

Biosurfactant and soil wetting bacteria amendments for
enhancing water retention in agricultural soils

by

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B.S., University of California, Riverside, 1996
M.S., Kansas State University, 2019

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

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KANSAS STATE UNIVERSITY
Manhattan, Kansas

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Abstract

Climate-driven drought and rising global population are driving increased demand for freshwater resources, particularly in agriculture. In the U.S. Central High Plains, irrigation increasingly relies on the rapidly depleting Ogallala Aquifer, highlighting the urgent need for sustainable soil and water management strategies. This dissertation investigates a microbial and biochemical approach to improving soil water retention, focusing on the biosurfactant-producing bacterium *Bacillus subtilis* and its secondary metabolite, surfactin. These agents offer a potentially sustainable, environmentally responsible approach to soil treatment in order to eliminate soil water repellency, enhance water retention, and improve agricultural water use efficiency, particularly in arid and semi-arid climates.

The research begins by reviewing the physicochemical basis of soil hydrophobicity, a key limitation to water infiltration and availability. The literature identifies amphiphilic organic compounds as a primary contributor to water-repellent soil, which becomes more pronounced under dry conditions. While synthetic surfactants can be added to the soil to transition these hydrophobic layers to hydrophilic layers, their environmental and economic drawbacks have prompted interest in microbially-produced biosurfactants like surfactin, which have high surface activity, low toxicity, and are stable under diverse conditions. The effect of soil texture in response to surfactin treatment is first presented. Soil textures were systematically varied by mixing sand and silty clay loam soils together at varied ratios, achieving mineralogically equivalent soils with a range of different textures. Contact angle measurements and dryout experiments revealed that soils with sandy loam textures, where water retention is often most problematic, were most responsive with respect to water retention improvement in lab-scale studies. Next, two delivery methods are discussed: (1) direct application of surfactin and (2) indirect delivery via *B. subtilis* inoculation. The findings demonstrate that *B. subtilis* inoculation can also reduce contact angles and enhance soil water retention. Experimental data and system dynamics modeling are combined to show that treated soils retain moisture longer, reduce evaporation under dry conditions, and maintain water availability through rainy cycles. Finally, the impact of amending the soil with nitrogen-based soil enrichments, such as NH_4NO_3 , to further promote *B. subtilis*-mediated soil water retention is reported in Chapter 5.

The final portion of this thesis describes the stable encapsulation of *B. subtilis* in polyethylene glycol (PEG) based hydrogels. This technique has the potential to enhance bacterial survival by forming a protective barrier around cells and to enhance biosurfactant production by localizing bacterial together. Bacteria encapsulation into PEG-based hydrogels was investigated for different step-growth polymerization chemistries that use Michael-type addition reactions for hydrogel crosslinking and PEG monomers of varied molecular weights. The survival and morphology of bacteria within the hydrogels were found to be dependent on crosslinking chemistry and monomer size. Microscopic analysis showed that thiol-acrylate crosslinking chemistries were most conducive to cell viability and single-cell encapsulation and that smaller mesh sizes also favored single-cell encapsulation. Other crosslinking chemistries favored encapsulation of aggregated cells and lower cell viability. These biohybrid systems have the potential to offer controlled and sustained microbial activity in various environments, including the soil.

In summary, this research advances bioinoculants towards the goal of improving water use efficiency in agriculture. While further field validation is needed, particularly with respect to biosurfactant production kinetics and soil microbiome interactions, this work lays a foundation for integrating microbial inoculants into sustainable, climate-resilient agricultural practices that can advance global food supply and freshwater security.

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Table of Contents

List of Figures	xii
List of Tables	xvii
Acknowledgments.....	xviii
Dedication	xx
Chapter 1 - Introduction.....	1
1.1 Characterization of Soil Water Repellency and Retention	4
1.1.1 Soil Water Repellency (SWR).....	4
1.1.2 Soil Water Content (SWC) and Wettability.....	5
1.2 The Use of Surfactants.....	7
1.2.1 Impact of the Surfactant on the Environment	8
1.3 The Use of Biosurfactants.....	9
1.4 Microorganisms Used in Soils	12
1.5 Using hydrogel for <i>in situ</i> applications.....	13
Chapter 2 - Objectives	15
2.1 Background	15
2.2 Motivation.....	15
Specific Aim One:.....	16
Specific Aim Two:.....	17
Specific Aim Three:.....	17
Specific Aim Four:.....	18
Chapter 3 - Impact of Soil Texture on Biosurfactant-Mediated Soil Wetting and Water	
Retention.....	19
Overview	19
3.1 Introduction.....	21
3.2 Materials and Methods.....	22
3.2.1 Soil Sampling and Preparation and Surfactin Amendment	22
3.2.2 Contact Angle and Dryout Experiments	23
3.2.3 Statistical Analysis.....	24
3.3 Results and Discussion	24

3.4 Conclusion	28
3.5 Acknowledgment	28
Chapter 4 - Effects of <i>Bacillus subtilis</i> and Surfactin on Evaporation Dynamics in Loam and Sandy Loam Soils	29
Overview	29
4.1 Introduction.....	31
4.1.1 Soil amendments for improved soil water retention	32
4.1.2 Synthetic Surfactants as Agricultural Wetting Agents	33
4.1.3 Biosurfactants as Agricultural Wetting Agents	34
4.1.4 Research objectives.....	36
4.2 Experimental Setup and Apparatus.....	36
4.2.1 Soils, Sampling, and Storage	36
4.2.2 Sample Preparation	36
4.2.3 Experimental Apparatus.....	38
4.2.4 Estimating Evaporation Rates and Soil Moisture Threshold	39
4.3 Results and Discussion	41
4.3.1 Soil Drying Experimental and Determination of Evaporation Rates.....	41
4.3.2 Evaporation Rates and Soil Moisture Threshold	44
4.3.3 Soil Moisture Simulations Using a System Dynamic Model	48
4.4 System Dynamic Model Results	51
4.5 Conclusions	54
4.6 Nomenclature	56
4.6.1 Variables	56
4.6.2 Subscripts	57
4.6.3 Symbols.....	57
4.7 Acknowledgments.....	57
Chapter 5 - Microbial Amendments Co-inoculated with Inorganic Nitrogen for Improved Soil Water Management.....	58
Overview	58
5.1 Introduction.....	59
5.2. Materials and Methods.....	61

5.2.1 Soil Samples.....	61
5.2.3 Evaporation Rate Experiments	62
5.2.4 SWC Measurements Using Different Cell Densities.....	63
5.2.5 Measurements of Nitrogen Extraction and Electroconductivity.....	65
5.2.5.1 Sample Preparation	65
5.2.5.2 KCl Nitrogen Extraction	65
5.2.5.3 Mesuaring the NH_3^+ , NO_3^- , NO_2^- using a Lachat Instrument	66
5.2.5.4 Electroconductivity (EC)	66
5.2.6 Data Analysis	67
5.3 Results and Discussion	67
5.3.1 Bacterial Growth Curves.....	67
5.3.2 Evaporation Rate Kinetics and SWC	70
5.3.3 Results on Consumption of Nitrates and Ammonium of the Amended Soils	78
5.4 Conclusion	81
5.5 Acknowledgments.....	82
Chapter 6 - Bacteria Encapsulation into Polyethylene Glycol Hydrogels Using Michael-Type	
Addition Reactions	83
Overview	83
6.1 Introduction.....	84
6.2 Materials and Methods.....	86
6.2.1 Preparation of hydrogel precursor solutions and cell encapsulation	86
6.2.2 Characterization of Cell Morphology and Viability	87
6.2.3 Bacteria Growth Kinetics.....	87
6.2.4 Statistical analysis.....	87
6.3 Results and Discussion	88
6.4 Conclusions.....	93
6.5 Acknowledgments.....	93
Chapter 7 - Encapsulation and Growth of Encapsulated <i>Bacillus subtilis</i> in PEG Hydrogels of	
Varied Mesh Sizes	94
Overview	94
7.1 Introduction.....	95

7.2 Materials and Methods.....	96
7.2.1 Hydrogel preparation and cell encapsulation.....	96
7.2.2 Live-dead assay.....	97
7.2.3 Bacteria Growth Tracking.....	98
7.2.4 Bacteria Growth Curve Analysis Determining Phase Shifts	98
7.2.5 Image Processing and Data.....	99
7.2.6 Statistical Analysis.....	100
7.3 Results and Discussion	100
7.4 Conclusion	105
7.5 Acknowledgments.....	106
Chapter 8 - Summary and Future Works	107
8.1 Summary and Future Work.....	107
8.2 Other Alternatives and <i>In Situ</i> Application.....	113
Chapter 9 - Reference	115
Appendix A - Acronyms	134
Appendix B - Supporting Information for Chapter 3.....	135
B.1 Preparation Methods	135
B.1.1 Soils and Sampling.....	135
B.1.2 Sample Mixing and Preparation.....	135
B.1.3 Surfactant Solutions Preparation.....	135
B.2 Data Tables.....	137
B.3 Soil Water Content (SWC) Curves	137
Appendix C - Supporting Information for Chapter 4.....	139
C.1 Determining the Soil Maximum Water Holding Capacity	139
C.2 System Dynamics Model parameterization.	140
Appendix D - Supporting Information for Chapter 5	141
Appendix E - Supporting Information for Chapter 6.....	146
E.1 Live-Dead assay calibration curve	146
Appendix F - Supporting Information for Chapter 7	149
Appendix G - Supporting Information for Chapter 8	150

List of Figures

Figure 1.1. Mechanisms of how amphiphilic molecules interact with soil particles by anchoring their hydrophobic, non-polar tails to the soil surface. This exposes the polar, hydrophilic ends of the molecules, allowing them to attract and retain water molecules as the soil dries. This dual affinity helps maintain moisture within the soil structure, playing a crucial role in soil-water interactions.	5
Figure 1.2 Molecular structure of surfactin, illustrating the amphiphilic properties.	10
Figure 3.1 Graphical Abstract for Impact of Soil Texture on Surfactin-Mediated Soil Wetting and Water Retention	20
Figure 3.2 CA measurements for soil samples treated with (A) 0 mg kg ⁻¹ , (B) 25 mg kg ⁻¹ , and (C) 50 mg kg ⁻¹ of surfactin. Textures connected with (*) are significantly different, $p < 0.05$. .	25
Figure 3.3 Evaporation rates (ξ) versus SWC (θ) during dryout experiments for (A) Sand(4):SCL(0) (sand texture), (B) Sand(3):SCL(1) (loamy sand texture), (C) Sand(2):SCL(2) (sandy loam texture), (D) Sand(1):SCL(3), (loam texture), and (E) Sand(0):SCL(4) (SCL texture).....	27
Figure 4.1. Diagram of the evaporation rate experimental apparatus.	38
Figure 4.2. Observed data showing loss of water mass over time for the three replicates in each combination of soil type and treatment. The inflection points for each regression line were chosen by minimizing the RMSE, the square root of the difference between observed and predicted values, across all values of the <i>post_{idt}</i> variable in Equation 4.1.	42
Figure 4.3. Effect of surfactin and <i>B. subtilis</i> treatment on constant and slower evaporation rates by soil type. Evaporation rates were calculated by converting the mass of water lost in five-minute intervals into millimeters of water evaporated per day. Observed evaporation rates display a 90-minute moving average to smooth extreme values.	45
Figure 4.4. System dynamic model of soil moisture during a season. Random precipitation events occur, then the feedback from the present soil moisture level causes infiltration to cease when the soil has reached field capacity. The feedback between the present soil moisture level and the evaporation rate determines whether constant or falling rate evaporation occurs according to the soil moisture threshold.	49

Figure 4.5. Histogram of daily simulated seasonal soil moisture from the system dynamics model by soil and treatment. Each treatment's density function represents simulated soil moisture values for a 100-day growing season. The Kernel density approximation indicates the expected probabilities as the number of models runs approaches infinity. Constant rate and slower rate evaporation occur above and below the soil moisture threshold for each graph, respectively. The percent of simulated days spent above the soil moisture threshold are displayed at the top right of each panel.....	52
Figure 5.1. Illustration of the cell density and evaporation rate experiments for Microbial Amendments for improved soil water management.	64
Figure 5.2. Illustration of the mercury (II) chloride (HgCl_2) sterilization samples and evaporation experiments, running side by side with fresh soil.	65
Figure 5.3. Illustration of the experiment measuring the nitrogen levels in the soil samples.	67
Figure 5.4 (A) Growth Curves for <i>B. subtilis</i> 168 1A1135 and (B) Growth Curves for <i>B. subtilis</i> ATCC 6051 using different sources of inorganic nitrogen.....	69
Figure 5.5 (A) Evaporation rate (ξ) as a function of SWC ratio (ϕ) for samples treated with nitrogen sources and inoculated with <i>B. subtilis</i> 168 1A1135. The dimensionless ratio (ϕ) was found using equation 5.1. All evaporation curves were higher than the control (water) except for the ammonium nitrate curve, which crossed the control curve and exhibited a lower evaporation rate as the samples dried. The first data point for each sample was excluded, as it reflected the system's initial equilibration period. (B) The final SWC ratio, where only the sodium nitrate ratio significantly differed from both ammonium-based media. (Data points with different letters are significantly different, $p < 0.05$).	71
Figure 5.6 (A) Evaporation rate as a function of SWC ratio (ϕ) for samples treated with nitrogen sources and inoculated with <i>B. subtilis</i> ATCC 6051. All evaporation curves were lower than the control (water). The first data point for each sample was excluded, as it reflected the system's initial equilibration period. (B) The final SWC ratio, where the control significantly differed from all nitrogen-treated samples. (Data points with different letters are significantly different, $p < 0.05$).	72
Figure 5.7 Water losses in samples treated with different nitrogen sources and inoculated with <i>B. subtilis</i> ATCC 6051. No significant differences were observed among samples treated with the same nitrogen source. However, all samples treated with ammonium and sodium nitrate	

exhibited significant differences from the controls (water and no-nitrogen media). Ammonium chloride-treated samples showed no significant differences from the controls. (Data points with different letters are significantly different, $p < 0.05$).	73
Figure 5.8 Water losses result in soil samples sterilized with mercury (II) chloride (HgCl_2) to inhibit microbial activity (Ste.), compared to fresh samples (Fre.) amended with nitrogen sources. No bacteria were inoculated in either treatment. Water losses in sterilized samples differed significantly from those in fresh soil samples. (Data points with different letters are significantly different, $p < 0.05$).	76
Figure 5.9. Results of 16S rRNA analysis are presented, showing (A) the absolute abundance of the top 10 taxa in the soil samples and (B) their relative abundance normalized to 100%. This analysis revealed the presence of <i>Bacillus spp.</i> , specifically gene groups associated with <i>Bacillus subtilis</i> . This suggests a high probability that native <i>Bacillus spp.</i> , without the inoculation of strain ATCC 6051, may contribute to improved water retention. However, further analysis is needed to identify the specific strains present in the soil and to assess their capacity to produce the biosurfactant surfactin.....	77
Figure 5.10. (A) $-\text{NO}_3$ Residual during the evaporation experiment and using different nitrogen sources and <i>B. subtilis</i> ATCC 6051, and (B) Ammonium ($+\text{NH}_4$) residual after the evaporation rate experiment using different nitrogen sources and <i>B. subtilis</i> ATCC 6051. The ammonium chloride residual did not decrease sufficiently, resulting in a significant difference from the no-ammonia media. (Data points with different letters are significantly different, $p < 0.05$).	79
Figure 5.11. Electrical conductivity (EC) measurements ($\mu\text{S}/\text{cm}$) of soil samples amended with different nitrogen sources: no nitrogen (No N_2), ammonium nitrate (NH_4NO_3), ammonium chloride (NH_4Cl), and sodium nitrate (NaNO_3). Ammonia chloride was significantly different from the control and both nitrate media. Different letters above the bars indicate statistically significant differences between treatments ($p < 0.05$).	81
Figure 6.1. Encapsulation of <i>B. subtilis</i> -GFP into PEG hydrogels using tetrafunctional PEG-thiol crosslinkers (MW = 10 kDa) and (a) PEG diacrylate, (b) PEG divinyl sulfone, or (c) PEG dimaleimide macromers (Mw = 3.4 kDa), forming thioether ester (red), thioether sulfone (blue), or succinimide thioether (green) bonds, respectively, at each crosslink through Micheal addition. Figure was prepared with assistance from Biorender software.	86

Figure 6.2. Cell morphology after encapsulation in PEG hydrogels. A Representative 10X (left column) and 20X (right column) fluorescent images of *B. subtilis*-GFP cells after encapsulation into PEGDA-PEGTT, PEGVS-PEGTT, and PEGMA-PEGTT hydrogels. B Average number and C percentage of encapsulated bacteria falling into size categories after particle size analysis (* $p < 0.05$). Darker shading in C) indicates a larger particle size range. Dashed white line in C) represent the cut-off area above which all particles are cellular aggregates. 10X images were used to quantify cell aggregation levels. Contrast was adjusted in Figure 6.2A to improve visualization. 89

Figure 6.3. *B. subtilis* cell viability after hydrogel encapsulation. A) Representative green-red fluorescent images of *B. subtilis* after encapsulation and live-dead staining (green denotes live cells, red denotes dead cells). B) Quantified cell viabilities (* $p < 0.05$). Contrast was adjusted in Figure 6.3A to improve visualization. 90

Figure 6.4. Growth kinetics of *B. subtilis* in bulk culture media (no hydrogel) and *B. subtilis* encapsulated in PEGDA-PEGTT, PEGVS-PEGTT, and PEGMA-PEGTT hydrogels. Triangles represent OD₆₀₀ readings from supernatant culture fluid outside of the hydrogels. Replicate trials were performed at $n \geq 3$ 92

Figure 7.1 Calculated mesh sizes for each hydrogel ($n \geq 3$). Results are reported as mean $\pm$ standard deviation. Asterisk (*) denotes statistically significant differences ($p < 0.05$) between formulations. 101

Figure 7.2. Effect of hydrogel mesh size on encapsulated *B. subtilis*. (i) PEGDA_{2k}-PEGTT_{5k} hydrogel shows a high frequency of small cell clusters ($\leq 13 \mu\text{m}^2$), as depicted by the histogram (left), corresponding fluorescence microscopy (middle), and 3D confocal reconstruction (right). (ii) Hydrogel PEGDA_{10k}-PEGTT_{10k}. (iii) In hydrogels formed by PEGDA_{10k}-PEGTT_{20k}, microcolony areas remain relatively small, but with a slight increase in diversity compared to the PEGDA_{2k}-PEGTT_{5k} hydrogel. Arrowheads indicate the peak distribution bin for the cell area. Fluorescence imaging confirms variations in microcolony organization as a function of mesh size. 103

Figure 7.3. Growth kinetics of *B. subtilis* encapsulated in PEGDA-PEGTT hydrogels with varying mesh sizes: PEGDA_{2k}-PEGTT_{5k}, PEGDA_{10k}-PEGTT_{10k}, and PEGDA_{10k}-PEGTT_{20k}. Optical density (OD₆₀₀) measurements reflect bacterial growth within the hydrogels over 72 hours.

Triangular markers denote OD ₆₀₀ readings from the supernatant, indicating minimal bacterial leakage. Error bars represent standard deviation ($n = 3$).	104
Figure 7.4. Effect of hydrogel mesh size on the growth kinetics of <i>B. subtilis</i> encapsulated cells. Bar segments represent the duration of each hydrogel's lag phase (darker shades) and exponential phase (lighter shades). A significant reduction in total growth time was observed when the MW of monomers increased, showing the longest lag and exponential phases. Error bars indicate standard deviation ($n = 3$). Asterisk (*) denotes statistically significant differences ($p < 0.05$) between conditions.	105

List of Tables

Table 3.1 Final and Threshold Soil Water Content for the Soil Mixtures	26
Table 4.1. Average evaporation rates during constant rate and slower rate periods, as well as the critical soil moisture threshold dividing the two periods (found by the method presented in section 4.2.4). Estimates and the corresponding standard errors represent the average across three trials for each soil type and treatment combination.	41
Table 4.2. Test results comparing the simulated effects of surfactin and <i>B. subtilis</i> . Treatments on soil moisture from the system dynamics model.....	54

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Dedication

I dedicate this work to the memory of my father,

Isidro Moises Gutierrez Chavez (1939–2018),

for his invaluable teachings and unwavering support.

To the memory of my brother,

Manuel Gutierrez Maldonado (1970),

who lost his life at birth.

To the memory of my godfather,

Francisco Xavier Ruiz Ruiz (1947–2014),

for his support and caring.

To the memory of my friend and colleague, a Chemical Engineer,

Héctor Carlos Ruiz Ruiz (1955–2023),

whose friendship I deeply cherished.

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whose friendship I also cherished.

And to my Lord, Jesus Christ,

for granting me the strength to complete this goal

Chapter 1 - Introduction

Global warming and environmental degradation are critical issues within the Food-Energy-Water nexus. Sustainable solutions are essential to meet the increasing global demands within this nexus. The interactions between food, energy, and water systems (FEWS) are complex and interdependent. For instance, water is directly or indirectly used in energy generation and food production. Simultaneously, energy is required to transport and treat wastewater and manufacture food. As the global population is projected to reach nine billion by 2050, the demand for food, energy, and water will rise proportionally, making the food-energy-water nexus even more intricate.¹

The need to supply food and energy significantly stresses the planet's freshwater resources. The effects of water scarcity are evident worldwide. Human societies rely on freshwater for numerous activities, but agricultural use far exceeds other types of freshwater consumption. Agricultural food and fiber production utilize about 82% of the freshwater used for human purposes.¹ Irrigation is critical to maintaining agricultural productivity. In 2018, D'Odorico et al. reported that 20% of the world's agricultural land is irrigated, contributing to 40% of crop production due to higher yields from irrigation systems. This situation increases the demand for irrigated land and, consequently, water.^{2,3}

Sustainable groundwater use is a crucial societal challenge for the future. Groundwater serves as a primary drinking water source for billions of people, plays a central role in irrigated agriculture, and supports the health of many ecosystems.^{4,5} The groundwater footprint, which represents the area of water tables required to sustain groundwater usage, is now estimated to be 3.5 times larger than the actual area of aquifers worldwide. This overuse threatens groundwater resources and ecosystems, placing billions of people at risk.⁴

Groundwater mean residence times are significantly longer than other parts of the hydrologic cycle, ranging from less than 10 years to over a million years for individual aquifers.⁶ Despite its importance, groundwater is often mismanaged, jeopardizing long-term sustainability. Approximately 80 percent of aquifers have a groundwater footprint smaller than their area, yet heavily overexploited aquifers skew the global average.⁶ This overexploitation is particularly critical in regions like Asia and North America, where groundwater is vital for agriculture.

A specific example is the Ogallala Aquifer in the High Plains of North America, a critical water resource supporting agriculture, power generation, and municipal usage in the western Great Plains.^{3,5} Spanning 174,000 square miles from South Dakota to the Panhandle region in Texas and New Mexico, it underlies an area responsible for around 20% of the United States wheat, corn, cotton, and cattle production.⁵ Central Nebraska, for example, has the most extensive groundwater stocks, measured in saturated thickness, which have remained relatively stable over time.

In contrast, the central and southern areas have lower levels of saturated thickness, leading to limited well capacity, which is the ability of a well to draw water for irrigation within a specific timeframe.^{3,5} In another case, the Ogallala Aquifer provides 90% of the water for irrigating 1.5 million acres in southwestern Kansas, making groundwater a crucial resource in the drier west, where surface water is scarce.⁷ Precipitation and temperature variability significantly impact dryland production, with annual precipitation ranging from less than 0.5 m/year in western Kansas to over 1.5 m/year in the southeast. Land elevation also varies, decreasing from over 1,200 meters in the west to 250–300 meters above sea level in eastern Kansas.^{8,9}

The Ogallala Aquifer supplies 30% of the US irrigated groundwater,⁸ with Kansas contributing significantly to the nation's agricultural market value.⁸ However, from 1950 to 2007, water demand in the region depleted 267.5 acre-feet or 8.2% of the total storage, and another 39% could be depleted in the next 50 years if current trends continue.^{3,8} This depletion poses a challenge

to maintaining agricultural production and slowing the decline in aquifer depth. Steward et al. estimated that recharge supplies for the Ogallala are about 15% of today's pumping, and it would take 500–1,300 years to refill the depleted aquifer fully.^{7,8} The annual depletion rate in the Central USA High Plains Aquifer peaked at $8.25 \times 10^9 \text{ m}^3/\text{year}$ in 2006, and it is projected to decrease to $4.0 \times 10^9 \text{ m}^3/\text{year}$ by 2110.⁷

In response to groundwater depletion, Kansas has adjusted irrigation practices by moving from extensive to limited irrigation rates and reducing irrigated acreage. Although hydrogeological and legal constraints exist, pumping rates have peaked, and withdrawals are projected to decline within 15–20 years. Reducing pumping by 20% would revert agricultural production to levels seen 15–20 years ago. Improved water use efficiency is expected to increase corn and cattle production over the next 30–40 years, extending peak agricultural production to the 2070s.⁸ However, as groundwater levels will continue to drop, adopting new irrigation technology and water conservation practices is essential to extend the aquifer's life, meet food demand, and enhance profitability⁹, especially with precipitation and temperature variations that alter soil water dynamics and make agriculture more dependent on groundwater.

Research into water reduction techniques has focused on fundamental soil-water studies and methods of altering soils to improve moisture content, which are now vital drivers in agricultural water management. Essential to these efforts are three soil properties: soil water repellency (SWR), soil water content (SWC), and soil wettability. These properties play a significant role in the overall surface and subsurface hydrology, dictating the productivity of soils.¹⁰ By understanding and manipulating these soil characteristics, we can develop more effective strategies for sustainable agriculture, a critical component of addressing the broader challenges within the Food-Energy-Water nexus.

1.1 Characterization of Soil Water Repellency and Retention

1.1.1 Soil Water Repellency (SWR)

Water repellency (hydrophobicity) is the characteristic of soil that rejects water. It influences rainwater penetration through the soil and water runoff paths, increasing variability in soil moisture levels.^{10–13} This property significantly affects agricultural water management, infiltration, contaminant transport, and soil erosion.¹³ Soil hydrophobicity is attributed to both coating soil particles with hydrophobic organic material and the presence of hydrophobic agents on mineral grain surfaces.^{10,11,13,14} The presence of hydrophobic organic materials on soil grains and aggregate surfaces comes from various sources, such as fire, organic matter from humus, microbial activity, soil temperature, land uses, and land management practices,^{12–15} and it develops in soils of varied textural composition and structural properties.¹⁶

Soil water repellency is not static but a dynamic property that responds to environmental conditions such as pH, temperature,^{17,18} and soil aggregation.¹⁹ Water repellency varies widely by soil type, location, time, and weather conditions. The severity of SWR depends mainly on the composition of organic matter, SWC, and wetting/drying history.^{13,20} Thus, some soils are expected to resist wetting when they are dry.¹⁰ The organic compounds identified from water-repellent soils can be divided into two groups. The first is the aliphatic hydrocarbons, which are non-polar and water-insoluble. The second group consists of amphiphilic molecules containing a hydrocarbon chain with one end and a hydrophilic functional group with a positive or negative charge on the other end,^{10,13} such as phospholipids, glycolipids and sphingolipids.

Soil hydrophobicity mechanisms are related to the configuration of amphiphilic organic matter compounds when they interact with water. When soils are wet, polar groups of the organic matter interact with water molecules, but as soils dry out, these polar groups interact with each other,^{13,18} suggesting that at higher ionic strength, there are low repulsion forces between the

hydrophilic group and high absorption of amphiphiles at the water-air interface, creating micelles and reconfiguring hydrophobicity; the opposite effect is seen at low ionic strength¹⁸, as illustrated in Figure 1.1. The soil hydrophobicity mechanisms become crucial in soil water retention since wetting agents can interfere by altering the mechanisms and enhancing water retention, delaying the soil from becoming hydrophobic.

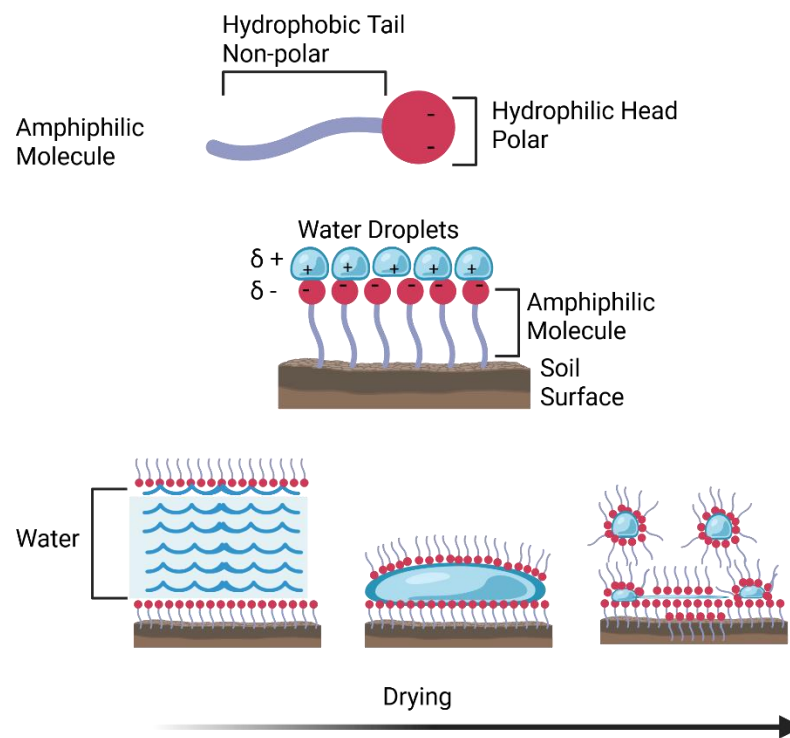


Figure 1.1. Mechanisms of how amphiphilic molecules interact with soil particles by anchoring their hydrophobic, non-polar tails to the soil surface. This exposes the polar, hydrophilic ends of the molecules, allowing them to attract and retain water molecules as the soil dries. This dual affinity helps maintain moisture within the soil structure, playing a crucial role in soil-water interactions.

1.1.2 Soil Water Content (SWC) and Wettability

Soil water content (SWC) and soil wettability are soil properties that describe water retention and the ability to absorb water in the soil. The following equations define soil water content:²¹

$$\theta_g = \frac{M_{water}}{M_{soil}} \quad (1.1)$$

where θ_g is the mass or gravimetric water content, and M_{water} and M_{soil} are the mass of the water and the mass of oven-dry soil, respectively. SWC can also be expressed as a volumetric water content, expressed as:

$$\theta_v = \frac{V_{water}}{V_{Total}} \quad (1.2)$$

where θ_v is the volumetric water content, V_{water} is the volume of the water, and V_{total} is the total volume occupied by the soil.

SWC and soil wettability are commonly studied due to their influence on water dynamics, particularly water infiltration and retention, and they are easily obtained from experimental measurements.¹⁵ SWC and wettability can also determine the fate of precipitation.¹² The effects of irrigation and precipitation are more effective in high retention capacity soils due to higher water storage. In these soils, water from an irrigation or precipitation event remains until plants use it. In contrast, soils with lower water retention capacity are evaporated, percolated into deeper layers, or lost by saturated overland flow.¹²

SWC is primarily determined by texture, structure, organic matter content, and soil bulk density. The moisture retained at a given texture increases with decreasing particle size and increasing organic matter content.¹² As mentioned before, soil condition changes with different environmental conditions, affecting SWC and wettability. The SWC characteristics largely determine the amount of water available for plant growth, which refers to the volume fraction of water present in the soil between field water capacity and wilting point, which is the minimum SWC level needed to have water available to plants. Water availability is limited beyond the wilting point because water tends to be firmly bound to the soil particles.¹² Vogelmann *et al.* determined that the threshold for hydrophobic soils to become hydrophilic lies within a soil water

content range of 0.36 to 0.57 cm³/cm³, depending on the soil type, texture, and organic matter. A soil becomes wettable once the SWC exceeds the threshold value, which is the critical moisture content at which the soil begins to exhibit water-repellency. Below this level, hydrophobicity is manifested, making the soil water-repellent, while above it, the soil become hydrophilic.²⁰ Hence, identifying the critical moisture content is essential to improve water retention.¹³

1.2 The Use of Surfactants

An immediate solution to preventing water repellency in soils is using wetting agents such as surfactants.^{22,23} Surfactants are essential in maintaining the soil water content (SWC) above the threshold critical value.^{10,22,24,25} These are amphiphilic molecules with both strongly hydrophilic and strongly hydrophobic groups. Due to their amphiphilic nature, surfactants accumulate at the solid-liquid interface, preventing their degradation and altering the surface tension of the solid, thereby converting hydrophobic soil into a hydrophilic one.^{10,26} When introduced to soils, the surfactant's hydrophobic (non-polar) end attaches to a non-polar water-repellent site on the soil particles. As water penetrates through the soil matrix, water molecules are attracted to the polar end of the surfactant, increasing water adsorption for all treated soils.¹⁰

Several studies have investigated using surfactants to improve soil water capacity and wettability and diminish soil water repellency. For instance, in 2008, Oostindie et al. reported the effects of a soil surfactant on reducing and preventing water repellency in sandy soil through a two-year field experiment located at Bosch en Duin near Utrecht, the Netherlands. In the experiment, one-half of the field was treated four times with a methyl-capped triblock copolymer soil surfactant over the entire year and for two consecutive years, and the soil's water repellency was assessed at different spots across the untreated and treated parts of the field under regular irrigation. Consequently, the surfactant applications resulted in a homogeneous wetting of the soil

profile and eliminated actual water repellency.¹⁴ Compared to the untreated field section, the SWC in the surfactant-treated section was consistently higher, with 4-32% SWC for the untreated and 28-36% SWC for the treated section. The study concluded that surfactant applications have several advantages: remediation and prevention of soil water repellency, more homogeneous and better soil wetting, and increased available water in the rhizosphere.¹⁴

In another study, Lehrs et al. found an increase in the volumetric water content by 3% ($p \leq 0.08$) at matric potentials from 0 to -20 kPa using an alkyl polyglycoside (APG) surfactant, which increased the volume of water held in pores with diameters $\geq 15 \mu\text{m}$.²⁶ However, despite these improvements, synthetic surfactants have introduced hazardous conditions to ecosystems. They are non-biodegradable, have high energy costs for manufacturing and are expensive for practical application. In addition, with high toxicity, there is a risk of introducing pollutants into the environment when overusing synthetic surfactants,^{27,28} as discussed in the next section.

1.2.1 Impact of the Surfactant on the Environment

Synthetic surfactant molecules can disrupt the soil ecosystems, including flora, fauna, and microbiomes. As non-biodegradable compounds, synthetic surfactants can also accumulate in the environment, reaching toxicity levels and enhancing the transport and proliferation of other pollutants, such as heavy metals.²⁹ In agricultural soils, using greywater with dissolved surfactants in irrigation can lead to acute toxicity to agrarian commodities.²⁹ Surfactants are also frequently used *in situ* soil flushing and *ex-situ* soil washing for remediation and have caused adverse effects.¹⁰ Due to the direct applications, synthetic surfactants can also end up in the aquatic environment. It has been documented that 7 million tonnes are released into the marine ecosystem annually.²⁹ These molecules also alter the aquatic ecosystem by forming a stable foam over the surface, reducing sunlight penetration, reducing oxygen absorption in the waters, disrupting the water cycle, and altering the fauna and flora of aquatic ecosystems.^{29,30} It has been shown that fish

mortality can rise to 50% in a surfactant concentration of 15 ppm and with fish eggs at a concentration of 5 ppm.³⁰

Another harm done to the environment by surfactant pollution is the disturbance of the microbiomes in ecosystems. Synthetic surfactants are toxic to some microorganisms, and as the concentration of surfactant contamination increases, the genetic and functional diversity of microbial communities decreases,³⁰ which could bring irreversible damage to an ecosystem. However, one of the most concerning ecological impacts is the long-term exposure of microorganisms to low concentrations of surfactants, which can generate resistance to the surfactant antimicrobial activity.²⁹

1.3 The Use of Biosurfactants

Biosurfactants are low molecular weight, surface-active compounds produced by bacteria, yeast, and fungi and thus serve as green surfactants. Biosurfactants have similar wetting effects on soils as their synthetic counterparts. The amphiphilic properties of these biosurfactants reduce the surface tension at the air/water interface and the interfacial tension at the soil/water interface.^{28,31,32} They are environmentally friendly bio-products, cost-effective, biocompatible, easy to produce, have low toxicity, are chemically diverse, are stable against changes in environmental conditions, and thus present unique solutions to the environment's drawbacks of their synthetic counterparts.^{28,32-34} They also have better activity in extreme environmental conditions such as a pH range of 2-12, a temperature range of 30 °C–100 °C, and a salinity level of up to 30%,^{28,32} and most notably, they are highly biodegradable.³²⁻³⁵

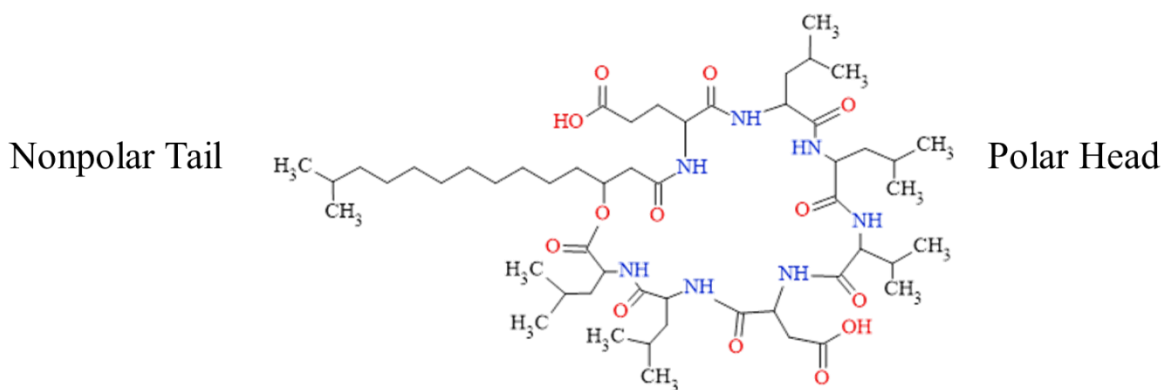


Figure 1.2 Molecular structure of surfactin, illustrating the amphiphilic properties.

Biosurfactants have a variety of applications in the agricultural industry, including promoting soil quality. They have found significant use in bioremediation, improving the quality of the soils by removing organic and metal pollutants to acceptable or tolerable levels, where healthy plants can grow with the necessary nutrients.²⁸ Because biosurfactants can reduce the surface tension at the water-soil interface, they can also cause soil aggregates to become more wettable to increase the soil moisture content. surfactin, displayed in Figure 1-2, is one of the most effective biosurfactants used as a bioremediation agent. Its amphiphilic properties reduce the surface tension of water from 72 to 27 mN/m,³⁵ and surfactin is used in environmental applications due to its efficient emulsification activity.³⁵ This molecule has also been applied in enhanced oil recovery applications.^{35,36}

The benefits offered by surfactin and other biosurfactants suggest they could potentially replace synthetic surfactants, mitigating environmental impact. Despite these bioremediation agents' capability to replace synthetic surfactants, biosurfactants still represent a minimal portion of total surfactant consumption. This lack of use is primarily due to the lack of cost-effective manufacturing methods. Low productivity and yields currently render large-scale production unfeasible. It is estimated that downstream processes—such as isolation, concentration, and

product purification- constitute about 60-80% of total production costs.³⁴ Despite recent innovations, few upgrades or optimization methods have been made to reduce downstream costs.

Several methods have been designed to address these costs, such as using waste as a substrate or employing *in situ* inoculation to produce biosurfactants at the point of application. For example, inoculating soil with bacteria such as *Bacillus subtilis*, which is a soil-dwelling bacterium that produces surfactin to improve motility, is a promising technique to increase soil water capacity and sustainably reduce soil water repellency.

The high amenability and the natural habitat have made *B. subtilis* an organism of choice for agricultural applications. Studies have shown mechanisms that *B. subtilis* utilizes for existence and competitive success in soil and the rhizosphere.³⁷ *B. subtilis*, as a spore-forming bacterium, has high adaptability to extreme conditions, including cycles of heat and cold, where it grows in a range of 5-55°C and resists freezing-thawing. It can also grow in physical abrasion environments and be exposed to corrosive chemicals; in a laboratory environment, *B. subtilis* can grow in nutrient broth at a salinity concentration of 7% NaCl.^{38,39} With the antimicrobial activity of the surfactin, *B. subtilis* has an inhibitory activity against microorganisms.^{40,41} Its extracellular degradative enzymes can prolong exposure to solar radiation.³⁸

B. subtilis growth is favored in agricultural soils; it can hydrolyze starch and use nitrate, increasing nitrite in soils, which is essential for plant growth.⁴² *B. subtilis* and its secondary metabolite, surfactin, balance ion and water movement between plants and soil⁴² and improve soil water content. This approach exemplifies how targeted innovations can make biosurfactants more viable for large-scale use.

1.4 Microorganisms Used in Soils

Some soil-dwelling microorganisms generate a range of chemically diverse, surface-active biosurfactants that improve soil health and help plants cope with environmental stress factors such as drought and contamination. Thus, microorganisms producing biosurfactants have been studied for their role in sustainable agriculture, as they offer a biological alternative to synthetic surfactants.^{43,44} Vurukonda et al. reviewed the benefits of plant growth-promoting rhizobacteria (PGPR), including biosurfactant-producing strains, highlighting their ability to enhance plant growth by improving nutrient uptake, regulating phytohormone levels and protecting plants from pathogens, thereby promoting overall plant health and soil quality.⁴⁵

Microbial biosurfactants, such as rhamnolipids, sophorolipids, and surfactin, can potentially enhance soil properties. For instance, rhamnolipids produced by *Pseudomonas aeruginosa* have been noted for their ability to degrade hydrophobic pollutants and improve soil hydrology.⁴⁶ Sophorolipids from *Candida bombicola* have been studied for their effectiveness in soil remediation and plant growth promotion.⁴⁷ Lipopeptide surfactants produced by *B. subtilis* have demonstrated significant potential in soil improvement and bioremediation.⁴⁸

Microbial bioinoculants can also improve soil health through mechanisms other than biosurfactant production. In 2019, Javeed et al. conducted a study that concluded that phosphorus-solubilizing bacteria *Enterobacter sakazakii* J129 could improve sandy soil characteristics and maize grain quality. The researchers found that the inoculation treatments, duration, and interactive effects significantly increased sandy soil water retention by more than 20% in some treatments. The study demonstrated that the incubation process improved soil structure and chemical properties by creating niches for microbial activities, thus enhancing soil water retention and nutrient availability.⁴⁹ These findings are consistent with a plethora of other studies that demonstrate that microbial inoculation can enhance soil water retention and fertility and make

soils more resilient to environmental stressors. Thus, using microbial soil inoculants is a promising area for developing sustainable farming practices and can ultimately reduce reliance on chemical inputs and improve crop resilience to environmental challenges.

A presumed drawback of biosurfactants is their potential to disrupt or shift the community structure of the soil microbiome, which could negatively impact beneficial microbial communities essential for soil health and plant growth. For example, rhamnolipids and surfactin can disrupt the cell membranes of some microbes, leading to cell lysis and the release of cellular contents, altering the microbial community structure.^{50, 40} Microbes can also respond to environmental stressors by altering their metabolism, which can cause undesired results, such as the production of less effective or even harmful metabolites under certain conditions.⁵¹ Similarly, biosurfactants can also impact nutrient availability, another factor that would affect the soil microbiome.⁴³ Thus, understanding microorganisms, their metabolite products, adaptation characteristics, and survival techniques in the soil is vital.

Ultimately, biosurfactants are considered a promising substitute for synthetic surfactants due to their eco-friendly nature and multiple benefits in agricultural practices. However, more research and a complete understanding of their interactions with the environment and microbial communities are required to optimize their use and eliminate potential adverse impacts.

1.5 Using hydrogel for *in situ* applications

Despite the limitations of *in situ* treatment, new technologies are addressing existing challenges. One promising approach is the use of hydrogels. These materials can encapsulate, protect, and release functional bacteria or compounds, offering advantages such as high water content, biocompatibility, and tunable properties. Hydrogels are three-dimensional polymer networks composed of hydrophilic, crosslinked macromolecules. Their unique structure allows

them to absorb water, aqueous solutions, and physiological fluids while remaining insoluble due to their network integrity.^{52,53} These properties create a stable environment that enhances bacterial viability, functionality, and resistance to external stressors. For example, encapsulated bacteria may be more resistant to environmental challenges, such as reactive oxygen species,⁵⁴ extreme pH conditions,⁵³ and biofouling by endogenous microorganisms. Thus, hydrogels can play a critical role in stabilizing and shielding functional microbes in bioinoculant applications.

Beyond microbial stabilization, hydrogels can facilitate various environmental bioremediation processes. For instance, encapsulated bacteria are increasingly used in oil recovery through biosurfactant production. Son et al. demonstrated a scalable approach using microfluidic droplet techniques, where *B. subtilis* was encapsulated in microgel particles to produce surfactin. Once introduced into the soil, the bacteria released biosurfactants through the hydrogel mesh, reducing surface tension at the oil-water interface. This process enhanced oil emulsification and mobilization, recovering 45% of residual oil from a model reservoir packed with silica beads.⁵⁵ Building on this approach, similar hydrogel encapsulation strategies could enhance bacterial survival in non-ideal environments and support *in situ* applications, including improving soil water retention. While challenges remain in optimizing encapsulation efficiency and bacterial viability, continued advancements in hydrogel-based materials are paving the way for innovative solutions to environmental bioremediation and agricultural applications.

Chapter 2 - Objectives

2.1 Background

Soil water retention and repellency are influenced by soil components' hydrophilic and hydrophobic characteristics, including organic carbon and microorganisms. Research has focused on understanding the factors contributing to soil water repellency and how these factors can be controlled. One promising approach involves using bacteria to produce biosurfactants within the soil through *in situ* application, ultimately improving soil water content and water retention capacity. *Bacillus species*, particularly *B. subtilis*, are well-known for producing biosurfactants, including surfactin. While microbial-derived biosurfactants have been successfully applied in bioremediation and oil extraction, their potential to improve the wettability of hydrophobic soils remains underexplored. However, it is crucial to consider that while surfactants can enhance the wettability of hydrophobic soils, they might also reduce water retention in already hydrophilic soils, underscoring the importance of careful application and consideration.

2.2 Motivation

The first section of this thesis investigates the effects of the surfactant-producing bacterium *B. subtilis* and its biosurfactant, surfactin, on water dynamics across different soil types. Given that soil textures vary in response to environmental disturbances, the diverse structures and physicochemical properties of soils play a crucial role in determining the success of any soil amendment. This study hypothesizes that soils with higher permeability and proper levels of organic carbon will benefit more significantly from surfactin application than those with lower permeability and inappropriate organic carbon content and other soil characteristics. To test this hypothesis, surfactin will be applied to soils with systematically controlled organic carbon and permeability levels. This study will assess changes in water dynamics, including hydrophobicity,

water retention, and overall soil moisture content, to understand surfactin's effectiveness with varied soil textures. The approach involves diluting soil samples with artificial sand to isolate and achieve varied soil textures with similar compositional properties.

The second section of this thesis investigates and develops optimal conditions for an *in situ* application of *B. subtilis* to produce surfactin. This research examines the feasibility of these amendments and aims to determine the conditions that maximize their benefits. A series of experiments are conducted in which soil samples are treated with bacteria and surfactin; evaporation rates and other water dynamics parameters are then analyzed and incorporated into a simulation to assess feasibility. Additionally, other samples undergo bacterial inoculation alongside environmental modifications aimed at optimization. These modifications include amending the soil with nutrients, incorporating inorganic nitrogen during fertilizer fabrication, and conducting nitrogen analysis to assess the impact of nutrient additions. This approach aims to increase soil water retention by promoting bacterial growth while preserving the integrity of the existing soil.

The findings from this research could have significant implications for optimizing the use of biosurfactants like surfactin in agricultural practices. Understanding the interaction between soil permeability and surfactin application could guide the selection of soil types that would most benefit from this treatment, leading to more efficient and targeted agricultural interventions.

The following goals define the objectives driving this research:

Specific Aim One:

The aim is to assess how directly applying surfactin enhances wettability and soil water retention and how different soil textures affect its efficiency.

This study evaluates various soils to determine how surfactin interacts with different textures and under which conditions it performs most efficiently. This objective is achieved by systematically measuring its effect and analyzing hydrophobicity, evaporation rates, and soil water content levels in treated and untreated soils.

Specific Aim Two:

The aim is to assess how *Bacillus subtilis* subsp. *subtilis*, a surfactant-producing bacterium, enhances water retention and wettability in two soil texture types, which responded more effectively to the surfactin treatments.

This study investigates how bacterial amendments influence soil water dynamics and compares the results to surfactin treatments. A feasibility assessment evaluates their performance under realistic precipitation conditions, focusing on how bacterial inoculation affects water retention during rainfall and subsequent dry periods and exploring the potential application of these treatments in real-world agricultural settings.

Specific Aim Three:

The aim is to determine the ideal conditions for the inoculation of two *B. subtilis* strains by analyzing how varying bacterial cell concentrations and nitrogen sources affect their effectiveness in improving soil water retention.

This study explores the optimal conditions for two *B. subtilis* strains—a high Surfactin producer and a non-producer—by assessing their impact on soil water dynamics. Key indicators include evaporation rate and residual soil moisture. The study also evaluates different cell densities and inorganic nitrogen sources with nutrients to determine the bacterial concentration that

maximizes soil moisture retention. Additionally, it investigates *B. subtilis*' potential role in influencing soil denitrification by analyzing nitrogen levels during the experiments.

Specific Aim Four:

This aims to assess how bacteria's encapsulation affects their viability, functionality, and potential for *in situ* applications.

This study explores how the functionality of *B. subtilis* is affected when encapsulated in hydrogels formed using different crosslinkers. Key indicators of cell growth, such as survival rate, morphology, mesh size, and carrying capacities, are analyzed to assess potential *in situ* applications.

The approach outlined in this thesis has the potential to significantly advance agricultural practices by providing a deeper understanding of sustainable solutions to soil management challenges. By focusing on the role of *B. subtilis* and surfactin in enhancing soil water retention, this research aims to address key issues affecting crop productivity under diverse environmental conditions and improve soil health. The findings are expected to contribute to developing more resilient and efficient practices, offering practical strategies for sustainable agriculture. Ultimately, this study lays the groundwork for future research and real-world applications that can help mitigate the impacts of climate change and resource scarcity on global food production.

Chapter 3 - Impact of Soil Texture on Biosurfactant-Mediated Soil Wetting and Water Retention

Overview

Increasing global food demand combined with more frequent and intense periods of drought necessitates new strategies to improve agricultural water use efficiency. Amending soils with biosurfactants provides a method to increase soil wettability and improve soil water retention, thereby reducing freshwater demand. This study evaluates the impacts of soil texture on soil water retention after amendment with the biosurfactant, surfactin. Texture effects were systematically investigated by mixing silty clay loam soil with Ottawa sand, ensuring chemically equivalent soil constituents. Sandy loam texture exhibited a significant response after surfactin treatment, indicated by increases in soil wettability and soil water retention after a 48-hour dryout period. In contrast, all other soil textures, including silty clay loam, loam, and loamy sand, had no significant improvements. These findings highlight the critical role of soil texture on biosurfactant efficacy for optimized application in agricultural soils.

Core Ideas

- Soil texture plays a critical role in biosurfactant amendment efficiency for improving soil water retention.
- Texture effects were isolated using mineralogically equivalent soils of varied textures.
- Sandy loam was the only texture with improvements in wettability and water retention after surfactin amendment.
- Biosurfactant amendments can increase the economic value of sandy loam soils by improving water retention.

Abbreviations

- CA contact angle
- ER evaporation rate
- OMC organic matter content
- SCL silty clay loam
- SWC soil water content
- SWR soil water repellency

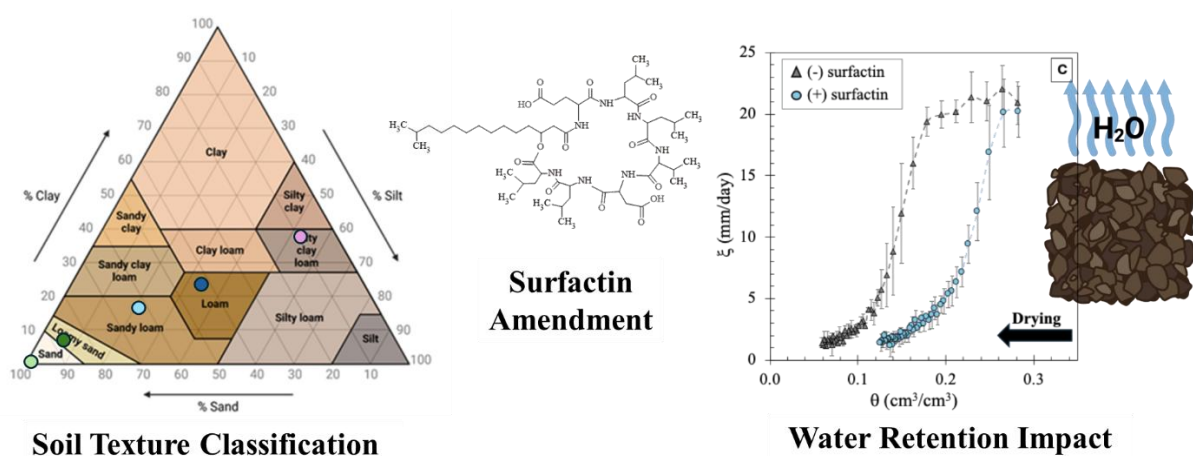


Figure 3.1 Graphical Abstract for Impact of Soil Texture on Surfactin-Mediated Soil Wetting and Water Retention

* Manuscript currently submitted for publication.

3.1 Introduction

Burgeoning global food demands combined with more frequent and intense periods of drought are diminishing freshwater resources in many arid and semi-arid agricultural regions. This trend is expected to intensify in the coming decades; by 2050, agricultural freshwater demand is expected to double while freshwater availability is expected to halve.⁵⁶ Improving agricultural water use efficiency is therefore crucial. One solution is to reduce soil water repellency (SWR) using surfactant amendments to improve soil wettability and achieve higher soil water retention. Due to their amphiphilic nature, surfactants can adhere to hydrophobic surfaces of soil particulates to reduce water surface tension and improve water infiltration and absorbance (Fernández-Gálvez & Mingorance, 2010).¹⁰ While synthetic surfactant amendments are infeasible for widespread use due to limitations in toxicity, cost, and scalability, biosurfactants present a practical alternative. They can be produced continuously in the soil by microorganisms such as *Bacillus subtilis*,⁵⁷ and circumvent the negative environmental impacts of synthetic surfactants.²⁸

Limited studies have explored the potential for soil water retention enhancement using biosurfactants. Early studies demonstrated that biosurfactants can enhance the rate of water infiltration and distribution within the soil.^{58,59} More recently, sterilized soils were amended with *B. subtilis* to break down SWR and retain significantly higher amounts of water than untreated soils. The study found that *B. subtilis* reduced SWR by producing the biosurfactant, surfactin.⁶⁰ In another study, evaporation dynamics after applying surfactin and *B. subtilis* separately to agricultural soil samples were coupled with a systems dynamic model that provided water input according to daily precipitation patterns to determine that these treatments could extend dryout periods in the field.⁶¹

While the potential benefits of biosurfactants are clear, the effects of texture on soil response to biosurfactant amendments have not been studied in a systematic manner and are,

therefore, not well understood. This is due to the structural, chemical, and biological heterogeneities in natural soils, which make it difficult to isolate and probe specific soil parameters.¹⁸ The objective of this research was to systematically investigate the effects of soil texture in response to biosurfactant amendments that aim to improve soil water retention. Textures were controlled by mixing Ottawa sand into silty clay loam (SCL) soil at varied ratios to achieve a range of soil textures with the same clay and silt composition. This approach normalizes for other complexities that can also influence biosurfactant-soil interactions, such as compositional differences in soil organic and inorganic constituents and pH.⁶² Changes in hydrophobicity and water retention with and without treatment are then characterized and compared across textures to provide insight into the textures that most benefit from biosurfactant amendment, knowledge important for agriculture and environmental soil management.

3.2 Materials and Methods

3.2.1 Soil Sampling and Preparation and Surfactin Amendment

Soil with SCL texture was collected from Kansas State University (KSU) experimental fields and then mixed with Ottawa sand at varied weight ratios to achieve three additional soil mixtures with textures equivalent to loam, sandy loam, and loamy sand (Figure B1). Ottawa sand was used because it had minimal mineralogical variability, organic carbon, and nutrient content, allowing for variation of texture while minimizing additional soil constituents introduced into the mixtures. The five soil samples used in this study, representative of five distinct soil textures, are referenced according to their mixing ratio as follows: Sand(0):SCL(4) (SCL texture), Sand(1):SCL(3) (loam texture), Sand(2):SCL(2) (sandy loam texture), Sand(3):SCL(1) (loamy sand texture), and Sand(4):SCL(0) (sand texture). Detailed preparation methods for the soil samples are described in supplemental materials (Section B.1). Physicochemical soil properties of

each sample were analyzed at the KSU Soil Testing Laboratory (Table B1). Surfactin solutions of 25 and 50 mg·kg⁻¹ water were applied to 26.0 g of soil sample in Petri dishes to yield final surfactin concentrations of 9.62 and 19.23 mg of surfactin per kg soil, which are comparable levels to those used in other surfactant-based soil amendment strategies.⁶³ A third treatment of pure water was used as a control. All samples were then placed in an air-forced convection oven for heating and wetting treatments following the established procedure for investigating soil water retention by Lowe et al.⁶⁰ Briefly, mimicking natural drying and irrigation processes, amended soils were kept at 30°C for three days. Ultra-pure water (10 mL) was then added every two hrs during the first six hrs of each day, followed by a final 10 mL addition six hrs later. This was then followed by a 12-hour drying period.

3.2.2 Contact Angle and Dryout Experiments

After heating and wetting treatments, contact angle (CA) measurements were performed using the sessile drop method with a goniometer (First Ten Angstroms® 206140, Allied Prosilica® GC Model # GC750). Prior to measurement, soil samples were sieved through a 1 mm mesh to remove large aggregates, then fixed on double-sided tape following the well-established method developed by Bachmann et al.¹⁵ While soil surface roughness can significantly affect CAs when wettable and non-wettable particles coexist,¹⁶ this method is particularly suitable for soils with CAs between 11° and 105°,⁶⁴ covering the CA range observed in this study.

The objective of dryout experiments was to measure evaporative water loss from partially saturated soils over time. Here, 2-inch tall columns (V=29.06 cm³) were packed with 40 g of each soil sample to achieve soils with a packing density of 1.4 g/cm³. All soils were brought to similar initial volumetric SWC levels (0.28-0.31 cm³/cm³), representative of typical soil moisture levels at field capacity.⁶⁵ Columns were placed on a series of digital scales (FX-1200i, 10 mg precision) in a convection oven at 30°C, and column mass was recorded every 5 min for 48 hrs. Kinetic mass

loss curves were used to calculate evaporation rates at each time point for every sample. A linear regression approach described by Gutierrez et al.⁶¹ was then used to delineate and quantify evaporation rates in the constant and falling phases and threshold SWC. Volumetric SWC at each time point was calculated using the soil water content method.²¹

3.2.3 Statistical Analysis

All statistical analyses were performed using SAS software (SAS Institute Inc.). Mean and standard deviations were calculated using $n=3$ independent trials in all experiments. Student's t-tests were used to compare data sets and to determine statistical differences, with a p-value < 0.05 considered statistically significant.

3.3 Results and Discussion

Increases in soil wettability were first investigated by comparing CA measurements with and without surfactin treatments for each soil texture. CAs were also compared across different soil textures treated with equivalent surfactin concentrations. Without surfactin treatment, all soils had statistically equivalent CAs, ranging from $28.3 \pm 3.0^\circ$ to $34.2 \pm 5.2^\circ$ (Figure 3.2A). No significant differences were identified when comparing CAs of untreated soils to CAs of equivalent soils treated with 25 mg kg^{-1} surfactin solutions. However, every sample except Sand(4):SCL(0) had a decreased average CA trend with treatment (Figure 3.2B), suggesting a mild increase in soil wettability. After treatment with 50 mg kg^{-1} surfactin, the Sand(2):SCL(2) mixture yielded the most hydrophilic soil (Figure 3.2C), and was the only soil to show a statistically significant CA decrease when compared to its untreated sample ($p<0.05$). While not statistically different from untreated samples, further decreases in average CA were observed when comparing 50 mg kg^{-1} surfactin treatments for all soils that contained SCL fractions. In contrast, the Sand(4):SCL(0) did not show a decreasing CA trend. Statistical differences were also found when

comparing 50 mg kg⁻¹ surfactin treated Sand(2):SCL(2) with 50 mg kg⁻¹ surfactin treated samples of either original texture (Sand(4):SCL(0) or Sand(0):SCL(4)) (Figure 3.2C). These findings identify sandy loam as the texture with the greatest increase in soil wettability.

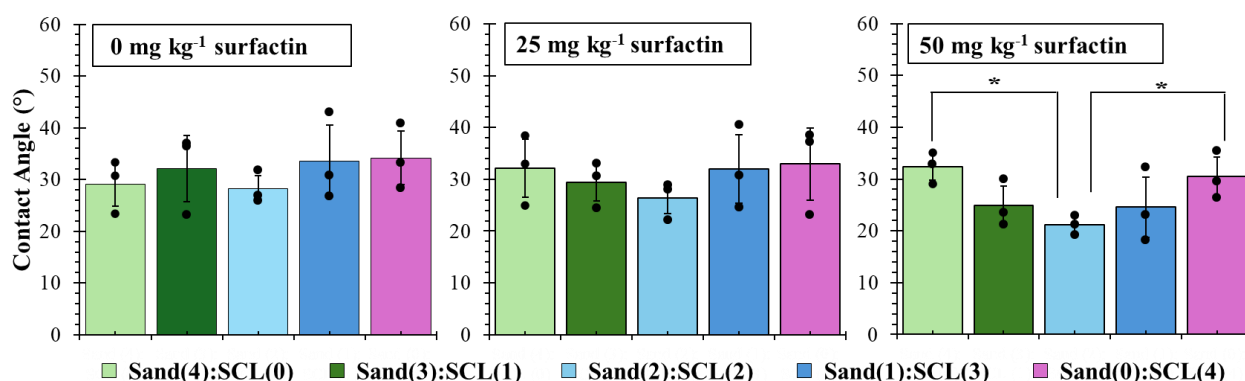


Figure 3.2 CA measurements for soil samples treated with (A) 0 mg kg⁻¹, (B) 25 mg kg⁻¹, and (C) 50 mg kg⁻¹ of surfactin. Textures connected with (*) are significantly different, $p < 0.05$.

Soils were next investigated in dryout experiments with and without surfactin treatments. Drying curves and final SWC levels at 48 hrs followed the expected trends for unamended control soils (Figure B2), with Sand(4):SCL(0) showing the lowest final SWC and Sand(0):SCL(4) showing the highest, while the mixed samples had intermediate water losses corresponding to the level of sand content. With surfactin treatment (Figures B3 and B4), Sand(2):SCL(2) increased in final SWC and had the highest water retention at 50 mg kg⁻¹ surfactin. In fact, surfactin-treated Sand(2):SCL(2) was the only sample to display the desired increase in final SWC when compared to the untreated control ($p < 0.05$). Table 1 provides comparisons of final SWC, as well as threshold SWC, for each sample. Constant and slower evaporation rates are also provided in Table B2. The key metric is threshold SWC, defined as the SWC level where the constant evaporation phase (driven by capillary action in macropores) shifts to the slower evaporation rate phase (driven by

vapor diffusion from micropores). A higher threshold SWC indicates enhanced water infiltration from soil macropores into micropores⁶⁶ and decreased capillary action in soil macropores, restricting upward water movement and breaking capillary pathways within the soil during evaporation.^{10,35,67} An increase in threshold SWC was observed with surfactin treatment only in Sand(2):SCL(2) samples, where transition to slower evaporation at higher SWC led to higher overall water retention throughout the dryout period. Water retention improved as surfactin concentration increased, findings consistent with the noted increases in soil wettability only for Sand(2):SCL(2) (Figure 3.2). Kinetic mass loss measurements were used to further compare evaporation rates with SWC levels for each sample with and without 50 mg kg⁻¹ surfactin treatment (Figure 3.3). Sand(2):SCL(2) was the only texture to maintain lower evaporation rates at equivalent SWC levels during the entire dryout process, while the other two mixtures (Sand(3):SCL(1) and Sand(1):SCL(3)) showed statistically equivalent evaporation rates during dryout.

Table 3.1 Final and Threshold Soil Water Content for the Soil Mixtures

Soil Mixture	Soil Water Content (cm ³ /cm ³) ± SD					
	Final SWC			Threshold SWC		
	0 mg·kg ⁻¹	25 mg·kg ⁻¹	50 mg·kg ⁻¹	0 mg·kg ⁻¹	25 mg·kg ⁻¹	50 mg·kg ⁻¹
Sand (4): SCL (0)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.01	0.01 ± 0.01	0.02 ± 0.03	0.03 ± 0.03
Sand (3): SCL (1)	0.04 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.09 ± 0.00	0.10 ± 0.02	0.09 ± 0.01
Sand (2): SCL (2)	0.06 ± 0.01	0.09 ± 0.01*	0.12 ± 0.02*	0.13 ± .001	0.17 ± 0.02*	0.20 ± 0.02*
Sand (1): SCL (3)	0.09 ± 0.01	0.10 ± 0.00	0.08 ± 0.02	0.19 ± 0.01	0.19 ± 0.02	0.19 ± 0.01
Sand (0): SCL (4)	0.11 ± 0.01	0.09 ± 0.03	0.06 ± 0.02*	0.22 ± 0.01	0.19 ± 0.02	0.17 ± 0.03*

Note: Data points marked with () denote a significant difference ($p < 0.05$) between the surfactin treatment and the control (0 mg·kg⁻¹).*

Abbreviation: SD, standard deviation.

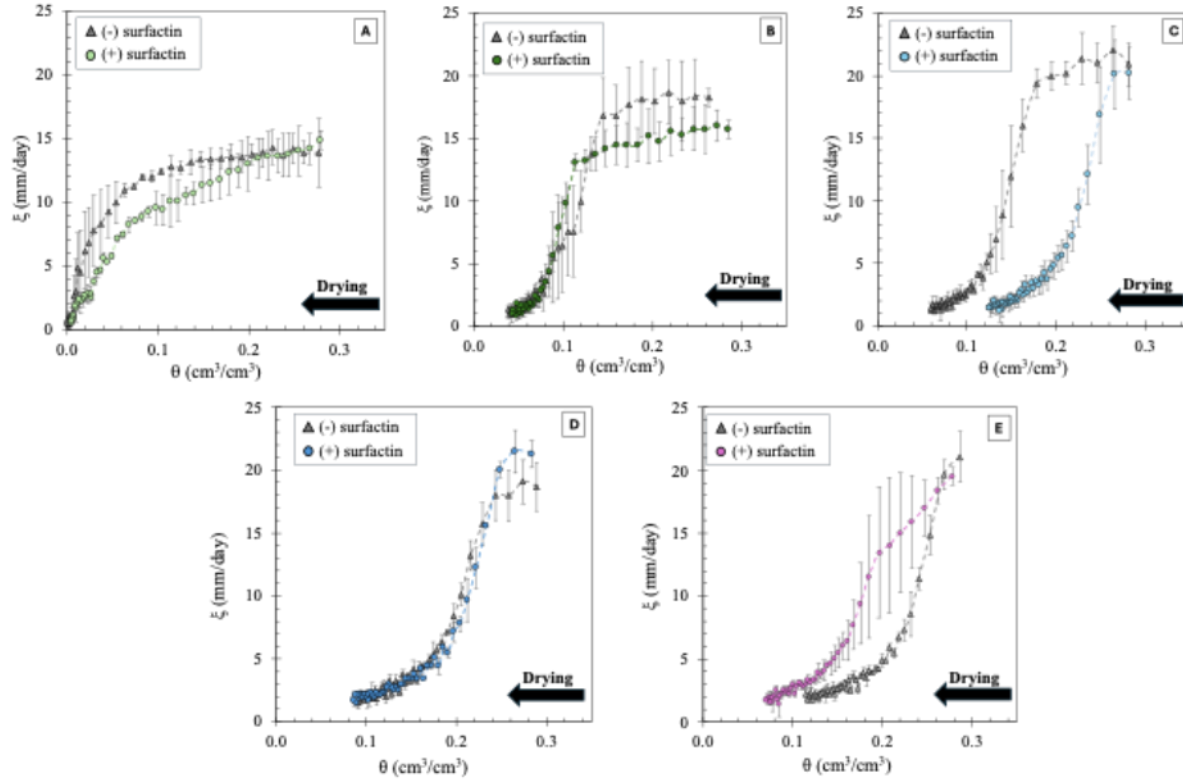


Figure 3.3 Evaporation rates (ξ) versus SWC (θ) during dryout experiments for (A) Sand(4):SCL(0) (sand texture), (B) Sand(3):SCL(1) (loamy sand texture), (C) Sand(2):SCL(2) (sandy loam texture), (D) Sand(1):SCL(3), (loam texture), and (E) Sand(0):SCL(4) (SCL texture).

It is notable that Sand(0):SCL(4) showed trends opposite to Sand(2):SCL(2); water losses instead increased with 50 mg kg^{-1} surfactin treatment (Figure 3.3E). The shift in threshold SWC towards lower moisture levels with surfactin treatment (Table 3.1) indicates that water infiltration into soil micropores became more inhibited, instead favoring water in larger pore spaces where capillary action is favored. This prolonged the constant evaporation phase and decreased SWC levels during the dryout period. This result was unexpected, as this sample had the highest organic matter content (OMC) and also the highest electrolyte composition (Table B1), both of which favor the association of anionic surfactants into the soil.⁶⁸ We hypothesize that this undesired effect was due to mass transport limitations during surfactin treatment, caused by the low permeability of silty and clay fractions.^{69,70} With limited pore diffusion, surfactin can aggregate on the exterior of

soil particles to form admicelles (monolayers) or hemimicelles (bilayers), the latter leaving the hydrophobic tail exposed⁷¹ to inhibit water infiltration into soil micropores.

3.4 Conclusion

These results highlight the critical role of texture in soil response to biosurfactant amendment. The Sand(2):SCL(2) samples showed improvement in soil wettability and water retention in a manner proportional to surfactin concentration. This provides evidence that sandy loam textures, which contain significant OMC in clay and silt fractions for surfactin association and larger pore sizes to promote surfactin mass transfer, are most responsive to treatment. Conversely, mineralogically equivalent soil textures with high OMC but lower pore size (SCL, loam) or low OMC (loamy sand, sand) did not display the desired effects of increased water retention after treatment with equivalent surfactin solutions. These findings can inform soil amendment strategies that use biosurfactants, or the microorganisms that produce them, to reduce SWR and retain higher soil moisture levels in effort to reduce agricultural freshwater demands. As sandy loam soil is typically less profitable in agriculture due to poor water retention,⁷² biosurfactant amendment may provide a route to increasing its economic value by improving water retention and reducing irrigation costs.

3.5 Acknowledgment

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Chapter 4 - Effects of *Bacillus subtilis* and Surfactin on Evaporation Dynamics in Loam and Sandy Loam Soils

Overview

Semi-arid regions faced with increasingly scarce freshwater resources must manage competing demands in the food-energy-water nexus. A possible solution modifies soil hydrologic properties using biosurfactants to reduce evaporation and improve water retention. In this study, two different soil textures representative of agricultural soils in Kansas were treated with a direct application of the biosurfactant, surfactin, and an indirect application via inoculation of *Bacillus subtilis*. Evaporation rates of the wetted soils were measured when exposed to artificial sunlight (1000 W/m²) and compared to non-treated control soils.

Experimental results indicate that both treatments alter soil moisture dynamics by increasing evaporation rates by when soil moisture is plentiful (i.e., constant rate period) and decreasing evaporation rates when moisture is scarce (i.e., slower rate period). Furthermore, both treatments significantly reduced the soil moisture content at which the soil transitioned from constant rate to slower rate evaporation. Out of the two treatments, inoculation with *B. subtilis* generally produced greater changes in evaporation dynamics; for example, the treatment with *B. subtilis* in sandy loam soils increased constant rate periods of evaporation by 43% and decreased slower rate evaporation by 49%. In comparing the two soil textures, the sandy loam soil exhibited a larger treatment effect than the loam soil.

To evaluate the potential significance of the treatment effects, a system dynamics model operationalized the evaporation rate results and simulated soil moisture dynamics under typical daily precipitation conditions. The results from this model indicate both treatment methods

significantly altered soil moisture dynamics in the sandy loam soils and increased the probability of the soil exhibiting constant rate evaporation relative to the control soils.

Overall, these findings suggest that the decrease in soil moisture threshold observed in the experimental setting could increase soil moisture availability by prolonging the constant rate stage of evaporation. As inoculation with *B. subtilis* in the sandy loam soil had the most pronounced effects in both the experimental and simulated contexts, future work should focus on testing this treatment in field trials with similar soil textures.

* Manuscript appearing in M.M. Gutierrez, M.V. Cameron-Harp, P.P. Chakraborty, E.M. Stallbaumer-Cyr, J.A. Morrow, R.R. Hansen, M.M. Derby, "Investigating a Microbial Approach to Water Conservation in Semi-Arid Regions: Effects of *Bacillus subtilis* and Surfactin on Evaporation Dynamics in Loam and Sandy Loam Soils", *Frontiers in Sustainable Food Systems*, 6:959592 (2022). doi: 10.3389/fsufs.2022.959591.

4.1 Introduction

Semi-arid regions, such as southwest Kansas, are at the center of the Food-Energy-Water nexus. Food, energy, and water are also linked to societal concerns, such as poverty and economic growth.^{1,73} Agriculture plays an important role in southwest Kansas, requiring energy and water for food production. The depleting Ogallala Aquifer, which this region relies on, is one of a select few aquifers absorbing the brunt of the Great Plains' freshwater demands.^{4,74-76} The Ogallala Aquifer depleted 267.5 ac-ft (i.e., 8.2%) of its total storage between 1950 and 2007.³ In Kansas alone, the aquifer lost 63 ac-ft of water (i.e., 19.7% of the total Kansas storage) in the same timeframe.^{77,78} As the water supply continues to deplete, the depths required to retrieve the water increase, requiring more energy to pump irrigation water.^{76,79,80} This water depletion and required pump energy create an even more convoluted and interdependent relationship between food, water, and energy.

Irrigation has the most significant impact on the depletion of the Ogallala Aquifer compared to any other water use sector. It is an essential part of increasing crop yields and has a significant economic impact on producers' profits.^{1,4,73,74} In Kansas, irrigation accounts for 83% of reported water use, with the Ogallala aquifer providing 90% of that water.⁸¹ In 2009, 70% of southwest Kansas' crop value came from irrigated land, even though the irrigated area only accounted for 44% of the total area.⁸² Water stress induced by climate change may also affect land value, potentially reducing land rental rates by \$3 billion annually over the next thirty years.⁸³

Methods of water application (e.g., irrigation methods and soil moisture monitoring) that reduce evaporation losses and increase the ratio of water applied to water consumed by crops are thoroughly researched and frequently adopted.^{73,77,82} These technologies utilized by producers can help in prioritizing higher soil moisture levels during critical growth stages, and, conversely, allowing drought during others.^{84,85} These methods focus on increasing the efficiency of water use

by changing the method of water application, which can accidentally result in higher water consumption by producers.^{73,86}

4.1.1 Soil amendments for improved soil water retention

Instead of focusing on water application, other methods of improving water use efficiency involve increasing soil water retention so that a greater portion of applied water effectively infiltrates into the soil and is used by crops. Mulching, for example, has been shown to improve the efficiency of water use in semi-arid growing regions by slowing the rate of evaporation.⁸⁷ Like other porous media, evaporation from soil can be classified into three periods: constant-rate, falling-rate, and slower-rate period.^{65,67,88–90}

In the constant rate period, water is evaporated from the top surface of the soil through forced or natural convection while the top layer is saturated, and a hydraulic connection is maintained between saturated and unsaturated parts through capillary action. The capillary action driving evaporation during the constant rate period is also the transport mechanism used by crops for transpiration. The breakdown of this capillary flow defines the lower limits of plant available water.⁹¹ When this hydraulic connection breaks down, the porous media experiences a sharp decrease in evaporation rate, defined as the falling rate period.

For crops, the breakdown of capillary flow and the associated reduction in hydraulic conductivity in the falling rate period indicates the beginning of water stress.⁹² During the falling rate period, liquid islands are formed between particles of the porous system and later, the evaporation enters into the diffusion dominated slower rate period with negligible evaporation when the liquid islands break down, and most of the porous sample becomes unsaturated.^{65,66,93,94} Zribi et al.⁹⁵ demonstrated that mulching reduces the evaporation rate during the constant rate stage, when soil moisture is relatively high, and suggest mulching will be most effective in high-frequency irrigation systems as a result. Liao et al.⁹⁶ also found that mulching reduces evaporation

in the constant rate stage, but they also reported reduced evaporation in the falling rate stage when soil moisture is relatively scarce. Additionally, Liao et al.⁹⁶ examined the soil moisture threshold separating the constant and falling rate stages of evaporation and found little difference between the bare soil and mulching treatments.

An alternative solution to improve water field capacity and reduce evaporation rates from the soil is the use of surfactants as soil amendments.^{22,23} As wetting agents, surfactants have the potential to modify soil hydrological characteristics and improve soil moisture conditions without requiring the infrastructure of irrigation technologies or the application of mulching material.²⁴ Furthermore, they can potentially prolong wetter soil moisture conditions and increase peak soil moisture after wetting events.^{60,97} Due to their amphiphilic nature, these surfactants can accumulate at a solid-liquid interface, preventing their degradation and reducing the surface tension between the water and the soil, thereby modifying soil wetting properties.^{10,26} Once the surfactant is introduced to soil, the surfactant's hydrophobic (non-polar) end associates with a non-polar or hydrophobic site on the soil particles. As water penetrates through the soil matrix, water molecules are attracted to the polar end of the surfactant. The polar end of the surfactant serves as an attachment site for water molecules, increasing water adsorption and reducing evaporation rates.^{10,32,36}

4.1.2 Synthetic Surfactants as Agricultural Wetting Agents

Several studies tested the use of surfactant amendments to improve soil water holding capacity and diminish soil water repellency (SWR), a characteristic of soils which are hydrophilic when saturated but then become increasingly hydrophobic as they dry out.⁹⁸ For example, surfactants can be applied to control the capillary evaporation process by changing the wettability of liquid in soils, reducing water in the unconfined spaces, and thereby reducing the evaporation area.⁹⁹ Oostindie et al.¹⁴ reported consistently higher soil water content in sandy soil by

mechanically applying a commercially available triblock co-polymer surfactant in a two-year field experiment.

The surfactant applications resulted in a 4–32% increase in soil water content for the untreated soil and a 28–36% increase in soil water content for the surfactant-treated soil.¹⁴ The study concluded that surfactant applications have additional advantages: remediation and prevention of SWR, more homogeneous and better soil wetting, and more water available to plant rhizosphere (i.e., the soil-root interface).¹⁴ Lehrs et al.²⁶ were able to obtain an increase in the volumetric water content by 3% ($p \leq 0.08$) at matric potentials from 0 to -20 kPa using an alkyl polyglycoside surfactant. The surfactant increased the volume of water held in pores with diameters $\geq 15 \mu\text{m}$.²⁶ However, many synthetic surfactant residues are considered hazardous to the environment and to humans and also persist in the environment because they are often non-biodegradable.²⁸ With these disadvantages, it has become necessary to identify alternative approaches that are environmentally sustainable.

4.1.3 Biosurfactants as Agricultural Wetting Agents

Surfactants of biological origin (i.e., biosurfactants) are low molecular weight, surface-active amphiphilic compounds produced by bacteria, yeast, and fungi. Although biosurfactants are used regularly in the pharmaceutical, cosmetics, and food industries,³⁶ they have received less attention in sustainable agriculture. However, biosurfactants can play an essential role in keeping the soil water content above the soil moisture threshold, separating the constant rate and falling rate stages.^{10,22,24,25,100} Because they are biodegradable, biocompatible, have low toxicity, and remain stable during environmental changes, they are an eco-friendly and inexpensive alternative to synthetic wetting agents.^{28,32,35} In addition, they can provide a variety of other benefits to agricultural soils by enhancing nutrient availability to plant-associated microbes or by eliminating plant pathogens and pollutants.³⁶

Many microbes in the rhizosphere are known to produce biosurfactants, indicating that these molecules can play a significant role in plant health and development.³² In semi-arid regions specifically, surfactant-producing microbes are a crucial component of soil crusts that influence soil hydrological processes, including infiltration and evaporation.¹⁰¹

Surfactin, a secondary metabolite produced by *Bacillus subtilis*¹⁰², is one of the most effective biosurfactants because it can reduce the surface tension of water from 72 to 27 mN/m with effective emulsification activity.³⁵ Surfactin has been demonstrated to improve soil wettability and soil water capacity,^{35,36} has been used as a bioremediation agent (i.e., an agent to break down pollutants in the soil), and has been applied for enhanced oil recovery. For example, Lowe et al.⁶⁰ studied the addition of *B. subtilis* to sandy soil to measure water soil dynamics and found that the production of surfactin increased the soil water retention in all treatments studied.⁶⁰ Significantly higher water content was measured in treated samples compared to control samples, with a mean of 0.33 cm³ of water per cm³ of soil and $p < 0.001$ for the treated samples.

In another observation, the control samples remained at low water content throughout the experiment, with a final soil water content of 0.064 cm³ of water per cm³ of soil. *B. subtilis* also produces biofilms in soils that are primarily composed of exopolymeric substances that could further modify the soil to a hydrophilic state. Biofilms are produced in low-nutrient environments and are enhanced when surfactin reaches a critical threshold level.¹⁰³ These findings suggest that inoculation of *B. subtilis* is a promising technique to increase soil water capacity and sustainably reduce evaporation rates.

Amending soils with *B. subtilis* could also be a more practical approach than direct application of the wetting molecule. *B. subtilis* production is highly scalable through culture-based methods, and agroindustry waste products can potentially be used as growth media for onsite biosurfactant production while providing the additional environmental benefit of recovering

waste.²⁹ Additionally, *B. subtilis* can be sporulated to be resistant to environmental stressors, making it shelf-stable and suitable for packaging, transport, and storage. Finally, *B. subtilis* may provide long-term wetting as it colonizes and establishes its niche in the soil.

4.1.4 Research objectives

The purpose of this research is to evaluate two treatments that alter the hydrologic properties of two types of soil found in Kansas. To that end, the first objective is to measure the evaporation rates of soils treated with surfactin and *B. subtilis* across soil types. The second objective is to evaluate the feasibility of the treatments for each soil type by testing whether the probability of the soil being above or below the soil moisture threshold is significantly affected by the treatments using a systems dynamics model.

4.2 Experimental Setup and Apparatus

4.2.1 Soils, Sampling, and Storage

Samples of two different soil textures were collected for this project from the Kansas State University experimental fields in Manhattan, Kansas. The chosen samples represent the distribution of soil hydraulic properties in Kansas's agricultural lands.¹⁰⁴ Loam and sandy loam soil textures were chosen due to their high potential for increased returns from improved soil water repellency.^{60,97} The soil collection occurred in two-pound batch sizes from 5 to 15 cm in depth and was categorized into loam and sandy loam, as listed in Table C1 in Appendix C. The samples were stored at 4°C before the analysis.¹⁰⁵ Table C2 summarizes the soil physicochemical characteristics for each texture.

4.2.2 Sample Preparation

The laboratory experiment was designed to evaluate the effects of two treatments on the evaporation rates and was compared to a set of control samples. The treatments are:

- I. Direct application of surfactin: Addition of surfactin directly to agricultural soils to amend hydrologic characteristics.
- II. Indirect application of surfactin: Inoculation of *B. subtilis* into agricultural soils to produce the biosurfactant, as shown by Lowe et al.⁶⁰ and Raddadi et al.⁹⁷, to amend hydrologic characteristics.
- III. Control: Addition of equivalent amounts of water.

The experimental treatments included the addition of surfactin at 1.29×10^{-5} moles of surfactin per kg of soil. For the surfactant applications, 1.667 mL of surfactin solution at 100 ppm were added to a 2-cm-diameter beaker with a 12.5 g soil sample, with an additional 1.51 g of ultrapure water. Surfactin (CAS # 24730-31-2) was obtained from Millipore-Sigma (Merck KGaA©, 2020). *B. subtilis* subsp. *subtilis* was obtained from the American Type Culture Collection (ATCC 6051), and maintained in a 25% glycol solution at -80°C. In the second treatment, the inoculation of the bacteria was performed as described by Lowe et al.⁶⁰ *B. subtilis* was inoculated in soil by adding 1.67 mL of a concentration containing 10^7 cells/mL in a 2-cm-diameter beaker with 12.5 ± 0.01 g of soil sample. During the microorganism preparation, production of a spore stock suspension occurred by growing *B. subtilis* in tryptic soy agar at 28°C, harvesting, and stored the cells in the dark at 4°C. Spores were heat-activated (70 °C for 30 min) followed by germination in a solution of 10 mM of Tris-HCl containing 10 mM of L-alanine for two hours at 37°C. Lowe et al.⁶⁰ described the preparation and spore's activation method. The solution was diluted to 1×10^7 cells/mL by OD₆₀₀ measurements (Epoch2 Microplate Reader, Biotek). For the final inoculation, 1.67 mL of cell solution were added to the soil samples with an additional 1.51g of ultrapure water for a final cell density of 1.33×10^6 cells/g of soil. In the last treatment, a control sample was run with 3.18 mL of ultrapure water for each texture. $n=3$ independent trials were run for each set of samples.

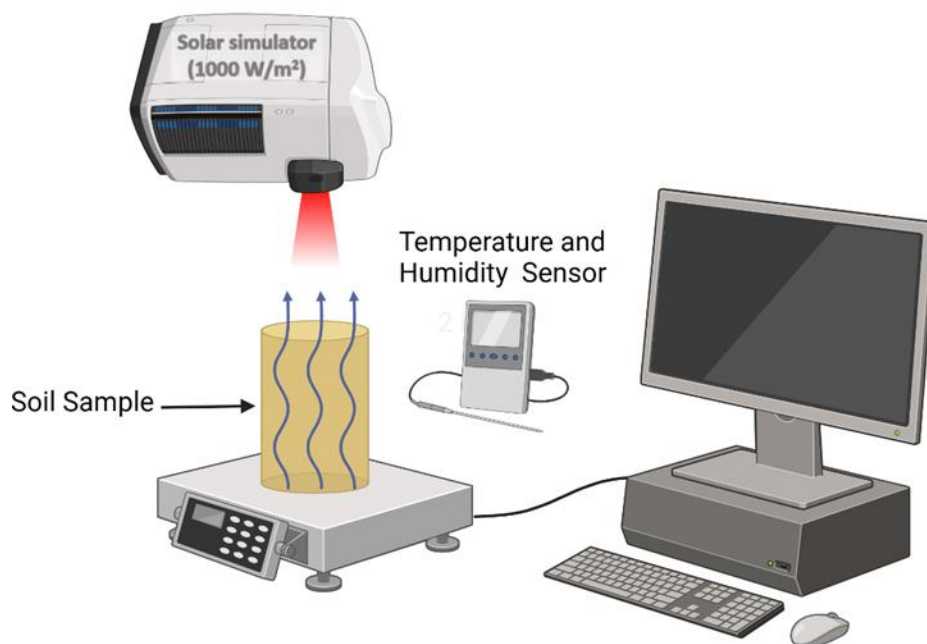


Figure 4.1. Diagram of the evaporation rate experimental apparatus.

4.2.3 Experimental Apparatus

To determine the evaporation rates, the treated soil samples were placed under a solar light simulator (Abet Technologies LS-10500), with a heat flux of $1,000 \text{ W/m}^2$ and a 22-mm beam diameter; a 90° beam tuner was used to project the beam vertically (Figure 4.1). The heat flux was measured with a 2.36-cm-diameter LI-COR LI-200R pyranometer with a sensitivity of $75 \text{ } \mu\text{A}$ per 1000 W/m^2 . The pyranometer's output was measured with an LI-2500A light meter (LI-COR®). The experimental trials were run during a four-month period between April and July. Each sample remained under the solar simulator for 48 hours, and the change in water mass was recorded every five minutes, providing the data for calculating the evaporation rate curves. The change in mass was recorded using a digital scale FX-1200i, maximum capacity of $1200 \pm 0.01 \text{ g}$ (A&D. 2020). The relative humidity (RH) was monitored with an OM-24 Multi-Use Temperature and Humidity Data Loggers (Omega Engineering®), with a specification of $\pm 0.5^\circ\text{C}$ of temperature and $\pm 3\%$ of RH sensitivity.

4.2.4 Estimating Evaporation Rates and Soil Moisture Threshold

The change in mass over time for each of the trials in every soil-treatment combination was used to estimate the evaporation rates, as well as the soil moisture threshold. The phases of evaporation were delineated using a regression approach with the recorded mass of water as the dependent variable in a single threshold model.¹⁰⁶ The estimating equation takes the following form:

$$y_{i,d,t} = \beta_0 + \beta_1 t + \beta_2 post_{i,d,t} + \beta_3 (t * post_{i,d,t}) + \varepsilon_{i,d,t} \quad (4.1)$$

where $y_{i,d,t}$ is the mass of water in grams, t is the time in minutes, $post_{i,d,t}$ is a binary dummy variable indicating whether the sample is currently experiencing constant or slower rate evaporation (a value of zero for constant rate period and a value of one during the slower period of evaporation), $t * post_{i,d,t}$ is an interaction of the t and $post_{i,d,t}$ variables, and $\varepsilon_{i,d,t}$ is the error term for each observation. The subscripts are $i \in 1,2,3$ to denote the trial, d to denote the treatment and soil combination (e.g., surfactin applied on loam), and t to denote the time. The coefficient β_0 is a constant, β_1 is the coefficient on time, β_2 is the coefficient for $post_{i,d,t}$, and β_3 is the coefficient for the interaction of time and the indicator variable, $post_{i,d,t}$.

To determine the evaporation rate during the constant rate and slower rate stages, as well as the soil moisture threshold separating them, Equation 4.1 was estimated iteratively using Ordinary Least Squares regression for each replicate in a soil and treatment combination across all possible delineations of constant and slower rate evaporation by varying the dummy variable, $post_{i,d,t}$.¹⁰⁷ Each iteration of the regression tested the goodness-of-fit for a different point in time when the evaporation rate changed from constant to slower by varying the time when $post_{i,d,t}$ changed from having a value of zero to one. The optimal value of the variable separating the constant and falling rate stages of evaporation for replicate i and soil-treatment combination d ,

defined as $post_{i,d,t}^*$, minimized the root mean squared error (RMSE) metric of model fit.¹⁰⁸ A similar procedure to select threshold values is presented in Gonzalo and Pitarakis.¹⁰⁶ The fitted regression lines for the optimal value of the soil moisture threshold are provided in Figure 4.2 for each set of replicates by soil and treatment type.

The point in time at which replicate i for soil-treatment combination d switched from the constant rate stage to the slower rate stage of evaporation is defined as: $t_{i,d}^* = t \in post_{i,d,t-1} = 0 \ \& \ post_{i,d,t} = 1$. The points at which the slope of the fitted regression lines in Figure 4.2 change correspond with the values of the optimal switching point, $t_{i,d}^*$. After using the density of water to convert the mass to volume in cm^3 , the mass of water at the soil moisture threshold, y_{i,d,t^*} , was used to generate the critical soil moisture threshold values in Table 1. The critical soil moisture threshold for each treatment and soil type combination, d , defined as θ_d^* , was calculated as follows:

$$\theta_d^* = \frac{1}{3} \sum_{i=1}^3 \frac{y_{i,d,t^*} * 1cm^3/g}{8.1cm^3} \quad (4.2)$$

where 8.1 cm^3 is the soil volume. To recover the rates of mass loss for constant rate evaporation and slower rate evaporation, the partial derivate of equation one is taken with respect to time after substituting $post_{i,d,t}^*$ for $post_{i,d,t}$:

$$\frac{\partial y_{i,d,t}}{\partial t} = \beta_1 + \beta_3(post_{i,d,t}^*) \quad (4.3)$$

By replacing the coefficients in Equation 4.3 with their corresponding estimates, $\hat{\beta}_1$ and $\hat{\beta}_3$, and setting $post_{i,d,t}^*$ equal to zero or one, the evaporation rates for each replicate in a soil and treatment combination during the constant rate and slower rate stages can be computed. The estimated rate of mass loss during the constant rate stage in grams of water lost per 5-minute interval. $\hat{\beta}_1$, was then converted into the corresponding evaporation rate, $E_{i,d}^{const}$, in millimeters of

water lost per day. Similarly, the estimated rate of mass loss during the slower rate stage, $\hat{\beta}_1 + \hat{\beta}_3$, was converted into the corresponding evaporation rate, $E_{i,d}^{slow}$. Within each treatment and soil combination, d , the arithmetic means of the two evaporation rates for each set of three replicates, E_d^{const} and E_d^{slow} , were then computed and are reported in Table 4.1. Defining $\widehat{se}_{d,i}^{const}$ and $\widehat{se}_{d,i}^{slow}$ as the standard errors for the estimated values of $E_{i,d}^{const}$ and $E_{i,d}^{slow}$, then the standard error for the evaporation rates across all three replicates, se_d^{const} and se_d^{slow} for the constant and slower rate stages respectively, were calculated using the following formula and reported in Table 4.1:

$$se_d^r = \sqrt{\frac{(\widehat{se}_{d,1}^r)^2 + (\widehat{se}_{d,2}^r)^2 + (\widehat{se}_{d,3}^r)^2}{3}} \quad \forall r \in (const, slow) \quad (4.4)$$

4.3 Results and Discussion

4.3.1 Soil Drying Experimental and Determination of Evaporation Rates

Following treatment with surfactin, either directly or indirectly through inoculation of *B. subtilis*, both the loam and sandy loam soils experienced similar changes in evaporation dynamics and soil water retention. The mass of water over time for each set of experiments is presented in Figure 4.2 and Table 4.1.

Table 4.1. Average evaporation rates during constant rate and slower rate periods, as well as the critical soil moisture threshold dividing the two periods (found by the method presented in section 4.2.4). Estimates and the corresponding standard errors represent the average across three trials for each soil type and treatment combination.

Soil type	Treatment	Constant evaporation rate (mm/day)	Slower evaporation rate (mm/day)	Soil moisture threshold (% soil volume)
Loam	Control	8.36 ± 0.03	2.56 ± 0.03	6.67%
	Surfactin	9.66 ± 0.04	1.93 ± 0.02	6.58%
	Bacteria	9.37 ± 0.04	1.44 ± 0.04	2.76%
Sandy loam	Control	5.01 ± 0.03	4.23 ± 0.01	24.50%
	Surfactin	6.70 ± 0.02	2.95 ± 0.03	11.10%
	Bacteria	7.16 ± 0.02	2.17 ± 0.03	5.76%

Estimates and the corresponding standard errors represent the average across three trials for each soil type and treatment combination.

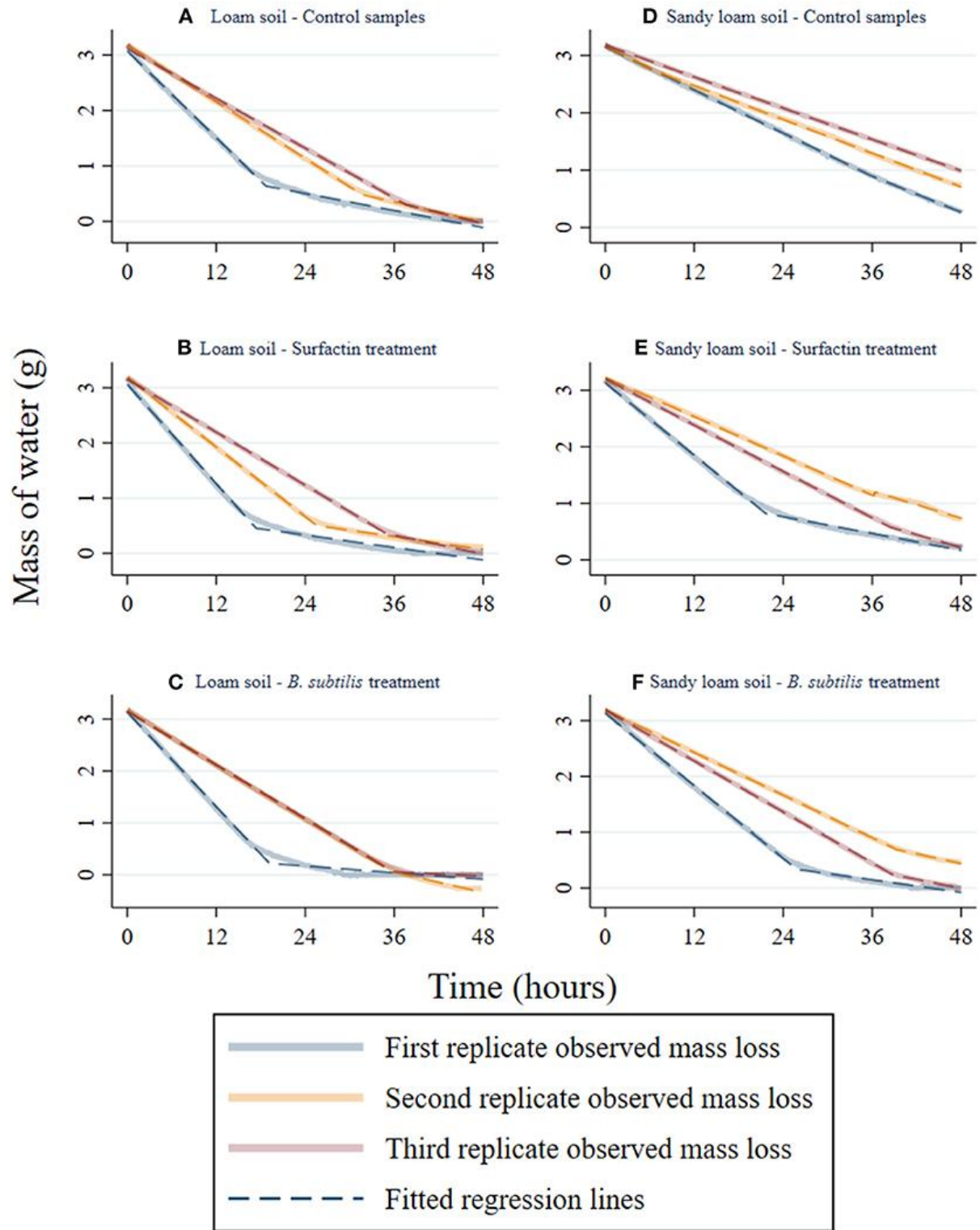


Figure 4.2. Observed data showing loss of water mass over time for the three replicates in each combination of soil type and treatment. The inflection points for each regression line were chosen by minimizing the RMSE, the square root of the difference between observed and predicted values, across all values of the $post_{idt}$ variable in Equation 4.1.

For the loam soil, treatment with surfactin caused the soil to transition from constant rate evaporation to slower rate evaporation earlier compared to the control samples. Treatment with *B. subtilis*, in contrast, shifted the transition between the constant and slower rate periods of evaporation to occur at lower soil moisture contents and later in time. The second and third replicates of the *B. subtilis* treatment on the loam soil, depicted in Figure 4.2C, displayed a negative mass of water values toward the end of the time series, potentially due to additional evaporation of residual moisture present in the soil before the drying experiments began. The earlier occurrence of a transition from the constant rate to slower rate evaporation periods for the loam soil replicates treated with surfactin, reflected in the change in slope of the mass of water curve in Figure 4.2B, suggests the treatment increased the rate at which water was initially evaporated from the soil.

This was likely due to surfactin's hydrophilic properties that increase the ease of readily evaporating bulk water from the soil.⁹⁹ However, as the water starts to become scarce in the slower evaporation stage, the evaporation rates decrease due to the hydrophilic properties of the surfactin associated with the soil, increasing the soil wettability and capillarity effects.⁹⁹

For the sandy loam soils, treatment with surfactin and *B. subtilis* displayed a change in the slope for the mass of the water curve, which was not present for any of the control replicates. Compared to the control samples for the sandy loam soil (Figure 4.2D), treatment with surfactin (Figure 4.2E) and *B. subtilis* (Figure 4.2F) indicate greater rates of evaporation during the constant rate period followed by decreased rates of evaporation during the slower rate period. Both treatments caused the transition from constant to slower evaporation rate periods to occur at lower soil moisture content. The difference between the control and treated samples for the sandy loam soils is most apparent in the control samples' mass of water curves displayed in Figure 4.2D. The control samples for the sandy loam soil lack an abrupt change in slope, meaning constant rate and slower rate evaporation are difficult to differentiate. This could be because the control replicates

did not dry out as quickly in comparison to the treated samples for the sandy loam soil. As such, the control replicates may not have reached the point at which the slope in the curve changed. While other wetting mechanisms by *B. subtilis* were possible, wetting was likely due to production of surfactin or other amphiphilic metabolites. Production of biosurfactants by *Bacillus spp* in soil is well known, particularly for remediation applications,³⁶ and soil provides a spatially confined environment that facilitates quorum-sensing processes, which includes surfactin expression.¹⁰⁹ Further, surfactants are also expressed in soil to provide enhanced motility of cells.³⁶ Finally, the observations made here are in agreement with those made by Lowe et al.,⁶⁰ where *B. subtilis* addition to sandy soil was used to break down soil water repellency and increase soil water retention⁶⁰

4.3.2 Evaporation Rates and Soil Moisture Threshold

Figure 4.3 shows the evaporation rate of the three experimental trials of each soil type and treatment, as well as the averaged constant rate and slower rate of evaporation estimated using the mass loss data depicted in Figure 4.2. The difference between the treated and the control samples is most apparent in the sandy loam soil displayed in Figures 4.3D-F; the loam soil, shown in Figures 4.3A-C, exhibits the same behavior but to a lesser degree. For either treatment, increased evaporation rates in the constant rate period and decreased evaporation rates in the slower rate period were observed in both soils compared to the control.

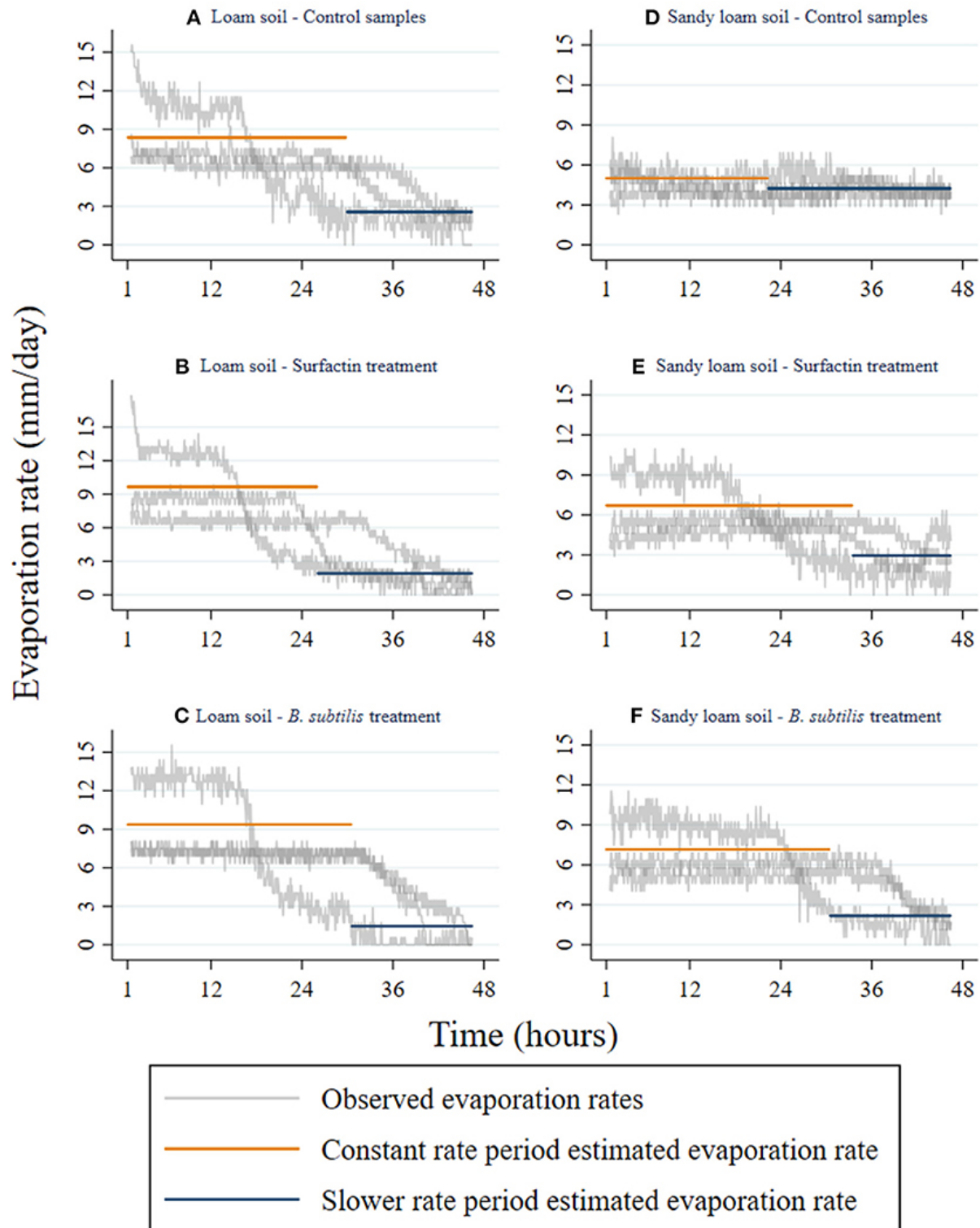


Figure 4.3. Effect of surfactin and *B. subtilis* treatment on constant and slower evaporation rates by soil type. Evaporation rates were calculated by converting the mass of water lost in five-minute intervals into millimeters of water evaporated per day. Observed evaporation rates display a 90-minute moving average to smooth extreme values.

As shown in Table 4.1, adding surfactin to the loam soils increased the average constant evaporation rate by 15.6% and decreased the average slower evaporation rate by 24.7% compared to the control; adding *B. subtilis* increased the average constant evaporation rate by 12.2% and decreased the average slower evaporation rate by 43.8% compared to the control. However, *B. subtilis* had a 2.95% lower average constant evaporation rate and a 25.4% lower average slower evaporation rate compared to the surfactin-treated samples. For sandy loam soils, adding surfactin increased the average constant evaporation rate by 34.0% and decreased the average slower evaporation rate by 30.3% compared to the control; adding *B. subtilis* increased the average constant evaporation rate by 43.0% and decreased the average slower evaporation rate by 48.6% compared to the control. However, *B. subtilis* had a 6.73% higher average constant evaporation rate and a 26.3% lower average slower evaporation rate compared to surfactin.

In both the loam and sandy loam soils, the increase in the constant evaporation rates was the anticipated impact of direct (i.e., surfactin) or indirect addition (i.e., inoculation with *B. subtilis*) of the amphiphilic surfactant. The loam soil experienced a greater increase in the constant rate evaporation period after the surfactin was directly applied to the soil, while the sandy soil experienced a greater increase with *B. subtilis* inoculation. However, the direct and indirect application of surfactin had a much larger effect on the sandy loam than on the loam soils. These differences in the treatment effects across soil types are also observed in studies of the effects of soil crusts on evaporation as soil crusts are known to modify soil water repellency as well.¹¹⁰ A potential explanation for this is the effects of the treatments on the soil moisture threshold; they reduce the threshold soil moisture value resulting in higher evaporation rates until a lower soil moisture value, compared to the control, is reached.

Loam and sandy loam soils exhibited a decrease in slower evaporation rates for the direct and indirect treatments with surfactin. In both soil types, soil inoculated using *B. subtilis* resulted

in a larger decrease in the slower evaporation rate than the directly applied surfactin. One possibility for the decrease continues from the SWR threshold explanation discussed with the constant rate of evaporation. The slower rate period will begin with a lower soil moisture value compared to the control. Given the positive relationship between evaporation and soil moisture, the reduced soil moisture values of treated samples entering the slower period of evaporation could cause the reduction in evaporation rates.

Alternatively, the biosurfactant's impact on surface tension in the soil throughout time could have played a role. This mechanism would be similar to the results found in He et al.,⁹⁹ which showed the evaporation rate can decline as the concentration of surfactant increases. In the soil samples, the surfactant would have increased in concentration as soil moisture declined. As such, the concentration of surfactant would be greater during slower periods of evaporation when soil moisture is relatively low. However, given the differences in working liquid and experimental designs between these experiments and those of He et al.,⁹⁹ this second explanation for the decreased slower period of evaporation rate is less likely.

While the increased evaporation rate during the constant rate stage may appear counterproductive to the goal of water conservation at first, it can be advantageous because of the positive relationship between surface soil moisture and evaporation lower in the soil profile.¹¹¹ The increased constant evaporation rate would hasten the formation of a dry surface layer, a shallow layer of dry soil wherein evaporation is driven by water vapor diffusion rather than capillary action.¹¹² The faster formation of this layer induced by the treatments could quickly shield lower portions of the soil profile from the more extractive capillary-action driven evaporation processes.^{113,114} Furthermore, the decreased slower evaporation rates indicate the treatments mitigate evaporation of the remaining moisture.

Finally, the increased constant evaporation rates suggest water would easily infiltrate into the root zone. As surfactin is produced naturally within the soil microbiome, this expectation conforms with studies of infiltration rates in soil crusts which find biological, rather than physical, soil crusts exhibit greater infiltration rates.¹¹⁵ Unlike the mechanical nature of physical soil crust formation due to the dispersion of fine soil particles, biological soil crusts are defined by symbiotic assemblages of species commonly including bacteria and fungi. As such, the hydrologic impacts of a biological soil crust are determined by the activity of the organisms involved. However, given the mixed results reported concerning the impact of soil crusts on infiltration, further experimental research is required to determine the effect of the treatments employed here on infiltration rates.¹¹⁶

4.3.3 Soil Moisture Simulations Using a System Dynamic Model

The value of additional soil moisture during the growing season is in constant flux due to growth-stage-dependent water demands for each crop, state-dependent inflow and outflow processes, and the random nature of rainfall events. For example, the value of water changes abruptly on either side of the soil moisture threshold, dividing the constant and falling rate stages of evaporation depending on the current objective. If an irrigator intends to seal soil moisture lower in the root zone, the value of additional water in the surface layer is negative because this may push the soil above the soil moisture threshold and increase evaporation. Given these complexities, rigorously evaluating whether the addition of surfactin or the inoculation of *B. subtilis* is desirable in an agricultural production environment requires an approach capable of accommodating these complexities. System dynamics (SD) was developed to address this sort of complex problem where stochastic processes, nonlinear equations of motion, and feedback mechanisms are present. Therefore, to test the feasibility of the surfactin and inoculation with *B. subtilis*, a system dynamics model of soil moisture in the upper soil profile was created and simulated using Vensim.¹¹⁷

The purpose of this treatment would be to modify soil hydrological characteristics in the upper layers of soil to retain soil moisture lower in the soil profile. To determine how effective the treatments may be at achieving this objective, the model is structured to simulate soil moisture dynamics in the upper ten centimeters of the soil for a one hundred-day growing season. Ten centimeters was chosen because of its similarity to the 10-15 cm depth of the soil sampling in the field and the existence of prior research studying soil hydrology with a 10 cm treated soil layer.¹¹⁸ As depicted in Figure 4.4, it is designed to parsimoniously mimic the fundamental soil moisture balance equation using one inflow (infiltration) and one outflow (evaporation).¹¹⁹ Built-in feedback mechanisms between the present soil moisture level and the two flows address the nonlinearities created by the soil moisture threshold separating the constant rate and slower rate evaporation periods.

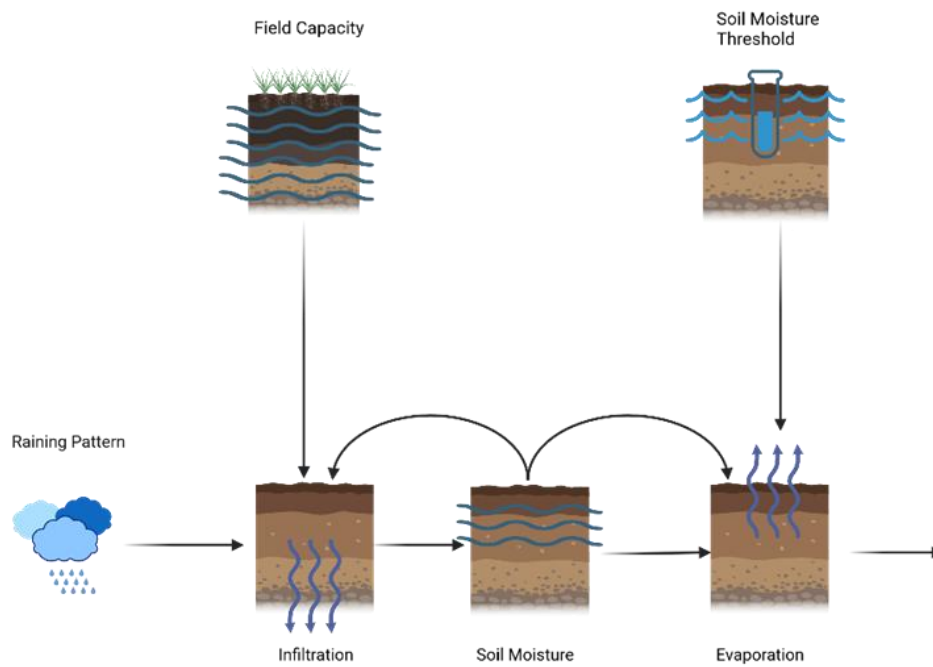


Figure 4.4. System dynamic model of soil moisture during a season. Random precipitation events occur, then the feedback from the present soil moisture level causes infiltration to cease when the soil has reached field capacity. The feedback between the present soil moisture level and the evaporation rate determines whether constant or falling rate evaporation occurs according to the soil moisture threshold.

The behavior for each combination of soil and treatment type was simulated four times, beginning with completely saturated soil and using model parameters drawn from the laboratory experimental results described in Section Evaporation rates and soil moisture threshold and System Dynamics Model parameterization in Appendix C. Within the simulation, the occurrence of rainfall on each day was determined by a Bernoulli trial, $p = 0.38$, such that the arrival of rainfall events over the course of the 100-day model run followed a Poisson process. If the outcome of the Bernoulli trial for a given day in the simulation produced a rainfall event, the intensity of the rainfall event was determined by a random draw from an exponential distribution with $\lambda = 105.45^{-1}$.¹²⁰ The probability of a rainfall event occurring and the rate parameter for the exponential distribution, λ , were calibrated using daily precipitation data during the summer months, June to August, of 2015 through 2020 from the Global Historical Climatology Network.¹²¹ The precipitation data were filtered to include 19 stations in Finney County, Kansas, because of the semi-arid nature of Southwest Kansas. Four precipitation patterns were produced using 100 realizations of the stochastic rainfall process described above and were then held consistent across each of the six combinations of soil and treatment. The exact parameterization of the precipitation regime and other variables is displayed in Table C3 in Appendix C.

The simulated soil moisture dynamics from the system dynamics model were compared across soil types and treatments to determine the feasibility of each potential amendment. To determine each treatment's efficacy, the daily soil moisture values across the 100-day simulated season were recorded for each model run, and then the values for each soil and treatment were combined across the four runs to produce a cumulative distribution. The Wilcoxon rank-sum test, or Mann-Whitney two-sample statistic, was used to detect significant differences between the treatment and control soil moisture distributions for each soil type.^{122,123} Then, to determine the efficacy of the treatment, the probability of a random draw from the treated soil's distribution

being above the soil moisture threshold while a random draw from the control soil's distribution is below the threshold was computed. Both the statistical tests and the probabilities comparing treatment and control distributions were calculated using Stata Statistical Software (StataCorp ®).

4.4 System Dynamic Model Results

The distributions produced from the simulated soil moisture dynamics displayed in Figure 4.5 demonstrate the implications of the treatment's effects for surface soil exposed to typical precipitation patterns in southwest Kansas. For the loam soils, displayed in Figures 4.5A-C, neither treatment caused a significant difference in the distribution of seasonal soil moisture as shown by the Wilcoxon rank-sum test results in Table 4.2. The null hypotheses that the soil moisture in the control soil, θ_{contr} , is equivalent to that of the surfactin-treated soil, θ_{surf} , or *B. subtilis* treated soil, θ_{bact} , were not rejected at a significance level of $\alpha < 0.10$. As such, the probabilities of the treated samples being above their respective soil moisture thresholds while the control samples are below them, 0.48 and 0.53 for the surfactin and *B. subtilis* treated soils, respectively, were not considered significant.

Both treatments resulted in significant differences between the control and treated sandy loam soils as the exact p-values, or Exact Prob in Table 4.2, were below the 0.10 significance level. The probability of the sandy loam soil treated with surfactin being above the soil moisture threshold, experiencing constant rate evaporation, while the control soil was below the threshold, experiencing slower rate evaporation, was estimated to be 0.54. The corresponding probability for the *B. subtilis* treated soil was estimated to be 0.55. This increased probability of the treatments experiencing constant rate evaporation is also evidenced in Figures 4.5D-F. While the increased evaporation rates during the constant rate period cause a leftward shift in sandy loam distributions in Figures 4.5E and 4.5F (toward drier soil moisture levels), the treatments' effect on lowering the

soil moisture threshold increases the portion of the simulated growing season spent above the threshold.

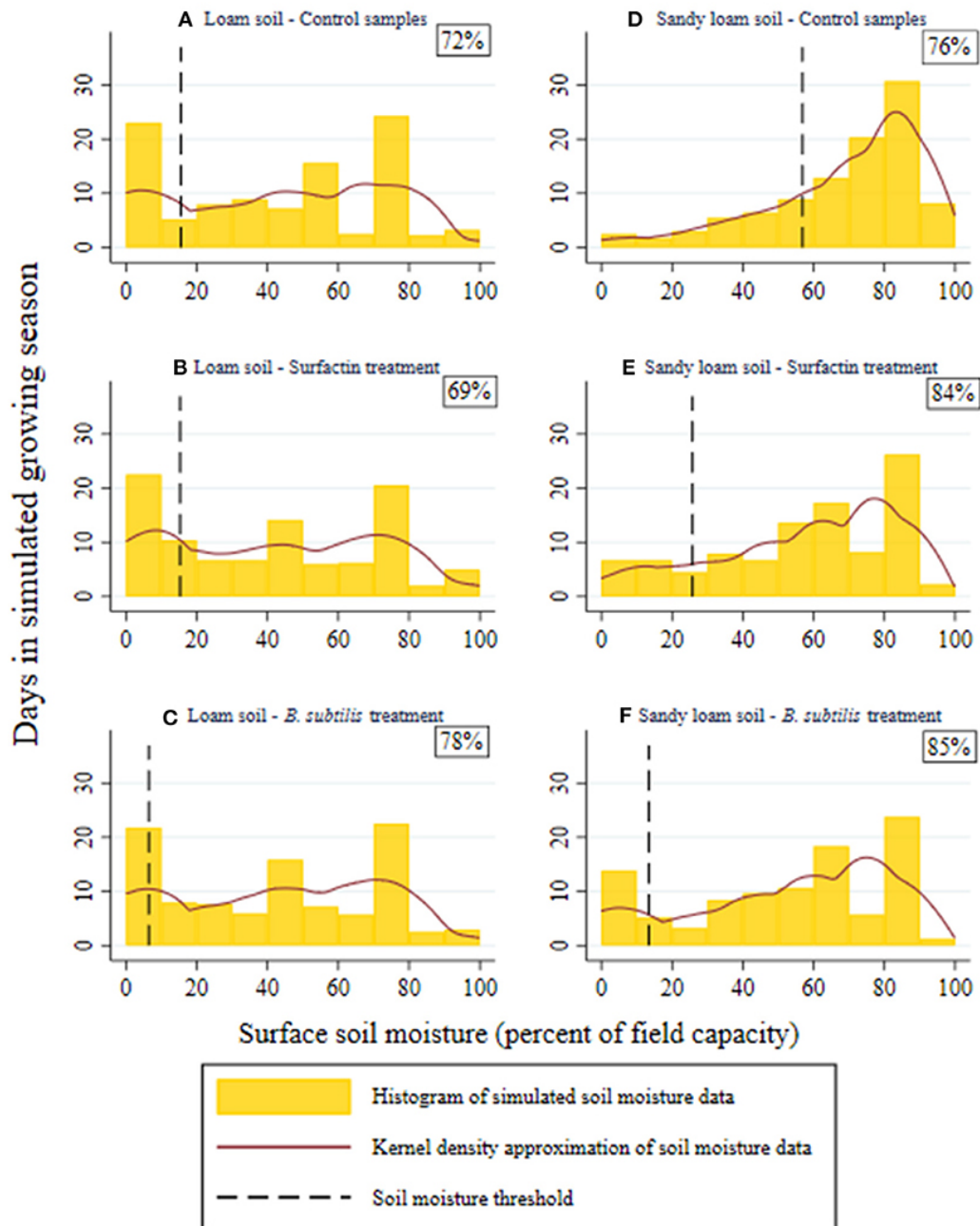


Figure 4.5. Histogram of daily simulated seasonal soil moisture from the system dynamics model by soil and treatment. Each treatment's density function represents simulated soil moisture values for a 100-day growing season. The Kernel density approximation indicates the expected probabilities as the number of models runs approaches infinity. Constant rate and slower rate evaporation occur above and below the soil moisture threshold for each graph, respectively. The percent of simulated days spent above the soil moisture threshold are displayed at the top right of each panel.

By including uncertainty in evaporation rates and randomly generated rainfall data, the system dynamics model simulates whether the treatments will induce a favorable shift in soil moisture dynamics and fulfil one of the principal roles of the microbiome for sustainable agricultural production, suppressing biotic and abiotic stress.¹²⁴ For the sandy loam soils, the simulated data indicate the treatments would reduce drought stress, given the significantly increased probability of the soil moisture being above the threshold separating the constant and slower rate stages. While, the significant difference in soil moisture distributions for the treated and control sandy loam soils is predominantly driven by treated soils' greater constant evaporation rates, the reduction in slower evaporation rates may play a more significant role in altering dynamics when rainfall is relatively scarce. For example, in a study of the impact of drought on wheat plants inoculated with two bacterial strains, Kasim et al.¹²⁵ found the benefits of inoculation with a different microbe in the *Bacillus* genus, *Bacillus amyloliquefaciens*, increased as the drought increased in duration.

Due to this research's focus on the effects of each treatment on evaporation dynamics in the absence of other mediating factors, these results do not account for the impacts of plant-microbe or microbe-microbe interactions on plants' ability to tolerate drought stress.¹²⁶ However, in isolating the impact of surfactin and *B. subtilis*, these results facilitate understanding of higher-order processes in the microbiome by highlighting a mechanism by which surfactant-producing microbes affect soil moisture conditions. Additionally, unlike the ability of another *Bacillus* species to mitigate salinity induced abiotic stress studied by Shahzad et al.,¹²⁷ the results in this study demonstrate *B. subtilis* can significantly alter soil moisture conditions even if it has not colonized a plant's root system. Lastly, while the effects of the treatments on infiltration rate and maximum water holding were not tested experimentally and remained consistent for each soil type in the system dynamics model as a result, any potential changes to these parameters are unlikely

to fundamentally alter these results for the surface soil layer. The implications of changes to either of these parameters are greater for soil moisture dynamics lower in the soil profile and require further investigation.

Table 4.2. Test results comparing the simulated effects of surfactin and *B. subtilis*. Treatments on soil moisture from the system dynamics model.

Feasibility criteria test results

Loam—Surfactin	Wilcoxon rank-sum test	$H_0 : \theta_{contr} = \theta_{surf}$	$z = 1.45$ $Prob > z = 0.15$ $Exact Prob = 0.15$
	$P(sm_{d,t} > \hat{\theta}_d^* d = \text{Surfactin, loam soil}$ $\& sm_{d,t} < \hat{\theta}_d^* d = \text{Control, loam soil}) = 0.48$		
Loam— <i>B. subtilis</i>	Wilcoxon rank-sum test	$H_0 : \theta_{contr} = \theta_{bact}$	$z = 1.22$ $Prob > z = 0.22$ $Exact Prob = 0.22$
	$P(sm_{d,t} > \hat{\theta}_d^* d = B. subtilis, \text{loam soil}$ $\& sm_{d,t} < \hat{\theta}_d^* d = \text{control, loam soil}) = 0.53$		
Sandy Loam—Surfactin	Wilcoxon rank-sum test	$H_0 : \theta_{contr} = \theta_{surf}$	$z = 7.04$ $Prob > z = 0.00$ $Exact Prob = 0.00$
	$P(sm_{d,t} > \hat{\theta}_d^* d = B. subtilis, \text{sandy loam soil}$ $\& sm_{d,t} < \hat{\theta}_d^* d = \text{control, sandy loam soil}) = 0.54^*$		
Sandy Loam— <i>B. subtilis</i>	Wilcoxon rank-sum test	$H_0 : \theta_{contr} = \theta_{bact}$	$z = 9.04$ $Prob > z = 0.00$ $Exact Prob = 0.00$
	$P(sm_{d,t} > \hat{\theta}_d^* d = B. subtilis, \text{sandy loam soil}$ $\& sm_{d,t} < \hat{\theta}_d^* d = \text{control, sandy loam soil}) = 0.55^*$		

The distribution of seasonal soil moisture values for the treatment and control groups are compared using Wilcoxon rank-sum tests, and then the probability of the treated soil being above the soil moisture threshold while the control soil is below it are reported. The (*) symbol indicates that the estimated probability is significantly different than random chance.

4.5 Conclusions

Direct and indirect addition of surfactin significantly modified soil moisture dynamics in both sandy loam and loam soils. The constant rate of evaporation was significantly higher relative to the control samples, while the slower evaporation rate was significantly lower. The effect was most pronounced in the sandy loam soils, which demonstrated little difference between constant and slower periods of evaporation without treatment. Results from the system dynamics model demonstrate the implications of these changes over a more extended time period with realistic precipitation patterns. The increase in constant rate period of evaporation caused the soil to dry

out more quickly in the treated samples despite intermittent rainfall events, and the reduced slower evaporation rate caused soil moisture to persist at lower levels for longer in the absence of precipitation.

The research findings suggest that the addition of surfactin, either directly or indirectly through inoculation of *B. subtilis*, could preserve precious soil moisture available to agricultural crops in semi-arid growing regions. This study shows the addition of surfactin affects evaporation dynamics in loam and sandy loam soils at the lab scale. Faster formation of a dry surface layer due to the increase in constant period of evaporation could preserve precious moisture lower in the root zone, and the reduction in slower evaporation rate would mitigate evaporation after the surface layer has dried. While both treatments may be effective in reducing evaporative demand, the effects may be most pronounced in sandier soils or those characterized by coarser textures. Given the more pronounced effects of the *B. subtilis* treatment in experimental settings and the similar efficacy in a simulated environment, indirect treatment with this microorganism could prove equally effective, while taking advantage of the benefits of its biological origin, including its rapid scale-up through culture and due to the fact that it is highly adaptable to agricultural soils,¹²⁸ where it can establish a niche in the soil microbiome to continuously produce surfactin. Developing an improved understanding of the soil environments that facilitate *B. subtilis* colonization and production of surfactin will be important for further advancement of this approach. For example, the presence of specific genera of bacteria in the soil, such as *Lysinibacillus*,¹⁰² may stimulate surfactin production from *B. subtilis* to facilitate wetting, while use of mineral fertilizers can have a negative impact on *B. subtilis* colonization¹²⁹ and therefore, would also be expected to impact its wetting potential.

Future work should investigate *B. subtilis* in a field trial setting, given the absence of plant-microbe and microbe-microbe interactions in this study. The impacts of inoculation with other

strains from the *Bacillus* genus should also be tested, given its higher prevalence of strains with resistance to drought and extreme temperature stress.¹³⁰ Similarly, due to the improved water retention following inoculation of maize plants with *Enterobacter sakazakii* observed by Javeed et al.,⁴⁹ the effects of inoculating soils with microbes from genera other than *Bacillus* may also be studied in the absence plants to determine the mechanisms by which each genus alters soil moisture dynamics. Finally, the optimal concentration and application rates of *B. subtilis* should be investigated as these will determine the financial feasibility of using inoculation in a production agriculture setting.

4.6 Nomenclature

4.6.1 Variables

y	Mass of Water in grams.
$\beta_0, \beta_1, \beta_2, \text{ and } \beta_3$	Coefficients for regression constant, time, indicator variable for constant or slower rate evaporation, and interaction term between time and indicator variable.
t	Time in minutes.
$post$	Binary dummy variable with value equal to 0 during constant rate evaporation and 1 during slower rate evaporation.
e	Error variance for each observation.
E_d^{const}, E_d^{slow}	Evaporation rate during constant or slower rate stages, respectively, in millimeters per day.
$se_d^{const}, se_d^{slow}$	Standard error for evaporation rate during constant or slower rate stages, respectively.
θ_d^*	Critical soil moisture threshold at which evaporation changes from constant rate to slower rate stage for each treatment and soil type combination expressed

as the water volume (cm^3) per total volume of soil (cm^3).

4.6.2 Subscripts

i	Trial number
d	Treatment and soil combination
t	Time

4.6.3 Symbols

$\hat{x}$	The accent, $\hat{}$, indicates the estimated value of the corresponding variable
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4.7 Acknowledgments

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Chapter 5 - Microbial Amendments Co-inoculated with Inorganic Nitrogen for Improved Soil Water Management

Overview

This study explores soil environments conducive to using *Bacillus subtilis* as a soil amendment to enhance water retention in agricultural soils, particularly under drought conditions. *B. subtilis* produces Surfactin, a secondary metabolite, which can reduce soil water repellency, creating hydrophilic conditions that improve the soil's water-holding capacity. Previous research demonstrated that inoculating sandy loam soils with *B. subtilis* in a lab-scale system decreased the soil moisture threshold, increasing soil moisture availability.

Here, the interaction between different forms of inorganic nitrogen (N) and *B. subtilis*-mediated soil wetting is investigated. Evaporation rate experiments were conducted on *B. subtilis*-inoculated soils treated with various nitrogen compounds, specifically NH_4NO_3 , NH_4Cl , and NaNO_3 , representing different nitrogen-based fertilizers. The soils were then heated in dryout experiments to measure evaporation rates and water retention levels. Results revealed that soils treated with NH_4NO_3 and NaNO_3 retained significantly more water than untreated soils and those treated with other forms of nitrogen. Quantification of inorganic nitrogen levels (ammonium and nitrates) via KCl extraction during the experiments showed that soils treated with NH_4NO_3 underwent the most intense consumption of nitrates, with nitrate levels dropping well below their initial concentrations despite the addition of nitrates during inoculation.

These findings suggest that NH_4NO_3 treatment of soils offers the most significant improvements with respect to water retention. Moreover, *B. subtilis* inoculation provides dual benefits for stressed agricultural soils by enhancing water-holding capacity and facilitating denitrification, potentially reducing nitrogen runoff and nitrogen losses, which could be highly beneficial in soil resilience under drought conditions while minimizing environmental impacts.

5.1 Introduction

Biosurfactants have become increasingly important in bioremediation, particularly in the petroleum, mining, and agricultural sectors. Agricultural improvements using biosurfactants enhance soil quality by eliminating metal pollutants, enriching it with organic matter, improving moisture retention, and promoting healthy plant growth.^{28,36} Studies show that surfactin, a secondary metabolite produced by *Bacillus subtilis*,¹⁰² is one of the most effective biosurfactants for improving soil water retention.^{35,36} It has been documented to boost soil moisture and reduce evaporation rates, demonstrating strong potential as a soil-wetting agent.^{60,61}

To harness these benefits in practical applications, *in situ* inoculation with *B. subtilis* is emerging as a promising strategy for localized surfactin production, significantly reducing processing costs.⁵⁵ However, fluctuating environmental soil conditions may impact consistency, posing a challenge for large-scale applications. Supplementing soil with targeted nutrients can help mitigate this variability by enhancing microbial activity and promoting the growth of beneficial soil bacteria responsible for biosurfactant synthesis.¹³¹

Building on these findings, extensive research has demonstrated significant variation among *B. subtilis* strains in the types and quantities of biosurfactants they produce. In addition to refining application methods, researchers have focused on optimizing surfactin production under controlled conditions, particularly in bioreactors. For instance, a study by Sen¹³² identified optimal concentrations of key media components—glucose, NH_4NO_3 , FeSO_4 , and MnSO_4 —to enhance surfactin yield in a biochemical reactor using the strain DZ10.¹³²

In 2017, Yaseen et al. studied biosurfactant production in *B. subtilis* BBG208, a fengycin-overproducing strain engineered with a green fluorescent protein (GFP). By varying nitrogen and carbon sources, they found that ammonium nitrate enhanced fengycin production but not surfactin.¹³³ In contrast, surfactin levels increased in the presence of urea and ammonium

bicarbonate NH_4HCO_3 .¹³³ These results highlight that biosurfactant yield, and even chemical structure, is influenced not only by the microbial strain but also by environmental and culture conditions. Factors such as nutrient sources, trace elements, temperature, oxygen availability, and pH can all induce structural changes in the biosurfactant molecule.^{134,135} The results from Yassen et al. support that the carbon-to-nitrogen ratio significantly impacts biosurfactant production, a finding echoed by Geissler et al., who identified a 10:1 C:N ratio as optimal in their review.¹³⁶

Inefficient nitrogen management, particularly losses from runoff and leaching, significantly threatens soil health by depleting essential nutrients.¹³⁷ Nitrification refers to the transformation of nitrogen in organic and inorganic compounds from a reduced to a more oxidized state, including the oxidation of ammonia to nitrate.^{138,139} Emerging microbial approaches that integrate nitrification and denitrification in a single system under uniform conditions offer a more efficient alternative, with benefits including higher growth rates, lower costs, and increased suitability for complex systems like wastewater and aquaculture treatment.¹⁴⁰ These treatment processes often involve autotrophic nitrification, where nitrifiers convert ammonium to nitrite or nitrate under aerobic conditions, and anaerobic denitrification, where denitrifiers reduce nitrate and nitrite to nitrogenous gas.^{141–143} Additionally, *B. subtilis* has been shown to metabolize nitrates and ammonium to produce surfactin,¹⁴⁴ which helps mitigate some drawbacks of heterotrophic nitrate processing, particularly the release of greenhouse gases.

Building on these microbial approaches, recent research has identified *Bacillus spp.* as participants in heterotrophic nitrification and aerobic denitrification processes,¹⁴⁵ offering a potential alternative to traditional nitrogen removal systems.¹⁴⁰ Several studies have investigated the role of *Bacillus spp.* in denitrification, particularly within wastewater systems and biochemical reactors. For instance, a study conducted by Sun et al. evaluated the use of *B. subtilis* as a biofertilizer for reducing ammonia (NH_3) emissions from soils. In this study, the bacteria were

used as a biofertilizer, and their performance was compared to traditional organic fertilizers. Unlike chemical fertilizers, incorporating *B. subtilis* biofertilizer reduced NH_3 volatilization by up to 71%, which is a 44% greater reduction than conventional fertilizers.¹⁴⁶ Although the role of *B. subtilis* in soil denitrification is not yet fully understood, emerging research suggests that this bacterium could significantly help manage nitrogen levels in soil. *Bacillus* spp., including *B. subtilis*, possess advantages such as a rapid growth rate and the ability to tolerate various environmental conditions, enabling them to act as an effective bioremediation agent.¹⁴⁰

This study hypothesizes that inoculating *B. subtilis* at higher cell densities, in conjunction with an inorganic nitrogen source, can enhance bacterial growth, increase surfactin production, and improve denitrification, ultimately contributing to greater soil water availability.

To test this hypothesis, experiments are carried out using sandy loam soil in a growth medium supplemented with various nitrogen sources, including (NH_4NO_3 , NH_4Cl , and NaNO_3). Key indicators, such as water retention, wettability, and denitrification efficiency, are assessed through measurements of soil water content (SWC), evaporation rates, KCl-extracted inorganic nitrogen, and electrical conductivity. The results may also support the potential replacement of the current medium with a urea-based fertilizer for future scale-up applications.

5.2. Materials and Methods

5.2.1 Soil Samples

To assess the impact of *B. subtilis* on soil water retention and evaporation rate, soil samples of sandy loam texture were collected from Ashland Bottoms, the Kansas State University experimental station. A three-kilogram batch was taken from 0 to 15 cm depth. The sandy loam texture was specifically chosen for its relevance to improving soil moisture dynamics, as suggested by the results of previous projects described in Chapters 3 and 4.

5.2.2 Microorganisms

Two strains of *B. subtilis* were selected to evaluate their impact on surfactin production in soil. The first strain, *B. subtilis* ATCC 6051, a high-yield surfactin producer, was obtained from the American Type Culture Collection (Marburg ATCC 6051). The second strain, *B. subtilis* 168 1A1135, a surfactin-negative strain, was obtained from the Bacillus Genetic Stock Center (168 1A1135). Both strain stocks were maintained in 50% glycol solution at -80°C. Bacteria were first grown in 2 mL of tryptic soy broth (TSB), centrifuged, and washed twice using an Eppendorf 5702 Centrifuge. The microorganisms were then resuspended in 2 mL of ultrapure water. The suspension was diluted to an OD₆₀₀ of 0.1 using Sen's optimized medium, composed of ammonium nitrate (4.5 g-L⁻¹), glucose (36.5 g-L⁻¹), FeSO₄ (4.0 mg-L⁻¹), MnSO₄ (275.0 mg-L⁻¹) and a phosphate buffer composed of KH₂PO₄ (4.07 g-L⁻¹), Na₂HPO₄ (5.678 g-L⁻¹), MgSO₄ (197.0 mg-L⁻¹), CaCl₂ (0.77 mg-L⁻¹), and EDTA (1.17 mg-L⁻¹).¹³² To study the effect of different nitrogen sources, ammonium nitrate was substituted with ammonium chloride (6.014 g-L⁻¹) and sodium nitrate (9.557 g-L⁻¹) based on equal nitrogen molar concentrations. A negative control medium was also prepared by eliminating the nitrogen source and maintaining the other nutrients. Finally, bacterial growth curves were generated by measuring the optical density (OD₆₀₀) every 10 minutes for 48 hours at 30°C using a BioTek Epoch2 Microplate Reader.

5.2.3 Evaporation Rate Experiments

To assess the evaporation rate of soil samples, a series of experiments was conducted in which bacteria were inoculated into 40 g samples of sandy loam soil packed into small columns with a total volume of 29.06 cm³, resulting in a bulk density of 1.22 ± 0.01 g/cm³. Inoculation is performed by adding 4.00 mL of the suspension at 0.1 OD index, containing a bacterial cell density

of 10^7 cells/mL,¹⁴⁷ to the soil samples and the media. This resulted in a final cell concentration of 10^6 cells/g of soil, consistent with the cell density used in Gutierrez et al.⁶¹ Then, the samples were incubated for 24 hours at 30°C and saturated in a 5 mM calcium chloride solution for 48 hours at room temperature to ensure maximum moisture levels across all samples. The evaporation rate experiments were initiated once the soil columns were prepared and saturated. The primary objective was to monitor the mass lost to evaporation over a 72-hour period. To achieve this, a series of digital scales with a capacity of 1,200 grams and a precision of ± 0.01 grams (FX-1200i from A&D Weighing®) were used. The mass of each soil sample was recorded every 5 minutes. After collecting the mass data, the evaporation rate for each sample was calculated and expressed in millimeters per day (mm/day) and as a function of a normalized volumetric expression of the SWC, given by the equation:

$$\varphi = \frac{\theta}{\theta_{max}} \quad (5.1)$$

where θ is the SWC at any time, and θ_{max} is the maximum SWC achieved by the sample during the saturation. The evaporation rate and soil water content were analyzed as functions of time, allowing us to observe how these parameters evolved throughout the 72-hour experiment. An illustration of the setup of the evaporation rate experiment is displayed in Figure 5.1.

5.2.4 SWC Measurements Using Different Cell Densities

In these experiments, similar to the evaporation rate experiments, bacteria were inoculated into 40 g samples of sandy loam soil packed into small columns with a total volume of 29.06 cm³, resulting in a bulk density of 1.22 ± 0.01 g/cm³. Inoculation is performed by adding 0.040, 0.400, and 4.00 mL of the suspension at an optical density of 0.1 (OD₆₀₀), containing a bacterial cell density of 10^7 cells/mL,¹⁴⁷ to the soil samples and the media. This results in final cell concentrations of 10^4 , 10^5 , and 10^6 cells per gram of soil, respectively. Negative control samples

were prepared by adding 4.0 mL of pure water to the soil. After inoculation, all samples, including the negative controls, were incubated for 24 hours at 30°C. After incubation, the samples were thoroughly saturated in a 5 mM calcium chloride solution for 48 hours at room temperature. Saturation was achieved through cohesive and capillary processes, with water penetrating the column from the bottom through a cheesecloth membrane. Once saturation was complete, the samples were weighed and placed in a conventional oven to evaporate the water (Figure 5.1)

This evaporation step was conducted over 72 hours at a temperature of 30°C. At the end of the experiment, the samples were reweighed to calculate water loss. The volumetric Soil Water Content (SWC) method, expressed in equation (1.2) from Chapter 1, was also employed to determine the soil water content in the experiments with different cell densities (Figure 5.1). In the case of sterilizing the soil, mercury (II) chloride was used by premixing at a rate of 500 mg-Kg⁻¹ of soil and incubating for 48 hours at room temperature. For safety, the samples' sterilization and evaporation experiments were conducted in an incubator within a chemical hood (Figure 5.2). This procedure was performed following Wolf and Skipper's soil sterilization method.¹⁴⁸

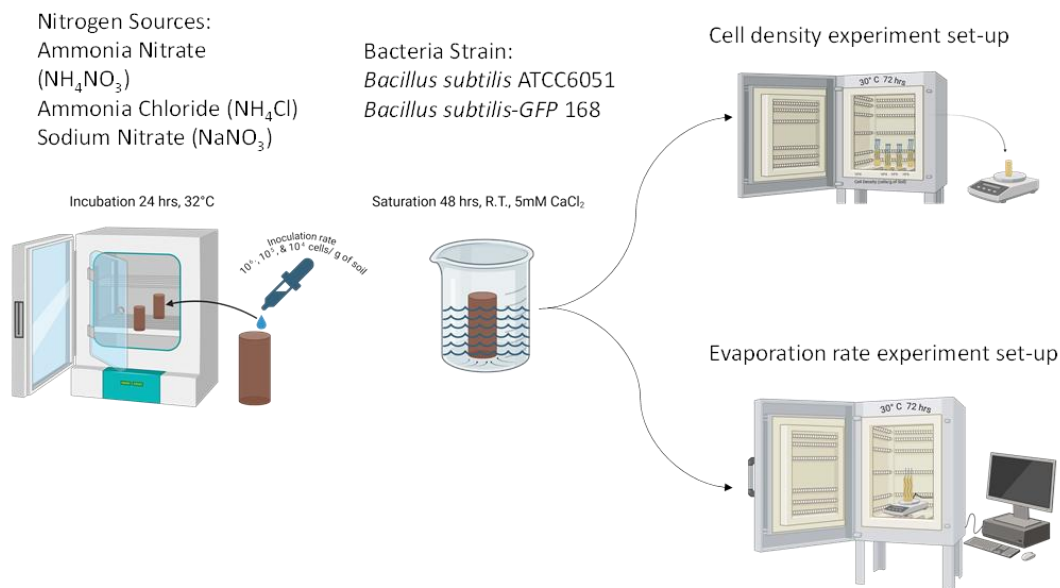


Figure 5.1. Illustration of the cell density and evaporation rate experiments for Microbial Amendments for improved soil water management.

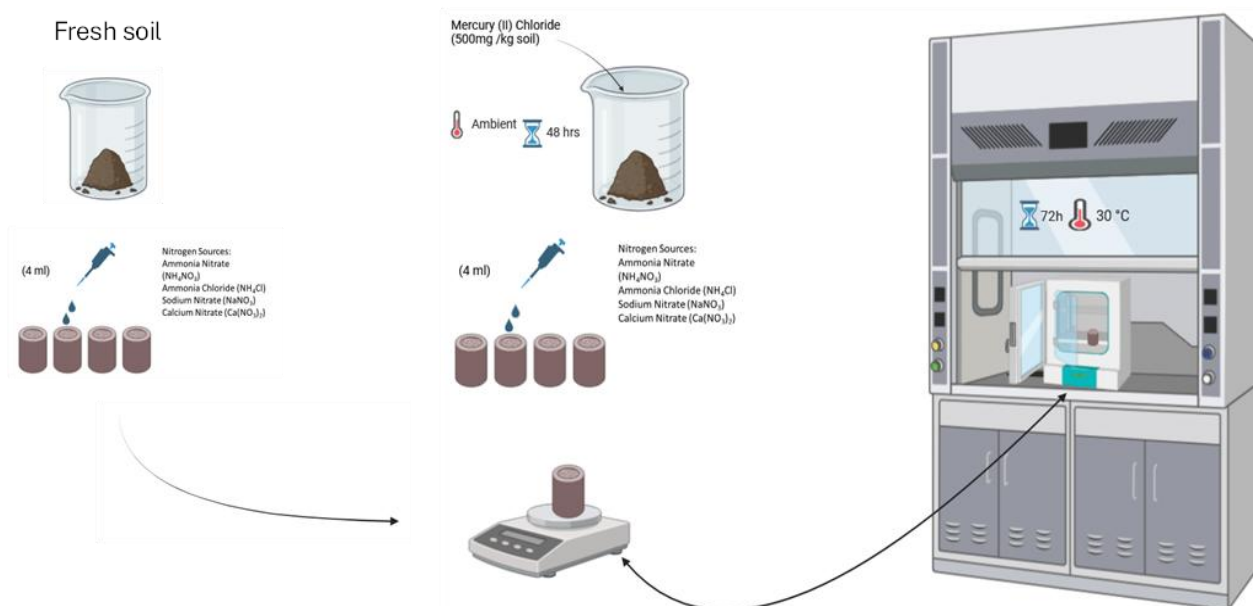


Figure 5.2. Illustration of the mercury (II) chloride (HgCl_2) sterilization samples and evaporation experiments, running side by side with fresh soil.

5.2.5 Measurements of Nitrogen Extraction and Electroconductivity

5.2.5.1 Sample Preparation

Samples were prepared for each nitrogen source, and *B. subtilis* ATCC 6051 was used to measure the final $-\text{NO}_3$ and $+\text{NH}_4$ and electrical conductivity. The samples were packed into 29.06 cm^3 columns at a bulk soil density of 1.22 g/cm^3 . Bacterial inoculation was carried out with a cell density of 10^7 cells- mL^{-1} , adding 4 mL of the inoculum to each column, resulting in a final concentration of 10^6 cells per g of soil. The samples were then incubated, saturated, evaporated, and collected for nitrogen extraction and to measure the soil's electrical conductivity.

5.2.5.2 KCl Nitrogen Extraction

A 50 mL aliquot of 1M potassium chloride (KCl) was added to 5 g of dried soil to extract nitrogen compounds from the soil. The mixture was shaken at 215 rpm for 1 hour to ensure thorough mixing and extraction of nitrogen compounds from the soil matrix. After shaking, the

suspension was filtered by gravity using a Whatman No. 42 filter paper to separate the solid particles from the liquid. The nitrogen compound filtrate was collected for further analysis using a Lachat spectrophotometer.

5.2.5.3 Mesuaring the NH_3^+ , NO_3^- , NO_2^- using a Lachat Instrument

Approximately 8 mL of the filtrate obtained from the KCl nitrogen extraction was placed in a 13 mm $\times$ 100 mm borosilicate glass tube and loaded onto the Lachat sampler. Solution and standards were prepared according to QuikChem® Methods 12-107-06-2-F and 12-107-04-1-J, as approved by the manufacturer.^{149,150} A calibration procedure is included in these instructions. To determine $(\text{NO}_2)^-$ and $(\text{NO}_3)^-$ levels in the soil samples, the samples were first run through the instrument at a wavelength of 520 nm to measure total $(\text{NO}_2)^-$ concentrations. The results were recorded. Next, the fluid flow path was adjusted to pass the sample through a cadmium reduction column. The sample was rerun, and levels of $(\text{NO}_3)^-$ and $(\text{NO}_2)^-$ were measured simultaneously. The final $(\text{NO}_3)^-$ concentration was determined by subtracting the initial reading of total $(\text{NO}_2)^-$ from the $(\text{NO}_3)^-$ and $(\text{NO}_2)^-$ combined reading.

For $(\text{NH}_3)^+$ analysis, the samples were processed separately through a dedicated column. Unlike the nitrate and nitrite measurements, the $(\text{NH}_3)^+$ concentration was determined directly from a single reading measured at a wavelength of 660 nm. An illustration of the measurement procedures is shown in Figure 5.3.

5.2.5.4 Electroconductivity (EC)

For electrical conductivity measurement, 1 g of dry soil was suspended in 10 mL of ultrapure water. The mixture was shaken at 215 rpm for 1 hour to ensure complete suspension and interaction between the soil particles and water. After shaking, each sample's electrical conductivity ($\mu\text{S}/\text{cm}$) was measured using a conductivity meter, providing insight into the ionic content of the soil solution.

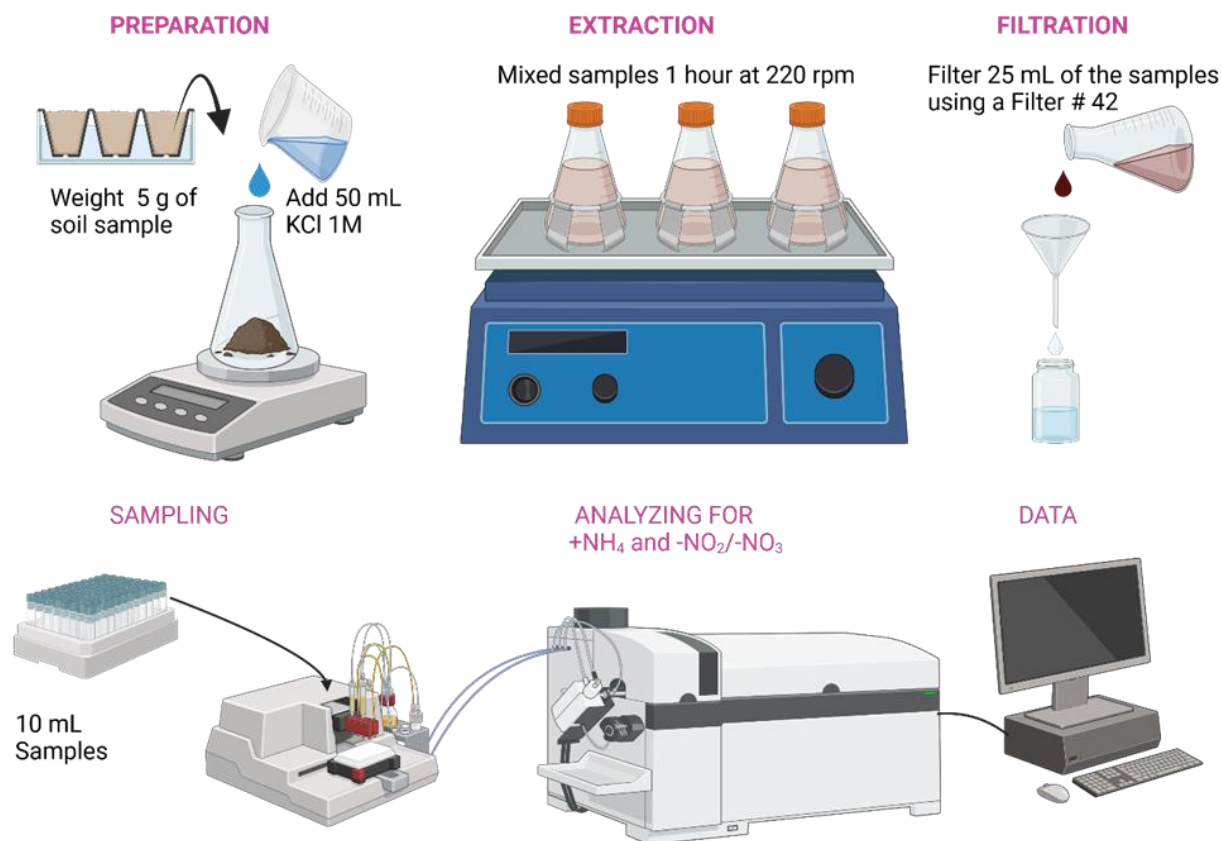


Figure 5.3. Illustration of the experiment measuring the nitrogen levels in the soil samples.

5.2.6 Data Analysis

All statistical analyses were performed using the SAS statistical software (SAS Institute Inc.). To ensure the reliability of the data, means and standard deviations were determined on at least three independent replicates ($n=3$) for each set of treatments. A p -value of less than 0.05 was considered statistically significant, indicating a difference between the data sets.

5.3 Results and Discussion

5.3.1 Bacterial Growth Curves

Two strains of *Bacillus subtilis* were tested to evaluate the efficacy of the media. *B. subtilis* 168 1A1135, which does not produce surfactin¹⁵¹ or a similar biosurfactant such as fengycin,^{152,153}

served as a comparative control. In contrast, *B. subtilis* ATCC 6051, a known surfactin-producing strain,¹⁵¹ was used as the primary experimental strain in this study.

Bacterial growth curves were generated to assess the impact of different nitrogen sources on each *B. subtilis* strain. As shown in Figure 5.4, the addition of inorganic nitrogen significantly enhanced the growth of both strains, with the strongest effects observed in treatments containing nitrate-based sources. Growth was notably higher in strain ATCC 6051, reaching maximum OD₆₀₀ values of 1.123 (NH₄NO₃), 1.390 (NaNO₃), and 0.933 (NH₄Cl).

In contrast, *B. subtilis* 168 1A1135 exhibited lower OD₆₀₀ values under the same conditions: 0.594 (NH₄NO₃), 0.840 (NaNO₃), and 0.700 (NH₄Cl). Remarkably, in nutrient-only media lacking inorganic nitrogen, the maximum OD₆₀₀ values reached very similar 0.528 for ATCC 6051 and 0.517 for strain 168 1A1135. The observed limited growth and extended lag phase are likely due to trace nitrogen from EDTA, as well as nitrogen recycling through cell lysis and bacterial cannibalism.¹⁵⁴

Strain ATCC 6051 responded more robustly to inorganic nitrogen sources, showing significant differences in growth and activity between nitrate-based media and non-nitrate conditions, such as ammonium chloride and the nutrient-only controls. This enhanced performance in nitrate-supplemented media suggests a metabolic preference or regulatory advantage for nitrate assimilation.¹⁴⁴ The stronger response also implies a higher biosurfactant production potential under nitrate-rich conditions, possibly due to more efficient nitrogen uptake supporting secondary metabolite synthesis.¹⁴⁴

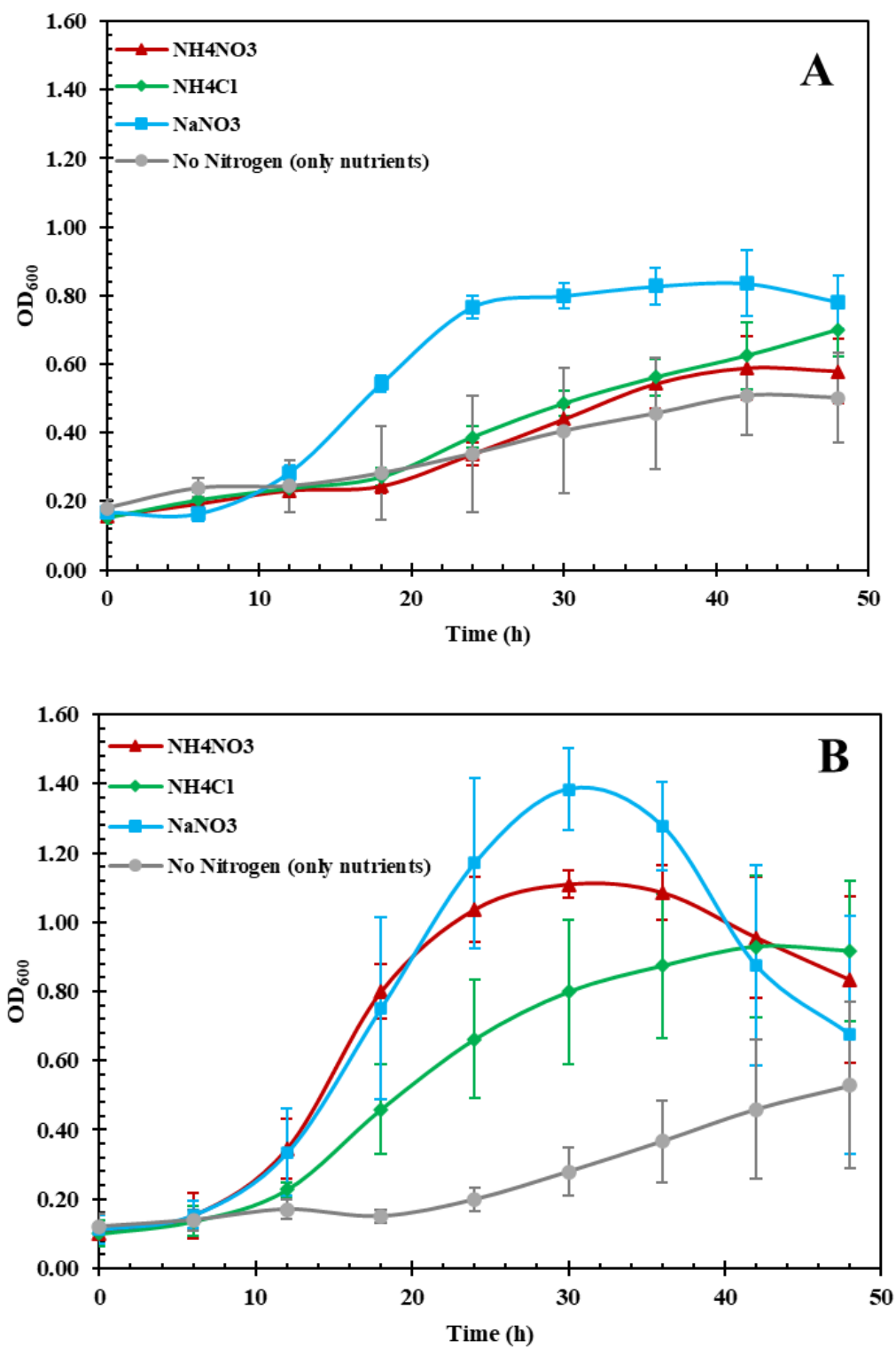


Figure 5.4 (A) Growth Curves for *B. subtilis* 168 1A1135 and (B) Growth Curves for *B. subtilis* ATCC 6051 using different sources of inorganic nitrogen.

5.3.2 Evaporation Rate Kinetics and SWC

Kinetic mass loss measurements were used to calculate and compare evaporation rates (ξ) with SWC (θ) levels using the highest cell density of 1×10^6 cells g^{-1} of soil. Figure 5.5 illustrates the evaporation rate for samples treated with *B. subtilis* 168 1A1135. Remarkably, the evaporation rate curves for nitrogen treatments were higher than the control (water) curve, except for ammonium nitrate, which dropped below the control as ϕ decreased below 0.55.

This indicates that the constant evaporation rate phase ended earlier for ammonium nitrate, causing the treated samples to retain more water, as described by Gutierrez et al.⁶¹ Figure D1 shows the evaporation rate (ξ) and SWC (θ) as a function of time (t) in hours. This trend is also evident in the SWC graph (Figure D1D). Finally, the final ϕ ratio for each treatment is shown in Figure 5.5, where samples treated with ammonium media retained a higher ϕ ratio than the control. In contrast, sodium nitrate-treated samples had a lower final ϕ .

The evaporation rates observed in *B. subtilis* ATCC 6051 samples exhibited a different response compared to *B. subtilis* 168 1A1135. Figure 5.6 presents the evaporation rate as a function of ϕ and the final ϕ ratios. All nitrogen source treatments resulted in evaporation rate curves below the control, indicating greater water retention. Additionally, all treatments showed a shorter constant evaporation phase, transitioning to a slower rate more quickly. This significantly reduced evaporation rates compared to the control, particularly during the transition stage (Figure D2). Once again, the faster transition contributed to higher water retention in treated samples compared to control samples.

The data demonstrate that nitrogen source treatments influence evaporation dynamics and soil water retention when inoculated with *B. subtilis*. Co-inoculating with strain 168 1A1135, ammonium nitrate slightly improved water retention, evidenced by a lower evaporation rate and an earlier end to the constant evaporation phase than the control. This was reflected in the higher

final water content ratio(ϕ). In contrast, sodium nitrate resulted in reduced water retention and higher evaporation rates.

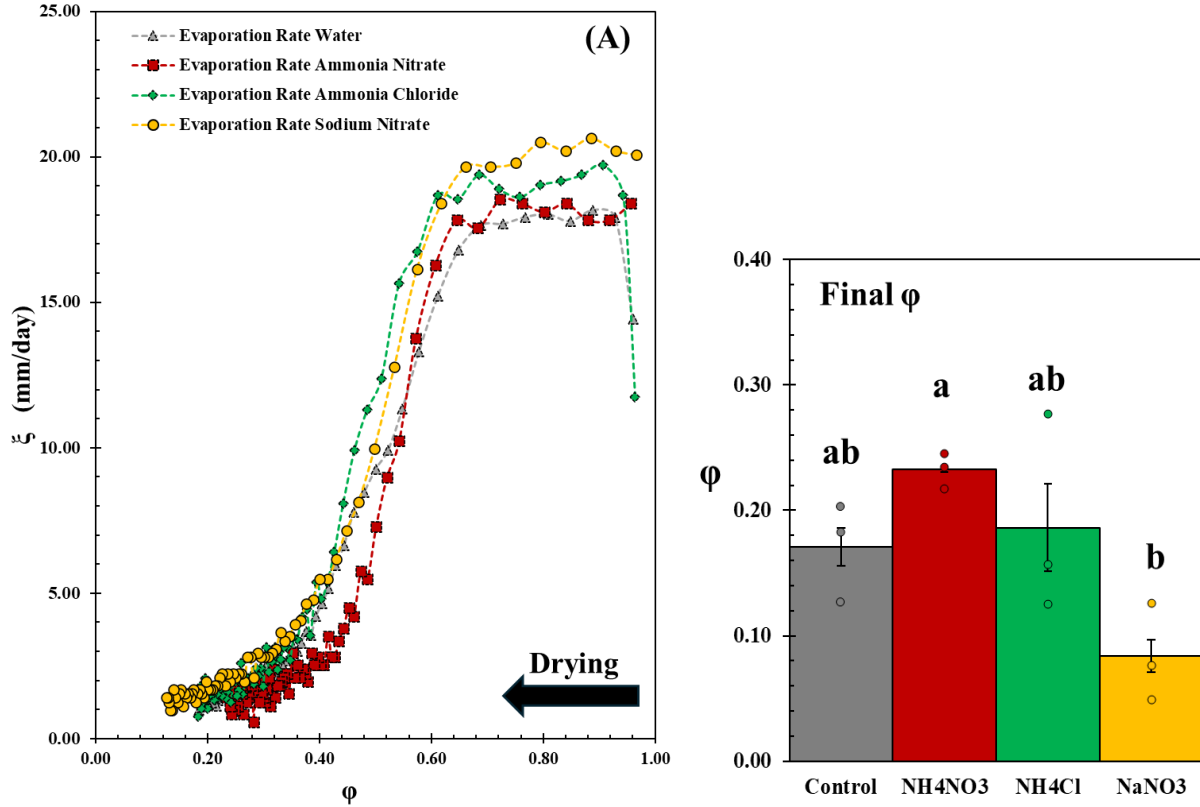


Figure 5.5 (A) Evaporation rate (ξ) as a function of SWC ratio (ϕ) for samples treated with nitrogen sources and inoculated with *B. subtilis* 168 1A1135. The dimensionless ratio (ϕ) was found using equation 5.1. All evaporation curves were higher than the control (water) except for the ammonium nitrate curve, which crossed the control curve and exhibited a lower evaporation rate as the samples dried. The first data point for each sample was excluded, as it reflected the system's initial equilibration period. **(B)** The final SWC ratio, where only the sodium nitrate ratio significantly differed from both ammonium-based media. (Data points with different letters are significantly different, $p < 0.05$).

Notably, *B. subtilis* ATCC 6051 demonstrated a consistently greater water-retention effect across all nitrogen treatments. In each case, the evaporation rate curves remained below those of the control and entered the slower evaporation phase at an earlier stage. These results indicate that ATCC 6051 more effectively enhanced soil moisture retention, suggesting that soil water

conservation is influenced by both strain-specific traits and the type of nitrogen source, likely mediated by microbial activity.

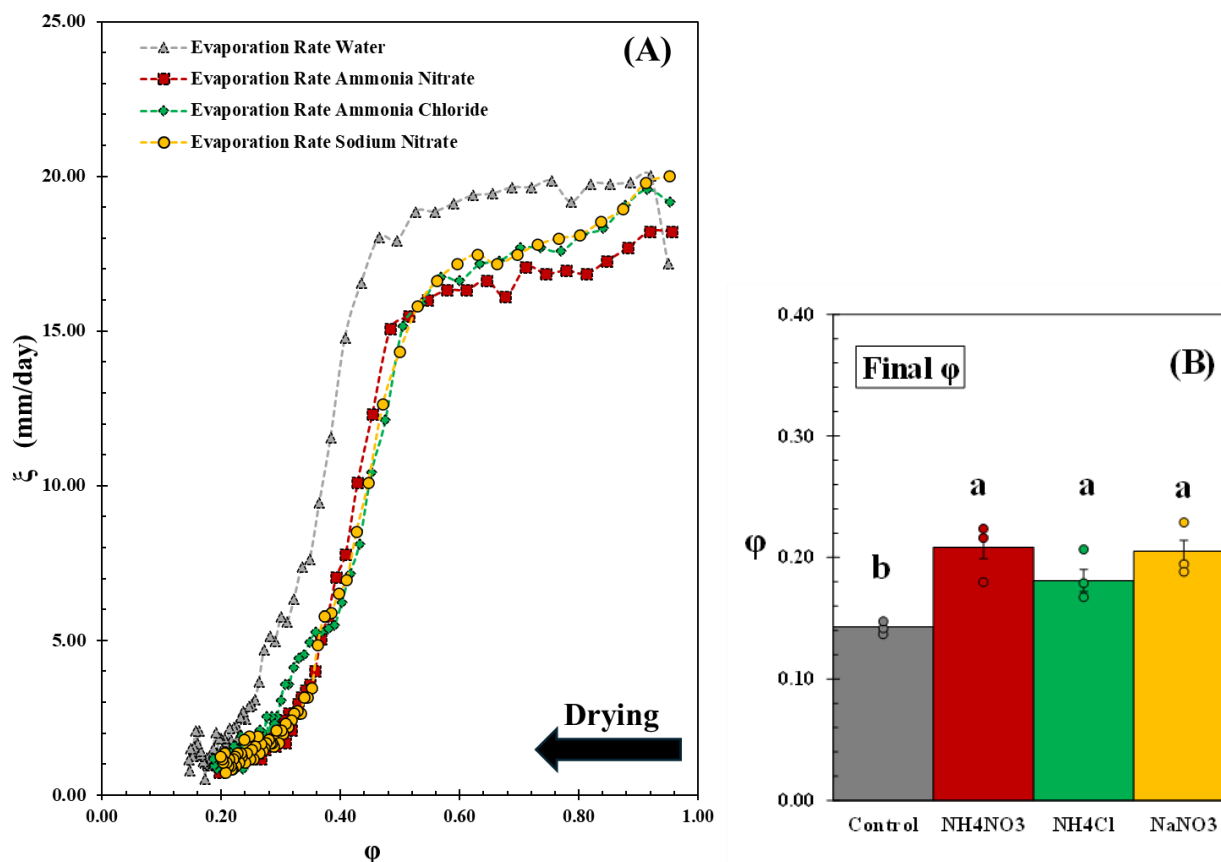


Figure 5.6 (A) Evaporation rate as a function of SWC ratio (ϕ) for samples treated with nitrogen sources and inoculated with *B. subtilis* ATCC 6051. All evaporation curves were lower than the control (water). The first data point for each sample was excluded, as it reflected the system's initial equilibration period. **(B)** The final SWC ratio, where the control significantly differed from all nitrogen-treated samples. (Data points with different letters are significantly different, $p < 0.05$).

To understand the role of *B. subtilis* ATCC 605, water loss measurements were used to estimate soil water content (SWC) across all media types, testing various cell densities. All treatments and controls were run concurrently during each independent trial. Initial and final SWC values, as well as total water loss, were quantified using mass balance and the gravimetric method to assess soil moisture retention performance directly.

Multiple control sets were included to isolate the effects of microbial presence and nitrogen availability. The first set consisted of columns containing nitrogen sources and nutrients, but no microorganisms. A second set included columns inoculated with *B. subtilis* at varying cell densities but without any added nitrogen source (NH_4NO_3 , NH_4Cl , or NaNO_3) while still containing the base nutrients. A final control group consisted of columns with microbial inoculation at all cell densities, using only pure water. These control configurations are displayed alongside the experimental treatments in Figure 5.7.

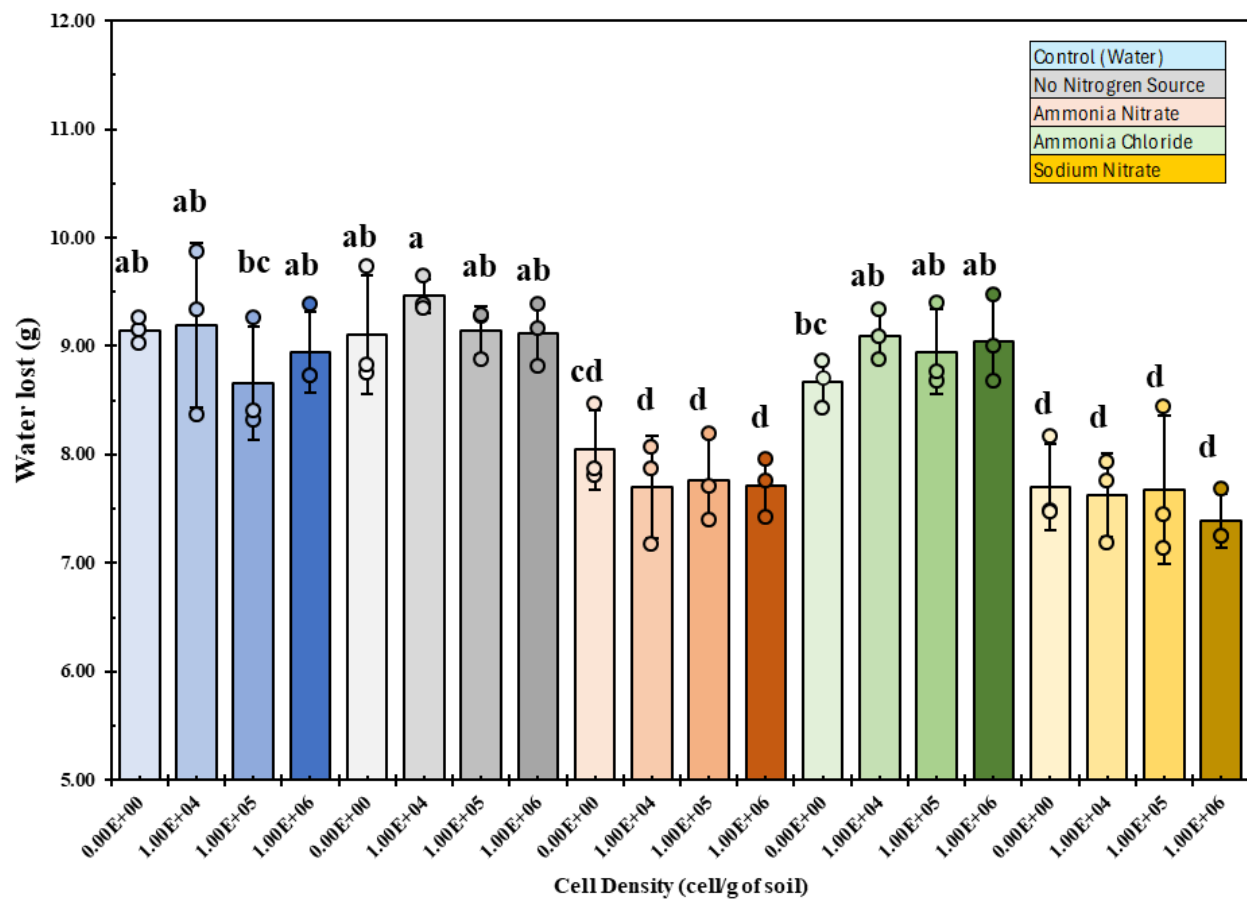


Figure 5.7 Water losses in samples treated with different nitrogen sources and inoculated with *B. subtilis* ATCC 6051. No significant differences were observed among samples treated with the same nitrogen source. However, all samples treated with ammonium and sodium nitrate exhibited significant differences from the controls (water and no-nitrogen media). Ammonium chloride-treated samples showed no significant differences from the controls. (Data points with different letters are significantly different, $p < 0.05$).

The soil columns inoculated with *B. subtilis* ATCC 6051 and a nitrate source enhanced water loss reduction. Ammonium nitrate-treated samples showed average water losses of 7.70 ± 0.47 g, 7.76 ± 0.40 g, and 7.71 ± 0.27 g for cell densities of 10^4 , 10^5 , and 10^6 cells/g of soil, respectively. In contrast, samples with ammonium nitrate without microorganisms had a water loss of 8.04 ± 0.36 g. While differences within the ammonium nitrate-treated samples were not statistically significant, they differed significantly from the water and no-nitrogen source controls ($p < 0.05$), except for the uninoculated sample.

Sodium nitrate-treated samples also exhibited significant differences from the controls. Water losses averaged 7.70 ± 0.40 g, 7.63 ± 0.39 g, 7.67 ± 0.68 g, and 7.39 ± 0.25 g for cell densities of 10^0 , 10^4 , 10^5 , and 10^6 cells/g of soil, respectively. In contrast, the ammonium chloride treatment did not improve water retention and showed no significant difference from the controls. Water loss for ammonium chloride-treated samples ranged from 8.67 g to 9.09 g, similar to the control samples, which ranged from 8.66 g to 9.19 g (with microbes and water) and 9.10 g to 9.46 g (with nutrients but no nitrogen source).

The response of each nitrogen source differs, and several factors could explain these results. Biosurfactant production by *B. subtilis* depends on cell density, which is primarily regulated through quorum sensing—a mechanism where accumulating signaling molecules, as the cell population grows, trigger gene expression involved in biosurfactant synthesis.^{155–157} While cell density is a key factor, environmental conditions such as nutrient availability, pH, and stress also influence overall biosurfactant production.

Column saturation must be taken into account when evaluating water loss. During the cell density experiment, the saturation levels of the columns were measured at the start and end of the test period to assess changes over time.

Additional controls were introduced to create a more complex situation. At initial saturation, no significant differences existed among the cell density treatments within the same media. However, samples with ammonium chloride and no-nitrogen sources reached a higher saturation level than those with nitrate sources or water alone.

By the end of the experiment, several treatments showed differences in residual soil water content (SWC) relative to their initial values. Figure D3 in Appendix D illustrates the initial and final SWC for each treatment. Specifically, samples treated with sodium nitrate and ammonium nitrate exhibited significantly higher final SWC compared to other nitrogen sources, with each showing statistical significance at $p < 0.001$. However, no significant differences were observed among the different cell densities within the same nitrogen treatment.

Water samples had the lowest final SWC, though the results were not significantly different from those of the ammonium chloride and no-nitrogen source samples. A similar pattern emerged when estimating Δ SWC: nitrate-treated samples exhibited a lower reduction in SWC compared to those treated with ammonium chloride or the controls. A statistically significant difference ($p < 0.001$) is exhibited between the nitrates and the non-nitrate samples. Figure D4 illustrates these differences across treatments. As a preliminary result, ammonium and sodium nitrates enhanced water retention.

Based on the cell density and evaporation rate experiments, it can be concluded that the high surfactin-producing strain *B. subtilis* ATCC 6051 enhances greater water retention when inoculated at higher cell densities in nitrate-rich media. The data suggest that samples inoculated with *B. subtilis* ATCC 6051 and treated with NH_4NO_3 or NaNO_3 developed a more significant microbiome, which potentially increased the water retention compared to those treated with NH_4Cl and the controls. This finding aligns with the results of pure culture growth, where high surfactin-

producing bacteria have more significant growth and, therefore, higher colonization in the soil (Figure 5.4).

However, an unexpected pattern emerged: water retention increased in samples with the nitrate-rich media and without *B. subtilis* ATCC 6051 inoculation. These findings suggest that the type of nitrogen source, rather than microbial cell density, played a more dominant role in influencing soil moisture retention under the tested conditions.

Additional experiments were conducted to investigate the role of the nitrogen source in water retention. One set of samples containing all media was sterilized with mercury (II) chloride to completely inhibit microbial activity. A second set, containing fresh, unsterilized soil, was also prepared. Equal amounts of water were applied to all samples to standardize soil water content (SWC), and equal saturation and final SWC levels were compared (Figure D5).

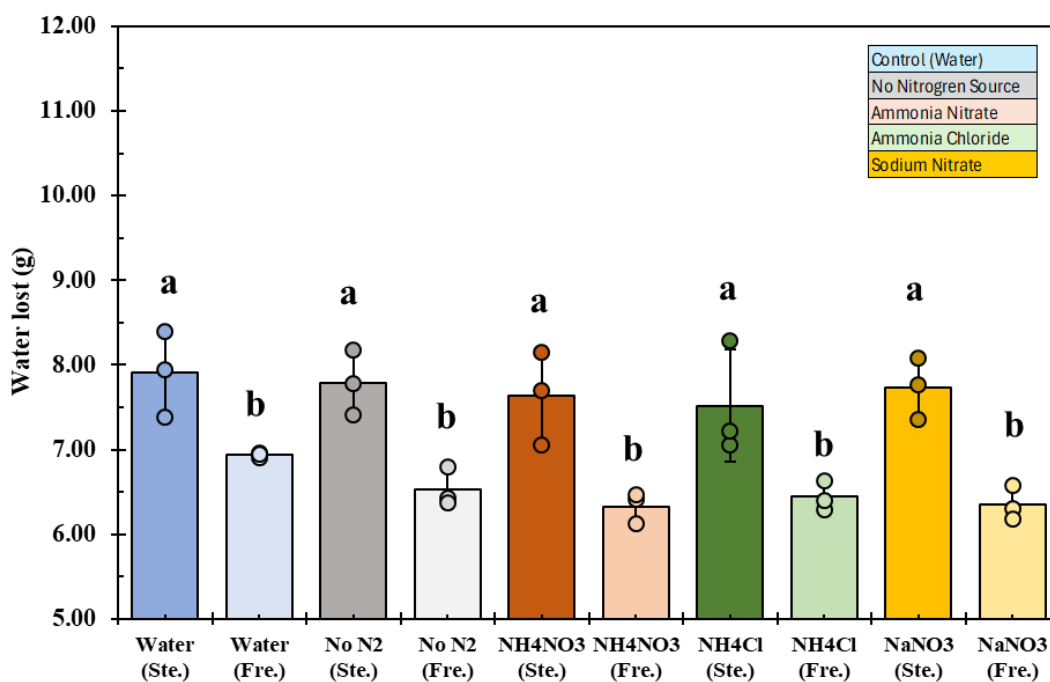


Figure 5.8 Water losses result in soil samples sterilized with mercury (II) chloride (HgCl_2) to inhibit microbial activity (Ste.), compared to fresh samples (Fre.) amended with nitrogen sources. No bacteria were inoculated in either treatment. Water losses in sterilized samples differed significantly from those in fresh soil samples. (Data points with different letters are significantly different, $p < 0.05$).

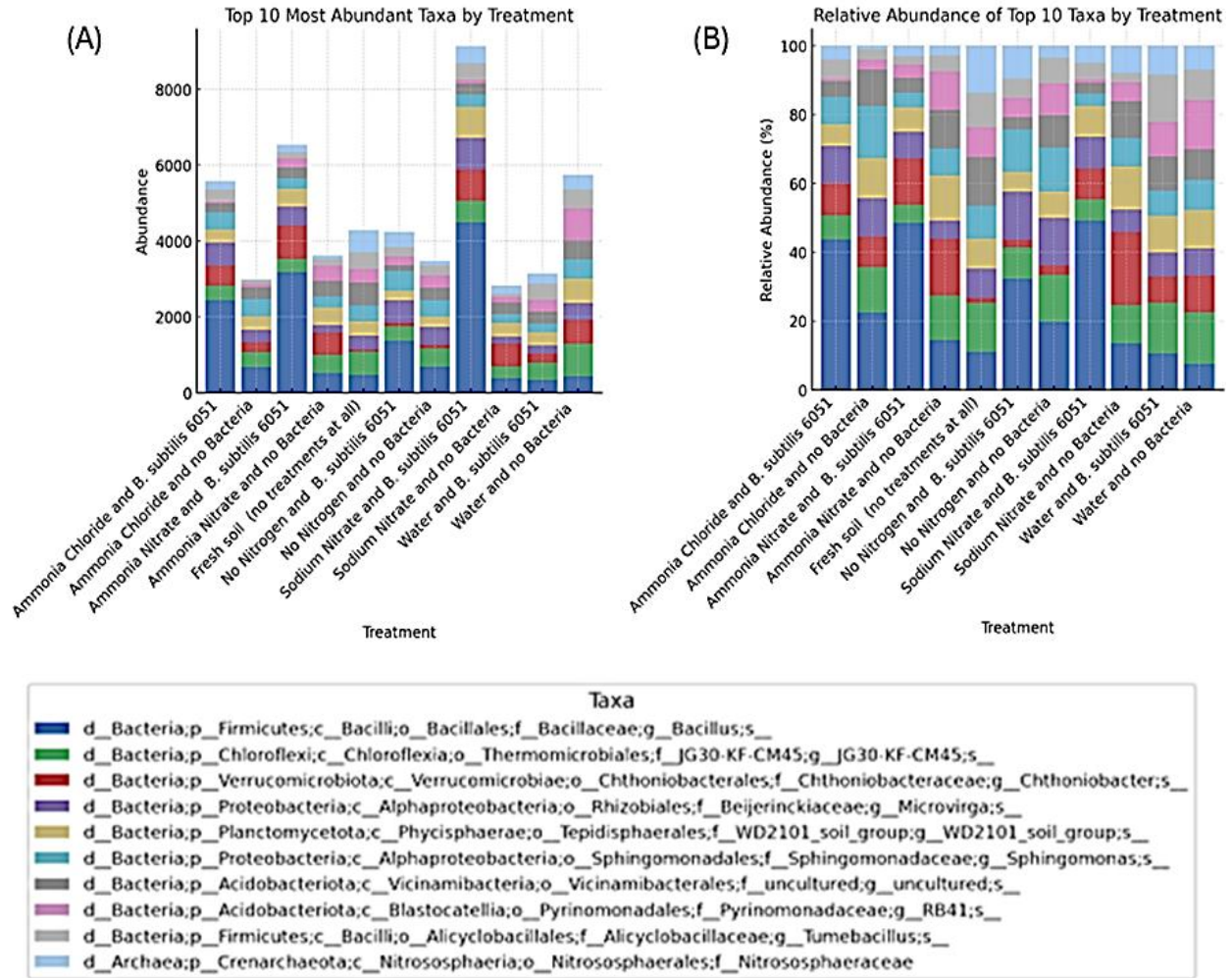


Figure 5.9. Results of 16S rRNA analysis are presented, showing (A) the absolute abundance of the top 10 taxa in the soil samples and (B) their relative abundance normalized to 100%. This analysis revealed the presence of *Bacillus spp.*, specifically gene groups associated with *Bacillus subtilis*. This suggests a high probability that native *Bacillus spp.*, without the inoculation of strain ATCC 6051, may contribute to improved water retention. However, further analysis is needed to identify the specific strains present in the soil and to assess their capacity to produce the biosurfactant surfactin.

The results indicate that the native microbiome contributes to water retention, as sterilized samples lost significantly more water than those with an intact microbiome (Figure 5.8). Samples receiving the same nitrogen treatment differed significantly in microbiome presence, and the statistical similarity among sterilized samples suggests that microbial activity was effectively inhibited. This result implies that nitrogen amendments alone are insufficient to improve water

retention without the contribution of active microbial communities. It underscores that nitrate-based media enhances water retention by supporting the inoculated *B. subtilis* and native microorganisms.

To confirm microbial presence, 16S rRNA gene sequencing and microbiome profiling were performed on soil samples. Preliminary results indicated the presence of *Bacillus spp.* in pristine (uninoculated) soils, suggesting the potential for native surfactin-producing bacteria (Figure 5.9). These findings raise the possibility that nitrogen amendments alone may stimulate the growth and activity of these indigenous microbes, potentially enhancing surfactin production without the need for external inoculation. However, to fully assess the role of nitrate-based media in influencing soil water retention, further investigation is necessary, including expanded microbiome profiling, diversity analysis, and quantification of microbial biomass.

5.3.3 Results on Consumption of Nitrates and Ammonium of the Amended Soils

Adding nitrates and ammonium alters the soil nitrogen cycle,¹³⁷ but *B. subtilis* has been identified as a heterotrophic nitrifier and aerobic denitrifier. To evaluate the impact of nitrogen amendments on this cycle, residual nitrate (NO_3^-) and ammonium (NH_4^+) levels were measured at the end of the heating stage. All samples were initially amended to achieve a carbon-to-nitrogen (C:N) ratio of 9.27, approximating the optimal 10:1 ratio.¹³⁶ Samples inoculated with *B. subtilis* ATCC 6051 showed variation in residual nitrate levels across the different nitrogen sources, though the differences were not statistically significant. Nevertheless, all treated samples, except those treated with sodium nitrate, demonstrated a notable reduction in nitrate compared to the untreated (pristine) soil (Figure 5.10A).

Interestingly, the no-nitrogen source and ammonium chloride treatments, which contained only the residual nitrates inherited from the fresh soil, did not result in greater nitrate reduction. In contrast, soils amended with ammonium nitrate and sodium nitrate introduced higher initial nitrate

levels into the system, yet ultimately reached nitrate concentrations comparable to those of the non-nitrate treatments. These preliminary findings suggest that *B. subtilis* strains play an active role in nitrate consumption and reduction. This is evidenced by the decline in nitrate levels in nitrate-amended soils, which reach levels comparable to those in unamended soils, and by the increase in growth shown in the culture growth curve (Figure 5.4).

The *B. subtilis* ATCC 6051 strain exhibited a similar response to consumption and reduction of ammonium ions, with significant residual levels (Figure 5.10B). Both non-ammonia media had low ammonium concentrations, which were not significantly different from each other. However, the ammonium chloride treatment resulted in a significantly higher residual concentration ($p < 0.035$) than the no-nitrogen source and sodium nitrate treatments, though it was not significantly different from the ammonium nitrate treatment. Meanwhile, ammonium nitrate residuals showed no significant differences across other media, including the non-ammonia media.

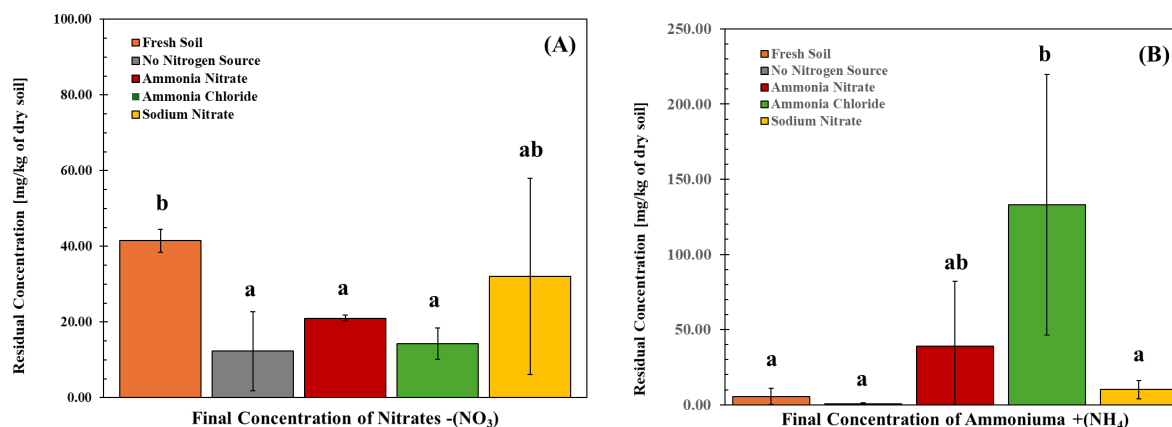


Figure 5.10. (A) -NO₃ Residual during the evaporation experiment and using different nitrogen sources and *B. subtilis* ATCC 6051, and (B) Ammonium (+NH₄) residual after the evaporation rate experiment using different nitrogen sources and *B. subtilis* ATCC 6051. The ammonium chloride residual did not decrease sufficiently, resulting in a significant difference from the non-ammonia media. (Data points with different letters are significantly different, $p < 0.05$).

B. subtilis ATCC6051, this strain can assimilate ammonium from both ammonia sources.¹⁴⁴ *B. subtilis* primarily utilizes glutamine as a nitrogen source,¹³³ however, in its absence,

it can rely on alternative nitrogen sources, including ammonium nitrate and chloride.¹⁵⁸ Nevertheless, ammonium nitrate provides both ammonium (NH_4^+) and nitrate (NO_3^-) ions, whereas ammonium chloride supplies NH_4^+ and chloride (Cl^-) ions. While NH_4^+ is readily assimilated, Cl^- is not a nutrient source and may disrupt cellular processes if it accumulates.¹⁵⁹ The presence of either nitrate or chloride anions likely influences nitrogen assimilation efficiency and overall growth. This was evident in bacterial growth curve assessments, where the strain exhibited a much higher carrying capacity in nitrate-based media (Figure 5.4B).

Although the final nitrate concentrations were not significantly different among all treatments, the results indicate that nitrates were reduced in the soils, even in treatments amended with nitrate. Conversely, the consumption of ammonium ions was observed, particularly in treatments with added ammonia. The ammonium nitrate treatment reduced (NH_4^+) levels to those comparable to those in pristine soil and the treatment with no nitrogen amendments.

Electrical conductivity (EC) measurements revealed distinct ion dynamics across treatments using *B. subtilis* ATCC 6051 (Figure 5.11). The control samples (no nitrogen amendment) exhibited the lowest EC, indicating a baseline ion concentration with minimal ionic contributions from the medium. In contrast, the high EC observed in NH_4Cl treatments suggests that while ammonium (NH_4^+) was consumed, chloride ions (Cl^-) remained unutilized, increasing conductivity. The lower EC recorded for NH_4NO_3 indicates efficient uptake of ammonium and nitrate, thereby reducing ion accumulation. Meanwhile, NaNO_3 treatments showed moderate EC levels, suggesting nitrate consumption while sodium ions (Na^+) persisted in the solution. Understanding these dynamics is crucial, as the presence of specific anions can affect nitrogen uptake and overall bacterial activity, which, in turn, may influence surfactin production, adsorption, and soil remediation.^{27,59,68,144}

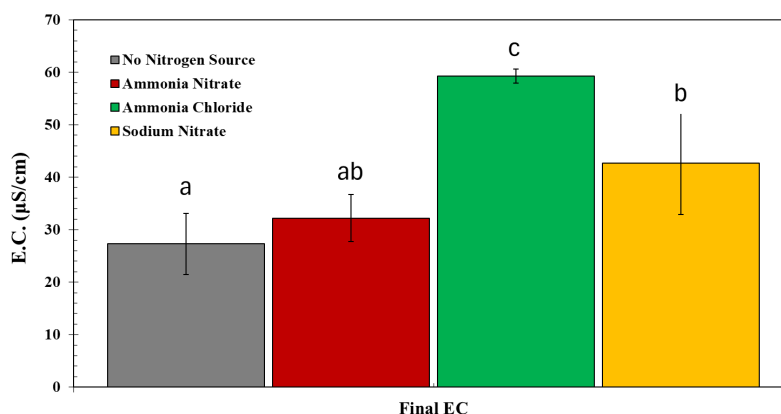


Figure 5.11. Electrical conductivity (EC) measurements ($\mu\text{S}/\text{cm}$) of soil samples amended with different nitrogen sources: no nitrogen (No N_2), ammonium nitrate (NH_4NO_3), ammonium chloride (NH_4Cl), and sodium nitrate (NaNO_3). Ammonia chloride was significantly different from the control and both nitrate media. Different letters above the bars indicate statistically significant differences between treatments ($p < 0.05$).

5.4 Conclusion

The results of these studies indicate that nutrient enrichment, particularly with nitrate-based compounds, stimulates the native soil microbiome and enhances water retention. Inoculation with *B. subtilis* ATCC 6051, a high surfactin producer, in media containing ammonium nitrate (NH_4NO_3) or sodium nitrate (NaNO_3) led to an additional reduction of water loss compared to treatments with ammonium chloride (NH_4Cl) and both controls, water and no-nitrogen media, suggesting that nitrate availability is a key driver of microbial activity, colonization, and biosurfactant production.

In contrast, ammonium alone (NH_4Cl) did not support the same level of microbial enhancement, indicating that NH_4^+ , even though *B. subtilis* can assimilate it, but it is not a key contributor to improved soil moisture retention. The combined uptake of ammonium and nitrate in mixed treatments may also help prevent excess ion accumulation, further supporting soil stability. Even though these findings underscore that nitrate-based nutrient amendments enrich the native microbiome and improve soil water dynamics, further validation is needed through

microbiome profiling, biomass quantification, and direct measurement of the biosurfactant in the soil to fully understand the overall effect of *B. subtilis* and surfactin.

B. subtilis inoculation with ammonia nitrate (NH_4NO_3) could benefit stressed agricultural soils by enhancing water-holding capacity and facilitating denitrification, reducing nitrogen runoff and loss. This dual action could be highly beneficial in improving soil resilience under drought conditions while minimizing environmental impacts. These studies also indicate that nitrogen assimilation efficiency varies depending on the presence of nitrate (NO_3^-) or chloride (Cl^-) ions, as suggested by electrical conductivity (EC) measurements, using *B. subtilis* ATCC 6051. However, nitrogen cycling in soil is a complex process influenced by bacterial activity, temperature, pH, aeration, organic matter, and moisture levels,¹³⁹ and the assimilation of nitrates and ammonium ions may depend on the soil conditions.

Further research is needed to quantify nitrogen consumption, production, and transport within the soil system. In addition, the identification of native denitrifying bacteria and other microorganisms in the soil is crucial for a comprehensive understanding of the complete role of *B. subtilis* in denitrification and nitrification processes.

5.5 Acknowledgments

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Chapter 6 - Bacteria Encapsulation into Polyethylene Glycol Hydrogels Using Michael-Type Addition Reactions

Overview

Hydrogel materials can be used to integrate bacteria cells into biohybrid systems. Here, we investigate the use of polyethylene-glycol-based hydrogels that employ different Michael-type addition crosslinking chemistries, including thiol-acrylate, thiol-vinyl sulfone, and thiol-maleimide click reactions, for covalent hydrogel network formation and bacteria encapsulation. All crosslinking chemistries generated hydrogels that provided stable encapsulation and culture of *Bacillus subtilis*; however, significant differences in cell viability and cell morphology after encapsulation were identified. Thiol-acrylate hydrogels provided the highest cell viability and favored encapsulation of single cells, while thiol-maleimide hydrogels had the lowest cell viability and favored encapsulation of larger aggregates. These findings demonstrate the impact of crosslinking strategies for encapsulation of microorganisms into hydrogel networks and suggest that thiol-acrylate chemistries are favorable for many applications.

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6.1 Introduction

Encapsulation of microorganisms within hydrogel materials has proven to be an effective strategy for preserving cellular function and for providing controlled cell release, enabling advancements in probiotics,¹⁶⁰ agriculture,¹⁶¹ and environmental applications¹⁶². Recent progress in synthetic biology has also facilitated the field of living materials, where a primary focus is on use of engineered cells to provide programmable control of material properties.¹⁶³ Prominent examples of living materials use embedded microorganisms to modulate hydrogel properties.^{164,165} These emerging research areas highlight the importance of hydrogel chemistries to provide stable encapsulation of viable bacteria.

The majority of hydrogels used for microorganism encapsulation employ naturally occurring polysaccharide-based materials, most notably alginate, chitosan, cellulose, starch, and hyaluronic acid. While used less frequently, synthetic hydrogels such as poly(ethylene glycol) (PEG), poly(vinyl alcohol), and poly(acrylamide) hold unique advantages over their naturally-occurring counterparts, particularly with respect to their flexible design options to achieve a wide range of tailored physicochemical properties. For example, hydrogels that use covalent PEG networks can be generated from a variety of commercially available PEG macromers with different end-group chemistries and molecular weights to yield highly tunable mesh sizes for control of mass transport.¹⁶⁶ Novel PEG macromers can also be used to generate hydrogels that are responsive to environmental stimuli such as light, pH, or enzymatic activity,⁵⁴ enabling dynamic control of the cellular microenvironment or triggered release of microorganisms at a desired time and location.^{167,168} Microbial encapsulation with PEG-based hydrogels have enabled recent advancements in anaerobic wastewater treatment systems,^{169,170} bioelectrochemical systems,¹⁷¹ and selection of bacteria from microbial communities or culture collections.^{172–174}

A variety of chain and step-growth polymerization strategies can be used to generate covalent PEG hydrogel networks. Step-growth polymerization mechanisms that use click reactions for covalent crosslinking, which include thiol-ene, copper-catalyzed and ring strain promoted azide-alkyne cycloaddition, inverse electron demand Diels-Alder cycloaddition, and Michael addition, can provide near stoichiometric conversion of crosslinking moieties for uniform network structure and precise control of hydrogel properties.^{175,176} While these novel crosslinking chemistries have received extensive use in tissue engineering and drug delivery, little attention has been given to its potential use for microorganism encapsulation. Michael addition relies on base-catalyzed conjugate addition between a thiolate anion and an electron-deficient vinyl group¹⁷⁷ and is an attractive option for microbe encapsulation as it does not generate free radicals or require initiators and external energy input for initiation, does not generate side products, and does not require catalysts that have antimicrobial properties. Finally, unlike free radical polymerizations, Michael addition coupling reactions are insensitive to oxygen,¹⁷⁷ making them equally amenable to encapsulation of aerobic and anaerobic microorganisms.

Here, we investigate the effects of crosslinking chemistry on bacteria encapsulation in PEG hydrogels when using three common Michael acceptors (acrylate, vinyl sulfone, and maleimide) present on difunctional PEG macromers. All groups are coupled with tetrafunctional PEG-thiol crosslinkers and use macromers with equivalent molecular weights to decipher the effects of end group chemistry on bacteria encapsulation, cell viability, and growth (Figure 6.1). *Bacillus subtilis* was used as a model bacteria as it is genetically tractable, produces endospores, and can provide host benefits in several biosystems, including the gut, plant rhizosphere, and soil. Encapsulated *B. subtilis* has garnered recent attention for controlled delivery^{178,179} and living hydrogel applications.¹⁸⁰

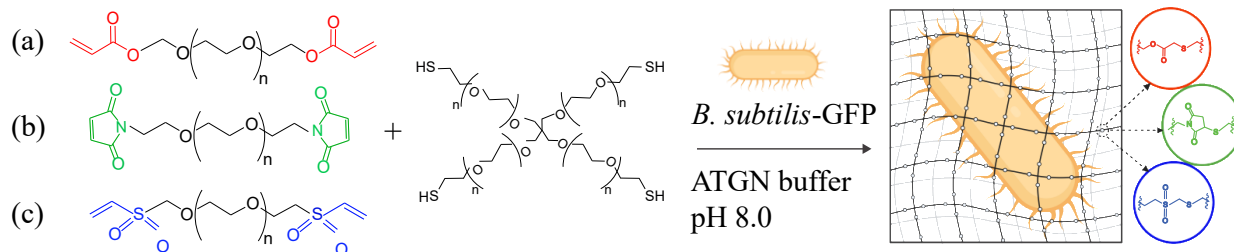


Figure 6.1. Encapsulation of *B. subtilis*-GFP into PEG hydrogels using tetrafunctional PEG-thiol crosslinkers (MW = 10 kDa) and (a) PEG diacrylate, (b) PEG divinyl sulfone, or (c) PEG dimaleimide macromers (Mw = 3.4 kDa), forming thioether ester (red), thioether sulfone (blue), or succinimide thioether (green) bonds, respectively, at each crosslink through Micheal addition. Figure was prepared with assistance from Biorender software.

6.2 Materials and Methods

6.2.1 Preparation of hydrogel precursor solutions and cell encapsulation

Bacillus subtilis or *B. subtilis* cells expressing GFP (*B. subtilis*-GFP) cells were grown in tryptic soy broth (TSB) at 28°C for 24 hours (hrs), followed by dilution into 2X ATGN minimal growth media in phosphate buffer (PBS)¹⁸¹ to an optical density (OD₆₀₀) of 0.1. Hydrogel precursor solutions consisted of cultured *B. subtilis*, a four-armed PEG thiol crosslinker (10 kDa PEGTT, 20 mM) and PEG diacrylate (3.4 kDa PEGDA, 49 mM), PEG vinyl sulfone (3.4k Da PEGVS, 49 mM), or PEG maleimide (3.4k Da PEGMA, 49 mM) macromers to form PEG diacrylate-PEG tetrathiol (PEGDA-PEGTT), PEG vinyl sulfone-PEG tetrathiol (PEGVS-PEGTT), and PEG maleimide-PEG tetrathiol (PEGMA-PEGTT) hydrogels, respectively. Hydrogel precursor solutions were prepared by first mixing *B. subtilis* with the difunctional PEG macromer, then combining the solution with PEGTT incubating over thiolated glass coverslips for microscopic imaging, following the procedure described by Fattahi et al.¹⁸² More details on this experimental protocol are provided in the Supplementary Material file. A minimum of three independent replicates were made for each hydrogel. Average hydrogel mesh size for each hydrogel was

measured as described by Canal and Peppas¹⁸³ and is further described in the Supplementary Material file (Table E1).

6.2.2 Characterization of Cell Morphology and Viability

Cell morphology and viability immediately after bacteria-hydrogel encapsulation were characterized using an inverted fluorescent microscope (Nikon Eclipse Ti-E). For cell morphology studies, *B. subtilis*-GFP was used for fluorescence imaging. ImageJ 1.54 Analyze Particle tool was used to quantify the cell aggregate sizes. Histogram plots with a bin size of 7 μm^2 were generated from the raw data; a final bin of >25 μm^2 was used for all larger aggregates. *B. subtilis* (non-GFP) was used for cell viability studies. Here, cells in each hydrogel were stained using a live/dead assay (Bacterial Viability Kit L7012). The percentage of live cells was determined by measuring the ratio of integrated green to red fluorescence intensity (RG/R) and using the live-dead calibration curve (Figure E1), following the manufacturer's recommended protocol.¹⁸⁴ All microscopic characterizations were performed for $n = 3$ independent replicates.

6.2.3 Bacteria Growth Kinetics

Kinetic growth studies were performed by encapsulating *B. subtilis*-GFP in hydrogels at the bottom of 96-well plate readers using the same monomer formulations as before but at 50 μL volumes. After hydrogel formation, 200 μL of ATGN minimal growth media was dispensed on top of the hydrogels, and cell growth was tracked by monitoring optical density (OD₆₀₀) for 72 hrs at 28°C (Epoch2 Microplate Reader, Biotek). To verify that the measured cell growth was due to cells confined in the hydrogel and not due to cell growth in external growth media, 300 μL was sampled from the supernatant growth media every 10-12 hrs for OD₆₀₀ measurement.

6.2.4 Statistical analysis

All statistical analyses were made using statistical software (SAS Institute Inc.). Mean and standard deviation were estimated based on a minimum of three independent replicates. Student t-

tests were used to compare data sets and determine statistical differences. A p-value < 0.05 was considered statistically significant.

6.3 Results and Discussion

The encapsulation and resulting morphology of *B. subtilis*-GFP cells into PEGDA-PEGTT, PEGVS-PEGTT, and PEGMA-PEGTT hydrogels was first investigated, and cell aggregation levels in each hydrogel were compared (Figure 6.2A). Assuming an average diameter and length of a *B. subtilis* cell to be 1 μm and 2-6 μm respectively,¹⁸⁵ it can be estimated that particles with areas greater than $\sim 19 \mu\text{m}^2$ represent aggregated cells, while particles with lower areas represent individual cells or only a small number of cells. Lowest levels of aggregation were identified in PEGDA-PEGTT hydrogels, where 20.9% of particles represented aggregates ($>19 \mu\text{m}^2$) and the majority of particles had areas representative of single cells. Encapsulated cells showed a shift towards higher aggregation levels in PEGVS-PEGTT hydrogels; here 28.2% of particles represented aggregated cells, while a lower fraction of particles representative of single cells was measured. Finally, PEGMA-PEGTT hydrogels showed a further shift towards higher aggregation; here 31.1% of particles were aggregates while a further decrease in single cells was noted. These trends are also observed in the raw data histogram plots output from the image analysis (Figure E2). In addition, sub-millimeter heterogeneities in cell distribution were observed across PEGMA-PEGTT hydrogels, whereas cell distribution in PEGDA-PEGTT and PEGVS-PEGTT hydrogels appeared relatively uniform (Figure 6.2A).

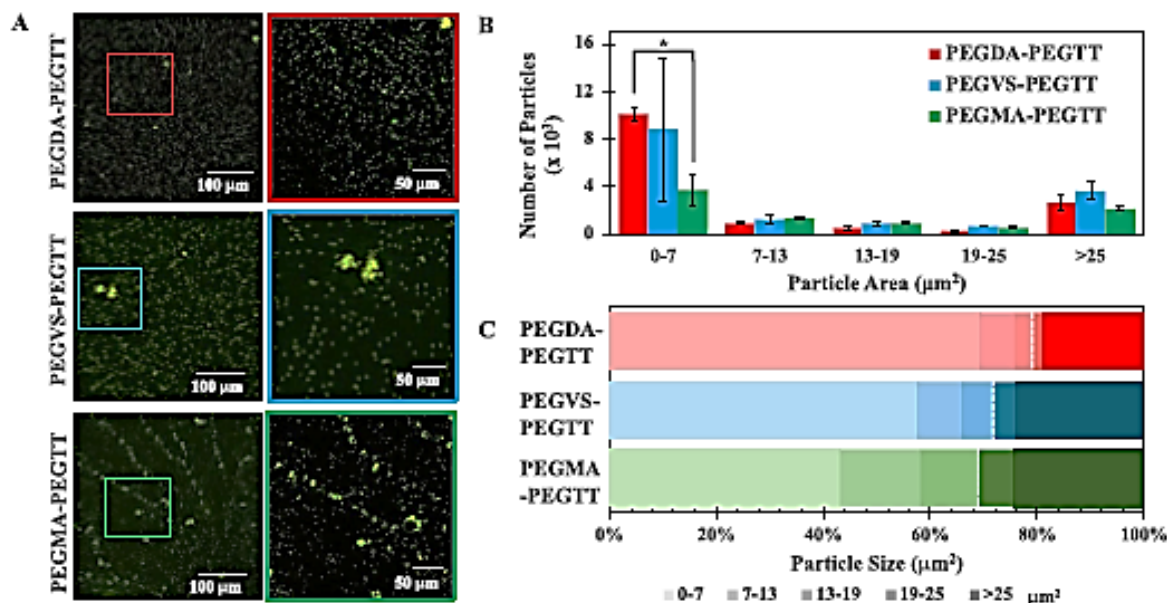


Figure 6.2. Cell morphology after encapsulation in PEG hydrogels. A Representative 10X (left column) and 20X (right column) fluorescent images of *B. subtilis*-GFP cells after encapsulation into PEGDA-PEGTT, PEGVS-PEGTT, and PEGMA-PEGTT hydrogels. B Average number and C percentage of encapsulated bacteria falling into size categories after particle size analysis (* $p < 0.05$). Darker shading in C) indicates a larger particle size range. Dashed white line in C) represent the cut-off area above which all particles are cellular aggregates. 10X images were used to quantify cell aggregation levels. Contrast was adjusted in Figure 6.2A to improve visualization.

We hypothesized that the noted differences in aggregation levels were due to the reactivity of the different Michael acceptors towards thiol groups. Maleimide-thiol reaction kinetics are rapid, while vinyl sulfone-thiol and then acrylate-thiol reaction kinetics are successively slower.^{176,186} Fast gelation times of less than 6 seconds were observed when forming PEGMA-PEGTT hydrogels, which limited mixing to cause higher levels of crosslinking heterogeneities and non-uniform cell distribution, ultimately leading to bacterial aggregation. Similar trends have been noted when using thiol-maleimide crosslinking strategies to encapsulate mammalian cell lines in tissue culture applications.¹⁸⁷ The higher reactivity of the difunctional maleimides can also lead to cross-reactivity with native thiols present on the cell surface¹⁸⁸ or in the extracellular polymer substance (e.g. cysteine residues), which may also be a contributing factor to aggregation. By contrast, PEGVS-PEGTT hydrogels showed more uniform dispersion of bacteria throughout the hydrogel and PEGDA-PEGTT hydrogels, which required ~25 min for gelation, showed highest

uniformity, which is potentially advantageous in applications where controlled release of beneficial bacteria is desired.

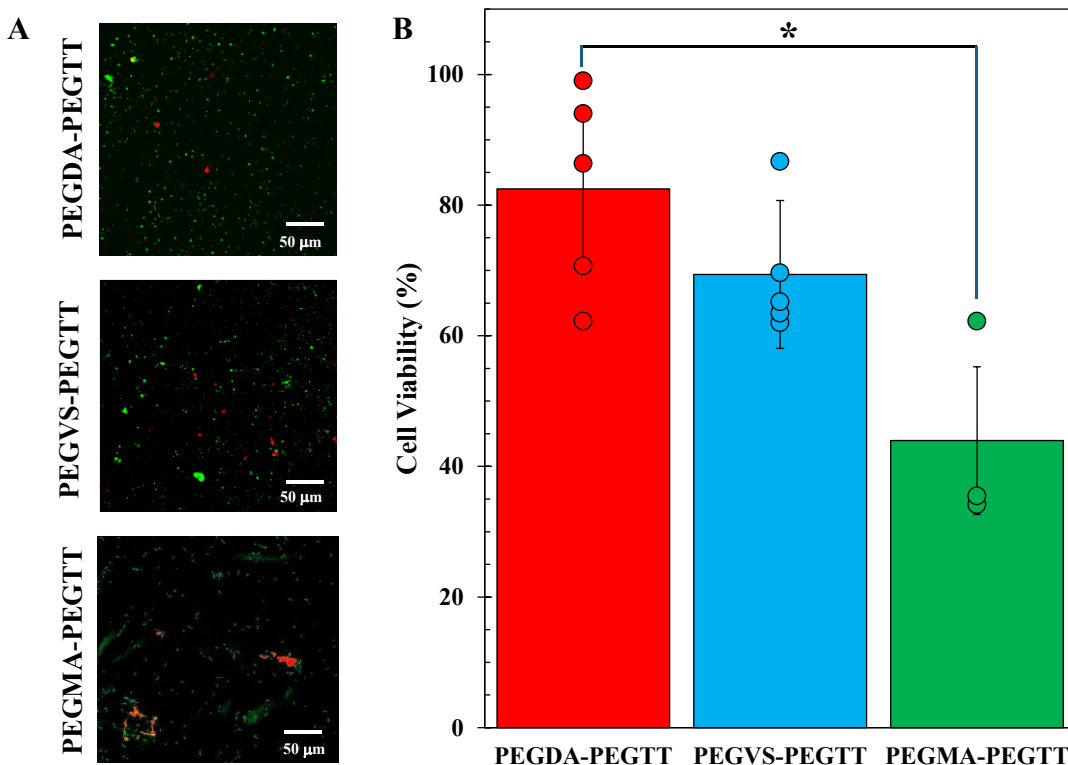


Figure 6.3. *B. subtilis* cell viability after hydrogel encapsulation. A) Representative green-red fluorescent images of *B. subtilis* after encapsulation and live-dead staining (green denotes live cells, red denotes dead cells). B) Quantified cell viabilities (* $p < 0.05$). Contrast was adjusted in Figure 6.3A to improve visualization.

Live-dead staining of *B. subtilis* encapsulated in PEGDA-PEGTT, PEGVS-PEGTT, and PEGMA-PEGTT hydrogels revealed variations in cell viability (Figure 6.3). Viability decreased with increased Michael acceptor reactivity, with PEGDA-PEGTT showing highest cell viability and PEGMA-PEGTT showing a statistically significant decrease. The findings are again comparable to tissue culture studies, where reactive crosslinking groups can have varied effects on cell toxicity due to cross-reactivity with mammalian cell lines¹⁸⁶ and where high cytotoxicity of maleimide crosslinkers has been reported.¹⁸⁸ Interestingly, many dead *B. subtilis* cells appeared within larger aggregates as opposed to single cells (Figure 6.3A, PEGMA-PEGTT), again

suggesting that cellular cross-reactivity and resulting cell aggregation was a cause of cytotoxicity in this system. By contrast, the uniform cell distributions within PEGDA-PEGTT hydrogels, as noted previously, appeared to promote cell survival.

Finally, as cell growth within hydrogels is a pre-requisite for most applications, the growth kinetics of encapsulated *B. subtilis* within the different hydrogels was studied. Here, *B. subtilis* was encapsulated into the respective hydrogels, and growth was monitored in 96-well plate format. Multiple controls were included for comparison. The first was culture of non-encapsulated *B. subtilis* present in bulk culture media (no hydrogel) of equivalent volume, which was used to decipher the effects of hydrogel confinement on growth. Second, free liquid media above *B. subtilis*-loaded hydrogel was sampled at 10-12 hr increments during culture to check for release of cells from the hydrogel into the supernatant solution. A final set of control samples included empty hydrogels to decipher any interference of the hydrogels on the optical density measurement. OD₆₀₀ readings in empty hydrogels during exposure to growth media remained unchanged through the entire experiment, indicating no interference or growth from contaminant microorganisms (Figure E3).

Hydrogel-encapsulated bacteria showed significant differences in growth kinetics and final growth levels compared to bacteria in bulk solution (Figure 6.4). Increases in the lag phase were noted for bacteria encapsulated in all hydrogels (lag phase: no hydrogel = 12.5 hr; PEGDA-PEGTT = 33.6 hr; PEGVS-PEGTT = 22.6 hr; PEGMA-PEGTT = 42.3 hr), as well as decreases in exponential phase growth rates (growth rate: no hydrogel = 0.129 hr⁻¹; PEGDA-PEGTT = 0.060 hr⁻¹; PEGVS-PEGTT = 0.096 hr⁻¹; PEGMA-PEGTT = 0.055 hr⁻¹). Both metrics indicate that nutrient diffusion limitations were prevalent in each hydrogel. Average mesh sizes determined by equilibrium swelling theory¹⁸³ were measured to be 12 ± 0.3 nm, 10 ± 0.3 nm, and 10 ± 0.8 nm for PEGDA-PEGTT, PEGVS-PEGTT, and PEGMA-PEGTT, respectively (Figure E4), which are

small enough to cause mass transport limitations during culture.¹⁶⁶ Despite this, cells were able to grow to significant and comparable levels within each hydrogel matrix during the 70 hr culture period, showing a 49% to 59% reduction in final carrying capacity compared to bulk culture in equivalent volume and growth media. It was also necessary to verify that hydrogels could provide stable encapsulation of bacteria during culture, as motile bacteria can potentially squeeze through non-uniformities within hydrogels or degrade liable hydrogel crosslinks for release. OD₆₀₀ readings taken from supernatant culture media (triangles, Figure 6.4) were low and did not increase with time, indicating that all hydrogels provided stable cell encapsulation during the growth period. Had cells escaped the hydrogels, OD₆₀₀ readings in the supernatant culture fluid would have increased in a manner comparable to bulk *B. subtilis* cultures (black line, Figure 6.4).

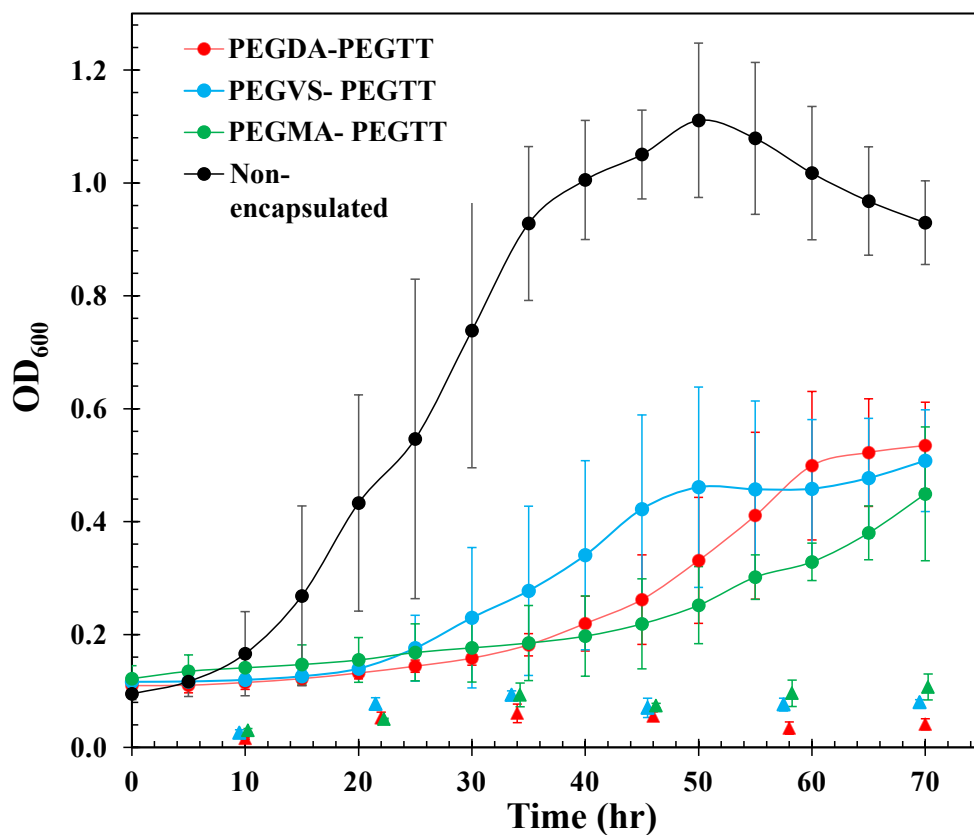


Figure 6.4. Growth kinetics of *B. subtilis* in bulk culture media (no hydrogel) and *B. subtilis* encapsulated in PEGDA-PEGTT, PEGVS-PEGTT, and PEGMA-PEGTT hydrogels. Triangles represent OD₆₀₀ readings from supernatant culture fluid outside of the hydrogels. Replicate trials were performed at $n \geq 3$.

6.4 Conclusions

These findings advance synthetic hydrogel materials formed with Michael addition click chemistries for integration of viable, functional bacteria into biohybrid systems. The highest levels of uniformity, viability, and single-cell encapsulation found in PEGDA-PEGTT hydrogels make it a strong candidate for use in many applications; however, other considerations may be necessary. For example, acrylate-thiol coupling generates thioester groups at each crosslink that are susceptible to acid or base hydrolysis. If pH stability is desired, PEGVS-PEGTT hydrogels that generate hydrolytically stable thioether sulfone bonds may provide a reasonable alternative. While PEGMA-PEGTT hydrogels in their current form are likely unfeasible for application, recent efforts to slow maleimide-thiol reactions have been reported,¹⁸⁵ which may improve this crosslinking chemistry for bacteria encapsulation.

6.5 Acknowledgments

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Chapter 7 - Encapsulation and Growth of Encapsulated *Bacillus subtilis* in PEG Hydrogels of Varied Mesh Sizes

Overview

This study investigates how the average mesh size of acrylate-thiol crosslinked hydrogels influences cell encapsulation and cell growth kinetics using *Bacillus subtilis* as a model organism. While hydrogels offer promise for *in situ* microbial applications, the effects of network mesh size on bacterial encapsulation and proliferation remain poorly understood. To address this, we systematically varied the molecular weights of PEGDA and PEGTT macromers to fabricate hydrogels with different mesh sizes within the range of 9-19 nm. Growth kinetics revealed statistically significant differences across hydrogels, with smaller mesh sizes supporting single-cell encapsulation and longer growth phases. Conversely, larger mesh sizes were associated with increased bacterial aggregation after encapsulation and shorter growth phases. These trends suggest that smaller mesh sizes slowed the growth of bacteria by lowering the diffusivity of nutrients throughout the matrix and by providing a stiffer network structure. All mesh sizes provided stable encapsulation of bacteria during the growth of cells within the hydrogel matrix.

7.1 Introduction

Hydrogels are three-dimensional, crosslinked polymer networks widely used in drug delivery and tissue engineering applications. They are also critical in cellular encapsulation, protecting and stabilizing functional microbes in bioinoculant applications. These microbes often face environmental stressors such as reactive oxygen species,⁵⁴ extreme pH conditions,⁵³ competition or antagonism from other microbes, and biofouling by endogenous microorganisms.¹⁸⁹ Most research on protective hydrogel coatings has focused on naturally occurring, polysaccharide-based materials, including alginate, chitosan, cellulose, starch, and hyaluronic acid.^{190,191} Synthetic hydrogels such as polyethylene glycol (PEG) and poly(vinyl alcohol)¹⁹² offer several distinct advantages for biomedical and biotechnological applications. They are highly valued for their excellent biocompatibility, chemical inertness, and ability to resist the nonspecific adhesion of proteins, cells, and bacteria.^{177,193–195} PEG-based hydrogels can be engineered as “smart” materials that respond to external stimuli such as light, temperature, or pH.¹⁹⁶

The versatility of PEG hydrogels stems mainly from the wide range of available molecular weights and end-group chemistries of PEG monomers.¹⁹⁰ These monomers can be crosslinked through various polymerization methods, including chain-growth polymerization, step-growth polymerization (such as Michael-type addition reactions), and other bio-orthogonal techniques, to form PEG-based hydrogel networks.^{176,196} However, the choice of polymerization method profoundly impacts the resulting network structure. Chain-growth polymerization often produces heterogeneous networks, where kinetic polymer chains crosslink monomers in a less controlled fashion, potentially introducing defects and variability.¹⁹⁷ In contrast, step-growth polymerization, including Michael-type addition, yields more homogeneous network architectures with tunable and predictable mechanical properties.¹⁷⁵ This approach is particularly advantageous for biological

applications, as it enables crosslinking through specific reactive sites without requiring initiators, catalysts, or external energy inputs and avoids the generation of free radicals. These features make step-growth polymerization especially well-suited for encapsulating sensitive cells and bacteria.^{54,177,195,197,198}

This study investigates how Michael-type addition chemistry and crosslinking densities in PEG-based hydrogels, specifically formed from acrylate-functionalized monomers (PEGDA) and thiol-reactive monomers (PEGTT), influence the encapsulation, growth, and morphology of *Bacillus subtilis*. Hydrogels were fabricated by crosslinking PEG monomers of varying molecular weights, resulting in a range of physical properties, including differences in swelling behavior and mesh size. The results reveal a clear correlation between hydrogel mesh size and cell morphology after encapsulation, where larger mesh sizes promote bacterial aggregation. Larger mesh size also led to faster growth, as measured by the duration of the lag phase and the exponential growth phase. This shows that structural variations within the hydrogel network significantly affected bacterial morphology and propagation.

7.2 Materials and Methods

7.2.1 Hydrogel preparation and cell encapsulation

The *Bacillus subtilis* strain 168 1S1135 expressing GFP (*B. subtilis*-GFP) used in these studies was obtained from the Bacillus Genetic Stock Center (Strain 1A1135, Bacillus Genetic Stock Center) and maintained in a 50% glycol stock solution at -80°C. For these studies, *B. subtilis* cells were cultured in tryptic soy broth (TSB) at 28°C for 24 hours. Afterward, the bacterial suspension was diluted with a phosphate-buffered saline (PBS) solution mixed with 2X ATGN to an optical density of 0.1 at 600 nm (OD₆₀₀) using a 96-well microplate reader (Epoch2 Microplate Reader, Biotek©). The PBS/ATGN solution was prepared by combining 1,506 mg of sodium

phosphate monobasic dihydrate with 95 mL of 2X AT minimal medium, supplemented with glucose and ammonium sulfate ((NH₄)₂SO₄), as described by Morton and Fuqua.¹⁸¹ The pH of the solution was adjusted to 8.0 using 5 M sodium hydroxide (NaOH).

Hydrogels were formed via a Michael-type addition reaction using PEG monomers of 2-kDa and 10-kDa of PEGDA and PEG monomers of 5-kDa, 10-kDa, and 20-kDa of PEGTT. For each 100 µL hydrogel, 50 µL of the diluted PBS/ATGN bacterial suspension was mixed with 22.4 µL of PEG diacrylate (PEGDA, 49 mM) in water. After thorough mixing, 27.6 µL of PEG tetrathiol (PEGTT, 20 mM) in water was added to achieve an equimolar ratio of acrylates to thiols. The reaction proceeded at room temperature for 25 minutes, forming stable hydrogels encapsulating the *B. subtilis* cells. The average mesh size for each hydrogel was measured as described in Chapter 6 and Gutierrez et al.¹⁹⁰

7.2.2 Live-dead assay

Cell viability after encapsulation was assessed using a Live/Dead Bacterial Viability Kit (Thermo-Fisher Scientific).¹⁸⁴ *B. subtilis* cells were encapsulated within hydrogels and incubated on thiolated glass coverslips for fluorescence imaging, following the procedure outlined by Fattahi et al.¹⁶⁷ After encapsulation, hydrogels were stained according to the manufacturer's instructions by adding 9 µL of the reagent mixture per 100 µL of hydrogel, followed by a 15-minute incubation at room temperature. Excess dye was removed by washing the samples with 0.85 wt% NaCl solution. Fluorescence intensity was then recorded using an inverted fluorescence microscope.

Two control suspensions of *B. subtilis* cells, equal in concentration to those encapsulated, were prepared to calibrate the assay. For the live control, untreated cells were used. For the dead control, cells were treated with 70% isopropanol for 20 minutes at room temperature. A calibration curve was generated by mixing different ratios of live and dead cells, producing a range of fluorescence reference points. Viability within hydrogels was quantified by calculating the ratio

of integrated green fluorescence (live cells) to integrated red fluorescence (dead cells), denoted as RG/R. This ratio was then mapped onto the calibration curve to estimate the percentage of viable cells.

7.2.3 Bacteria Growth Tracking.

After suspending the cells in PBS/ATGN, 50 μ L of hydrogel was formed in each well of a 96-well microplate to monitor bacterial growth using an OD₆₀₀ reader (Epoch2 Microplate Reader, Biotek). To create the hydrogels, 11.2 μ L of PEGDA was mixed with 25 μ L of the cell suspension, followed by 13.8 μ L of PEGTT to initiate the crosslinking reaction. The hydrogels were incubated at room temperature for 25 minutes to allow for complete formation. Afterward, 200 μ L of ATGN was dispensed on top of each hydrogel. The 96-well plate was then placed in the OD₆₀₀ reader (Epoch2 Microplate Reader, Biotek), where bacterial growth was tracked over 72 hours at 28°C, with optical density measurements recorded every 10 minutes. The molecular weight of the crosslinkers was varied to examine its impact on encapsulation by altering the hydrogel's mesh size. PEGDA molecular weights of 2 kDa and 10 kDa were tested, along with PEGTT molecular weights of 5 kDa, 10 kDa, and 20 kDa.

7.2.4 Bacteria Growth Curve Analysis Determining Phase Shifts

Bacterial growth curves typically consist of distinct phases: lag, exponential, stationary, and death. These durations are influenced by factors such as bacterial species, temperature, nutrient availability, pH, and water activity.¹⁹⁹ Given the dynamic nature of bacterial growth and the continuous changes in environmental conditions, analyzing the lag and exponential phases is essential for evaluating the impact of bacterial encapsulation within hydrogels. Growth data were fitted to the Gompertz equation¹⁹⁹ using Mathematica (Wolfram, Inc.), which models the bacterial growth curve as an asymmetrical sigmoidal function,¹⁹⁹ allowing for precise estimation of lag duration and growth rate;

$$L(t) = a + ce^{-e^{-\{b(t-m)\}}} \quad (7.1)$$

where $L(t)$ is the log number of bacteria at time t , a is the initial log bacterial count, c is the increase in bacterial numbers, m is the time at which the maximum growth rate is reached, and b is the growth rate at time m .

According to Buchanan & Cygnarowicz,¹⁹⁹ plotting the rate of change in population density versus time yields a curve equivalent to the first derivative of the Gompertz equation. Similarly, plotting growth velocities versus time generates a curve corresponding to the second derivative, where the maximum and minimum points indicate the end of the lag and exponential phases, respectively. Alternatively, the end of each phase can be determined by setting the third derivative of the Gompertz equation to zero. This approach results in two equations that simplify the estimation of the bacterial growth curve's phases:¹⁹⁹

$$e^{-b(t_1-m)} - e^{-\left(\frac{b}{2}\right)(t_1-m)} - 1 = 0 \quad (7.2)$$

$$e^{-b(t_2-m)} + e^{-\left(\frac{b}{2}\right)(t_2-m)} - 1 = 0 \quad (7.3)$$

Here, t_1 represents when the lag phase ends, and t_2 represents when the exponential phase concludes

7.2.5 Image Processing and Data

Images of the hydrogel aggregates were captured using an inverted fluorescent microscope (Nikon Eclipse Ti-E) and a confocal microscope (Zeiss LSM 700) to analyze bacterial growth within the hydrogels. Image and particle analysis software (ImageJ 1.54d) was employed to calculate the size and distribution of microbial aggregates. Three independent replicates were prepared for each hydrogel. Histogram plots with a bin size of $13 \mu\text{m}^2$ were generated from the aggregate size data, with an overflow bin set to include all aggregates bigger than $50 \mu\text{m}^2$.

7.2.6 Statistical Analysis

Statistical analyses were performed using statistical software (SAS Institute Inc.). Means and standard deviations were calculated based on at least three replicates ($n=3$). A Student's t-test was conducted to compare datasets, with a p-value < 0.05 considered statistically significant.

7.3 Results and Discussion

Hydrogels were synthesized using PEGDA and PEGTT macromers of varying molecular weights (MW) to modulate network mesh size.^{176,200,201} The estimated mesh sizes were 9 nm for PEGDA_{2k}–PEGTT_{5k}, 15 nm for PEGDA_{10k}–PEGTT_{10k}, and 19 nm for PEGDA_{10k}–PEGTT_{20k}. These mesh sizes were significantly different from one another, ensuring the formation of structurally distinct hydrogel networks. Figure 7.1 illustrates the measured mesh sizes for each hydrogel formulation.

Despite having different hydrogel structures, the encapsulation process and network architecture did not significantly impact *B. subtilis* survival. Viability across the different hydrogel formulations remained relatively consistent (Figure F1), with an overall average viability of $76.62 \pm 16.6 \%$, indicating that all formulations supported bacterial survival to a comparable extent. These findings are consistent with the results of Gutierrez et al. 2024,¹⁹⁰ where hydrogels crosslinked with PEGDA and PEGTT achieved a cell viability of $82.49 \pm 12.36\%$. Similarly, in 2019, Mainardi et al. reported high survival rates for *B. subtilis* encapsulated in alginate hydrogels, demonstrating extended culture viability for up to 60 days with the periodic addition of fresh media.²⁰² Together, these results suggest that hydrogel encapsulation can effectively preserve bacterial viability, supporting their use in long-term applications.

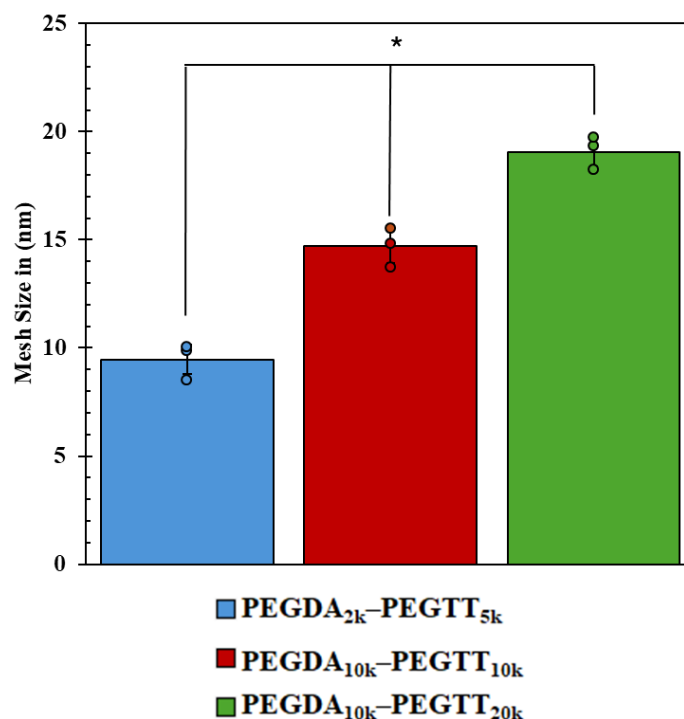


Figure 7.1 Calculated mesh sizes for each hydrogel ($n \geq 3$). Results are reported as mean $\pm$ standard deviation. Asterisk (*) denotes statistically significant differences ($p < 0.05$) between formulations.

The morphology of cells after encapsulation was studied with methods similar to those presented in Chapter 6 and in Gutierrez et al.¹⁹⁰ The analysis presented in Figure 7.2 demonstrates that the molecular weight of the monomers significantly influences the spatial distribution and aggregation of encapsulated *B. subtilis* cells. Hydrogels with smaller monomers (Panel i) primarily confined cells as single entities, as evidenced by the high frequency of small-area objects in the histogram and the dispersed pattern observed in both 2D and 3D fluorescence images. In contrast, as the MW of the monomers increased (Panels ii and iii), there was a noticeable shift toward larger measured areas, indicating increased cell aggregation. These observations were made with a 2D inverted microscope and a 3D confocal microscope, both of which revealed denser bacterial populations and more extensive aggregation in hydrogels with larger mesh networks. Aggregation is undesirable in most applications, as it can lead to localized nutrient depletion and limited access

for individual cells, particularly in dense clusters or early biofilm-like structures. Additionally, irregular crosslinking during hydrogel formation, which is more likely in less densely crosslinked networks, may create heterogeneities that further promote aggregate formation. Overall, the data suggest that mesh size plays a critical role in modulating bacterial confinement and aggregation, which may have important implications for designing encapsulation systems for microbial growth, viability, and activity regulation.

Growth kinetics of *B. subtilis* after hydrogel encapsulation were next monitored in a 96-well plate format, as shown in Figure 7.3. The hydrogel mesh size was found to influence the growth kinetics of encapsulated *B. subtilis*. In PEGDA_{2k}–PEGTT_{5k} hydrogels, the bacteria exhibited an overall lag phase and exponential growth phase of approximately 47 hours. Increasing the pore size with PEGDA_{10k}–PEGTT_{10k} hydrogels shortened the lag phase by six hours and the exponential phase by four hours, reducing the total growth duration to 37 hours. For PEGDA_{10k}–PEGTT_{20k} hydrogels with the largest pore size, both the lag and exponential phases further decreased, resulting in a total growth time of approximately 29 hours. A statistically significant difference was observed between the total growth times of the hydrogels ($p \leq 0.02$).

To confirm that the hydrogels provided stable bacterial encapsulation during culture, OD₆₀₀ measurements were taken from the supernatant of each sample (triangles, Figure 7.3). However, OD₆₀₀ values in the supernatant remained consistently low and did not increase over time, indicating that all hydrogel formulations effectively retained the encapsulated cells throughout the growth period. In contrast, if cells had escaped, OD₆₀₀ readings would have increased similarly to those observed in bulk cultures.

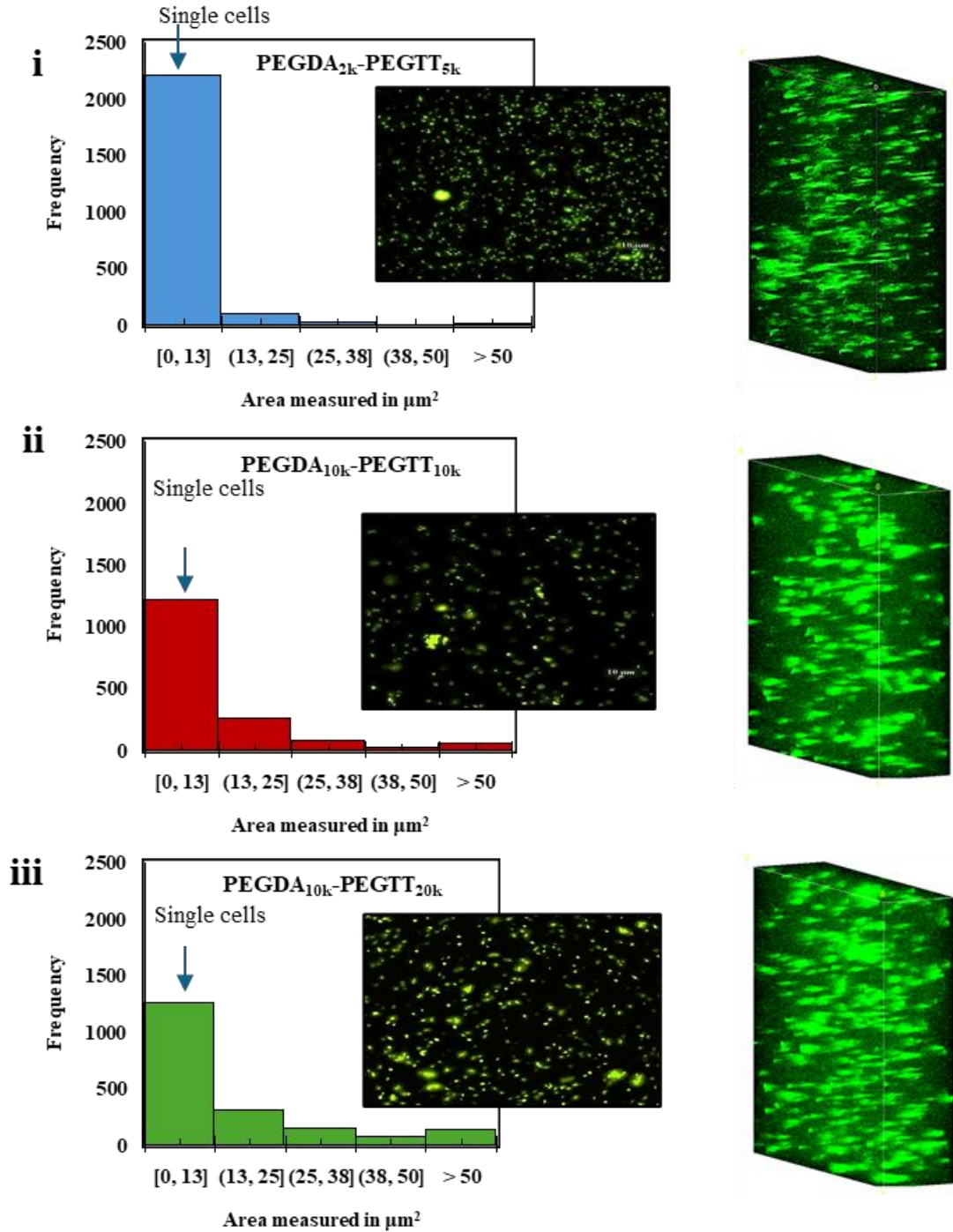


Figure 7.2. Effect of hydrogel mesh size on encapsulated *B. subtilis*. (i) PEGDA_{2k}-PEGTT_{5k} hydrogel shows a high frequency of small cell clusters (≤ 13 μm²), as depicted by the histogram (left), corresponding fluorescence microscopy (middle), and 3D confocal reconstruction (right). (ii) Hydrogel PEGDA_{10k}-PEGTT_{10k}. (iii) In hydrogels formed by PEGDA_{10k}-PEGTT_{20k}, microcolony areas remain relatively small, but with a slight increase in diversity compared to the PEGDA_{2k}-PEGTT_{5k} hydrogel. Arrowheads indicate the peak distribution bin for the cell area. Fluorescence imaging confirms variations in microcolony organization as a function of mesh size.

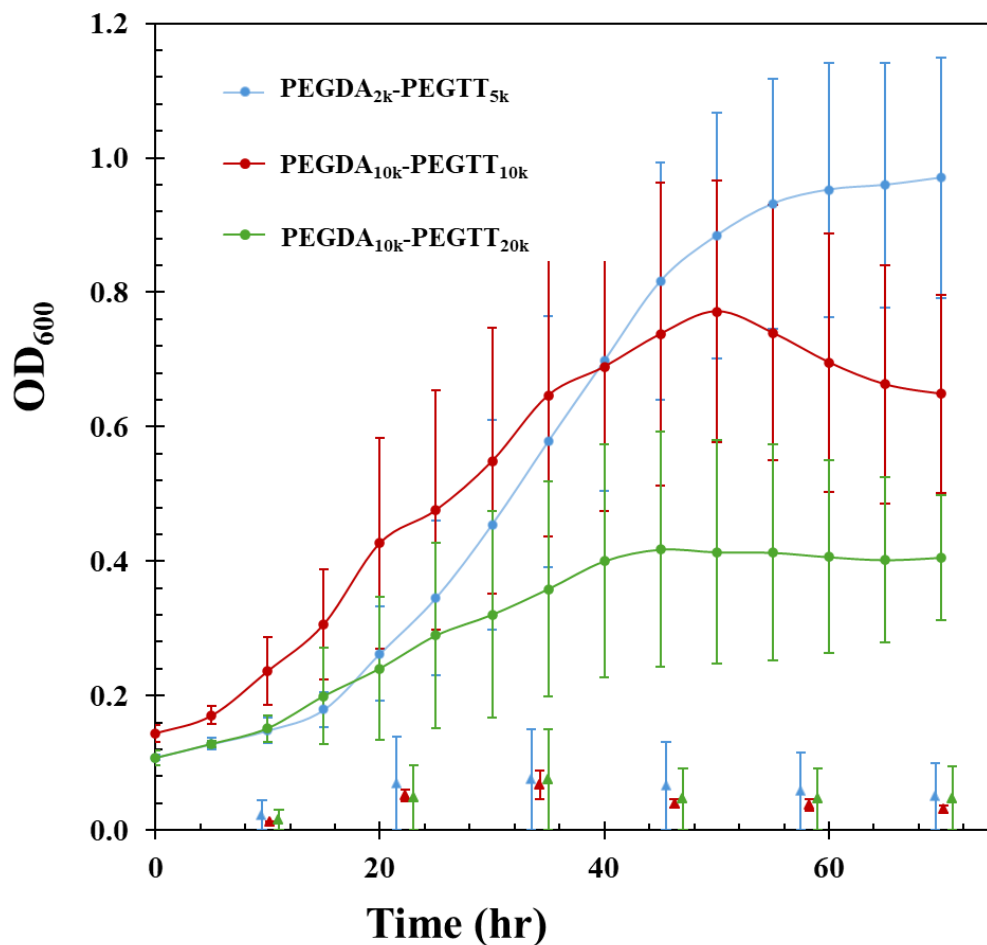


Figure 7.3. Growth kinetics of *B. subtilis* encapsulated in PEGDA–PEGTT hydrogels with varying mesh sizes: PEGDA_{2k}–PEGTT_{5k}, PEGDA_{10k}–PEGTT_{10k}, and PEGDA_{10k}–PEGTT_{20k}. Optical density (OD₆₀₀) measurements reflect bacterial growth within the hydrogels over 72 hours. Triangular markers denote OD₆₀₀ readings from the supernatant, indicating minimal bacterial leakage. Error bars represent standard deviation ($n = 3$).

Figure 7.4 presents the results for the lag and exponential phases across all mesh sizes. Notably, the PEGDA_{2k}–PEGTT_{5k} hydrogel with the lowest mesh size supported the most extended lag phase and overall growth duration. This is likely caused by lower substrate diffusivity and a stiffer network structure. These results indicate that smaller mesh sizes may create a microenvironment conducive to prolonged bacterial growth. The observed differences in bacterial growth may also be partially attributed to differences in cellular aggregation behavior in the different hydrogels. As mesh size increased, a corresponding rise in bacterial aggregation was noted. These structural

effects highlight the importance of controlling mesh size and network uniformity when designing encapsulation systems to sustain microbial proliferation.

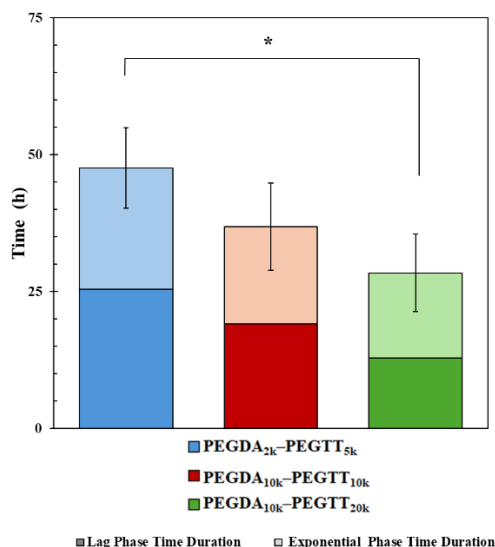


Figure 7.4. Effect of hydrogel mesh size on the growth kinetics of *B. subtilis* encapsulated cells. Bar segments represent the duration of each hydrogel's lag phase (darker shades) and exponential phase (lighter shades). A significant reduction in total growth time was observed when the MW of monomers increased, showing the longest lag and exponential phases. Error bars indicate standard deviation ($n = 3$). Asterisk (*) denotes statistically significant differences ($p < 0.05$) between conditions.

7.4 Conclusion

This study demonstrates that hydrogels of different mesh sizes formed through Michael-type addition chemistry using PEGDA and PEGTT macromers of varying molecular weights are critical in shaping the morphology, spatial distribution, and growth behavior of encapsulated *B. subtilis*. While all hydrogel formulations supported high bacterial viability and stable cell encapsulation, mesh size significantly influenced both the morphology of cells after encapsulation and the duration of growth phases. Smaller mesh sizes were associated with single-cell encapsulation and prolonged cell growth, likely due to the more confined, stiffer microenvironment, whereas larger mesh sizes led to aggregate formation and shorter growth phases. These findings suggest that hydrogel mesh size can be controlled to regulate microbial

behavior. This insight has important implications for developing encapsulation systems in biotechnology, including applications such as microbial delivery, probiotic formulations, and bioreactor optimization, where precise control over bacterial growth and spatial organization is essential.

7.5 Acknowledgments

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Chapter 8 - Summary and Future Works

8.1 Summary and Future Work

8.1.1 Effects of Different Soil Textures on Surfactin Efficiency in Wettability and Soil Water Retention- (Specific Aim One, Chapter 3)

Surfactin plays a critical role in altering surface properties, particularly by modifying hydrophobicity and hydrophilicity, thereby influencing soil water dynamics. However, this study demonstrated that surfactin affects soil evaporation in a non-uniform way, with responses varying depending on soil type and conditions. In earlier controlled experiments (Specific Aim One, Chapter 3), sandy loam was the only soil texture that exhibited a significant increase in water retention and a decrease in contact angle in response to surfactin treatment. Nevertheless, when surfactin treatments were applied to natural soils (Figure G1), loam soil also responded significantly, alongside sandy loam, by reducing hydrophobicity (Figure G2).

These highlight the importance of underlying soil characteristics in mediating the effects of biosurfactants. Natural soils exhibit considerable variation in their physical and chemical properties, even among soils of the same texture classification. Differences in mineral composition, organic matter content and type, and particle distribution all influence the capacity of surfactin to be adsorbed and interact with the soil matrix, including environmental conditions²⁰³. This variability, as shown in Table G1, underscores the complexity of applying biosurfactant-based amendments across heterogeneous field conditions.

Mechanistically, surfactin reduces surface tension and increases soil wettability, promoting water infiltration and retention^{60,61} within the soil matrix.¹⁸ Soils with moderate to high permeability⁷⁰ and balanced particle size distribution,⁶⁵ such as loam and sandy loam, responded most positively to treatment. In contrast, loamy sand, which lacks structural balance, demonstrated limited responsiveness. Clay-rich soils, despite their fine structure and high total porosity,

exhibited minimal improvements. Their small pore sizes and strong, cohesive forces promote matric potential–driven retention,⁶⁵ but these same characteristics likely inhibit the added benefits of biosurfactant action. These soils have lower permeability⁷⁰, and their tight structure may limit the diffusion and mobility of surfactin within the matrix. In contrast, sandy loam, which has high porosity and gravitational forces that primarily drive soil water dynamics and allow for better diffusion, behaves differently. Similarly, loam soils exhibit a balance of gravitational forces and matric potential, allowing for better water movement and retention regulation.⁶⁵

A key limitation in the application of surfactin lies in its interaction with fine soil fractions. Similar behavior has been observed with synthetic surfactants, as reported by Lee et al.,²⁰³ where interactions with charged soil components alter solubility equilibria and inhibit performance. These point to the need for site-specific strategies when using biosurfactants for soil remediation or moisture retention. Because soil texture, composition, and mineralogy vary significantly across landscapes and even within single fields, tailoring treatments to local soil characteristics is essential to maximize their effectiveness.²²

8.1.2. Future Work (Specific Aim One, Chapter 3)

While this study has shown that surfactin can significantly enhance water retention and reduce hydrophobicity in sandy loam soils, further studies should investigate a broader range of soil types with varying textures and mineral compositions to determine the thresholds at which surfactin remains effective. In particular, research should focus on clay and silty contents, where the adsorption of surfactin to soil particles may limit its availability and function. Mechanistic studies examining how surfactin interacts with specific soil components, such as clay minerals, organic matter, and pore structures, could clarify the physical and chemical barriers to its performance.

8.2.1 The Role of *Bacillus subtilis* in Modifying Soil Water Dynamics Loam and Sandy Loam Texture Types- (Specific Aim Two, Chapter 4)

This study aimed to evaluate the feasibility of *B. subtilis* and surfactin enhancing water retention and soil wettability, particularly in loam and sandy loam soils, which were found to respond most effectively to surfactin treatments in Specific Aim One. Through a combination of laboratory experiments and modeling under simulated rainfall conditions, this work demonstrated that both direct application of surfactin and indirect application via bacterial inoculation significantly influenced soil moisture dynamics by increasing evaporation rates during the constant rate period (when water is plentiful) and reducing them during the slower rate period (when water is scarce).

Treated soils exhibited a faster transition to a dry surface layer (increased constant evaporation rate) and a slower rate of water loss thereafter, suggesting deeper moisture retention in the root zone. These effects were especially pronounced in sandy loam soils, highlighting the importance of soil texture. The findings indicate that surfactin-based treatments, especially those delivered biologically via *B. subtilis*, offer a promising strategy for conserving soil moisture in semi-arid agricultural systems.

8.2.2. Future Work (Specific Aim Two, Chapter 4)

Future research should build on these findings by exploring the mechanisms and conditions that optimize *B. subtilis* colonization and *in situ* surfactin production in different soil textures. While sandy loam soils demonstrated the most pronounced response in this study, further investigation is needed to understand how soil structure, organic matter content, and native microbial communities influence treatment effectiveness across a broader range of textures. Field-scale trials in semi-arid agricultural regions are essential to validate the laboratory and modeling results under realistic weather, irrigation, and cropping conditions. Additionally, future work

should assess the long-term persistence of *B. subtilis* in soil microbiomes and its capacity for repeated surfactin production under seasonal environmental variability.

Another important direction is to examine the interaction between biosurfactant-producing bacteria and common agricultural inputs, such as fertilizers or pesticides, which may enhance or inhibit microbial activity and soil wettability. Finally, the development of delivery systems (e.g., encapsulated inoculants or co-formulated soil conditioners) and techno-economic analyses will be critical for scaling up the use of *B. subtilis* as a biologically based soil amendment for moisture retention in vulnerable agrosystems.

8.3.1 Evaluating the Influence of Cell Density and Nitrogen Source on *B. subtilis* Efficacy- (Specific Aim Three, Chapter 5)

This study investigated the role of *B. subtilis* as a biologically based soil amendment to enhance water retention in agricultural soils, with a particular focus on improving conditions for inoculation using different nitrogen sources. The experiments demonstrated that *B. subtilis*, co-inoculated with ammonia nitrate, can reduce soil water repellency, increase water-holding capacity and maintain low nitrogen levels in sandy loam soils, a soil texture known for its lower water retention potential.²⁰⁴

Among the nitrogen sources tested, soils treated with ammonium nitrate (NH_4NO_3) and sodium nitrate (NaNO_3) retained significantly more water than those treated with ammonium chloride (NH_4Cl) or controls. The improved performance associated with nitrate-based treatments suggests that nitrate availability enhances microbial activity and facilitates *B. subtilis* colonization, as well as surfactin production. In particular, NH_4NO_3 treatments not only improved water retention but also induced significant denitrification, as evidenced by reduced nitrate concentrations during the dryout experiments. Electrical conductivity measurements further

supported differences in nitrogen assimilation efficiency among treatments, indicating that the form of nitrogen present influences ion dynamics and microbial interactions in the soil.

In summary, these findings suggest that the dual function of *B. subtilis* inoculation under nitrate-rich conditions offers a promising strategy for improving soil water retention and reducing nitrogen losses in drought-prone agricultural systems. The combined effect of enhanced wettability and microbial-driven denitrification suggests a sustainable approach to managing soil health in the agricultural system.

8.3.2 Future Work (Specific Aim Three, Chapter 5)

Future work should prioritize validation, microbiome profiling, and colonization to deepen the understanding of how *B. subtilis* interacts with sandy and loamy soil systems under diverse environmental conditions. This knowledge will be essential for developing scalable, biologically based strategies for soil water conservation and nutrient management. In parallel, the complexity of nitrogen cycling, shaped by variables such as pH, temperature, moisture, and organic matter, warrants further investigation. Additional studies should aim to quantify nitrogen transformation and transport processes, identify key microbial contributors to denitrification and nitrification, and directly measure surfactin levels within soil matrices.

8.4.1 Evaluating the Effects of Encapsulation on Bacterial Viability and Functionality- (Specific Aim Four, Chapters 6 & 7)

The survival and growth of *Bacillus subtilis* were investigated through hydrogel-based encapsulation as part of the fourth research aim. This study had two primary objectives: first, to evaluate how different polymerization chemistries affect *B. subtilis* during the encapsulation process, and second, to understand key bacterial properties such as growth kinetics, aggregation behavior, and interactions with hydrogel with different mesh sizes and morphologies. Both aspects

of the study revealed critical factors for achieving effective encapsulation and site-specific application.

Bacterial aggregation was observed during the process, limiting culture growth and reducing cell viability, thereby impacting the potential for long-term storage and shelf stability. These aggregations were linked to tunable parameters within the encapsulation process. For instance, rapid polymerization led to the formation of hydrogel networks before the bacteria could disperse uniformly, resulting in clumping. In contrast, slower polymerization rates allowed more time for even bacterial distribution, often encapsulating cells individually.

Mesh size was also found to play a key role. Smaller mesh sizes typically reduced aggregation and supported better encapsulation outcomes. Notably, optimizing mesh size within a specific range increased the lag and exponential phases without compromising viability or promoting excessive clustering. Overall, the successful encapsulation of *B. subtilis* in hydrogels depends on carefully controlling polymerization kinetics, crosslinking chemistry, and hydrogel architecture to balance bacterial viability, uniformity, and application potential.

8.4.2 Future Work (Specific Aim Four, Chapters 6 & 7)

Building on the insights from hydrogel encapsulation, future research should also explore the application of encapsulated *B. subtilis* for *in situ* soil treatments using natural polymers. Controlled release systems using optimized hydrogel formulations could enhance bacterial survival, maintain viability during storage, and allow precise delivery of surfactin directly into target soil zones such as the rhizosphere. This approach has the potential to improve the consistency and effectiveness of microbial amendments in the field, particularly under challenging environmental conditions such as drought. Encapsulation strategies tailored to soil texture, moisture availability, and microbial compatibility will be critical for advancing this next phase of application.

8.2 Other Alternatives and *In Situ* Application

While these studies have demonstrated the potential of *B. subtilis* and surfactin in enhancing soil water retention and modifying hydrophobicity, several avenues remain open for further investigation. The limited efficiency of current production methods for bacterial bioproducts has constrained their full-scale application, making industrial-scale implementation a persistent challenge. To address this, two potential strategies warrant further investigation.

The first approach involves encapsulating quorum-sensing *B. subtilis* within micro- or nano-structured polymeric hydrogel beads, which can then be introduced into a bioreactor system. Encapsulation modulates the bacterial microenvironment, allowing fine-tuned control over growth phases and metabolic activity. By adjusting nutrient flow and encapsulation conditions, the system can be directed toward phases that favor the synthesis of secondary metabolites, such as surfactin, even while overall bacterial proliferation remains controlled. This strategy could provide a scalable and cost-effective method for enhancing bioproduct yields, with implications for industrial bioprocessing and bioformulation technologies.

However, relying solely on the direct application of purified biosurfactants may miss the additional ecological and agricultural benefits of live *B. subtilis* cells. These include promoting plant growth under stress,²⁰⁵ supporting long-term root colonization,²⁰⁶ and enhancing soil microbial processes such as denitrification, as discussed in this work. Therefore, encapsulation approaches that retain live bacterial activity and boost metabolite production may offer a more comprehensive solution for environmental and agricultural applications.²⁰⁷

In addition to manufacturing challenges, bioproducts' transportation and application could also limit the efficient use of bacteria. However, these limitations can be addressed *in situ* applications, where bacteria function within living environments. Similar to processes in

bioreactors, encapsulated quorum-sensing bacteria can modulate their growth phases and regulate metabolite production over time, releasing bioproducts in controlled quantities exactly when and where they are needed. In addition, encapsulated bacteria also present a source of protection against any hostile environment, prolonging the culture's life. This strategy can enhance control over bioproduct timing and dosage, significantly boosting the overall efficiency of the environmental processes. Besides, since the production of surfactin is a quorum-mediated process, the encapsulation will stimulate the biosurfactant's output. Ultimately, surfactin is a good substitute for synthetic wetting agents in agricultural soils with the potential for increased water retention. However, further field research would be required to understand and implement this innovative technique, which could alleviate the impact of water scarcity and relieve the stress on the FEW nexus.

Chapter 9 - Reference

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Appendix A - Acronyms

CA	Contact Angle
EC	Electrical Conductivity
ER	Evaporation Rate
FEWS	Food Energy Water Systems
GSW	Gravimetric Soil Water Method
OD ₆₀₀	Optimal Density at 600 nm wavelength
OM(C)	Organic Matter (Content)
MW	Molecular Weight
PEG	Polyethylene Glycol
PEGDA	Poly(ethylene glycol) Diacrylate
PEGMA	Poly(ethylene glycol) Maleimide
PEGTT	Poly(ethylene glycol) Tetrathiol
PEGVS	Poly(ethylene glycol) Vinyl Sulfone
PGPR	Plant Growth-Promoting Rhizobacteria
ppm	Parts Per Million
RMSE	Root Mean Squared Error
SCL	Silt Clay Loam
SDM	Sessile Drop Method
SWC	Soil Water Content
SWR	Soil Water Repellency

Appendix B - Supporting Information for Chapter 3

B.1 Preparation Methods

B.1.1 Soils and Sampling

Soil samples were collected from the Ashland Bottoms, Agricultural Experimental Station, Kansas State University (39° 7'31" N 96° 38'8" W). A one-kilogram batch was taken from a 5 to 15 cm depth, and the soil texture was Silty Clay Loam. The soil samples were heat-sterilized before treatment. To avoid altering soil hydraulic properties through autoclaving,⁶⁰ Tyndallization Heat Treatment—a method involving intermittent heating and cooling cycles—was used. This process neutralized native microbes and prevented unwanted interactions with the biosurfactant.^{97,208} In addition to the natural soil sample, a one-kilogram batch of hydrophilic Ottawa Sand was obtained from Humboldt Manufacturing Company H-3820BX.

B.1.2 Sample Mixing and Preparation

Three soil mixtures were prepared by combining Ottawa Sand and Silty Clay Loam (SCL) soil in different proportions. Five mixtures were created, with Ottawa Sand-to-SCL ratios of 0:4, 1:3, 2:2, 3:1, and 4:0, respectively. These ratios corresponded to soil textures classified as Loam, Sandy Loam, and Loamy Sand, as well as the original pure Sand and SCL textures. After preparation, the mixtures were analyzed at the Kansas State University Soil Testing Laboratory to confirm their soil textures. The percentages of sand, clay, and silt in each mixture were determined by using the hydrometer method²⁰⁹ and are listed in Figure B1.

B.1.3 Surfactant Solutions Preparation

Surfactin solutions were prepared by dissolving 25 mg and 50 mg of surfactin (CAS # 24730-31-2, MW 1,036.34 g/mol) obtained from Millipore-Sigma (Merck KGaA) in one liter of

ultrapure water. The surfactant was prepared by diluting the powder in ethanol at a ratio of 10 mg per 1 mL of ethanol,²¹⁰ then diluting it to a 200 mg-kg⁻¹ solution. Solutions were further diluted to the proper concentrations prior to the experiments.

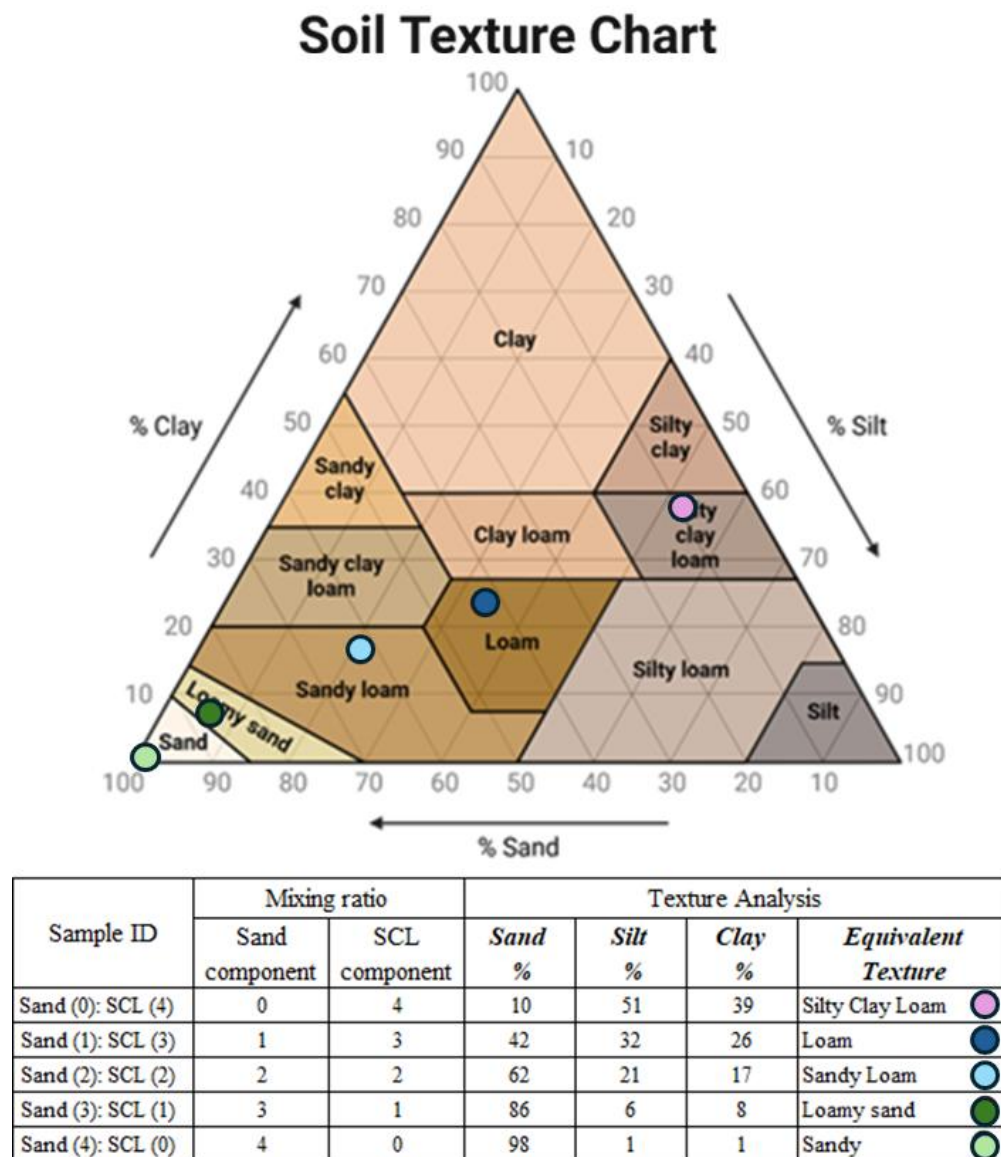


Figure B1. Dilution of silty clay loam soil with Ottawa sand to achieve soil mixtures with textures equivalent to loam, sandy loam, and loamy sand. The figure was prepared with assistance from Biorender software.

B.2 Data Tables

Table B1 Physicochemical Properties of the Soil Mixtures

Mixture	Texture Equivalent	Sand %	Silt %	Clay %	Total N %	TOC %	pH	OM MWB %	NO ₃ -N ppm	K ppm	NH ₄ -N ppm	Ca ppm	Mg ppm	Na ppm
Sand (0): SCL (4)	Silty Clay Loam	10	51	39	0.12	1.11	6	2.30	8.4	212.0	6.5	2995.0	548	23
Sand (1): SCL (3)	Loam	42	32	26	0.08	0.78	6	1.70	8	180.0	5.6	2590.0	475	22
Sand (2): SCL (2)	Sandy Loam	62	21	17	0.06	0.48	6.2	1.60	5.9	129.0	4.3	1822.0	332	20
Sand (3): SCL (1)	Loamy Sand	86	6	8	0.02	0.14	6.8	0.50	3.5	62.0	2.8	855.0	155	16
Sand (4): SCL (0)	Sand	98	1	1	0.02	0.01	7.25	0.00	0.7	6.0	1.5	49.0	14	10

* Analysis performed by Kansas State University Soil Testing Laboratory.

Table B2 Constant and Slower Evaporation Rate for the Soil Mixtures

Soil Mixture	Evaporation rate (mm/day) $\pm$ SD					
	Constant ER			Slower ER		
	0 mg-Kg ⁻¹	25 mg-Kg ⁻¹	50 mg-Kg ⁻¹	0 mg-kg ⁻¹	25 mg-kg ⁻¹	50 mg-kg ⁻¹
Sand (4): SCL (0)	12.83 $\pm$ 0.60	10.22 $\pm$ 0.44	10.57 $\pm$ 1.71	0.85 $\pm$ 0.07	0.52 $\pm$ 0.25	0.66 $\pm$ 0.12
Sand (3): SCL (1)	16.58 $\pm$ 3.50	13.33 $\pm$ 3.12	14.67 $\pm$ 1.77	1.63 $\pm$ 0.21	1.98 $\pm$ 0.24	1.60 $\pm$ 0.20
Sand (2): SCL (2)	18.63 $\pm$ 1.19	14.95 $\pm$ 0.40 *	12.59 $\pm$ 0.65 *	2.35 $\pm$ 0.28	2.02 $\pm$ 0.08	2.24 $\pm$ 0.42
Sand (1): SCL (3)	15.97 $\pm$ 0.24	15.07 $\pm$ 1.75	15.96 $\pm$ 1.52	2.90 $\pm$ 0.34	2.67 $\pm$ 0.21	3.13 $\pm$ 0.46
Sand (0): SCL (4)	17.70 $\pm$ 1.80	16.98 $\pm$ 1.28	17.05 $\pm$ 2.11	3.13 $\pm$ 0.18	2.69 $\pm$ 0.49	3.06 $\pm$ 0.18

Abbreviation: SD, standard deviation.

B.3 Soil Water Content (SWC) Curves

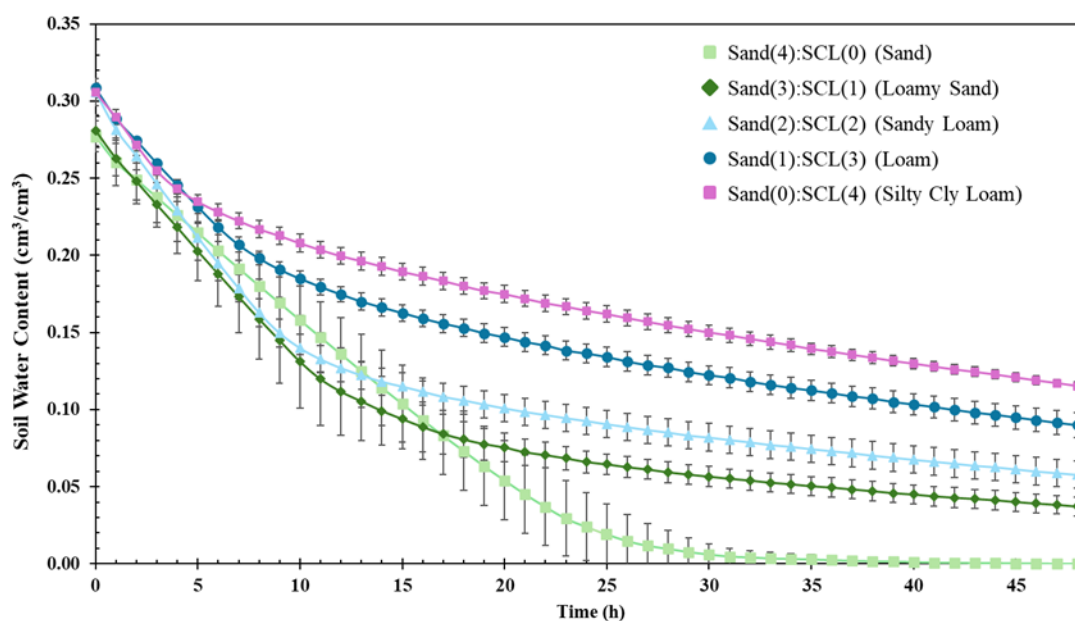


Figure B3. SWC during evaporation for control soil mixtures with no added surfactin.

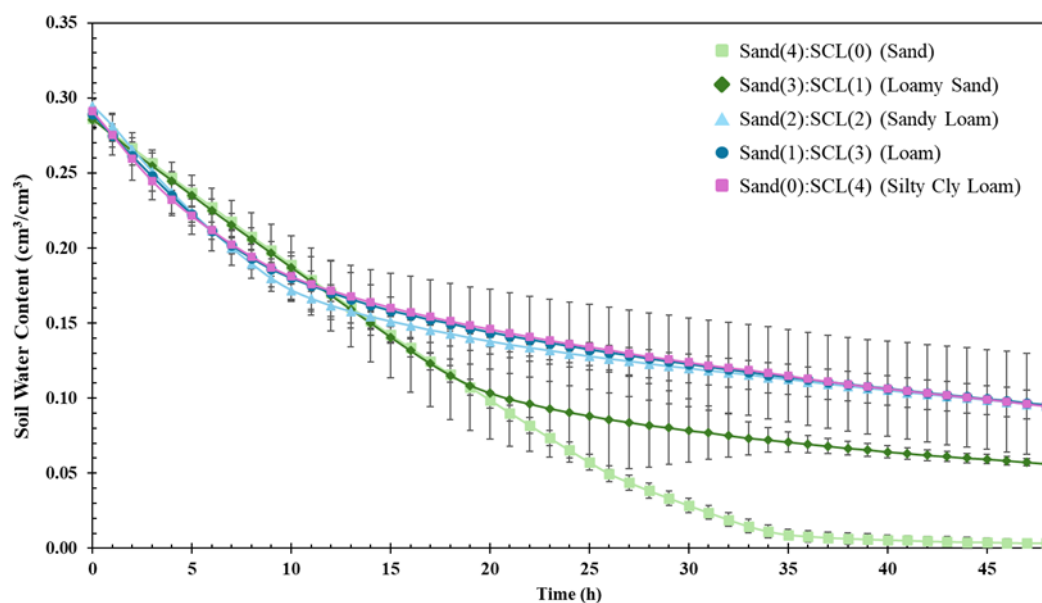


Figure B3. SWC during evaporation for the soil mixtures treated with 25 mg·kg⁻¹ surfactin.

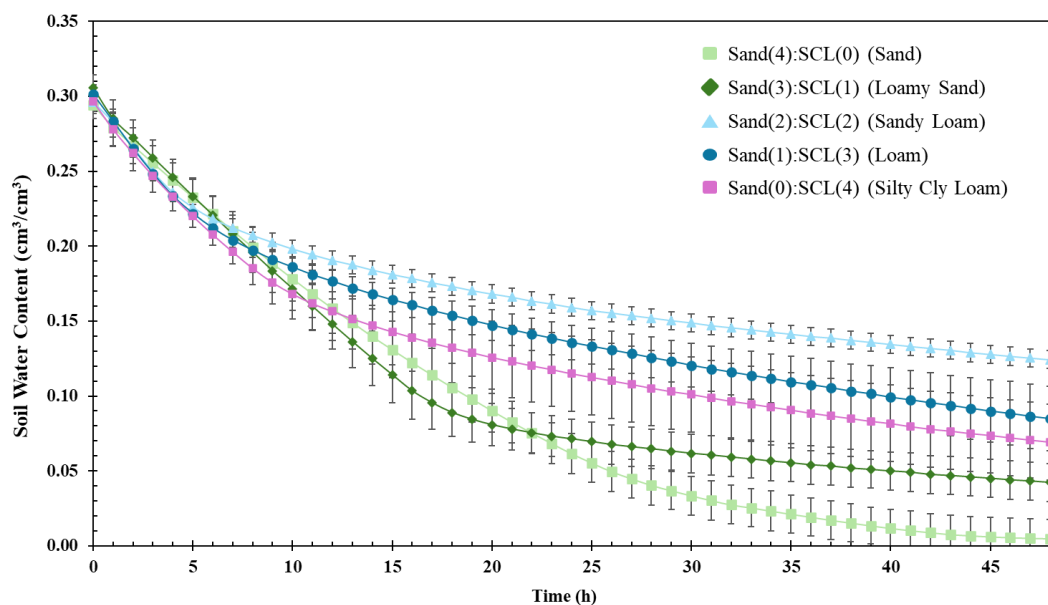


Figure B4. SWC during evaporation for the soil mixtures treated with 50 mg·kg⁻¹ surfactin.

Appendix C - Supporting Information for Chapter 4

Table C1. Soil Texture Analysis

	Sand %	Silt %	Clay %	Coordinates
Loam	32	50	18	39° 8' 17" N 96° 38' 25" W
Sandy Loam	60	26	14	39° 8' 20" N 96° 38' 14" W

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Table C2. Soil Physicochemical Analysis

	pH	Electrical Conductivity dS/cm	Total Nitrogen %	Total Organic Carbon %	Organic Matter (MWB) %	Total Phosphorus ppm
Loam	8	0.55	0.11	1.02	1.58	459.67
Sandy Loam	7.8	0.52	0.1	0.84	1.26	522.66

	NO ₃ -N ppm	NH ₄ -N ppm	K ppm	Ca ppm	Mg ppm	Na ppm
Loam	5.9	14.8	287	3,282.00	89.6	9.2
Sandy Loam	4.2	9.5	199.2	2,304.50	51.1	8.5

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C.1 Determining the Soil Maximum Water Holding Capacity

Soil water-holding capacity is the amount of water that a given soil holds for crop use. Field capacity is when the soil's water holding capacity has reached its maximum. The field capacity was determined experimentally by placing 50 g of soil in a filter funnel with a Whatman filter paper ® (No.1, 185 mm), attaching a rubber hose to the funnel with closed clips, adding 50 mL of ultrapure water to the soil in the funnel and allowing it to stand for 30 minutes. After 30 minutes, the clips were removed, and water was allowed to drain for the following 30 minutes. Finally, the total amount of water drained was recorded, and the percentage of the absorbed water was then calculated.

C.2 System Dynamics Model parameterization.

System dynamics model parameters for static and stochastic variables. Static variables operate identically in each time period, while values for stochastic variables are generated in each time step according to their governing distribution.

Table C3. System Dynamics Model parameterization

Static variables					
Variable	Value and description				
Soil moisture threshold	Displayed in Table 1.				
Maximum water holding capacity (MWHC)	Experimentally determined using the protocol outlined above. Sandy loam soil: 43.08% of soil volume. Loam soil: 35.43% of soil volume.				
Infiltration	100% of precipitation if the soil is below MWHC. Infiltration ceases once soil moisture reaches MWHC.				
Stochastic variables					
Variable	Governing distribution	Interval		Condition	
Precipitation occurrence	Uniform	$D \sim U(0,1)$		$D \leq 0.38$ $\rightarrow$ Rainfall event occurs	
Variable	Governing distribution	Mean	Standard Deviation	Minimum	Maximum
Precipitation magnitude	Exponential	40.5 cm	95.8 cm	0 cm	831 cm
Constant evaporation rate and slower evaporation rate	Normal	Estimates are displayed in Table 1.	Standard errors in Table 3 are divided by the square root of the sample size, 575.	Mean minus 3 times the standard deviation.	Mean plus 3 times the standard deviation.

Appendix D - Supporting Information for Chapter 5

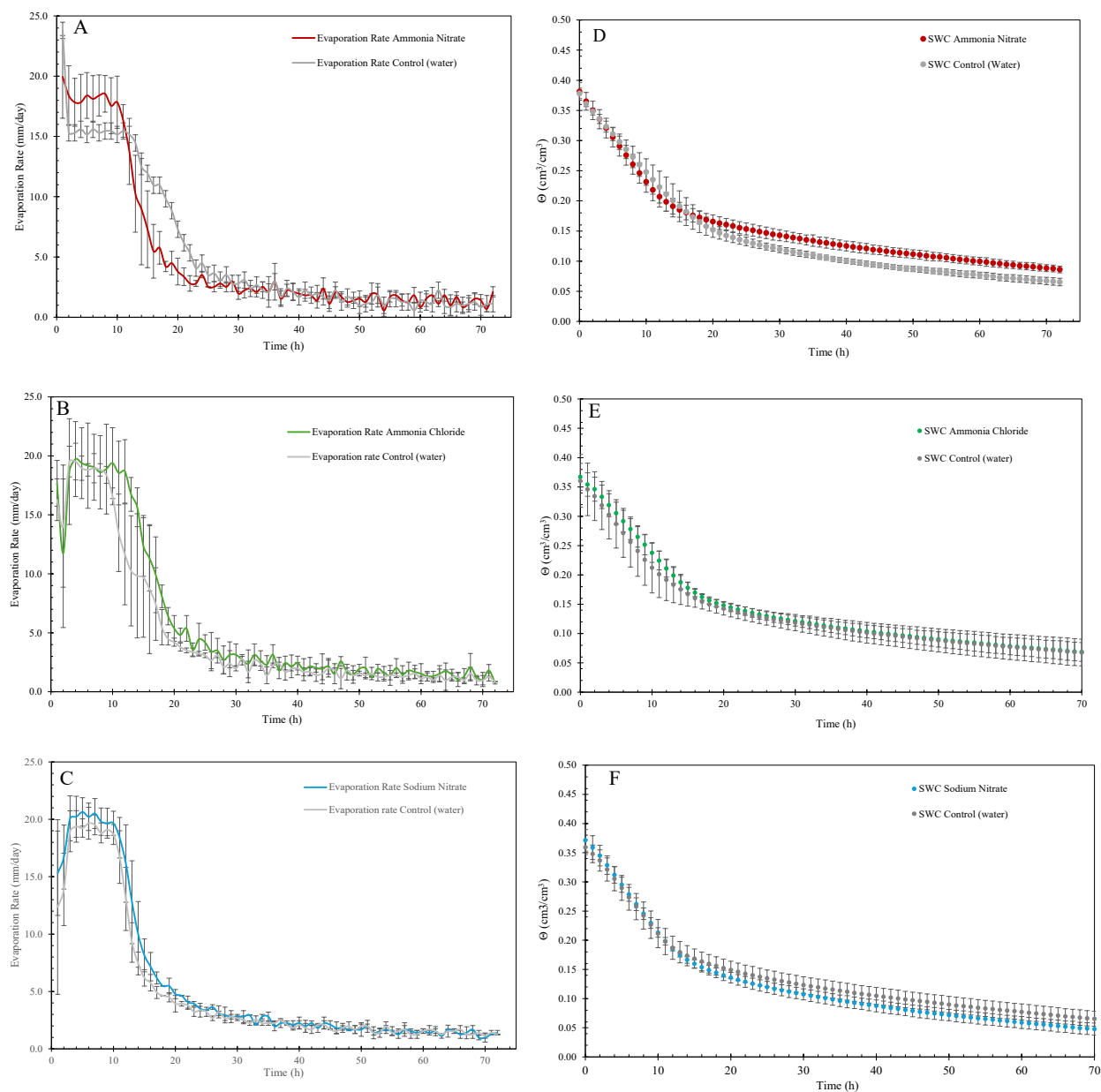


Figure D1. Evaporation Rate and Soil Water Content as a function of time, inoculated with *B. subtilis* 168 1A1135. (A) and (D) Ammonium Nitrate, (B) and (E) Ammonium Chloride and (C) and (F) Sodium Nitrate.

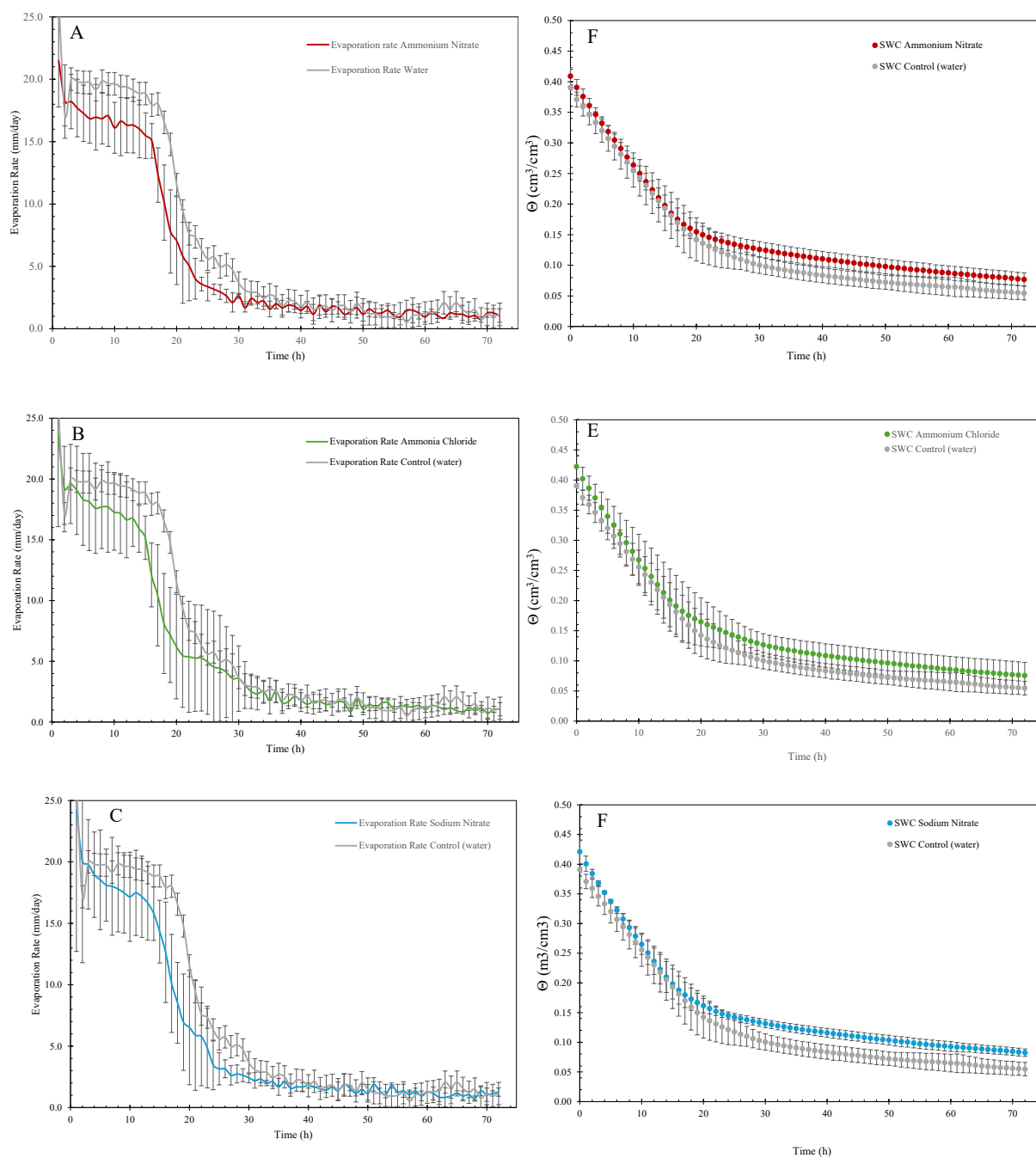


Figure D2. Evaporation Rate and Soil Water Content as a function of time, inoculated with *B. subtilis* ATCC 6051. (A) and (D) Ammonium Nitrate, (B) and (E) Ammonium Chloride and (C) and (F) Sodium Nitrate.

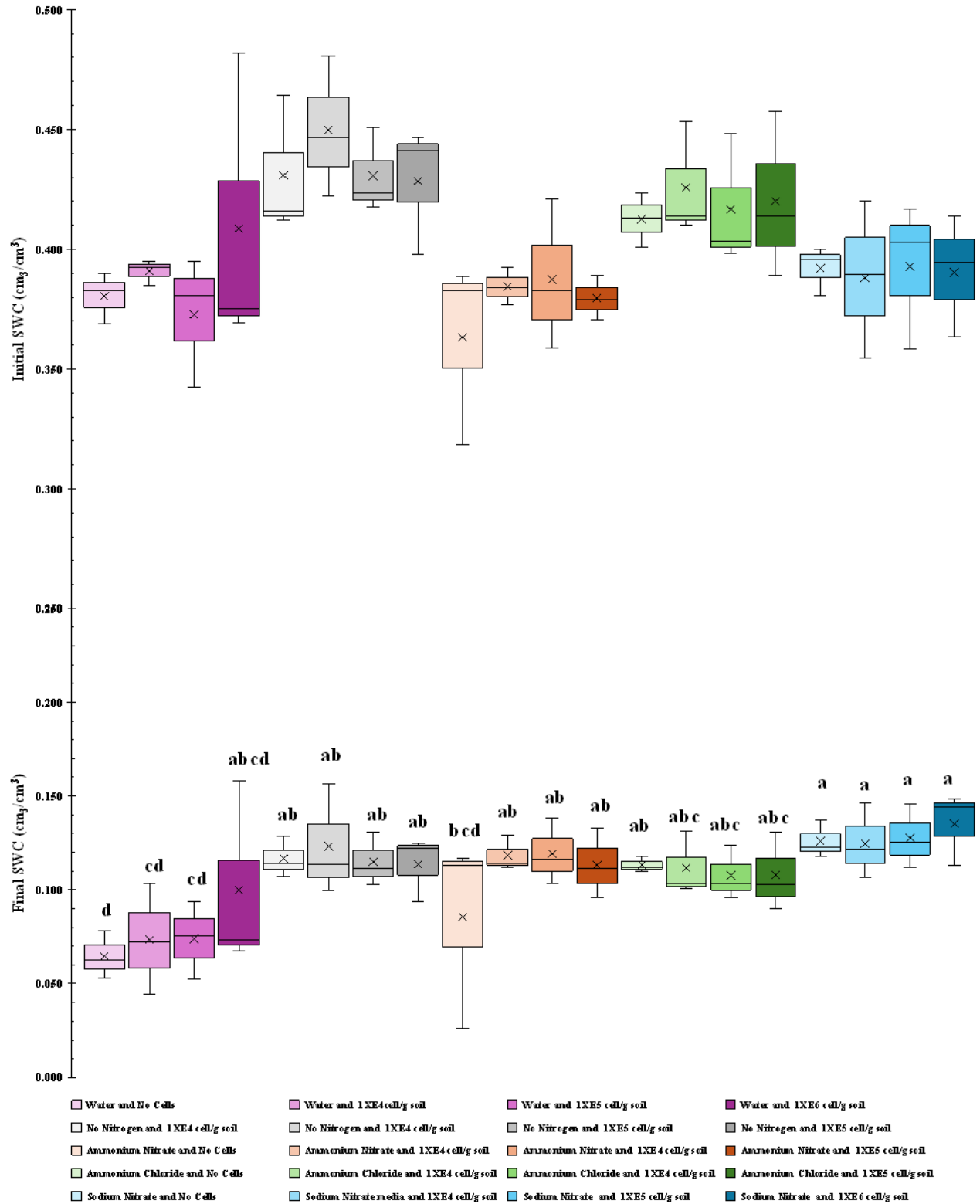


Figure D3. Initial and Final Soil Water Content (SWC) for samples treated with *B. subtilis* ATCC 6051 in the cell density experiments. (Data points with different letters are significantly different, $p < 0.05$.)

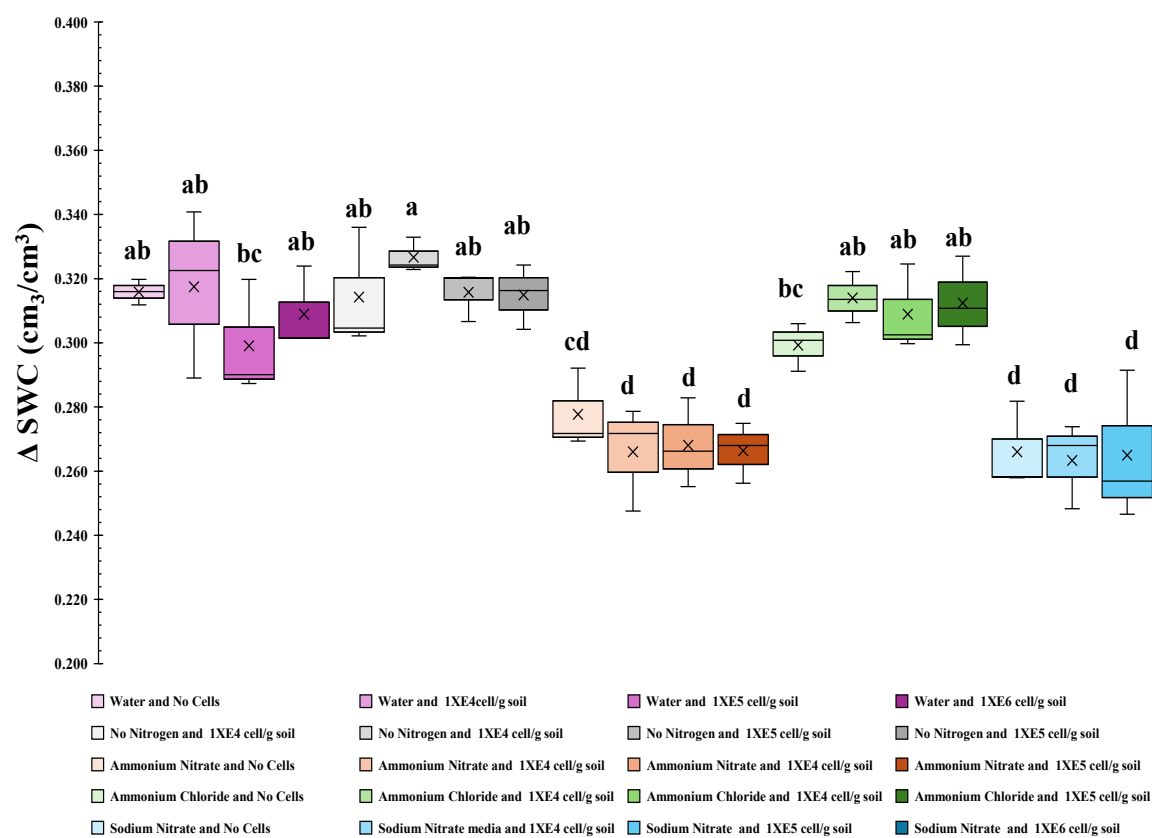


Figure D4. Difference between the initial and final soil water content (ΔSWC) for samples treated with *B. subtilis* ATCC 6051 in the cell density experiments. (Data points with different letters are significantly different, $p < 0.05$.)

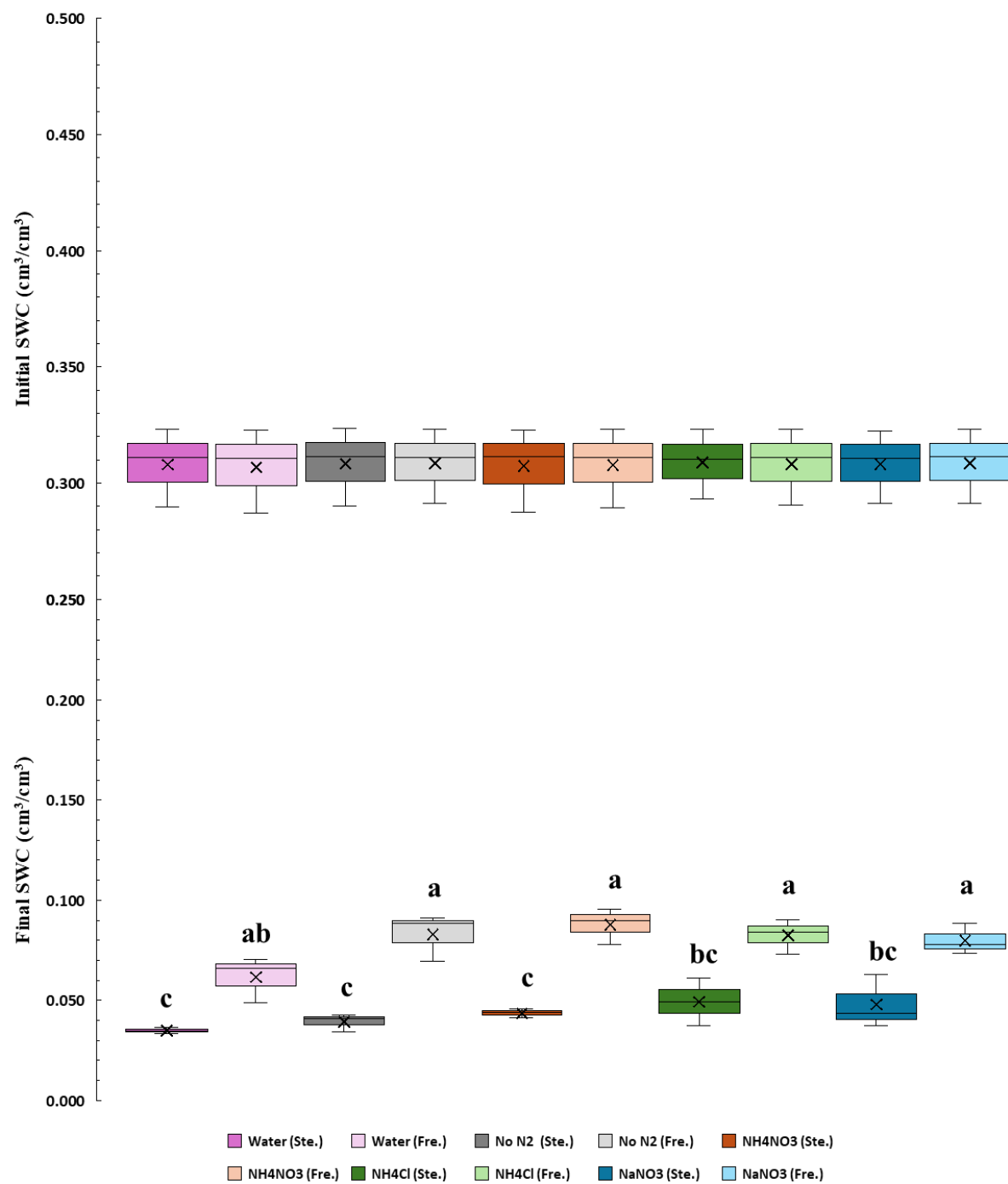


Figure D5. Initial and final SWC in soil samples sterilized with mercury (II) chloride (HgCl_2) and fresh samples amended with nitrogen sources. All samples were initially saturated to the same level, with no significant differences. The final SWC in sterilized samples differed significantly from that in fresh soil samples. (Data points with different letters are significantly different, $p < 0.05$.)

Appendix E - Supporting Information for Chapter 6

E.1 Live-Dead assay calibration curve

The calibration curve was prepared following the live/dead assay kit instructions manual.¹⁸⁴ *Bacillus subtilis* subsp. *subtilis* (ATCC 6051) was used in all live-dead experiments. To generate the calibration curve, four cell suspensions were used, representing 0%, 33%, 67%, and 100% of living cells in solution. Standards were made by preparing two 600 μ L solutions with cells suspended with an OD₆₀₀ index of 0.1. The first solution contained 0% live cells, and the second contained 100% live cells. For this first solution, *B. subtilis* cells were killed by incubating in 70% isopropanol at room temperature for 20 minutes, then centrifuged and resuspended in water. The other two standards for the calibration curve points were made using a combination of the 0% and 100% live cell suspensions. To prepare the 67% standard, 100 μ L of the 0% live cell suspension was mixed with 200 μ L of the resuspended 100% live cell solution. Similarly, the 33% standard was made by mixing 200 μ L of the 0% live cell suspension with 100 μ L of the 100 % live cell solution. Syto 9 was used to label intact live and compromised dead cells, while propidium iodide was used to label the compromised cells. Green and red images of cell populations were measured on a Nikon Ti-U inverted microscope with a 10X, NA 0.3 objective using FITC and TRITC filters (Figure E1).

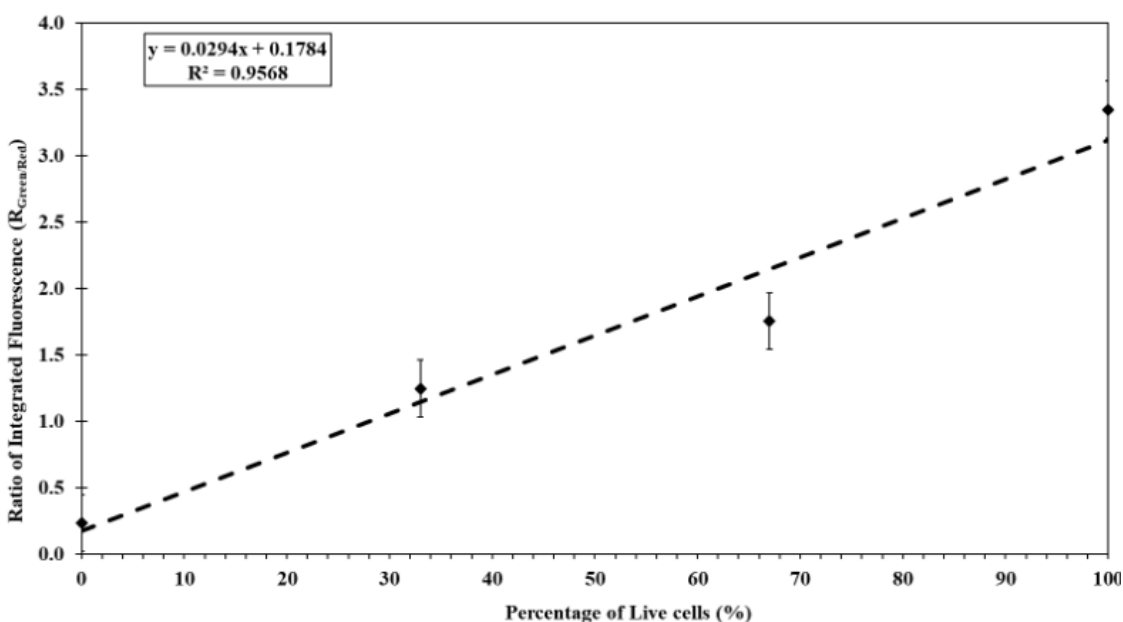


Figure E1. Calibration Curve for Live/Dead Assay.

Table E1. Constant used to calculate the hydrogel average mesh size using equilibrium swelling theory¹⁸³

Constant	Symbol	Value	Reference
Average bond length (nm)	l	0.146	Zustiak and Leach ¹⁹⁴
Flory Characteristic ratio of the polymer	C_n	4.0	Rehmann et al. ¹⁶⁶
Molecular weight of the repeat unit (g/mol)	M_r	44.0	
Molar volume of the solvent (cm ³ /mol)	V	18.0	
Polymer-solvent (PEG-water) interaction parameter	χ	0.426	Zustiak and Leach ¹⁹⁴ Cruise et al. ²¹¹
The number-average molecular weight of each monomer between crosslink points *	M_n	8400	
Density of polymer (g/cm ³)	ρ_p	1.125	
Density of solvent (g/cm ³)	ρ_s	1.000	
The specific volume of the polymer (ρ_s/ρ_p)	v	0.889	

*3,400 kDa for the 2-arm monomer and two 2,500 kDa for the 4-arm monomer

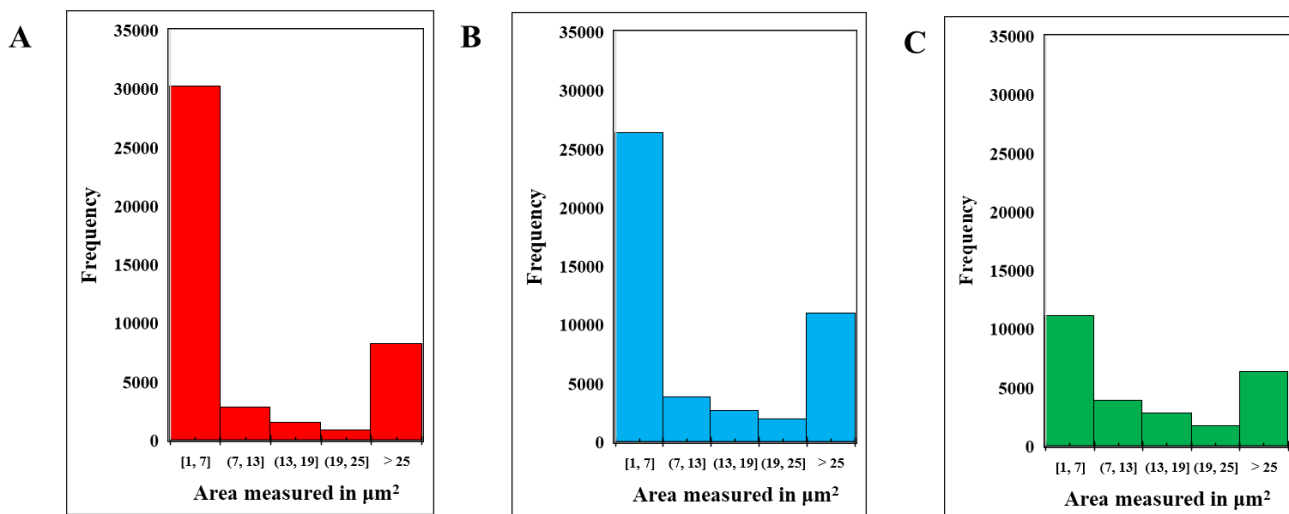


Figure E2. Histograms of particle size distributions for *B. subtilis* – GFP encapsulated in (A) PEGDA-PEGTT hydrogels, (B) PEGVS-PEGTT hydrogels, and (C) PEGMA-PEGTT hydrogels. Histograms contain the combined number of particles for all images analyzed.

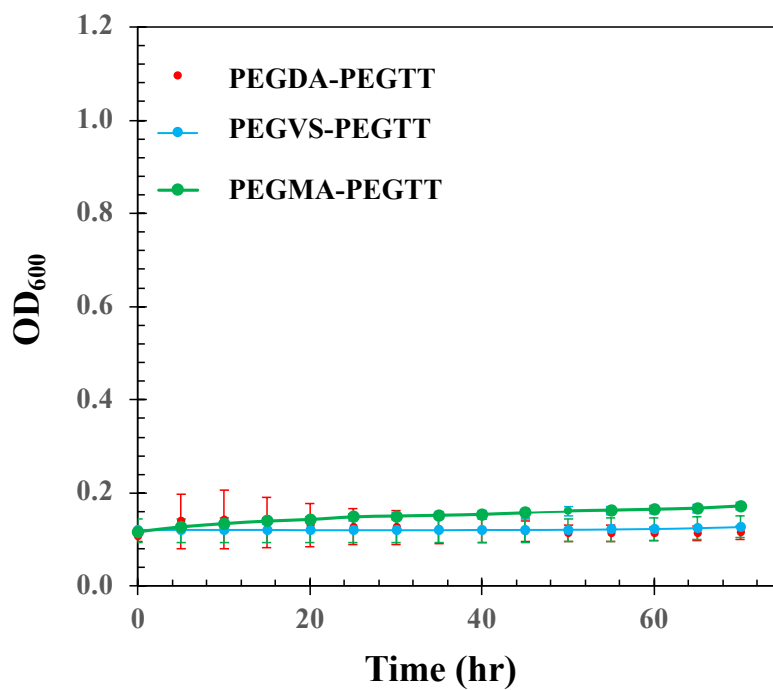


Figure E3. Growth profiles for empty hydrogel control (no bacterial cells) during incubation in ATGN growth media ($n \geq 3$).

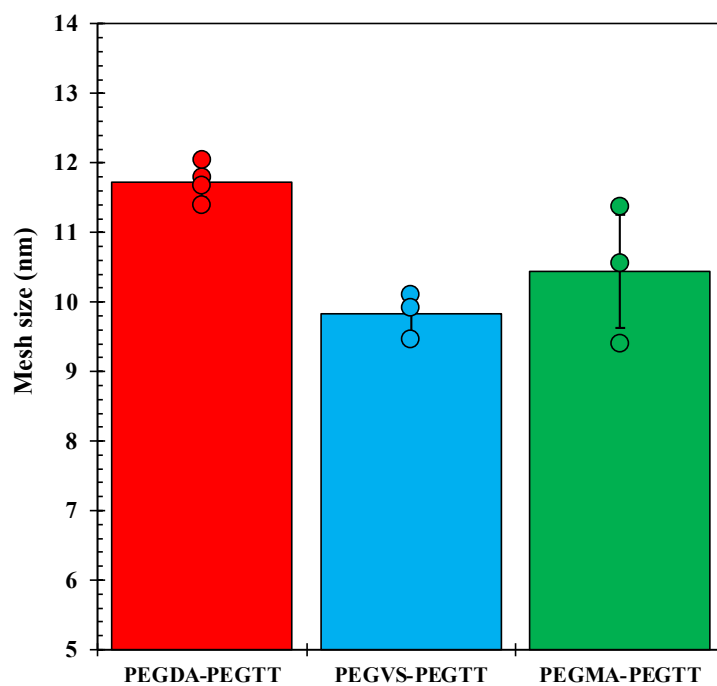


Figure E4. Calculated mesh sizes for each hydrogel ($n \geq 3$). Results are reported as mean $\pm$ standard deviation.

Appendix F - Supporting Information for Chapter 7

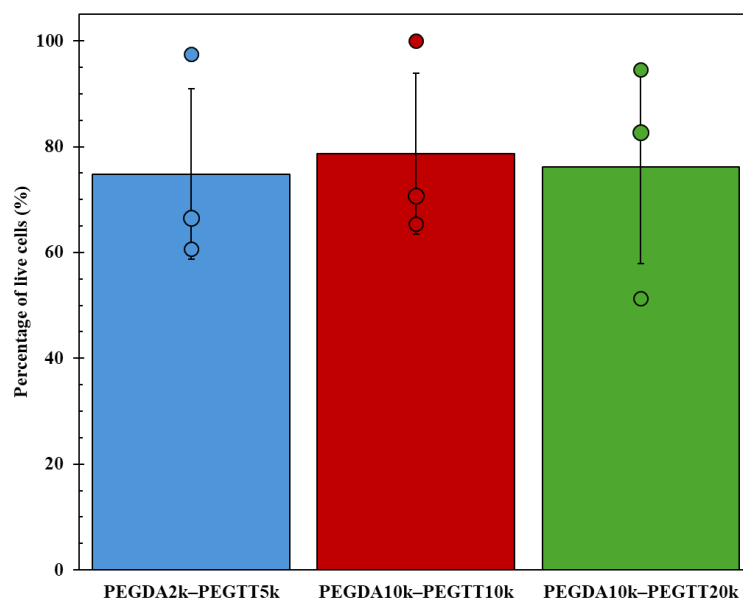


Figure F1. Quantified cell viabilities ($n = 3$). Results are reported as mean $\pm$ standard deviation (* $p < 0.05$).

Appendix G - Supporting Information for Chapter 8

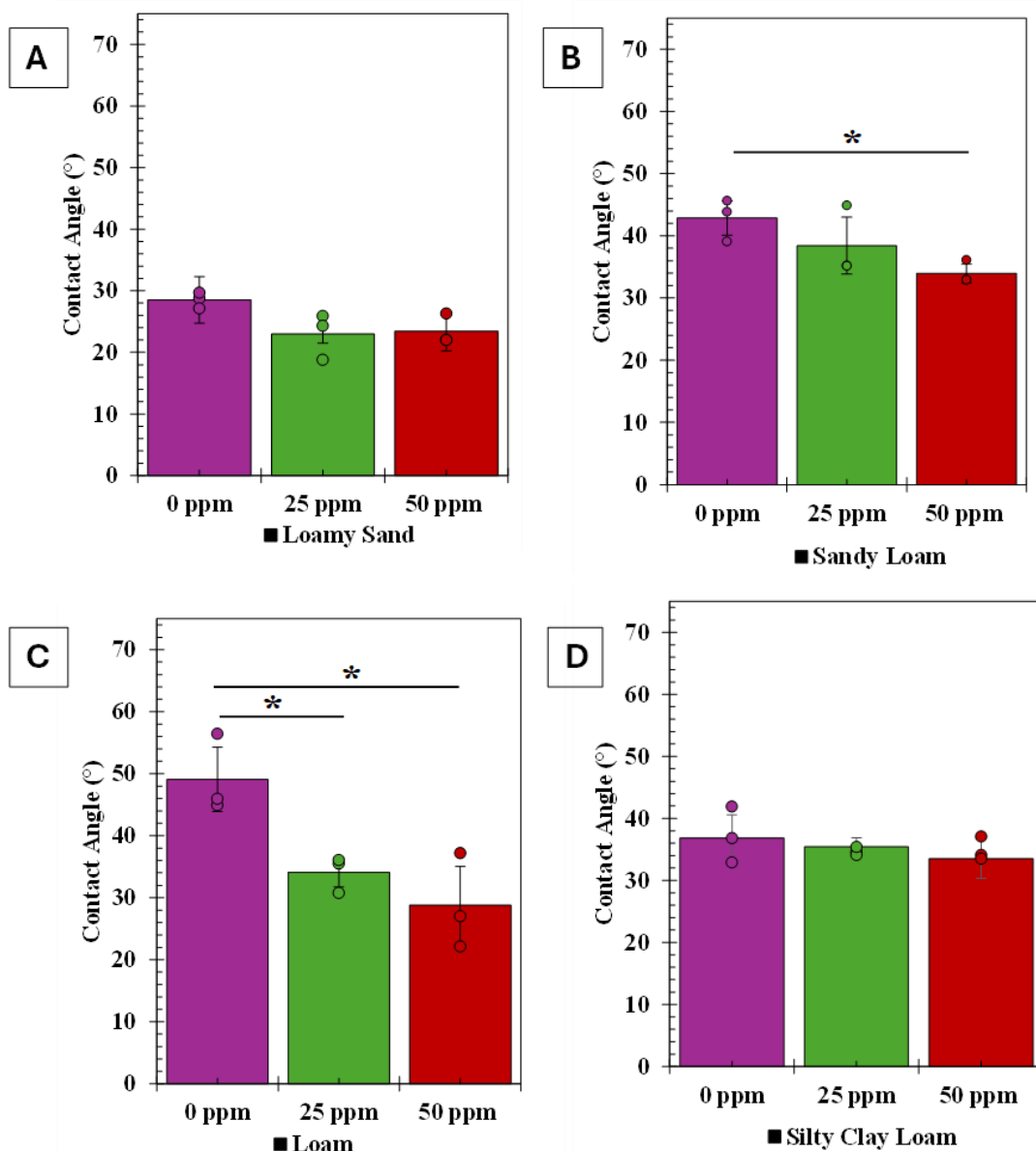


Figure G1. The contact angle (CA) measurements were taken for natural soil samples treated with surfactin. (A) Despite its higher organic matter in Loamy Sand, surfactin treatment did not significantly increase hydrophobicity. In contrast, (B) Sandy Loam showed a significant increase in hydrophobicity at 50 ppm compared to the control (0 ppm). (C) Loam exhibited a stronger response to surfactin, with both the 25 ppm and 50 ppm treatments showing significant differences from the control. Finally, (D) Silty Clay Loam showed no significant changes in hydrophobicity after treatment. Asterisks (*) indicate statistically significant differences ($p < 0.05$).

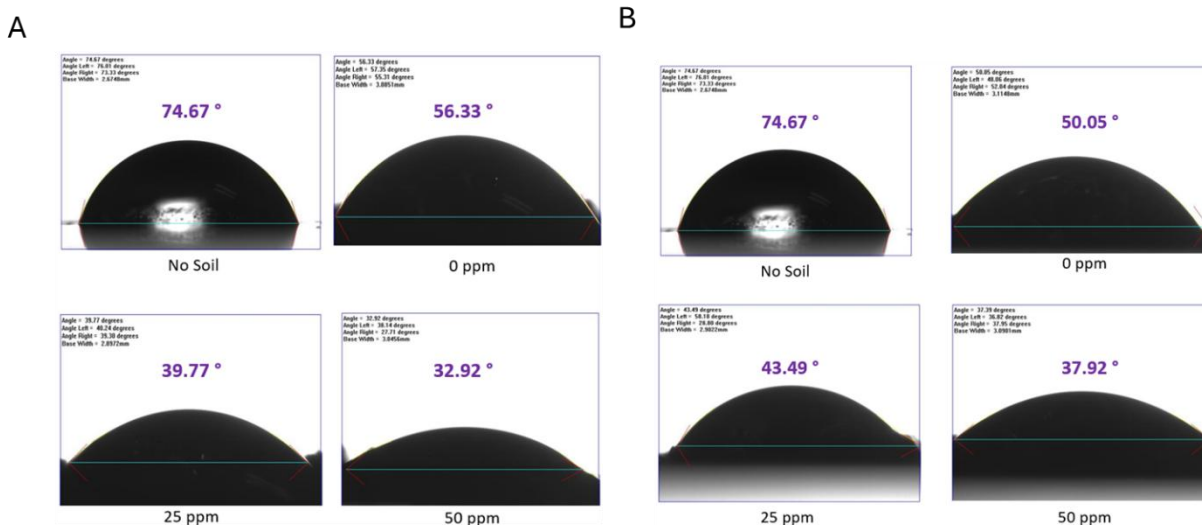


Figure G2. Some contact angle measurements for **(A)** Loam soil texture and **(B)** Sandy Loam soil texture. A decrease in contact angles can be viewed as the concentration of surfactin increases.

Table G1. Physicochemical Properties of the Different Natural Soil Textures

<i>Texture</i>	<i>Sand %</i>	<i>Silt %</i>	<i>Clay %</i>	<i>Total N %</i>	<i>TOC %</i>	<i>pH</i>	<i>OM MWB %</i>	<i>NO₃-N ppm*</i>	<i>NH₄-N ppm*</i>	<i>K ppm**</i>	<i>Ca ppm**</i>	<i>Mg ppm**</i>
Loamy Sand	82	12	6	0.12	1.02	7.60	2.40	2.59	6.30	315.00	1246.00	163.00
Sandy Loam	60	26	14	0.10	0.84	7.80	1.26	4.20	9.50	199.20	2304.50	51.10
Loam	32	50	18	0.11	1.02	8.00	1.58	5.90	14.80	287.00	3282.00	89.60
Silty Clay Loam	16	52	32	0.17	1.32	6.50	1.89	2.50	6.40	323.80	2580.90	353.40

* Extractable, ** Non-exchangeable quantities.

Analysis performed by Kansas State University Soil Testing Laboratory.