

**Proteins in Geobiology: New Approaches for Enzyme Field Detection and
Biomarker Visualization**

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Abstract

Proteins are the biomolecules that carry out most functions of the cells, yet their impacts and potentials to understand current and past Earth-life interactions are understudied. Here, I attempt to exploit the use of proteins—in the form of antibodies and enzymes—to answer fundamental questions in geobiology through two different approaches: (1) Using field assays to identify enzyme activity in the field that promotes calcium carbonate precipitation in modern environments, and (2) using antibodies to visualize lipid biomarkers in the fossil record. The first objective explores in-field analysis of urease and carbonic anhydrase (CA) activity, two enzymes known to promote carbonate precipitation. Microbes are attributed to drive environmental changes that explain geological phenomena, yet the mechanisms underlying these transformations are poorly studied. Simple, fast, and economical field tests were developed to identify on-site CA and urease in biofilms. The tests were used in microbial samples from calcareous fens, ferruginous springs, and an alkaline lake (Salt Lake) in Minnesota, along with carbonate tufa biofilms from an alkaline lake (Big Soda Lake) in Nevada. Relatively high *in situ* microbial urease activity in lake samples also promoted carbonate precipitation in microcosm incubation experiments. Active enzymes in tufas, together with community characterization by 16S rRNA gene sequencing and shotgun metagenomics, provided a plausible new mechanism to understand tufa biogenicity and its rapid growth. The second objective explores immunological techniques—antibody-based methods used in life sciences—here used to detect lipid biomarkers in the rock record. A previously known antibody that binds squalane was used to detect biomarkers in crude oil samples and to

identify *in situ* micro-scale signatures in organic-rich rocks and potentially in fish fossil remains from the Eocene Green River Formation. A novel chromatographic method developed to separate and immunodetect hydrocarbon fingerprints showed that the antibodies also bind other soluble hydrocarbons in Green River Formation rocks, likely acyclic isoprenoids. The use of antibodies proved useful for identifying biomarker distributions in the sedimentary fabric that are otherwise difficult to study by current techniques. Future methods aimed at investigating the role of microbial proteins in the environment and their value as detection tools will benefit our understanding of the past and current biosphere and its interactions with the geosphere.

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Chapter 1

Introduction

The microbial activity—the combination of metabolic reactions that organisms require to survive and reproduce in a specific environment—is capable of driving environmental changes that not only affect local ecosystems, but that has also drastically changed our planet through time. Despite the importance of microbially-mediated processes, environmental microbiology studies rarely evaluate the functional units of microbial metabolisms that are responsible for environmental change – namely proteins. Instead, the presence, absence or activity of microbial proteins is generally assumed from the genomic assembly of microbial communities. There is a great opportunity, however, to understand environmental microbial functions by analyzing the microbial protein repertoire. Proteins are responsible for most of life's ability to metabolize substrates, generate gradients useful to drive energetically challenged reactions, catalyze redox transformations, and assist in the transport of molecules among several other functions that potentially affect the environment. Thus, we should focus our efforts in analyzing not only the genomes of microbial communities, but also specific proteins—particularly enzymes—that catalyze key reactions and potentially modify the environment around microbes.

In addition to their environmental impact, the ability of enzymes to catalyze certain reactions, or the capability of specialized proteins to bind to specific structures, has been extensively exploited as tools that are used by a variety of methods in life sciences. Antibodies, for example, are proteins produced by the immune system of mammals that bind specific pathogen molecules to help combat infections. Antibodies can be designed to bind to a particular molecule and produced *in vitro*, so they can be applied as molecular probes to visualize specific proteins within cells and cell extracts.

Molecular detection and visualization using antibodies has revolutionized biomedical research over the past decades. Although antibodies are mainly designed to target proteins, there are few examples of antibodies designed to bind lipids (Alving & Swartz, 1991), some of which are also found in sedimentary organic matter (Matyas *et al.*, 2000; Spier *et al.*, 2009; Li *et al.*, 2016a). Those sedimentary lipids can be preserved in the geologic record for millions of years in the form of molecular fossils that presumably derived from ancient organisms, providing valuable molecular information of Earth's past life (Brocks & Summons, 2003; Grice & Eiserbeck, 2014). Analogously to the antibody-based detection methods in life sciences, antibodies could be used as a tool to bind and identify molecular fossils preserved in ancient rocks; a strategy that has not been evaluated in the field of organic geochemistry.

This dissertation research shows two examples where protein use and analysis can be exploited to complement studies in geobiology. The first example, described in **Chapters 2 & 3**, incorporates field assays to evaluate the enzymatic activity of urease and carbonic anhydrase, two enzymes that have been associated with environmental precipitation of calcium carbonates promoted by microbes. Field evaluations of urease and carbonic anhydrase may provide information about the mechanisms involved in the formation of modern and ancient carbonaceous structures. The second example, described in **Chapters 4, 5, & 6**, explores the use of antibodies to detect sedimentary hydrocarbons, introducing a novel tool that can be useful for directly visualizing specific molecular fossils in organic-rich rocks.

1.1. Chapter Overview

Each of the following chapters incorporates methods based on protein analysis to illustrate the potential use of enzymes or antibodies in geobiology.

- **Chapter 2** details the development of a rapid field assay to detect the activity of urease and carbonic anhydrase from environmental samples that can be used to assess potential microbially-induced carbonate precipitation.
- In **Chapter 3**, the method developed in **Chapter 2** is used to explore the potential biogenicity of modern carbonate tufa towers from Big Soda Lake (Nevada, USA),

complementing the characterization of tufa microbial communities, tufa carbonates, and lake water geochemistry.

- **Chapter 4** describes the use of antibodies that bind squalane, a hydrocarbon found in the sedimentary record, to visualize potential molecular fossils preserved in crude oils of different origins, and in rocks from the Green River Formation (Wyoming, USA).
- Antibody-based detection of soluble lipids and hydrocarbons is further expanded in **Chapter 5** by exploring the potential of a simple planar chromatographic technique that can be coupled with immunodetection.
- The subsequent **Chapter 6** follows up on the potential use of antibodies that bind squalane to visualize hydrocarbon signals that may be directly associated with the skeletal structure of fish fossil remains from the Green River Formation (Wyoming, USA).
- The final **Chapter 7** briefly summarizes some concluding points and key findings from the previous sections.

Chapter 2

Field detection of urease and carbonic anhydrase activity using rapid and economical tests to assess microbially induced carbonate precipitation

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2.1. Synopsis

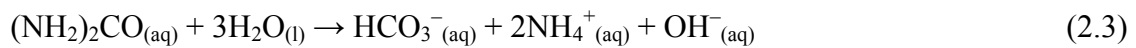
Microbial precipitation of calcium carbonate is a widespread environmental phenomenon that has diverse engineering applications, from building and soil restoration, to carbon sequestration. Urease-mediated ureolysis and CO_2 (de)hydration by carbonic anhydrase (CA) are known for their potential to precipitate carbonate minerals, yet many environmental microbial community studies rely on marker gene or metagenomic approaches that are unable to determine *in situ* activity. Here, we developed fast and cost-effective tests for the field detection of urease and CA activity using pH-sensitive strips inside microcentrifuge tubes that change color in response to the reaction products of urease (NH_3) and CA (CO_2). The urease assay proved sensitive and useful in the field to detect *in situ* activity in biofilms from a saline lake, a series of calcareous fens, and ferrous springs, finding relatively high urease activity in lake samples. Incubations of lake microbes with urea resulted in significantly higher CaCO_3 precipitation compared to incubations with a urease inhibitor, showing that the rapid assay indicated an on-site active metabolism potentially mediating carbonate precipitation. The CA assay, however, showed less sensitivity compared to the urease test. While its sensitivity limits its utility, the assay may still be useful as a preliminary indicator given the paucity of other means for detecting CA activity in the field. Field urease, and potentially CA, activity assays complement molecular approaches and facilitate the search for carbonate-precipitating microbes and their *in situ* activity, which could be applied toward agriculture, engineering and carbon sequestration technologies.

2.2. Introduction

Microbially-induced calcium carbonate precipitation (MICP) is a widespread environmental process that affects the carbon cycle and that has been explored as an alternative solution for several engineering and environmental challenges, such as restoration of concrete structures, soil consolidation, pollutant bioremediation, and CO₂ sequestration (Sarayu *et al.*, 2014; Li *et al.*, 2015; Bose and Satyanarayana, 2017; Krajewska, 2018; Reeksting *et al.*, 2020). Despite its environmental relevance and multiple applications, MICP can be difficult to assess and identify in a given environment. Current environmental microbiota analyses commonly characterize communities via 16S rRNA gene sequencing and metagenomic sequencing. High throughput sequencing techniques, however, fail to determine active metabolisms, unless they are complemented with challenging and labor-intense transcriptomic or culture-based analysis, which do not necessarily reflect *in situ* activity. It is possible, however, to directly test the activity of certain enzymes in the field and identify active metabolisms on-site, therefore assessing the microbial function in a particular environment. Here, we developed a method for the field detection of urease (EC 3.5.1.5) and carbonic anhydrase (CA; EC 4.2.1.1) activity, two enzymes associated with MICP (Achal and Pan, 2011), to reveal microbial communities that potentially promote carbonate precipitation *in situ*.

Metabolisms such as photosynthesis, methane oxidation, nitrate reduction, bicarbonate transport, and ureolysis can locally increase carbonate saturation and promote MICP (Zhu and Dittrich, 2016; Seifan and Berenjian, 2019). Two of these metabolisms rely on enzymes whose activity can potentially be detected in the field:

ureolysis and CO₂ (de)hydration via CA. Ureolysis, catalyzed by urease, allows microorganisms to use urea as a nitrogen and carbon source (Krausfeldt *et al.*, 2019). CA facilitates rapid carbon transport into the cell via CO₂-HCO₃⁻ interconversion (Kumar and Ferry, 2014). Both enzymes generate carbonate anions and increase the pH as a product of their activity. Urease generates ammonia and CO₂ from urea (Eq. 2.1), which coupled to ammonia hydrolysis and CO₂ hydration, a reversible reaction catalyzed by CA (Eq. 2.2), produces one mol of hydroxide and one mol of bicarbonate per mol of urea (Eq. 2.3):



The production of hydroxide and bicarbonate increases the saturation state with respect to calcium carbonates, promoting their precipitation. Urease and CA have shown to drive MICP in multiple environments (Dhami *et al.*, 2013; Seifan and Berenjian, 2019), yet their effects are commonly evaluated via culture-based approaches, disregarding the activity of complex communities *in situ*. An alternative approach is to test the enzymatic activity from microbial biofilms directly in the field. Existing urease and CA assays, however, are not efficient for field testing. For example, ammonia detection by the Berthelot reaction is commonly used to evaluate urease activity by a color change in solution (e.g., Kandeler and Gerber, 1988; Sinsabaugh *et al.*, 2000; Cordero *et al.*, 2019), which is dramatically affected by the inherent components of environmental samples (e.g., pigments in photosynthetic biofilms preclude the reading of the assayed solutions), requiring high sample dilution, several individual steps, long

incubation times, and laboratory equipment, which prevent its application in the field. In the case of CA, most activity measurements are dependent on a solution-based colorimetry of its esterase activity (e.g., Baliukynas *et al.*, 2020), a characteristic known to only some CA isoforms from few organisms (Innocenti and Supuran, 2010). Methods that instead evaluate the CA hydratase activity are based on a pH change in solution, which could also be affected by the inherent buffer capacity of environmental samples. These urease and CA assays were intended to evaluate the enzymatic activity from protein extracts of cultured organisms or from laboratory-processed samples, rather than complex environmental samples, and cannot be implemented in the field without sophisticated equipment. To circumvent these inconveniences, we used a pH-sensitive strip to detect the gaseous products of urease and CA reactions, NH_3 and CO_2 , respectively, which diffuse from the enzymatic reaction site and can therefore be detected with minimal interference from other components of an aqueous environmental sample. Separating the gas detection site from the enzymatic reaction site can be achieved inside a microcentrifuge tube, which makes the tests suitable for field deployment. We evaluated on-site urease and CA activities using this strategy in samples from calcareous fens, iron-rich springs, and a saline lake. In these settings, the environmental conditions may lead to carbonate precipitation, however, the contribution of MICP is difficult to assess in the absence of a field assay. By using a field test, we obtained a preliminary assessment of potential MICP, which can be applied to identify environments with relevant microbial carbonate mineralization, and to facilitate the management of urease- and CA-based technologies.

2.3. Experimental Procedures

2.3.1. Rapid urease field test

Urease activity was evaluated using a modification of the rapid urease test for the detection of *Helicobacter pylori* in clinical samples (Thillainayagam *et al.*, 1991; Ross and Behar, 2012). The test consists of a sample-loading strip containing urea (urease substrate), and a pH-sensitive indicator strip. Sample-loading strips were prepared by immersing a cellulose fiber sheet paper P8 (Thermo Fisher Scientific, Cat. 09-795D, Waltham, MA, USA) in a fresh 0.6 M urea, 0.4 mM EDTA solution (50 mL per 100 cm² of paper sheet) using a rocking flat glass pan to impregnate the paper thoroughly for 30 seconds per side. The impregnated paper sheet was extended on a tilted (~45°) dry flat glass surface to drain excess solution, and left drying at room temperature (~10 h). Indicator strips were similarly prepared by impregnating a paper with fresh 0.02 % phenol red solution adjusted to pH 6.0 with 50 mM HCl. After the papers dry completely, circles of 6.5 mm diameter (or alternatively, strips of 4 × 8 mm) were cut from both the urea- and indicator-impregnated papers.

To assemble the test, individual sample-loading strips were introduced in Seal-Rite 0.5 mL microcentrifuge tubes (USA Scientific, Inc., Ocala, Florida, USA), and indicator strips were placed inside the seals of the caps (**Figure 2.1A**). Urease activity was detected by adding standard solutions of urease from *Canavalia ensiformis* (one unit, U, was defined as the quantity of enzyme that liberates 1 μmole of ammonia from urea per minute at pH 7.0 and 25 °C; product number U1875, Sigma-Aldrich, St. Louis, Missouri, USA) or wet biofilm samples in direct contact with the sample-loading strip before tightly closing the tube. A positive reaction was visualized by a color change from

yellow to red in the indicator strip after ~5 to 60 minutes of incubation at room/field temperature (**Figure 2.1B**). Volatile ammonia released from urea (Eq. 2.1) increases the pH in the indicator strip, changing its color (**Figure 2.1C**). During the test, it is important to prevent any contact of the sample with the indicator strip to avoid a color change derived from alkaline or acidic samples that may turn the indicator. The dry format of this test yields a faster reaction compared to other liquid- or gel-based assays (Tseng *et al.*, 2005), and makes it conducive to field deployment.

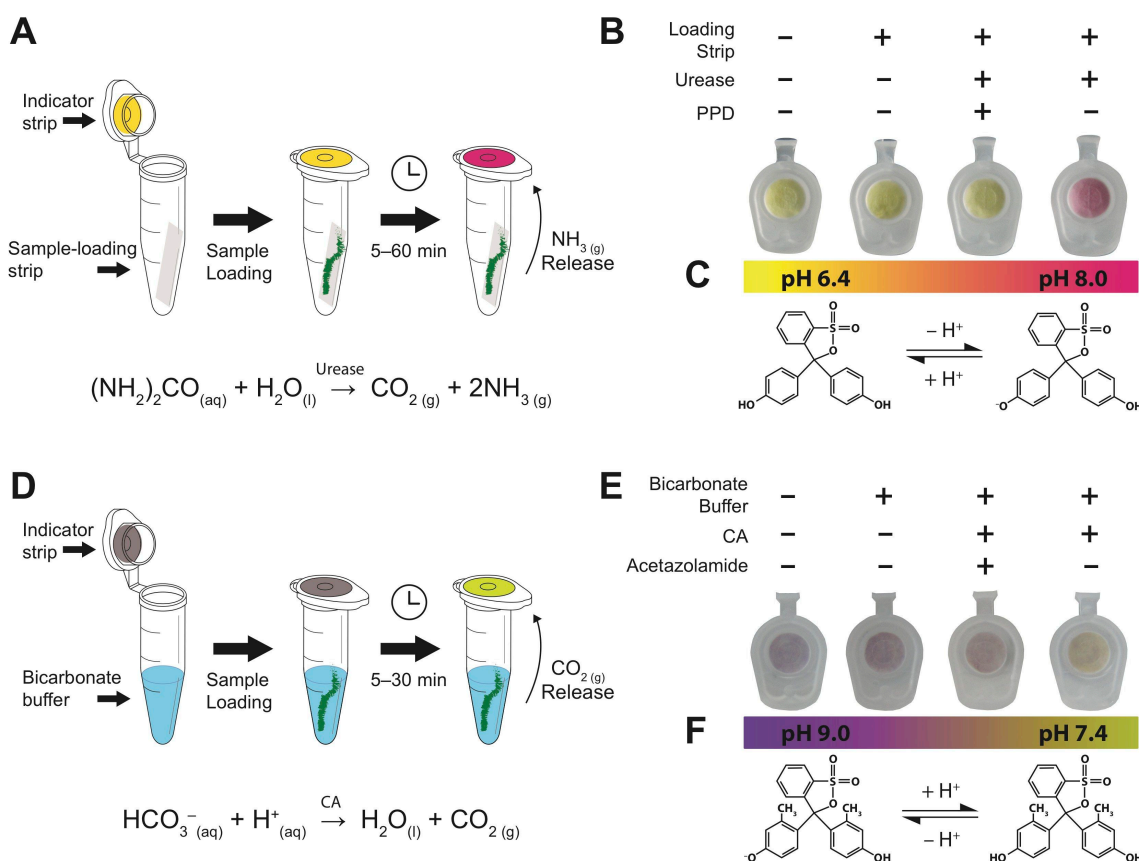


Figure 2.1. Urease and CA field tests. (A) Representation of a positive urease assay after adding a biofilm sample in direct contact with the loading strip. (B) A switch to red in the tube cap (indicator strip) indicates a positive urease reaction. If no urease is present or if a

loading strip with urease inhibitor (PPD) is assayed, the indicator is expected to remain yellow. (C) Color transition of phenol red (pKa 7.4, pH range 6.4–8.0) used for urease tests. (D) CA assay scheme after adding a sample in the tube with bicarbonate solution. (E) Faster development of a yellow color in the indicator than a negative control (no sample added or using a CA inhibitor) indicates positive CA activity. (F) Color transition of metacresol purple (pKa 8.32, pH range 7.4–9.0) used for CA assays.

A negative control was prepared under the same conditions and additionally adding 1 mM phenyl phosphorodiamidate (PPD)—a urease inhibitor—in the soaking solution of sample-loading strips. A positive reaction without the inhibitor and a negative reaction with PPD strongly indicate urease activity. The test, prepared as described here in closed microcentrifuge tubes, lasts at least one year stored at room temperature and protected from light with no decrease in sensitivity. The negative control containing PPD, however, expires after ~2–3 months, because the PPD inhibitor is sensitive to oxygen. After three months, the PPD-impregnated strips are unable to inhibit high urease concentrations and may result in a false positive reaction, although yielding a considerably less intense color change than the urease test without inhibitor. We recommend preparing the inhibitor-impregnated loading strips from a fresh PPD solution a few days prior to use, or alternatively, storing the loading strips under anoxic conditions and protected from light.

2.3.2. Rapid CA field test

CA assays were based on a CO₂ detection strip intended for proper endotracheal catheter introduction (Fehder, 1988). CO₂-sensitive strips were prepared, similarly to the urease field test strips, by soaking a cellulose fiber paper grade P8 (Thermo Fisher Scientific, Cat. 09-795D, Waltham, MA, USA) with a fresh solution containing 0.0065 M Na₂CO₃, 0.01% metacresol purple, and 50% glycerol diluted with N₂-purged distilled water. Impregnated papers were immediately dried on a flat glass surface by a stream of hot air (~15 min) and circles of 6.5 mm (or strips of 4 × 8 mm) were cut and placed inside the cap seals of 0.5 mL microcentrifuge tubes (USA Scientific, Inc., Ocala, Florida, USA; **Figure 2.1D**). Inside microcentrifuge tubes, the bluish-purple indicator gradually turns purplish-yellow after three days of exposure to atmospheric CO₂, which limits the extent of using this method for field applications. We recommend using indicator strips prepared no more than 2 days in advance.

CA activity was detected by introducing 80 µL of cold 1 M NaHCO₃ in the tubes and immediately adding the samples, or 15 µL of standard CA (isozyme II from bovine erythrocytes; Sigma-Aldrich, St. Louis, Missouri, USA) solutions. The tubes were immediately closed and incubated on ice. Bicarbonate dehydration (catalyzed by CA, Eq. 2.2) produces volatile CO₂, which reacts with glycerol-absorbed water in the indicator lid and generates acidity that turns the indicator from bluish-purple to purplish-yellow (**Figure 2.1E**). Non-enzymatic bicarbonate dehydration proceeds rapidly and therefore the indicator color change is observed within minutes, even without the enzyme. For this reason, it is important to use cold solutions and incubate the assay tubes on ice. Cold incubation limits non-enzymatic CO₂ hydration to a larger degree than the CA-mediated reaction, and is essential for visualizing a reaction time difference between negative

controls and CA-incubated reactions. Wilbur and Anderson (1948) noted the utility of cold incubations, which has since been applied in most CA hydratase activity assays to date. By using the CO₂-sensitive strips in microcentrifuge tubes we found a subtle, but reproducible, color change difference between CA-incubated (15–85 mM CA) and negative controls assayed simultaneously. Negative controls were prepared under the same conditions and adding 5 µL of fresh 1 mM acetazolamide—a CA inhibitor—to the bicarbonate buffer. A faster color change compared to negative controls is indicative of CA activity.

2.3.3. Microbial sampling locations

Samples were taken near calcareous fens in the Minnesota River Basin, from ferrous springs and from Salt Lake, MN, during July and August 2019 (see detailed locations in **Figure A.S1** and **Table A.S1**). Salt Lake is an alkaline sulfate- and sodium-dominated saline lake. The lake alkalinity (234 ± 2 mg/L CaCO₃, pH 8–9), together with calcium carbonates in its sediments (Dean *et al.*, 1993), indicates favorable conditions for carbonate mineral precipitation, which could be stimulated by microbial metabolisms. A sample of buoyant green biomass was collected from Salt Lake during a bloom event in July 2019. Submerged green filaments attached to shoreland rushes were also collected following the bloom in August 2019 (**Figure A.S2**).

Calcareous fens, peatlands in which surficial calcium carbonate precipitates (Almendinger and Leete, 1998), are also environments where microorganisms may contribute to carbonate mineralization. Samples were collected from green biofilms growing on peat exposed by a creek at Black Dog Lake Fen, and from surficial green

filaments suspended on water ponds during flood events in Nicols Meadow Fen, and between Fontier 8 Fen and Sioux Nation WMA Fen (locations shown in **Table A.S1** and **Figure A.S3**). Additionally, samples from iron-oxidizing microbes were collected from orange precipitates at a creek in Nicols Meadow Fen, from groundwater seeping to the Mississippi River at Saint Mary's Spring, and from a sulfide seep at a roadside near Soudan, MN (**Figure A.S4**). A small portion of biomass (enough to wet the sample-loading strip of the urease assay) was evaluated on-site in triplicate (with the exception of the Salt Lake bloom sample, where only two samples were evaluated) for urease and CA activity using the rapid field test. Additional triplicate aliquots for each sample were obtained for protein extraction and microscopic observations (see detailed extraction methods in the supplementary file).

2.3.4. Calcium depletion kinetics

Salt Lake filaments (Sample ID 02 in **Table A.S1**) were incubated (0.5 g wet weight) in 20 mL of 0.2 μm -filtered lake water with the addition of 0.8 M urea in closed 50-mL glass serum bottles with agitation (90 rpm, orbital shaker MaxQTM 2000, Thermo Fisher Scientific) at room temperature and under natural light cycles for 12 days. Four different incubation conditions were evaluated in triplicate: lake water without inhibitors, lake water with 1 mM acetazolamide, lake water with 1 mM PPD, and lake water with both inhibitors (1 mM each). Aliquots of 0.5 mL were taken over time and titrated with EDTA (HAC-DT, Hach, Loveland, CO, USA) to quantify calcium. Solid residues after incubations were evaluated by powder micro X-ray diffraction (micro-XRD) using a Bruker D8 Discover micro-diffractometer with a CoK α source ($\lambda = 1.78899 \text{ \AA}$) equipped

with a graphite monochromator and a 2D Vantec 500 detector. Samples were mounted on vacuum grease and three frames (30° 2 θ width) centered at 20°, 45° and 70° were collected for 900 s at 40 kV and 35 mA. Phase identification was conducted using Match! (v3.8.3.151) and the Crystallographic Open Database (COD-Inorg REV218120 2019.09.10) reference patterns for aragonite (96-901-6601), calcite (96-900-0971), monohydrocalcite (96-901-2074), quartz (96-901-0145), thenardite (96-900-4093) and vaterite (96-150-8972). Ikaite diffraction pattern was obtained from Hesse *et al.* (1983).

2.3.5. Image and Statistical analysis

To compare and semi-quantify the rapid test results, we followed color changes using hue values. The hue represents color pigmentation by a single number, disregarding saturation and brightness, therefore, minimizing color differences resulting from light and exposure time changes in the field (**Figure A.S5**). Average hue values were calculated from RGB colors of standard circle areas over photographs of the indicator strips using the NIH ImageJ 1.49v software. Statistical significance of hue values from the rapid assays was assessed via mixed-effects models (considering the reaction time, as well as the presence or absence of inhibitor for each site, as nominal variables) with Geisser-Greenhouse correction using GraphPad Prism 8.3.0. Differences between each assay at a given incubation time and its respective control containing an enzyme inhibitor were evaluated by Bonferroni's multiple comparisons tests. Statistical analysis for the calcium depletion kinetic experiments was similarly evaluated via a two-way ANOVA, and Bonferroni correction to compare between experimental conditions.

2.4. Results and Discussion

2.4.1. Rapid test sensitivity and reproducibility

A pH-sensitive dye encapsulated within a cellulose matrix was used as a detector of NH_3 or CO_2 inside the cap of microcentrifuge tubes. Urease activity was followed by an ammonia-mediated pH increase, whereas CA was detected by a CO_2 -driven pH decrease. The urease test format in **Figure 2.1A** turns a phenol red indicator from yellow to red in less than 15 minutes when > 30 mU urease is assayed (**Figure 2.2A**). The hues in the indicator are significantly affected by the incubation time ($F = 429.6$, $DFn = 3.2$, $DFd = 125.8$, $p < 0.0001$) and by the incubation condition ($F = 258.9$, $DFn = 7$, $DFd = 40$, $p < 0.0001$). After 30 minutes, the method sensitivity is ~ 3 mU when compared to a PPD-containing negative control (**Figure 2.2B**), which is comparable to the response using the Berthelot reaction in a canonical urease quantification assay (**Figure A.S6**).

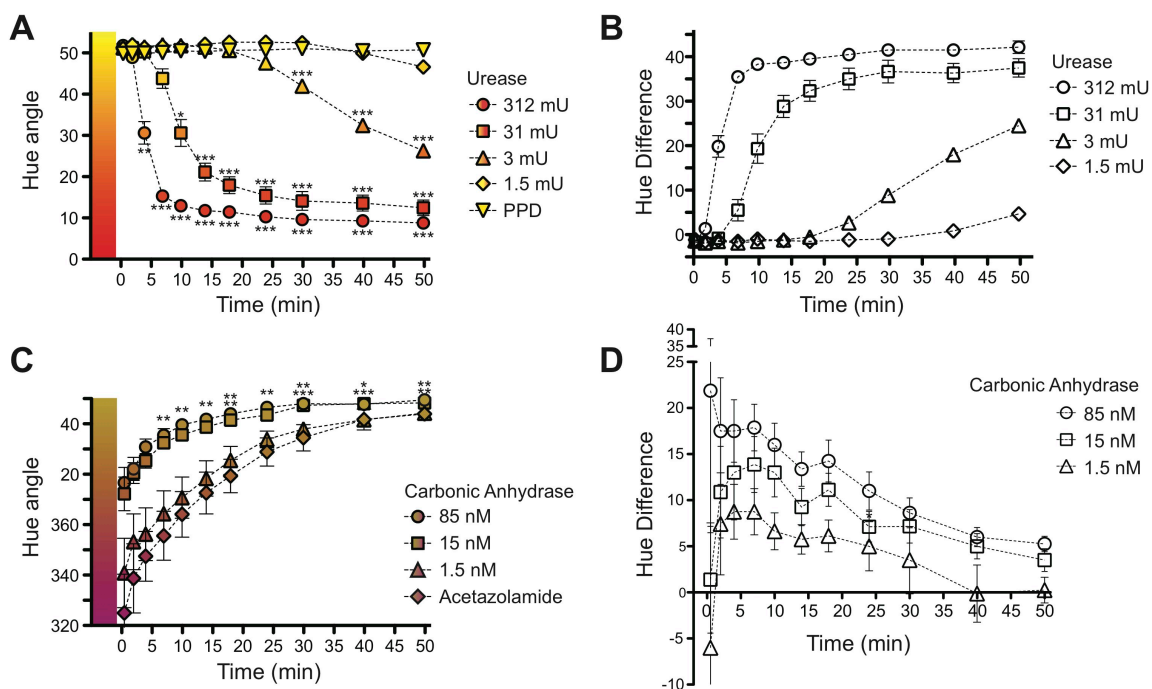


Figure 2.2. Field test color change kinetics and sensitivities. (A) Assay color (expressed as average hue values) as a function of incubation time for the urease test using different urease standards (1.5–312 mU), and the average of their negative controls containing a urease inhibitor (PPD). (B) Hue difference of urease assays compared to their respective negative controls. (C) CA assay hue as a function of incubation time after adding different CA concentrations (1.5–85 nM), and the average of their negative controls in the presence of a CA inhibitor (acetazolamide). (D) Hue difference of CA standards compared to their respective negative controls. All conditions were assayed using six replicates. Error bars represent standard error (if not shown, the error is smaller than the symbols). * $0.1 < p < 0.05$, ** $0.05 < p < 0.01$, *** $p < 0.01$ between each condition and its respective negative control assessed by Bonferroni's multiple comparisons test.

In contrast to the urease test, the CA assay yields only a subtle color change when compared to acetazolamide-containing negative controls, because the rapid and spontaneous bicarbonate dehydration, even without the enzyme, turns metacresol purple indicator to yellow within 30 minutes (**Figure 2.2C**). Detection of the CA hydratase activity by any method, however, is limited by its fast non-enzymatic reaction, therefore always requiring a comparison with a negative control at a given reaction time. CA-containing assays show hues significantly affected by the incubation time ($F = 117$, $DFn = 2.22$, $DFd = 93.2$, $p < 0.0001$) and by the incubation condition ($F = 5.8$, $DFn = 5$, $DFd = 42$, $p = 0.0004$). Pairwise comparisons between conditions with and without inhibitor assayed simultaneously, however, show subtle hue differences, mostly significant ($p <$

0.05) at 15 nM CA or higher (**Figure 2.2D**). By contrast, a canonical CA hydratase assay shows sensitivities as low as 0.15–0.3 nM CA (**Figure A.S6**).

2.4.2. Field detection of urease and CA activity

Urease and CA tests were used for on-site enzymatic activity detection in samples from a saline lake, ponds from calcareous fens, and iron oxide precipitates from ferrous springs. Biomass collected during a bloom event in Salt Lake and a sample of green filaments collected after the bloom were urease-positive, where the incubation time significantly affected the hues ($F = 13.8$, $DFn = 1.4$, $DFd = 2.8$, $p = 0.036$, and; $F = 23$, $DFn = 1.1$, $DFd = 4.4$, $p = 0.0064$, respectively), and finding a significant difference ($p < 0.05$) in less than 35 minutes when compared to negative control assays (**Figure 2.3A, B**).

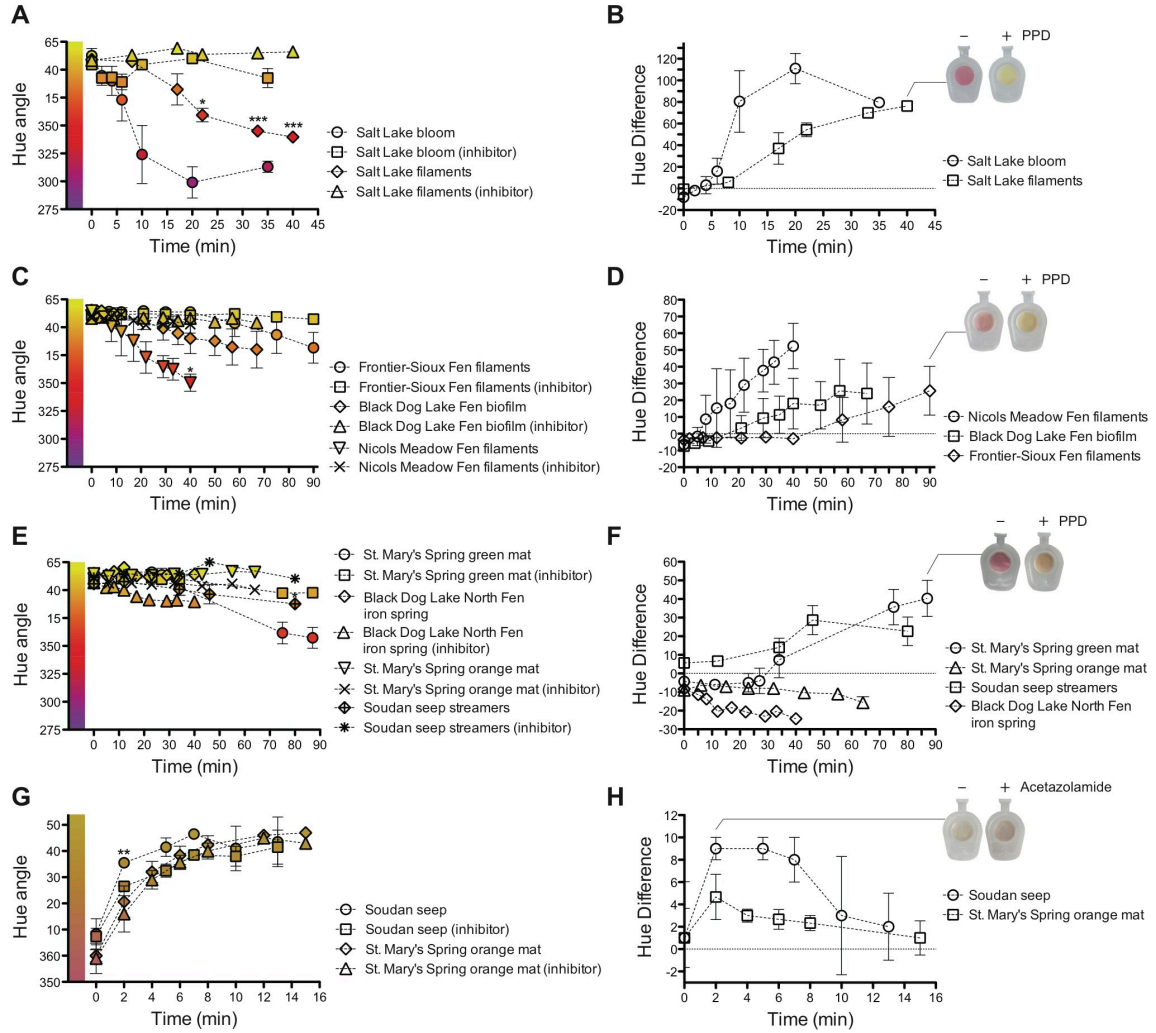


Figure 2.3. Field test color change using environmental samples. Hue, and hue difference from on-site urease assays compared to their respective negative controls of Salt Lake (A, B), calcareous fen (C, D), and iron spring (E, F) samples. CA assay hue (G), and hue difference (H) of ferric precipitates is also shown, along with representative pictures of tubes without (–) and with (+) inhibitors for particular time points and samples. When shown, error bars are larger than their respective symbols, representing the standard error.

* $0.1 < p < 0.05$, ** $0.05 < p < 0.01$, *** $p < 0.01$ between each sample and its respective negative control according to Bonferroni's multiple comparisons test.

Nicols Meadow Fen samples were also positive for urease, with hues significantly affected by the incubation time ($F = 13.3$, $DFn = 1.2$, $DFd = 4.9$, $p < 0.014$), although showed less color change intensity than the samples from Salt Lake (**Figure 2.3C, D**). Green photosynthetic sheaths from Fortier-Sioux Nation Fen and green biofilms from Black Dog Lake Fen showed comparatively less urease activity, where the incubation time did not significantly affect their hues ($F = 3.4$, $DFn = 1.1$, $DFd = 4.2$, $p = 0.134$, and; $F = 4.3$, $DFn = 1.1$, $DFd = 4.2$, $p = 0.101$, respectively), despite showing a slight positive red coloration after one hour of incubation (**Figure 2.3C, D**). In organisms that do not constitutively express urease, its expression is likely induced when urea is available (Mobley *et al.*, 1995; Zhou *et al.*, 2019). The higher urease activity in Salt Lake may therefore reflect urea accessibility and correlate with the bloom event observed in July 2019. Agriculture promotes eutrophication (Paerl *et al.*, 2016), and in particular urea—the major worldwide fertilizer (Glibert *et al.*, 2014)—can be used by microbes as both N and C source (Krausfeldt *et al.*, 2019). Salt Lake is located in close proximity to farmland and its microbial communities may be sensitive to nearby fertilization practices. By contrast, less urease activity found in calcareous fen samples, in particular Fortier-Sioux Nation Fen, which is located near a State Wildlife Management Area, may reflect a lower agriculture impact (**Figure A.S7**).

Soudan seep biofilms showed slight urease-positive reactions with hues significantly influenced by the incubation time ($F = 6.05$, $DFn = 1.6$, $DFd = 6.6$, $p = 0.036$; **Figure 2.3E, F**). Biofilms at St. Mary's Spring have a combination of green cyanobacterial filaments that were slightly positive for urease, with hues significantly

affected by the incubation time ($F = 40.4$, $DFn = 1.8$, $DFd = 7.2$, $p = 0.0001$), and stalks of iron-oxidizing bacteria surrounded by few cyanobacteria and iron oxides, which were urease-negative (**Figure 2.3E, F**). Iron oxides from Black Dog Lake North Fen creek were also urease-negative. PPD-containing controls in these ferric precipitates, however, showed a slight color change (represented by low hues, and a negative hue difference in **Figure 2.3E, F**), attributed to ammonia release from urea (which may be less stable in the presence of PPD) or from PPD degradation. The P–N bonds in phosphoramidates are unstable in aqueous solutions (Kafarski and Talma, 2018) and may release ammonia. Moderate transitions to red may lead to false positives, although its intensity was not comparable to the positive reactions observed in other environments. Several other urease inhibitors not tested in this study (Amtul *et al.*, 2002) may prevent false positives, however, phosphoramidates (such as PPD) are among the most potent and specific urease inhibitors (Kafarski and Talma, 2018) and were therefore selected for this assay.

In contrast to urease, the CA assay did not show color differences in Salt Lake and calcareous fen samples. Only Soudan seep samples showed subtle visual differences compared to negative controls that were significant (Bonferroni-corrected p -value = 0.037) at 2 minutes of assay (**Figure 2.3G, H**). It is generally expected to find CA in photosynthetic biofilms as part of their CO₂ concentrating mechanisms (Kumar and Ferry, 2014), and thus CA levels in most analyzed samples were possibly below the detection limit of the field assay. While the urease assay was shown to be robust and shown to have low detection limits, the CA assay was limited by the nature of its catalyzed reaction (Eq. 2.2), which always requires a rigorous comparison with a negative control at a particular time. In addition, carbonic anhydrases include at least six

enzyme classes with no structural or sequence homology and, consequently, the inhibitor choice may have varied effects. Although acetazolamide is a potent wide-spectrum CA inhibitor (Zimmerman *et al.*, 2004; Zimmerman *et al.*, 2007), it is possible that certain microbial CA were not effectively inhibited, which would yield higher negative control rates and thus lower assay sensitivities when compared to conditions without inhibitors. We failed, however, to obtain environmental protein extracts with significant soluble CA activity in the laboratory, using a conventional assay (**Figure A.S8**), even in photosynthetic cells. We also found very low soluble urease activity from protein extracts (**Figure A.S8**), indicating that most on-site activity found may have been the result of extracellular, possibly membrane bound or extracellular polymeric substances (EPS)-associated urease, which was observed as residual urease activity of cell debris after protein extraction.

The different factors affecting the quality of the CA field detection method (such as ice incubation, rigorous comparison with a negative control at a particular time, color change of the detection strip with atmospheric CO₂ within days of storage, and lower sensitivity compared to conventional laboratory assays) limits its reliable application in a field setting. To our knowledge, however, there are no reported CA assays for field detection, and thus the strategy described here could provide preliminary semi-quantitative information of CA activity in the environment. The applicability of the field CA assay for environmental samples may be improved by using more sensitive and stable CO₂ detectors, along with negative controls containing a cocktail of different CA inhibitor types.

2.4.3. Carbonate precipitation induced by urease and CA

Differences in intensity and time to obtain a positive reaction serve as parameters to compare relative activities among sites, where the strongest urease activity was found in Salt Lake samples. To determine potential urease- and CA-dependent MICP, we incubated Salt Lake filaments with lake water containing urea, showing a significant decrease in its Ca^{2+} concentration ($14.4 \pm 0.5 \text{ mM}$) through time ($F = 118.8$, $DFn = 4.1$, $DFd = 33.2$, $p < 0.0001$) starting at Day 4, and depleting at Day 9 (**Figure 2.4A**). The calcium drop is interpreted as calcium carbonate precipitation, which was also observed by the solution turbidity (attributable to CaCO_3) starting at Day 3–4 (**Figure 2.4B–D**). By contrast, incubations in the presence of PPD depleted only around one-third of the initial calcium after 12 days, having significantly higher calcium than incubations without PPD after Day 4 (**Figure 2.4A**).

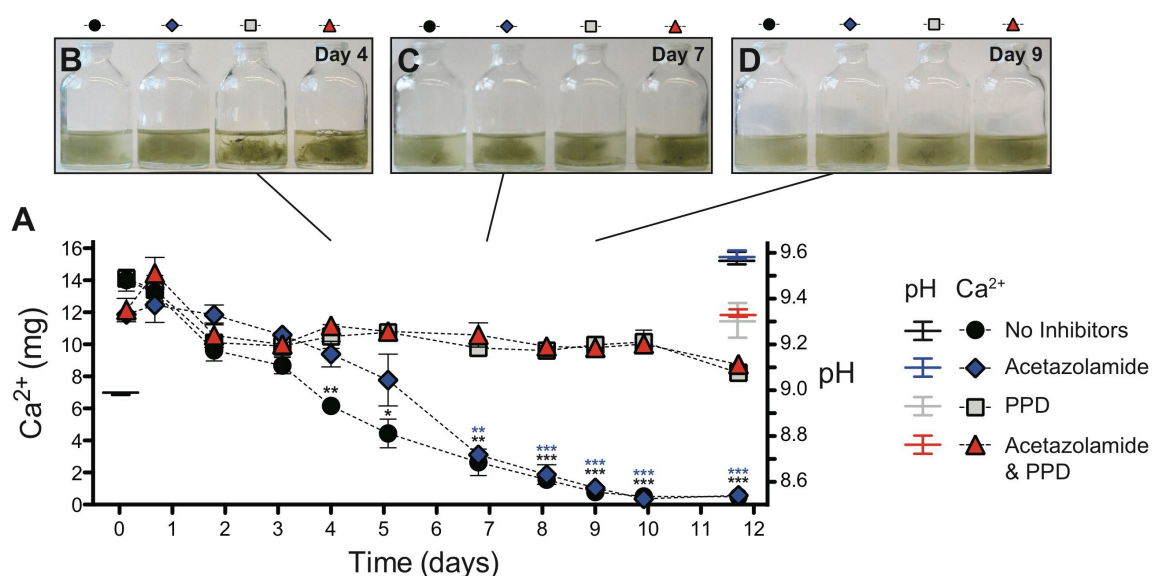


Figure 2.4. Calcium depletion kinetics of Salt Lake water in the presence of its microbial biomass. (A) Calcium quantification (left axis) from aliquots taken over 12 days for

triplicate incubations with a CA inhibitor (acetazolamide), a urease inhibitor (PPD) and conditions with and without both inhibitors. A pH increase (right axis) is observed at the end of the incubations. Standard error of triplicate assays is represented by the error bars, unless the error is smaller than the respective symbol. Bonferroni's p -values between each condition and the experiment with both inhibitors are shown with asterisks: * $0.1 < p < 0.05$, ** $0.05 < p < 0.01$, *** $p < 0.01$. Photos of representative incubations at Day 4 (B), 7 (C) and 9 (D) show hazy solutions attributable to mineral precipitation.

Filament incubations in the presence of acetazolamide, however, did not prevent calcium depletion (**Figure 2.4**). Therefore, urease, and not CA activity, is most likely responsible for carbonate precipitation under the studied conditions, consistent with the enzymatic activity observed in the field. Between Days 4 and 7, however, a delay in calcium depletion with acetazolamide compared to the condition without inhibitor may indicate a possible CA influence on CaCO_3 precipitation dynamics. Both enzymes may synergistically affect carbonate precipitation, as suggested previously (Dhami *et al.*, 2014), via rapid bicarbonate generation by CA (Eq. 2.2) from CO_2 produced by urease (Eq. 2.1), providing CO_3^{2-} and neutralizing the acidity produced by CO_2 hydration. The mechanism by which urease promoted CaCO_3 precipitation is attributable to a pH increase during incubations compared to the lake water initial pH (**Figure 2.4A**). Without PPD, the pH rises more than 0.5 units in 12 days, increasing the saturation with respect to carbonate minerals (**Figure A.S9**).

Following 12 days, the filaments were covered by a white precipitate, extensively found in incubations without inhibitors or with acetazolamide only. Under the

microscope, green trichomes were encrusted by precipitates, which appeared white under phase contrast, blackish under bright field, and were autofluorescent (**Figure 2.5A**). The encrusting particles may correspond to magnesium-containing calcium carbonates, which have been observed to emit wide-spectrum fluorescence (Yoshida *et al.*, 2010). Additionally, characteristic signals of calcium carbonate polymorphs, such as vaterite, monohydrocalcite, calcite, ikaite, and aragonite, along with thenardite (Na_2SO_4 , likely the result of high Na and SO_4 in Salt Lake) and quartz (presumably from diatom frustules) were detected in the precipitates by micro-XRD (**Figure 2.5B**).

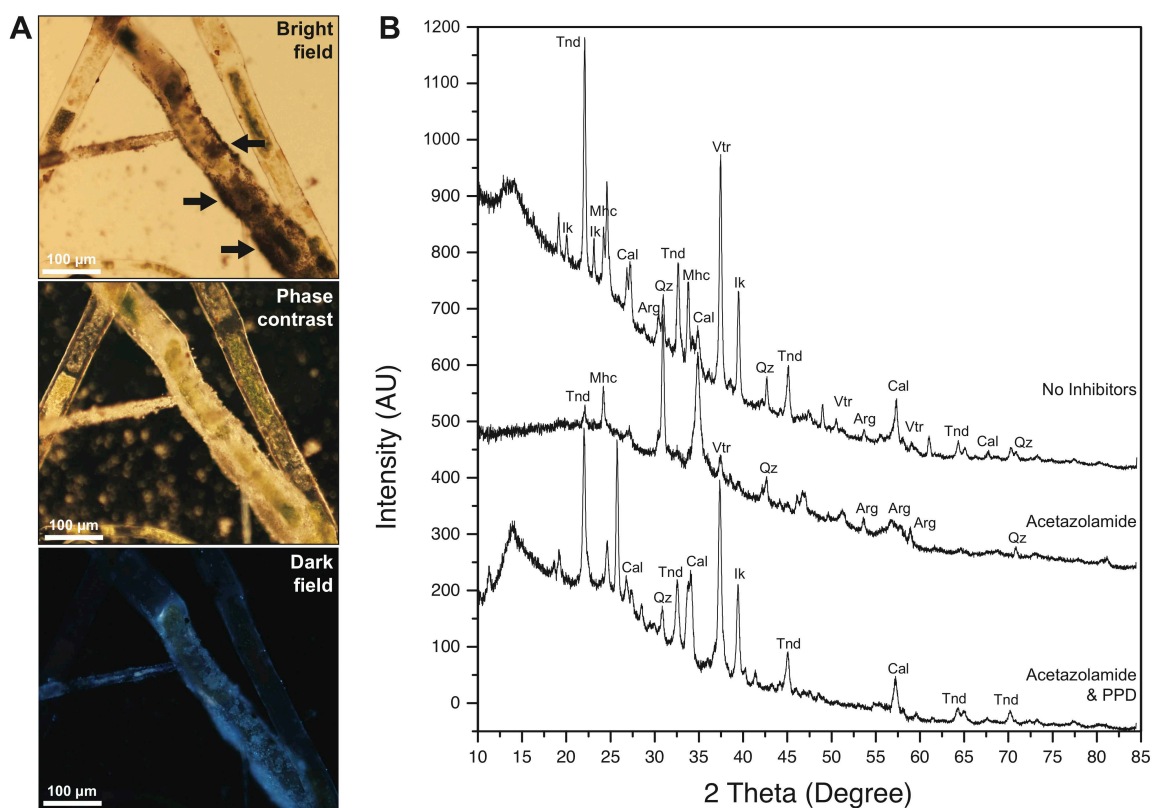


Figure 2.5. Salt Lake microbial sheaths after incubations. (A) Filament

photomicrographs after 12 days of incubation, showing black precipitates covering the sheaths under bright field (black arrows, top panel) that appeared white under phase

contrast (middle panel) and have wide-spectrum autofluorescence (lower panel, dark field showing 420 nm-excited 450 nm long-pass emission). (B) Micro-XRD of precipitates after incubations without inhibitors, with acetazolamide, and with both inhibitors. Mineral abbreviations: Arg, aragonite; Cal, calcite; Ik, ikaite; Mhc, monohydrocalcite; Qz, quartz; Tnd, thenardite; Vtr, vaterite.

2.4.4. Potential applications of urease field tests

The field detection of urease activity could prove useful for evaluating the predictability of fertilizer efficiency. Urea-based fertilizers are hydrolyzed by soil bacteria, resulting in volatilization and nitrogen loss to the atmosphere, which is not utilized by crops (Cantarella *et al.*, 2018; Modolo *et al.*, 2018). Although not evaluated in this study, the use of a simple test that could be employed by farmers to determine on-site urease activity from soils may help decide appropriate fertilizer types for a given region (Ouyang and Norton, 2020), without the need of experienced personnel or laboratory equipment to perform the analysis. Although different laboratory assays intended to quantify soil urease activity are available (Kandeler & Gerber, 1988; Sinsabaugh *et al.*, 2000; Cordero *et al.*, 2019), they require several steps with hours-long incubations using different reagents and equipment (plate reader, centrifuge), making their implementation difficult—if not impossible—in the field. Field samples are transported, stored, and analyzed in a laboratory setting after days or weeks from their collection, which may result in urease activity changes derived from sample manipulation, temperature fluctuations, freeze-thaw cycles, and time until its analysis (Lee *et al.* 2007). By contrast, once assembled in microcentrifuge tubes, the rapid urease detection method provides a

cheap and simple, one-step assay that could be carried out by inexperienced individuals, obtaining a qualitative result at the sampling site within minutes.

The field test may also be useful for rapid and cheap screening of environmental organisms with high urease activity that can be used for engineering applications. Urease-driven MICP has been explored in recent years for cementation and restoration of diverse structures, from art sculptures to buildings, as well as bedrock plugging for enhanced oil recovery (Dhami *et al.*, 2013; Krajewska, 2018). Application of ureolytic bacteria, however, requires evaluating the presence of urease activity, hopefully at the site of application, to provide evidence that the restoration approach is likely to work under the intended conditions. Rapid on-site evaluation of urease activity could help predict the efficiency of restoration approaches, instead of waiting for long-term reactive solutions.

A simple and economical test to detect enzymatic activity in the field may also help us understand the microbial processes that contribute to the chemistry and mineralogy of poorly studied sites. For example, though calcareous fens and other peatland ecosystems are extensive in some regions and are relevant carbon sinks (Lunt *et al.*, 2019), little information exists about the activity of their microbial communities, in particular the activity of urease. We showed here not only that calcareous fen microbes have the potential to express urease, but also that their urease is active *in situ* in certain environments, where urease-driven MICP could occur. Though the increasing use of fertilizers has been linked to ecosystem damage, it may be possible to encourage the use of urea-based fertilizers in regions that are hydrologically connected to calcium-rich areas (such as calcareous fens) where indigenous microbes are known to drive MICP, resulting in a sustainable carbon sequestration alternative (Mitchell *et al.*, 2010).

An economical test may be useful for educational demonstrations, and also could prove valuable to determine temporal variations of environmental metabolisms along different seasons, days, or even hours, which may otherwise prove difficult because of budget constraints. In-field tests may also be convenient to assess the influence of MICP on microbialites, particularly where bicarbonate transport and urease-related genes are known to be present (Warden *et al.*, 2016), which could help us understand the elusive metabolisms involved in ancient microbialite formation (Bosak *et al.*, 2007).

Top-down molecular studies of microbial communities and their environmental effects have exploded in the past decade, increasing our understanding of uncultivable microorganisms as well as the diversity of distinct environments in a microbe-dominated Earth. The information currently obtained via high-throughput sequencing of environmental microbes should be complemented with field activity assays to assess not only metabolic potential, but also microbial activity in a given environment. Enzymatic activities not only represent protein expression, but also the direct microbial effects on the environment, which in this study has been related to carbonate precipitation. We anticipate that field-based bottom-up approaches will aid in addressing challenging questions, such as determining the microbial role in mineral formation, as well as providing new eco-friendly technologies for engineering challenges. To our knowledge, this is the first field environmental urease and CA evaluation using an inexpensive and fast method developed with conventional laboratory materials. We expect that a variety of other environments can be tested using this method by other researchers, expanding our knowledge of environmental protein expression and its effects on MICP.

2.5. Acknowledgement

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Chapter 3

Active microbial metabolisms influence both carbonate precipitation and microfabric of rapidly formed modern tufas

The contents of this chapter are under preparation for submission to the journal *Geobiology*. This work corresponds to a collaborative research that started in the 2016 International Geobiology Course, co-authored by Fernando Medina Ferrer, Michael R. Rosen, Jayme Feyhl-Buska, Virginia V. Russell, Fredrik S nderholm, Sean Loyd, Russell Shapiro, Blake W. Stamps, Victoria Petryshyn, Cansu Demirel-Floyd, Jake V. Bailey, Hope A. Johnson, John R. Spear, and Frank A. Corsetti.

3.1. Synopsis

Modern carbonate tufa towers from Big Soda Lake (BSL), an alkaline lake in Nevada, exhibit rapid precipitation rates (exceeding 3 cm/yr) and host diverse microbial communities. The role of microbes in BSL tufa formation is, however, unknown. Based on lake water and groundwater carbonate saturation, along with carbon and oxygen isotope compositions of water and tufas, carbonate precipitation is primarily driven by the mixing of calcium-rich groundwater and carbonate-rich lake water, though microbial activity may also play a role. Here, we characterize the BSL microbial communities and evaluate their potential effects on carbonate precipitation that may influence fast carbonate precipitation rates. Small subunit rRNA gene surveys indicate a diverse microbial community living endolithically, in interior voids, and on tufa surfaces. Metagenomic DNA sequencing shows that genes associated with metabolisms that are capable of increasing carbonate saturation/precipitation (e.g., photosynthesis, ureolysis, and bicarbonate transport) are present. Enzyme activity assays showed that urease and carbonic anhydrase, two microbial enzymes that promote carbonate deposition, were found active *in situ* and also affected carbonate precipitation rates in laboratory incubations. We propose that, although BSL tufas form primarily as a result of abiogenic water mixing, tufa-inhabiting microbes may promote rapid carbonate authigenesis via ureolysis, and potentially via bicarbonate dehydration and CO₂ outgassing by carbonic anhydrase. Microbially-induced calcium carbonate precipitation in BSL tufas may generate signatures preserved in the carbonate microfabric, such as micrite replacement of abiogenic carbonates and stromatolitic layers, that could serve as models for developing potential biosignatures on Earth and elsewhere.

3.2. Introduction

Tufa—porous, non-marine, non-hydrothermal carbonate accumulations—form in mixing zones that saturate waters with respect to calcium carbonate minerals, and has therefore been typically interpreted as abiogenic in origin (e.g., Bischoff, Stine, Rosenbauer, Fitzpatrick, & Stafford, 1993; Buchardt, Israelson, Seaman, & Stockmann, 2001; Andrews, 2006). Diverse microbes colonize tufas, and growing evidence in the past decade suggests that microorganisms actively participate in the tufa formation process (e.g., Pedley, Rogerson, & Middleton, 2009; Guo, & Chafetz, 2012; Trampe, Larsen, Glaring, Stougaard, & Kühl, 2016; DeMott, Napieralski, Junium, Teece, & Scholz, 2020). Lacustrine tufa mounds, a type of vertical carbonate deposition that can reach several meters high, are found tightly associated with microbial mats in an environment where the formation of such structures cannot be explained by abiogenic mechanisms only (Brasier *et al.*, 2018). The microbial relevance in tufa growth is, however, difficult to assess in natural systems (Riding, 2000, 2011; Capezzuoli, Gandin, & Pedley, 2014). If microbial activity affects their formation, certain tufa characteristics could be considered a biosignature on Earth or elsewhere (e.g., Mars; Bosak, Souza-Egipsy, Corsetti, & Newman, 2004; Petryshyn, Corsetti, Frantz, Lund, & Berelson, 2016). Here, we characterize the microbial communities and attempt to elucidate their role in the rapid formation of active columnar tufas from Big Soda Lake (BSL), Nevada (**Figure 3.1**).



Figure 3.1. Big Soda Lake location and its actively growing tufa mounds. (a) Satellite image of BSL, Nevada (highlighted contour in North America), showing the location of the tufa shoreline (inset, white arrows show visible subaerial tufas) at the sampling site (39°31'45.6"N 118°52'54.2"W). Satellite image from the USGS Geospatial Data Sources, available at <https://earthexplorer.usgs.gov/>. (b) Wide-angle view of subaerial tufas at the northwestern shore of BSL. (c) Thermal image of visible sublacustrine springs associated with BSL tufas (insets), showing a colder upwelling plume in the summer (left panel) and a temperature reversal relative to the lake water, where warmer vents emerge during

winter (right panel). (d) Modern tufas at the northwestern shore of BSL showing evidence of active carbonate precipitation from 2016 to 2018 on automobile (inset) metal pieces.

BSL is an alkaline lake that hosts extensive tufa mounds along its margin. BSL tufa towers are thought to result from sub-lacustrine springs of groundwater infiltrating into the lake (Rosen, Arehart, & Lico, 2004), in a similar construction process to the tufa mounds from other modern and ancient alkaline lakes, such as Mono Lake (California, USA; Bischoff *et al.*, 1993), Pyramid Lake (Nevada, USA; Arp, Thiel, Reimer, Michaelis, & Reitner, 1999), and Searles Lake (California, USA; Guo, & Chafetz, 2012). When Ca^{2+} -bearing groundwater emerges into the alkaline, carbonate-rich lake waters, mixing in the infiltration vicinity saturates the lake water with respect to calcium carbonate minerals, promoting tufa formation. Carbonate precipitation, which in BSL tufas have predominantly been found in the form of calcite and monohydrocalcite (Rosen *et al.*, 2004), may result abiotically under such conditions (Bischoff *et al.*, 1993; Buchardt *et al.*, 2001). Yet, additional factors, such as temperature fluctuations, the presence of other ions/dissolved organic compounds, and the metabolic activity of microbes may influence authigenic precipitation and constrain tufa morphology and growth rate in BSL (Rosen *et al.*, 2004; Reddy, & Leenheer, 2011).

BSL tufas have an upper-estimate growth rate of 30 mm/year, resulting in 2–3 m structures that have precipitated over no more than the last 100 years (Rosen *et al.*, 2004). Although the water level in BSL has fallen in the last 20 years, exposing several tufas, some still remain submerged and exhibit continuous groundwater discharge, along with evidence of recent carbonate precipitation (**Figure 3.1c, d**). In addition, the presence of

pervasive photosynthetic mats on the surfaces of BSL tufas (**Figure 3.2**) suggests a possible microbial influence on tufa formation. The abundance of microbial mats covering actively growing tufas at BSL offer a natural laboratory to test their potential biogenicity and to study the microbial communities thriving in active lacustrine tufa mounds.

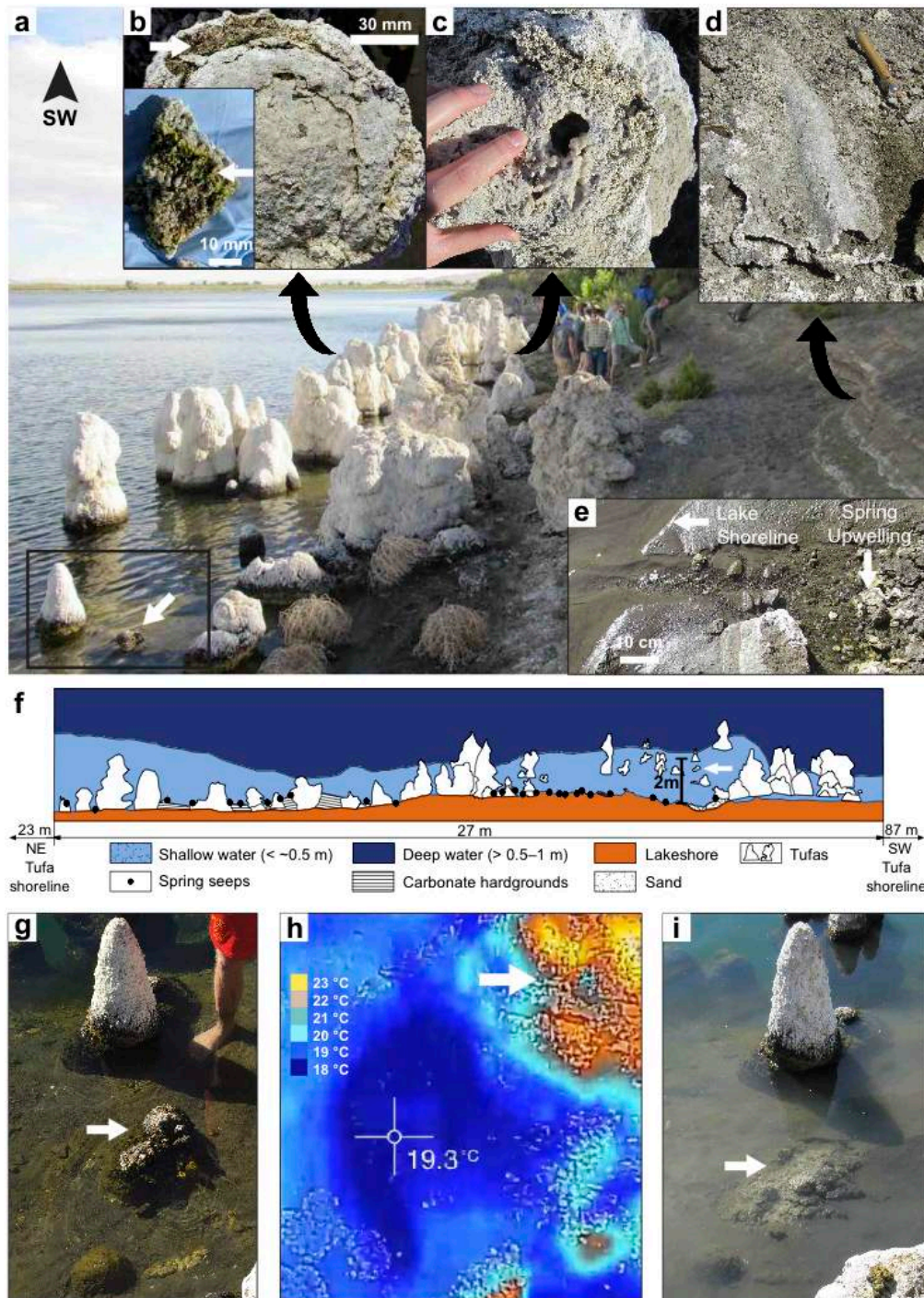


Figure 3.2. Tufa and water sampling at BSL as on 19 June 2016. (a) Picture of the northwestern shore of the lake showing a high density of exposed tufas where our water and tufa sampling took place. Members of the 2016 International Geobiology Course for

scale. Note the brownish mats on the surface of submerged portions of tufas. Some tufas have visible green endoliths ~1–2 cm beneath subaerial carbonates (b, white arrows pointing to a broken piece of tufa), and internal conduits that presumably correspond to groundwater channels (c). Calcified conduits are also observed at some exposed hardgrounds (d) as a consequence of spring discharges toward the lakeshore (e). Multiple freshwater upwellings were mapped and represented in (f), a cartoon showing the tufa sampling location. Inset at the lower left corner in (a) indicates the submerged tufa sampled (a, f–i, white arrow), which had visible groundwater seeping from its sides (h, a thermal image showing a colder upwelling plume left to the tufa). Groundwater discharge was still visible after removing the tufa (i, white arrow).

Microbes have been associated with a variety of carbonate depositional environments and actively participate in mineral precipitation (Dupraz *et al.*, 2009). Certain microbial metabolisms, such as photosynthesis, ureolysis, methane oxidation, bicarbonate transport, and nitrate reduction, can locally increase carbonate saturation by raising the pH and shifting dissolved inorganic carbon (DIC) species toward CO_3^{2-} , therefore indirectly promoting calcium carbonate precipitation at the biofilm microenvironment (Pedley, 2014; Zhu & Dittrich, 2016). Microbial cell walls and extracellular polymeric substance (EPS), abundant in photosynthetic biofilms, also provide nucleation sites for crystal growth. In addition, EPS may chelate, concentrate and release calcium after heterotrophic degradation of organic matter, accelerating or inhibiting mineral precipitation by controlling calcium availability (Arp *et al.*, 1999; Arp, Reimer, & Reitner, 2001; Dupraz, Visscher, Baumgartner, & Reid, 2004). We

investigated the geochemical conditions that promote the formation of tufas in BSL and whether these tufas preserve indicators of microbial influence. We also studied the microbial community composition in BSL waters and tufas, and used enzyme activity assays to quantify ureolysis and carbonic anhydrase (CA)-mediated CO₂ degassing, two metabolic processes that potentially increase the pH within the biofilm microenvironment thereby providing a mechanism of carbonate precipitation in BSL tufas.

3.3. Materials And Methods

3.3.1. Big Soda Lake description

BSL is a ~1.6 km² and ~63 m deep maar lake located northwest of Fallon, Nevada, USA (**Figure 3.1a**). Maar lakes are low relief volcanic craters, formed when hot magma contacts groundwater, resulting in violent explosions with production of tephra. The BSL crater is composed of alkali basaltic lapilli and bombs, along with lacustrine sediments dated as old as late Pleistocene in age ($14,740 \pm 1,945$ cal yr BP; Rosen, Reidy, Starratt, & Zimmerman, in press). From 1907 to 1930 the lake water level rose ~18 m as a consequence of irrigation of neighboring farmlands (Rosen *et al.*, 2004). Since then, groundwater effluents into the lake have provided favorable conditions to form columnar tufas, which mostly locate at the northwestern and southeastern edges of the lake (**Figure 3.1a–c**). Rosen *et al.* (2004) showed that tufas rooted at 4 m water depth could not have formed when the lake was 18 m lower, concluding that BSL tufas have rapidly precipitated within the last 100 years. In addition, around the tufa deposition site we found presumably automobile metal pieces (thus, less than 100 years) that are covered

with carbonates—a coating that seem to have increased from 2016 to 2018 (**Figure 3.1d**), indicating recent carbonate precipitation events at BSL.

Surface lake waters are mesosaline (TDS > 25 g/L), alkaline (pH 9.2–9.8), and have high (30–70 mM) DIC (Kharaka, Robinson, Law, & Carothers, 1984), whereas regional groundwaters are usually carbonate-depleted and calcium-rich relative to the lake (**Table B.S1**; Rosen *et al.*, 2004). Regional groundwater flows eastward (Smith, Buto, & Welborn, 2016) and it is likely that groundwater discharge into BSL comes from the southwest, associated with the Carson River, and also from unlined irrigation canals located east and north of the lake (**Figure B.S1**). In addition to the tufa mounds, precipitated salts, carbonate hardgrounds, and nearshore crusts that are characteristic of saline lakes are visible along the shoreline of BSL (**Figure 3.2a–d**).

3.3.2. Water sample collection

Samples were collected in June 2016 and October 2018 at the northwestern shore of BSL (39°31'45.6"N 118°52'54.2"W), where abundant subaqueous and subaerial tufas are located (**Figure 3.2**). In 2016, three lake water samples were taken near a subaqueous tufa (39°31'45.6"N 118°52'54.2"W) that was subsequently removed for microbial sampling (**Figure 3.2g**). The lake water samples were collected within centimeters of the tufa ("proximal" samples), at ~0.5 m ("medial" samples) away from the tufa toward the center of the lake, and at ~1 m ("distal" samples) distance toward the same direction. Spring water samples were collected from two different freshwater springs discharging into the lake and located above the lake water level near the tufa sampling location. By October 2018, the lake water level had risen ~0.5 m, covering some previously exposed

tufas and the springs sampled in 2016. Three additional water samples were taken in 2018; one of nearshore lake water outside the tufa deposition site (outside nearshore; 39°31'40.6"N 118°52'57.3"W), and two from tufa porewater using a syringe to extract visible groundwater and lake water mixing in interior channels of two subaqueous tufas. Four lake water samples from the tufa deposition site (39°31'45.6"N 118°52'54.2"W) and one sample from an irrigation canal immediately northwest of BSL (39°31'52.3"N 118°53'09.6"W) were collected in triplicates in August 2020 to quantify urea using a modified version of the ammonia quantification method by the Berthelot reaction after urease-mediated hydrolysis of urea (McCarthy, 1970). A sample aliquot of 150 µL was diluted to 300 µL with nanopure water in a microcentrifuge tube and 200 µL were added of a solution containing 1 U/mL urease (Sigma-Aldrich, St. Louis, MO, USA, product number U1875) diluted in 100 mM Na₂HPO₄, 200 mM NaCl pH 7.0 (or an equivalent glycerol dilution without urease as a non-enzymatic control was added instead; Larsen, Dalsgaard, & Pedersen, 2015). The reaction was incubated for 90 min at room temperature and stopped by adding 250 µL of a phenol nitroprusside solution and 250 µL of alkaline hypochlorite solution (Sigma-Aldrich), incubated for additional 30 min at 37 °C and read in a spectrophotometer (Hach DR1900, Loveland, CO, USA) at 630 nm.

All water samples from June 2016 were filtered (0.2 µm) and collected in triplicate for anions, cations, DIC, and isotopic analysis as previously described (Kraus *et al.*, 2018). Water samples collected in October 2018 were filtered (0.2 µm) and analyzed (no replicates) for anions, cations, and titrated for carbonate quantification. Saturation indices (SI) for most phases were calculated using the water chemistry obtained for each sample (**Table B.S1**) and WEB-PHREEQ (www.ndsu.edu/webphreeq). SI of the

metastable calcium carbonates vaterite, monohydrocalcite, and ikaite, were calculated using the solubility constants from Plummer and Busenberg (1982), Kralj and Brečević (1995), and Bischoff, Fitzpatrick, and Rosenbauer (1993), respectively.

3.3.3. Carbonate tufa and microbial sampling

Carbonate samples were collected in June 2016 at BSL from a removed subaqueous tufa (**Figure 3.2a, g–i**; 39°31'45.6"N 118°52'54.2"W), and from a removed subaerial tufa (39°31'45.7"N 118°52'54.1"W). The tufas were transported to a laboratory and sliced to prepare thin sections and obtain solid carbonates for subsequent elemental and isotopic ($\delta^{13}\text{C}$ and $\delta^{18}\text{O}$) analysis conducted as previously described (Kraus *et al.*, 2018). Microbial samples for microscopy and DNA sequencing were obtained in June 2016 from lake water filtered residues (0.2 μm), from an ~3.5 cm deep sediment core taken at one site of a sampled freshwater spring, and from carbonate-associated microbial biofilms on the surface, interior, and endolithic portions of the sampled subaqueous and the subaerial tufa. Surficial microbial samples consisted of brownish-green biofilms from the surface of the subaqueous tufa, and green material from crevices of the subaerial tufa. The top ~5 to ~20 mm of the surface carbonates was excavated in the field to obtain endolithic microbes, and a grey/white microbial mat was obtained from the base of the uprooted subaqueous tufa (interior sample) and from internal voids in the subaerial tufa. Microbial samples were divided for microscopy and high-throughput sequencing in triplicate. Two lake water filtered residues (distal and proximal), one sediment core from a freshwater spring, along with interior, endolithic, and exterior biofilm samples from the subaqueous and the subaerial tufa were analyzed by small subunit (SSU) rRNA gene

sequencing. Only two samples—exterior biofilms from the subaqueous tufa and endolithic microbes from the subaerial tufa—were analyzed in triplicate by shotgun metagenomic sequencing. The carbonate samples for microscopic analysis were crushed, homogenized, stained with DAPI (4',6-diamidino-2-phenylindole) and imaged on an Olympus BX41 microscope (Olympus Corporation, Shinjuku, Tokyo, Japan). In addition, surficial biofilm samples were also taken in October 2018 from 14 subaqueous tufas and in August 2020 from two subaqueous tufas and from green filaments in the lake water collected adjacent to, but not directly on tufas, and analyzed on-site to determine urease and carbonic anhydrase (CA) activity (see section 3.3.5) using the method described by Medina Ferrer, Hobart, and Bailey (2020).

3.3.4. Microbial DNA sequencing

The microbial samples for high-throughput DNA sequencing were suspended in 705 µL of Xpedition™ Lysis/Stabilization Solution (Zymo Research Co., Irvine, CA, US) and homogenized by bead-beating in the field with a modified reciprocating saw (Kraus *et al.*, 2018). DNA extraction and SSU rRNA gene amplification from water, sediments, and carbonate-associated biofilms were done in triplicate as previously described (Kraus *et al.*, 2018; Stamps *et al.*, 2018). Primers for PCR amplification (forward primer 515F-Y; 5'-GTAAAACGACGGCCAGCCGTGYCAGCMGCCGCGGTAA-3', and reverse primer 926R; 5'-CCGYCAATTYMTTTRAGTTT-3') spanned the V4 and V5 hypervariable regions of the 16S rRNA gene between positions 515 and 926, yielding a ~400 bp fragment for Bacteria and Archaea, and a ~600 bp fragment for the Eukarya 18S rRNA

gene (Kraus *et al.*, 2018; Stamps *et al.*, 2018). These primers have shown to amplify a portion of the SSU rRNA gene to reflect all three domains of life, and most of bacterial and archaeal diversity yielded by the V4 and V5 regions in environmental samples (Parada, Needham, & Fuhrman, 2016; Zoss *et al.*, 2019). Amplified libraries were sequenced on an Illumina MiSeq (Illumina Inc. San Diego, CA, USA) at the Oklahoma Medical Research Foundation (OMRF) Clinical Genomics Center. SSU rRNA gene reads were analyzed using QIIME v1.9.1 (Caporaso *et al.*, 2010) as previously described (Kraus *et al.*, 2018), clustering operational taxonomic units (OTU) at 97% similarity using USEARCH v6.1. Shotgun metagenomic DNA sequencing for the surficial biofilm of the subaqueous tufa and for the endolithic biofilm of the subaerial tufa was performed using the Nextera XT library kit (Illumina Inc., San Diego, CA, USA) following the manufacturer's instructions, and then sequenced on an Illumina MiSeq (Illumina Inc. San Diego, CA, USA) at the OMRF Clinical Genomics Center. Downstream metagenomic analysis was performed according to Kraus *et al.* (2018) to identify the potential function from annotated predicted proteins.

3.3.5. Field evaluation of enzymatic activities and calcium depletion kinetic assays

To determine whether the enzymes urease and carbonic anhydrase (CA) were active *in situ*, surficial biofilms from submerged tufas were field tested using the assay described by Medina Ferrer *et al.* (2020). The tests consist of a pH-sensitive indicator strip (a cellulose paper impregnated with a 0.02% phenol red pH 6.0 solution for the urease assay, and with a 6.5 mM Na₂CO₃, 0.01% metacresol purple, 50% glycerol for the CA assay) situated in the cap of a microcentrifuge tube, and the substrate of the

respective enzyme (a cellulose paper impregnated with a 0.6 M urea solution as urease substrate, and a cold 1 M NaHCO₃ solution as CA substrate) located in the main body of the tube. The reaction was initiated by adding a portion of tufa biofilm inside the tube and directly in contact with the enzyme substrate before closing the microcentrifuge cap. A positive reaction was indicated by a color change from yellow to red in the indicator strip of the urease assay (produced by a pH increase after NH_{3(g)} release in the reaction tube), or by a faster color change from purple to yellow (produced by a pH decrease after CO_{2(g)} release in the reaction tube) compared to a negative control for the CA assay. Negative control assays were evaluated under the same conditions, but additionally adding a specific inhibitor of urease, phenyl phosphorodiamidate, (PPD; Alfa Aesar, Ward Hill, Massachusetts, USA; Kafarski, & Talma, 2018), or a specific inhibitor of CA, acetazolamide (Sigma-Aldrich, St. Louis, Missouri, USA; Zimmerman *et al.*, 2004) to the corresponding enzyme substrate as previously described (Medina Ferrer *et al.*, 2020). The progression of the enzymatic reactions was followed by taking photographs in the field of the indicator strips and semi-quantifying their hue values (section 3.3.6).

In addition to the field analysis, surficial microbial mats from one subaqueous tufa collected in August 2020 were also kept on ice for two days prior to laboratory microcosm incubation experiments to determine calcium depletion over time as an indicator of calcium carbonate precipitation. Triplicate biofilm samples (0.5 g wet weight) were incubated in 20 mL of 0.2 µm-filtered Big Soda Lake water adding 0.8 M urea in closed 50-mL serum glass bottles with constant agitation (90 r.p.m., orbital shaker MaxQTM 2000, Thermo Fisher Scientific) for 11 days at room temperature and under natural light cycles. As negative controls, incubation experiments under the same

conditions, but without microbial mats (abiotic control), or additionally containing either 1 mM PPD (urease inhibitor; Alfa Aesar, Ward Hill, Massachusetts, USA) or 1 mM acetazolamide (CA inhibitor; Sigma-Aldrich, St. Louis, Missouri, USA) were conducted as urease and CA inhibiting controls, respectively. Calcium concentration was quantified by titration with EDTA (HAC-DT, Hach, Loveland, CO, USA) from 1 mL aliquots sampled from the incubation solutions over time.

3.3.6. Image and statistical analysis

In situ urease and CA assay results were followed by semi-quantifying their hue changes over time from photographs taken in the field as previously described (Medina Ferrer *et al.*, 2020). Average hues were calculated from RGB measurements over a standard circle area of the assay color indicator using the NIH ImageJ 1.49v. The obtained hues were analyzed via mixed-effects models (including the reaction time and the presence or absence of the corresponding inhibitor as nominal variables) with Geisser-Greenhouse correction using GraphPad Prism 8.3.0. Statistical significance at a given reaction time between each assay and its respective inhibiting negative control was assessed by a Bonferroni's multiple comparisons test. Statistical analysis of calcium depletion in the microcosm incubations was assessed by two-way ANOVA and the significance of pair-wise comparisons at a given incubation time was obtained by Bonferroni correction.

3.4. Results

3.4.1. BSL waters are saturated with respect to calcium carbonate minerals

BSL waters have relatively high surface DIC (30–70 mM), with pH values fluctuating between 9.2 and 9.8 (**Table B.S1**). Under these conditions, all the water samples taken were saturated (Saturation Index, $SI > 0$) with respect to major calcium carbonate minerals, such as calcite, aragonite, vaterite, and dolomite (**Figure 3.3a**; **Table B.S1**). Groundwater discharge springs are also saturated and richer in calcium compared to lake water. Freshwater emergence, evidenced by field observations (**Figures 3.1c**, **3.2e**) and by a reduced salinity in the vicinity of tufas (**Figure 3.3b**), may increase calcium carbonate saturation near tufa deposition sites. We found, however, that the SI of spring and lake waters does not dramatically differ for most carbonate phases, with the exception of dolomite (**Figure 3.3a**). By contrast, isotopic compositions ($\delta^{18}\text{O}_{\text{water}}$, $\delta^2\text{H}_{\text{water}}$ and $\delta^{13}\text{C}_{\text{DIC}}$) of spring samples and lake water vary to a greater extent (**Figure 3.3c**). Groundwater from spring samples is isotopically light compared to the lake waters, where water $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ from DIC are around -11‰ and -1.5‰ for groundwater and lake water, respectively, and $\delta^2\text{H}$ averages -91‰ and -45‰ .

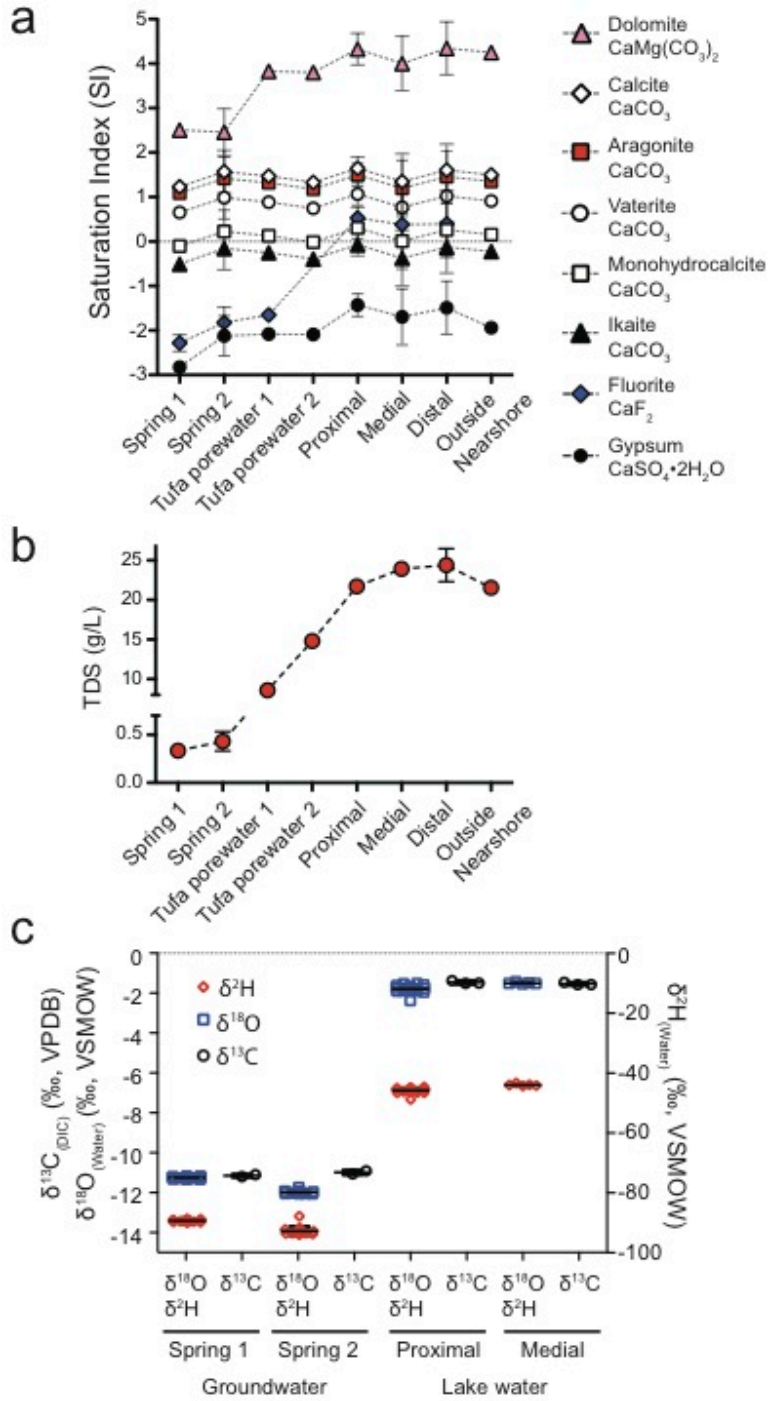


Figure 3.3. BSL water chemistry. (a) Saturation index (SI) of calcium phases in samples representing a mixing gradient from groundwater (spring samples) to mixed waters (tufa porewater) to lake water at proximal, medial (0.5 m), and distal (1 m)

distance from the subaqueous tufa sampled, and from a location outside the tufa deposition site (outside nearshore). (b) Total dissolved solids (TDS) of BSL water samples showing the mixing gradient. (c) Spring and lake water $\delta^{13}\text{C}_{\text{DIC}}$, $\delta^2\text{H}_{\text{water}}$ and $\delta^{18}\text{O}_{\text{water}}$.

3.4.2. Tufa geochemistry and microfabric show both possible abiogenic and biogenic signatures

Spring and lake water isotopic compositions are spatially reflected in tufa carbonates. Lighter isotopic values correlate within tufa interiors, especially in samples taken close to interior voids, which presumably correspond to groundwater conduits (**Figure 3.4a, b**). The isotopic values of all tufa carbonates analyzed fall between spring and lake water isotopic values (**Figure 3.4c; Table B.S2**), indicating carbonate precipitation from groundwater and lake water mixing, in agreement with Rosen *et al.* (2004).

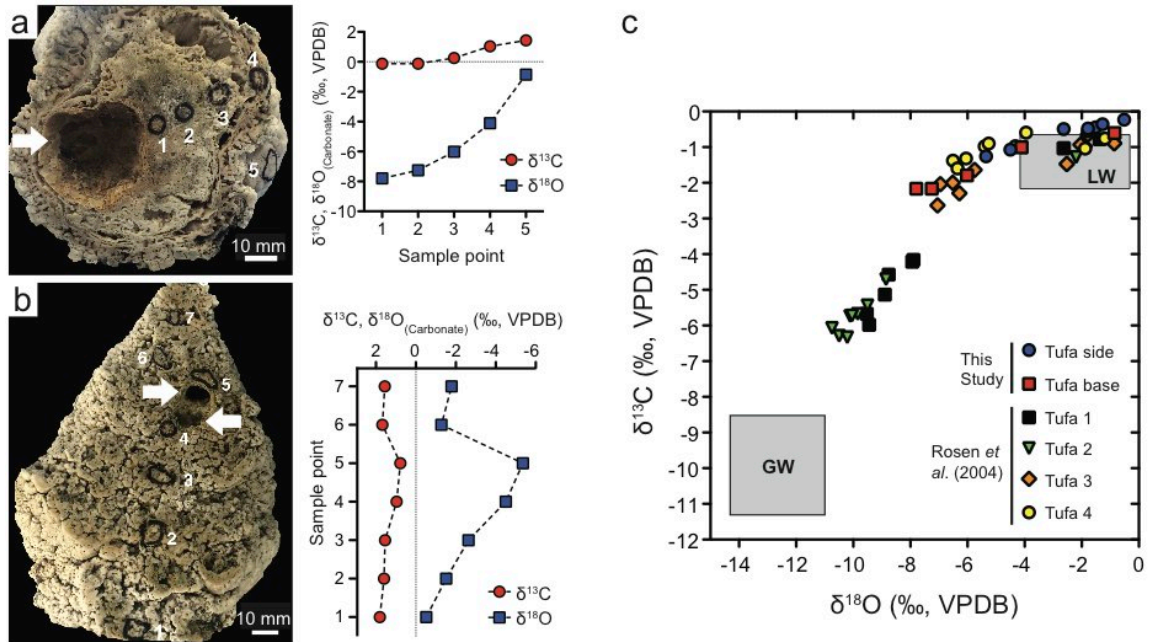


Figure 3.4. Tufa fabric and carbonate geochemistry. Transverse (a) and longitudinal (b) sections of a subaerial tufa indicating sampling sites (left panel, numbered circles) and their $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ values (right graphs). White arrows show interior cavities where groundwater seeped. (c) Mixing diagram of carbon and oxygen isotopic ratios from Big Soda Lake carbonate tufas. $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ fractionation values were corrected to represent the isotopic composition of waters (at 15 °C) from which carbonate precipitated according to O'Neil, Clayton, and Mayeda (1969), and Deines, Langmuir, and Harmon (1974). Lake water (LW) and groundwater (GW) isotopic ranges are estimated from Rosen *et al.* (2004) and this study. Data values of tufa carbonate $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ from Rosen *et al.* (2004) are also shown.

The tufa microfabric contains abundant, likely abiogenic, carbonate botryoids (**Figure 3.5a–c**). Botryoids preferentially locate in tufa interiors and are progressively micritized toward the exterior (**Figure 3.5c**). The tufas are composed mostly of

monohydrocalcite and calcite with trace aragonite and an unidentified Mg-rich clay (Rosen *et al.*, 2004). Based on petrographic observations of our samples, the tufa botryoids correspond mostly to calcite with minor aragonite in a porous fabric that incorporates volcanoclastic debris along with spherulites (Chafetz, Barth, Cook, Guo, & Zhou, 2018), and other biogenic-like structures, such as laminated micrite, and possible fungal boring (**Figure 3.5c–e**).

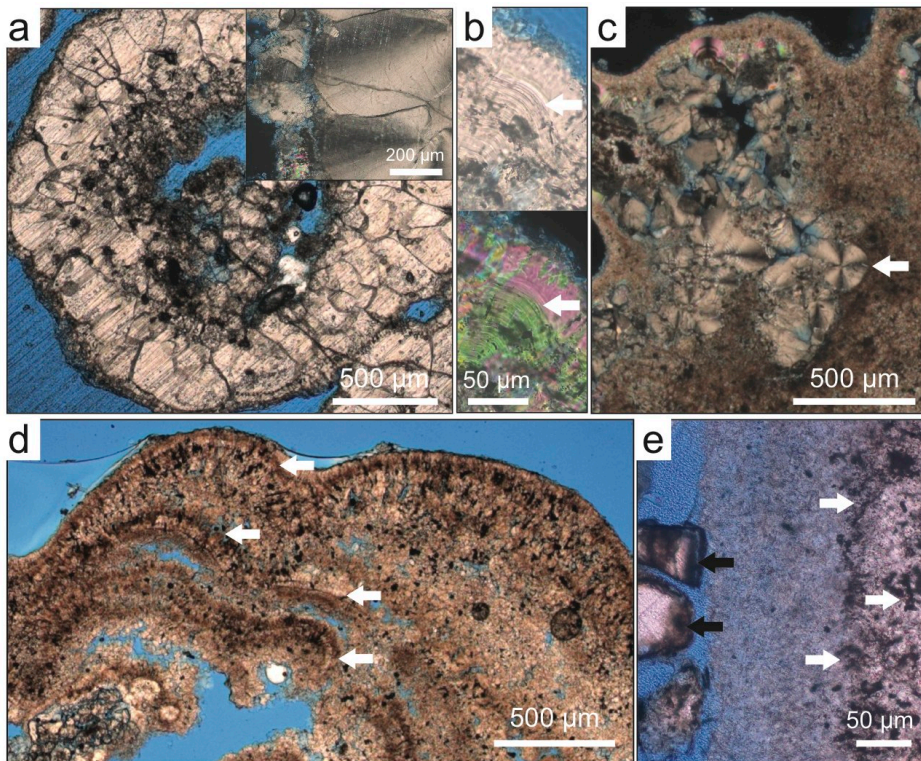


Figure 3.5. BSL tufa microfabric. Transverse thin sections (a, b) showing potential abiogenic carbonate botryoids. Inset shows an enlarged botryoid under cross-polarized light. Some botryoids display fine abiotic-like laminations (b, white arrow), whereas other sections show replacement by micrite (c), spherulites (c, white arrow), and stromatolitic fabric (white arrows at d, a photomicrograph from a longitudinal thin

section). Some portions show burrowing structures, probably by fungal hyphae (white arrows in e, a photomicrograph from a subaqueous tufa), along with abundant volcanoclastic debris (e, black arrows). Blue areas indicate pore space by dyed epoxy in thin section preparation.

3.4.3. BSL tufas host diverse microbial communities

Green and brown biofilms are pervasive on the surface of subaqueous tufas, whereas conspicuous microbial mats were absent on subaerial portions (**Figure 3.2**). Subaerial tufas, however, contained microbes in tufa crevasses and inside carbonates (**Figure 3.2b**), forming endolithic communities (e.g., Walker, Spear, & Pace, 2005; Guida, Bose, & Garcia-Pichel, 2017; Trampe, Castenholz, Larsen, & Köhl, 2017). Light and fluorescence microscopy shows abundant, likely photosynthetic, green filaments attached to mineral grains on surficial biofilms from subaqueous tufa samples, along with diatoms, nematodes, and small motile bacteria (**Figure B.S2**). The spring sediments, the interior of the subaqueous tufa, and the subaerial tufa, were dominated by mineral grains containing fewer microorganisms, with the exception of the subaerial endolithic portion. Tufa endoliths comprised large branched filaments (presumably hyphae; **Figure B.S2**), cyanobacteria and multiple cocci, rods, and motile bacteria.

SSU rRNA gene sequencing resulted in hundreds of bacterial OTUs (**Figure 3.6a**; **Figure B.S3**), whereas eukaryotic and archaeal sequences composed only 0.03% and 0.8% of the total OTUs of combined samples, respectively (**Figure B.S4**). Cyanobacteria prevail on the exterior of the subaqueous tufa (> 50%), in endolithic communities (~30%), and lake waters (10–35%). Lake waters are characterized by the presence of the

phyla Tenericutes and Actinobacteria, which are not well represented in tufa or sediment samples. By contrast, representatives of the Alphaproteobacteria and Acidobacteria dominate spring sediments and subaqueous tufa interiors (Figure 3.6a). Community similarity between the subaqueous tufa interior and the spring sediments is visualized by PCoA ordination (Figure 3.6b). Interior, endolithic, and exterior samples from the subaqueous tufa locate in ordination space between spring sediment and lake water communities, evidencing a microbial gradient that spans the geochemical gradient between lake water and the groundwater upwelling through the tufa (Figure B.S5). Microbial communities from the subaerial tufa cluster separately from the subaqueous tufa (Figure 3.6b).

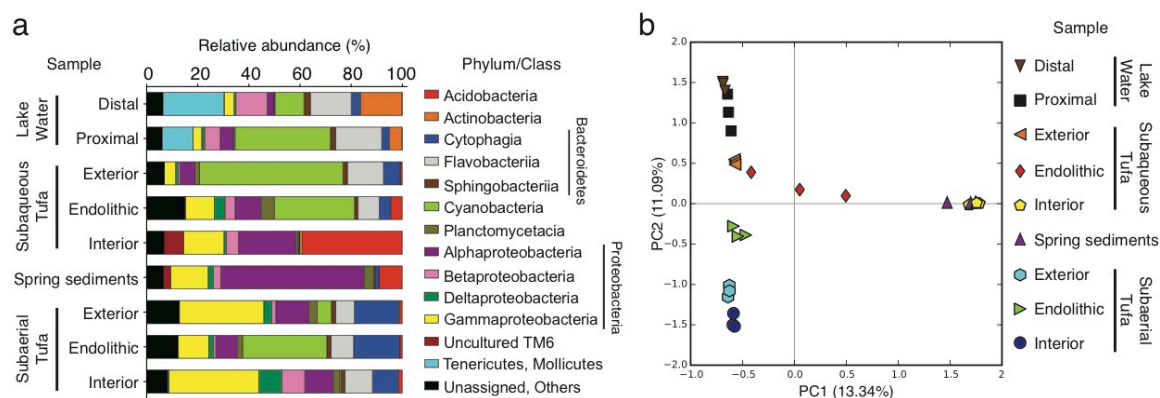


Figure 3.6. BSL microbial communities. (a) Relative abundances of bacterial classes in lake waters and tufa microbial mats. Biofilms were taken from surficial (exterior), excavated (endolithic), and internal voids (interior) from subaqueous and subaerial tufas. Bars represent averaged biological triplicates. (b) PCoA of chi-squared distances among bacterial communities from triplicate samples of water, sediments, and tufas.

3.4.4. BSL microbes host active metabolisms potentially driving carbonate precipitation and tufa growth

Genes of major metabolic pathways involved in microbially-induced calcium carbonate precipitation were found by metagenomic sequencing of the surficial and endolithic biofilm samples from the subaqueous and subaerial tufa, respectively (**Figure 3.7**). Photosynthesis genes, along with ureolysis and bicarbonate metabolism, predominate. Methane oxidation and nitrate reduction pathways are also present, although some enzymes are either missing or were sequenced in low abundance (**Figure 3.7; Table B.S3**).

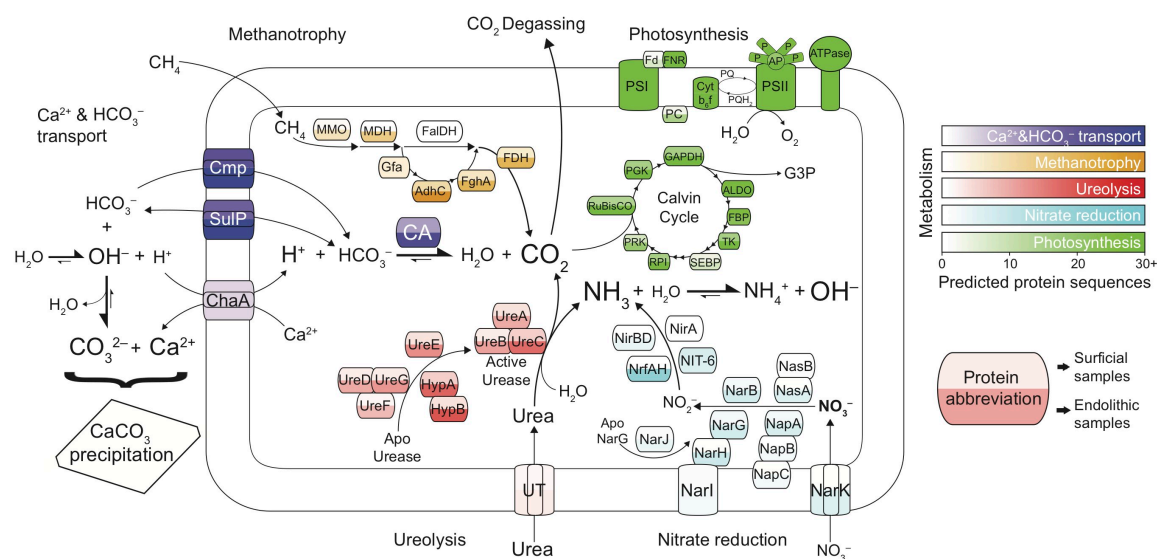
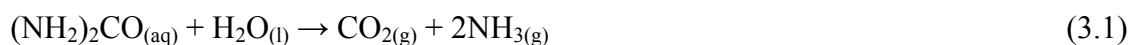


Figure 3.7. Potential carbonate-precipitating metabolisms in BSL tufas. Metabolic map of microbially-induced carbonatogenesis showing color-coded enzymes according to their pathway and number of annotated predicted proteins found by metagenomic sequencing from averaged triplicate surficial and endolithic samples. Pathways may

represent extracellular, periplasmic or cytoplasmic reactions. Protein abbreviations are listed in Table B.S3.

While metagenomic analyses indicate the metabolic potential of a community, we also investigated the actual activity of the key enzymes urease and CA, which potentially promote carbonate precipitation by increasing the pH of the biofilm microenvironment (Zhu & Dittrich, 2016; Seifan & Berenjian, 2019). Urease catalyzes the hydrolysis of urea into NH₃ and CO₂ (Eq. 3.1), which microbes can use as nitrogen and carbon sources (Krausfeldt *et al.*, 2019), whereas CA catalyzes the interconversion of CO₂ and bicarbonate that facilitates rapid carbon transport (Eq. 2; Kumar & Ferry, 2014):



Both reactions potentially increase the pH at the biofilm microenvironment—thus, promoting carbonate precipitation—by generating ammonia, in the case of urease, or by increasing the rate of CO₂ degassing, in the case of CA. Using an enzymatic field detection assay (Medina Ferrer *et al.*, 2020), we found *in situ* active urease and CA in surficial mats from subaqueous tufas (**Figure 3.8a, b; Figure B.S6**). Preliminary assessments in the field showed multiple urease-positive tufa biofilms in October 2018, which were re-evaluated and semi-quantified after a second sampling campaign in August 2020 (**Figure 3.8a**). The urease field assay hues changed over time according to their incubation condition, showing a significant ($P < 0.05$) positive reaction when compared to their respective inhibited negative control assays (in the presence of PPD) at ~10 min of incubation for the biofilms associated with BSL tufas (**Figure 3.8a**).

Similarly, the CA field assay hues were also affected by their incubation conditions, although a significant ($P < 0.05$) positive reaction, when compared with the respective inhibited negative control assay (in the presence of acetazolamide), was observed only in one tufa surficial biofilm sample (Figure 3.8b, Figure B.S6).

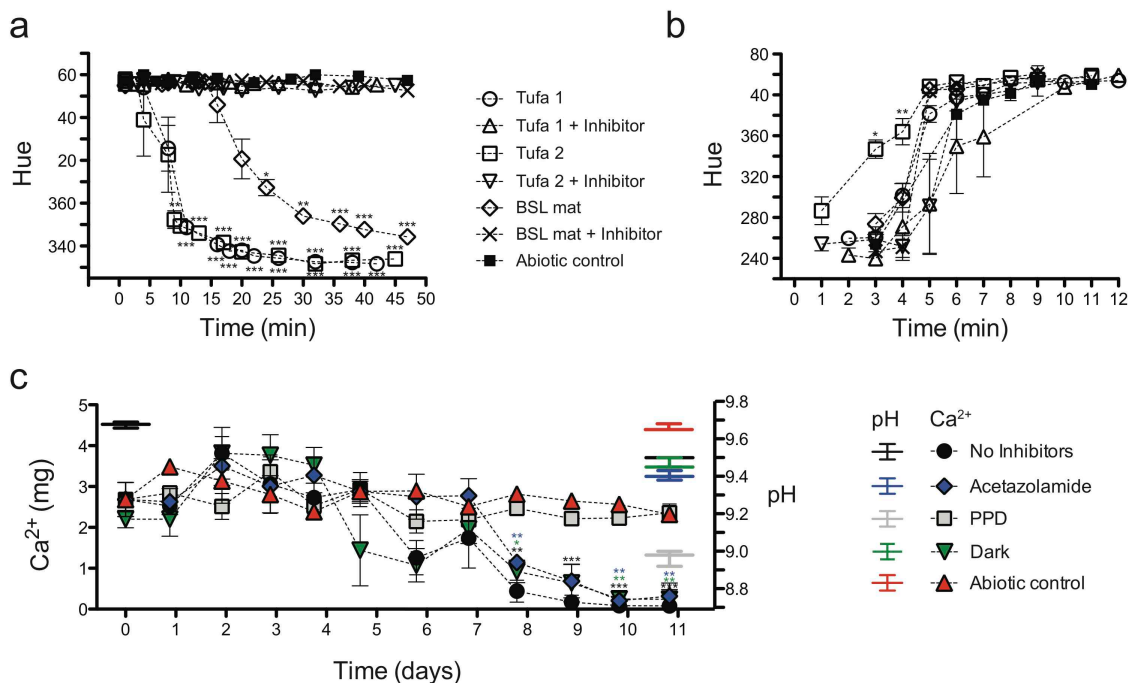


Figure 3.8. Activity of urease and CA in BSL. Field test color change represented by the hues obtained from *in situ* assays of urease (a) and CA (b) over time, corresponding to the surficial biofilms of two subaqueous tufas, and lake mats not directly associated with the tufa carbonates (BSL mat). (c) Calcium depletion (left axis) over time from incubations of BSL water with tufa microbes in the presence of a urease or a CA inhibitor (PPD and acetazolamide, respectively), without inhibitors, without inhibitors under the dark (dark), or without microbial mats (abiotic control). The lake water pH at the beginning and the end of the incubations is indicated on the right axis. Error bars show

the standard error of triplicate assays (unless the error is smaller than the symbols). Statistical significance is represented by $*0.1 < P < 0.05$, $**0.05 < P < 0.01$, or $***P < 0.01$ between each condition and its respective inhibiting control assay (a, b) or each condition compared to the abiotic control (c) at a particular incubation time analyzed by a Bonferroni's multiple comparisons test.

In addition to the field enzymatic tests, laboratory microcosm incubations of tufa surficial mats in lake water containing urea showed a significant decline of its endogenous soluble calcium over time (**Figure 3.8c**). The drop of dissolved calcium is interpreted as Ca^{2+} sequestration by CaCO_3 precipitation. Microcosm incubations without inhibitors, or with a CA inhibitor (acetazolamide) showed a significant ($P < 0.05$) Ca^{2+} reduction compared to similar incubations in the presence of a urease inhibitor (PPD) or without microbial mats (abiogenic control) after one week of incubation (**Figure 3.8c**), indicating that microbial urease from BSL tufa biofilms promotes carbonate precipitation (soluble calcium decrease) when an additional source of urea is included. Urea was not detected in BSL water samples themselves, but was detected at low concentrations ($1.5 \pm 0.2 \mu\text{M}$) in a neighboring irrigation canal that is thought to discharge into the tufa-hosting flank of BSL.

3.5. Discussion

3.5.1. Abiogenic and biogenic influence in BSL tufa formation

Regardless of microbial influence, BSL water chemistry alone may be sufficient for carbonate precipitation based on its supersaturation with respect to calcium carbonate minerals, such as calcite, aragonite, vaterite, monohydrocalcite, and dolomite. Other calcium-sequestering minerals, such as gypsum and fluorite, are either undersaturated or have lower saturation indexes compared to most calcium carbonate phases (**Figure 3.3a**), with the exception of hydroxyapatite, for which precipitation seems negligible at BSL (**Table B.S1**; Kharaka *et al.*, 1984). Groundwater influx, however, does not dramatically increase the SI of most calcium carbonate minerals near tufa accretion sites, with the exception of dolomite (**Figure 3.3a**). Nevertheless, dolomite is not present in BSL tufas (Rosen *et al.*, 2004) and its low-temperature precipitation in natural environments is kinetically constrained and therefore unusual, even under highly over-saturated conditions (Petrash *et al.*, 2017). The relatively similar SI of calcite, aragonite, and monohydrocalcite among spring water and lake water samples observed in **Figure 3.3a** suggests that, in addition to providing Ca^{2+} , groundwater upwelling into BSL may promote tufa formation in other ways, such as by decreasing the salinity of tufa porewaters and their surroundings (**Figure 3.3b**), and by providing a continuous flux of limiting nutrients (e.g., urea) for microbial growth (Warden *et al.*, 2016, 2019).

A primarily abiogenic precipitation mechanism in BSL is supported by tufa isotope compositions (**Figure 3.4a, b**) as well as the microfabric of calcite fans with high-inheritance laminae (**Figure 3.5a, b**), a feature that can potentially be interpreted as abiogenic in origin (Riding, 2008; 2011). In addition, carbonate $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ values agree well with spring and lake water isotopic compositions, which is also consistent with abiogenic precipitation (**Figure 3.4c**; Rosen *et al.*, 2004). Yet, evidence of microbial

activity and possible microbially-induced carbonate precipitation is observed in the tufa microfabric as stromatolitic laminae or as possible secondary reworking of carbonate botryoids and spherulites (**Figure 3.5c, d**). Therefore, even though abiogenic carbonate precipitation primarily results from groundwater and lake water mixing, the tufa microfabric also show signs of biologic influence. Similar microscopic features that are potentially biogenic in origin have been found in modern microbialites (e.g., Warden *et al.*, 2016; Wacey, Urosevic, Saunders, & George, 2018), as well as modern and fossil tufa mounds intimately associated with microbial biofilms, such as tufas from Mono Lake (Brasier *et al.*, 2018), Pyramid Lake (Arp *et al.*, 1999), Searles Lake (Guo & Chafetz, 2012), and Lake Lahontan (DeMott *et al.*, 2020). Although the microbial biofilms living in tufas seem to affect the carbonate microfabric morphology, it is not entirely clear whether microbes also enhance carbonate precipitation and promote tufa growth.

3.5.2. Potential microbial influence on BSL tufa formation

Microbes have the potential to locally increase the saturation with respect to calcium carbonate minerals by raising the concentration of calcium and/or carbonate ions (Dupraz *et al.*, 2009; Pedley, 2014; Zhu & Dittrich, 2016). Microbial factors controlling the availability of soluble calcium has been proposed to be a major mechanism of microbially-induced calcium carbonate precipitation in high-pH environments (Arp *et al.*, 1999; 2001; Dupraz *et al.*, 2004), and may therefore constitute an important mechanism for tufa formation in BSL. EPS and bacterial walls provide nucleation sites (Dupraz *et al.*, 2009) and chelate calcium via negatively charged groups (Fein, 2017), locally

concentrating Ca^{2+} . Alternatively, calcium chelation sequesters available ions, inhibiting calcite precipitation in a mechanism proposed for BSL fulvic acids by Reddy and Leenheer (2011). Organic matter degradation by heterotrophs could release chelated Ca^{2+} and generate local supersaturation (Arp *et al.*, 1999; Dupraz *et al.*, 2004), highlighting the importance of a diverse community in microbially-induced mineral precipitation processes (Gat, Tsesarsky, Shamir, & Ronen, 2014; Zhu & Dittrich, 2016). Photosynthetic cyanobacteria can also accumulate Ca^{2+} , increasing calcium by pumping it to the exterior (Waditee *et al.*, 2004), or even by precipitating carbonates intracellularly (Benzerara *et al.*, 2014). It is likely that photosynthetic cyanobacterial biofilms, known to produce high amount of EPS and abundant in endolithic and surficial mats of BSL tufas (**Figure 3.6a & Figure B.S2**), facilitate the precipitation of micrite and shape tufa microfabrics with features associated to biogenic input, such as the laminated- or stromatolite-like layers shown in **Figure 3.5d**, resulting in carbonates incorporating or exhibiting distinctive textures that may serve as biosignatures.

In addition, the diverse microbial communities found in BSL have the potential to accelerate carbonate precipitation by increasing the pH via diverse metabolisms and consequently increasing the activity of CO_3^{2-} (**Figure 3.7 & 3.8**). Although bicarbonate is abundant in BSL, the availability of soluble carbonate species relies on maintaining high pH values that favor the speciation of HCO_3^- into CO_3^{2-} , which in turn increases the saturation with respect to calcium carbonate minerals. BSL waters already have high pH values that fluctuate between 9.2 and 9.8, depending on the sampling site and the year sampled (**Table B.S1**). The pH may locally increase by a mechanism that promotes CO_2 degassing via abiotic factors, such as turbulent flow in riverine systems (Zhang, Zhang,

Zu, & Cheng, 2001; Andrews, 2006), which is unlikely in BSL. Alternatively, a biotic mechanism can also mediate a pH increase, such as rapid CA-mediated dehydration of HCO_3^- to CO_2 , which is likely in BSL given the active CA found in tufa biofilms (**Figure 3.8b**), or carbon dioxide uptake by photosynthesis in the abundant cyanobacterial BSL tufa biofilms. Calcium carbonate precipitation in alkaline environments, however, is less likely to be promoted by microbial metabolisms that increase the pH than by microbial factors controlling calcium availability (Arp, Hofmann, & Reitner, 1998; Dupraz *et al.*, 2004; Arp *et al.*, 2012), because high-pH waters would already have most DIC ions in the form of CO_3^{2-} , leaving somewhat less potential to increase the saturation with respect to carbonate phases. However, in BSL waters, the SI for calcite, aragonite, and monohydrocalcite increases linearly until pH ~10, reaching a plateau above pH 10.5 (**Figure B.S7**). It is therefore expected that at pH waters above ~10.5, the effect of pH-increasing microbial metabolisms on carbonate precipitation would be negligible. BSL water pH, however, fluctuates in the range 9.2–9.8, and consequently a local pH increase could still raise the SI of calcium carbonate minerals in BSL tufas.

Photosynthesis, ureolysis, and CO_2 degassing catalyzed by CA, are potentially involved in tufa CaCO_3 precipitation by increasing the pH at the biofilm vicinity. CA catalyzes a reversible reaction that either provides bicarbonate ions by hydration of CO_2 (reverse of Eq. 3.2), or increases the pH by promoting CO_2 degassing, depending on the system disequilibrium (Kumar & Ferry, 2014). In environments with low DIC, CA-mediated CO_2 hydration provides soluble carbonate ions that sustain calcium carbonate precipitation (e.g., Müller *et al.*, 2013). In the carbonate-rich waters of BSL, however, where the DIC pool is already high, CA increases the rate of HCO_3^- dehydration (Eq.

3.2), mediating rapid CO₂ outgassing and providing a mechanism of pH increase that potentially promotes CaCO₃ precipitation (Seifan & Berenjian, 2019). Via a different reaction, urease also increases the pH by producing ammonia as a product of urea hydrolysis (Eq. 3.1), a mechanism known to promote calcium carbonate precipitation in a variety of environments (Achal & Pan 2011; Zhu & Dittrich, 2016; Seifan & Berenjian, 2019).

In situ urease and CA activity found in BSL tufa biofilms (**Figure 3.8a, b**) indicate that tufa microbes potentially accelerate precipitation rates by generating ammonia and promoting CO₂ degassing, thereby increasing the pH and consequently the saturation with respect to calcium carbonate minerals at the biofilm microenvironment (Achal & Pan, 2011; Zhu & Dittrich, 2016; Seifan & Berenjian, 2019; Medina Ferrer *et al.*, 2020). Saturation increase associated with the microbial mats could in turn contribute to the observed fast growth rate of BSL tufas (Rosen *et al.*, 2004). In the laboratory, microcosm incubations of tufa mats depleted more Ca²⁺ compared to incubations having a specific urease inhibitor (PPD) and to incubations without microbial mats (abiotic control; **Figure 3.8c**), indicating that urease accelerates carbonate precipitation under the studied conditions. Although incubations with a CA inhibitor seem to have delayed calcium depletion compared to incubations without inhibitors and to the abiotic control, CA inhibition did not prevent calcium depletion over the course of 10 days (**Figure 3.8c**). The results show that microbial urease—found active *in situ* in BSL tufa biofilms—accelerates carbonate precipitation in BSL waters containing an external source of urea. Although we did not detect urea in BSL waters near the tufa deposition site, which could be explained by the detected relatively high microbial urease activity that readily

hydrolyzes urea in the lake, we did find urea in waters from an adjacent irrigation canal, which likely contributes to the groundwater discharge observed at the tufa deposition site. Therefore, groundwater influx provides a potential continuous source of urea, contributing to carbonate authigenesis at BSL.

Although the microcosm incubation experiments in **Figure 3.8c** do not necessarily reflect the actual conditions for calcium carbonate precipitation in BSL, they can still highlight the potential relevance of microbial pH-increasing metabolisms, even in alkaline systems. For example, even though the ureolysis catalyzed by tufa biofilms did not increase the bulk lake water pH (~9.6), it still maintained a highly alkaline environment at the end of the incubations, whereas in the presence of a urease inhibitor (PPD) the pH dropped to ~9.0 (**Figure 3.8c**), likely as a result of other microbial metabolisms, possibly heterotrophic degradation and fermentation of dead biomass and polysaccharides. Therefore, despite the fact that microbially mediated pH-increase mechanisms have not been considered relevant to explain carbonate precipitation in alkaline environments (Arp *et al.*, 1998; 1999; 2001; Dupraz *et al.*, 2004), the activity of urease and, to a lesser extent, CA and possibly photosynthesis, seem to counteract the effect of acid-producing metabolisms that may limit the extent of calcium carbonate precipitation at the BSL biofilm microenvironment. It is possible that a complex interplay of certain microbial metabolisms, such as heterotrophic degradation of EPS that increases local calcium concentrations (but may decrease the pH), and local increases in pH mediated by photosynthesis, ureolysis or CA, may promote calcium carbonate precipitation and tufa formation in BSL.

3.5.3. A proposed formation model for BSL tufa mounds

Columnar tufas form in waters saturated with respect to calcium carbonate minerals and associated with subaqueous freshwater springs providing an additional source of calcium or carbonate (Bischoff *et al.*, 1993; Buchardt *et al.*, 2001). An increase in local saturation at the site of groundwater discharge, along with a continuous upward flow of low-density freshwater into a denser water body facilitates the construction of calcium carbonate towers. It has been noted, however, that under such environments the stabilization of recently precipitated calcium carbonates as a benthic structure potentially requires the presence of biofilms associated with the spring vent sites (Brasier *et al.*, 2018). Initial stabilization of microbialites may also come from calcium carbonate replacement of Mg-silicate precursors, which may serve as early templates for crystal growth and this has been proposed as a mechanism for modern microbialite formation in other alkaline lakes, such as the Great Salt Lake, USA (Pace *et al.*, 2016), Lake Thetis, Australia (Wacey *et al.*, 2018), and Lake Clifton, Australia (Burne *et al.*, 2014). However, Tutolo and Tosca (2018) showed that the timescales over which carbonates grow and the degree to which their solubility is governed by thermodynamic equilibrium are considerably shorter and greater, respectively, than for Mg-silicates. While microbes may increase Mg-silicate precipitation, the rapid growth of BSL tufas and the rarity of authigenic Mg-silicates in their crystal fabric, similar to the columnar tufas from Mono Lake (Brasier *et al.*, 2018), indicate that a mechanism governed by initial precipitation of Mg-rich clays is, at best, minor in BSL.

We propose that initially, microcrystalline calcite and/or monohydrocalcite precipitate at groundwater upwelling sites as a consequence of abiogenic water mixing

and local supersaturation with respect to carbonate minerals. Subsequently, the positively charged carbonaceous surfaces (Song *et al.*, 2017) serve as adhesion sites for BSL bacteria with negatively charged membranes, evidenced by the similarity between the lake water microbial communities and the tufa surface biofilms (**Figure 3.6**). The abundant biofilms growing on subaqueous calcium carbonate minerals potentially stabilize the formation of a coherent columnar structure rooted at the sites of sublacustrine spring plumes. Photosynthetic biofilms, fed by groundwater discharge, have the potential to locally increase the pH by CO₂ consumption during photosynthesis, increasing the saturation and precipitation rates of CaCO₃. In addition, active microbial urease and CA from BSL tufa biofilms (**Figure 3.8a, b**) may promote carbonate authigenesis, especially when their substrates are available. Bicarbonate is abundant in BSL and urea, a major fertilizer common in agricultural land waters and found here in a neighboring canal, is likely transported by groundwater to BSL (**Figure B.S1**), where its hydrolysis by microbial urease in tufas may contribute to keeping highly alkaline conditions upon calcium release from heterotrophic degradation of EPS, thus accelerating carbonate accretion. Subsequent precipitation events entomb the initial biofilms inside the tufa, where microbes reflect the groundwater community composition (**Figure 3.6**). As tufas grow from continuous precipitation, the geochemical gradient from fresh groundwater to saline lake water becomes wider and the microbial communities change over time, establishing a microbial gradient that spans the tufa salinity gradient. The microbial gradient is reflected by the beta-diversity ‘horseshoe effect’ in the ordination of subaqueous tufa samples (**Figure B.S5**; Morton *et al.*, 2017). Similar to other community gradients visualized by ordination analysis in stratified mats (e.g., Harris *et al.*, 2013), the

‘horseshoe effect’ in BSL tufa communities likely represents niche differentiation of taxonomic units in a gradient that provides a wider range to host a diverse community. After the water level drops, tufas still provide an environment where microbes thrive, represented by endolithic bacteria from the subaerial tufas. Consequently, even if they remain inactive, tufas serve as geochemical reservoirs of microbial diversity that house potential biosignatures preserved in the sedimentary record.

We have shown that metabolisms that can promote the precipitation of carbonates via urease and CA were present *and active* in BSL tufas, but the extent to which microbial activity accelerates tufa growth is unclear. To our knowledge, this is the first time that urease and CA have been found active *in situ* and potentially promoting carbonate precipitation in tufas. A similar formation mechanism may also occur in other tufa mounds, such as Mono Lake tufas (Brasier *et al.*, 2018), and modern thrombolites (e.g., Burne *et al.*, 2014; Warden *et al.*, 2016; Wacey *et al.*, 2018; DeMott *et al.*, 2020).

3.6. Conclusion

Lake and groundwater mixing favor the formation of BSL tufas by both abiogenic and biogenic mechanisms. Lake water supersaturation with respect to calcite, aragonite, and monohydrocalcite composing BSL tufas, promotes mineral precipitation while groundwater discharges and carbonate biofilms potentially control the tufa precipitation rate and morphology. BSL tufas have shown to host diverse and active microbiota, which stratify from the tufa surface to its interior following the water mixing gradient, therefore providing wider ecological niches where BSL microbes thrive. Photosynthesis, ureolysis and CA-mediated CO₂ degassing—demonstrated not simply by the presence of their

genes, but by *in situ* measurement of their activity—potentially increase growth rates by maintaining a highly alkaline microenvironment in concert with EPS-degrading metabolisms that release calcium.

The microbial activity in tufa biofilms alters the carbonate microfabric and may reflect specific metabolisms that preserve biogenic signatures of rapid mineral precipitation or carbonate reworking on otherwise abiogenic deposits. Elucidating the microbial mechanisms of calcium carbonate precipitation and their influence on fossil carbonates could help us understand groundwater-surface water interactions and their connections with microbial activity relevant to the formation of preserved carbonate biosignatures on Earth and elsewhere.

3.7. Acknowledgements

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Chapter 4

Assessing biomarker syngeneity: An in situ approach using monoclonal antibodies

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4.1. Synopsis

Lipid biomarkers preserved in ancient rocks have the potential to reveal much about ancient ecosystems. However, establishing that the compounds of interest are syngenetic has proven to be an analytically challenging task. Traditional biomarker analyses rely on extraction of large quantities of powdered rock, making the association of molecules with sedimentary fabrics difficult, if not impossible. As an alternative approach, here we show that monoclonal antibodies that bind specifically to geolipids can be used as molecular probes for in situ detection and localization of such compounds. Monoclonal antibodies that bind to squalene and cross-react with the biomarker squalane were evaluated for labeling sediment-associated hydrocarbons. The anti-squalene antibodies were shown by dot immunoblotting with composed standards to cross-react also with other isoprenoids, such as phytol and its diagenetic products, suggesting reactivity towards acyclic isoprenoids. Then, the anti-squalene antibodies were shown to react with naturally occurring crude oils and, via an immunofluorescence-labeling approach, to bind to isolated organic-rich lamina in rocks from the Eocene Green River Formation known to contain squalane among other linear isoprenoids. These results suggest that squalane, or structurally similar organic biomarkers that cross-react with the antibodies, are confined to discrete organo-sedimentary fabrics within those rocks, providing evidence for their syngeneity. Depending on the specificity and sensitivity of the antibody/geolipid pair, an in situ antibody detection approach may be useful for establishing biomarker syngeneity in older rocks.

4.2. Introduction

Lipid biomarkers are organic compounds with hydrocarbon skeletons that can be related to biochemical precursors that are presently thought to be restricted to specific taxonomic groups (Brocks and Summons, 2003; Peters et al., 2005). Many lipid biomarkers are stable over geologic time, and the identification of these so-called geolipid compounds in rocks and sediments has provided an alternative source of evidence for the appearance of specific taxa in the rock record (Moldowan and Talyzina, 1998; Brocks et al., 1999; Brocks and Summons, 2003; Brocks and Pearson, 2005). However, biomarker studies are commonly complicated by the possibility of contamination, which introduces non-syngenetic biomarkers to the host rocks (Brocks, 2011; French et al., 2015). Here we propose the utilization of monoclonal antibodies as molecular probes to in situ visualize specific biomarkers and address syngeneity by relating their spatial distribution to the sedimentary fabric of the host rock.

4.2.1 Biomarker syngeneity

A biomarker is taken to be syngenetic if it was introduced from the primary depositional environment. However, the mobilization of petroleum can introduce non-indigenous hydrocarbons into sedimentary rocks along the pathway of migration (Curiale and Bromley, 1996). In addition, anthropogenic petroleum products can contaminate samples during collection and storage (Hoering, 1965; 1966). For these reasons, establishing the syngeneity of geolipids is a critical prerequisite to their use as paleobiological indicators (Meinschein, 1965; Nagy, 1970; Brocks et al., 2003). Brocks et al. (2003) presented a number of analytical approaches to provide evidence of biomarker

syngeneity in Archean rocks. Such approaches include matching molecular indicators of thermal maturity with the age and lithological maturity of the host rocks, comparing the stable isotope values of sample kerogen and bitumen, assessing the geological context of the host rocks for potential contamination by migrating hydrocarbons, evaluating the spatial distribution of hydrocarbons within the sample and analyzing the pyrolytic degradation products from kerogen. However, these approaches are not always possible, nor are their results fully conclusive (French et al., 2015).

One alternative approach to assessing biomarker syngeneity would be to evaluate the association between specific organic biomarkers and primary or secondary organo-sedimentary fabrics, as indicators of endemicity or contamination, respectively. In this paper, we show that the distribution of specific compounds within rock fabrics may, in certain cases, be visualized through a new in situ detection method using monoclonal antibodies that bind to specific classes of geolipids (or to geolipids in a broad sense). Association of the biomarkers with the primary sedimentary fabric (e.g., organic rich laminae vs. secondary veins) would provide additional evidence for the syngeneity of antibody-binding hydrocarbons.

4.2.2. Antibodies

The immune system of vertebrates uses antibodies as part of an adaptive response to recognize and disable potentially harmful compounds and pathogens (e.g., Abbas et al., 1991; Roitt, 1991; Birch and Lennox, 1995). Because antibodies bind to specific sites (epitopes) on the target molecules (antigens), custom antibodies are commonly developed and used as probes for the detection of specific molecules in modern life sciences

applications, ranging from cancer treatment (Berinstein and Levy, 1987; Scheinberg, 1991; Biela et al., 2003) to microbial ecology (Lin and Carpenter, 1996; Faude and Höfle, 1997; Caron et al., 2003). Immunodetection approaches have even been used in paleontological studies. For example, Muyzer and Westbroek (1989) raised polyclonal antibodies against the organic matrix of recent bivalve shells. The antibodies also reacted with biopolymers preserved in Pleistocene bivalve shells suggesting a chemical similarity between the modern and ancient shell matrices (Muyzer et al., 1984; Muyzer and Westbroek, 1989; Collins et al., 1991; 2003).

Antibodies commonly bind to epitopes with remarkable selectivity. When used in immunolabeling applications, usually a secondary antibody that is conjugated to a detection probe is added. The secondary antibody is designed to bind the primary antibody, allowing the antibody-antigen interaction to be detected, either in situ or in an extract (Blanchette-Mackie et al., 1989; Compère et al., 1995). Biological antigens are typically proteins and polysaccharides that have epitopes derived from their complex functional groups and three-dimensional stereochemistry (Liddell and Cryer, 1991; Li et al., 1998). Although lipids, especially in their saturated hydrocarbon form, are structurally simple molecules compared to other antigenic biomolecules, antibodies that exhibit specific binding to lipids are well documented (Fogler et al., 1987; Swartz et al., 1988; Maneta-Peyret et al., 1992). For example, human blood is known to contain antibodies against cholesterol, which is thought to act as a mechanism for regulating low-density lipoprotein production (Swartz et al., 1988; Alving et al., 1989; Alving and Swartz, 1991). Naturally-occurring antibodies, however, likely bind epitopes containing polar groups, such as the hydroxyl group on cholesterol. Antibodies binding hydrophobic

portions of lipids are less likely to occur and therefore the immunological study of biomarkers in sedimentary rocks have been poorly documented thus far.

4.2.3. Antibodies to target lipid biomarkers

Maule et al. (2003) produced a polyclonal antibody serum that showed some reactivity to hopanes for use as a stand-alone biomarker detection tool for potential life on Mars. In the context of Mars exploration, other attempts have been made to produce polyclonal antibodies in the developing of the Life Marker Chip, an antibody-based tool to detect small hydrophobic molecules in situ, such as the lipid biomarkers coprostane, hopane, phytane and squalane among others (Sathe et al., 2011; Rix et al 2011; Sephton et al., 2013). However, the heterogeneous nature of polyclonal antibodies, which consist of a mixture of multiple antibodies that target different epitopes and potentially different molecules, fundamentally limits confidence in their capability to bind only to the target compound in complex molecular assemblages of structurally-similar hydrocarbons, such as those found in sedimentary bitumen and petroleum. In addition, polyclonal antibodies would likely bind to polar functional groups not usually found in sedimentary biomarkers.

More promisingly for studies of natural organic matter, Matyas et al. (2000; 2002) produced several mouse monoclonal antibodies against the lipid squalene. In contrast to polyclonal antibodies, monoclonal antibodies derive from an isolated clone of a single antibody-producing cell. Therefore, the antibodies that bind to polar moieties on biomarker structures can be selected from the population of antibodies comprising the immune response. Among the antibody-producing clones obtained by Matyas et al.

(2000), the hybridoma¹ SQE#14 (PTA6538) secreted antibodies shown by ELISA not only to bind squalene, but also to bind its saturated form, squalane, a relatively-common sedimentary geolipid (Matyas et al., 2000; 2002; Alving et al., 2005).

Squalene is the biochemical precursor for the synthesis of all polycyclic triterpenes and sterols. Therefore, squalene is synthesized by all domains of life and its saturated analog, squalane, is ubiquitously found in sediments and known to occur in ancient rocks as a sedimentary hydrocarbon (Gardner and Whitehead, 1972; Peters et al., 2005). The cross-reactivity of the antibodies from the clone SQE#14 was exploited in this study to detect sedimentary squalane (or other hydrocarbons the antibody may bind to) and test the ability of monoclonal antibodies to target lipid biomarkers in the rock record. In this study, the antibodies produced by Matyas et al. (2000) were evaluated for their molecular specificity by measuring their binding affinity, first to standard solutions and crude oil samples containing other common sedimentary hydrocarbons to test the feasibility of the antibody-geolipid binding. Then, as a test case, anti-squalene antibodies were applied to squalane-containing mudstones from the Eocene Green River Formation for biomarker visualization in rock-associated sedimentary organic matter.

4.3. Materials and methods

4.3.1. Hybridoma culturing and antibody production

¹A hybrid cell line obtained by the fusion of an immortal myeloma cell line and the antibody-producing cells from the spleen of the mouse that was injected with the desired antigen. The hybridoma can be cultured *in vitro* indefinitely and, when isolating a particular cell clone, the same specific antibodies can be collected from the culture media.

Hybridoma clone PTA6538-SQE14 cells were obtained from the American Type Culture Collection and were cultured in hybridoma growth medium (GIBCO; Formula number 06-5012EL supplemented with 3% characterized fetal calf serum). Cells were grown initially in T75 flasks. When confluent, the cells were further expanded to T250 flasks and then added to 3 L spin flasks. When cells reached a concentration of 1×10^6 cell/mL, the flasks were aerated for additional 3–5 days of growth. After cultivation, the cell suspension was poured into 500 mL conical centrifuge tubes and spun at 2,400 rpm for 15 minutes at 4 °C. The supernatant was then filtered through a 0.2 µm 1 L bottle top filter unit into a 5 L sterile glass bottle. Several aliquots of the filtered supernatant were stored at –20 °C until they were thawed and used as the primary antibody. Anti-CD75 IgM antibodies from the clone LN-1, which react with the human surface proteinaceous lymphocyte antigen CD75, were similarly obtained from culture supernatants and used as an irrelevant isotype-control antibody in all immunolabeling experiments.

4.3.2. Immunoblotting on PVDF membranes

Biomarker standards and crude oil samples were evaluated for their binding affinity to the antibodies by dot immunoblotting (Stott, 2000). The isoprenoids squalene, phytane, stigmasterol, phytol (Sigma-Aldrich, St. Luis, Missouri, USA), squalane, pristane and cholestane (Acros Organics, Geel, Belgium) together with the alkanes *n*-pentadecane, *n*-icosane and *n*-tetracosane (Alfa Aesar, Ward Hill, Massachusetts, USA) were used as biomarker standards. The crude oil samples (ONTA Inc., Toronto, Ontario, Canada) were numbered from one to eight in increasing order of density, corresponding to: 1, paraffinic oil from the Appalachian Basin, USA (827 mg/mL); 2, paraffinic oil

from Louisiana, USA (839 mg/mL); 3, paraffinic oil from Qua Iboe, Nigeria (846 mg/mL); 4, paraffinic-naphthenic oil from Edmonton, Alberta, Canada (861 mg/mL); 5, naphthenic oil from Hoops, Texas, USA (869 mg/mL); 6, aromatic-intermediate oil from Saudi Arabia (870 mg/mL); 7, aromatic-asphaltic oil from Oriente, Ecuador (910 mg/mL); 8, aromatic-asphaltic oil from Merey, Venezuela (968 mg/mL).

One microliter of biomarker or crude oil solution dissolved in *n*-hexane (with the exception of stigmasterol, which was dissolved in tetrahydrofuran; Sigma-Aldrich) at different concentrations were spotted onto 0.2 μ m PVDF membranes (Thermo Fisher Scientific, Waltham, Massachusetts, USA) without previous activation of the membrane with alcohol. The biomarker-coated membranes were dried overnight at room temperature and then incubated for one hour under orbital shaking at 25 °C with 2% bovine serum albumin (BSA; Fraction V from Roche Diagnostics GmbH, Mannheim, Germany) dissolved in TBS solution (Tris-buffered saline, 20 mM Tris Base pH 7.6, 150 mM NaCl). Membranes were then incubated for one hour under orbital shaking at 25 °C with undiluted culture supernatants of either the hybridoma clone SQE#14 (PTA6538) or the hybridoma clone LN-1, both cell lines that secrete mouse antibodies of the isotype IgM. After the primary antibody incubations, the membranes were washed three times for 15 min each under orbital shaking at 25 °C with TBS and then incubated for one hour under orbital shaking at 25 °C with goat anti-mouse IgM horseradish peroxidase-conjugated antibodies (Invitrogen, Waltham, Massachusetts, USA) diluted 1:1000 in TBS. The membranes were subsequently washed three times for 15 min each under orbital shaking at 25 °C with TBS and the secondary antibody binding was revealed by a chromogenic precipitate developed after the addition of CN/DAB (4-chloro-1-

naphthol/3,3'-diaminobenzidine) substrate, according to the manufacturer's instructions (PierceTM CN/DAB substrate kit, Thermo Fisher Scientific).

It is important to note that because the biomarkers used to coat the membranes are hydrophobic, they are susceptible to diffusion after coating and may elute from PVDF membranes if: (1) the membranes are pre-activated by immersion in alcohol and maintained wet prior to antigen spotting, as is typically required for activation of PVDF membranes when proteins are blotted, or (2) the membranes are washed with solutions containing detergent-like solvents, such as polysorbate 20 or other non-ionic detergents that are frequently used to reduce non-specific antibody interactions in immunoblotting applications (Alving and Grabenstein, 2000). Therefore, no pre-activation or washing solutions containing detergents were used here to avoid diffusion and removal of the antigens spotted on PVDF membranes.

ImageJ (Schneider et al., 2012) was used to subtract the background of the dot blot images and quantify the chromogen reciprocal intensities (Nguyen and Nguyen, 2013). Each dot intensity obtained was normalized to the intensity of the two-microliter dot of anti-CD75 culture supernatant diluted 1:4 spotted together with the samples on every membrane. For comparison among biomarker standards and crude oil samples, the normalized intensities obtained by anti-squalene blotting were subtracted from the normalized intensities of the corresponding dot obtained by anti-CD75 blotting (isotype control) and the values were expressed as arbitrary units in **Figures 4.1** and **4.2**. Graphs and statistical analyses between experimental values (anti-squalene) and the isotype control (anti-CD75) were performed using the Mann-Whitney *U* test on GraphPad Prism 5.0.

4.3.3. Immunofluorescence of organic matter in natural rock samples

Unweathered hand samples of lacustrine kerogen-rich mudstones from the Green River Formation, outcropping in Fossil Basin, were collected from a quarry near Kemmerer, Wyoming (Lat. 41.798; Lon. -110.732, **Figure C.S1**) and wrapped in pre-combusted aluminum foil for transport and storage. Rock samples were slabbed with a water-cooled unlubricated rock saw. One face was used for immunofluorescence and the other for conventional GC-MS analysis (see Section 4.3.4). Press-on immunoreagent wells were applied to the slab surfaces to keep control reagents from mixing with experimental reagents. Slab surfaces used for immunofluorescence were etched in 0.2% HCl before being rinsed three times with Milli-Q water. The acid treatment removes carbonates potentially blocking organic remains, therefore increasing the exposure of organic antigenic surfaces to antibodies. Rock surfaces were subsequently blocked with 2% BSA/PBS (bovine serum albumin dissolved in phosphate-buffered saline) and allowed to incubate in a humid chamber to prevent dehydration. Blocking solution was aspirated before 60 μ L of antibody-containing supernatant diluted 1:10 in 2% BSA/PBS was added to each well and allowed to incubate in a humid chamber for one hour at ~25 °C. Upon aspiration, each well was washed ten times with 260 μ L drops of 2% BSA/PBS with aspiration between washes. Twenty μ L of secondary goat anti-mouse IgM FITC-conjugated antibody-containing solution diluted 1:1000 in 2% BSA/PBS was then added to each well and allowed to incubate in the dark for one hour. After ten washings with 20 μ L drops of 2% BSA/PBS, 20 μ L of VectaShield antifade mounting medium (Vector Laboratories Inc., Burlingame, California, USA) was added and a coverslip attached.

FITC-conjugated antibodies were visualized using a Zeiss Axioscope equipped with Zeiss Filter Set 09 (BP) 450–490, (BMS) 510, (LP) 515, Zeiss, Jena, Germany. Image post processing was restricted to uniform whole-image level adjustments.

Two types of controls, with two replicate wells for each control, were contained on each immunofluorescence slide. First, all reagents were applied to un-etched portions of each section. Second, etched portions of the slide received a control treatment identical to the experimental treatment, but with an irrelevant primary antibody (anti-CD75, described in Section 4.3.1).

4.3.4. Gas chromatography–mass spectrometry (GC–MS)

For this study, cuttings from the same hand sample used in the immunofluorescence detection experiment were powdered and their solvent extracts were analyzed by GC–MS to provide independent confirmation of squalane in the samples used for immunofluorescence. Powdered rock was extracted using dichloromethane. The saturate fraction was isolated by silica gel chromatography using hexane as a mobile phase. A branched/cyclic fraction was obtained using silicalite (ZSM-5) chromatography with isooctane as a mobile phase. GC–MS analysis of the rock extract was performed on a VG Autospec-Q mass spectrometer coupled to an HP Series II gas chromatograph fitted with an Agilent DB-1 silica capillary column (60 meters long, 0.25 mm ID, 0.25 μ m film thickness). The temperature program started with 150 °C for one minute, followed by an increase of two degrees per minute until 320 °C was reached and held for an additional 20 minutes.

Crude oil sample number two (described in Section 4.3.2) was evaluated by selected ion monitoring (SIM)–GC–MS on an Agilent 5975c MSD with a 7890A GC front end coupled to an Agilent DB-1 silica capillary column (60 meters long, 0.25 mm ID, 0.25 μ m film thickness). The chromatographic temperature program started with 35 °C, increasing at two degrees per minute until 80 °C was reached, followed by an additional increase of three degrees per minute until 320 °C was reached and held for 15 minutes. The quantification of squalane was calibrated with an internal standard of squalane and detected using the m/z 113 ion. Other acyclic isoprenoids were also identified in the analysis and their approximate concentrations were calculated using the m/z 113 ion, assuming the same sensitivity response as squalane (**Table C.S1**).

4.4. Results

4.4.1. Evaluation of anti-squalene antibody specificity

Squalene, an acyclic terpenoid composed of six isoprene subunits (triterpene, C₃₀), was used as immunization antigen by Matyas et al. (2000) for the production of several antibody-secreting hybridomas. One of the obtained hybridomas, the clone SQE#14 (PTA6538), secretes antibodies that have been previously shown by ELISA to also bind squalane (Matyas et al., 2000). The binding promiscuity of these antibodies may be useful for sedimentary hydrocarbon detection, because it is possible that the antibodies bind to other biomarkers along with squalane and squalene. We tested the cross-reactivity of hybridoma supernatants from the clone SQE#14 against other relevant lipid biomarkers that are structurally similar to squalene (see **Figure C.S2** for structure comparison amongst the hydrocarbons used in this study).

Antibody binding reactivity was evaluated by an immunoblotting procedure adapted to detect biomarker standards by dot blot on PVDF membranes. Standard solutions of the acyclic isoprenoids squalane (C₃₀), squalene (C₃₀), phytol (C₂₀), phytane (C₂₀) and pristane (C₁₉) along with the cyclic isoprenoids cholestane (C₂₇) and stigmasterol (C₂₉) as well as the alkanes *n*-pentadecane (C₁₅), *n*-icosane (C₂₀) and *n*-tetracosane (C₂₄) were used to coat PVDF membranes with one microliter at concentrations ranging from 5 to 200 nmoles/μL. After anti-squalene antibody incubation, binding was detected by the addition of peroxidase-conjugated anti-IgM antibodies and CN/DAB substrate, which forms a blackish precipitate on the membrane (**Figure 4.1A**).

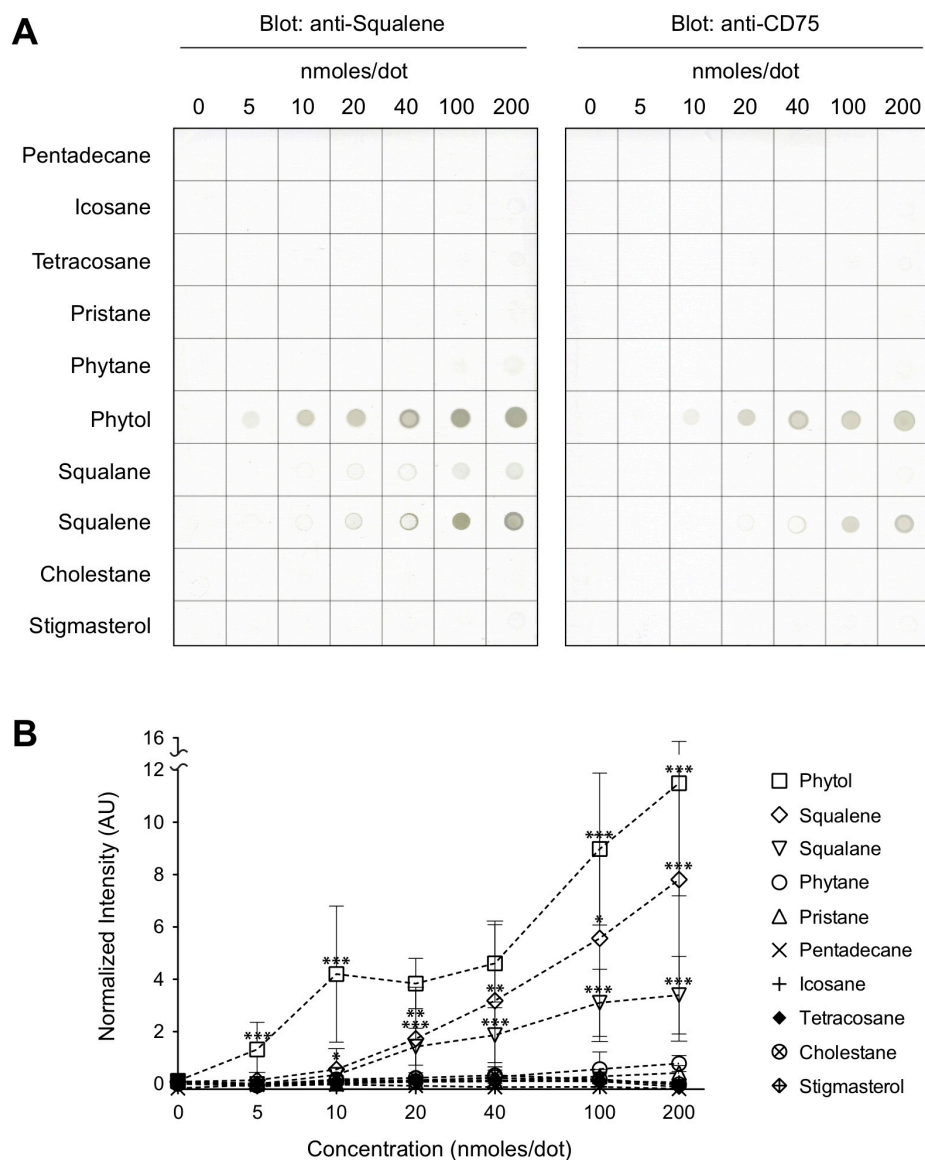


Figure 4.1. Immunoblotting of biomarker standards on PVDF membranes. (A) Representative dot blot of biomarker-coated PVDF membranes using anti-squalene antibodies (left) and anti-CD75 antibodies as isotype control (right). The *n*-alkanes pentadecane, icosane and tetracosane, the acyclic isoprenoids pristane, phytane, phytol, squalane and squalene, along with the sterols cholestane and stigmasterol were used to coat the membranes at different concentrations. (B) Semi-quantification of the dot blots

shown in A. Data are expressed as arbitrary units (AU) of the mean of quintuplicate pixel intensities of each anti-squalene dot normalized with respect to an IgM antibody-coated dot and subtracted to the corresponding anti-CD75 normalized pixel intensities. Significantly higher values that are at least twofold above the background signal are indicated (* $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$).

In addition to squalene and squalane, the antibody derived from the clone SQE#14 displays binding affinity to phytol and, to a lesser extent, phytane and pristane (**Figure 4.1A**). Immunoblotting using an irrelevant IgM antibody (anti-CD75) is also shown in **Figure 4.1A** to reveal the background signal intensity derived from the lipid coatings, non-enzymatic reaction and potential non-specific binding of both primary and secondary antibodies. Particularly for phytol and squalene, the background developed was significantly higher than other lipids. To compare the signal intensities among each hydrocarbon tested, the anti-CD75 control signal was subtracted from the anti-squalene intensities, so the semi-quantitative results in **Figure 4.1B** reflect specific binding of the antibodies from the clone SQE#14. By comparing to the background intensities, the anti-squalene antibody provides significantly higher values for phytol starting at 5 nmoles/dot² ($p < 0.01$), squalene at 10 nmoles/dot ($p < 0.1$) and squalane at 20 nmoles/dot ($p < 0.01$; **Figure 4.1B**). Considering the statistical significance and at least a twofold intensity of the experimental condition over the background signal, the detection

²For convenience, lipid concentrations on the membrane are expressed in nmoles per dot. Each dot area was lipid- and concentration-dependent, with an average phytol dot of $11.1 \pm 0.2 \text{ mm}^2$, squalane dot of $8.5 \pm 0.03 \text{ mm}^2$ and squalene dot of $8.8 \pm 0.05 \text{ mm}^2$.

limit for squalane ranges from 10 to 20 nmoles/dot (1.2 to 2.4 nmoles/mm²) under the tested conditions. Phytane and, to a lesser extent, pristane show significant increase of intensities over 100 nmoles/dot; however, their values remain comparatively low and do not rise twofold above the background. The immunoblottings also show no twofold increase above the background and no significantly higher intensities for coatings of the *n*-alkanes and the cyclic isoprenoids (**Figure 4.1B**). Even at higher hydrocarbon concentrations of 2000 nmoles/dot, the alkanes and cyclic isoprenoids tested showed no apparent signal increase (data not shown), suggesting binding affinity to linear isoprenoids mostly.

4.4.2. Biomarker detection in crude oils by immunoblotting

In addition to assessing the specificity of anti-squalene antibodies to bind biomarker standards, immunoblotting of crude oil samples was also applied to evaluate the binding capacity of the antibody in a complex mixture of biomarkers. Eight crude oil samples from different field locations were spotted on PVDF membranes at increasing concentrations from five to 320 µg per dot. On their own, the crude oil membrane coats appear as a blackish-brown color. Therefore, to assess the binding affinity of anti-squalene antibody by immunoblotting, the color intensities were compared to PVDF membranes incubated with the anti-CD75 isotype control antibody, as described above (**Figure 4.2A**).

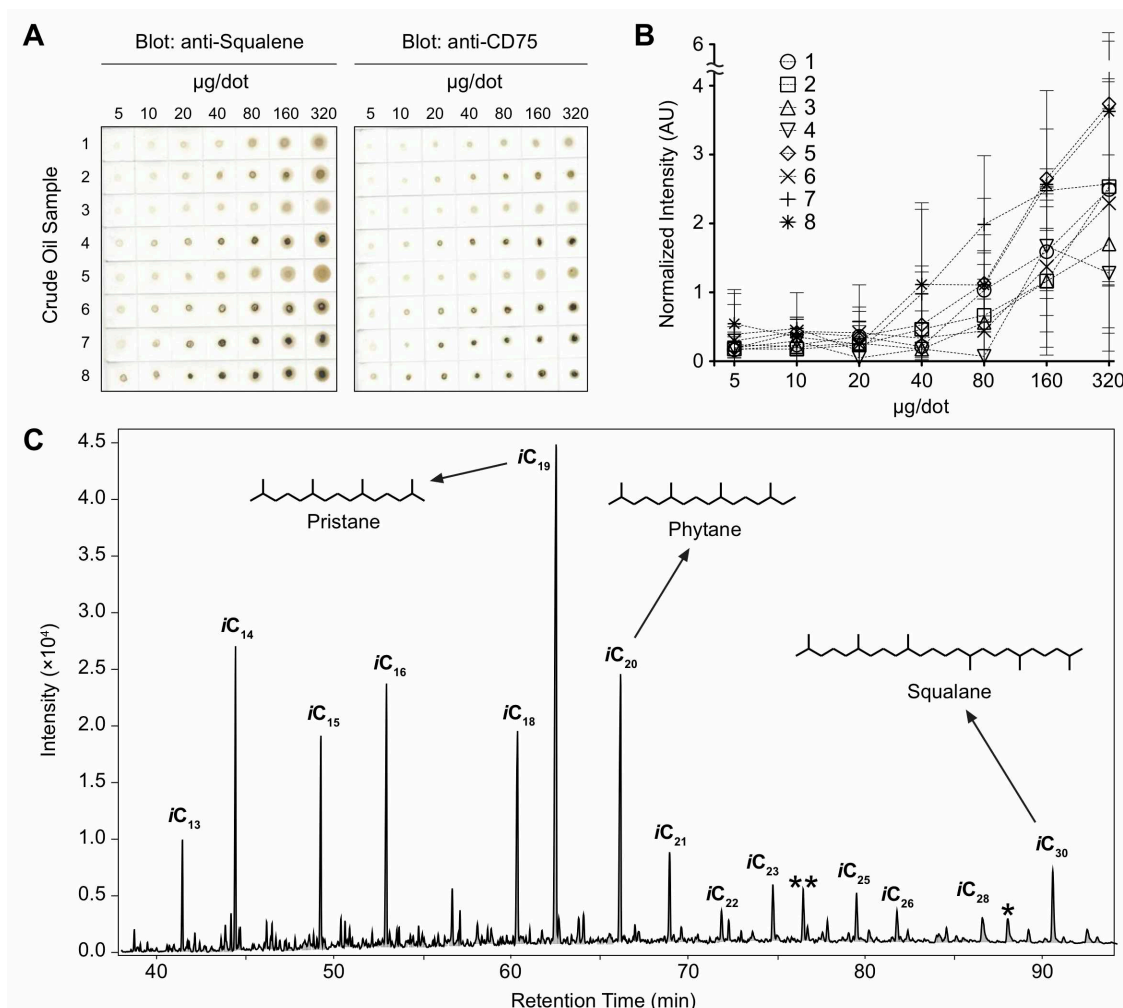


Figure 4.2. Immunoblotting of crude oil samples on PVDF membranes. (A)

Representative dot blot of crude oil samples used to coat PVDF membranes that were incubated with anti-squalene antibodies (left) and anti-CD75 antibodies as isotype control (right). Membranes were coated with different concentrations of eight crude oil samples: 1, paraffinic oil from the Appalachian Basin, USA; 2, paraffinic oil from Louisiana, USA; 3, paraffinic oil from Qua Iboe, Nigeria; 4, paraffinic-naphthenic oil from Edmonton, Alberta, Canada; 5, naphthenic oil from Hoops, Texas, USA; 6, aromatic-intermediate oil from Saudi Arabia; 7, aromatic-asphaltic oil from Oriente, Ecuador; 8, aromatic-asphaltic oil from Merey, Venezuela. (B) Semi-quantification of the dot blots

shown in A. Data are expressed as arbitrary units (AU) of the mean of duplicate pixel intensities corresponding to each dot normalized with respect to an IgM antibody-coated dot and subtracted to the anti-CD75 normalized pixel intensity. (C) Mass chromatogram m/z 113 of the oil sample 2, showing the identification of squalane by GC–MS at concentrations of 516 mg/L (0.6 mg/g). Other isoprenoids were also identified using the m/z 113 ion as shown in the chromatogram.

Quantification of immunoblot signal intensities shows increasing tendencies towards higher concentrations for all crude oil samples analyzed, indicating greater intensity of the anti-squalene antibody signal compared to the isotype control (**Figure 4.2B**). Analysis of the crude oil sample number two by GC–MS indicates the presence of several isoprenoids along with squalane (**Figure 4.2C** and **Table C.S1**). The more abundant isoprenoids correspond to pristane (iC_{19}) and phytane (iC_{20}) with estimated concentrations of 2631 and 1248 $\mu\text{g/mL}$ (3.1 and 1.5 mg/g), respectively. Squalane, although not as abundant as pristane and phytane, was found at concentrations of 516 $\mu\text{g/mL}$ (0.6 mg/g). Therefore, a maximum of 3.7, 1.7 and 0.5 nmoles of pristane, phytane and squalane, respectively, were spotted per dot on the membrane for the crude oil sample 2, which are below the detection limits found in **Figure 4.1**. The anti-squalene antibody seems not only to bind squalane, but likely binds other isoprenoids. The cumulative effect of other acyclic isoprenoids (and perhaps other hydrocarbons not tested in this study) may explain the signal in **Figure 4.2A**.

4.4.3. In situ detection in kerogen-rich marlstone using immunofluorescence

Rock sections from the Eocene Green River Formation were examined using petrographic and fluorescence microscopy after acid etching and antibody incubations. The examined rock sections consisted of kerogen-rich laminated mudstones characterized by alternating calcite-rich and kerogen-rich laminae. The mineralogy of the examined rocks consists primarily of calcite, with minor amounts of dolomite, quartz, feldspar, and clay, as previously reported by Buchheim (1994) and Buchheim and Eugster (1998). Kerogen occurs primarily as continuous, wavy organic-rich laminae, ~10-50 μm thick. Many of the laminae in the sampled rocks are discontinuous and evidence for soft-sediment deformation is present in hand samples.

In the rock sections, anti-squalene antibody binding was visualized by the addition of fluorescent FITC-conjugated anti-IgM antibodies followed by fluorescence microscopy. Of the more than 1,100 individual laminae examined, only 14 total regions showed extensive antibody binding. These segments of antibody binding accumulation present as convolute undulations within the organic lamina, connecting small irregularly-shaped signal “patches” ~5 μm in diameter (**Figure 4.3A-C**, Arrows). In total, antibody binding was observed in fourteen individual spots – although in all but three cases, these occurred as small patches rather than single horizons that followed bedding several hundred microns in length. Antibody binding was not observed in control wells that examined subjacent portions of the same laminae containing un-etched surfaces (**Figure 4.3D**). Examination of the control wells in which an irrelevant primary antibody was used on the same rock coupons as the test wells, also revealed no FITC signal (**Figure 4.3E**).

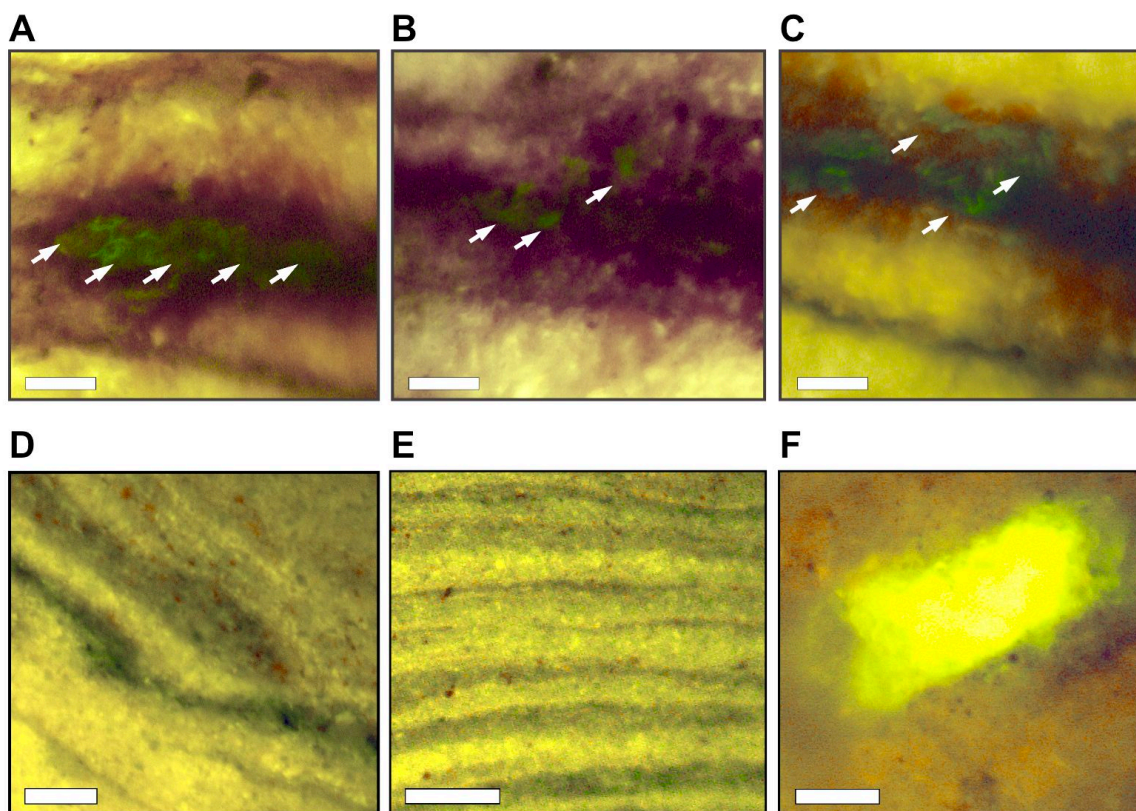


Figure 4.3. Immunofluorescence of organic laminae from the Green River Formation.

(A-C) Imaging of FITC-conjugated antibodies on three different sections of organic-rich slabs that were incubated with anti-squalene antibodies revealing discrete signal accumulation (arrows) and suggesting the presence of squalane/isoprenoids associated with individual laminae. Negative controls of acid un-etched sections incubated with anti-squalene (D) and acid etched sections incubated with an irrelevant anti-CD75 primary antibody (E) did not exhibit an immunofluorescence signal. Mineral autofluorescence was present in the imaged sections as a dull yellow background and diffuse bright yellow associated with certain mineral grains (F). Autofluorescence is distinct from the sharply-localized green fluorescence of antibodies shown in A-C. Scale bar = 50 μm .

Carbonate minerals within the marls tested commonly exhibited a dull greenish-yellow background autofluorescence with rare isolated grains showing bright yellow autofluorescence with diffuse margins (**Figure 4.3F**). Kerogenous laminae and organic macerals such as microfossils were not autofluorescent. The yellow autofluorescence of the minerals and the green FITC antibody label showed spectral overlap that was clearly differentiated by visualizing the color signal under a FITC longpass filter. In addition, the FITC probe showed distinctive sharp margins and photobleached during observation, whereas the mineral autofluorescence did not. Therefore, the green fluorescence within organic-rich laminae of acid etched surfaces incubated with anti-squalene antibodies is attributable to antibody binding. A variety of linear isoprenoids contained in the organic laminae may also bind the anti-squalene antibody, specifically pristane and phytane, which have been shown to dominate in Green River Formation Rocks (Eglinton et al., 1966; Robinson 1979; Olcott Marshall and Cestari, 2015). Squalane, less studied in Green River samples, was evaluated by GC–MS performed on sample cuttings removed from the same slabs used for the immunofluorescence analysis. **Figure C.S3** shows the chromatogram indicating the presence of squalane in extracts from the cuttings at concentrations of ~100 ppb (0.1 µg/g) as well as other acyclic isoprenoids found, such as pristane and phytane.

4.5. Discussion

4.5.1. Antibody binding specificity

The potential for antibodies to be used as probes for specific biomarker compounds (or structurally-related classes of biomarkers) in ancient rocks is directly

related to the antibodies' binding affinity for the target compound, as compared to other hydrocarbons that are also common in extracts from ancient organic matter. The antibodies used in this study, secreted by the hybridoma clone SQE#14, were not originally intended to bind biomarkers, yet their capacity to bind squalane and other geolipids could be useful for analyzing biomarker syngeneity in rock extracts.

The immunoblotting assay in **Figure 4.1** shows the relatively-strong binding affinity of the anti-squalene antibody to some biomarkers, as compared with the weak signal observed in the isotype control. This background signal is prominent for the standards of squalene and phytol, and it is a contribution of the intrinsic opacity of each hydrocarbon coating over the PVDF membrane, the non-enzymatic reaction of the CN/DAB substrate with the lipids and non-specific molecular interaction of the different components of the supernatant medium along with non-specific binding of primary and secondary antibodies with the lipids. Among the above-mentioned factors, non-specific binding of the primary antibody is the most sensitive problem, because it may give false positive signals in complex matrices. In this study, non-specific binding may be produced because of the mild conditions used in the incubation and washing steps during the immunoblotting that involve the usage of non-detergent containing solutions (see Section 4.3.2). Nonionic surfactants are usually incorporated in the washing solutions to avoid non-specific hydrophobic interactions between proteins; however, they would also remove the lipids used in this study and the immunoblotting results would not be reproducible (Alving and Grabenstein, 2000). Therefore an isotype control should be carried out on each blotting procedure to compare binding affinities and acknowledge false positive signals, as shown in **Figure 4.1A**.

Even though the isotype control signal was subtracted from the anti-squalene, cross-reactivity of the anti-squalene antibodies against phytol was observed. The quantified binding intensities for phytol are comparable and even higher than the intensities of the cognate antigen, squalene, suggesting binding multiplicity (**Figure 4.1B**). It is well recognized that proteins, among them enzymes and antibodies, can present functional promiscuity (James and Tawfik, 2003). In the case of antibodies, promiscuous binding may constitute the cause of most allergic reactions and autoimmune diseases, and is also thought to provide a first line of defense against pathogens (Parnes, 2004). Despite the relevance of promiscuous antibody binding for health and disease, the molecular mechanisms by which antibodies bind to multiple antigens are poorly understood (Kaur and Salunke, 2015; Laffy et al., 2017). The anti-squalene antibody immunoblots presented here suggest that the antibody binds at least to certain acyclic isoprenoids. The anti-squalene antibodies also seem to cross-react with phytane and, to a lesser extent, pristane, at concentrations one order of magnitude higher than squalane. Even though the molar quantities of acyclic diterpanoids tested in **Figure 4.1** show comparatively less antibody binding, higher concentration of these biomarkers may account for a stronger antibody signal on natural samples, where pristane and phytane are usually dominant. The response of highly concentrated alkanes may also be important given their relative high abundance, along with other hydrocarbons not tested in this study. Additional analyses that are beyond the scope of this study would be necessary to determine the full range of cross-reactivity of this antibody.

Binding affinity of the antibodies towards pristane and phytane is also supported by its binding to their parent hydrocarbon in sediments, phytol (**Figure 4.1**). Strong

intensities obtained with phytol may be interpreted as a cause of its rigid stereochemistry and double bond that better reflect the structural conformation of squalene. Because of its double bonds, squalene presents a relatively rigid structure, whereas the saturated hydrocarbon structures of squalane, pristane and phytane have more flexible three-dimensional configurations. Comparison of the Tanimoto structure similarity calculated from two-dimensional structure fingerprints of the hydrocarbons (Bajusz et al., 2015) yields a higher similarity score when squalene is compared with phytol (0.59) rather than with squalane or phytane/pristane (0.43; **Figure C.S2A**). Non-evident structure similarities between phytol and the cognate antigen observed by Tanimoto score comparisons may be relevant to understanding the antibody cross-reactivity. Three-dimensional comparison between possible structure configurations of squalene and phytol also shows the conformational similarity, although non-significant, that may illustrate the flexibility of the antibody's binding site to accommodate diverse epitopes (**Figure C.S2B**), as previously shown by binding surface analysis of homology models for isolated anti-squalene single-chain variable fragments (Al Qaraghuli et al., 2015). The paratope (the antibody binding site) is therefore likely composed of a hydrophobic binding pocket that can accommodate at least two adjacent isoprenoid subunits in *cis* configuration, as represented by phytol's structure stereochemistry. On the other hand, the antibody binding of saturated forms therefore likely requires folding of the hydrocarbons into a specific three-dimensional configuration. Consequently, the anti-squalene antibody affinity is weaker towards squalane, pristane and phytane, consistent with the results shown in **Figure 4.1**. The relevance of the conformational configuration of the hydrocarbon to supporting binding affinity with the antibody is also highlighted by

the high affinity towards phytol, even though it has a hydroxyl group, suggesting that conformational arrangement is more important for antibody binding than the overall hydrophobicity of the hydrocarbon (**Figure C.S2B**).

Regardless of its binding promiscuity, the antibody is able to bind biomarkers in complex matrices of hydrocarbons, represented by the crude oil samples analyzed in **Figure 4.2**. Even though the crude oils contain hydrocarbons not tested in this study, the identification of squalane by GC–MS suggests that antibody binding to squalane in crude oil samples is possible. However, the binding intensities observed by immunoblot were not consistent with the concentration of squalane alone. According to GC–MS quantification, the crude oil coat of 320 μg in **Figure 4.2** only contains about 0.5 nmoles of squalane, which is below the detection limit of the immunoblot for squalane (10–20 nmoles per dot; **Figure 4.1**). High intensities of immunoblotting signals at low squalane concentration compared to the squalane standards analyzed in **Figure 4.1** suggest that the antibody is also binding to something else, likely other isoprenoids present in the crude oils, such as the diterpanes derived from phytol.

Pristane and phytane are thought to originate after diagenesis of the phytol group of chlorophylls and therefore are commonly the most abundant isoprenoid biomarkers in petroleum (Peters et al., 2005). Substantial presence of pristane and phytane observed by GC–MS analysis in one of the crude oil samples suggests binding of the anti-squalene antibody to these biomarkers as well, consistent with the concentration-dependent increase of antibody binding to crude oils shown in **Figure 4.2**. Quantities of pristane and phytane in the membrane coatings of the crude oil sample 2 range from 0.03 to 3.7 nmoles/dot, which are also below the detection limits of the immunoblot for pristane and

phytane (**Figure 4.1**). It is therefore likely that the antibodies cross-react with other isoprenoids from the crude oil samples, which sum up over 16 nmoles in the most concentrated coat of the crude oil sample number two. On the other hand, we cannot discount the possibility that the antibodies bound to other acyclic or irregularly branched isoprenoids such as botryococcane, crocetane and lycopane, that were not tested in this study and that may be present in the crude oil samples, contributing to the observed signal intensity. Preliminary analysis of lycopene and carotene extracts (Roh et al., 2013) also suggests antibody binding. However, the intense coloration of extract coatings over PVDF membranes precludes their quantification by the chromogenic detection method used (data not shown). Other detection methods, such as chemiluminescence substrates or the incubation with fluorescent-tagged antibodies, used here for geolipid detection in the rock sections, would provide a possible future alternative approach that could increase resolution and detection limits of immunolabeling techniques.

4.5.2. In situ detection

As part of their efforts to evaluate biomarker syngeneity in Archean rocks, Brocks et al. (2003) noted that the spatial distribution of contaminants in a rock sample should differ from the distribution of syngenetic compounds. For example, some Hamersley and Fortescue Group drill cores contain decreasing concentrations of low-molecular-weight hydrocarbons from the center to the surface of the rock sample, while the concentration of high-molecular-weight compounds increases with distance from the center of the core. Brocks et al. (2003) attribute this heterogeneous spatial distribution to either surficial contamination or migration of the low-molecular-weight compounds during a pressure

release brought about by drilling and core extraction (e.g., live oil effect; Brocks et al., 2011). Certain gross heterogeneities may indeed indicate contamination, however we suggest that given the proper detection tools, the spatial distribution of molecules relative to sedimentary fabrics can provide more detailed information that could be used for the purposes of testing syngeneity and detecting contamination. For example, by analogy to Steno's Principle of Inclusion, molecular fossils included within primary sedimentary fabrics or macerals would suggest that the molecules themselves are syngenetic (**Figure 4.4A-B**). Although preferential incorporation of contaminants into sedimentary organic matter is possible, it is unlikely that contamination, natural or anthropogenic, would deposit molecules only within isolated laminae or macerals. Similarly, spatial associations with secondary features such as fractures or drill core margins would seem to suggest that the molecules of interest were secondarily emplaced (**Figure 4.4C-D**). The homogenous distribution of hydrocarbons within a uniform rock matrix may present a case in which spatial analysis would be unhelpful for establishing the origins of included hydrocarbons.

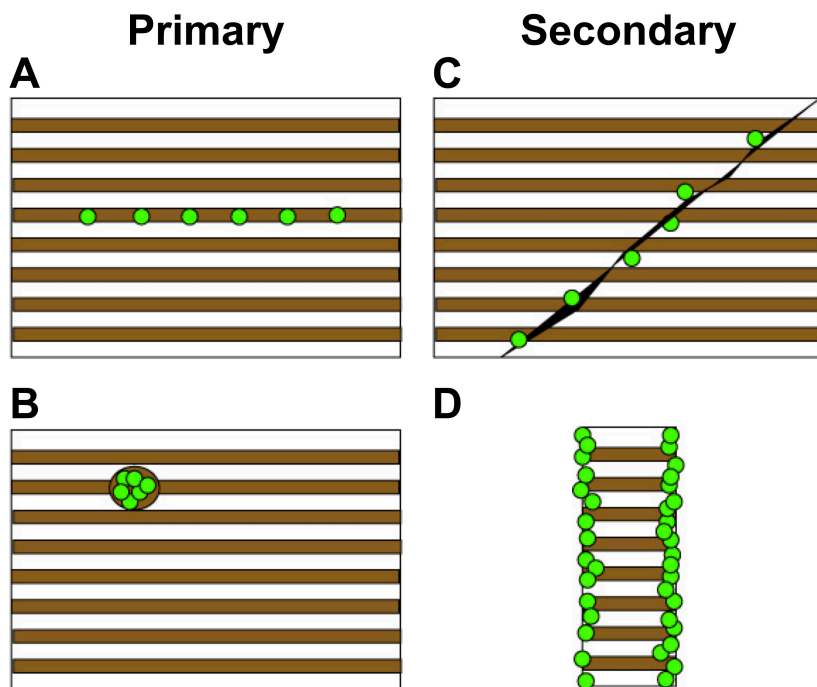


Figure 4.4. Schematic representation of hypothetical biomarker syngeneity visualized by immunofluorescence. The localization of biomarkers within primary organo-sedimentary rock fabrics and macerals, such as organic-rich laminae (A) and (micro)fossils (B) is consistent with biomarker syngeneity. Conversely, contamination is suggested by the association of biomarkers with secondary features such as fractures (C), or patterns which are suggestive of diffusion, such as those in illustration D - a cartoon of a core sample contaminated by drilling fluids.

GC–MS analyses typically require large sample sizes (grams to tens of grams) to produce a detectable signal, which precludes the possibility of spatial analysis by selectively analyzing organic matter from discrete macerals or horizons by conventional techniques. As a result, syngeneity cannot generally be demonstrated by establishing an association between molecules and organo-sedimentary fabrics in the host rock. Time of

flight secondary ion mass spectrometry (TOF–SIMS), which allows for the analysis of molecules ablated from a sample, offers one possible solution – although TOF–SIMS analyses are commonly plagued by surface effects and the absence of standardized spectra (Toporski and Steele, 2004).

The immunodetection approach described here is another possibility, yet a more robust antibody characterization would still be required for its application on rocks where biomarker syngeneity is controversial. Variability in the distribution of organic matter in laminated organic rich sediments commonly exists at the sub-millimeter scale; therefore, traditional GC–MS approaches are unsuitable for fine-scale identification of stratigraphic biomarker associations. The detection of squalane within organic-rich laminae in rocks from the Green River Formation (**Figure 4.3**) demonstrates that a monoclonal antibody detection approach may be an effective means of visualizing the distribution of biomarkers or hydrocarbon types within a rock sample at the scale in which they were deposited. The low number of laminae that exhibited binding, as well as the absence of signal from negative control wells, suggests that the antibodies are binding to squalane, and/or structurally-similar acyclic isoprenoids, that are concentrated within the partially mineral-bound organic matter. If antibody binding had been observed in many or all laminae, this most likely would have resulted from non-specific binding. The relatively low concentrations of extractable squalane in these rocks, as detected by GC–MS, is consistent with the immunofluorescence results that suggest squalane is restricted to only a few discrete macerals. Given the cross-reactivity of these antibodies to concentrated pristane and phytane, they may also have reacted with other isoprenoids, or similar polymer-bound structures within the kerogen matrix. This possibility seems reasonable,

given the low concentration of small area of organic matter exposed for binding on each etched slab face.

Ancient rocks that contain organic matter of questionable provenance generally have low concentrations of biomarkers and it is possible that these anti-squalene antibodies would not be sensitive enough to provide an in situ detectable signal of syngenetic compounds at the microscale (Brocks et al., 2003). Antibody binding requires surface exposure of the lipids at concentrations higher than the detection limit of the technique, which may explain the low number of laminae showing a signal. According to **Figure 4.1**, the limit of detection for squalane is 20 nmoles/dot (2.4 nmoles/mm^2), which translates to the microscale as $2.4 \text{ fmoles}/\mu\text{m}^2$ ($1 \text{ pg}/\mu\text{m}^2$). Therefore, the visualization of contaminants at the microscale may be limited to above-femtomolar concentrated spots and not to low-abundant lipids homogeneously distributed at the macroscale. Unfortunately, the spatial heterogeneities of potential non-sygeneic biomarkers in controversial samples are far from understood. The future production of other monoclonal antibody lines and their characterization coupled with more sensitive reporters may yet provide important clues regarding biomarker sygeneity of ancient rocks.

4.6. Conclusions

Antibodies are powerful tools for molecular detection. Targeted antigens for biological applications are typically complex macromolecules, such as proteins. However, numerous studies document the production of antibodies to lipids and membrane-rigidifying lipid compounds such as cholesterol. This study demonstrates, for

the first time, that monoclonal antibodies can be used for the in situ detection and visualization of geolipids within partially mineral-bound organic matter. Immunoblotting showed that anti-squalene antibodies bind to squalane and other structurally-similar acyclic isoprenoids. Although we cannot discount antibody binding to other compounds, it appears that the binding to other common hydrocarbons, such as the *n*-alkanes and steroids tested in this study, is negligible. In addition, the antibodies were able to bind biomarkers within a complex hydrocarbon matrix represented by the crude oil samples analyzed. Immunofluorescence analysis of squalane-containing, organic-rich mudstones from the Eocene Green River Formation demonstrated that anti-squalene antibodies bind to isolated portions of organic-rich laminae. These findings are significant for organic geochemical studies in that they allow for the association of molecules with organo-sedimentary fabrics, providing critical evidence of syngeneity. Furthermore, paleontological or sedimentological features of the host organic accumulation may provide important clues about the biological source of certain biomarkers. In situ detection could provide evidence of syngeneity, or even show an association between organic-walled microfossils and biomarkers that could be used to establish their phylogenetic affinities.

Our results suggest that certain monoclonal antibodies have the potential to bind to broad classes of hydrocarbons, including some of those preserved in the rock record. Antibodies that bind to lipids are poorly studied relative to protein-specific antibodies. If anti-lipid antibodies can attain the sensitivity and specificity of their anti-protein counterparts, then they may also serve as tools for the in situ detection of specific lipid biomarkers. Because the potential exists for non-specific binding, immunodetection

should be used to augment, rather than replace, conventional GC–MS techniques. Future development of this technique could include the production of antibodies against other biomarkers that could then be used to detect these compounds in sediments, rocks, fossils and modern cells.

4.7. Acknowledgements

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Chapter 5

Planar chromatography and immunodetection of hydrocarbons on PVDF membranes

The contents of this chapter are in preparation for submission, and have been developed by Fernando Medina Ferrer, and Jake V. Bailey.

5.1. Synopsis

Fast, cheap, and simple chromatographic separation of lipids and hydrocarbons can currently be achieved using thin layer chromatography (TLC). Here, we describe an alternative separation method of planar chromatography using polyvinylidene difluoride (PVDF) membranes as the stationary phase. The procedure has the same advantages of TLC over other better resolving—but expensive and time-consuming—techniques, such as HPLC or GC. Unlike TLC, however, PVDF membranes provide an immediate support for spot development via immunodetection. Although antibodies are typically developed to target proteins, there are some examples of antibodies able to bind lipids and hydrocarbons. We show that lipids and hydrocarbon extracts can be separated and further detected by immunoblotting on PVDF membranes. PVDF is compatible with a variety of solvents that can migrate over retained hydrocarbon samples loaded on a membrane. Continuous solvent flow redistributes the sample components according to their relative affinities between the hydrophobic PVDF membrane and the solvent, providing a chromatographic separation. The PVDF membranes are also useful immobilizing supports for immunoblotting, providing high binding capacity and ease of use. We illustrate the chromatographic separation using crude oils hydrocarbons and plant lipid extracts, by planar chromatography on PVDF membranes and their subsequent immunoblotting using anti-squalene antibodies that cross-react with acyclic isoprenoids. To our knowledge, this is the first time PDVF membranes have been used as a stationary phase for planar chromatography and shown to be useful for the immunodetection of the separated compounds using antibodies that bind hydrocarbons.

5.2. Introduction

Analytical separation using thin layer chromatography (TLC) provides a relatively simple, cost-effective, and reliable high throughput method for the analysis of diverse lipids and hydrocarbons. The capability of analyzing all sample components, without the need for sample pre-treatment, using different detection methods applied to the same chromatographic run, has conferred TLC (and its high performance application—HPTLC) a major analytical advantage compared to liquid or gas chromatography, despite its lower resolution (Fuchs *et al.*, 2011; Cebolla *et al.*, 2015). Developed TLC plates serve as archives to which intrinsic or induced optical characteristics (Cebolla *et al.*, 2011), chemical staining, effect-directed analysis (Weiss *et al.*, 2017), immunostaining (Suetake & Yu, 2003; Conti *et al.*, 2012), and mass spectrometric (MS) methods (Nakamura *et al.*, 2006; Fuchs, 2012) can be directly applied to identify the resolved analytes and determine their physicochemical properties, biological effects, and structure. Most of these detection strategies can be improved by prior lipid transfer from the developed TLC plate to a polyvinylidene difluoride (PVDF) membrane (Taki *et al.*, 1994a, 1994b; Goto-Inoue *et al.*, 2008). The lipid transfer procedure from TLC plates to PVDF membranes, referred to as TLC-Blot or Far-Eastern Blot (Taki & Ishikawa, 1997), has been applied and optimized for the analysis of glycolipids, sphingolipids, and phospholipids (Taki, 2015), and requires the application of direct heating (180 °C). Heating, however, may affect lipid properties (e.g., by inducing thermal degradation and modification) and volatilize low molecular weight hydrocarbons. In addition, lipid transfer to PVDF membranes may prove rather inefficient for species not yet evaluated, as well as certain hydrocarbons—particularly

resin and asphaltic hydrocarbons—that have reduced mobilities or permanently bind to TLC plates. Here, we show that chromatographic separation of lipids and hydrocarbons could be directly applied on PVDF membranes instead of TLC plates, thus avoiding the transfer step and gaining the capability to analyze all non-volatile petroleum hydrocarbons.

Downstream analysis of resolved samples on PVDF membranes has shown several advantages compared to TLC plates. Extraction of separated lipids from PVDF membranes is more efficient than extraction from TLC silica gel plates, providing higher yields for preparative purification (Taki *et al.*, 1994b). MALDI-TOF and imaging MS analyses showed less background interference, better sensitivity, and better resolution when performed on PVDF membranes than silica gel plates (Guittard *et al.*, 1999; Goto-Inoue *et al.*, 2008, 2009; Valdes-Gonzalez *et al.*, 2011). Immunodetection on PVDF membranes incubated with monoclonal antibodies also showed improved sensitivities compared to incubations using silica gel plates (Taki *et al.*, 1994a). Although antibodies are not usually developed to target small haptens, such as lipids and hydrocarbons, some antibodies have shown to bind specific PVDF-bound lipids transferred from TLC plates (Taki & Ishikawa, 1997; Taki, 2015). Other antibodies have shown to specifically bind compounds with constrained molecular moieties found in hydrocarbons and organic extracts (Matyas *et al.*, 2000; Li *et al.*, 2016b), opening the possibility to characterize petroleum hydrocarbon classes and lipid biomarkers by immunostaining on PVDF membranes (Medina Ferrer *et al.*, 2018).

PVDF membranes have several engineering applications for pollutant removal and water treatment (Orr *et al.*, 2013). In life sciences, PVDF membranes are mostly used

for filtering media and as a protein immobilizing support for immunoblotting techniques (e.g., dot blot and western blot; Komatsu, 2015). PVDF membranes have also proved useful for the chromatographic purification of certain proteins, in a mechanism proposed to be governed by ion exchange and hydrophobic interactions (Ghosh, 2001, 2003; Yu *et al.*, 2008; Fan *et al.*, 2017). We propose expanding the utility of PDVF membranes by using them directly as a stationary phase for planar chromatography (planar PVDF Membrane Chromatography, referred herein as PMC). The application of PMC is especially useful for the separation and downstream analysis of certain petroleum hydrocarbon type groups, such as asphaltic fractions that permanently bind to TLC silica plates and thus cannot be transferred to PVDF membranes. Lipid and hydrocarbon samples separated by PMC can directly be evaluated by MS, immunodetection, or other effect-directed analysis (e.g., Fuchs, 2012; Weiss *et al.*, 2017). Here we show that PMC can be used as a direct substrate for hydrocarbon and lipid separation, followed by their analysis using immunoblotting. We use monoclonal antibodies that bind squalene and other acyclic isoprenoids to evaluate their separation by PMC, showing a potential use for the analysis of petroleum hydrocarbons and lipid extracts.

5.3. Material and Methods

5.3.1. PDVF membrane chromatography (PMC)

The PMC procedure follows the same operational format as paper chromatography and TLC (e.g., Fuchs *et al.*, 2011; Cebolla *et al.*, 2015). Samples were loaded using a 10- μ L glass syringe (Hamilton, Reno, Nevada, USA) as spots of 1 μ L (~ 0.07 mm²) aligned 8 mm away from the bottom edge of a 40 $\times$ 50 mm PVDF

membrane (0.2 μm pore size, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The spotted samples were left to dry out for at least 1 h at room temperature, after which the chromatographic development was initiated by immersing ~ 5 mm of the bottom edge of the membrane in the developing solvent contained in a closed flat-bottomed glass chamber. The solvent migrates upwards and through the loaded sample spot by membrane capillarity. Solvents with different polarities were used for isocratic elution or for development using a stepwise sequence of eluants (gradient elution) migrating to different distances. The mobile phase front was followed until reaching different migration distances (8 to 34 mm) according to the condition tested, taking ~ 2 –25 min for development. The membranes were visualized with a UV-fluorescence imager (Gel Doc XR+ System, Bio-Rad Laboratories, Hercules, CA, USA) and dried for at least 1 h at room temperature before the immunoblotting (section 5.3.4). TLC runs were performed on activated (80 $^{\circ}\text{C}$ for 10 h) silica gel plates (40 $\times$ 80 mm polyester sheets coated with a 0.2 mm thick layer of unmodified silica gel with UV254 fluorescence indicator; Macherey-Nagel, Düren, Germany) under similar PMC conditions. The samples used corresponded to composed standards of phytol, squalene, and squalane (Sigma-Aldrich, St. Louis, Missouri, USA) diluted in hexane, crude oils and their fractions (section 5.3.2), and pigment extracts of lycopene, carotene, and chlorophyll. Lycopene and carotenoid fractions were extracted from freeze-dried tomatoes as previously described (Roh *et al.*, 2013), and chlorophyll extracts were obtained from *Amelanchier* sp. leaves by sequential extractions with methanol followed by dichloromethane.

5.3.2. Crude oils and SARA fractionation

The crude oils tested (ONTA Inc., Toronto, Ontario, Canada) having different characteristics and origins were labeled according to their density: 1, paraffinic oil from the Appalachian Basin, USA (827 mg/mL); 2, paraffinic oil from Louisiana, USA (839 mg/mL); 3, paraffinic oil from Qua Iboe, Nigeria (846 mg/mL); 4, paraffinic-naphthenic oil from Edmonton, Alberta, Canada (861 mg/mL); 5, naphthenic oil from Hoops, Texas, USA (869 mg/mL); 6, aromatic-intermediate oil from Saudi Arabia (870 mg/mL); 7, aromatic-asphaltic oil from Oriente, Ecuador (910 mg/mL); 8, aromatic-asphaltic oil from Merey, Venezuela (968 mg/mL). Oils were diluted in a solution of dichloromethane:methanol 3:1 before loading on PVDF membranes or TLC plates.

The Crude Oil #3 was separated into its saturate, aromatic, resin, and asphaltene (SARA) fractions as previously described (e.g., Gaspar *et al.*, 2012; Bissada *et al.*, 2016). The oil volatiles were first eliminated by vacuum centrifugation (5–6 Torr; Thermo Fisher Scientific) until reaching a constant sample weight (~60 min). The asphaltic fraction was then precipitated from 0.2 g of oil by adding n-heptane to obtain a 1:100 petroleum/heptane ratio, incubated for 48 h at room temperature, and separated from the maltene fraction by vacuum filtration using glass fiber filter. The maltenes were separated using sequential elutions in a gravity flow column (1.9 mm ID × 250 mm) packed with silica gel (Sigma-Aldrich, St. Louis, Missouri, USA) previously activated at 200 °C for 10 h. Saturates were eluted with heptane followed by extraction of the aromatic fraction by toluene and final elution of the resin fraction using a solution of dichloromethane:methanol 3:1. Excess solvent from each collected fraction was evaporated by vacuum centrifugation (5–6 Torr) and gravimetrically quantified. All

solvents were ACS reagent grade and previously dehydrated with molecular sieves (type 4A, 8–12 mesh beads; Thermo Fisher Scientific, Waltham, Massachusetts, USA).

5.3.3. Hybridoma culturing and antibodies

Anti-squalene antibodies were obtained from the hybridoma clone PTA6538-SQE14 (American Type Culture Collection; Matyas *et al.*, 2000), which secrete mouse antibodies of the isotype IgM. Cells were grown in hybridoma medium (GIBCO; Formula number 06-5012EL supplemented with 3% characterized fetal calf serum) initially in T75 flasks, further expanded to T250 flasks when confluent, and then transferred to 3 L spin flasks that were aerated for 3-5 days of growth after reaching a concentration of $\sim 10^6$ cell/mL. The cell suspension was then centrifuged at 2,400 r.p.m. for 15 minutes at 4 °C in 500 mL conical centrifuge tubes. The obtained culture supernatant was filtered (0.2 μ m), NaN₃ was added to a final concentration of 0.02%, and aliquots were stored at -20 °C until they were thawed and used as the primary antibody. Anti-CD75 IgM antibodies (clone LN-1) that react with the human surface lymphocyte antigen CD75 were similarly obtained from culture supernatants and used as isotype-control antibodies. Antibody clones BAP-13 and 10C10 against pyrene were obtained from Sigma-Aldrich (St. Louis, Missouri, USA) and Santa Cruz Biotechnology (Dallas, Texas, USA), respectively.

5.3.4. Immunoblotting detection

The chromatographed membranes were incubated under orbital shaking at 25 °C for one hour with 2% bovine serum albumin (BSA; Fraction V from Roche Diagnostics

GmbH, Mannheim, Germany) dissolved in TBS solution (Tris-buffered saline, 20 mM Tris Base pH 7.6, 150 mM NaCl). Membranes were subsequently rinsed with TBS and incubated with orbital shaking at 25 °C for one hour using either culture supernatant of the hybridoma clone SQE14 (PTA6538), culture supernatant of the hybridoma clone LN-1, or a solution of anti-pyrene antibodies diluted in TBS (dilutions 1:500, 1:1000, 1:4000, and 1:8000 were tested). Membranes were quickly rinsed and washed three times for 5 min with TBS under orbital shaking at 25 °C, after which they were incubated for one hour with orbital shaking at 25 °C with goat anti-mouse IgM horseradish peroxidase-conjugated antibodies (Millipore, Burlington, Massachusetts, USA) diluted 1:4,000 in TBS. The membranes were again rinsed and washed three times for 5 min with TBS under orbital shaking at 25 °C. The binding signal was chromogenically revealed after adding a substrate (CN/DAB; 4-Chloro-1-naphthol/3,3'-Diaminobenzidine) according to the manufacturer's instructions (PierceTM CN/DAB substrate kit, Thermo Fisher Scientific).

5.4. Results

5.4.1. Hydrocarbon separation using PMC

PMC can be used to separate hydrocarbons, some of which can be detected by immunoblotting. Immunoblotting characterization of the anti-squalene antibody clone SQE14 developed by Matyas *et al.* (2000) showed that the antibody not only binds its cognate antigen—squalene—but also cross-reacts with other acyclic isoprenoids, such as phytol and squalane (Medina Ferrer *et al.*, 2018). Antibody binding to acyclic isoprenoids is visualized by dot immunoblotting on PVDF membranes (**Figure 5.1A**). Binding to

other hydrocarbons, such as alkanes and cyclic isoprenoids was negligible, suggesting that this anti-squalene antibody preferentially binds acyclic isoprenoid moieties (Medina Ferrer *et al.*, 2018). When crude oil samples are loaded on PVDF membranes for dot immunoblotting, a halo is visualized by UV-fluorescence only (**Figure 5.1B**), indicating differential mobility of fluorescent hydrocarbons during sample loading, and hence differential partition properties that could be exploited for chromatographic separations of hydrocarbons on PVDF membranes.

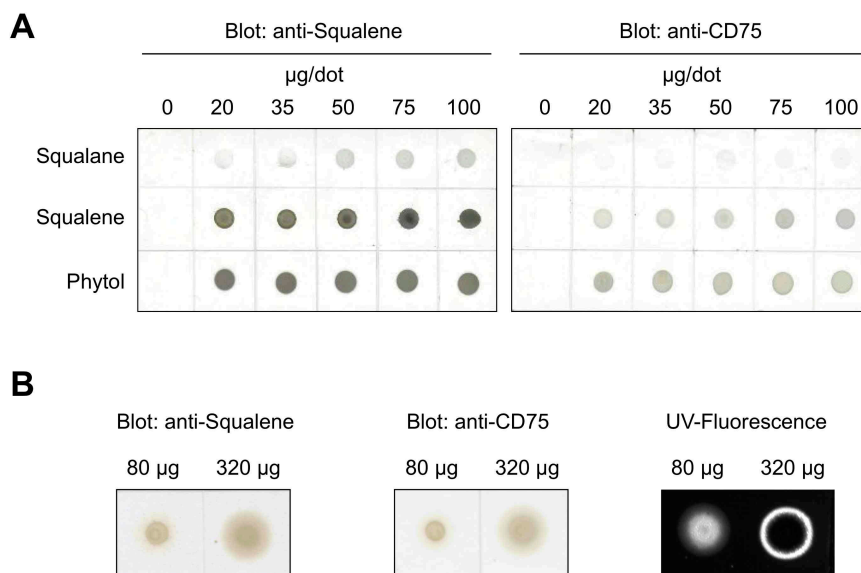


Figure 5.1. Dot immunoblotting on PVDF membranes. (A) Standard solutions of phytol, squalene, and squalane at different concentrations loaded on PVDF membranes and visualized by immunoblotting with either anti-squalene antibodies or anti-CD75 (isotype control) antibodies. (B) Crude Oil #3 visualized by dot immunoblotting or by UV-fluorescence on PVDF membranes.

Mobile phase alternatives to separating acyclic isoprenoids by PMC were evaluated using stepwise additions of solvents of increasing polarity to the same chromatographic run. The migration pattern of standard solutions of phytol, squalene, and squalane detected by immunoblotting shows that their separation occurs in the polarity range between acetone and methanol (**Figure 5.2A**). Phytol, squalene, and squalane migrate along acetone, whereas methanol promotes the migration of polar isoprenoids (phytol and, to a lesser extent, squalene) only (**Figure 5.2B**). A 2:1 methanol:acetone ratio better resolves among phytol, squalene, and squalane (**Figure 5.2D**), and an optimized stepwise gradient elution can also be used to separate them according to their polarity (**Figure 5.2C**). The obtained relative mobility (R_f) follows the polarity of the separated compounds (phytol > squalene > squalane), indicating a partitioning mechanism between the mobile (solvent) and the stationary phase (PVDF membrane) similar to a reversed phase chromatography.

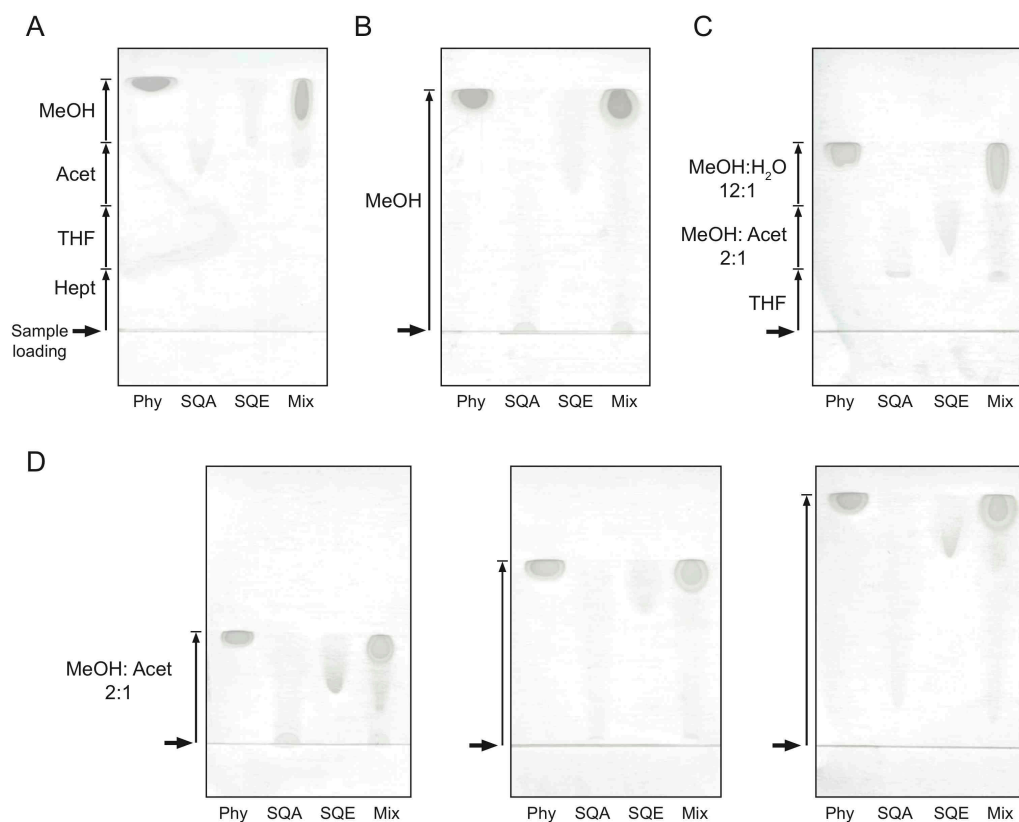


Figure 5.2. PMC immunoblotting of acyclic isoprenoids. Standards of phytol (Phy), squalane (SQA), squalene (SQE), and a mixture of them (Mix) were separated by PMC (100 nmoles/dot for each loaded standard) and visualized by immunoblotting using anti-squalene antibodies. (A) Stepwise gradient using sequential developments with heptane (Hept), tetrahydrofurane (THF), acetone (Acet), and methanol (MeOH) in the same direction. (B) Isocratic elution using methanol. (C) Stepwise development with THF, methanol:acetone 2:1, and methanol:water 12:1. (D) Development sequence from 10 to 30 mm length using 2:1 methanol:acetone. Vertical arrows indicate the progression of the solvent fronts. Horizontal arrows indicate the sample loading site.

5.4.2. PMC separation of petroleum hydrocarbons

PMC of crude oils from different origins using separating solvent conditions to distinguish among phytol, squalene, and squalane (e.g., **Figure 5.2**) show the presence of hydrocarbons having similar relative mobilities than phytol and squalene, and that are detected by immunoblotting using anti-squalene antibodies (**Figure 5.3**). In addition, the conditions tested seem to separate at least three hydrocarbon types, one at higher elution distances (yellow compounds) and probably corresponding mostly to saturated hydrocarbons that are reactive with the anti-squalene antibody, a second that does not elute (**Figure 5.3A**) or that elutes only with hydrophobic solvents (dark brown hydrocarbons; **Figure 5.3B**), and a third that does not elute and remains in the loading spot (black hydrocarbons), probably corresponding to resin and asphaltic fractions. Although the chromatography of oils in **Figure 5.3** separates some hydrocarbons, most hydrocarbon group types migrate poorly on the PVDF membrane and remain unresolved under these conditions.

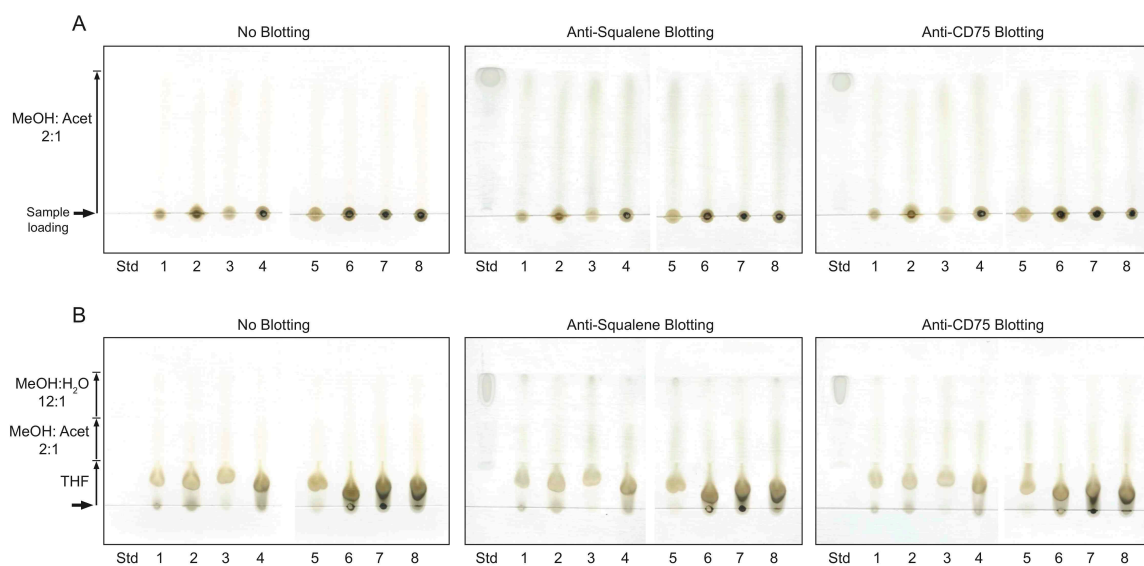


Figure 5.3. PMC immunoblotting of crude oil samples. Crude oils (1–8; 320 µg/dot) and a composed standard of phytol, squalene and squalane (Std) were analyzed by PMC immunoblotting using anti-squalene antibodies and an isotype control antibody (anti-CD75). (A) Isocratic elution with 2:1 methanol:acetone. (B) Stepwise development gradient with tetrahydrofurane, 2:1 methanol:acetone, and 12:1 methanol:water. Vertical arrows indicate the progression of the solvent fronts. Horizontal arrows indicate the sample loading site.

To identify potential elution gradients resolving particular hydrocarbon fractions by PMC, a paraffinic crude oil sample (Crude Oil #3) was separated into saturate, aromatic, resin, and asphaltic (SARA) fractions (**Figure 5.4A**). The fractions were assayed by PMC immunoblotting under different elution conditions (**Figure 5.4B, C**). Separation patterns of saturate, aromatic, and resin fractions produced by a stepwise gradient of increasing polarity (**Figure 5.4B**), or by using selective focusing fronts of decreasing distance applied sequentially in the same migration direction (**Figure 5.4C**), show distinctive patterns that seem reflected in the whole crude oil and that better resolve aromatic and resin components of the sample.

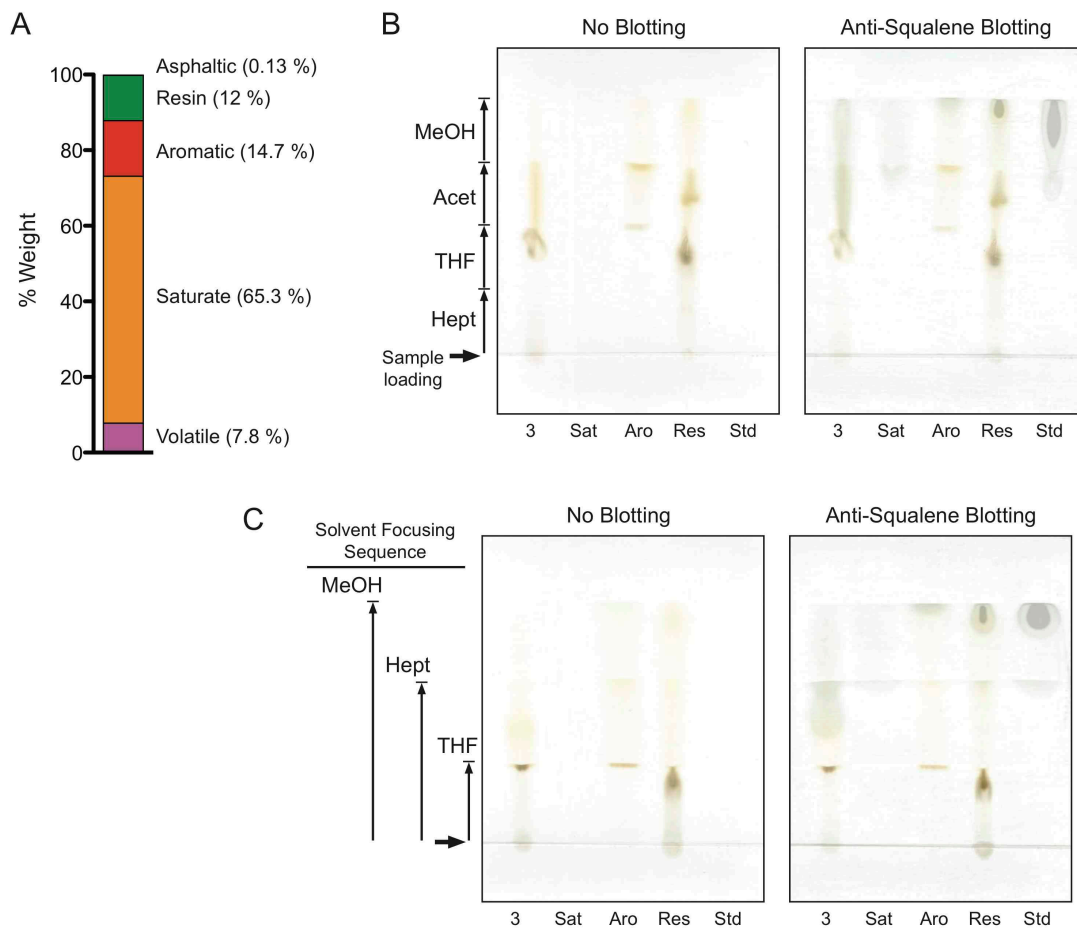


Figure 5.4. PMC immunoblotting of SARA fractions. Saturate, aromatic, resin, and asphaltic (SARA) fractions by percentage weight of the crude oil #3 (A) and their separation by PMC using a stepwise gradient of heptane (Hept), tetrahydrofuran (THF), acetone (Acet), and methanol (MeOH; B) or sequential focusing fronts of decreasing migration distances with methanol, heptane, and tetrahydrofuran (C). A composed standard (Std) of phytol, squalene, and squalane (100 nmoles/dot of each hydrocarbon) is also shown. Vertical arrows indicate the progression of the solvent fronts. Horizontal arrows indicate the sample loading site.

When using the solvent systems of **Figure 5.4**, each oil sample shows distinctive patterns that can be associated with their oil type, particularly to their proportions of resin and asphaltic fractions, which have lower mobilities compared to saturate and aromatic fractions (**Figure 5.5**). Although the PMC migration patterns of oils show some differences when compared to the migration patterns on TLC silica plates using the same developing systems (**Figure 5.5C**), both chromatographic strategies resolve certain hydrocarbon group types. Saturate and aromatic hydrocarbons mostly distribute at higher migration distances, while resins have lower mobilities and asphaltics remain at the loading site. In addition, immunodetection using anti-squalene antibodies on developed PMC membranes shows the presence of immunoreactive hydrocarbons in crude oils with similar mobilities as phytol, and squalene/squalane.

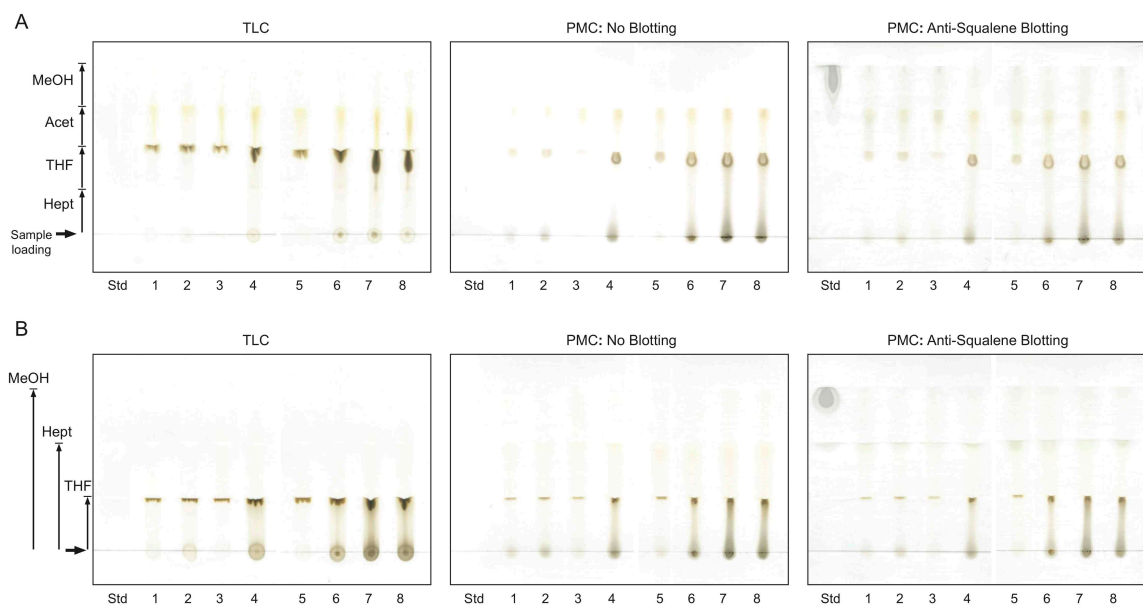


Figure 5.5. TLC and PMC immunoblotting of petroleum hydrocarbons using different developing systems. Crude oils (1–8; 80 $\mu\text{g}/\text{dot}$) and a composed standard of

phytol, squalene and squalane (Std; 100 nmoles/dot of each hydrocarbon) assayed by PMC immunoblotting or by TLC using a stepwise gradient of heptane (Hept), tetrahydrofurane (THF), acetone (Acet), and methanol (MeOH; A) or consecutive focusing fronts of decreasing migration distances with methanol, heptane, and tetrahydrofuran (B). Intrinsic color and immunoblotting using anti-squalene antibodies after PMC is indicated. Vertical arrows show the progression of the solvent fronts. Horizontal arrows indicate the sample loading position.

5.4.3. Additional applications of squalene PMC immunoblotting

By using anti-squalene antibodies, it is possible to study squalene in natural samples and its physical properties by means of a simple PMC assay. For example, it has been reported that squalene reacts with atmospheric ozone, degrading into several reactive intermediaries and species of different polarities (Wells *et al.*, 2008; Fu *et al.*, 2013; Fooshee *et al.*, 2015). Separation of squalene by PMC shows its degradation kinetics and the development of oxidation products that are also immunoreactive under the blotting conditions (**Figure 5.6**). Most of the squalene ozonolysis, visualized after one day of squalene exposure on the PVDF membrane, which is seemingly complete in the course of two weeks (**Figure 5.6A**), may be attributed to UV-catalyzed ozone formation from atmospheric oxygen, as incubations in the dark seem to delay the extent of squalene degradation (**Figure 5.6B**) and incubations in an atmosphere devoid of O₂ completely preserved squalene over the span of two weeks (**Figure 5.6C**).

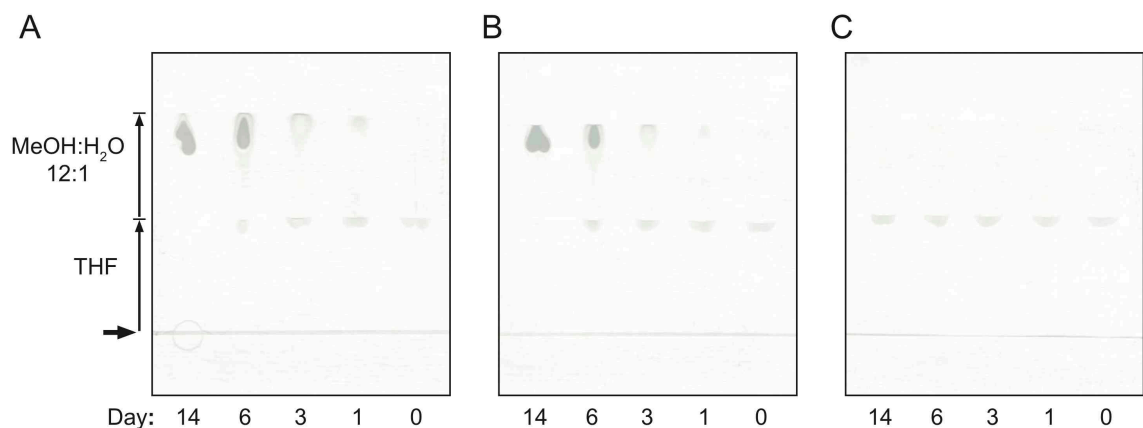


Figure 5.6. Squalene degradation kinetics. PMC immunoblotting of squalene spotted on PVDF membranes (100 nmoles) and resolved by a stepwise gradient of tetrahydrofuran (THF) and methanol:water 12:1 (2) after 14–0 days of membrane exposure to atmospheric conditions with natural light (A), in the dark (B), or under an anoxic atmosphere (C).

Another example of lipid analysis using anti-squalene antibodies can be applied to characterize squalene, phytol, and other related acyclic isoprenoids present in plant extracts. Pigment extracts are frequently studied by TLC (Fuchs *et al.*, 2011), and PMC can have similar applications for pigment analysis, where the PVDF membrane could additionally be assayed by effect-directed analysis or immunoblotting. **Figure 5.7** shows the PMC separation and detection by anti-squalene immunoblotting of chlorophyll extracts from *Amelanchier* sp. leaves, and lycopene and carotenoids extracts from tomatoes. Developing gradients of increasing polarity show the separation of diverse carotenoid and chlorophyll species (**Figure 5.7A, B**), which can be optimized by applying sequential focusing fronts of decreasing migration distances (**Figure 5.7C**).

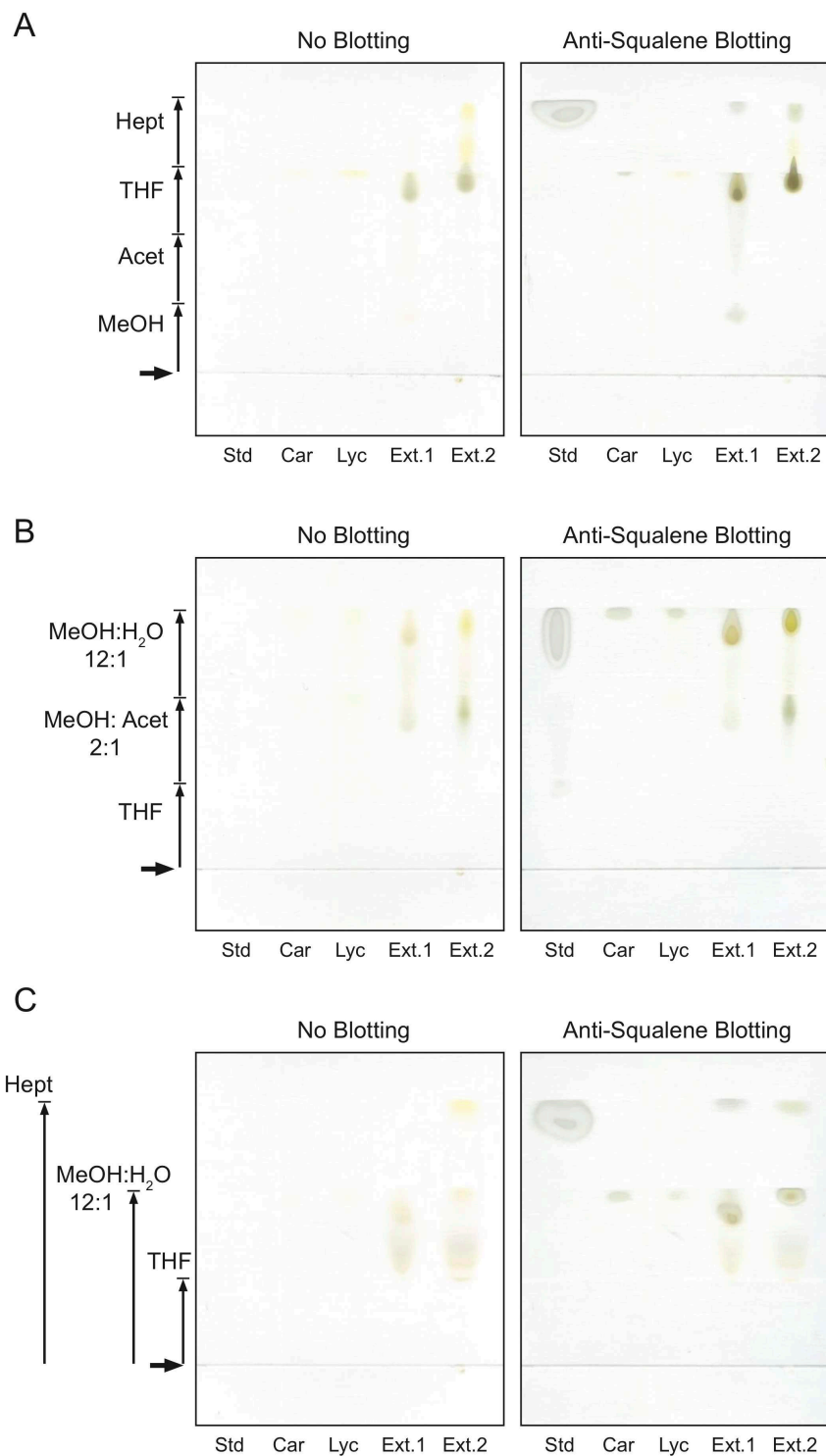


Figure 5.7. PCM immunoblotting of plant extracts. (A) PMC separation using a stepwise gradient of decreasing polarity with methanol (1), acetone (2), tetrahydrofuran

(3), and heptane (4). (B) PMC development with a stepwise gradient of increasing solvent polarity using tetrahydrofuran (1), 2:1 methanol:acetone (2), and 12:1 methanol:water. (C) Sequential focusing fronts of decreasing distance with heptane (1), 12:1 methanol:water (2), and tetrahydrofuran (3) applied in the same migration direction. The spotted samples correspond to a composed standard of phytol, squalene, and squalane (Std; 100 nmoles/dot of each hydrocarbon), methanol extracts (Ext.1) and dichloromethane extracts (Ext.2) from *Amelanchier* sp. leaves, and carotenoid (Car) and lycopene (Lyc) extracts from tomatoes.

5.5. Discussion

5.5.1. PMC performance

PVDF membranes have already proved useful as immobilizing media for lipid purification (Taki *et al.*, 1994b), direct MS analysis of lipids (Guittard *et al.*, 1999; Goto-Inoue *et al.*, 2008), and have been widely used for immunodetection of proteins (e.g., Komatsu, 2015). In addition, PVDF membranes are resistant to several solvents, have high affinity and binding capacity, and its surface can be functionalized to gain different physical properties (Kang & Cao, 2014), such as a broad range of polarities, conferring great advantages when used as substrate for planar chromatography. We showed that different developing solvents redistribute lipids on PVDF membranes according to their relative solubilities, potentially resolving them by PMC. The appropriate conditions to resolve specific compounds are empirical and should be tested based on defined objectives. For example, PMC separation of the acyclic isoprenoids phytol, squalene, and squalane (**Figure 5.2**) show effective resolution between phytol and squalane using a

broad range of solvents, likely because of their different relative polarities. Squalene, however, has a polarity that is relatively-close to squalane, which makes their separation difficult (e.g., no narrow signals) under the studied conditions (**Figure 5.2**). The resolving power of PMC may also be affected by matrix effects, evidenced by signal-broadening of phytol and squalene when chromatographed as a sample mixture (**Figure 5.2**), a phenomenon known in TLC and other chromatographic approaches (Cebolla *et al.*, 2015). Increasing of the migration distance can potentially improve the separation, however, it also increases the diffusion of the analyte on the membrane, affecting the signal resolution (**Figure 5.2D**).

Solvent systems that separate specific hydrocarbons can be used to correlate their migration distance to complex samples, such as the crude oil migration patterns showing the presence of hydrocarbons with similar *R_f* to phytol and squalene (**Figure 5.3**). Different developing solvents and gradient systems increase the resolution with respect to a larger spectrum of hydrocarbons found in crude oils (**Figure 5.3, 5.5**), a strategy that has been used to characterize hydrocarbon group types by TLC (e.g., Cebolla *et al.*, 2000; Matt *et al.*, 2002, 2003; Wang *et al.*, 2010; Jarne *et al.*, 2011; Vorapalawut *et al.*, 2012; Nowak *et al.*, 2017). Comparison of the petroleum migration patterns between PMC and TLC shows that PVDF membranes have the potential to separate hydrocarbon group types based on their polarities, obtaining a comparable separation to silica plates (**Figure 5.5**).

5.5.2. Advantages and disadvantages of PMC

As a planar chromatography assay, PMC shares many of the advantages and disadvantages of TLC compared to column-based chromatographic methods, such as liquid and gas chromatography (reviewed in Cebolla *et al.*, 2003, 2015). For the analysis of petroleum hydrocarbons, for example, it is particularly useful that previous separation of asphaltic compounds from the samples (deasphalting)—which will not elute or volatilize in a column, possibly damaging it—is not required in PMC. TLC plates and PVDF membranes are disposable and allow most sample analytes to be analyzed, even those that will not elute and permanently bind to the stationary phase. For example, **Figure 5.5** shows a fraction of hydrocarbons (likely asphaltenes) that did not elute and remained in the sample application site, but can still be visualized on PVDF membranes and evaluated by downstream analysis, in this case by immunoblotting and by their native color. Planar chromatography, however, may be inefficient for studying highly volatile analytes that can evaporate from the stationary phase under the running conditions (usually atmosphere pressure and room temperature). In addition, PMC requires simple instrumentation, uses low quantities of solvents, is cheaper than column chromatographic methods, and can serve for preparative separations, showing increased recovery yields when analytes are eluted from PDVF membranes compared to silica plates (Taki *et al.*, 1994b). Planar chromatographic methods also have the potential to separate several samples simultaneously in a short period of time, which can be later analyzed under the same conditions, an advantage that is particularly useful when comparing different samples by immunodetection.

Compared to TLC plates, using PVDF membranes to separate hydrocarbons has an improved potential for downstream analyses, such as immunostaining of phytol,

squalene, and squalane using monoclonal antibodies (Matyas *et al.*, 2000, 2002; Medina Ferrer *et al.*, 2018). Although immunoblotting can be directly applied to TLC plates (Magnani *et al.*, 1980; Schweppe *et al.*, 2010), its overall performance is increased on PVDF membranes in terms of sensitivity and handling of the operational procedure (Taki *et al.*, 1994a, 1994b). In addition, it has been shown that certain compounds, such as glycosphingolipids (Goto-Inoue *et al.*, 2008), are more stable on PVDF membranes compared to silica plates, which is relevant during the immunoblotting procedure. Although the anti-squalene antibodies developed by Matyas *et al.* (2000) have shown binding reactivity to some acyclic isoprenoids, the extent to which these antibodies potentially bind other hydrocarbons (either as a consequence of structural similarity or via non-specific interactions) have been not fully tested. PMC immunoblotting of complex samples can help evaluate the specificity of hydrocarbon-binding antibodies. For example, this anti-squalene antibody cross-reacts toward squalane (Matyas *et al.*, 2000), and petroleum samples containing squalane have shown positive immunoreaction signals via dot immunoblotting, presumably via squalane binding (Medina Ferrer *et al.*, 2018). The PMC immunoblotting of crude oils in **Figure 5.3** using anti-squalene antibodies show, however, that most binding signals have similar migration patterns to those of phytol and squalene, indicating that the antibody binds mostly to these acyclic isoprenoids in the crude oil samples, or to hydrocarbon group types that have similar polarities (and thus similar migration distances) than phytol and squalene. The binding signal against compounds of broad polarities from complex natural hydrocarbon samples indicates that the anti-squalene antibody Clone 14 (Matyas *et al.*, 2000) cross-reacts with other hydrocarbons, possibly acyclic isoprenoids (Medina Ferrer *et al.*, 2018).

Immunodetection with anti-squalene antibodies, however, generates a background signal that complicates the identification of specific binding reactivity in complex hydrocarbon samples (e.g., Matyas *et al.*, 2000, 2002; Medina Ferrer *et al.*, 2018). The incorporation of control assays to subtract the background signal, such as blotting with an isotype control antibody (**Figure 5.1, 5.3**), aids in identifying specific antibody binding and can be used to semi-quantify the antibody response (e.g., **Figure 5.3**; Medina Ferrer *et al.*, 2018). Analyte quantification in planar chromatography assays, however, has a limited calibration range compared to column chromatographic methods, because the signal response is usually obtained by scanning densitometry (Ebel & Kang, 1990; Ebel, 1996) that does not follow the Lambert-Beer law as opposed to spectrophotometric detection in liquid chromatography (Ferenczi-Fodor *et al.*, 2010; Renger *et al.*, 2011), for example. Another disadvantage of PMC is that efficient separations occur before reaching a certain migration distance (~30–40 mm), after which the mobile phase dramatically slows down and increases analyte diffusion on the membrane (e.g., **Figure 5.2D**), a known phenomenon in planar chromatography (Guiochon *et al.*, 1979; Cebolla *et al.*, 2015). The capillary-driven solvent front is faster in silica layers and can rapidly reach over 70 mm, whereas a similar solvent front in PVDF membrane would reach half of that distance in about the same time, leaving less theoretical plates for analyte separation. In addition, TLC spotting and elution can be automated with the possibility to run reproducible multiple development separations (e.g., Cebolla *et al.*, 2011), which could increase analyte resolution in complex samples. Given its planar chromatography format, however, PMC may be adapted to fit automated multiple development systems in the future.

5.5.3. Potential applications of PMC

TLC has been used to quantitatively determine hydrocarbon group types and to obtain petroleum fingerprints using multiple detection techniques (Cebolla *et al.*, 2003; Matt *et al.*, 2003; Wang *et al.*, 2010). Although SARA fractionation on silica gel columns provides a non-specific quantitative characterization of hydrocarbon group types from petroleum samples (e.g., **Figure 5.3A**), the procedure is time-consuming, labor-intensive, and requires large amount of sample and solvents, making it impractical when analysis of several samples is required (Gaspar *et al.*, 2012; Vorapalawut *et al.*, 2012; Bissada *et al.*, 2016). Therefore, analytical alternatives using TLC and fluorescence scanning densitometry, and TLC coupled with flame ionization detection (TLC-FID) have been developed to estimate hydrocarbon group types (Matt *et al.*, 2002, 2003; Cebolla *et al.*, 2003; Wang *et al.*, 2010). Similarly, different gradient elution systems on PMC immunoblotting show that the studied oils have distinguishing patterns that can be associated with their hydrocarbon group types, particularly to their proportions of resin and asphaltic fractions, which have lower mobilities with respect to saturate and aromatic fractions (**Figure 5.4, 5.5**). Although not evaluated in this study, sample derivatization with fluorescence-inducing agents, such as berberine (Cossío *et al.*, 2000), to visualize saturated hydrocarbons lacking UV fluorescence (e.g., alkanes and naphthenes) could be used to quantify the relative proportions of hydrocarbon types by PMC, similarly to the estimation of hydrocarbon type groups by TLC (Cebolla *et al.*, 2003; Matt *et al.*, 2003).

Profiling analysis can also be used to characterize other complex natural samples, such as botanical extracts. Two sequential *Amelanchier* leaf extracts analyzed by PMC

immunoblotting in **Figure 5.7** show different patterns associated to the analyte solubility from each extract. Similarly, particular PMC profiles may help identify temporal changes or differences among species that could indicate environmental stress, such as soil and water contamination. PMC immunoblotting could then be applied to follow chlorophyll degradation by immunodetection of phytol with anti-squalene antibodies, in addition to targeting other plant acyclic isoprenoids that potentially bind anti-squalene antibodies, such as lycopene and carotenoids. Squalene is also a main component of leaf surface lipids that protects plants from atmospheric oxidizers and acts as an important ozone sink (Wells *et al.*, 2008; Fu *et al.*, 2013; Fooshee *et al.*, 2015). Squalene ozonolysis produces species with O-containing functional groups (such as carbonyl, carboxyl, peroxy, hydroxyl, and ether groups), all of which are more polar than squalene and therefore have a higher chromatographic migration when visualized with anti-squalene antibodies (**Figure 5.6**). It is possible that most squalene oxidation products bind anti-squalene antibodies because they preserve acyclic isoprenoid moieties, showing atmospheric squalene degradation through time on PVDF membranes (**Figure 5.6**). PMC immunoblotting analysis could be used to evaluate potential factors affecting squalene degradation by ozone (or other agents), and to characterize the wide variety of produced ozonides.

In addition to immunodetection, PDVF membranes are suitable immobilizing-matrices for several types of effect-directed analysis, where separated extracts can be screened for compounds having specific biological activities, such as inhibitory effects on enzymes, antibacterial and fungicidal properties, and toxic activity, among others reviewed in Weiss *et al.* (2017). Analyte identification could then be evaluated using MS

methods, such as SIMS and MALDI-TOF, which can be directly applied on PVDF membranes (Taki *et al.*, 1995; Guittard *et al.*, 1999). Compared to direct MS analysis on TLC plates, MS detection on PVDF membranes show several advantages, such as improved sensitivity and resolution, in addition to facilitate the handling of the overall procedure and avoiding possible contamination of the MS instrument with silica (Goto-Inoue *et al.*, 2008, 2009; Valdes-Gonzalez *et al.*, 2011).

Another possible applications of PMC include exploiting the binding properties of other antibodies that target lipids (e.g., Alving & Swartz, 1991; Tanaka *et al.*, 1997; Katagiri *et al.*, 2011; Schellenberger *et al.*, 2012) or that bind environmentally relevant hydrocarbons. For example, some antibodies developed against pyrene have shown binding to a broad spectrum of polycyclic aromatic hydrocarbons (PAH), which are known carcinogenic pollutants (Li *et al.*, 1999, 2016a; Spier *et al.*, 2009; Stec *et al.*, 2018). Different immunodetection strategies have been studied to potentially monitor dissolved PAH in drinking and environmental waters using rapid and cheap assays (Zhang *et al.*, 2010; Meng *et al.*, 2015; Li *et al.*, 2016b; Sun *et al.*, 2017). PMC separates some PAH; however, we have not yet obtained an immunoreactive signal by immunoblotting on PVDF membranes using two commercial anti-PAH antibodies. Distinct PAHs have different carcinogenic potential, thus a simple detection by PMC immunoblotting may help identify specific PAH from a complex mixture, which is relevant when assessing the potential risk of contaminated water sources.

5.6. Conclusion

PVDF membranes share several advantages of silica gel and other adsorbents used in TLC applications, such as their resistance to a variety of solvents, high affinity to lipids and hydrocarbons, and the possibility to be applied in a planar chromatographic assay. Consequently, similar TLC applications and advantages are shared by PMC, such as the possibility to study all non-volatile analytes in a sample, apply different detection techniques and effect-directed analysis to the same chromatographic run, reduced cost, high sample throughput, and overall simplicity compared to column chromatographic methods (Cebolla *et al.*, 2015; Weiss *et al.*, 2017). Some applications are improved on PVDF membranes compared to silica plates, such as preparative purification of lipids (Taki *et al.*, 1994b), MS analysis (Goto-Inoue *et al.*, 2008), and immunological detection (Taki *et al.*, 1994a). We showed that it is possible to separate hydrocarbons and lipids from complex samples by PMC, obtaining a separation comparable to TLC. PMC is particularly useful for analyzing samples that may not efficiently transfer to PVDF membranes by Far-Eastern Blot (Taki & Ishikawa, 1997; Taki, 2015), such as petroleum hydrocarbons, which can be separated according to their group types by PMC, similarly to hydrocarbon separations by TLC (Cebolla *et al.*, 2000; Matt *et al.*, 2003). Lipid and hydrocarbon analysis by TLC is, however, a well-developed technique that has been used for chromatographic separations for over 50 years (Sherma, 1977). We believe that PMC may complement lipid and hydrocarbon separations from complex samples, especially when immunoblotting or other detection methods that are improved in PVDF membranes are used. Future evaluation of the performance of different mobile phases, the possibility for automating sample spotting and elution, and the continued analysis of complex samples from diverse origins will expand the full potential of PMC separations.

5.7. Acknowledgments

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Chapter 6

Hydrocarbon immunodetection in fish fossils from the Eocene Green River Formation

The contents of this chapter contain preliminary data in preparation for submission, and have been developed by Fernando Medina Ferrer, Cécile Bidaud, and Jake V. Bailey.

6.1. Synopsis

Sedimentary hydrocarbons preserved in body fossil remains can provide information about the biological lineage of particular fossils, as well as the possible origins of specific biomarkers found in sedimentary strata. Hydrocarbon biomarkers associated with particular fossils may also potentially reveal the mechanisms of organic matter preservation. However, associating specific biomarkers with the depositional context of fossils proves challenging using current biomarker analysis techniques. Here, we evaluate the possibility of using monoclonal antibodies that bind squalane to directly visualize hydrocarbons preserved in the fossiliferous structure of fish fossils from the Green River Formation, WY, USA. These antibodies not only bind squalane from extracted hydrocarbons of slab layers containing *Knightia* fossils, but also bind other unknown soluble hydrocarbons on the basis of dot immunoblotting assays and the binding patterns after chromatographic elutions on PVDF membranes. Antibody binding assays performed directly on fish fossil structures show weak signals that likely correspond to background intensity, with the possible exception of one fossil showing intense binding signal associated with sediments confined to the fish vertebrae. If the detected hydrocarbons are indeed preferentially preserved in specific fish fossil structures, then the evaluation of fossil remains using antibodies may provide taphonomic information that could help identify potential mechanisms of organic matter preservation.

6.2. Introduction

After organismal decay and under specific environmental conditions, certain biological lipids may evade decomposer degradation and will remain in the sediments (Peters *et al.*, 2005). Following subsequent burial and diagenesis, the functional groups of sedimentary lipids modify, but their overall structure may still preserve basic information that can be used to trace back their origin. These resilient lipids—or biomarkers—preserved in the geological record are specific to certain biological lineages and therefore can provide molecular information about the organisms that lived in a particular environment and time (Brocks & Summons, 2003; Brocks & Pearson, 2005; Peters *et al.*, 2005; Grice & Eiserbeck, 2014). Canonical biomarker analysis relies on mass spectrometric methods, typically GC-MS, requiring biomarker extraction from grams of rocks, some of which preserve only trace extractable biomarkers that can potentially be confused with non-syngenetic contaminants (Brocks, 2011; French *et al.*, 2015). An alternative biomarker analysis explores the direct visualization of sedimentary hydrocarbons from the rocks, without having to extract them, by using antibodies (Medina Ferrer *et al.*, 2018). Antibodies that bind the biomarker squalane (Matyas *et al.*, 2000) have been used to bind biomarkers that correlate with depositional layers of organic matter from rocks of the Green River Formation (GRF; Medina Ferrer *et al.*, 2018). We show here that these antibodies may be used to directly visualize squalene and other biomarkers associated with the sedimentary structure of GRF fish fossils.

The GRF is a group of three Eocene sedimentary basins covering regions of Utah, Wyoming, and Colorado in the USA. Sections of the GRF, such as Fossil Lake in southwest Wyoming, a well-known Lagerstätte deposit, contain layers showing a variety

of exceptionally preserved fossils (Dayvault & Bersch, 1983; Buchheim, 1994), in addition to sedimentary hydrocarbons, such as squalane (Medina Ferrer *et al.*, 2018). The fossil source of sedimentary squalane, thought to originate from deposition and diagenesis of the lipid squalane, may be diverse. Squalene occurs in organisms from all three domains of life, where it acts as a molecular precursor for the biosynthesis of steroids, terpenoids, and carotenoids (Kannenbergh & Poralla, 1999; Peters *et al.*, 2005); however, these precursors are generally transient, and only a handful of organisms bioaccumulate squalene. One possible source of concentrated squalene-bearing biomass in the GRF is fish debris. Green River mudstones are well known for their extremely common preservation of fish fossils, particularly *Knightia* sp., a small fish of the family Clupeidae—the fish family comprising sardines and herrings among others. Squalene can be a substantial constituent of fish oils, such as herring oil (Gershbein & Singh, 1969), and *Knightia* tissues may well have contained concentrated squalene (Ferber & Wells, 1995).

One alternative to determine whether GRF *Knightia* fossils are associated with squalane, contributing to a possible source of these biomarkers, is to directly visualize squalane in fish fossils using antibodies. Unfortunately, visualizing biomarkers with fluorophore-conjugated antibodies, as previously proposed (Medina Ferrer *et al.*, 2018), is complicated by the autofluorescence of abundant carbonate minerals when imaging GRF rocks, which dramatically increases the background of the analysis. We adapted the fluorophore-based immunological detection of squalane by using a chemiluminescence reporter system instead, which can be visualized by a CCD camera in the dark. The chemiluminescence detection allows the observation of macroscopic signals that can be

associated with the exposed fish fossil and its sedimentary context. The direct visualization of hydrocarbons preserved in the body of fish fossils may prove useful for determining the potential provenance of GRF squalane biomarkers, and the potential mechanisms of organic matter preservation in GRF fish fossils.

6.3. Material and Methods

6.3.1. GRF fish fossils

Fish fossil samples were collected from mass mortality beds of two quarries at Fossil Basin, near Kemmerer, Wyoming, USA (Lat. 41.798; Lon. -110.732). Most fish fossils in the quarries correspond to *Knightia* and *Diplomystus* spp., and the collected specimens were likely *Knightia* based on its abundance, although the species identification was not evaluated. The depositional layers were carefully opened to look for unweathered fish specimens that were wrapped in pre-combusted aluminum foil for transport and storage. Rock samples were sized down and the fish structure exposed (if covered) with a pre-combusted (24 h at 250 °C) chisel.

6.3.2. Immunoblotting and PVDF membrane chromatography

One microliter of standard solutions (squalene, squalene, or phytol; Sigma-Aldrich, St. Louis, Missouri, USA) and hydrocarbon extracts from rocks containing fossil remains (section 6.3.3, Fish Sample 5) were spotted with a 10- μ L glass syringe (Hamilton, Reno, Nevada, USA) on polyvinylidene difluoride (PVDF) membranes (0.2 μ m pore size, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and left to dry at room temperature for ~8 h. The membranes were visualized by dot immunoblotting

(Medina Ferrer *et al.*, 2018), or by directly separating the samples using planar chromatographic development on the PVDF membrane surface (PVDF membrane chromatography; PMC; Chapter 5) before the immunoblotting. The membranes were then incubated for one hour under orbital shaking at 25 °C with a solution of 2% bovine serum albumin (BSA; Fraction V from Roche Diagnostics GmbH, Mannheim, Germany) dissolved in TBS (Tris-buffered saline, 20 mM Tris Base pH 7.6, 150 mM NaCl). The membranes were rinsed with TBS before incubation for one hour under orbital shaking at 25 °C using either culture supernatant of the hybridoma clone SQE14 against squalene (PTA6538, American Type Culture Collection; Matyas *et al.*, 2000) or culture supernatant of the hybridoma clone LN-1 against CD75, which were obtained as previously described (Medina Ferrer *et al.*, 2018). Membranes were subsequently rinsed and washed three times for 5 min each with TBS at 25 °C under orbital shaking, following an incubation with secondary goat anti-mouse IgG+IgM horseradish peroxidase-conjugated antibodies (Abcam, Cambridge, UK) diluted 1:50,000 in TBS for one hour with orbital shaking at 25 °C. The membranes were then rinsed and washed three times for 5 min with TBS under orbital shaking at 25 °C before visualizing antibody binding signals by adding a chemiluminescence substrate (SuperSignal™ West Femto substrate, Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions, and imaged in a dark chamber (Gel Doc XR+ System, Bio-Rad Laboratories, Hercules, CA, USA) during 20–99 sec of exposure time.

6.3.3. Biomarker immunodetection in fish fossils

Slab layers containing fish fossil remains were either washed twice with 0.2% HCl (30 min at 25°C with orbital shaking) and rinsed three times with Milli-Q water (Fishe Samples 1–4), or sequentially washed with tetrahydrofuran first (80 min at 25°C with orbital shaking), then with 0.2% HCl (80 min at 25°C with orbital shaking) followed by three five-minute washings with Milli-Q water and a final wash with tetrahydrofuran for 80 min at 25°C with orbital shaking (Fish Samples 5 and 6). The HCl washing was used to etch and expose the fish organic matter from its carbonate matrix, while the tetrahydrofuran washing was used to extract soluble hydrocarbons. The fish fossils were dried at 25°C for ~10 h before following a similar immunodetection procedure to the immunoblotting from Section 6.3.2. Rock slabs were incubated with a 2% BSA dissolved in TBS for 1 h at 25°C with orbital shaking, rinsed once with TBS and immediately incubated with culture supernatant containing either anti-squalene or anti-CD75 antibodies for 1 h at 25°C with orbital shaking. After incubation, the rock slabs were rinsed and washed three times with TBS for 5 min at 25°C with orbital shaking, and incubated for 1 h with goat anti-mouse IgG+IgM peroxidase-conjugated antibodies (Abcam, Cambridge, UK) diluted 1:50,000 with TBS at 25°C with orbital shaking. The rocks were rinsed and washed with TBS three times for 5 min each at 25°C with orbital shaking before revealing antibody binding signals using a chemiluminescence substrate (SuperSignalTM West Femto substrate, Thermo Fisher Scientific) following the manufacturer's instructions. The reaction signal was imaged in a dark chamber (Gel Doc XR+ System, Bio-Rad Laboratories) during 20–99 sec of exposure time. Fish Samples 1–4 were used twice for immunodetection; therefore, after the first incubations and visualization, the antibodies were dehybridized by washing the rock slabs twice with

0.2% HCl (30 min at 25°C with orbital shaking) and rinsing them three times with Milli-Q water before continuing with the next immunodetection procedure.

6.4. Results

6.4.1. Biomarker visualization via immunoblotting

Squalane, squalene, and phytol standards were detected at concentrations ranging 10–100 nmoles/dot using anti-squalene antibodies by dot immunoblotting with chemiluminescence detection (**Figure 6.1A**). Although the method shows some background signal visualized by immunoblotting using an irrelevant isotype control antibody (anti-CD75), the response associated with anti-squalene antibodies shows a higher intensity that can be interpreted as hydrocarbon binding (**Figure 6.1A**). Immunoblotting was used to identify antibody-binding signals of soluble hydrocarbons extracted from sequential washes of rock slabs containing fish fossil remains. Both extracts with an organic solvent (tetrahydrofuran) and a 0.2% HCl aqueous solution showed positive chemiluminescence signals by dot immunoblotting (**Figure 6.1B**).

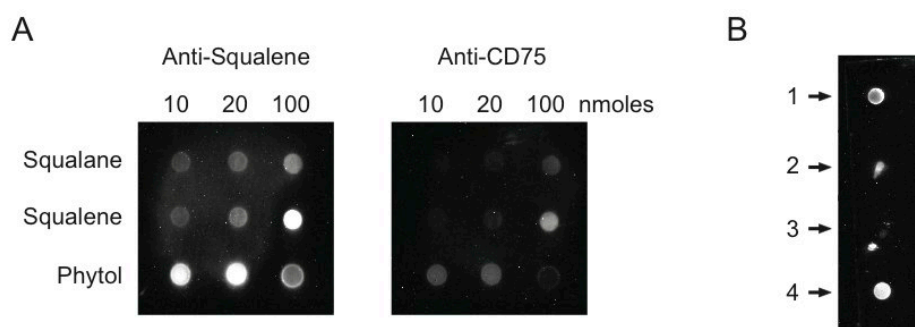


Figure 6.1. Immunoblotting with chemiluminescence detection. (A) Dot immunoblotting using either anti-squalene antibodies (left panel) or anti-CD75 antibodies

(right panel) of squalane, squalene, and phytol at 10, 20, or 100 nmoles/dot. (B) Dot immunoblotting of extracts from rock slabs containing fish remains using anti-squalene antibodies. The extractions correspond to sequential washings, first with tetrahydrofuran (1), then with 0.2% HCl (2) followed by water washings (3), and a final extraction with tetrahydrofuran (4).

Separation of the extracted biomarkers by PMC and their visualization by immunoblotting shows that the extracts contain antibody-binding hydrocarbons of different polarities (**Figure 6.2A, B**). Tetrahydrofuran extracts show two distinct signals for hydrocarbons migrating along with fronts of dichloromethane and methanol when a stepwise development gradient of increasing polarity is used (**Figure 6.2A**). Extracts with 0.2% HCl show an antibody-reactive signal having a similar migration distance to a squalane standard in a gradient elution (**Figure 6.2A**), although it that does not elute with a dichloromethane:methanol 19:1 solution (**Figure 6.2B**), indicating that the HCl extract signal does not correspond to squalane and that the anti-squalene antibodies bind to other hydrocarbons contained in fish fossil rocks.

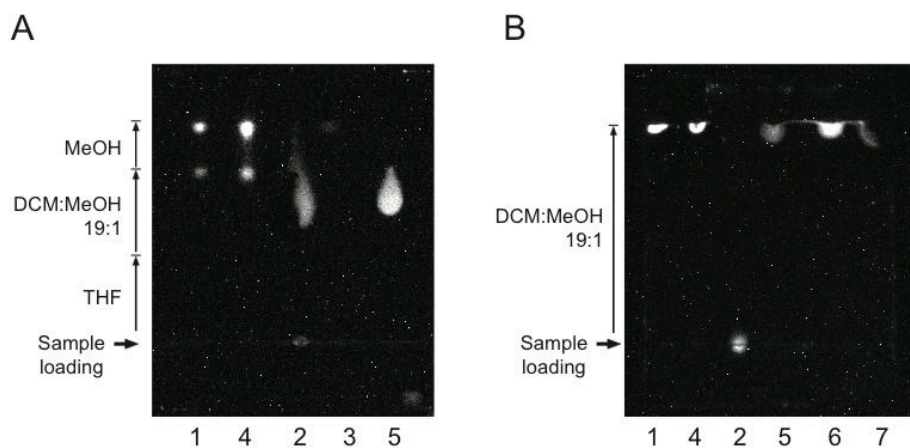


Figure 6.2. Chromatographic separation of fish fossil extracts by PMC. Sequential stepwise gradient elution (A) with tetrahydrofuran (THF), dichloromethane:methanol 19:1 (DCM:MeOH), and methanol (MeOH), and isocratic elution (B) with dichloromethane:methanol 19:1 on PVDF membranes followed by immunoblotting with anti-squalene antibodies. Lanes correspond to a first sequential extraction with tetrahydrofuran (1), a 0.2% HCl extract (2) followed by water washings (3), and a final extraction with tetrahydrofuran (4) of rock slabs containing fish remains, along with squalane (5), squalene (6), and phytol (7) standard solutions (100 $\mu\text{g}/\text{dot}$).

6.4.2. GRF fish fossil immunodetection of biomarkers

Rock slabs containing fish fossils were used as direct binding substrate for chemiluminescence immunodetection. Four fish fossils that were previously washed with 0.2% HCl (fishes 1–4) showed no specific signals using anti-squalene antibodies when compared with incubations with an irrelevant anti-CD75 antibody (**Figure 6.3**). Although incubations with anti-squalene antibodies showed higher intensities, most of the response can be attributed to background signal that was not directly associated with skeletal

structures in the fossils. By contrast, rock slabs containing fish fossil remains that were previously incubated with sequential washings of tetrahydrofuran in addition to HCl (Fish Samples 5 and 6) show some immunoreactive signals that seem associated with sediments inside vertebrae cavities of the fish spine (**Figure 6.4**).

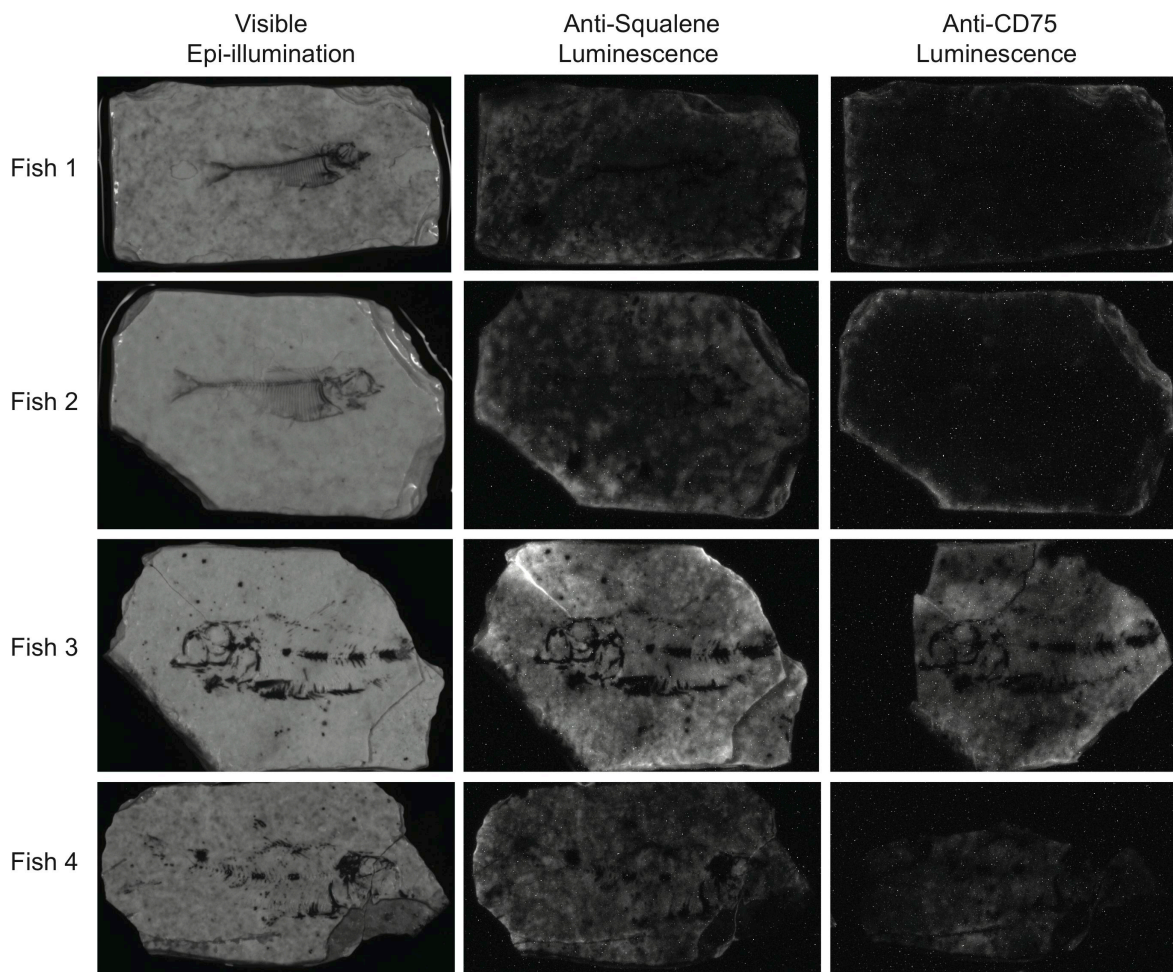


Figure 6.3. Hydrocarbon immunodetection in GRF fish fossils. Rock slabs containing fish fossils that were previously etched with 0.2% HCl and incubated with either anti-squalene or anti-CD75 antibodies. The left panels show photographs (grayscale) of the

fossils illuminated with white light, whereas the central and right panels show photographs under the dark exhibiting visible chemiluminescence signals.

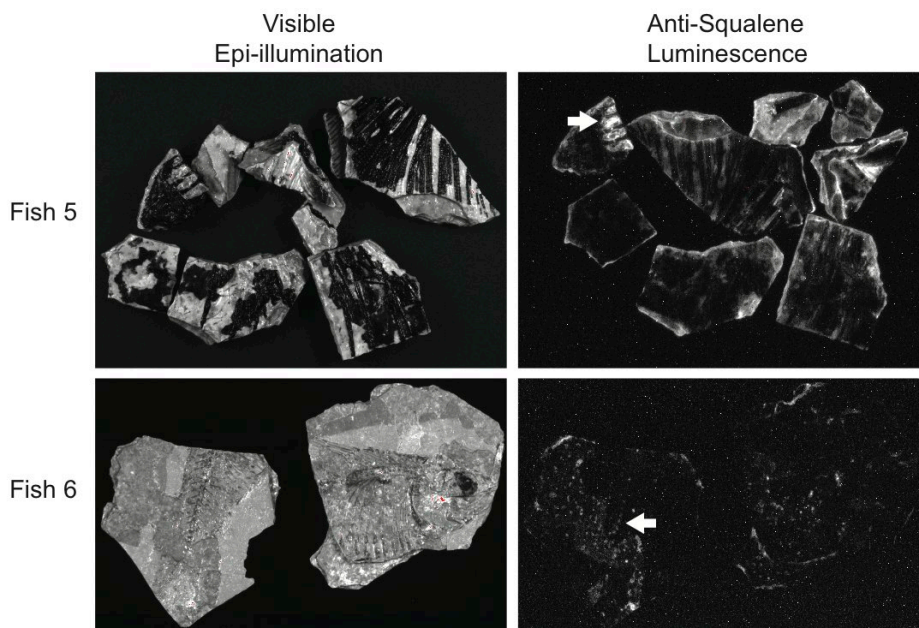


Figure 6.4. Hydrocarbon immunodetection in rock slabs containing fish fossil residues. Fossil remains that were sequentially washed with tetrahydrofuran and HCl. The left panels show photographs (grayscale) of the fossils under white light, and the right panels show photographs under the dark exhibiting visible chemiluminescence signals, some of them associated to the fish backbones (white arrows) after incubations with anti-squalene antibodies.

6.5. DISCUSSION

Although the anti-squalene antibodies bind squalane detected by immunoblotting with chemiluminescence detection (**Figure 6.1**), they also bind soluble hydrocarbons that are different from squalane based on their migration patterns on PMC. Fish extracts with

tetrahydrofuran may contain detectable amounts of squalane that migrate along a dichloromethane front during PMC separation (**Figure 6.2A, B**). Antibody-binding signals, however, are also observed for hydrocarbons that migrate with a subsequent methanol front during a stepwise gradient elution. Given that squalane does not elute with methanol on PVDF membranes (**Figure 6.2A**), the tetrahydrofuran-extracted hydrocarbons showing the highest migration distance in **Figure 6.2A** correspond to compounds different from squalane that are less hydrophobic. In addition, fossil washing with HCl also has immunoreactivity and does not elute with a mobile phase containing 19:1 dichloromethane:methanol (**Figure 6.2B**). The signal observed in the HCl extract having a comparable migration distance to squalane in the PMC gradient elution of **Figure 6.2A** must have migrated during the last sequential elution with methanol, mobilizing relatively polar compounds. Therefore, the antibodies not only bind squalane, but also other unidentified hydrocarbons extracted from the fish fossil. Given that these antibodies seem to bind other acyclic isoprenoid moieties (Medina Ferrer *et al.*, 2018), it is possible that a variety of acyclic isoprenoids with polar functional groups, such as phytol, are detected from the fish extracts and potentially contribute to some binding signals observed via direct immunodetection of *Knightia* fossils. GC-MS analysis of the extracted solutions or direct MALDI-TOF MS on the different migration zones developed by PMC (e.g., Goto-Inoue *et al.*, 2008, 2009; Valdes-Gonzalez *et al.*, 2011) may help determine the potential biomarkers to which the anti-squalene antibody binds in the rock extract.

Fish fossil remains that were only washed with HCl prior to the immunodetection did not show distinctive antibody signals. Although immunodetection with anti-squalene

antibodies showed chemiluminescence reaction, most of the observed signal likely corresponds to background intensity that is visualized by the incubation with an irrelevant isotype control antibody (anti-CD75; **Figure 6.3**) resulting from non-specific antibody binding or non-enzymatic reaction of the chemiluminescence substrate. Although no isotype control antibody was used for the analysis of the fish pieces that were washed with tetrahydrofuran in addition to HCl (**Figure 6.4**), a more intense signal compared to the background matrix is observed in sediments inside fish vertebrae. No distinct signal was associated with preserved skeletal structures. However, antibody-binding signal found associated with vertebrae sediments opens the possibility that squalane, and other hydrocarbons visualized by the antibody, preserve at specific locations of the fossil matrix. If particular biomarkers are indeed concentrated in certain fossil areas, such as *Knightia* spine, it is possible that an abundant source of these hydrocarbons is associated with specific original fish tissues, or that the organismal environment aided in the preservation of their lipid biomarkers. For example, the interior of the bones may provide a protective microenvironment that inhibits microbial infiltration (e.g., McNamara *et al.*, 2006) or maintains undisturbed, anaerobic, conditions that ultimately limits biomarker degradation, therefore increasing its preservation potential.

By contrast, it is also possible that initial decomposition of the fish organic matter promoted rapid microbial growth (Liebenau *et al.*, 2015) and consequently the preserved hydrocarbons correspond mostly to the lipids from microbial decomposers, as opposed to the lipids from the original skeletal fossil (e.g., Thiel *et al.*, 2014). A potential source of microbial acyclic isoprenoid biomarkers are archaea, where squalene is a primary lipid membrane constituent in methanogens, halophiles, and thermoacidophiles, among others

(Tornabene, 1978; Tornabene *et al.*, 1978, 1979; Ten Haven *et al.*, 1988). Halophilic archaea, such as *Halobacterium*, *Haloarcula*, *Halococcus*, and *Haloferax*, are found in great abundance in many hypersaline lakes and brines (Oren, 1994; Arahall *et al.*, 1996), and the saturated form of squalene, squalane, is common in sediments and sedimentary rocks deposited in hypersaline settings where it has been interpreted as a sedimentary hydrocarbon biomarker for halophilic archaea and hypersaline environments (Ten Haven *et al.*, 1988; Grice *et al.*, 1998). The closed basin lake system leading to the deposition of the Green River Formation was subjected to periods of evaporation indicated by the precipitation of evaporative minerals, such as sodium carbonates and halides, and by changes in $\delta^{18}\text{O}$ recorded from carbonaceous stromatolites (Frantz *et al.*, 2014). Therefore the waters may have also contained halophilic archaea characteristic of hypersaline environments, some of which could potentially colonize dead biomass and contributed to the diagenetic biomarker prints of the fish fossils (Andrei *et al.*, 2012).

In addition to the environmental conditions, the specific mineralogy of the sedimentary layers in which the fishes were buried can contribute to the preservation of biomarkers associated to the dead biomass. Sedimentary organic matter may bind mineral surfaces that can assist with the preservation process (Keil *et al.*, 1994; Torn *et al.*, 1997; Kennedy *et al.*, 2002). Chemical and physical protection of organic matter may occur in response to the mineral composition of the sediments, where for example, high surface area minerals (e.g., secondary phyllosilicates, metal-oxyhydroxides) are thought to provide protection against degradation of organic matter via sorption on mineral surfaces (Kennedy *et al.*, 2014; Posth *et al.*, 2014). It is possible that *Knightia* organic matter—including squalene and other biomarkers—mostly binds burial sediments and clay

minerals that contribute to the preservation of specific biomarkers. Both the depositional environment and the mineralogy of the burial site may be important for determining the distribution of sedimentary hydrocarbons that may remain associated with the skeletal fossil. Biomarker-binding antibodies may provide an alternative technique for directly visualizing the organic matter of body fossils in their sedimentary depositional context. To our knowledge, this is one of the few examples where biomarkers have been directly associated with a body fossil (e.g., Edwards *et al.*, 2014; Bobrovskiy *et al.*, 2018a, 2018b). However, given the binding of the anti-squalene antibodies to multiple hydrocarbons preserved in the fossil matrix, the precise nature of the biomarkers observed is not known. The future development of specific antibodies against sedimentary biomarkers to visualize their association with respect to fossilized remains may prove useful for understanding taphonomic and diagenetic processes leading to preservation of organic matter in the geological record.

6.6. CONCLUSION

An antibody-based detection of sedimentary organic matter may be used to identify the distribution of biomarkers in fossils. By using an antibody that binds squalane (Matyas *et al.*, 2000) as well as other hydrocarbons preserved in rocks from the GRF (Medina Ferrer *et al.*, 2018), we identified antibody-binding signals in extracts from GRF fish fossils and potentially also in the sediments associated with fish skeletal structures, particularly the fish vertebrae. The signals detected by the antibodies, however, may correspond with squalane and possible other preserved hydrocarbons of unknown identities. Although direct biomarker visualization in the fish body fossil may

provide evidence that those biomarkers originated from the primary fish tissue, it is also possible that later biomass decomposition introduced microbial-derived hydrocarbons that contributed to the biomarker signals observed from the GRF fossils. Future development of hydrocarbon-specific antibodies may help determine the potential mechanisms associated to organic matter preservation in the structure of fossilized organisms.

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Chapter 7

Concluding Remarks

The contents of this dissertation show two main examples of tools for protein analysis in the life sciences that were adapted and used to address questions in geobiology. By complementing these newly developed techniques with information obtained from the field, petrographic analyses, geochemical characterizations, DNA sequencing and bioinformatics, among others, it was possible to conclude the following:

- Although the mechanisms of microbially-induced carbonate precipitation are well known, it is hard to evaluate them in their indigenous environment. Urease and carbonic anhydrase field assays provide a simple and economic alternative to test *in situ* the activity of microbial enzymes associated with carbonate precipitation.
- Field testing of urease and carbonic anhydrase may help determine the elusive mechanisms involved in the formation of tufa mounds, which may potentially serve as biosignatures of life on Earth and elsewhere.
- Antibodies can be used as tool to bind and visualize sedimentary biomarkers. Although the anti-squalene antibodies used in this research also bind other compounds, the technique can be applied to detect hydrocarbons in oils and to

visualize biomarker distributions in rocks—a tool that can be useful to evaluate biomarker syngeneity.

- Lipids and hydrocarbons can be separated by planar chromatography using PVDF membranes, which can be directly coupled with immunoblotting and other potential downstream techniques.
- Antibodies can also be used to visualize potential biomarkers preserved in the structure of fossilized organisms, such as the fish fossils of the Green River Formation. The future development of antibodies capable of binding specific lipid biomarkers may prove useful for determining biomarker provenance and the potential mechanisms of organic matter preservation.

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Appendix A

Supporting Information for Chapter 2

A.1. Supplementary Methods

A.1.1. Microscope examination

Biomass portions obtained in the field were kept at 4–8 °C for microscopic observation using an Olympus BX61 (Olympus Corporation, Tokyo, Japan) fluorescence microscope after DAPI (4',6-diamidino-2-phenylindole) staining. Fluorescent red light (584–664 nm) emission was used to observe chlorophyll autofluorescence and detect potential photosynthetic organisms.

A.1.2. Microbial protein extraction

Approximately 4 mL of wet biomass was lysed in the field using a modification of the method for obtaining environmental proteins described by Ogunseitan (1993), which has shown the highest protein recovery and stability compared to other commonly used environmental microbial lysis methods (Singleton *et al.*, 2003). The wet biomass was mixed in a 15 mL centrifuge tube with 2 mL 50 mM Tris, 10 % sucrose, 4 mM EDTA, 1.2 mg lysozyme and a protease inhibitor cocktail (Thermo Fisher Scientific, Cat. 87786, Waltham, MA, USA), adjusted to pH 8.0. The tube was incubated for 1 h on ice and then 2 mL of a solution containing 50 mM Tris pH 8.0, 10 % sucrose, 4 mM EDTA, 1 % Triton X-100 and 1× protease inhibitor cocktail was added, mixed with the previous

solution and immediately frozen in dry ice for transportation to the laboratory. The frozen aliquot was then subjected to 4 freeze-thaw cycles (-80 °C to 25 °C) and centrifuged at $7,000 \times g$ and 4 °C for 1 h. A total of 10 mM MgSO₄ and 2.5 U DNase I (Thermo Fisher Scientific, Cat. 89836) were added to the lysate supernatant and incubated in ice for 1 h with occasional, gently mixing. A Bradford assay was used for protein quantification following the manufacture's instructions (Bio-Rad Laboratories Inc., Hercules, California) and the lysates were then used for urease and CA activity assays.

A.1.3. Canonical Urease and CA activity assays

CA activity of standard enzyme solutions (**Figure A.S6**) and environmental protein extracts (**Figure A.S8**) was evaluated using a modification of the Wilbur and Anderson (1948) electrometric method. Different CA concentrations (0.05–1.5 nM CA in the final reaction volume; isozyme II from bovine erythrocytes, product number C2522, Sigma-Aldrich, St. Louis, Missouri, USA) or ten micrograms of protein extract were added to 6 mL of cold 0.02 M Tris buffer, previously adjusted to pH 8.0 with H₂SO₄ at room temperature, and agitated in a magnetic stirrer at 700 rpm (Thermo Fisher Scientific, Fisherbrand™ Isotemp™) while kept at 0–4 °C. The enzymatic reaction was initiated by introducing 4 mL of cold CO₂-saturated water using a five-mL syringe. The time for the pH drop from 8.3 to 6.3 was recorded and compared to a control assay in the presence of 0.1 mM of the CA inhibitor acetazolamide (**Figure A.S6**) or to a blank assay without protein lysate (**Figure A.S8**). One unit of CA activity was defined as $2(t_0 - t)/t$, where t is the time for the pH drop from 8.3 to 6.3 in the presence of the lysate and t_0 is the same time recorded by the blank.

Urease activity was assayed in microplates (Thermo Fisher Scientific, CorningTM Cat. 07-200-98) using a modification of the phenol-hypochlorite method (Natarajan, 1995; Achal *et al.* 2009). Urease standard solutions (1.5–31 mU urease in the final enzymatic reaction volume; product number U1875, Sigma-Aldrich, St. Louis, Missouri, USA) were mixed with 0.2 M Urea in 50 μ L of phosphate buffer (100 mM Na₂HPO₄ pH 7.0, 200 mM NaCl) and incubated for 20, 60, or 120 min at 25 °C. Protein extracts were similarly assayed, by mixing ten micrograms of protein with 0.2 mM Urea in 50 μ L of phosphate buffer and incubating for 20 min. The reaction response was determined by adding 100 μ L phenol nitroprusside solution and 100 μ L alkaline hypochlorite solution (Sigma-Aldrich, St. Louis, Missouri, USA), incubated for 30 min at 37 °C and quantified at 630 nm relative to a control without urea (**Figure A.S8**) or compared to a control containing 0.1 mM of the urease inhibitor phenyl phosphorodiamidate (PPD; **Figure A.S6**). Ammonia was quantified using an ammonium chloride standard curve. One urease unit (U) was defined as the quantity of enzyme that will liberate 1 μ mole of ammonia from urea per minute at pH 7.0 and 25 °C.

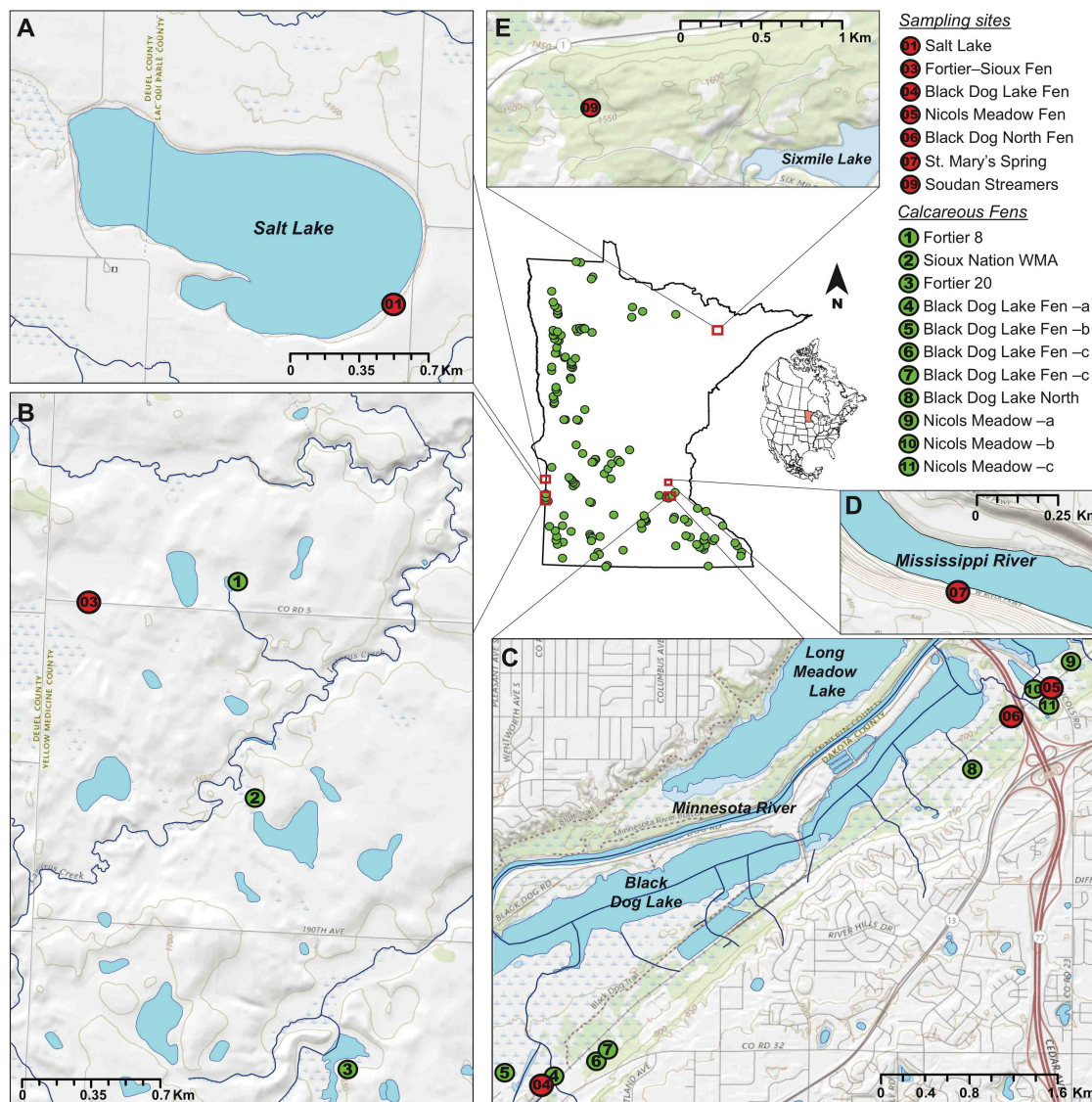
In-gel enzymatic activity assays were performed following the protonography method described for CA (De Luca *et al.*, 2015) in non-reducing SDS-PAGE, using 100 mM Tris pH 8.5, 0.1 % metacresol purple as a pH indicator solution. The in-gel activity or urease was assayed similarly, using a 100 mM Tris pH 6.5, 0.1 % phenol red as indicator and a 4 % urea, 0.4 mM EDTA solution as substrate.

A.2. Supplementary Table

Table A.S1. Description of the environmental samples obtained in this study.

Sample ID	Sample Name	Site Name	Coordinates	Description	Site reference
01	Salt Lake cyanobacterial bloom	Salt Lake, East shore	44°57'39.7" N 96°26'12.4" W	Bright green surficial biomass suspended at the lake shore.	(Dean <i>et al.</i> , 1993)
02	Salt Lake filaments	Salt Lake, East shore	44°57'39.7" N 96°26'12.4" W	Submerged dark green filaments attached to vegetation at the lake shore.	(Dean <i>et al.</i> , 1993)
03	Fortier-Sioux filaments	Fortier 8 & Sioux Nation WMA Fen	44°41'21.6" N 96°27'03" W	Green floating filaments containing abundant air bubbles at shallow inundation ponds.	(Almendinger and Leete, 1998)
04	Black Dog peat biofilm	Black Dog Lake Fen	44°47'16.5" N 93°16'35.7" W	Green biofilm intermixed with semi-consolidated peat clays and sand at an eroded bank of a fen stream.	Minnesota Department of Natural Resources
05	Nicols Meadow filaments	Nicols Meadow Fen – c	44°49'18.4" N 93°13'14.6" W	Abundant green filaments at an inundation pond next to Nicols Meadow Fen – c.	(Almendinger and Leete, 1998)
06	Black Dog North orange stream	Black Dog Lake North Fen	44°49'09.3" N 93°13'28.7" W	Muddy bright orange precipitates at a spring between Nicols Meadow Fen and Black Dog North Fen.	(Almendinger and Leete, 1998)
07	St. Mary's Spring orange mat	St. Mary's Spring	44°58'05.5" N 93°14'05.2" W	Abundant orange precipitates at the emergence of an iron-rich seep.	(Anderson <i>et al.</i> , 2011)
08	St. Mary's Spring green mat	St. Mary's Spring	44°58'05.5" N 93°14'05.2" W	Green microbial mat beneath abundant orange precipitates.	(Anderson <i>et al.</i> , 2011)
09	Soudan seep streamers	Highway near Soudan, MN	47°50'30.5" N 92°08'56" W	Green and white filaments in a sulfidic stream with orange precipitates and black sediments.	This study

A.3. Supplementary Figures



shore of the Mississippi River (D), and from a sulfidic seep roadside Highway 169, near Soudan, MN (E). Calcareous fen locations and water bodies were obtained from the Minnesota Department of Natural Resources. Topographic maps were obtained from the USGS National Geospatial Program, The National Map.

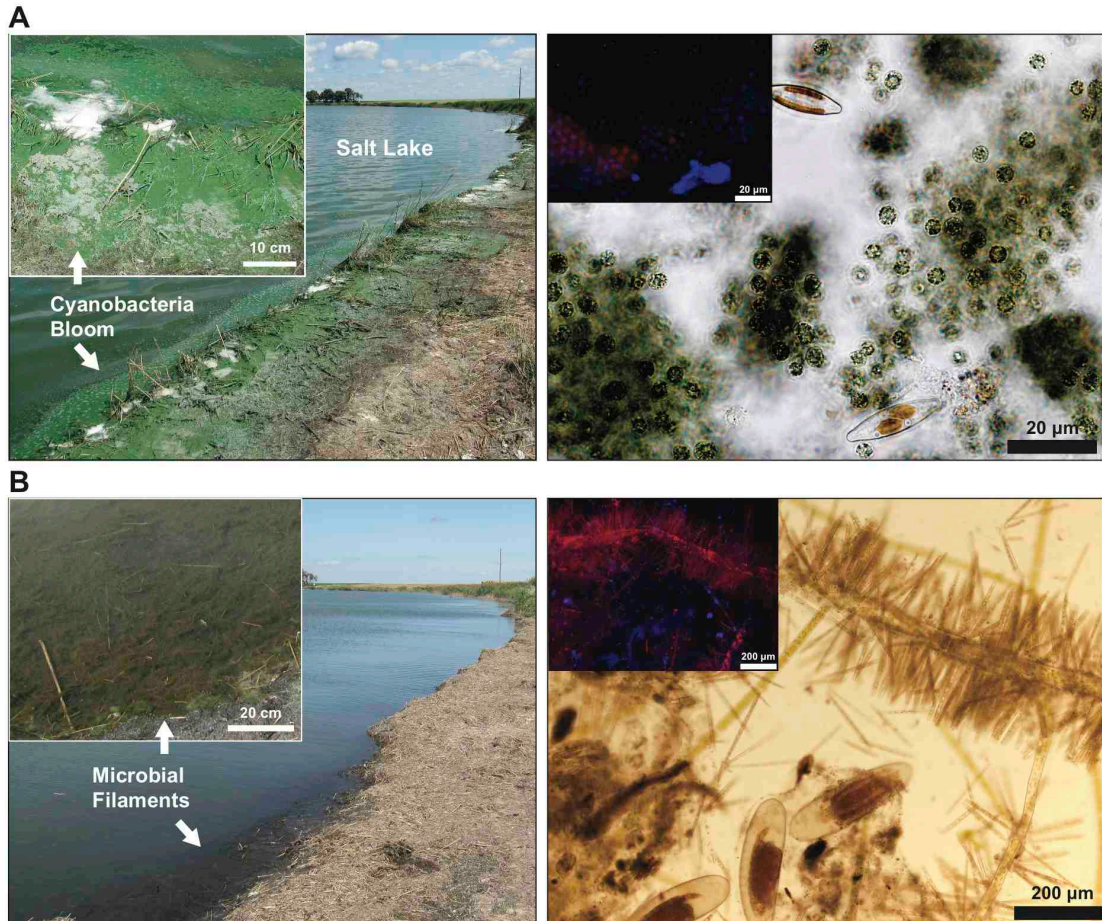


Figure A.S2. Microbial biomass in Salt Lake, MN. Lake shore showing a bright green cyanobacterial bloom (A) sampled in July 2019 and its decline in August 2019 (B), where dark green microbial filaments at the lake shore were sampled instead. Insets show a close-up of the bloom and microfilaments sampled (left panels). Right panels show bright field photomicrographs of the sampled biomass with a merged fluorescence image (insets) of chlorophyll (red) and DAPI (blue) emission. Note that the bloom event in A is dominated by a cyanobacterium resembling *Microcystis* spp., whereas the sample taken after the bloom decline is dominated by large green trichome sheaths, probably *Microcoleus* spp., adorned with numerous photosynthetic diatoms.

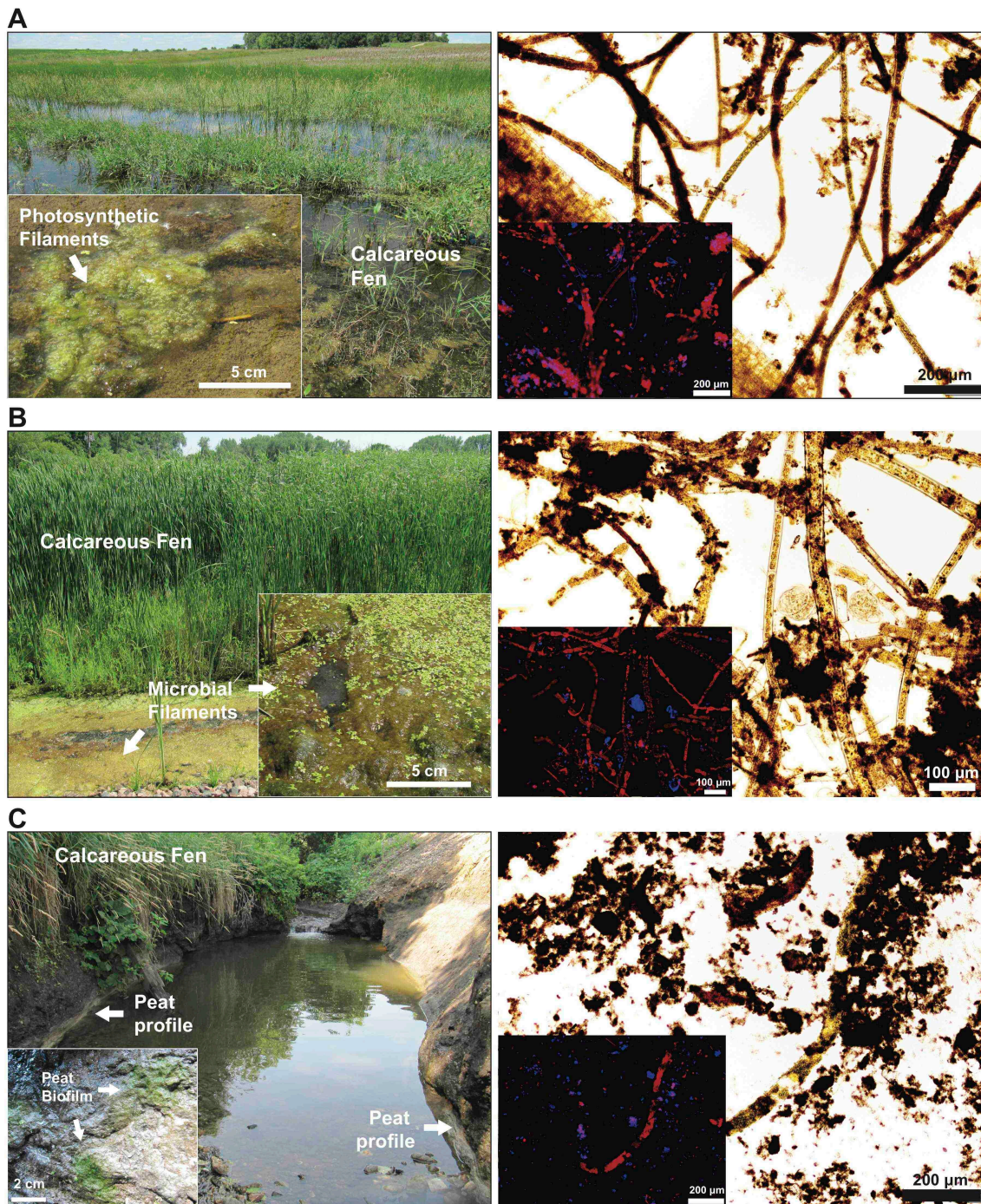


Figure A.S3. Microbial filaments at calcareous fens. Inundation ponds showing green surficial filaments with bubbles at Fortier-Sioux Nation Fens (A) and abundant filaments mixed with aquatic plants (*Spirodela* spp.) at Nicols Meadow fen (B). Green biofilms also colonize the peat exposed in a stream-eroded cliff at Black Dog Lake Fen (C). Insets

show a close-up of the biomass sampled (left panels). Right panels show bright field photomicrographs for each sample, with insets of merged fluorescence images of chlorophyll red emission and blue fluorescence from DAPI.

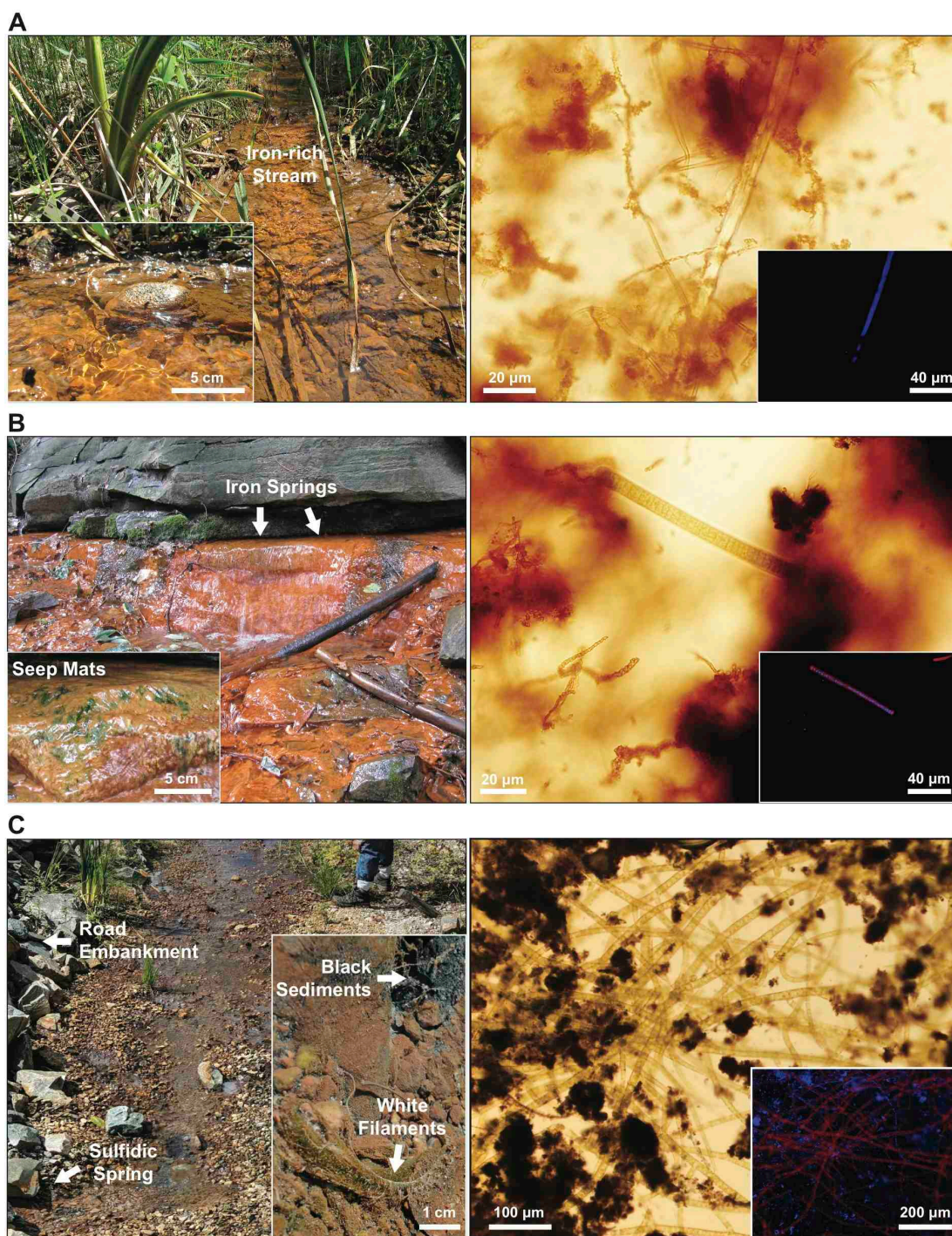


Figure A.S4. Microbial mats at ferrous seeps. Orange precipitates from a stream between Nicols Meadow Fen and Black Dog Lake North Fen (A), from St. Mary's Spring ferrous seeps (B), and from a sulfidic seep near Soudan, MN (C, field photo credit to

Tanner Barnharst). Insets show a close-up of the orange precipitates and microbial mats sampled. Right panels show bright field and fluorescence microscopy (insets) of chlorophyll (red) and DAPI (blue) emission. Note the stalks of iron-oxidizing bacteria in A and B, and abundant cyanobacterial filaments in B and C.

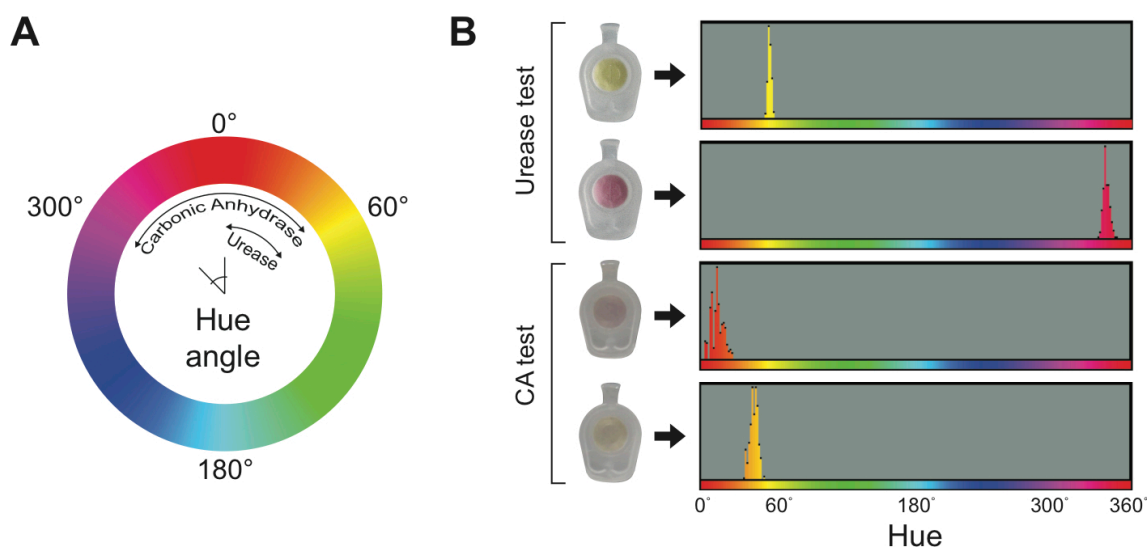


Figure A.S5. Quantification of hue values. (A) Color wheel diagram showing the hue range of the carbonic anhydrase and urease tests. (B) Histograms of hue pixels for representative indicator strip (test tube caps) assays during the enzymatic reaction. The hue was expressed as the average value from assay photos over time.

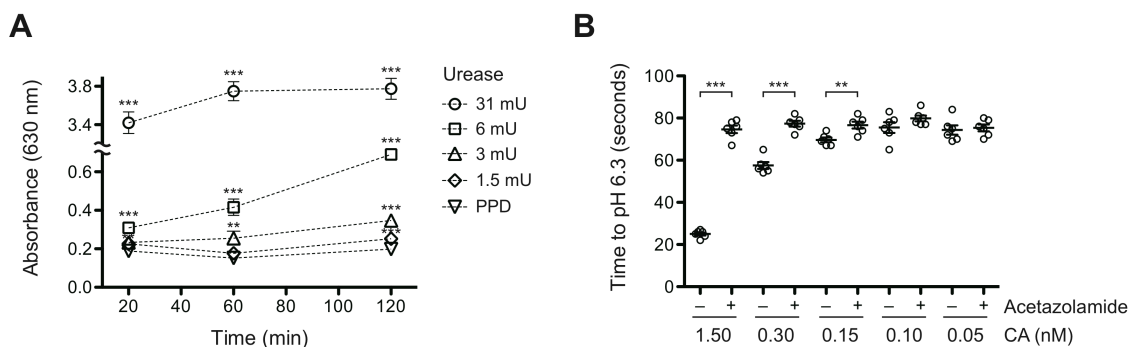


Figure A.S6. Urease and carbonic anhydrase standard assays. (A) Colorimetric response after 20, 60, and 120 minutes of urease reaction measured by the phenol-hypochlorite method using urease standards (1.5–31 mU) with or without a urease inhibitor (PPD). (B) CA hydratase response (time for pH drop from 8.3 to 6.3) based on the Wilbur and Anderson (1948) method of standard CA solutions (0.05–1.5 nM) with or without its inhibitor, acetazolamide. All conditions were assayed with six replicates. Error bars represent standard error (when not shown, the error is smaller than the symbols). The urease colorimetric response was analyzed by a mixed-effects model, and the CA time response was evaluated by a two-way ANOVA, where * $0.1 < p < 0.05$, ** $0.05 < p < 0.01$, and *** $p < 0.01$ between each condition and its respective negative control assessed by Bonferroni's multiple comparisons test.

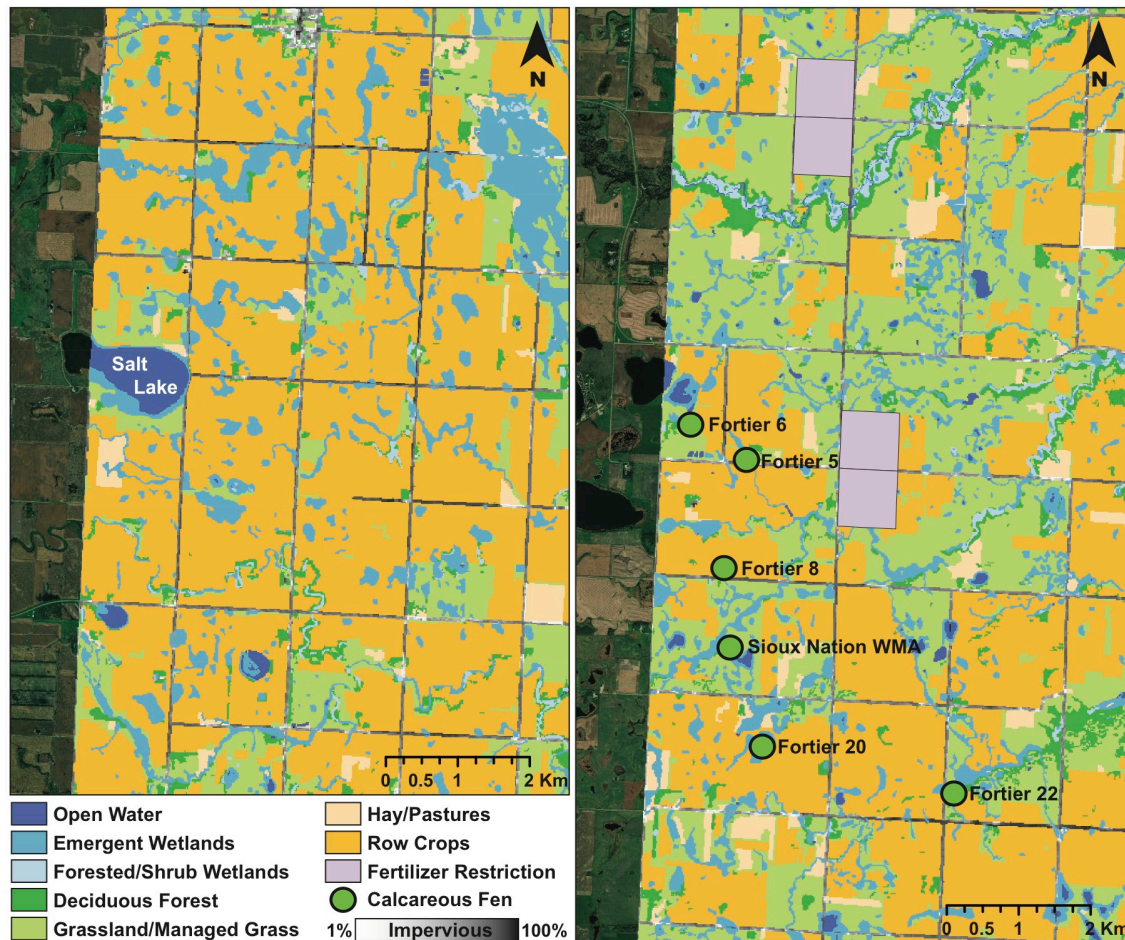


Figure A.S7. Land use near Salt Lake and Sioux Nation WMA Fen. Minnesota land cover classification maps around Salt Lake (A) and Sioux Nation-Fortier fens (B).

Classifications according to 2013 Landsat and Lidar data (Rampi *et al.*, 2013). Note the abundance of agriculture areas near Salt Lake compared to the calcareous fens. Quadrants with nitrogen fertilizer (anhydrous ammonia and urea) application restrictions are based on vulnerable groundwater areas according to the Minnesota Department of Agriculture. Calcareous fen locations were obtained from the Minnesota Department of Natural Resources. South Dakota areas are represented by USGS satellite images, Geospatial Data Sources (2019).

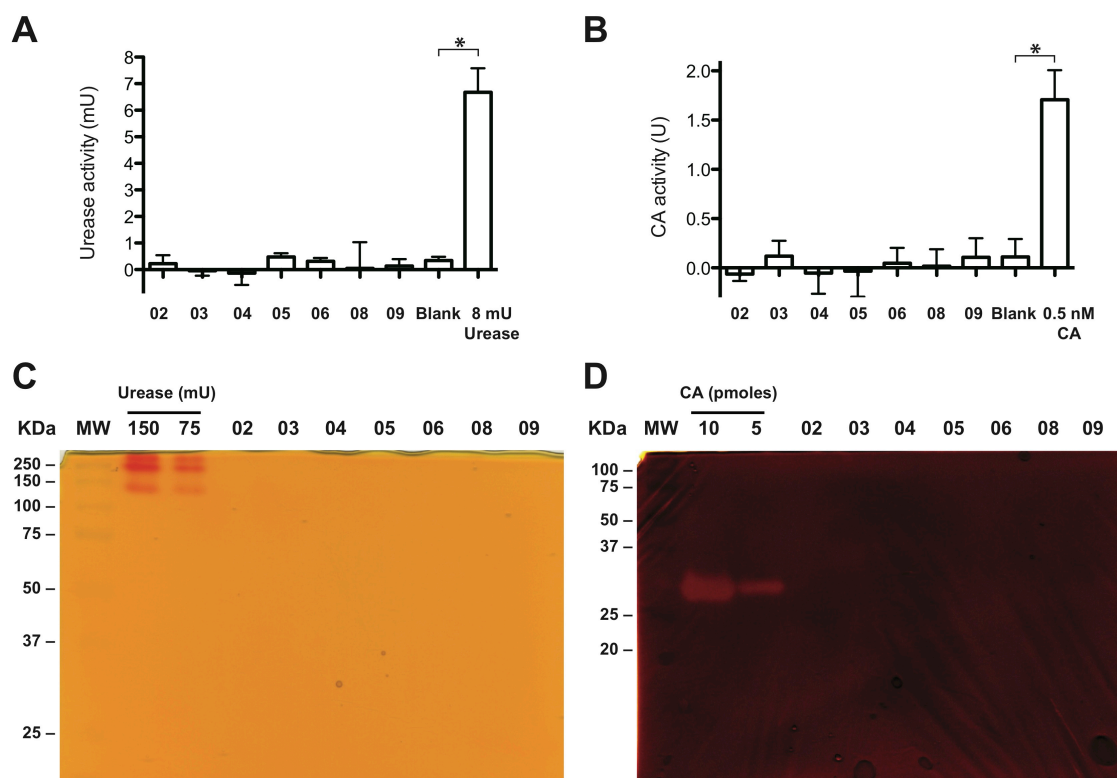


Figure A.S8. Urease and carbonic anhydrase activity from protein extracts. Urease activity using the Berthelot reaction method (A; Achal *et al.*, 2009) and CA activity after the Wilbur and Anderson (1948) assay (B). A total of 1 μ g of protein extracts from Salt Lake filaments (02), Fortier-Sioux filaments (03), Black Dog peat green biofilm (04), Nicols Meadow filaments (05), Black Dog Lake North orange precipitates (06), mixed orange and green mats from St. Mary's Spring (08), and Soudan streamers (09) were used. None of the extracts were significantly higher than a blank assay ($*p < 0.01$, Welch's t-test), which is also observed using in-gel activity assays (C & D; De Luca *et al.*, 2015). A red band on a yellow background indicates urease activity (C), whereas a yellow band on a purple background indicates CA activity (D).

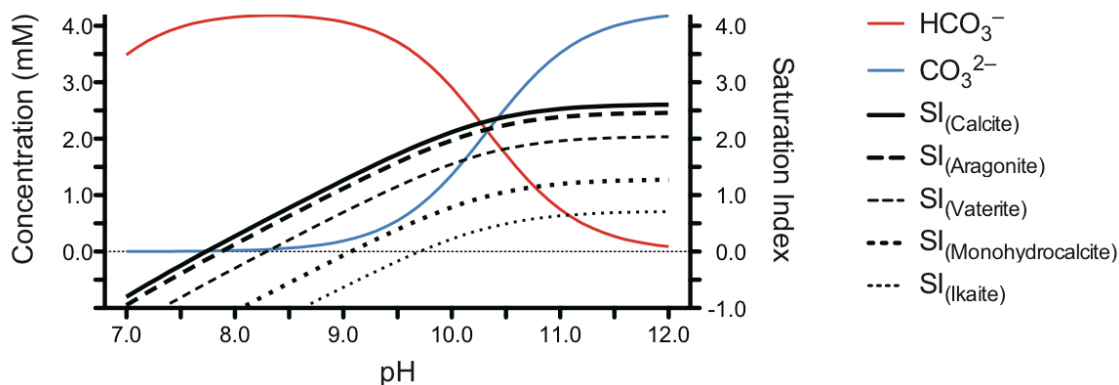


Figure A.S9. Salt Lake carbonate speciation and carbonate mineral solubilities.

Bicarbonate and carbonate concentration as a function of pH, considering the alkalinity (234 mg/L as CaCO_3) and calcium concentration (16 mM) of Salt Lake. Assuming constant calcium and carbon concentrations, the saturation index (SI) for calcium carbonate minerals increases linearly from pH 9.0 (lake water pH) to 9.6 (pH at the end of microbial incubations without urease inhibitors). Note that above pH 9.6, all metastable calcium carbonate phases are saturated. Solubility values at 25 °C were obtained from Plummer and Busenberg (1982), Kralj and Brečević (1995), and Bischoff *et al.* (1993).

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Appendix B

Supporting Information for Chapter 3

B.1. Supplementary Figures

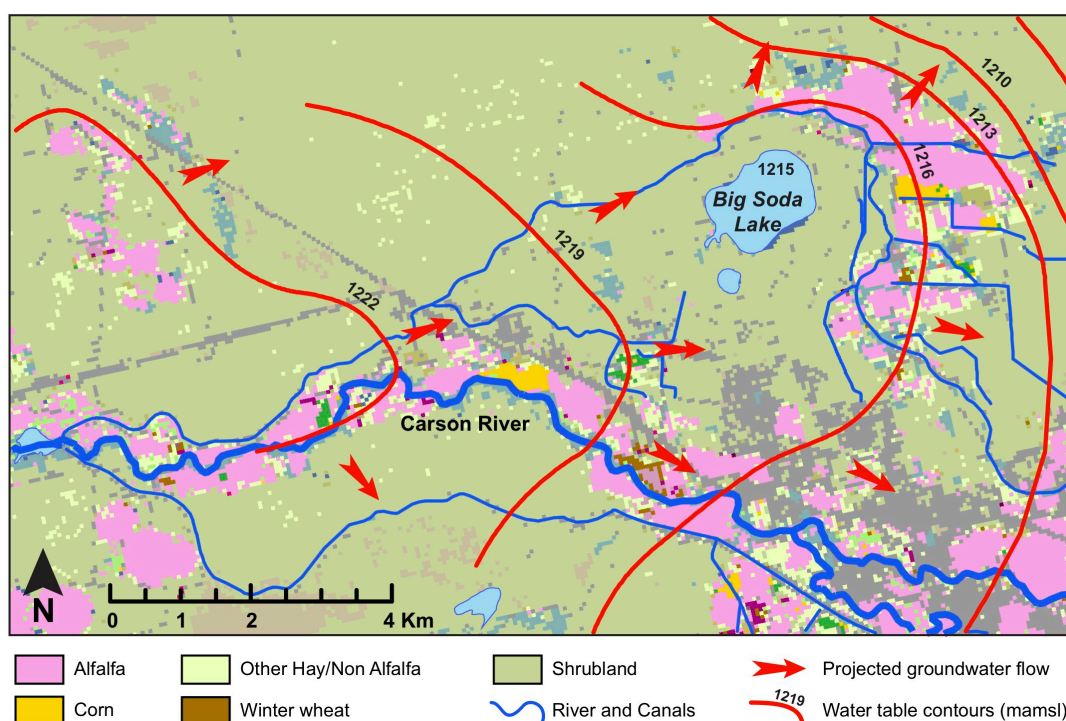


Figure B.S1. Hydrogeological and agricultural map of the Carson River basin near BSL, Churchill County, Nevada. Groundwater flows from agricultural land west of the lake, upstream of the Carson River. Groundwater feeding the lake could also come locally from the immediate irrigated lands north and east of the lake, and leaking canals that distribute irrigation water to the fields. Most of these canals are unlined. Water table contours after Smith *et al.* (2016). Crops and land use obtained from the USDA National Agricultural Statistics Service, Cropland Data Layer, available at

<https://nassgeodata.gmu.edu/CropScape>. Canal distributions after Leu, Hanser, and Knick (2008).

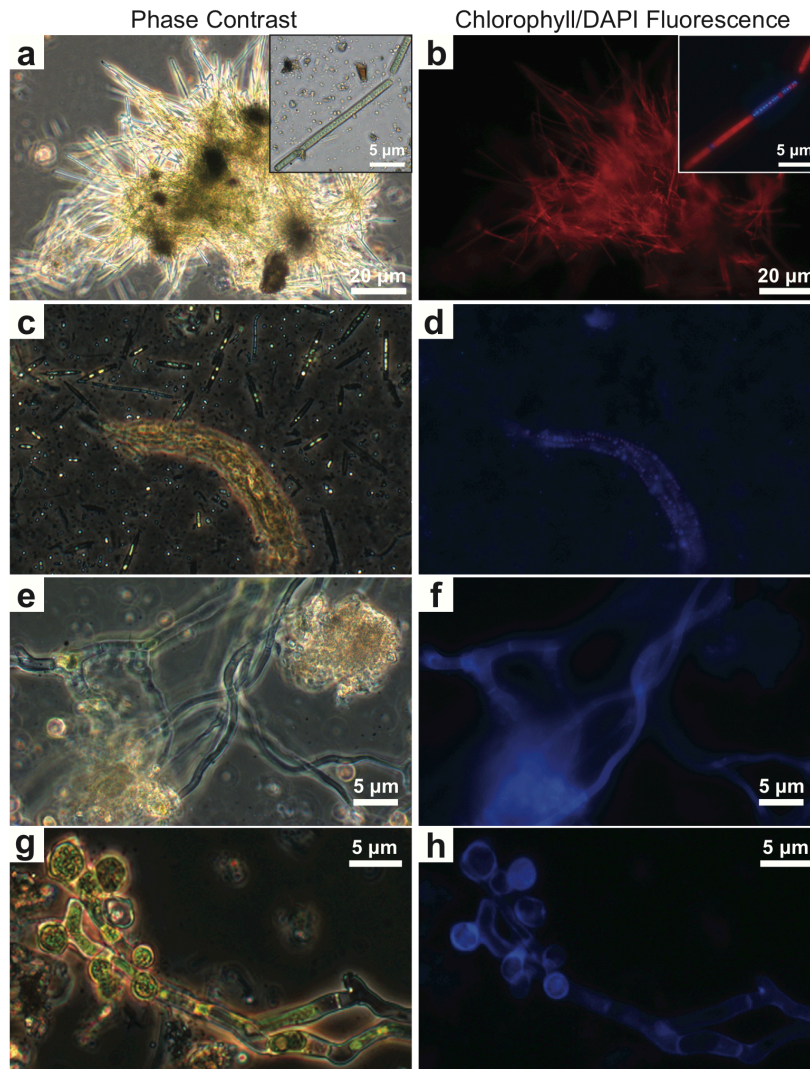


Figure B.S2. Photomicrographs of BSL tufa biofilms. Optical and fluorescence microscopy of microbial mats from the surface of subaqueous tufas showed abundant possible photosynthetic filaments attached to carbonate grains (a). Red fluorescence indicates chlorophyll emission (b); blue fluorescence shows DAPI-stained DNA (b, inset). Surficial biofilms had also abundant diatoms and nematodes (c, d). Endolithic communities presented hyphae (e, f) and conidia (g, h) characteristic of fungi.

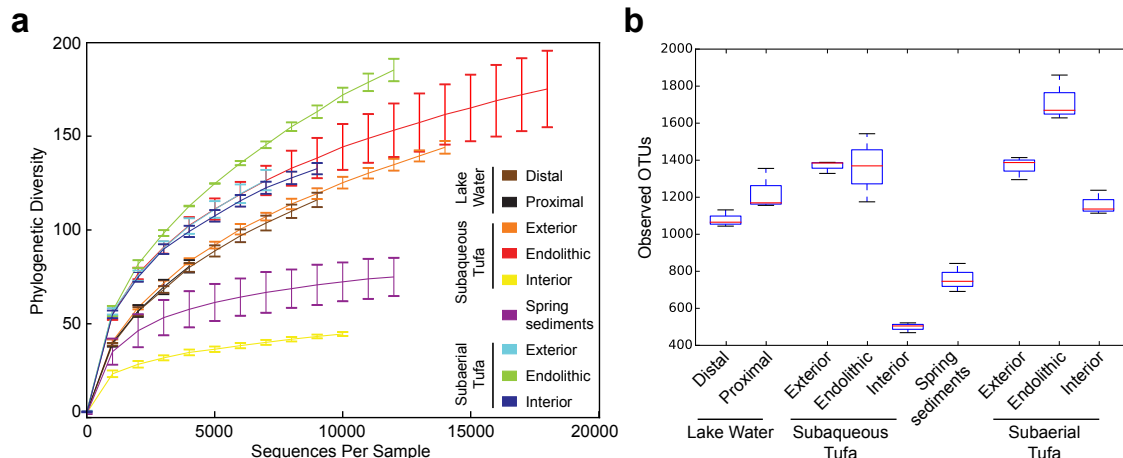


Figure B.S3. Alpha-diversity of microbial communities from Big Soda Lake. (a)

Rarefaction curves of the phylogenetic diversity for each microbial sample. (b)

Comparison of the observed OTUs for each sample at an even rarefaction depth of 4900 sequences. Data represent SSU rRNA gene sequencing for water, sediment, and tufa samples.

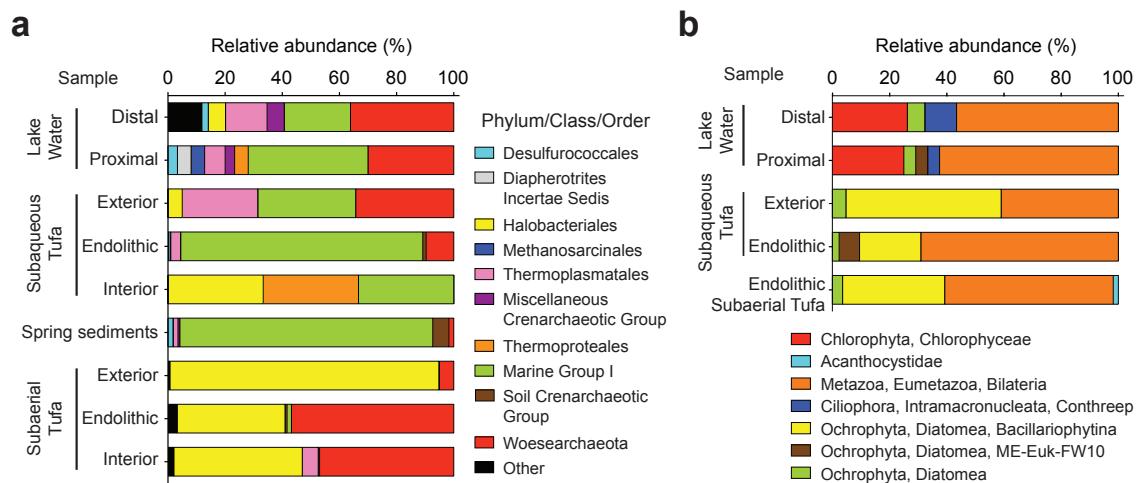


Figure B.S4. Archaeal and eukaryote communities at Big Soda Lake. Relative

abundance of archaea (a) and eukaryotes (b) obtained by 16S and 18S rRNA gene

sequencing. Each sample represents biological triplicates from lake waters, shore

sediments in contact with spring water, and microbial biofilms from a subaqueous and a subaerial tufa.

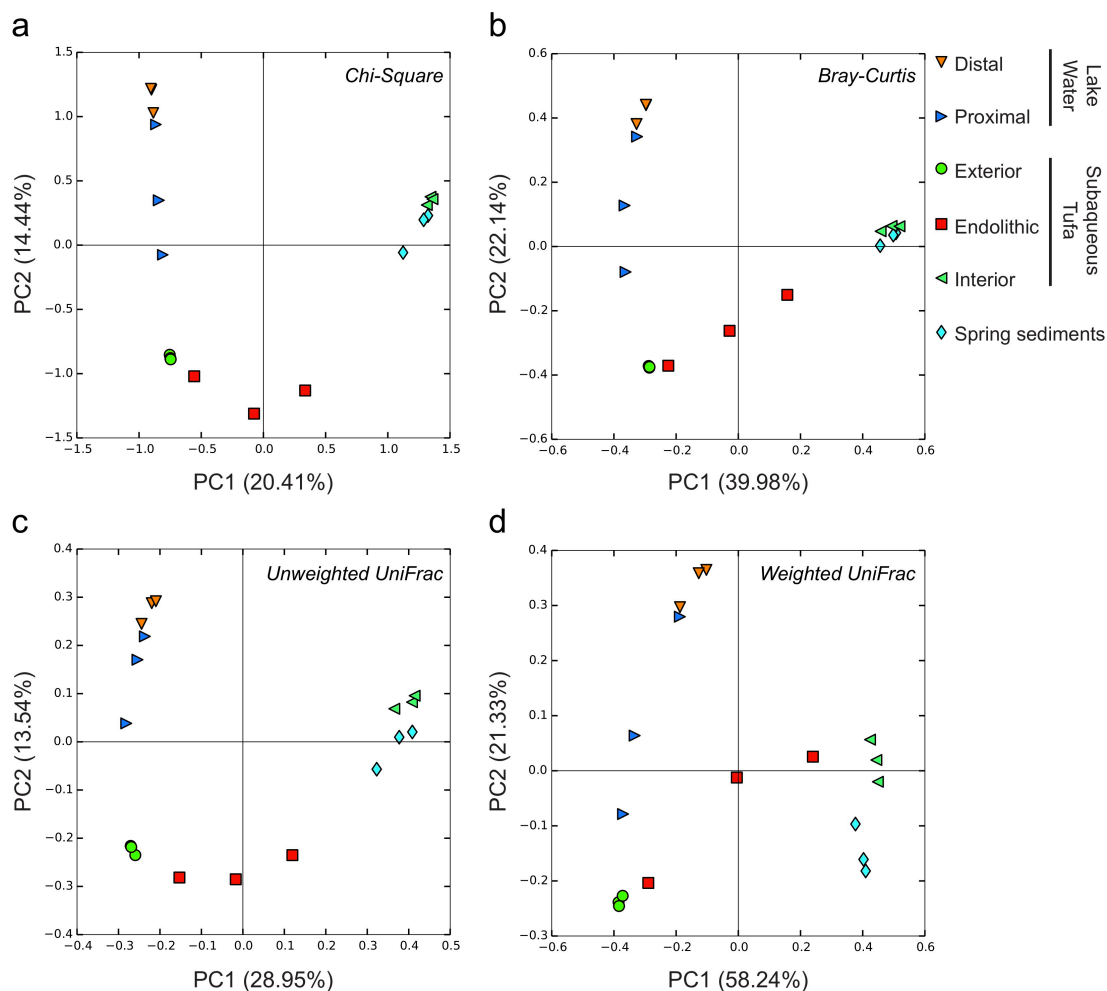


Figure B.S5. Beta-diversity of microbial communities from Big Soda Lake.

Comparison of different distance metrics of triplicate samples from lake water, spring water, and the submerged tufa visualized via PCoA. Note the ‘horseshoe effect’ that better resolves on the PC1 axis by chi-square (a) compared to Bray-Curtis (b), unweighted UniFrac (c) and weighted UniFrac (d) distances, indicating a biological gradient from lake water through the tufa interior.

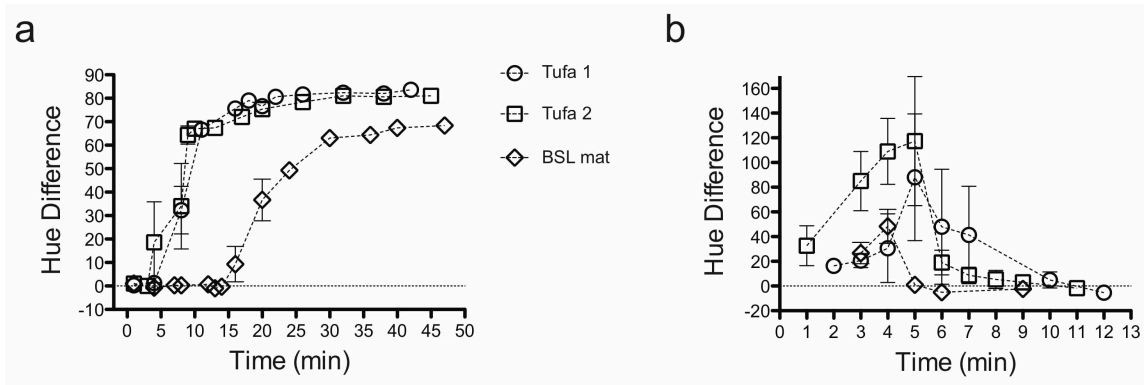


Figure B.S6. Field urease and CA hue differences. Hue difference of the assayed urease (a) and CA (b) compared to their respective negative inhibiting control over time. Samples correspond to the surficial biofilms of two subaqueous tufas, and lake mats that were not directly associated with the tufa carbonates (BSL mat). Error bars show the standard error of triplicate assays (unless the error is smaller than the symbols).

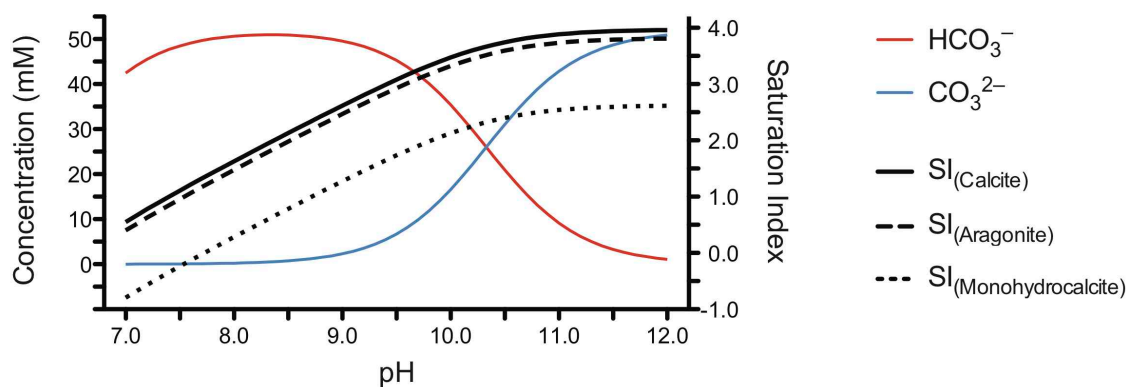


Figure B.S7. DIC speciation and carbonate mineral saturation indices in BSL.

Modeled DIC species concentration (left axis) and saturation index (right axis) as a function of pH at 15 °C. Assuming only fixed calcium (0.7 mM) and DIC (52 mM) concentrations in BSL waters, the saturation index (SI) for calcite, aragonite and monohydrocalcite increases linearly in the BSL pH water range (9.2–9.8), reaching a

plateau at waters above pH ~10.5. Solubility values were obtained from Plummer and Busenberg (1982), and Kralj and Brečević (1995).

B.2. Supplementary Tables

Table B.S1. Water chemistry parameters measured from spring water, tufa porewater (mixed water), and lake water at the site of tufa deposition and outside (nearshore) of the tufa formation site.

	Groundwater*		Tufa porewater ⁺		Lake water ⁺	Lake water (distance from sampled tufa)*		
	Spring 1	Spring 2	Tufa1 ^a	Tufa2 ^b	Outside tufas ^c	Proximal	Medial	Distal
Major anions (mM)[#]								
Br ⁻	BDL	BDL	0.062	0.138	0.175	0.346 ± 0.002	0.370 ± 0.002	0.373 ± 0.004
Cl ⁻	0.67 ± 0.003	0.45 ± 0.01	56.4	107.2	152.3	149.8 ± 1.1	173.0 ± 1.0	178.7 ± 1.5
F ⁻	0.04 ± 0.01	0.02 ± 0.004	0.15	BDL	BDL	1.30 ± 0.08	1.36 ± 0.06	1.30 ± 0.25
NO ₃ ⁻	0.064 ± 0.001	0.039 ± 0.0004	0.012	0.014	0.001	0.44 ± 0.05	0.45 ± 0.02	0.52 ± 0.10
NO ₂ ⁻	BDL	BDL	NA	NA	NA	BDL	BDL	BDL
PO ₄ ³⁻	BDL	0.0003 ± 0.0002	0.055	0.103	0.226	BDL	BDL	BDL
SO ₄ ²⁻	0.539 ± 0.002	0.366 ± 0.003	17.70	33.31	46.84	49.07 ± 0.28	55.55 ± 0.32	57.22 ± 0.37
Major cations (mM)[†]								
Ca ²⁺	0.25 ± 0.03	2.5 ± 2.2	0.34	0.25	0.35	0.95 ± 0.56	0.9 ± 1.2	1.2 ± 1.1
Mg ²⁺	0.21 ± 0.04	0.29 ± 0.12	1.81	2.51	4.52	7.2 ± 1.9	6.8 ± 0.4	7.3 ± 0.1
K ⁺	0.17 ± 0.01	0.24 ± 0.02	3.32	4.86	8.18	2.0 ± 1.6	2.4 ± 0.9	3.6 ± 2.3
Na ⁺	3.37 ± 0.11	2.69 ± 0.01	126.1	213.1	330.6	375.4 ± 16.3	404.4 ± 18.2	391.0 ± 80.1
Minor elements (μM)[†]								
Al	NA	NA	BDL	BDL	BDL	NA	NA	NA
Sb [§]	NA	NA	BDL	BDL	BDL	NA	NA	NA
As	1.5 ± 0.2	0.4 ± 0.1	7.7 [§]	11.2 [§]	20.0 [§]	29.1 ± 1.2	33.0 ± 1.4	32.6 ± 2.9
Ba	NA	NA	BDL	BDL	0.4	NA	NA	NA
Be	NA	NA	BDL	BDL	BDL	NA	NA	NA
Bi	NA	NA	BDL	BDL	BDL	NA	NA	NA
B	NA	NA	1480	2220	3700	NA	NA	NA
Cd	NA	NA	BDL	BDL	BDL	NA	NA	NA
Cr	NA	NA	BDL	BDL	BDL	NA	NA	NA
Co	NA	NA	BDL	BDL	BDL	NA	NA	NA
Cu	NA	NA	BDL	BDL	BDL	NA	NA	NA
Ga	NA	NA	BDL	BDL	BDL	NA	NA	NA
Fe	2.8 ± 1.4	20.1 ± 10.7	BDL	BDL	BDL	11.2 ± 5.0	6.8 ± 2.8	10.3 ± 8.2
Pb [§]	NA	NA	BDL	BDL	BDL	NA	NA	NA
Li	NA	NA	43.2	63.4	112.4	NA	NA	NA
Mn	0.02 ± 0.01	0.47 ± 0.44	BDL	BDL	BDL	0.27 ± 0.15	0.27 ± 0.09	0.18 ± 0.02

Mo	0.06 ± 0.02	0.09 ± 0.11	2.9	4.5	7.2	6.04 ± 0.37	6.81 ± 0.51	6.86 ± 0.76
Ni	NA	NA	BDL	BDL	BDL	NA	NA	NA
Sc	NA	NA	BDL	BDL	BDL	NA	NA	NA
Se [§]	NA	NA	BDL	BDL	BDL	NA	NA	NA
Si	NA	NA	324.0	302.6	338.2	NA	NA	NA
Ag	NA	NA	BDL	BDL	BDL	NA	NA	NA
Sr	NA	NA	5.6	7.5	12.6	NA	NA	NA
Sn	NA	NA	BDL	BDL	BDL	NA	NA	NA
Tl [§]	NA	NA	BDL	BDL	BDL	NA	NA	NA
Ti	NA	NA	BDL	BDL	BDL	NA	NA	NA
U	0.015 ± 0.001	0.010 ± 0.001	NA	NA	NA	0.37 ± 0.03	0.41 ± 0.03	0.42 ± 0.01
V	3.50 ± 0.08	2.08 ± 0.06	7.5	11.0	15.7	13.6 ± 0.9	14.6 ± 0.8	15.0 ± 0.6
Zn	NA	NA	BDL	BDL	BDL	NA	NA	NA
Other conditions								
DIC (mM)	2.806 ± 0.002	2.81 ± 0.01	34.5	52.0	70.2	40.0 ± 0.1	41.2 ± 0.1	46.4 ± 0.2
TDS (g/L)	0.334 ± 0.005	0.433 ± 0.10	8.59	14.83	21.54	21.71 ± 0.57	23.91 ± 0.58	24.40 ± 2.08
pH	9.91	9.22	9.86	9.57	9.57	9.24	9.24	9.25
DO (%)	83	89	NA	NA	NA	99.9	99.3	100.4
T (°C)	19.6	19	NA	NA	NA	18.8	19	19.2
Isotopic ratios[¶]								
δ ¹³ C	-11.16 ± 0.07	-10.99 ± 0.11	NA	NA	NA	-1.46 ± 0.08	-1.53 ± 0.06	-1.34 ± 0.06
δ ¹⁸ O	-11.22 ± 0.04	-11.96 ± 0.08	NA	NA	NA	-1.74 ± 0.19	-1.45 ± 0.04	NA
δ ² H	-89.2 ± 0.3	-92.7 ± 1.5	NA	NA	NA	-45.6 ± 0.9	-43.8 ± 0.4	NA

*Samples from June 2016. Measured in triplicate. Ranges indicate standard deviation.

⁺Samples from October 2018. Measurements from a single sample.

^aUSGS SITE ID: 393146118525401.

^bUSGS SITE ID: 393145118525501.

^cUSGS SITE ID: 393142118525601.

[#]Measured using ion exchange chromatography.

[†]Measured using ICP-AES, unless otherwise noted.

[§]Measured using ICP-MS.

[¶]VSMOW (O, H) and VPDB (C) corrected values.

BDL, below detectable limit; NA, not analyzed.

Table B.S2. Element quantification and isotopic ratios of carbonate tufa samples from transversal and longitudinal sections of a subaerial tufa.

	Tufa Base cross section					Tufa Side longitudinal section						
	1	2	3	4	5	1	2	3	4	5	6	7
Element concentration normalized to Ca + Mg (ppm)*												
Al	BDL	BDL	BDL	BDL	BDL	9.26	3.65	8.19	BDL	BDL	21.12	BDL
As	0.85	BDL	BDL	1.42	BDL	0.82	0.77	BDL	BDL	BDL	0.82	0.66
B	6.82	4.28	BDL	13.78	8.41	6.61	20.03	17.92	15.69	20.92	11.77	9.06
Ba	1.58	1.61	1.48	0.80	0.93	0.60	0.58	0.58	1.02	0.78	0.69	0.60
Be	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Ca	9604	9591	9539	9813	9976	9919	9864	9851	9842	9780	9920	9919
Cd	0.04	0.02	0.03	0.04	0.02	0.03	0.03	0.02	0.05	BDL	0.04	0.03
Co	0.09	0.17	0.30	0.22	BDL	0.05	BDL	BDL	0.22	0.18	BDL	0.17
Cr	BDL	BDL	BDL	BDL	BDL	0.06	BDL	BDL	BDL	BDL	0.06	0.12
Cu	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Fe	1.56	1.64	1.09	1.30	BDL	1.32	2.29	2.16	1.13	1.16	1.00	1.31
K	10.91	8.22	BDL	7.72	4.04	4.78	10.87	BDL	BDL	20.67	BDL	7.81
Li	0.16	0.22	0.22	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	0.17
Mg	198.5	206.1	230.1	93.2	12.3	40.8	68.5	74.8	79.6	110.2	40.3	40.9
Mn	7.29	7.47	4.54	1.77	1.05	0.87	0.95	1.07	1.13	1.11	1.01	0.81
Na	77.70	70.81	58.56	87.06	75.47	70.3	83.28	61.90	69.26	167.1	66.21	95.0
Ni	0.11	0.11	0.19	0.19	BDL	0.08	BDL	0.11	0.22	0.19	0.08	0.31
P	20.71	18.85	25.17	31.87	64.20	35.3	31.92	28.15	30.19	28.01	38.54	32.8
Pb	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
S	345.4	372.8	287.5	224.9	220.2	219	217.9	220	229.8	247.2	219.2	220
Se	BDL	BDL	BDL	BDL	BDL	6.12	BDL	BDL	BDL	BDL	BDL	BDL
Si	8.85	5.03	6.88	15.59	3.33	10.2	80	43.48	30.30	35.89	25.32	18.2
Sr	34.73	37.75	38.35	28.47	23.86	23.5	22.95	24.24	33.87	25.60	24.18	23.1
Tl	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
V	1.24	1.18	1.05	1.55	BDL	1.17	1.36	2.24	0.92	0.73	0.48	0.75
Zn	1.80	1.01	1.42	2.00	1.02	1.31	1.52	1.66	1.99	2.75	1.65	1.39
Sn	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Mo	BDL	BDL	BDL	BDL	BDL	BDL	0.34	BDL	BDL	BDL	BDL	BDL
Sb	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Ti	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Isotopic ratios⁺												
$\delta^{13}\text{C}$	-0.12 ± 0.01	-0.12 ± 0.01	0.25 ± 0.01	1.03 ± 0.01	1.44 ± 0.01	1.81 ± 0.01	1.6 ± 0.004	1.55 ± 0.01	0.97 ± 0.01	0.78 ± 0.01	1.68 ± 0.01	1.56 ± 0.01
$\delta^{18}\text{O}$	-7.80 ± 0.08	-7.25 ± 0.02	-6.02 ± 0.03	-4.10 ± 0.04	-0.85 ± 0.06	-0.51 ± 0.01	-1.51 ± 0.02	-2.63 ± 0.02	-4.50 ± 0.02	-5.35 ± 0.02	-1.27 ± 0.04	-1.78 ± 0.03

*Measured using ICP-AES from a single sample per site.

⁺VPDB corrected values. BDL: below detectable limit.

Table B.S3. List of the metabolic pathways and their representative proteins (showing their corresponding orthologs) shown in Figure 6, along with their identified counts obtained by shotgun metagenomic sequencing of surficial and endolithic tufa biofilm samples.

KEGG orthology entry	Name	Definition	Enzyme Commission number (EC)	Surficial Sample	Endolithic Sample
Methane oxidation, KEGG module M00174 and pathway ko00680					
MMO; Methane monooxygenase					
K10944	pmoA-amoA	Methane/ammonia monooxygenase subunit A	1.14.18.3 1.14.99.39	0	0
K10945	pmoB-amoB	Methane/ammonia monooxygenase subunit B		0	0
K10946	pmoC-amoC	Methane/ammonia monooxygenase subunit C		0	0
K16157	mmoX	Methane monooxygenase component A alpha chain	1.14.13.25	0	0
K16158	mmoY	Methane monooxygenase component A beta chain	1.14.13.25	0	0
K16159	mmoZ	Methane monooxygenase component A gamma chain	1.14.13.25	0	0
K16160	mmoB	Methane monooxygenase regulatory protein B		0	0
K16161	mmoC	Methane monooxygenase component C	1.14.13.25	1	7
K16162	mmoD	Methane monooxygenase component D		0	0
MDH; Methanol dehydrogenase					
K14028	mdh1, mxaF	Methanol dehydrogenase (cytochrome c) subunit 1	1.1.2.7	0	5
K14029	mdh2, mxaI	Methanol dehydrogenase (cytochrome c) subunit 2	1.1.2.7	0	0
K16254	mxaj	Methanol oxidation protein MxaJ		0	0
K16255	mxag	Cytochrome c-L		0	0
K16256	mxaa	MxaA protein		0	0
K16257	mxac	MxaC protein		0	0
K16258	mxak	MxaK protein		0	0
K16259	mxal	MxaL protein		0	0
K16260	mxad	MxaD protein		0	0
K17066	MOX	Alcohol oxidase	1.1.3.13	0	0
K00093	mdh	Methanol dehydrogenase	1.1.1.244	0	8
FalDH; Formaldehyde dehydrogenase					
K00148	fdhA	Glutathione-independent formaldehyde dehydrogenase	1.2.1.46	0	0
Gfa; Glutathione-dependent formaldehyde-activating enzyme					
K03396	gfa	S-(hydroxymethyl)glutathione synthase	4.4.1.22	4	4
AdhC; S-(hydroxymethyl)glutathione dehydrogenase					
K00121	frmA, ADH5, adhC	S-(hydroxymethyl)glutathione dehydrogenase / alcohol dehydrogenase	1.1.1.284 1.1.1.1	9	33
FghA; S-formylglutathione hydrolase					
K01070	frmB, ESD, fghA	S-formylglutathione hydrolase	3.1.2.12	6	19
FDH; Formate dehydrogenase					
K00123	fdoG, fdhF, fdwA	Formate dehydrogenase major subunit	1.17.1.9	2	9

K00124	fdoH, fdsB	Formate dehydrogenase iron-sulfur subunit		0	0
K00125	fdhB	Formate dehydrogenase (coenzyme F420) beta subunit	1.17.98.3 1.8.98.6	1	6
K00126	fdsD	Formate dehydrogenase subunit delta	1.17.1.9	0	0
K00127	fdoI, fdsG	Formate dehydrogenase subunit gamma		0	3
K22515	fdwB	Formate dehydrogenase beta subunit	1.17.1.9	0	0
K22516	fdhA	Formate dehydrogenase (coenzyme F420) alpha subunit	1.17.98.3 1.8.98.6	2	1
Photosynthesis and antenna protein KEGG pathways ko00195 and ko00196; Calvin Cycle Module M00165					
PSII; Photosystem II subunits					
K02703	psbA	Photosystem II P680 reaction center D1 protein	1.10.3.9	12	19
K02706	psbD	Photosystem II P680 reaction center D2 protein	1.10.3.9	7	11
K02705	psbC	Photosystem II CP43 chlorophyll apoprotein		7	19
K02704	psbB	Photosystem II CP47 chlorophyll apoprotein		7	12
K02707	psbE	Photosystem II cytochrome b559 subunit alpha		5	9
K02708	psbF	Photosystem II cytochrome b559 subunit beta		4	10
K02713	psbL	Photosystem II PsbL protein		1	3
K02711	psbJ	Photosystem II PsbJ protein		3	6
K02709	psbH	Photosystem II PsbH protein		4	8
K02710	psbI	Photosystem II PsbI protein		1	6
K02712	psbK	Photosystem II PsbK protein		2	8
K02714	psbM	Photosystem II PsbM protein		1	4
K02718	psbT	Photosystem II PsbT protein		2	6
K02724	psbZ	Photosystem II PsbZ protein		4	7
K02723	psbY	Photosystem II PsbY protein		3	9
K08902	psb27	Photosystem II Psb27 protein		1	8
K08903	psb28	Photosystem II 13kDa protein		9	8
K02716	psbO	Photosystem II oxygen-evolving enhancer protein 1		2	7
K02717	psbP	Photosystem II oxygen-evolving enhancer protein 2		0	0
K02720	psbV	Photosystem II cytochrome c550		4	11
K02719	psbU	Photosystem II PsbU protein		3	10
K02722	psbX	Photosystem II PsbX protein		2	4
K08904	psb28-2	Photosystem II Psb28-2 protein		0	0
PSI; Photosystem I subunits					
K02689	psaA	Photosystem I P700 chlorophyll a apoprotein A1		6	10
K02690	psaB	Photosystem I P700 chlorophyll a apoprotein A2		18	18
K02691	psaC	Photosystem I subunit VII		5	9
K02692	psaD	Photosystem I subunit II		6	7
K02693	psaE	Photosystem I subunit IV		4	9
K02694	psaF	Photosystem I subunit III		4	8
K02696	psaI	Photosystem I subunit VIII		4	8
K02697	psaJ	Photosystem I subunit IX		4	9
K02698	psaK	Photosystem I subunit X		5	14

K02699	psaL	Photosystem I subunit XI		3	7
K02700	psaM	Photosystem I subunit XII		4	5
K02702	psaX	Photosystem I 4.8kDa protein		1	2
Cyt b6f; Cytochrome b6/f complex					
K02634	petA	Apocytochrome f		9	18
K02635	petB	Cytochrome b6		12	29
K02636	petC	Cytochrome b6-f complex iron-sulfur subunit	7.1.1.6	11	32
K02637	petD	Cytochrome b6-f complex subunit 4		4	8
K02640	petG	Cytochrome b6-f complex subunit 5		2	7
K02642	petL	Cytochrome b6-f complex subunit 6		1	6
K02643	petM	Cytochrome b6-f complex subunit 7		4	7
K03689	petN	Cytochrome b6-f complex subunit 8		0	0
PC; Plastocyanin					
K02638	petE	Plastocyanin		2	14
Fd; Ferredoxin					
K02639	petF	Ferredoxin		1	6
FNR; Ferredoxin-NADP⁺ reductase					
K02641	petH	Ferredoxin-NADP ⁺ reductase	1.18.1.2	27	42
AP; Allophycocyanin					
K02092	apcA	Allophycocyanin alpha subunit		4	11
K02093	apcB	Allophycocyanin beta subunit		5	12
K02094	apcC	Phycobilisome core linker protein		3	7
K02095	apcD	Allophycocyanin-B		4	15
K02096	apcE	Phycobilisome core-membrane linker protein		3	12
K02097	apcF	Phycobilisome core component		0	0
P; Phycocyanin/Phycocerythrocyanin/Phycocerythrin					
K02284	cpcA	Phycocyanin alpha chain		3	5
K02285	cpcB	Phycocyanin beta chain		1	7
K02286	cpcC	Phycocyanin-associated rod linker protein		1	8
K02287	cpcD	Phycocyanin-associated, rod		2	6
K02288	cpcE	Phycocyanobilin lyase subunit alpha	4.4.1.32	1	8
K02289	cpcF	Phycocyanobilin lyase subunit beta	4.4.1.32	1	7
K20713	rpcG	R-phycocyanin alpha-cysteine-84 phycocourobilin lyase/isomerase	4.4.1.33	0	0
K02290	cpcG	Phycobilisome rod-core linker protein		4	18
K02628	pecA	Phycocerythrocyanin alpha chain		0	0
K02629	pecB	Phycocerythrocyanin beta chain		0	2
K02630	pecC	Phycocerythrocyanin-associated rod linker protein		2	7
K02631	pecE	Phycocerythrocyanin alpha-cysteine-84 phycocourobilin lyase/isomerase subunit PecE	4.4.1.31	2	5
K02632	pecF	Phycocerythrocyanin alpha-cysteine-84 phycocourobilin lyase/isomerase subunit PecF	4.4.1.31	1	7
K05376	cpeA, mpeA	Phycocerythrin alpha chain		1	1
K05377	cpeB, mpeB	Phycocerythrin beta chain		1	1

K05378	cpeC, mpeC	Phycoerythrin-associated linker protein		1	1
K05379	cpeD, mpeD	Phycoerythrin-associated linker protein		0	1
K05380	cpeE	Phycoerythrin-associated linker protein		0	0
K05381	cpeR	Phycoerythrin-associated linker protein		0	0
K05382	cpeS	Phycoerythrin-associated linker protein		1	8
K05383	cpeT	CpeT protein		4	5
K05384	cpeU, mpeU	Bilin biosynthesis protein		0	0
K05385	cpeY	Bilin biosynthesis protein		0	0
K05386	cpeZ	Bilin biosynthesis protein		0	0
RuBisCo; ribulose-bisphosphate carboxylase large chain					
K01601	rbcl	Ribulose-bisphosphate carboxylase large chain	4.1.1.39	6	19
K01602	rbcS	Ribulose-bisphosphate carboxylase small chain	4.1.1.39	7	11
PGK; Phosphoglycerate kinase					
K00927	PGK, pgk	Phosphoglycerate kinase	2.7.2.3	15	32
GAPDH; Glyceraldehyde 3-phosphate dehydrogenase					
K00134	GAPDH, gapA	Glyceraldehyde 3-phosphate dehydrogenase	1.2.1.12	22	61
K00150	gap2	Glyceraldehyde-3-phosphate dehydrogenase (NAD(P))	1.2.1.59	2	8
K05298	GAPA	Glyceraldehyde-3-phosphate dehydrogenase (NADP ⁺) (phosphorylating)	1.2.1.13	0	0
ALDO; Fructose-bisphosphate aldolase					
K01623	ALDO	Fructose-bisphosphate aldolase, class I	4.1.2.13	10	22
K01624	FBA, fbaA	Fructose-bisphosphate aldolase, class II	4.1.2.13	6	10
FBP; Fructose-1,6-bisphosphatase					
K03841	FBP, fbp	Fructose-1,6-bisphosphatase I	3.1.3.11	25	22
K02446	glpX	Fructose-1,6-bisphosphatase II	3.1.3.11	0	0
K04041	fbp3	Fructose-1,6-bisphosphatase III	3.1.3.11	0	2
SEBP; Sedoheptulose-1,7-bisphosphatase					
K11532	glpX-SEBP	Fructose-1,6-bisphosphatase II / sedoheptulose-1,7-bisphosphatase	3.1.3.113.1.3.37	3	7
K01086	fbp-SEBP	Fructose-1,6-bisphosphatase I / sedoheptulose-1,7-bisphosphatase	3.1.3.113.1.3.37	0	0
K01100	SBPase	Sedoheptulose-bisphosphatase	3.1.3.37	0	0
TK; Transketolase					
K00615	tktA, tktB	Transketolase	2.2.1.1	15	49
RPI; Ribose 5-phosphate isomerase					
K01807	rpiA	Ribose 5-phosphate isomerase A	5.3.1.6	7	22
K01808	rpiB	Ribose 5-phosphate isomerase B	5.3.1.6	8	7
PRK; Phosphoribulokinase					
K00855	PRK, prkB	Phosphoribulokinase	2.7.1.19	7	18
Active calcium and Bicarbonate transport, CO₂ hydration					
ChaA; Ca²⁺:H⁺ antiporter					
K07300	chaA, CAX	Ca ²⁺ :H ⁺ antiporter		4	4
SulP; Bicarbonate:Na⁺ symporter					

K03321	TC.SULP, BicA	Sulfate permease, SulP family, SulP-type bicarbonate transporter		17	32
Cmp; ABC-type bicarbonate transporter					
K11950	cmpA	Bicarbonate transport system substrate-binding protein		1	3
K11951	cmpB	Bicarbonate transport system permease protein		8	27
K11952	cmpC	Bicarbonate transport system ATP-binding protein	3.6.3.-	7	24
K11953	cmpD	Bicarbonate transport system ATP-binding protein	3.6.3.-	5	25
CA; carbonic anhydrase					
K01673	cynT, can	Beta-class carbonic anhydrase	4.2.1.1	9	20
K01674	CA, cah	Alpha-class carbonic anhydrase	4.2.1.1	1	4
K01743	CA	Gamma-class carbonic anhydrase	4.2.1.1	0	0
Ureolysis					
UT; Urea transporter					
K03191	ureI	UreI; acid-activated urea channel		0	1
K08717	utp	Urea transporter		1	0
K11959	urtA	urtA; urea transport system substrate-binding protein		0	0
K11960	urtB	urtB; urea transport system permease protein		0	0
K11961	urtC	urtC; urea transport system permease protein		0	0
K11962	urtD	urtD; urea transport system ATP-binding protein		0	0
K11963	urtE	urtE; urea transport system ATP-binding protein		0	0
UreEDFG; Urease accessory proteins					
K03187	ureE	Urease accessory protein UreE		3	12
K03188	ureF	Urease accessory protein UreF		1	7
K03189	ureG	Urease accessory protein UreG		2	11
K03190	ureD, ureH	Urease accessory protein UreD		2	9
HypAB; Hydrogenase/Urease maturation factor HypAB					
K04651	hypA, hybF	Hydrogenase/Urease nickel incorporation protein HypA/HybF		5	17
K04652	hypB	Hydrogenase/Urease nickel incorporation protein HypB		3	19
UreABC; Urease subunits					
K01428	ureC	Urease subunit alpha	3.5.1.5	5	16
K01429	ureB	Urease subunit beta	3.5.1.5	2	10
K01430	ureA	Urease subunit gamma	3.5.1.5	3	9
Assimilatory nitrate reduction, KEGG module M00531					
NarK; NarK-like Nitrate channels					
K02575	NRT, narK, nrtP	MFS transporter, NNP family, nitrate/nitrite transporter		0	5
NarB, NasAB; Nitrate reductase					
K00367	narB	Ferredoxin-nitrate reductase	1.7.7.2	2	7
K10534	NR	Nitrate reductase (NAD(P)H)	1.7.1.1 1.7.1.2 1.7.1.3	0	0
K00372	nasA	Assimilatory nitrate reductase catalytic subunit	1.7.99.-	0	5
K00360	nasB	Assimilatory nitrate reductase electron transfer subunit	1.7.99.-	0	1
NirA, NIT-6; Nitrite reductase					

K00366	nirA	Ferredoxin-nitrite reductase	1.7.7.1	0	0
K17877	NIT-6	Nitrite reductase (NAD(P)H)	1.7.1.4	6	5
Dissimilatory nitrate reduction, KEGG module M00530					
NapABC; Periplasmic Nitrate reductase					
K02567	napA	Periplasmic nitrate reductase NapA	1.7.99.-	2	6
K02568	napB	Cytochrome c-type protein NapB		0	1
K02569	napC	Cytochrome c-type protein NapC		0	1
NarGHIJ; Nitrate reductase					
K00370	narG, narZ, nxA	Nitrate reductase / nitrite oxidoreductase, alpha subunit	1.7.5.11.7.99.-	2	7
K00371	narH, narY, nxB	Nitrate reductase / nitrite oxidoreductase, beta subunit	1.7.5.11.7.99.-	0	4
K00373	narJ, narW	Nitrate reductase molybdenum cofactor assembly chaperone NarJ/NarW		1	3
K00374	narI, narV	Nitrate reductase gamma subunit	1.7.5.1 1.7.99.-	0	0
NirBD, NrfAH; Nitrite reductase					
K00362	nirB	nitrite reductase (NADH) large subunit	1.7.1.15	0	2
K00363	nirD	nitrite reductase (NADH) small subunit	1.7.1.15	0	1
K03385	nrfA	Nitrite reductase (cytochrome c-552)	1.7.2.2	0	10
K15876	nrfH	Cytochrome c nitrite reductase small subunit		0	10

B.3. References for The Supplementary Material

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Appendix C

Supporting Information for Chapter 4

C.1. Supplementary Tables

Table C.S1: Mass chromatographic quantification of acyclic isoprenoids from the oil sample #2 using the m/z 113 ion response.

Compound	Retention Time	Area	Height	mg/L	mg/g
iC11	30.35	296989	76445	222	0.26
iC13	41.47	447284	112726	335	0.40
iC14	44.46	1274419	305324	954	1.14
iC15	49.28	995905	215981	745	0.89
iC16	53.00	1357118	272121	1016	1.21
iC18	60.39	1335273	218551	999	1.19
iC19	62.58	3515608	526607	2631	3.14
iC20	66.22	1667033	275192	1248	1.49
iC21	68.97	556021	94089	416	0.50
iC22	71.92	220417	33985	165	0.20
iC23	74.82	407350	61663	305	0.36
iC24	76.52	348647	56355	261	0.31
iC25	79.54	348042	51291	260	0.31
iC26	81.82	244925	33350	183	0.22
iC28	86.64	317287	26284	237	0.28
iC29	88.07	261714	25701	196	0.23
iC30	90.63	688857	95939	516	0.61
iC31	92.55	193462	15869	145	0.17
iC33	96.68	160649	12613	120	0.14
iC34	97.89	182037	13609	136	0.16
iC35	99.70	54651	5608	41	0.05
iC35	100.12	152670	12510	114	0.14
iC36	101.79	113116	7927	85	0.10
iC37	103.39	82812	5453	62	0.07
iC38	105.45	122002	7115	91	0.11

iC38	104.48	104957	7282	79	0.09
iC39	106.65	169576	10437	127	0.15
iC40	109.05	105127	4460	79	0.09

C.2. Supplementary Figures

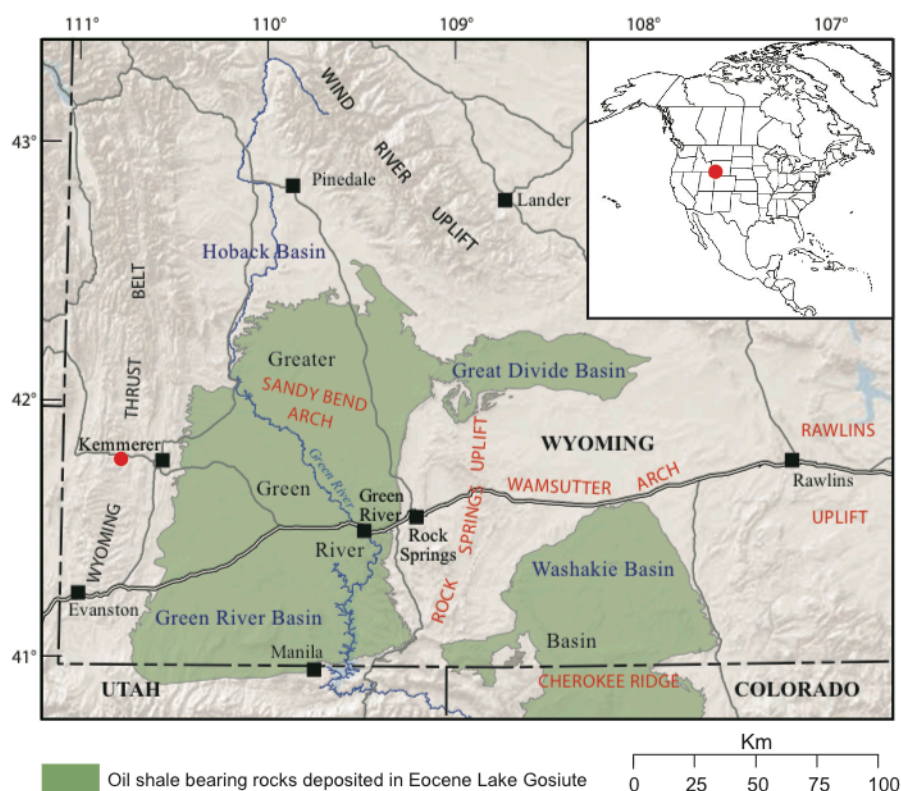


Figure C.S1. Sampling site location. Map of the Green River Basin indicating the location of the kerogen-rich rock samples (red dot; Lat. 41.798; Lon. -110.732) from the Green River Formation taken at Fossil Basin, Wyoming (modified from Mercier and Johnson 2012).

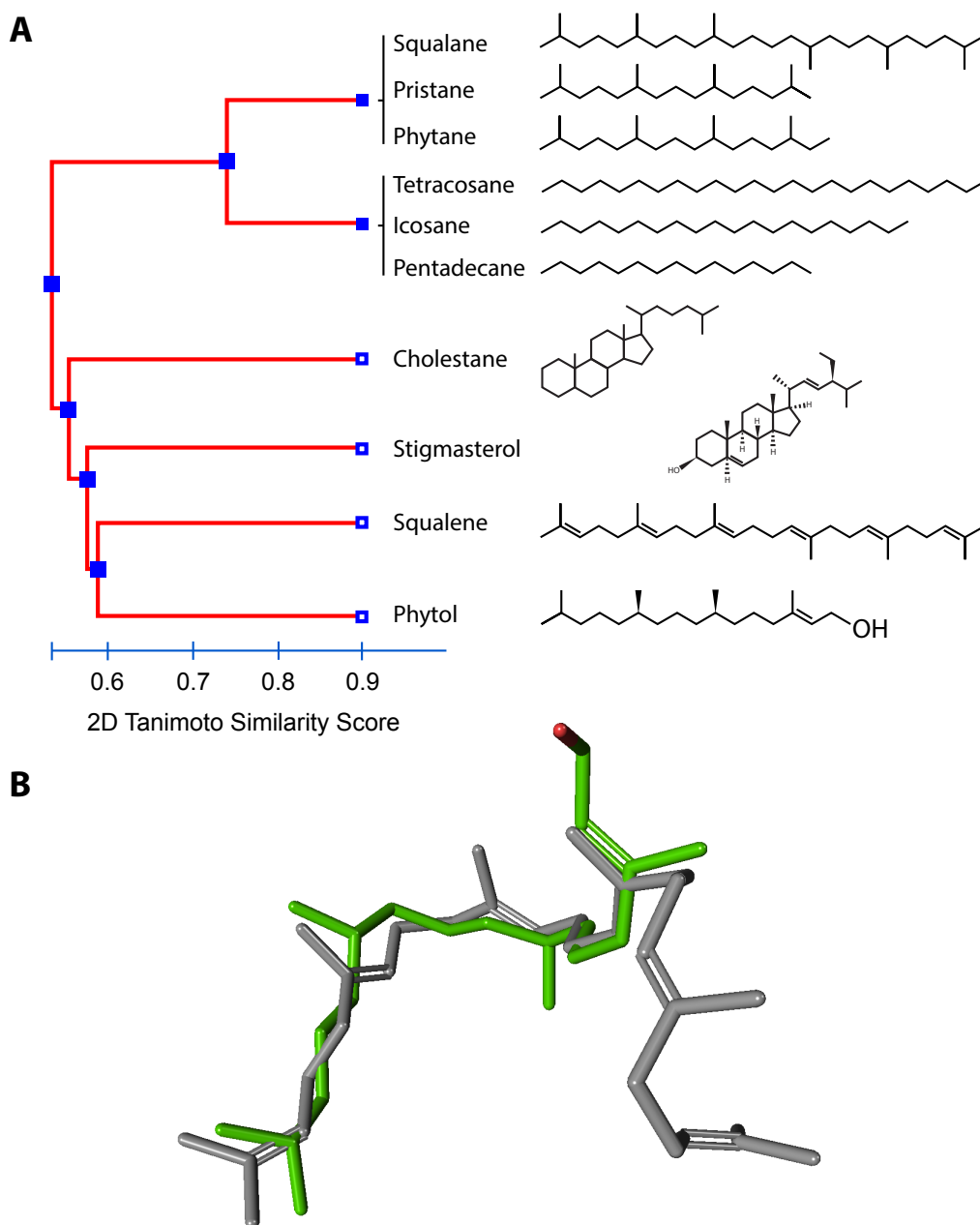


Figure C.S2. Chemical structure of relevant hydrocarbons used in this study. (A) Wire-frame formulae representation of the *n*-alkanes tetracosane (C₂₄), icosane (C₂₀) and pentadecane (C₁₅), the linear isoprenoids squalane (C₃₀), squalene (C₃₀), pristane (C₁₉), phytane (C₂₀) and phytol (C₂₀), and the cyclic isoprenoids cholestane (C₂₇) and stigmasterol (C₂₉) clustered based on the Tanimoto structure similarity score calculated from their two-

dimensional structure fingerprints. Cluster representation was performed using the online structure clustering tool from The PubChem Project. (B) Visualization of one possible three-dimensional arrangement of superimposed conformers of squalene (grey) and phytol (green). In this conformer superposition case, the phytol's hydroxyl group (red) is positioned outside the main hydrocarbon chain of squalene that likely fits the paratope of the anti-squalene antibody. The representation was created using PubChem3D, yielding a shape similarity score of 52% (Bolton et al., 2011). PubChem database unique chemical structure identifiers CID are: phytol, 5280435; squalene, 638072; squalane, 8089; pristane, 15979; phytane, 12523; tetracosane, 12592; icosane, 8222; pentadecane, 12391; cholestane, 2723895; stigmasterol, 5280794 (Kim et al., 2016).

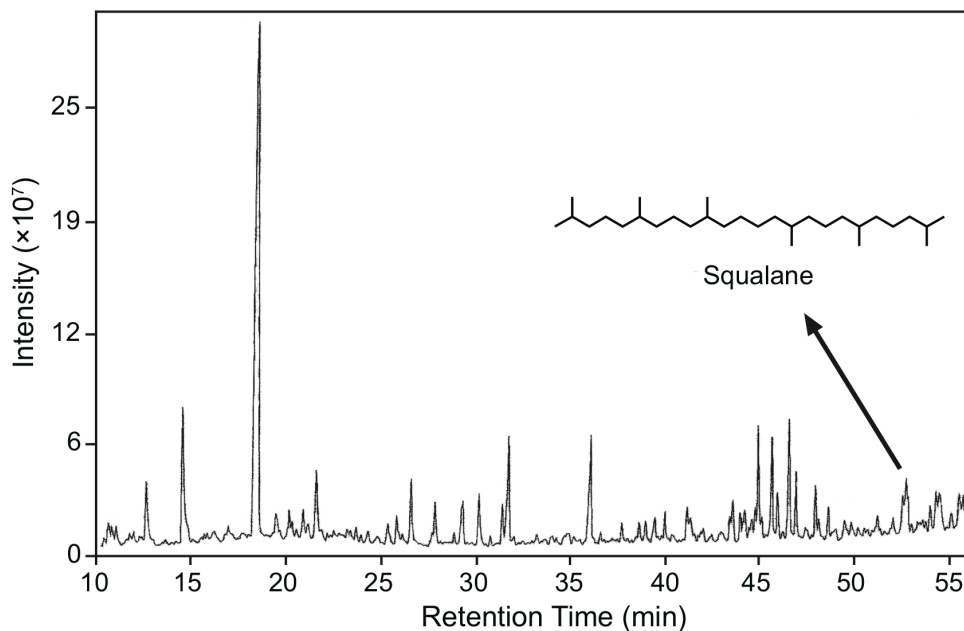


Figure C.S3. Squalane analysis in the slab sections from the Green River Formation. The mass chromatogram of extractable hydrocarbons indicates the presence of squalane at relatively low concentration of ~100 ppb (0.1 $\mu\text{g/g}$).

C.3. References for The Supplementary Material

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