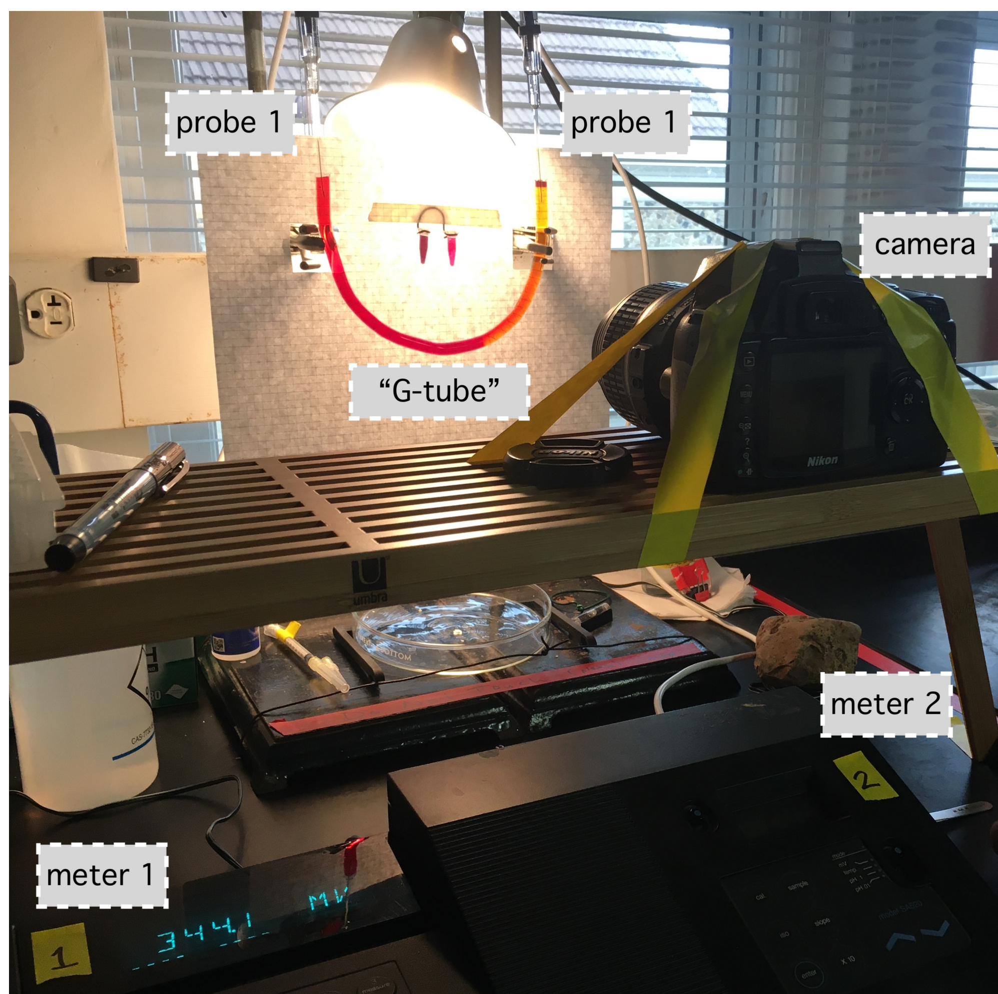



Figure 4.6: Experimental arrangement for the "Grotthuss-Mitchell experient", measuring proticity (1) visually by the acid-sensitive dye thymol blue and (2) potentiometrically by co-equilibrated glass probes.

#### 4.5.2 Results

The experiment progressed in roughly four stages (Figure 4.7.A-D).

A. The denser HCl solution dropped through the leftmost tube length and to the bottom of the G-tube, but the HCl did not ascend the rightmost length of the tube. This created a gap between the bolus of HCl and Probe 2 of apprximately $10$ [cm]. Probe 1 clearly shows the acidity greatly increase.

B. The HCl at the bottom of the G-tube began to mix with the rightmost solution, causing a visible change in color at the interface but no response from Probe 2.

C. Although the HCl could only diffuse at a rate predicted by Fick's law, which is on the order of only several millimeters per hour, both Probe 2 began to show the solution around its bulb increased in acidity and the entire column itself changed color from blue to yellow in less than one minute.

D. The solution surrounding Probe 2 continued to increase in acidity, asymptotically approaching the acidity of the solution surrounding Probe 1.

Acidity, as detected simultaneously by both an acidity-sensitive and two glass microprobes, was transferred across $10$ [cm] of dilute electrolyte solution at a rate several hundred times faster than diffusion of chloride according to Fick's law. This suggests the flow of positive charge outpaced diffusion, drawing into question the applicability of Fick's law—as well as pH as an activity of hydrogen ions—to acidity. Both Fick's law and pH are defined in terms of change in concentration or activity, whereas proticity is a model that describes acidity in terms of the response of protons to charge density (i.e. undergoing protonations). The results suggest that a charge imbalance across the tube induced a charge transfer (current), whereby electric charge (electricity) traveled from Probe 2 to Probe 1 and the complementary positive charge (proticity) traveled from Probe 1 to Probe 2, demonstrated by the shift in potential far outpacing chemical diffusion.

### 4.6 Conclusions

Theories founded on the deprecated notions of "pH", "acids", and "bases" do not characterize acidity as well as ready alternatives, and I have presented acidity as proticity within the framework

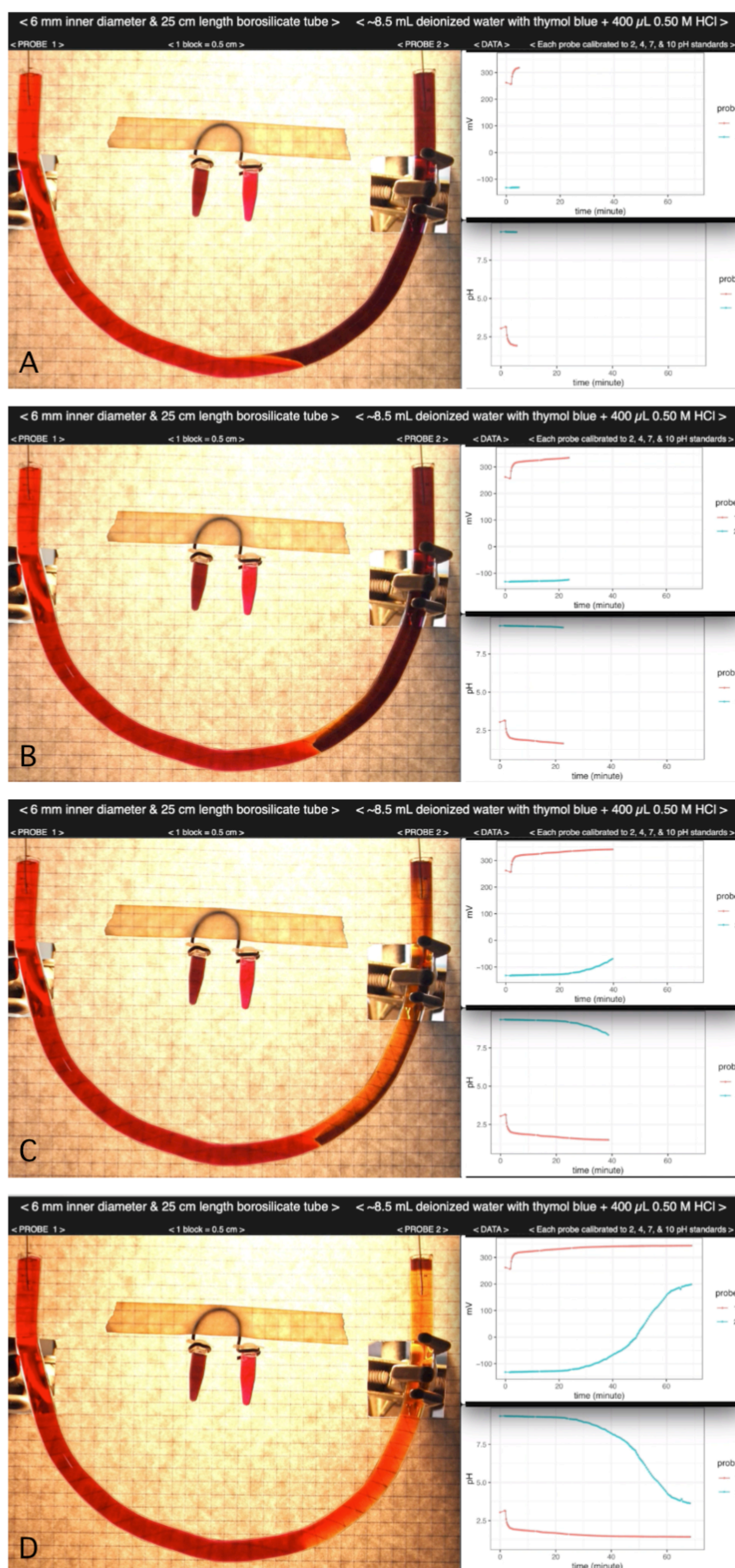



Figure 4.7: Four stages of the “Grotthuss-Mitchell experient”.

of temporal bond state theory. "pH" describes solutions of HCl up to $0.1$ [M] but little else, and the continued use of negative logarithms is unnecessary, if not cruel to students and researchers alike, in order to describe acidity. The theory of "pH" masks the fact that the proton, as it exists in protic mixtures, has no single chemical entity but rather has an ineluctable motion and transfer resulting from a large positive charge density. Proticity and bulk proticity are extensive, non-notional, unitful physical properties of all aqueous solutions, from dilute to concentrated.

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# 5 The Iplate Suite: Affordable and Customizable Implementations of Ichip Technology

## 5.1 Overview

Small innovations in instrumentation can have large impacts on experimental research. Julius Richard Petri's container, composed of two flat glass disks with interlocking edges (Petri 1887), was a brilliant novelty during the late 19th century, simultaneously preventing the open-air contamination that plagued microbiologists working in military and medical research organizations and making the chamber of a portable size and shape for use with a microscope to inspect colony growth and structures. Petri's dish did not spring from nothing but was rather a modified version of the existing container used by Petri's supervisor, Robert Koch, c. 1881. Koch's container used one flat dish holding the "nutrient gelatin", complete with a glass bell-shaped cover that was stationary and unwieldy to handle, especially with one hand. Petri's minor alteration of Koch's incubation chamber to be flat, mobile, and contained enabled the Petri dish to become a workhorse in microbiological laboratories across the world today.

Soil microbiologists have typically begun their experiments with such Petri dishes, or "plates", which also present several key obstacles (Lagier et al. 2015; Gao et al. 2013), owing to the many taxa of bacteria that are uncultivable on plates, colloquially known as the "Great Plate Count Anomaly" (Amann, Ludwig, and Schleifer 1995). The counts of colonies on Petri dishes has not kept pace with cultivating the microbial taxa known to live in the environment, especially soils. To greatly improve upon plates, a diffusion microchamber device called the "ichip" (Nichols et al. 2010)[4] has proved very successful, continuing the progress initiated by Koch and Petri. Much as Petri's dish was a minor but not insignificant improvement upon Koch's dish, the purpose of this study is to present small alterations of the ichip device such that the technology can be both more customizable and more affordable. One does not wish "to reinvent the wheel" but, rather, to facilitate the incorporation of diffusion microchambers into the work of scientists and students of soil microbiology, many of whom wish to test the growing number of hypotheses emerging from new cultivation-independent methods (Amann, Ludwig, and Schleifer 1995). Many schools also have the software and machines necessary to produce the devices described here in "fab labs" and "makerspaces". Soil microbiology today is closer than ever to solving the problem of creating realistic *in situ* soil conditions of directly soil-derived microbial growth factors while additionally

[4] The ichip has also been spelled "Ichip" and "iChip", but here I have chosen to use "ichip" simply as a noun whose first letter is capitalized only at the beginning of a sentence.

isolating target microorganisms, and our intent is that the use of diffusion microchambers will further improve discovery and education in scientific outreach programs, such as Jo Handelsman's Small Worlds Initiative (Davis et al. 2017).

The ichip is an invention of the research group of Dr. Slava Epstein (Nichols et al. 2010) to enable free exchange of isolates with soil solution—or any other sol- or gel-like media—without contaminating the culture. The device allows the investigator to create soil microhabitats and control the conditions and inoculum added while still allowing for internal and external soil solution to exchange freely with the cells. The dimensions of the original device are roughly $2.5$ [cm] in width, $7$ [cm] in length, and $3$ [cm] in depth, composed of three parts: a thin hard-plastic plate of polyoxymethylene (POM), polyethylene glycol (PEG), or equivalently hard plastic, sandwiched by two thicker plates of the same plastic, or aluminium (Figure 5.1). All pieces have $4$ to $6$ through-holes for fitting screws and inset nuts plus two arrays of very small ($< 1$ [mm]) through-holes (approximately $192$ per array) grouped together in the diameter of standard $0.02[\mu$m] filters. By applying pressure on these filters covering the microchambers of the internal plate, the array of microchambers allows the diffusion of dissolved organic carbon and other components of the soil solution but disallows the movement of microbial cells to or from the soil or from one microchamber to another. Cells extracted from soil are diluted into an agar or alginate solution and injected into the through-holes of the inner flat rectangular plate. One cell inhabits each microchamber, suspended between two filter membranes in contact with moist soil, thereby simultaneously isolating and cultivating microorganisms, without preparation of a defined media such as nutrient agar. The ichip is incubated directly in soil in the field and recovered for analysis via microscopic inspection, transference to plate-cultivation, and genetic sequencing assays.

Although highly innovative, the ichip is somewhat restricted in its implementation. There are great advantages to using a diffusion-based system of isolating while cultivating microorganisms, but there are also challenges with this device that inspired the creation of the various iplate extensions of the technology. These challenges include the following: (1) the minute size of the plugs on/in which microbes grow, (2) the density of plugs as they are arrayed on the device, (3) inoperability at the scale of standard pipettes, plates, etc. in most life science laboratories, (4) unwieldy assembly and disassembly, with high risk of contamination, and (5) unaffordable manufacture with customization for most investigators. I propose here three types of modified ichip devices called the iplate suite (with the arbitrary names "Type 1", "Type 2", and "Type 3"). The suite of iplates is both a series of designs that build on the technology of the ichip ("Type

0") as well as the philosophy of each investigator or research group making their own diffusion microchambers with regard to their specific research needs and system(s) of interest.

## 5.2 Approach

The novel types of iplates presented in this work can be produced using a 3D printer or laser-cutter (see Supplemental Figures 5.8-9) and inexpensive materials (silicone, Delrin, thumb screws, and dialysis tubing). There are three types, which together were nicknamed the "iplates" because the first type originally resembled a 96-well-plate. Iplate devices have fewer microchambers than the ichip, but the array of microchambers can be modeled after a standard 96-well plate, be one single chamber instead of an array, or any other desired shapes and sizes. For example, the iplate modeled after 96-well plates has the advantage of allowing interoperable use with multichannel pipettes and multi-pronged replicators ("froggers"). All variants of iplates also have the advantage of using larger parts, allowing for more rapid and careful assembly and disassembly by hand while simulaneously lessening the risk of cross-contamination pre- and post-incubation. The larger microchambers are more easily viewable under a microscope as well, having much larger areas into and across which isolates may freely grow.

The shape of iplate devices is purposefully vague because they are not not manufactured by a company or ordered from a distributor, but instead customized and created from simple materials by investigators themselves with their own exact needs in mind. We have listed the specifications for using a 3D printer or laser-cutter and provided referrals to open-source software as well as several templates, the machines for which are increasingly ubiquitous and available today in the workshops and laboratories of engineers, academic scientists, and industrial researchers.

The following redesign of the ichip device makes several changes: (1) more rapid transfers aligned directly over multi-well plates, (2) wider spacing lowering risks of cross-contamination between microchambers, (3) the addition of pools above the microchambers for loading soil for in-lab incubation without sacrificing *in situ* growth conditions, and (4) wider availability for future customization and manufacture for experimentation with a maximum variety of media, microbes, and protocols.

## 5.3 Membranes

Membrane-bound diffusion microchambers offer investigators highly increased ability to incubate isolated soil microorganisms without contaminating simulated soil microhabitats with soil particles

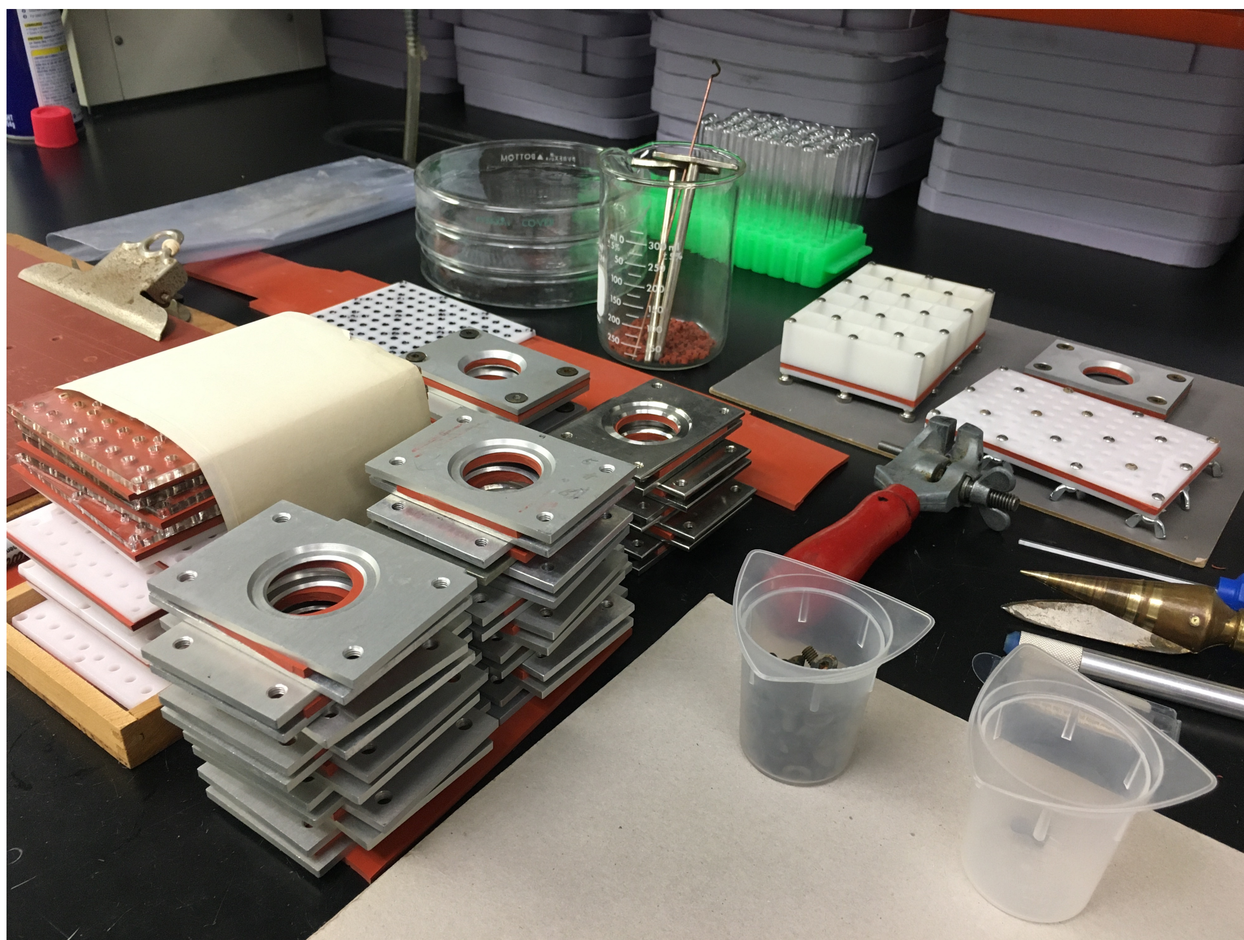

Figure 5.1: Ichip/Iplate devices workshop, or “microchamber makerspace”. The inexpensive materials for iplate devices include sheet(s) of Delrin (1/8”), sheet(s) of silicone (1/8”), 4-40 or 9/16 button cap bolts, and nuts (button, wing, thumb, etc.).

or non-target microorganisms. Here we will describe the exact functionality of this technology, which utilizes diffusion, dialysis, and osmosis of gels, sols, and membranes. Familiarity with the options available to enable these processes is required to design and customize microchamber array devices for soil microbial ecologists and other microbiologists with diverse research goals. In the following specifications, a cellulose dialysis tubing membrane allows for the passage of small molecules to travel between the inside and outside of the membrane.

Standard medical-grade dialysis tubing (Aldon Corp or Ward's Science+ Dialysis Tubing, LOT AD-17157) the simplest and most affordable cellulosic membrane. This material is composed of highly purified crystalline cellulose, which, when dry, acts like a tough plastic sheet and, when wet, is very soft and pliable but inelastic. The membrane, when it is chemically saturated, allows the passage of molecules that are approximately $< 14$ kDa, which would include vitamins, disaccharides, very small complex carbohydrates, amino acids, small peptides, nucleic acids, and small RNA/DNA. Drawbacks to this material include the fact that numerous soil taxa produce enzymes that target and degrade cellulose, and therefore its use in long-term incubations may be limited. An additional disadvantage is the fact that purified cellulose will chemically adsorb soluble compounds in the system. Therefore the membrane may greatly deplete solutes from soil solution. To reduce the potential robbery of nutrients from the bacterial cell intended to grow and readily divide at the upper surface of the microchamber plug, one may saturate the membrane in a mixture of biomolecules commonly found in soil solution before inserting it into the device and incubating. This "growth factor bath" ideally includes amino acids, nucleid acids, a variety of disaccharides, and a small amount of albumin to mimic soil glomalin.

Depending on the procedure of its preparation, a cellulosic membrane can become a threat to the success of an organism inside of an isolation-cultivation microchamber device. One must consider cellulosic membrane as less of a 2-dimensional boundary and more of a very thin 3-dimensional section of denser material. Dialysis tubing is not a sheet with perfect throughhholes but is instead a firm gel when saturated. This material is sufficiently thick that will interact physically and chemically with both the soil solution outside a microchamber and with the gel solution inside a microchamber. I note this distinction not for semantic argumentation, but for the practical consideration of ensuring sufficient moisture and substrate leaching from the soil via diffusion and dialysis to the microchamber gel, which is the sole source of nourishment to microbial isolates. The membrane will perform absorption of the weaker physical kind, whereby the solute can diffuse from one region to another somewhat freely, and the membrane will perform adsorption of the

stronger chemical kind, whereby solutes will bind to the many chemical groups of higher charge-density that compose surfaces of cellulose crystals that essentially "stain" the membrane rather than diffuse across to the gel as desired.

## 5.4 Description of Ichip/Iplate Devices

### 5.4.1 Type 0: Original ichip

The ichip is a device that creates very, very small diffusion microchambers, so small they become unwieldy to load and unload. The ichip is also referred to in this work as "Type 0" and has the following specifications:

- Thin black polyethylene glycol (PEG) "middle plate" with $2$ side-by-side arrays of $179$ through-holes each.
- Aluminum housings ("upper and lower plates") for containing the membrane-bound plugs.
- Hardware of bolts (the upper and lower plate housing has internal threaded bolts).
- Dimensions when assembled: $1.4$ [cm] x $7.2$ [cm] x $2.4$ [cm].

The original ichip is a device that was presented by Nichols et al. (2010), and operates by the following protocol:

1. Sterilize all parts of ichip with a combination of autoclaving, alcohol, flame, and/or UV.
2. Inoculate warm agar (as cool as possible but still molten/liquid) with bacteria or other cells such that there is roughly $1$ cell per volume of each microchamber.
3. Dip central plate into agar, let cool a moment, and scrape away excess to leave the surface of plugs in the through-holes flush.
4. Apply membrane to each side of the central plate.
5. Place the membrane-bound central plate in the upper and lower plate housing.
6. Insert and tighten screws with sufficient pressure to prevent cross-flow between microchambers.
7. Bury in a pot of soil/sediment in the laboratory/incubator or the field for any desired length of time.
8. Disassemble the device in reverse order, being careful to prevent cross-contamination between chambers. This can be assisted by using clips to hold the membranes flat to the central plate while it dries, but this does not guarantee zero contamination.
9. Use the tip of a straightened paper clip to push the plugs out of the central plate into a tube or well-plate for analysis or resuspension for another incubation.

10. Clean and refurbish the metal and plastic parts of the device for storage and later use.

### 5.4.2 Type 1: "Deep-96" Iplate

The "Deep-96" iplate device (Figure 5.2 and Figure 5.3) suspends a bolus of media (liquid, solid, gas, colloid, suspension, soil, or any other material) maintained above $4$ microchambers.

- Upper plate: Laser-cut Delrin.
- Middle plate: Laser-cut Delrin or punched silicone.
- Lower plate: Laser-cut Delrin.
- Membrane: Cellulose dialysis tubing or plastic nuclear pore.
- Hardware of long bolts and thumb-nuts or wing-nuts.
- Through-holes dimensions: thickness of $1/8$" ($3.175$ [mm]) and diameter of $3.0$ [mm].
- Dimensions assembled: $11.5$ [cm] x $3.5$ [cm] x $7.75$ [cm].
- Microchamber volume $= \pi r^2 h$, where $h = 3.175$mm and $r = 1.5$cm $\rightarrow 22.4[\mu$L]. (Note: The laser cutter causes a slight cone-shape instead of a perfect cylinder as the melted Delrin slightly pools as it cools, so the real volume may be $5\%$ to $10\%$ smaller, approximately $20[\mu$L].)

Assembly & Incubation:

1. Sterilize the plates, hardware, and membranes using a combination of autoclaving, flame, soaking in alcohol, or UV treatment (the latter works well on the membrane).
2. Partially assemble the iplate using the lower plates that have screw-holes but no through-holes, one sheet of membrane, and the central plate. This can be held together with strong clips, clamps, or with the nuts and bolts.
3. Pipette sterile molten agar or other gel medium, with or without added nutrients or buffers, into the open through-holes of the central plate.
4. Remove the lower plate and replace with a lower plate that has through-holes, then pipette dilute inoculum onto the top of the resulting plugs situated in the central plate.
5. Apply a membrane to the central plate and finally the "deep-well" upper plate with partitioned through-holes to the assembly, inserting its longer bolts to tighten with wing nuts.

Disassembly:

1. Remove the device from its incubation cabinet, any parafilm covering it, and all hardware. Keep the plates flush and apply a little pressure with your fingers or clips/clamps as it is

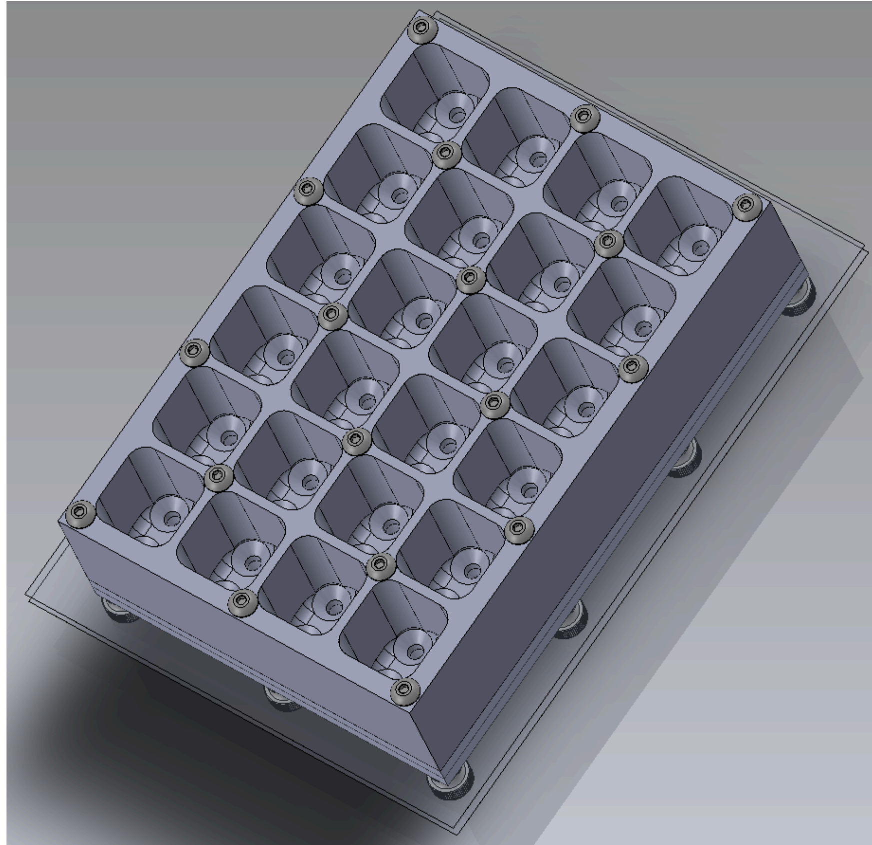

Figure 5.2: “Deep-96” iplate device parts and assembly. Note the 4 chambers housed in each “well”, which holds a bolus of media.

Figure 5.3: Applying water to assembled and loaded Type 1 “Deep-96” iplate device. This assembly can then be wrapped in parafilm or put in a cabinet or incubator.

disassembled.

2. Keeping the upper membrane flattened to the top of the middle plate, remove the upper plate (96-well with deep pools).
3. Peel away the membrane from the upper side of the middle plate. You need to allow these membranes to dry a little to prevent a flowing meniscus that travels across the plate as the membrane is peeled away. This can cause significant contamination, which makes this the most crucial step in the protocol, especially if the chambers of this plate each have different isolates.
4. Remove the lower plate from the middle plate while keeping the membrane flattened to the bottom of the middle plate.
5. Peel away the membrane from the bottom of the middle plate, first allowing the membrane to dry a little to prevent the "flowing meniscus".
6. Check that the plugs are intact, and note whether any plugs have dried out or are misshapen from intermittent drying during incubation.

#### 5.4.3 Type 2: "Flat-96" Iplate

This device is assembled and disassembled in a virtually identical manner to Type 1 (see above and Figures 5.4 and 5.5). This type cannot hold soil the way the "deep-well" iplate can, but it is more adaptable to burying in porous media like soil or suspending in a liquid medium if desired. It is recommended, however, that the Type 2 iplate be loaded into soil on a $45^{\circ}$ angle, because the water that is added neither pools in the through-holes of the upper plate, as it would if loaded into soil horizontally, nor flows off entirely, as it would if the device were loaded into soil vertically.

- Upper plate: Laser-cut Delrin.
- Middle plate: Laser-cut Delrin or punched silicone.
- Lower plate: Laser-cut Delrin.
- Membrane: Cellulose dialysis tubing or plastic nuclear pore.
- Hardware of long bolts and thumb-nuts or wing-nuts.
- Through-holes dimensions: thickness of $1/8$" ($3.175$ [mm]) and diameter of $3.0$ [mm].
- Dimensions assembled: $3/8$" ($9.525$ [mm]) x $11.5$ [cm] x $7.75$ [cm].
- The volume of the microchambers is the same as the "deep-well" plate (specs listed above in that section) because the silicone middle plate is identical here.

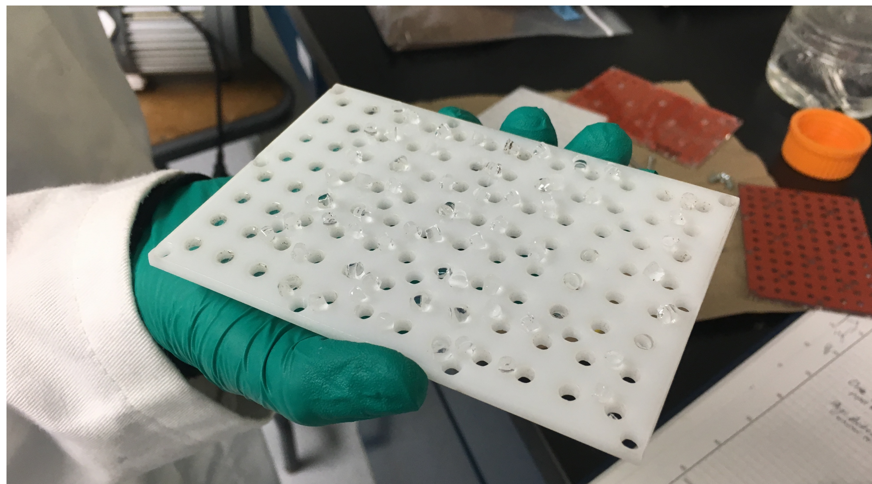

Figure 5.4: A test of agar plug shape, size, and durability when removed or transported from the central plate of the Type 2 iplate (“Flat-96”). When loaded with agar, this design allows for the use of a multichannel pipettte and produces plugs of an optimal size for inoculation and subsequent manipulation, microscopy, etc.

Figure 5.5: Type 2 Iplate assembled and buried in soil.

#### 5.4.4 Type 3: "Flat-Coin" Iplate

This device is an adaption of the "Rose chamber", which is a device used largely in phycology to observe the real-time growth or behavior of algae or protists. Originally, the upper and lower housing plates held a silicone sheet with a "coin" of material removed, and the central silicone plate was contained with large glass cover slips. These glass parts have a tendency to crack, so the pressure applied only gently holds the system together. Once one removes the glass and replaces this with a membrane, such as dialysis tubing, for use as a diffusion chamber, the microbes, or "bugs", can be grown inside the chamber with the soil or medium on the outside, or the bugs can be grown outside the chamber with the soil or medium on the inside.

- Upper and lower plates: steel or aluminum $\approx 1/8$" $(3.175$ [mm]).
- Central plate: silicone with thickness of $1/8$" $(3.175$ [mm]).
- Dimensions assembled: $7.5$ [cm] x $5.0$ [cm] x $0.9$ [cm].
- The volume of the internal chamber is $\pi r^2 h$ where $h = 3.175$ [mm] and $r = 1.25$ [cm] $\rightarrow$ $1.6$ mL.

##### 5.4.4.1 "Soil Inside, Bugs Outside" (Protocol)

1. Assemble the lower plate, membrane, and middle plate with the nuts and bolts (or clips/clamps). A washer may help as the head of the bolt is directly on the surface of the silicone middle plate.
2. Load soil into the coin-shaped hollow of the middle plate, gently tamping it down. It helps to partially wet the soil before loading, which also prevents clays and silts from blowing onto the outer surface of the middle plate, which can cause problems for later sealing via pressure from the upper membrane and upper plate.
3. Remove hardware (or clips/clamps if you used those instead) and apply another membrane and upper plate.
4. Insert and tighten the nuts and bolts such that the internal chamber is sealed from its exterior environment.
5. Inoculate the top and/or bottom of the "coin" of soil. Streaking may be possible as well, just as one would on the gel of a Petri dish. Alternatively, a thin inoculated gel may be applied to the surface instead, which would allow for the diffusion of growth factors from the soil to the gel, but this is not guaranteed to function because the diffusion will be very, very slow (see Fick's law).

6. Incubate in a humid space and alternately wet the surface if it appears to dry out. Some soils can be very absorbent, even after initial wetting to help load the soil into the device.

#### 5.4.4.2 "Bugs Inside, Soil Outside" (Protocol)

1. Assemble the lower plate, membrane, and middle plate with the nuts and bolts (or clips/clamps). A washer may help as the head of the bolt is directly on the surface of the silicone middle plate.
2. Load liquid/molten agar into the empty space, slightly overfilled, and allow to cool. Scrape across with a clean scalpel such that the gel surface is flush with the middle plate's upper surface.
3. Inoculate the top and/or bottom of the "coin" of gel. Streaking may be possible as well, just as one would on the gel of a Petri dish.
4. Remove hardware (or clips/clamps if you used those instead) and apply another membrane and upper plate.
5. Insert and tighten the nuts and bolts such that the internal chamber is sealed from its exterior environment.
6. Incubate buried in soil or with soil piled on top, and alternately wet this soil if it appears to dry out.

## 5.5 Co-Opting Similar Equipment: Slide-A-Lyzer

In addition to the iplates described here, there are also many possible options for pre-made hardware and equipment in the laboratory to function as diffusion microchambers. One such example is the "Slide-A-Lyzer" dialysis cassette. The "molecular weight cutoff", meaning the maximum molecular weight of molecules allowable across the membrane, can be selected from $2$ kilodaltons to $20$ kilodaltons. The chamber inside is loaded with any material, even a gel if one keeps the whole system warm, via a syringe inserted into one of the septa situated in the corners. Likewise, sterile gel may be loaded into the chamber and then may be inoculated with another syringe after cooling. If one is very careful not to puncture either membrane, cells may be spread somewhat evenly across the membrane just inside the chamber, which, when buried or added to a soil or liquid medium, will receive the highest density of diffusing growth factors from soil solution. One may also inject a soil slurry into the chamber and grow microbes on the surface of the membrane. Following the overall philosophy of this project, one can be very inventive with

simple materials to recreate and simulate the complex microenvironments in which soil bacteria and other microorganisms grow.

## 5.6 Source Code

All files will be made available on the Github repository devoted to this project.

## 5.7 Funding

Funding for this project was provided by the Wisconsin Institutes for Discovery and the UW-Madison Soil Ecology Laboratory.

## 5.8 Supplemental Materials

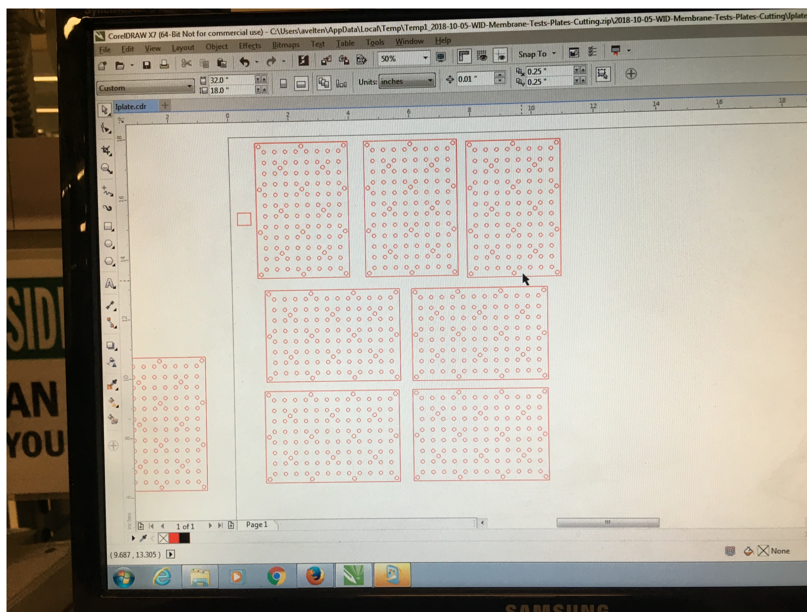


Figure 5.6: Type 2 Iplate 96-chamber plate design file that is submitted to the laser cutter to cut the through-holes and plates from Delrin.

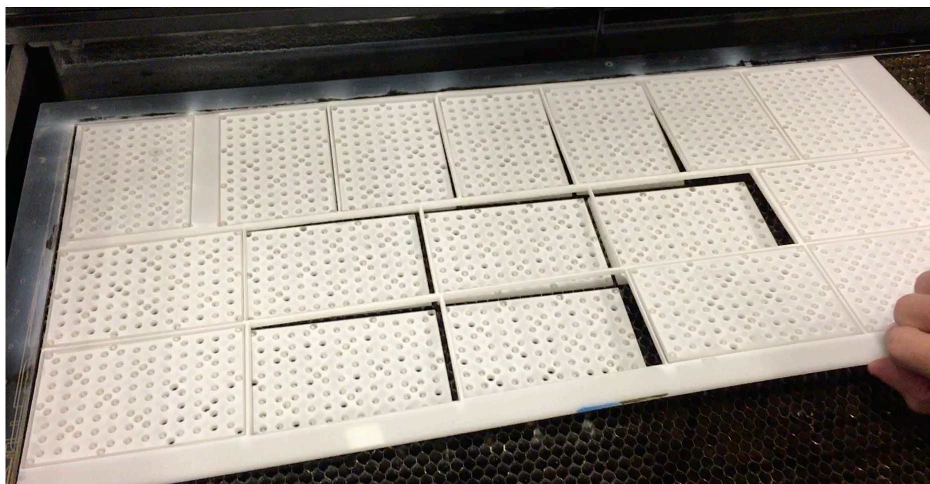

Figure 5.7: Type 2 Iplate 96-chamber plates laser-cut from Delrin.

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# 6 Concluding Remarks

I performed my first scientific experiment as a boy of roughly seven years old, when I inserted the plug of a vacuum cleaner into an electrical outlet (*sans* personal protective equipement) after suspending a penny between the prongs of the plug. Connecting the ciruit caused a gout of shimmering molten metal to seer the wall above the outlet as I leaped back, shaken but thrilled. I subsequently performed many more experiments and studies with colleagues from around the world and, most recently, unearthed century-old literature to produce new theories and experimental evidence for the underlying nature of soil acidity. I learned over the last few years that soils are not simply nutrient reservoirs or growth media but intensely reactive matrices of colloidal interfaces that are electrochemically interconnected via briny hydrofilms and muggy organic skins. Life abounds in the dark.

Such is the beauty of science, to look honestly at evidence from the past with growing questions to answer in the future, all to understand the present to one's maximum capability (and delight). Across my past studies, research, and other touchstones, at least three tangible lessons have surfaced in my progression from a reckless boy to a careful scientist. First, life forms biogeochemical cycles and ecological symbioses to encourage and maintain themselves such that, for example, when soils are stripped of their biomass, the land irremediably bleeds red and yellow into the sea, curiously analogous to a human bleeding out. Second, chemistry is not "the central science" at all but rather the lexicon for naming the many entities of reality, to which physics is neither inferior nor superior but simply the necessary lucid grammar. My third lesson has been that humans' beliefs change much like soil profiles change—very, very slowly—and "soil pH" is a prime example. As for the future, from exploding outlets to tropical soundscapes to protonmotive force to soil acidity and everything I have studied in between, a persistent peculiarity has slowly amplified in my mind: "Physical reality emerges from mass arranged in fields of charge." Now I ask, what are these fields of charge, fundamentally?

This thesis proposed that soil pH, protonmotive force, redox, and many other biogeochemical processes in soils, waters, and microorganisms trace back to the fundamental dynamic states of substances and chemical groups as protonated or deprotonated over time and space. The potentiometric, unitless, and highly context-dependent metric of "pH" does not apply well to the moisture in soils—or any briny solution such as seawater, mitochondria, and blood—without severe error. I have proposed means by which soil scientists may mitigate these errors, and ultimately

I have formalized a new metric, "proticity", that is stoichiometric, unitful, and commensurable. In light of the evidences above, it is tempting to throw out "pH" altogether, but some of its usefulness remains. We can still use pH values with great effectiveness, but "soil pH" provides insufficient information to understand the lives and histories of soil microorganisms. For this reason, we can and must continue to explore and improve the measurement of acidity in soils, the wider environment of Earth, and among planets throughout the universe with potential for life.

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*END*

# 7 Appendices

## 7.1 Appendix A: The Dilute Solution Assumption

*Michael J. Braus*: To convey the seriousness with which I wish to communicate that "pH" does not apply to most of the systems where it is currently being mesured, namely those with ionic strength greater than 100 millimolar, I have listed below many references supporting the adherence of pH to this dilute solution assumption. Each of the following subsections of this supplementary section is a direct quotation, and the subheading provides each passage's citation listed in the reference list.

### 7.1.1 Truog (1915, 506)

It must be carefully noted that this law [of chemical affinity of acids] only holds when all the reacting substances are in a true solution, or if there are partially soluble substances formed, then in any series of comparisons, the solubility of the corresponding substances must be of the same order. The opportunity for secondary or side reactions must also be eliminated or made comparable. In the soil there is almost unlimited opportunity for these side reactions to occur. Most previous investigators of soil acidity and absorption have entirely overlooked and ignored these most important considerations and hence have accordingly arrived at erroneous conclusions.

* * *

### 7.1.2 Bjerrum and Gjaldbæk (1919, 17), translated by J. K. Rundo at the Atmoic Energy Reserach Establishment (Harwell Laboratory, Oxfordshire, 1956)

It is now required to use the values of pH in Table 4 to calculate $K$. We shall first shew how one can obtain an approximately correct result by using equation (5) and putting $\mathrm{pH} = -\log C_{\mathrm{H}^+}$ Taking logarithms in equation (5) and putting in pH we obtain

$$-\log K = \mathrm{pH} + \frac{1}{2}\log C_{\mathrm{Ca}++} + \frac{1}{2}\log p_{\mathrm{CO}_2} \;..... \qquad (14)$$

The calculation of $\log p_{\mathrm{CO}_2}$ does not present any difficulties. On the other hand by calculating the calcium ion ccncentration we cannot just set this equal to the concentration of the calcium chloride since, as known, calcium carbonate in the presence of carbon dioxide dissolves slightly as bicarbonate. It is however easy to calculate the amount of the dissolved calcium carbonate from Schlösing's measurements of the solubility of calcium carbonate in water at different carbon dioxide pressures. Bodlander has given the theory for Schlösing's measurements. In the following we give a new calculation of Schlösing's measurements since this has become necessary by our altered views in recent times of the activity conditions of ions.

In a solution which is saturated with calcium carbonate at a definite carbon dioxide pressure there must be equilibrium between the bicarbonate ions on the one side and the carbon

dioxide and the solid calcium carbonate on the other side according to the equaticn:

$$Ca^{++} = 2HCO_3^- = CaCO_3 + CO_2 + H_2O$$

Where we igncre the change in the activity of the water, which is very small in the dilute solutions under consideration here, the law of mass action gives

$$\frac{a_{Ca^{++}} \cdot a^2_{HCO_3^-}}{p_{CO_2}} = K'....... \quad (15)$$

* * *

#### 7.1.3 Debye and Hückel (1923, 197), translated by Michael J. Braus (2020)

Theorem 1.

For all electrolytes, in the limit for low concentrations, the percentage deviation of the freezing point depression from the classical value is proportional to the square root of the concentration.

It is possible to state this law as a general law because all the electrolytes for large dilutions can be considered as completely dissociated into ions. Of course, only the strong electrolytes practically reach that area of complete dissociation.

Secondly, equation (39) makes a statement about the influence of ion valence, which can be formulated as follows:

Theorem 2.

If the dissolved molecule dissociates into $\nu_1,\ ...\nu_i,\ ...\nu_s$ different ions of types $1,\ ...i,\ ...s$ with the valences $z_1,\ ...z_i,\ ...z_s$, then, for low concentrations, the percentage deviation of the freezing point depression from the classical value is proportional to a valence factor $w$, which is calculated from

$$w = \left( \frac{\sum \nu_i z_i^2}{\nu_i} \right)^{3/2}.$$

As an example for the calculation of this valence factor, Table 2 is presented, where in the left column an example of the type of salt is given, and in the right column the value of $w$ is given:

The influence of the ions therefore increases considerably with increasing valence, which also corresponds to the qualitative findings.

Thirdly, the solvent has an influence, in the sense of Nernst's well-known suggestion for explaining the ionizing force of solvents with a high dielectric constant. Following equation (40), one finds

Theorem 3.

For low concentrations, the percent deviation of the freezing point depression from the classical value is inversely proportional to the 3/2th power of the dielectric constant of the solvent.

* * *

#### 7.1.4 MacInnes (1939, 148)

General Remarks Concerning the Debye-Hiickel Theory. Experimental studies concerning the validity of the Debye-Hückel theory have been made using freezing-point data, measurements of the solubilities of electrolytes, and the results of determinations of the potentials of concentration cells. The discussion in this book will be limited to the last of these methods since the interpretation of the first two types of data will be considered, somewhat arbitrarily, to be outside the field of electrochemistry. Although the interionic attractions postulated in the theory would be expected to exist at all concentrations in soluions of ions, and to be even more effective in determining the properties of such solutions at high concentrations than in dilute solutions, the validity of the theory as developed in the preceding pages is limited to dilute solutions. At higher concentrations various complicating effects must be considered. For instance there is probably a change of the dielectric constant of the solvent, due to the presence of the charged ions. Also the mathematical approximations that were made in the derivation as given must be considered in connection with the range of applicability of the theory to actual solutions.

There is no detail of the derivation of the equations of the Debye-Hückel theory that has not been criticized. Its incompleteness mathematically is evident, since only the first term of the expansion of equations (3) and (29) is used. The extensions of the theory to overcome this deficiency are, however, briefly considered below. A possibly more serious deficiency of the theory as given is that it does not take account of "fluctuation terms." This amounts to the statement that the Boltzmann equation does not yield a correct average potential if, this being subject to wide variations for which allowance should be made in the theory.

* * *

#### 7.1.5 Ashcraft (1947, 29)

DISCUSSION

Mr. Martin Kilpatrick − There are four things that I should like to point out:

1. Assigning a value to $f_{\mathrm{H}^+}$ on the basis that $f_{\mathrm{K}^+} = f_{ce^-}$ is reasonable when the solvent salt is largely potassium chloride. When the solvent salt is largely sodium chloride or sodium acetate or other solvent salt the assumption is no longer correct.
2. The Lewis rule that a given ion has the same activity coefficient in all solutions at the same ionic strength is only true as a limiting law, that is, as one approaches infinite dilution.
3. In 1926 it was shown that measurements of cells with or without liquid junction do not yield any information on ionic free energies. For references and discussion see Mary Kilpatrick and Martin Kilpatrick.
4. A quantity cannot be measured more accurately than it can be defined, and it is uselss to talk about pH in terms of thousandths of a unit.

...

Mr. D. S. McKinney. — ... The points mentioned by Mr. Kilpatrick are, we believe, adequately discussed in the paper. It is obvious that such terms as pH or individual ion activities cannot be defined on thermodynamic grounds, without resort to some solution theory. Since out present theories are in the nature of limiting laws, exact agreement cannot be expected at finite concentrations. However, this should not prevent us from making pH measurements at higher concentrations, if such measurements serve a useful purpose.

* * *

#### 7.1.6 Feldman (1956, 1865)

COLLOIDS AND SUSPENSIONS

The existence of the “suspension effect” on the pH of clays, soils, and ion exchange resins has been known for some time. In general, the pH of suspensions and pastes appears to be lower than the pH of their supernatant liquids.

For instance, Jenny and associates reported that a pH of 9.2 was obtained for a 10% potassium bentonite suspension, whereas a 1 to 1 potassium bentonite-water paste gave a pH of 5.8. They attributed the suspension effect to the liquid junction potential at the point of contact between the potassium chloride bridge and the suspension. As evidence, they presented the following results. For an ion exchange resin sediment in contact with its supernatant liquid, a pH of 6.0 was measured when both the glass and calomel electrodes were immersed in the supernatant, but a pH of 2.0 was indicated when the electrodes were immersed in the sediment. The e.m.f. equivalent of this pH difference, 240 mv., was obtained between two calomel electrodes when one was suspended in each phase, but when a glass electrode was suspended in each phase no potential difference existed between the two glass electrodes.

Although there is disagreement ([many references listed]) as to whether the suspension effect is due to the liquid junction potential or is a true membrane potential at a Donnan system, there is no question that the effect exists and that, as a result, pH measurements on suspensions of highly charged particles are meaningless. The effect is not significant, however, for solutions containing mobile colloidal ions or proteins of high equivalent weight because of the efficiency of the potassium chloride bridge. For instance, the author detected no difference between the pH of whole blood, the sedimented cells obtained on centrifugation, and the supernatant plasma. The meaning of the pH near cell surfaces or of dental plaques, however, may be questionable.

* * *

#### 7.1.7 Ashcraft (1957, 3)

These fundamental applications of measured pH values arc permissible for many rather dilute aqueous systems. However, there is always the temptation to extend the interpretation beyond the limited range of its validity, to systems that are not dilute or not aqueous. It must always be borne in mind that the experimental (that is, operational) pH value in such media as 50 per cent alcohol, molasses, and glacial acetic acid cannot be interpreted in terms of hydrogen ion concentration or the conventional hydrogen ion activity.

—ROGER G. BATES

* * *

### 7.1.8 Sena (1972), Appendix 3

pH Index

The activity of electrolyte solutions depends on the concentration of ions in them. This relationship, however, is not quite single-valued owing to the interaction between ions. For this reason concentration can serve to describe the activity of a solution only when it is greatly diluted. At high concentrations the concept of equivalent concentration is introducted, which is the product of the actual concentration and an activity factor less than unity. Since both the actual and the equivalent concentrations of ions can change within very broad limits, a logarithmic scale is used. The idex measured in this scale (designated pH) is equal to the common logarithm, with sign reversed, of the activity or equivalent concentration of hydrogen ions measured in gram-equivalents per litre. Since the concentration of hydrogen ions in water (and chemically neutral soutions) is $10^{-7}$, then for water $pH = 7$. In acid solutions the hydrogen ion concentration is higher, and, accordingly $pH < 7$, and in alkaline solutions, on the contrary, $pH > 7$.

* * *

### 7.1.9 Pourbaix (1974, 14)

When the metal forms soluble complexes of great stability with other substances (such as cyanides or ammonia), the equilibrium diagrams for the binary system metal-water must be modified: one must then take into account the equilibrium conditions of these two complexes, for example by plotting equilibrium diagrams for a ternary system. This may modify appreciably the domains of relative predominance of the dissolved species and the domains of thermodynamic stability. In these cases one must therefore be very careful, particularly because only when a dissolved species is greatly predominant, in the case of dilute solutions at least, can one assume, as has been in this Atlas, that the activities are virtually the same as the molarities.

Use of the diagrams therefore renders necessary corrections of activities with respect to molarities (or molalities): these corrections, which may be important for all the diagrams when one considers concentrated solutions, may also be important when one envisages the use of binary diagrams for studying ternary systems which involve stable complexes.

* * *

### 7.1.10 Volk and Rozen (1977), [p. 1569]

Eqn. (4) allows us to calculate the analytical concentration of hydrogen ions at any point of the isotherm from the known values of $\Delta_1$pH and $\Delta_2$pH. We can see from this equation that in the presence of the salting-out agent the conventional assumption that $[H^+] = 10^{-pH}$

is quite incorrect, the error increasing with $\Delta_1$pH, i.e. with the concentration and with the degree of hydration of the salting-out agent (see Fig, 2 of Ref . 1). For example, for a $2m$ solution of $Ca(NO_3)_2$ at pH 5 we find that $[H^+] = 3.05 \times 10^{-5}$M $\neq 10^{-5}$M (300% error).

* * *

#### 7.1.11 Butler (1998, 462–63)

pH in Brines: Limitations and Ambiguities

pH measurements are less certain in brines than in dilute solutions for several reasons.

- The usual pH electrode-calomel reference electrode combination does not measure hydrogen ion activity (i.e., pH) alone, but rather measures a combination of hydrogen ion activity, counter-ion activity, membrane potentials, and liquid junction potentials.
- In the standard two-electrode or combination-electrode pH setup, liquid junction potentials arise between the calibration or test solution and the salt bridge leading to the reference electrode. When the calibration and test solutions are similar in composition, the liquid junction potentials are close in value, and errors are small. If the calibration and test solutions are very different in composition, however, the difference in liquid junction potentials can also be large—introducing an error equivalent to one or more pH units.
- The standard buffers developed by the National Bureau of Standards, and widely available commercially, have much lower ionic strength($< 0.1$ M) than brines ($4-6$ m), and standard buffers of composition similar to the brines are not available.
- Liquid junction potentials have traditionally been eliminated by using the a "Harned cell," a hydrogen gas/platinum electrode with a silver/silver chloride electrode (see Chapter 2, p. 53). The Hamed cell has been used to measure the activity of HCl in a wide variety of multicomponent solutions, and is still the method of choice for the most accurate potentiometric measurements on acid-base systems. A variant on this theme, using a glass electrode and a chloride ion-selective electrode, has been investigated, and some of the results are described below.
- However, even a cell without liquid junction measures the combination of hydrogen and chloride activities, not the hydrogen ion activity alone. To establish a pH scale, some theoretical assumptions must be made to evaluate the activity coefficient of at least one of the ions. For the NBS scale, the Bates-Guggenheim convention 8 for the activity coefficient of chloride ion was chosen:

  $$\log(\gamma_{\text{Cl}}) = -\frac{A\sqrt{I}}{1 + 1.5\sqrt{I}}$$

  but this convention was not intended to apply at ionic strengths greater than 0.1. Other possibilities for calculating activity coefficients theoretically include Guggenheim's and Pitzer's equations, which are extensions of the Debye-Hückel theory.

* * *

#### 7.1.12 Galster (1991, 16)

1.5.7 Activities in Concentrated Solutions

Electrolyte solutions having concentrations greater than 1.0 mol/L no longer conform to the Debye-Hückel theory, because none of the simplifying assumptions in Section 1.5.3 for dilute solutions is applicable any longer. Glueckauff and Stokes and Robinson attribute the large variations in the ac-tivity coefficients in concentrated solutions to the fact that there is no longer sufficient water present for all ions to form complete hydration sheaths. At higher concentrations further difficulties are often encountered as a result of the formation of complex compounds with differing charge numbers, so that smaller ionic concentrations are often measured than would be expected from the amounts of substance dissolved in the solution.

The increase in the activity coefficients in acid solutions corresponds to an increase in acid strengths. According to Schwabe the addition of neutral salt in the range between $b = 0.5$ mol/kg and saturation causes the pH to fall almost linearly and can be expressed by the empirical formula

$$\mathrm{pH} = \mathrm{pH}_0 - 5,55bz(\frac{h_+}{r_+} - \frac{h_-}{r_-}).$$

Here $\mathrm{pH}_0$ is the pH with no added salt, $b$ the concentration of added neutral salt and $z$ the electrochemical charge number of the added salt, $h$ the hydration numbers of the added ions and $r$ the radius of the ions in pm plus the radius of the water molecule ($r_{\mathrm{H2O}} = 138$ pm). The number 5.55 is a theoretically based constant.

The factor

$$K = 5,55z(\frac{h_+}{r_+} - \frac{h_-}{r_-})$$

represents the increase in the acidity and is characteristic of the neutral salt added.

* * *

#### 7.1.13 Anderegg and Kholeif (1994, 1521)

CONCLUSIONS

If enough values for the examined equilibrium constants are known for a given system ($N \geq 10$), and are well distributed on the $I$ [ionic strength] scale particularly atlow $I$ values near 0.01 to 0.1,then the problems discussed here can be treated without difficulty. When $N$ is reduced to 5 with three points at $Z < 0.1$, the accompanying increase in error is not dramatic as far as the constants are not affected by large errors. This is the casefor acetic acid but probably not for NTA. These last inconsistenciescan be becauseof the large $\Delta z^2$ that makes ${}^*\beta_{1,0,1}$ more dependent on the $I$ values. If the values of the constants are only known for $I \geq 0.5$, the error of ${}^*\beta^0_{n,p,m}$ can be very large but the values of the constants can be well extrapolated if they are properly distributed on the $I$ scale.

* * *

#### 7.1.14 Sparks (1998, 112)

Some confusion exists regarding the term “extended Debye-Hilckel equation” since the more detailed equation:

$$\log \gamma_{\pm} = -Az^2 \frac{I^{0.5}}{1 + BaI^{0.5}}$$

is sometimes called the Debye-HUckel equation26 in contrast to the Debye-Hilckel limiting law… This equation is considered to give acceptable results to about $I = 0.1$.

The extended form of this equation:

$$\log \gamma_{\pm} = -\frac{Az^2 I^{0.5}}{1 + BaI^{0.5}} + bI$$

is considered the extended Debye-Hückel equation by Robinson and Stokes… usable to about $I = 1.0$, but this is very dependent on the mixed electrolyte under consideration.

* * *

#### 7.1.15 Baucke (2002, 774)

The incorporation of pH values into the SI system necessitates one to state a numerical value of the uncertainty of the Bates-Guggenheim convention. This is accomplished by estimating the possible range of the conventional product $aB = 1.5$ in Eq. (6). The recommended estimate is $1.0 \leq aB \leq 2.0$ corresponding to varying the ion size parameter a from 0.3 to 0.6 nm, which yields a range from $\pm 0.012$ (at $I = 0.1$ mol kg ) to $\pm 0.007$ (at $I = 0.05$ mol kg ) for $\lg \gamma^0_{\text{Cl}}$. The 0.01 uncertainty of $\lg \gamma^0_{\text{Cl}}$ thus covers this variation Cl and must be included in the uncertainty of pH values if they are to be regarded as traceable to SI. pH values stated without this contribution to their uncertainty are conventional pH values without a numerical link to the SI system, which, however, will suffice in most cases.

* * *

#### 7.1.16 Wright (2007, 382)

10.8 Shortcomings of the Debye-Hückel model

When the Debye-Hückel equation is tested against experimental results it is very successful in accounting for behaviour at low concentrations, and it is believed that the theory is basically correct for low concentrations (see Section 10.10). Having to test the theory rigorously at very low concentrations proved a great stimulus in developing precision techniques for deriving experimental values of $\gamma_{\pm}$. At moderate and higher concentrations deviations from theoretical behaviour become apparent, and ways of dealing with these problems are described later in the chapter.

It is constructive to look again at the physical basis of the simple Debye-Hückel model and its mathematical development to see where both could be modified, and to consider whether this would be mathematically possible. What has been written in Chapter 1 on ions and solvent structure shows that the Debye-Hückell model is painfully naïve and cannot even approach

physical reality. A brief reassessment of the features 1–7 of the simple Debye-Hückell model is given below, along with indications as to how these problems have been tackled.

10.8.1 Strong electrolytes are completely dissociated

...

10.8.2 Random motion is not attained

...

10.8.3 Non-ideality results from coulombic interactions between ions

...

10.8.4 Ions are spherically symmetrical and are unpolarisable

...

10.8.5 The solvent is a structureless dielectric

...

10.8.6 Electrostriction is ignored

...

10.8.7 Concept of a smeared out spherically symmetrical charge density...

This is an absolutely crucial part of the model and has been dealt with by statistical mechanical averaging procedures. But only spherical symmetry has been assumed. And so for large non- spherical ions a modification to the smearing out procedure is needed, but see Sections 10.17.3 and 10.19.

Any one given distribution of ions around a spherically symmetrical central j-ion need not necessarily be spherically symmetrical, but on average all possible arrangements will correspond to spherical symmetry. A charge density necessarily corresponds to an average distribution of ions, so conversion of the Poisson-Boltzmann equation to spherical symmetry is purely formal.

But as has been hinted at above, there is one important limitation to this when considering large complex electrolytes such as are found in aqueous solutions of biological materials. Here the central ion is non-spherical. An ion which is not spherically symmetrical may impose a non-spherically symmetrical distribution of charge around it, and this ought to be taken care of, but is not, in the theory. The Debye-Hückell theory can thus only be approximate for non-spherical ions.

* * *

#### 7.1.17 Levie (2010)

Where do those limits originate? A pH meter with an indicator electrode, plus an external reference electrode (i.e., separated from the sample solution by a liquid junction), and calibrated with the usual reference or standard buffer solutions, can only yield an approximation for $-\log(a\mathrm{H}^+)$ as currently defined by IUPAC, for the following reasons.

- The ionic activity coefficient is, admittedly, an immeasurable quantity and therefore needs an approximation. Well beyond the estimated range of validity of the chosen Bates-Guggenheim approximation, which IUPAC set at $I = 0.1$ mol kg$^{-1}$, the method becomes powerless. At $I = 0.1$ mol kg$^{-1}$, it already has an estimated uncertainty of at least $\pm 10\%$.
- The measurement specifically includes a liquid junction to make the measured response maximally independent of the anions in the sample. Unfortunately, the resulting liquid junction potential is also immeasurable and can only be estimated by approximate models that neglect the difference between ionic concentration and activity.

...

Using Popper's criterion (36), the hydrogen ion activity as defined by IUPAC is not falsifiable and therefore falls outside the demarcation that separates science from non-science. That is why the problem lies with IUPAC rather than with the original Sørensen definition.

* * *

#### 7.1.18 Spitzer and Pratt (2011, 75)

The work on the traceability and dissemination of pH is not yet complete. The Bates–Guggenheim convention is only valid at ionic strengths up to 0.1 mol kg$^{-1}$. For applications in clinical chemistry and in environmental samples (e.g., rainwater, seawater), pH reference buffer solutions with ionic strengths more similar to these samples are expected to improve the comparability of measurement results in these matrices. Further investigations into solution theory and into the concept of single ion activity are necessary to overcome present limitations for the primary procedure for measurement of pH.

* * *

#### 7.1.19 Levie (2014, 615)

To summarize, within the constraints that all measurements are restricted to samples with $I \leq 0.1$ mol kg$^{-1}$ , $2 \leq$ pH $\leq 12$, and $5 \leq t \leq 50$ C, the assumptions currently adopted by IUPAC are:

1. Ionic activity coefficients are fully and solely determined by the absolute ionic valency $|zi|$ of the ions considered, and by the ionic strength $I = 1/2\Sigma z_j^2 c_j$ of the solution.
2. The activity coefficient of chloride ions is given by the Bates-Guggenheim approximation with $Ba = 1.5$ kg$^{1/2}$ in the Debye-Hückel expression, which then reads (in 10-based logarithmic format) as $\log \gamma_{\mathrm{Cl}} = -(A\sqrt{I})/(1 + 1.5\sqrt{I})$, regardless of the nature of the counterion(s), where $A \approx 0.5108$ kg$^{1/2}$ mol$^{1/2}$ at 25 C.
3. Moreover, for actual pH measurements, the liquid junction potential is assumed to be constant for the cell in contact with either the sample or the two bracketing pH standards.

* * *

#### 7.1.20 Covert and Hore (2016, 235–38)

Several models exist to describe charge distribution and electric fields at solid–liquid interfaces. One of the earliest models was proposed by Gouy in 1910 and Chapman in 1913. This model describes the distribution of charged species in the region adjacent to a surface by an exponential decay function. As an example, let us consider a negatively charged surface in contact with a dilute NaCl solution. With respect to the bulk ionic concentrations of this solution, there are, immediately adjacent to the surface, a net depletion of Cl and a net accumulation of Na . The interfacial concentrations are determined by the surface potential, $\Psi_0$, that decays exponentially with distance from the surface. The characteristic thickness of this layer, termed the Debye length, is roughly given (in dilute solutions) by

$$\kappa^{-1} \approx \sqrt{(0.09\text{nm}^2\text{mol L}^{-1} I^{-1})}$$

where $I$ is the ionic strength of the bulk electrolyte. Notice that the Debye length decreases with increasing ionic strength. This is a result of a screening of the surface charge by the mobile charges in solution, thereby decreasing the penetration of $\Psi_0$. This description of the interface is appropriate for dilute electrolyte solutions ($< 0.1$ mol $\text{L}^{-1}$) but is not applicable at higher concentrations. At higher electrolyte concentrations, the Stern model of the interface is more appropriate. This model considers the interface as two layers. The inner layer is characterized by contact adsorption to the charged surface of counterions in solution, and the outer layer is described by the exponential decay function of the Gouy–Chapman model. The physical size of the ions sets a lower limit on the proximity to the surface that the screening charges can inhabit. At this limit, the surface may be modeled as a capacitor—two charged plates separated by a finite distance.

* * *

#### 7.1.21 Dobrovolskii et al. (2018, 87)

$$\text{pH} = -\log(a_\text{H}\gamma_\text{Cl})_{m_{\text{Cl}\to 0}} + \log(\gamma_\text{Cl})_{m_{\text{Cl}\to 0}},$$

where the component $(a_\text{H}\gamma_\text{Cl})_{m_{\text{Cl}\to 0}}$ is called the acidity function and is denoted by $pa_0$.

The coefficient $\gamma_\text{Cl}$ (see (2)) was calculated according to the Bates–Guggenheim convention:

$$\log\gamma_\text{Cl} = -A\sqrt{I/m^0}\Big/\left(1 + 1.5\sqrt{I/m^0}\right)$$

where $I$ is the ionic strength of the solution; $A$ is the Debye–Hückel constant. Equation (3) is justified when $I < 0.1$ mol/kg.

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### 7.2 Appendix B: Original Translation of Debye and Hückel (1923)

I have created a new translation (2020) with updated language and clear typesetting of "*Zur Theorie der Elektrolyte. I. Gefrierpunktserniedrigung und verwandte Erscheinungen*", written by Peter Debye and Erich Hückel and published in 1923 in the journal *Physikalische Zeitschrift*. Much of electrochemistry, acid-base chemistry, and many other scientific fields were inspired by this single paper, and a highly legible English edition with typeset equations was warranted and overdue. The document is available for download at `MINDS@UW` at the following web address:

`http://digital.library.wisc.edu/1793/79225`

The original German publication has also been made available at the following web address:

`https://archive.org/details/1923-debye-huckel-theory-zur-theorie-der-elektrolyte-1/`

Please refer to Appendix B for a guide to better understanding the many symbols the authors used, some of which were endemic to Germany in the beginning of the 20th century. One of the best guides to the content of this publication can be found in Chapter 7 of *The Principles of Electrochemistry* by MacInnes (1939, 137–51), entitled "The Debye-Hückel Method for the Theoretical Calculation of Activity Coefficients".

## 7.3 Appendix C: Mathematical Symbols Used by Debye & Hückel (1923)

Below I have provided a list of the mathematical symbols used by the German authors, and each definition has additional information to assist readers.

### 7.3.1 Latin

$a \equiv$ the mean radius of the ions of a solution, when considered as hard spheres, or the mean distance up to which surroundings of postivie and negative ions can approach.

$A \equiv$ a constant determined from the boundary conditions at the surface of the sphere of ions.

$B \equiv$ a constant determined from the potential generated at the center of an ion sphere by the surrounding ionic atmosphere.

$c \equiv$ the concentration. However, the definition of "concentration" in the middle of page 8 suggests this is a fractional value, more precisely the number of a species of particle as a percentage of the total particles that constitute the solution. For equation 54, the authors call this symbol "molar concentration", which referes to a mole-per-mole unit. This is different from the contemporary "conentration" that is measured in mole-per-liter or mole-per-kilogram units.

$c_p \equiv$ the specific heat of the liquid solvent at constant pressure.

$c'_p \equiv$ the specific heat of the frozen solvent at constant pressure.

$C_p \equiv$ the specific heat per mole of the liquid solvent at constant pressure.

$C'_p \equiv$ the specific heat per mole of the frozen solvent at constant pressure.

$d \equiv$ the differential symbol.

$D \equiv$ the dielectric constant of the solution.

$f \equiv$ any defined function, different from the coefficients of osmotic and activity.

$f_a \equiv$ the activity coefficient relating the chemical concentration under complete dissociation to the real or observed chemical activity.

$f_o \equiv$ the osmotic coefficient relating the osmotic pressure under complete dissociation to the real or observed osmotic pressure.

$G \equiv$ the total potential.

$G_e \equiv$ the "electrical component" of total potential.

$G_k \equiv$ the "classical component" of total potential.

$i \equiv$ an index, such as that of each particle in solution.

$j \equiv$ an index, used for a summation function.

$k \equiv$ the Boltmann constant, which the authors list as $1.346 * 10^{-16}$ erg, where $1$ erg $= 10^{-7}$ joules.

$K \equiv$ the dissociation or equilibrium constant.

$n \equiv$ the number of an ionic species per unit volume, usually cm$^3$.

$N \equiv$ the number of individual particles in a system, or Loschmidt's number, which is how early 20th century German scientists referred to the Avogadro constant.

$N_o \equiv$ the number of individual particles in the solution referring specifically to the solvent.

$N'_o \equiv$ the number of individual particles in the solution referring specifically to the solvent in the frozen phase.

$p \equiv$ the pressure.

$p_e \equiv$ the "electrical pressure".

$P \equiv$ the osmotic pressure under incomplete dissociation, or real osmotic pressure. $P$ was also used to signify a point in space.

$P_k \equiv$ the osmotic pressure under complete dissociation.

$q \equiv$ the heat of fusion of the frozen solvent.

$Q \equiv$ the melting heat of a mole of substance.

$r \equiv$ the mean distance between two point charges.

$R \equiv$ the "gas constant", referring to the ideal gas constant.

$s \equiv$ the index of each species in a solution.

$S \equiv$ the entropy.

$T \equiv$ the absolute temperature.

$T_o \equiv$ the freezing point of the pure solvent.

$u \equiv$ the potential energy. This symbol was also used briefly during integration by substitution for equation 24.

$U \equiv$ the total energy.

$U_e \equiv$ the "electical component" of total energy.

$U_k \equiv$ the "classical component" of total energy.

$v \equiv$ an undefined symbol, but it was implied that this is the volume of a particle.

$V \equiv$ the volume of the system.

$w \equiv$ the "valence factor", defined at equations 39, equation 49, and elsewhere, measuring the influence of the ion valences.

$x \equiv$ a variable used during an integration by substitution.

$z \equiv$ the integers of positive and negative valances (charges), or, briefly just before equation 12', a coordinate perpendicular to an electrode surface.

#### 7.3.2 Greek

$\gamma \equiv$ the measured concentration in moles per liter (review the definition of symbol "$c$" above), not the activity coefficient as it was later defined and used in the Debye-Hückel equation by electrochemists today. For equation 54, the authors define this symbol as "volume concentration".

$\gamma' \equiv$ the measured concentration in moles per $1000$ grams of water, or molality, for use where moles per liter was made impossible in the absense of measurements of the density of salt solutions.

$\delta \equiv$ an infinitesimal change.

$\mathit{\Delta} \equiv$ the real freezing point depression.

$\mathit{\Delta}_k \equiv$ the expected freezing point depression.

$\varepsilon \equiv$ the magnitude of electric charge (negative or positive), listed as $4.77 * 10^{-10}$ electrostatic units.

$\Theta \equiv$ the deviation of the osmotic coefficient from its limiting value of $1$.

$\kappa \equiv$ the "characteristic length" introduced in this publication to replace Ghosh's "mean distance between the ions". The authors state the inverse of this length is the thickness of the ionic atmosphere or Helmholtz double-layer.

$\mu \equiv$ an undefined symbol, but one can assume it signifies the stoichiometry of each ion, as in Guldberg-Waage's law of mass action.

$\nu \equiv$ the number of ions in a solution.

$\pi \equiv$ the ratio of circumference to diameter.

$\varrho \equiv$ the density of electricity in a medium.

$\sigma \equiv$ a temporary abbreviation defined in equation 33.

$\varphi \equiv$ the thermodynamic potential.

$\Phi \equiv$ the total potential at constant pressure (unlike $G$).

$\Phi_k \equiv$ the "classical component" of total potential at constant pressure.

$\Phi_e \equiv$ the "electrical component" of total potential at constant pressure.

$\chi \equiv$ a function for relating $x_i$ with $\kappa a_i$ such that $\chi$ approaches $1$ (referred to as unity) in infinitesimal concentrations.

$\psi \equiv$ the mean electrical potential with respect to time.

$\Psi \equiv$ a quantity that is not explicitly defined.

$\Omega \equiv$ the molar volume of water.

#### 7.3.3 Miscellaneous

$\partial \equiv$ the partial differential.

$\mathfrak{Sin} \equiv$ a deprecated symbol for "sinh", the hyperbolic sine function.

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