Soil Column Design for Studying Interkingdom Networks in Soil

Major Qualifying Project

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ABSTRACT

The goal of this project was to design and construct a column of appropriate dimensions to test chemical detection with interacting bacteria and fungi. The report also investigates mass transfer equations involving the diffusivity of acetone, benzene, methane, ethanol, toluene, and TNT. Concentrations of the compounds through a one meter section of soil were graphed. It was determined that methane had the highest total flux over a period of three days. Laboratory experiments with the designed column show the effects of iron and PVC caps on the efficiency and effectiveness of the column sterilization process. We found that iron caps were the better choice when using the autoclave.

1. INTRODUCTION

Our project is part of a larger endeavor to investigate bacterial migration and chemical diffusion along fungal highways for detecting and responding to chemicals underground. Fungal highways could deliver a microbial biosensor deep in the soil to detect and report chemicals faster than they diffuse, propagating a signal to the top of the soil. Here, we designed a column for testing such systems. We tested its ability to be sterilized, which is difficult with soil, and we estimated diffusion rates of several organic molecules. This column and the diffusion calculations could be used for future analytical testing of fungal or bacterial experiments and diffusivity determinations.

Our objectives were:

1. Design and manufacture a column of appropriate dimensions to replicate volatile chemical detection through a mycorrhizal network of fungi and bacteria.
2. Perform a lab demonstration with the designed column.
    a. Determine if the packed soil column withstands autoclave sterilization.
    b. Determine if the autoclave was successful in sterilizing the soil within the soil column.
3. Deduce mass transfer equations related to the diffusivity of seven organic compounds with varying volatility in soil.

1.1 The Problem

Underground chemicals are difficult to assess, yet they impact various processes, including agriculture, the built environment, and the military. Current detection approaches have limitations and do not allow for efficient and safe detection. For example, on-site methods such as electromagnetic induction and animal based detection are slow and not distributed. Methods such as spectrography work, but are not sensitive enough to detect low levels of chemicals underground. The careful balance between timeliness, accuracy, and safety could be relieved through a microbial-based detection system. Prototyping these systems requires columns that can be fully sterilized.

1.2 Fungi in Soil

Soil is host to numerous microorganisms, including fungi and bacteria. These microbes can form a range of physical relationships dependent on molecular communication for their function and development. The connection between fungi and bacteria makes this interaction an area of interest for many biological questions in environmental science, medicine and food production[1]. Natural filamentous fungal “highways” with bacteria dispersed along a network enable signal propagation from a depth of one meter from the surface. Filamentous fungi encompass a large group of eukaryotes that form simple tube-like hyphae as they grow. This hyphae forms a filamentous network by extending the ends of their branches throughout the soil and are capable of reaching depths that organisms such as bacteria wouldn’t be able to reach on

their own[2]. When the bacteria and fungi make a symbiotic connection, this forms a "mycorrhizal network" throughout the soil. The bacterial strain *Pseudomonas putida* (*P. putida)* can be a desirable candidate in a mycorrhizal network with fungi in soil. This gram-negative strain is naturally motile and capable of travelling mycelium in soil at a rate of 1 cm per day. This makes it an ideal chassis organism as it can additionally survive in soil for the desired timeframe for the program metrics and beyond. Depicted below in Figure 1 is the proposed schematic for the fungal and bacterial interaction.

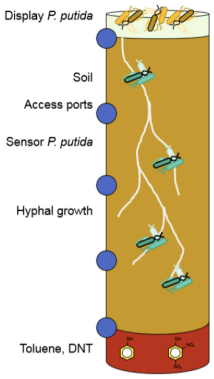


Figure 1. Schematic for the mycorrhizal network in our project application

The mycorrhizal network formed by the fungal hyphae acts as a bacterial highway to communicate biosignals throughout the soil. In this highway, bacteria disperse along the fungal mycelium as it travels downward into the soil[3]. The synaretic film formed around the hyphae mimics properties of a liquid to allow bacteria to move along it and to reach microhabitats in the soil that were previously unreachable. In most cases, the bacteria can't reach soil microhabitats when it is a water unsaturated environment[4]. Without saturation, soil acts as a porous environment with air voids that bacteria can't travel through, however, fungal hyphae can grow across these voids. Lukas Wick and his colleagues at the UFX Centre for Environmental Research demonstrated that the fungal mycelia *Pythium ultimum* can bridge simulated air-filled pores, using glass beads[4]. This enabled the bacteria *P. putida* to travel across this continuous network and continue traveling through the soil. The mycorrhizal network formed between fungi and bacteria allows the bacteria to travel into the soil and towards other chemicals, like chemoattractants[5].

There are four signals in the fungal network: propagation by cell-to-cell volatile organic compound (VOC) activation, diffusion of VOC through hyphae and soil, propagation on hyphae and soil, and a chemoattractant concentration gradient. The primary propagation mode is feed-forward VOC signal transmission between systems. This is aided by the diffusion of butanol through hyphae and soil. Figure 2 depicts the methodology of how the mycorrhizal network is formed and carries chemical signals down into the soil.

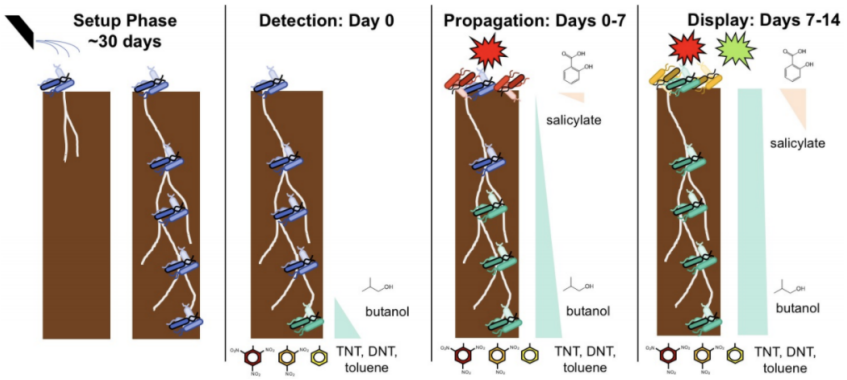


Figure 2. Schematic of propagation methodology

1.3 Sensing Volatile Organic Compounds

The development of a system to accurately, safely, and efficiently detect chemicals underground would be extremely beneficial. Our specific project focuses on the detection of chemicals one meter deep in soil. The microbial biosensor will not only detect the chemical, but also propagate a signal to the surface through fungal hyphae and display a fluorescent signal that can be observed from 10 meters away by a camera-based detector. When designing our soil column for testing, it is important to know the diffusion properties of the chemicals involved.

We chose to study a variety of organic chemical contaminants and one explosive - acetone, benzene, methane, ethanol, toluene, and TNT.

TNT, pictured in Figure 3, is one of the most common explosives. It is not susceptible to shock or friction, which makes it convenient to transport and lowers the risk of accidental detonation. Additionally, the melting point is far below the temperature at which it detonates, making it easy to pour or safely combine with other explosives. Various research teams have published data on solubility, among other properties, of TNT. Our group is specifically interested in TNT's miscibility in water and how it behaves in a soil environment.

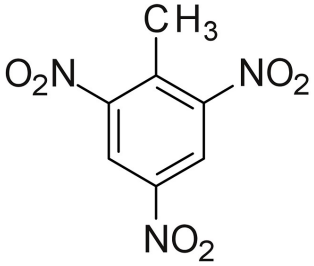


Figure 3. 2,4,6-trinitrotoluene, more commonly known as TNT [6]

TNT is known to be weakly soluble in water. Similar to most compounds, as the temperature of water increases, the solubility of TNT increases as well. One research team determined a solubility range of roughly 52 mg/L at 6 °C to 205 mg/L at 42 °C. The same group reported an increase in solubility with a higher pH as well. At high temperatures and pH values

however, a high performance liquid chromatography analysis showed three unknown peaks, suggesting the formation of new, unwarranted compounds. It is important to regulate the conditions of the column so that TNT remains in a stable state[7].

Since TNT is slightly soluble in water, it is able to migrate through subsurface soil. TNT, along with many of its amine products like 2-NT, 3-NT, 4-NT, 2,6-DNT, 2,5-DNT, 2,3-DNT, 2,4-DNT, 3,5-DNT, 3,4-DNT, 4-ADNT and 2-ADNT, were found in a groundwater sample taken from a ditch close to a TNT manufacturing plant. The detection of these compounds illustrates their ability to diffuse through subsurface soil and contaminate groundwater[8].

Previous research shows that an engineered *P. putida* sensor strain is a viable option for TNT detection. Because inducible genetic circuits can be engineered with synthetic biology, it might be possible for *P. putida* to be engineered to detect a wide variety of organic compounds.

1.4 Column Design

Our proposed column design is made of stainless steel with a length of 1.15 meters to allow for soil to be placed in the column at a depth of one meter, with an allowance of 15 centimeters split between the top and bottom of the column. Two half meter columns will be threaded together with an iron coupling to reach the total length of 1.15m. The diameter of the column will be 5.08 centimeters (2 inches). The column will have access ports every 10.16 centimeters (4 inches), providing eight access ports for the column. The access ports will have associated silicon stoppers to cover the port when not in use, therefore avoiding unnecessary chemical diffusion and outside contamination. The material of choice is stainless steel to enable the column to be autoclaved without issue and to provide increased durability for prolonged use. We found it needless for the column to be clear due to the dense packing of the soil making it improbable to see fungi grow to any significant observation. An example of the column design can be seen below in Figure 4.

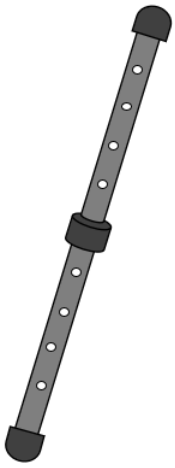


Figure 4. Potential Column Design

Our proposed column design allows us to simulate a soil environment and selectively control various variables contributing to chemical diffusion. As seen in Figure 5 below, soil has many components in it such as organic matter, air, water, and minerals.

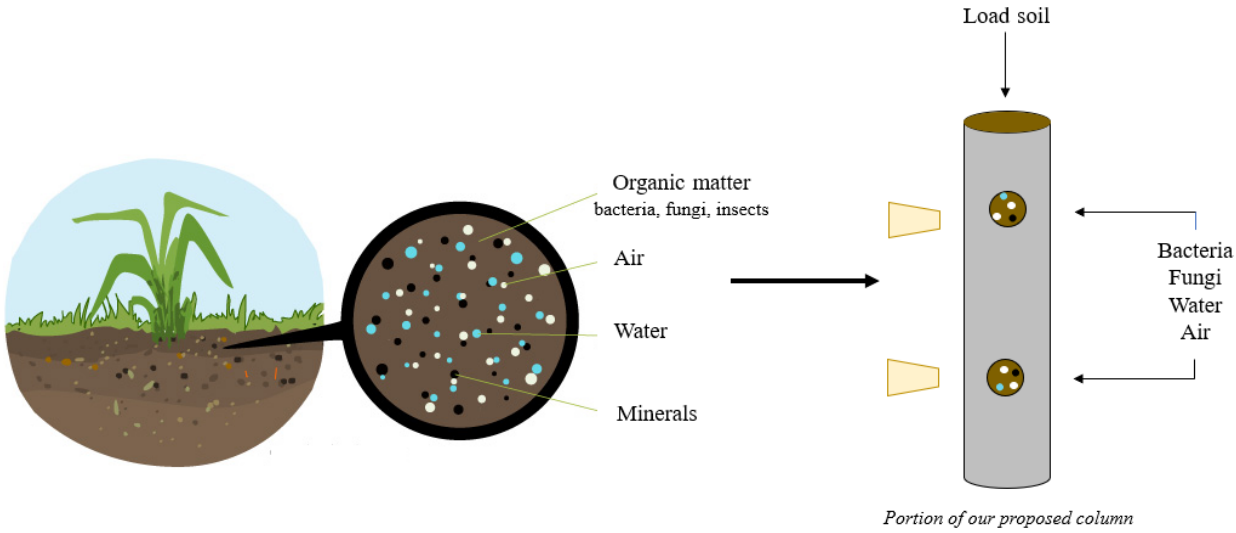


Figure 5. Comparison of soil environment and our simulated column system

With the use of equally interspaced port openings in our column, we can introduce the various soil components and control quantity and which height or "depth" they are administered. Starting with a sterilized column will provide a consistent baseline, allowing for an array of comparative analytical tests that can be used to study the mycorrhizal network or the VOCs diffusion times.

1.5 Mass Transfer

To estimate movement of chemicals through soil without any fungal highways we applied mass transfer concepts. Mass transfer is the movement of mass from one location to another by concentration gradients. In this project, mass transfer concepts will be used to estimate the concentration of chemicals 2 inches below the top of the soil column at steady state.

The mass transfer environment of interest is pictured below in Figure 6. Throughout the project the column design was adjusted to allow for a wider range of testing, as seen in Figure 4 above. For mass transfer calculations, the idealized version of this system, seen in Figure 5 below, was used to provide baseline results.

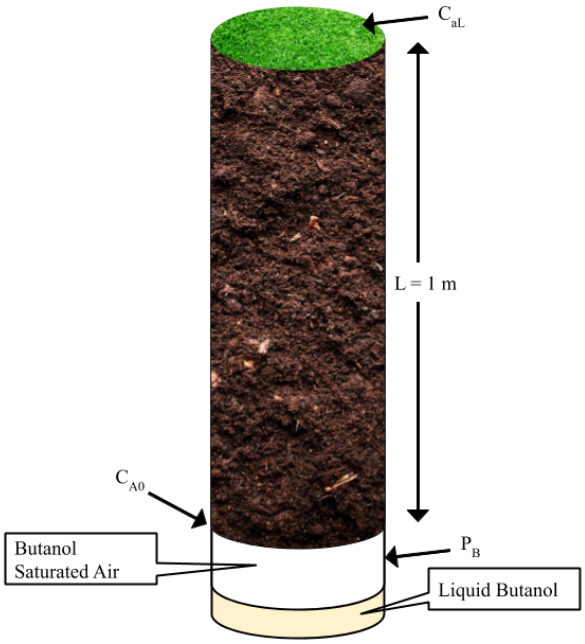


Figure 6. Diagram of mass transfer system

For example, the butanol is provided as a liquid, so the vapor pressure data needs to be considered. Assuming a room temperature of 25°C, the vapor pressure of butanol in the air is 0.905 kPa[9]. Using the ideal gas law, as seen below in Equation 1, this gives a molar concentration of 0.365 $\frac{mol}{m^3}$ in the saturated air.

$$PV = nRT \rightarrow \frac{n}{V} = \frac{P}{RT} \qquad \textit{Eqn. 1}$$

$$\frac{0.905\ kPa}{0.008314\frac{kPa*m^3}{K*mol}*298.15K} = 0.365\frac{mol}{m^3}$$

With the environment well defined, the governing mass balance equation, Equation 2, can begin to be simplified.

$$\frac{dC_a}{dt} + v \cdot \nabla C_a = cD_{ab}\nabla^2 x_a + R_a \qquad \textit{Eqn. 2}$$

This system can be assumed to be at steady-state, meaning $\frac{dC_a}{dt} = 0$ because there is excess butanol present.

The dimensionless Peclet number, as defined in Equation 3, indicates if mass transfer in a system is advection or diffusion driven.

$$\text{Pe} = \left(\frac{advection\ rate}{diffusion\ rate}\right) = \frac{V_y L}{D_{AB}} \qquad \textit{Eqn. 3}$$

There is no bulk motion within the domain so the Peclet number becomes much less than 1, allowing the stationary medium approximation to be made, meaning $v \cdot \nabla C_a = 0$. There is no reaction occurring in the soil so the reaction term $R_a = 0$. These two assumptions show that the mass transfer system is diffusion driven. The diffusion of interest is only in the upwards, y, direction, so the governing mass balance equation simplifies to Equation 4 below.

$$0 = D_{ab} \frac{\partial^2 C_a}{\partial y^2} \qquad \textit{Eqn. 4}$$

Next, the boundary conditions must be determined. The dimensionless Biot number determines if advection or diffusion dominates at the surface of the domain. If the Biot number is greater than 10, diffusion dominates, if it is less than 1, advection dominates. The Biot number is given in Equation 5 below.

$$B_{im} = \frac{k_m * Lc}{D_{ab}} \qquad \textit{Eqn. 5}$$

Since there is very little motion in the air at the surface, the Biot number is much greater than 10, meaning diffusion dominates at the surface. With this conclusion the first boundary condition can be determined as; $BC_1: C_{a_L} = 0$. Since the butanol is assumed to be in excess, the concentration of butanol at the bottom of the column is equivalent to the concentration of butanol in the air. This gives the second boundary condition of $BC_2: C_{a_0} = 0.365 \frac{mol}{m^3}$.

Next, diffusivity data must be determined. It is difficult to find diffusivity data for chemicals in soil, especially for soil that matches exactly to that to be used in the column. To overcome this difficulty, the Millington and Quirk Model will be used. This model solves for a chemical's diffusivity in soil based on the chemicals diffusivity in water, the soil's porosity, and the soil's water content, as seen in Equation 6.

$$D_e = D_o \frac{\theta^{10/3}}{\theta_s^2} \qquad \textit{Eqn. 6}$$

Using $9.3 \times 10^{-6}\ \frac{cm^2}{s}$ as the diffusivity of butanol in water, 0.45 soil porosity and 45% water content, the diffusivity of butanol in wet soil is $3.21 \times 10^{-6} \frac{cm^2}{s}$[10]. This value is of the same magnitude of other researched diffusivities in soil, such as TNT's diffusivity of $1.18 \times 10^{-6}\ \frac{cm^2}{s}$, or methyl ethyl ketone's diffusivity of 2.31×10^{-6} [11].

With this data, the governing mass balance equation is simplified and solved for constants as seen in Appendix A.

$$C_a = \frac{-1.172*10^{-10}\frac{mol}{m^2 s}*z}{3.21*10^{-10}\frac{m^2}{s}} + 0.365\frac{mol}{m^3} \qquad \textit{Eqn. 7}$$

With the specific equation solved, the concentration profile could be determined, as seen in Figure 7. Because the system is at steady state, the concentration profile does not change over time.

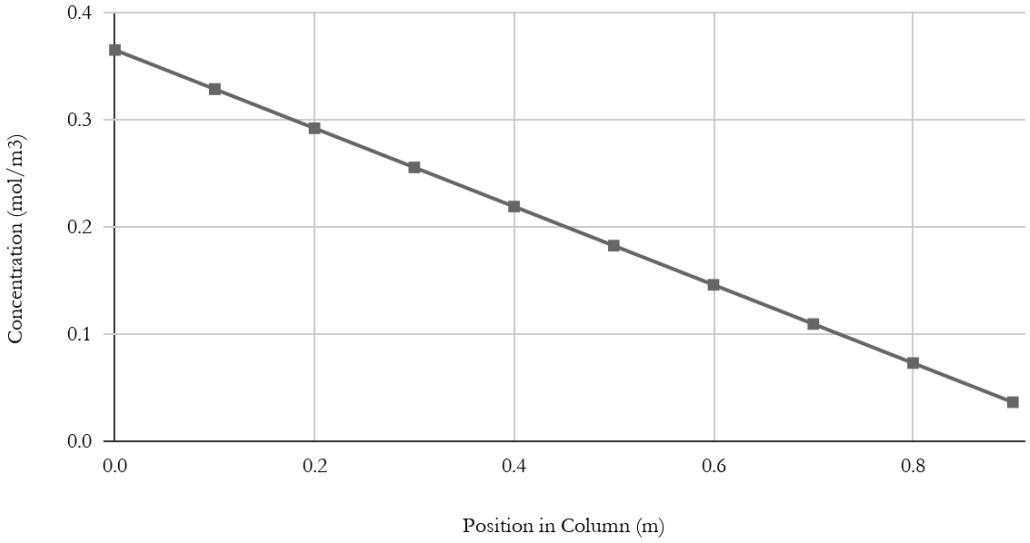


Figure 7: Concentration profile of butanol through soil

Using the concentration profile, the concentration of butanol in the soil 2 inches below the surface was determined to be 0.018 mol/m^3. The concentration at other points in the column can be easily determined using Equation 7.

This data is a rough estimate of the actual concentration and concentration profile, as we used an idealized system. In the environment the system would not be ideal, as soil content and wind conditions would affect the diffusivity. Additionally, the adjusted column provides import opportunities for butanol directly into the soil at varying heights, to provide more flexibility for experimental results.

2. METHODOLOGY

The primary challenge with the current column systems being used in the lab for testing is that the researcher has to sterilize each component by hand with ethanol in a fume hood to ensure sterility. The columns are made out of PVC and although they should be able to withstand the temperature and pressure of an autoclave, the columns, being 1m long, are too long to fit into the autoclave. This method is tedious and does not ensure sterility to the extent autoclaving would.Additionally, soil could not be loaded into the column until after the PVC was sterilized with ethanol and the soil was autoclaved separately. For ease and sterility, we sought to build our column out of autoclavable materials so soil could be loaded into the column before autoclaving and both column and soil could be autoclaved together in one run. We also were cognizant of dimension limitations of the autoclave when choosing column sizing. This would increase efficiency and decrease chances for contamination. Therefore, our column is made of autoclavable materials like black iron, black steel, silicone and Sch-40 PVC.

2.1 Column Construction

The column consists of two, 18-inch black steel pipes threaded together with a 2-inch black iron coupling, as seen in Figure 8. Each column has 8 drilled ports that are ½-inch in diameter and spaced out every 4 inches. 11mm-15mm silicone stopper plugs are used to seal the ports and can be removed to collect samples. In the first round of experiments, 2-inch PVC schedule 40 socket caps were placed on each end of the pipe. For the second round, the PVC caps were replaced with 2-inch black iron pipe caps.

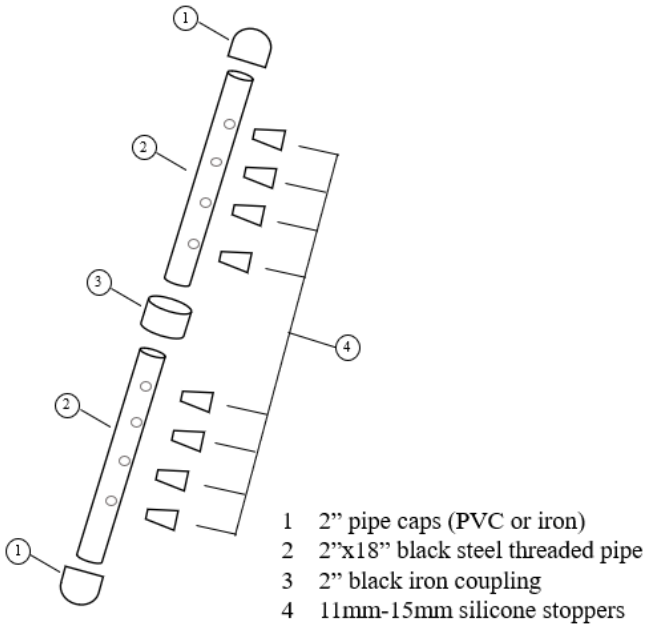


Figure 8. Schematic of column construction components

2.2 Soil Preparation and Sterilization

Materials

1. Two 2”x18” black steel threaded pipes
2. Two 2” pipe caps (PVC or iron)
3. Eight 11mm-15mm silicone stoppers
4. Soil
5. Conical tube

Method

1. Load column with soil
    i. Prep a sterile area with 70% ethanol and lay down paper towels to catch stray soil.
    ii. Obtain soil and place in staging area
    iii. Cap bottom of pipe with cap

iv. Place silicone stopper plugs in port holes
v. Using a conical tube, load the column with soil
vi. Once soil is fully loaded, cap top of pipe

2. Autoclave both capped pipes following WPI autoclaving procedures. The autoclave ran at a temperature of 121℃ and pressure of 15lbs with a 30 minute dry cycle.
3. Safely remove all components from the autoclave and carefully transfer to a biosafety hood.
4. Sample and culture on oat flake agar (OFA) plates (see Section 2.3)

2.3 Plating Soil Samples

Figure 9 below depicts the following method for plating the soil samples,

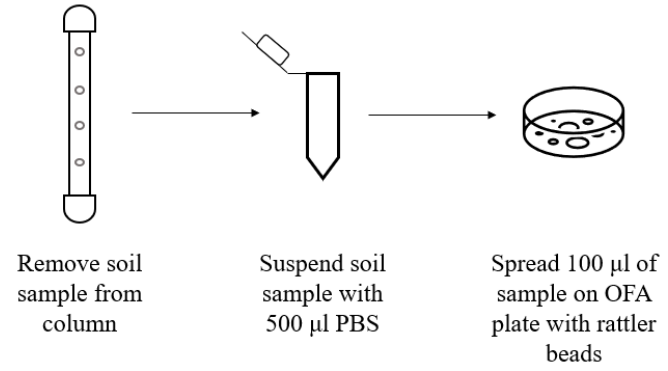


Figure 9. Method for plating soils samples for sterilization observations

Materials

1. Filter (0.2 µm) sterilized phosphate buffer saline (PBS)
2. Oat flakes agar (OFA) plate
3. 1.5 ml microcentrifuge tubes
4. Rattler plating beads (Zymo Research, USA)

Method

1. Collect soil samples from a sampling port of the soil column using 1 ml pipette tip.
2. Resuspend soil sample in 500 µl PBS and mix it rigorously.
3. Collect 100 µl liquid from resuspended sample and pour on OFA plate.
4. Spread the poured liquid on a plate using Rattler beads.
5. Seal the plate using parafilm tape and incubate at room temperature for 2-3 days.

Note: The procedure should be performed in a Biosafety hood.

2.4 Diffusivity Simulation Protocols

To best understand the diffusion of different chemicals through the soil column, it was decided that modeling would be implemented. The environment of interest is the same as the theoretical idealized column presented in Figure 6 of the background section. To model the different chemicals the governing mass balance equation, Equation 2, was used. Using the same methods as described in the background, the governing mass balance equation can be simplified to Equation 8 below.

$$C_a = \frac{C_1 * z}{D_e} + C_2 \qquad \textit{Eqn. 8}$$

Where C_1 and C_2 are constants found using the boundary conditions, and D_e is the diffusion coefficient of the chemical through the soil. To solve this equation for each chemical, the diffusion coefficient through soil and vapor pressure are required.

In-depth research was required to find the diffusion coefficients of the six chemicals in soil. We utilized the ScienceDirect resource to find peer-reviewed articles for the chemical's diffusion in soil. When possible, multiple sources were consulted to ensure the data was accurate. The data were also compared to previous knowledge, such as the butanol diffusion coefficient, to understand if the values were reasonable. Often the diffusion coefficients were for very specific environments, so we sought out coefficients found for soil around 0.45 porosity, 0.45 water content, and at room temperature.

However, even with thorough research, some chemicals, such as acetone and ethanol, only had available data for extreme conditions. For example, we were only able to find acetone data for zero degrees Celsius, or for transport in very porous media. Ethanol is strongly affinitive to staying in the water phase, so aqueous-phase diffusion is realistic. For these chemicals, we used the Millington and Quirk Model as outlined in Equation 6 of the background.

Determining vapor pressure for the chemicals was a much simpler process, with most data coming from common reliable chemical description websites, like PubChem.

Once the vapor pressures and diffusion coefficients were determined, the general equation was solved for constants using the boundary conditions. The boundary conditions, similar to the butanol example, are listed below.

$$BC_1 : C_{a_L} = 0$$

$$BC_2 : C_{a_0} = \frac{P}{RT}\frac{mol}{m^3}$$

These boundary conditions gave rise to six mass transfer equations as listed in Table 1 below. These equations were then plotted from 0-1 meter, representing the soil column, which can be seen in Figures 19-24 in Appendix C.

Table 1. GMB Equations for Chemicals of Interest

Chemical	GMB Equation
TNT	$C_a = \frac{-1.96*10^{-15}\frac{mol}{m^2 s}*z}{1.8*10^{-10}\frac{m^2}{s}} + 1.09 * 10^{-5}\ \frac{mol}{m^3}$
Ethanol	$C_a = \frac{-7.35*10^{-10}\frac{mol}{m^2 s}*z}{4.2*10^{-10}\frac{m^2}{s}} + 1.75\frac{mol}{m^3}$
Toluene	$C_a = \frac{-1.52*10^{-11}\frac{mol}{m^2 s}*z}{1.3*10^{-11}\frac{m^2}{s}} + 1.17\frac{mol}{m^3}$
Methane	$C_a = \frac{-5.98*10^{-5}\frac{mol}{m^2 s}*z}{1.79*10^{-5}\frac{m^2}{s}} + 3.34\frac{mol}{m^3}$
Benzene	$C_a = \frac{-4.15*10^{-11}\frac{mol}{m^2 s}*z}{8.04*10^{-12}\frac{m^2}{s}} + 5.16\frac{mol}{m^3}$
Acetone	$C_a = \frac{-3.88*10^{-9}\frac{mol}{m^2 s}*z}{3.93*10^{-10}\frac{m^2}{s}} + 24.5\frac{mol}{m^3}$

Next, the concentration of a given chemical two inches below the surface of the column was determined for the six chemicals. This was determined by simply inputting the z value of 0.0508 meters into each equation. These values can be seen in Table 2 in the results section.

Additionally, the molar flux of chemicals coming through the top of the soil was to be determined. This data will allow us to further understand the diffusion of the chemicals through soil over a period of time. The equations for molar flux and total flux are given in Equations 9 and 10 below and an example is given in Appendix B.

$$N_a = -D_{AB}\frac{dCa}{dz} \qquad \textit{Eqn. 9}$$

$$W_a = SA * N_a \qquad \textit{Eqn. 10}$$

The data was evaluated to find a total flux over a three day period of time based on the two inch diameter column's top surface area.

3. RESULTS

3.1 Structural Integrity

3.1.1 Column with PVC Caps

Our first round of experimentation was conducted using PVC caps with our column in the autoclave, as seen in Figure 10 to the right. After being autoclaved, the steel pipe and silicone stoppers withheld their shape and structural integrity. However, the PVC caps appeared to deform while in the autoclave, making it difficult to remove them without compromising the column. After safely using a hand saw to cut a small "X" in the PVC, the caps were able to come off without significant effort. This led us to believe that although the autoclave was not hot enough to fully melt the PVC, the material was susceptible to pressure changes, leading to the caps being suctioned onto the pipe and creating an airtight seal. Because of those observations, our team conducted a second round of sterilization using 2" black iron caps instead of PVC caps.

Figure 10. Columns with PVC caps after autoclaving

3.1.2 Column with Iron Caps

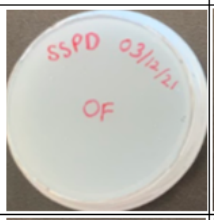
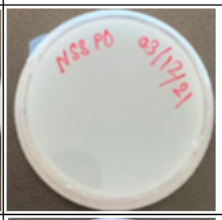
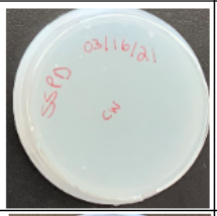
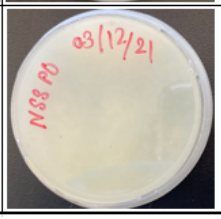
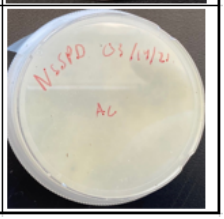
Our second round of experimentation was conducted using iron caps with our column in the autoclave, as seen in Figure 11. Unlike the PVC caps, the iron caps did not deform and were easily removed from the column. Due to these observations, our group recommends using iron caps during the sterilization process in future work.

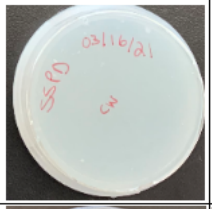
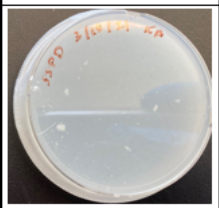
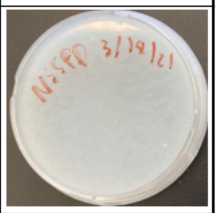
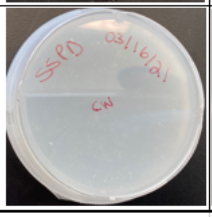
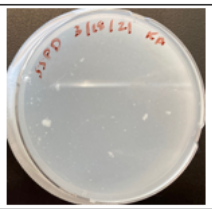
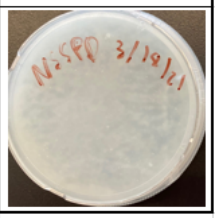
Figure 11. Columns with iron caps after autoclaving

3.2 Soil Sterilization

3.2.1 Column with PVC Caps

The first round of experimentation was done with the PVC caps on the pipe during autoclaving. In addition to testing the structural capabilities of our column design to withstand the autoclave, we also needed to consider how well the column and the loaded soil would be sterilized. It is important that the soil be completely sterile after autoclaving because as exploratory experiments are being conducted regarding fungal-bacterial pathway interactions, unknown contaminants in the soil would make data inconsistent and difficult to replicate or analyze. Figure 12 below, shows the OFA plate images of our soil samples collected on alternate days for a week after autoclaving. For this round of experimentation, the columns were autoclaved on 3/12/2021 and final observations were made on 3/20/2021.

Date Plated	3/12/2021		3/14/2021	
Days of incubation	10		8	
	Sterilized	Non-Sterilized	Sterilized	Non-Sterilized
Innoculated				
Observed growth on 3/22/21				

Date Plated	3/16/2021		3/18/2021	
Days of incubation	6		4	
	Sterilized	Non-Sterilized	Sterilized	Non-Sterilized
Innoculated				
Observed growth on 3/22/21				

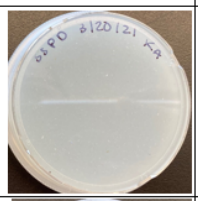
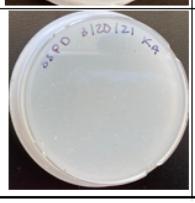
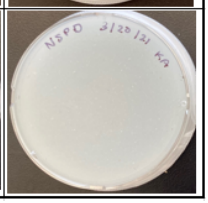
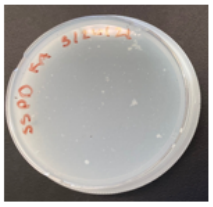
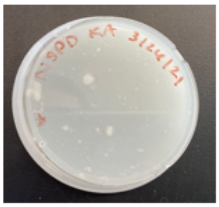
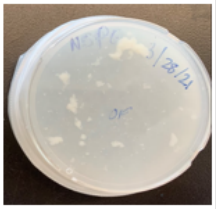
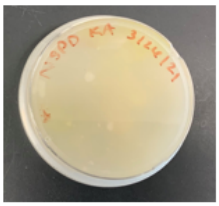
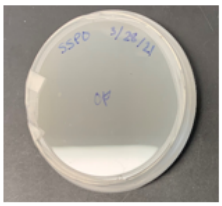
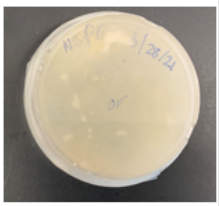
Date Plated	3/20/2021	
Days of incubation	2	
	Sterilized	Non-Sterilized
Innoculated		
Observed growth on 3/22/21		

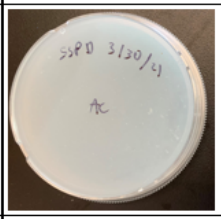
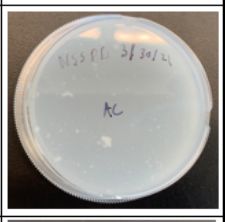
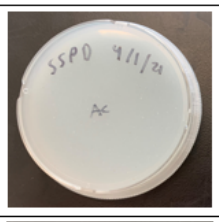
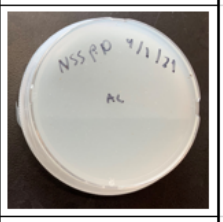
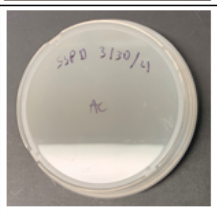
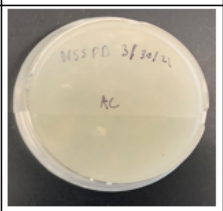
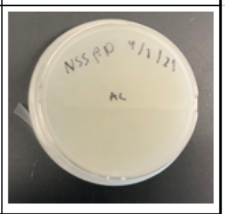
Figure 12. Plate results from PVC capped sterilization tests, observed on date of inoculation and on 3/22/2021
Note: 3/14 inoculation images are from 3/16 plates, we don't have an image for 3/14 but wanted to show the change in growth from an initial inoculation

As seen above, there is an overall trend that the non-sterilized samples had substantially greater growth observed. When looked at closer by the team, the plates appeared to have fungal and bacterial growth on the non-sterilized samples. However, in the sterilize samples from 3/12/2021 and 3/14/2021, there were small bacterial colonies on the plate. In some images in Figure 12, it is difficult to denote organismal growth versus inconsistencies in the oat flake agar. Some OAF plates had oat particles in them, such as 3/16/2021 and 3/18/2021, so when looking at a picture, they can be mistaken for bacterial growth. However when observed in the lab, bacterial growth can be denoted by the shiny risen colonies on the plate.

3.2.2 Column with Iron Caps

The second round of experimentation was done with the iron caps on the pipe during autoclaving. Structurally, the iron caps were superior to the PVC caps due to the fact the iron caps experienced no deformation and could be removed from the column easily post autoclave. In terms of sterilization, the iron caps produced better results as well. We see slightly less bacterial growth in the sterilized iron capped trials compared to the sterilized PVC samples. Figure 13 below, shows the OFA plate images of our soil samples collected on alternate days for a week after autoclaving. For this round of experimentation, the columns were autoclaved on 3/24/2021 and final observations were made on 4/5/2021.

Date Plated	3/26/2021		3/28/2021	
Days of incubation	10		8	
	Sterilized	Non-Sterilized	Sterilized	Non-Sterilized
Innoculated				
Observed growth on 4/5/2021				

Date Plated	3/30/2021		4/1/2021	
Days of incubation	6		4	
	Sterilized	Non-Sterilized	Sterilized	Non-Sterilized
Innoculated				
Observed growth on 4/5/2021				

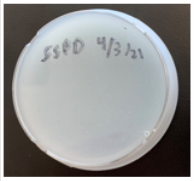
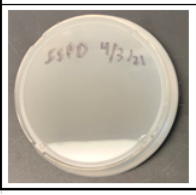
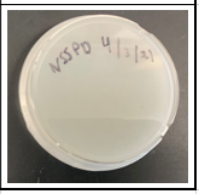
Date Plated	4/3/2021	
Days of incubation	2	
	Sterilized	Non-Sterilized
Innoculated		
Observed growth on 4/5/2021		

Figure 13. Plate results from iron capped sterilization tests, observed on date of inoculation and on 4/5/2021

Much like the PVC capped trials, we notice that the non-sterilized samples had significantly more bacteria growth than the sterilized samples. This being said, there was a small amount of bacterial growth observed in the sterilized dishes. This was most noticeable on days 3/26/2021 and 3/30/2021. The later dates show less growth in both the sterilized and unsterilized samples.

3.3 Diffusivity Simulations

Diffusivity calculations were run for a variety of different chemicals to estimate and compare their movement through the soil. These values were also compared to the calculations for butanol transport to see if a different chemical may be a better option as a chemoattractant in the mycorrhizal network.

Table 2. Concentration of Chemicals in the Soil (2 inches below ground level)

Chemical	Concentration (mol/m3)
TNT	$5.53*10^{-7}$
Ethanol	0.0889
Toluene	0.0592
Methane	0.170
Benzene	0.262
Acetone	0.502
Butanol	0.018

Table 2 shows the concentration of the various chemicals in the soil column 2 inches below the surface. Full concentration profiles for each chemical can be found in Appendix C. The table shows that all of the tested chemicals, except for TNT, had high concentrations in the soil compared to butanol, with acetone being the most concentrated.

Next, the flux for the chemicals through the top of the soil column was calculated over a 3 day period. The following figures show the flux comparison of the various chemicals, with outliers being removed in some graphs to better show the data.

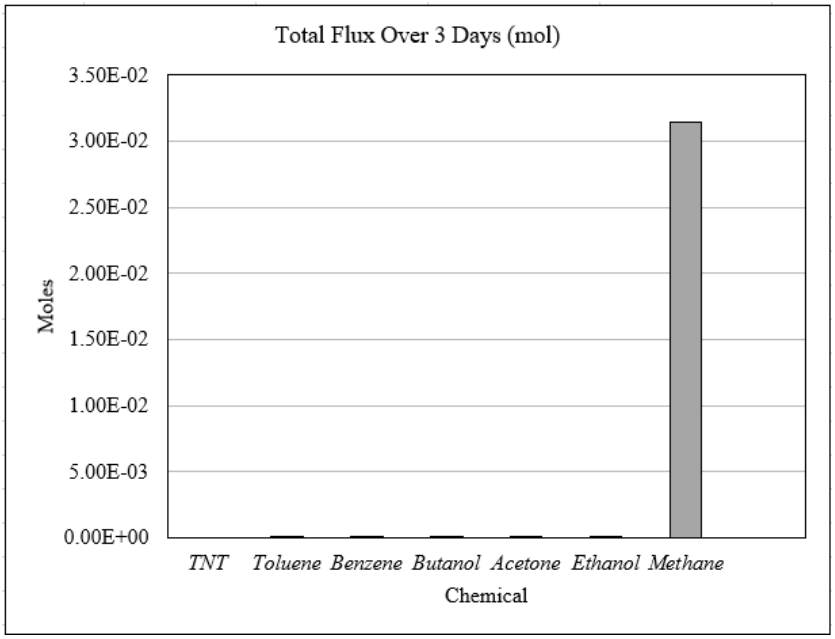


Figure 14. Comparison of total fluxes through the top of the soil column over 3 days (including methane)

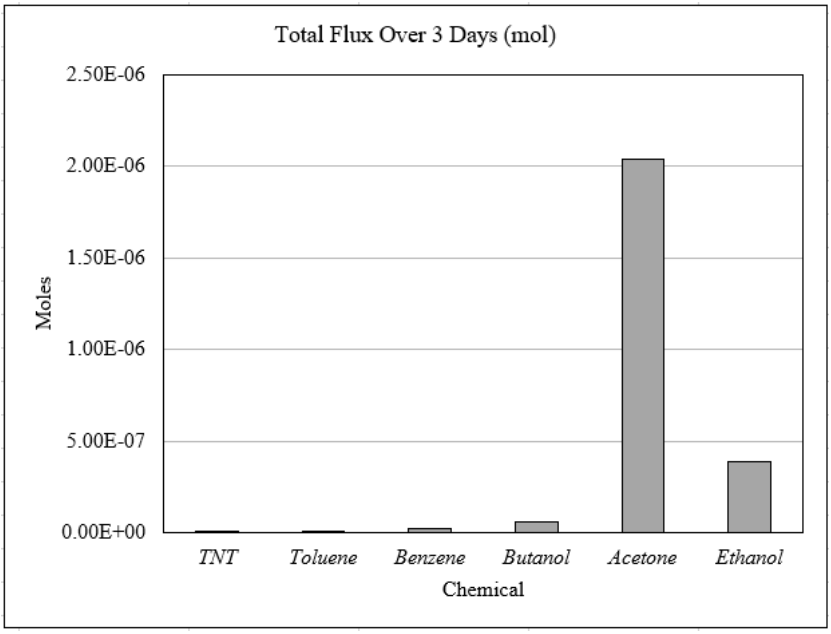


Figure 15. Comparison of total fluxes through the top of the soil column over 3 days (excluding methane)

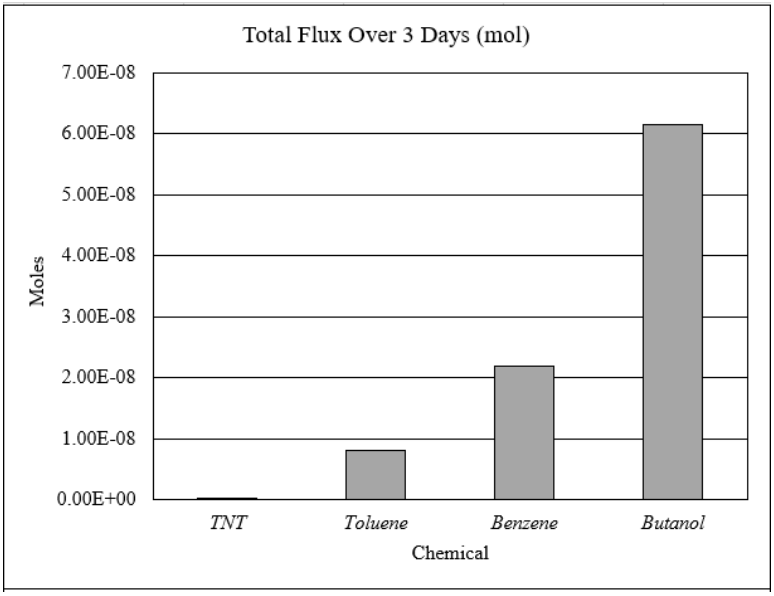


Figure 16. Comparison of total fluxes through the top of the soil column over 3 days (excluding methane, acetone, ethanol)

Figures 14-16 show that methane has the highest flux rate by many magnitudes. Acetone and ethanol have flux rates that are significantly higher than the others, including butanol. Butanol has a higher flux than benzene, toluene and TNT, with TNT being miniscule in comparison.

4. DISCUSSION

4.1 Structural Integrity

4.1.1 Column with PVC Caps

When the columns were placed in the autoclave using PVC caps to contain the soil, the PVC deformed slightly, and created a tight seal on the column that could not be broken until a hand saw was used to relieve some of the pressure. From previous experiments conducted by the graduate student leading the column design aspect of the grant, we were told that PVC can withstand the autoclave. Although the PVC did not melt in the autoclave, the heat was high enough to cause the material to become slightly malleable and become susceptible to pressurized changes. This phenomena can be explained using the Ideal Gas Law seen below,

$$PV = nRT \qquad Eqn.\ 1$$

In this equation, temperature is proportional to pressure. Therefore as temperature increases, pressure does as well. As the temperature in the autoclave increased, the pressure of the surrounding air increased and therefore pushed the non-rigid PVC caps inward slightly, suctioning them onto the pipes. This theory was supported when a handsaw was used to saw a small opening into the top of the PVC cap. Without any further cutting, the cap was able to easily come off the pipe. The inside of the pipe had some threading indentations, as seen in Figure 17 below.

Figure 17. PVC cap after being removed from the steel pipe

The striations on the inside of the caps show that the PVC caps deformed slightly. Repeated use of these caps would lead to additional imperfections, causing possible inconsistencies in sterility.

4.1.2 Column with Iron Caps

Sealing the column with iron caps is the preferred way to conduct sterilization in an autoclave. The caps can be twisted off after sterilization which allows for easier threading of the half meter pipes and lower risk of soil contamination. Additionally, using the iron caps allows for greater reproducibility between experiments because of their resistance to structural changes in the autoclave. Although purchasing iron caps are more expensive than using PVC, future researchers might spend more money on investing in multiple PVC caps as they deform and no longer become suitable. Whereas the iron caps will last longer and are more durable.

4.2 Soil Sterilization

4.2.1 Column with PVC Caps

As seen in Figure 12 in the previous section, we can confirm that sterilizing the soil column in the autoclaves diminished organismal growth when compared to a non-sterilized column. All of the non-sterilized samples had filamentous growth with filiform margins, indicating the presence of fungal strains. When looked at “agar side up” as the pictures are taken, the fungal growth yields a yellow, cloudy hue on the petri dishes. There were also off-white bacterial colonies observed on these plates. In the images taken and presented in the figure, this growth pattern is not as clear. However when observed “agar side down” in the lab, this growth is apparent. These observations support our claim in the importance of sterilizing the columns. When running analytical tests, the organisms present in the soil could interact and interfere with the strains being analyzed or tested for a specific experiment. Proof-of-concept experimentation must be done first with the sensor and chassis strains to confirm pathway mechanisms. Once there is enough experimentation to support the ability of the fungal/bacterial pathway proposed in the original grant, then the column can be used non-sterilized to observe how native soil components change the pathway interactions.

Overall, the sterilized samples had little to no growth. Plates from 3/12/2021 and 3/14/2021 have small, off-white, circular, raised colonies with entire margins on them. This could be a result of human error in becoming more familiar with the plating technique, or this could mean that the autoclave was able to kill the fungal strains present in the soil, but not the bacterial strains. It takes about one week for there to be noticeable growth on oat flake agar plates. Because the bacterial growth was observed on the earlier days, this could mean that the other samples plated at a later date could have the potential for bacterial growth. When looked at closely, the plates did not appear to have the beginnings of bacterial colony formation. Proper autoclave treatment should be able to kill all resistance bacterial spores in addition to fungi, bacteria, and viruses[12]. However as seen in the following section, this phenomena is also present in the iron cap experiments, although not as severe.

4.2.2 Column with Iron Caps

As stated previously, we noticed an increased amount of bacterial growth in the non-sterilized samples compared to the samples taken from the autoclaved column. Much like the column with PVC caps, all of the non-sterilized samples had filamentous growth with filiform margins. Comparing Figures 12 and 13 we see that for the sterilized samples, the PVC cap trials seem to have slightly more bacterial growth than the iron cap samples. This supports our hypothesis that using iron caps would both increase efficiency and decrease risk of contamination. This being said, there was still bacterial growth observed in the sterilized iron capped samples, specifically on days 3/26/2021 and 3/30/2021. These plates exhibited off white colonies and a yellow coloring. As mentioned previously, this is most likely attributed to human error.

Although there was bacterial growth in the early platings of both the PVC-capped columns and the iron-capped columns, plates from the latter had more bacterial growth. A possible reasoning for the severity differences in bacterial growth could be attributed to the air-tight seal created in the PVC-capped column. Although the autoclave heats up to extremely high temperatures, the inability of the hot air to move freely in the PVC-capped column could contribute to less effective sterilization, thus more bacterial growth. The course of the bacterial growth is still unknown. Additional investigation into the strain of bacteria present is needed to deduce why it survives the autoclave. For example, there could be an extremophile present in the soil that can withstand the high temperature and pressure of the autoclave.

4.3 Diffusivity Simulations

Factors Affecting Concentration Profile Results

Soil Porosity and Pore Space

Pore space is the fraction of soil volume unoccupied or isolated by solid mass[13]. Pore space is essential for air, nutrients, and water movement through soil [13,14]. It is similarly necessary for the diffusion and reaction of chemicals[13]. Pore space is treated as single and contiguous in a body of soil[13]. For this reason, isolated areas of nonsolid material in soil are precluded from consideration in pore space[13]. The pathways through soil, where the movement of fluids occur, are highly connected but constricted due to the presence of solid material[13]. Figure 18 is a visual for this concept of pore space as pathways through soil.

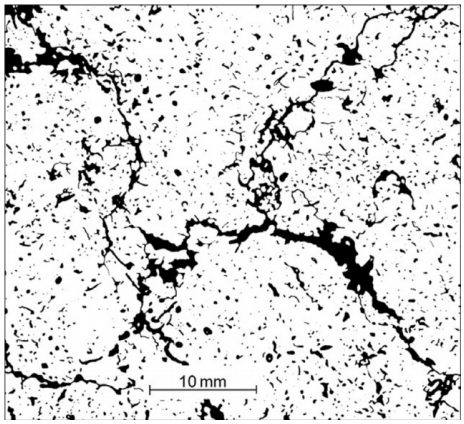


Figure 18. Typical soil cross section with pore space identified in black [13]

Porosity refers to the ratio between the nonsolid volume, or pore space, and total volume of soil[14]. Porosity values have a range between 0 and 1 as they are a fraction of total soil volume. Porosity is given the symbol ø. Soil values for ø are most frequently between the range 0.3 and 0.7[13]. The smaller ø, the less pore space is present, and therefore the less pore space accessible to, and accommodating of, fluid [13]. Soil porosity is affected by factors such as packing density, breadth of particle size distribution, particle shape, and cementing[13]. Breadth of particle size distribution refers to the shape of the individual spheres within the soil material. An example of uniform, or monodisperse, idealized soil spheres is sand with individual sand grains of the same

size[13]. Polydisperse soil refers to soil with spheres of varying sizes [13]. Irregular particle shapes, diverting from the idealized spheres, have bigger gaps in between individual particles. These larger gaps create greater soil porosity[13]. Cementing refers to the cementing of particles together by material such as clay, forming many aggregates that have large gaps in the soil, thus increasing ø [13]. Peat soil has a ø between 0.8 and 0.9 due to cementing from a large presence of organic matter[13].

Soil porosity is significant to the volumetric water content and its limit in a body of soil as ø represents the maximum fraction of space available to water in soil. ø also is valuable when determining the gas content in soil.

The accurate measurement of pore space and pore size distribution has been debated as individual pores are not discrete objects in soils and require artificial and therefore subjective distinction[13]. Regardless of this subjectiveness, pore space can effectively describe behavior in the soil-water-air system. Pores should be thought of as fluid conduits and are therefore important in the discussion of chemical diffusion through soil that takes place in the pore space[13].

The chosen soil porosity was 0.45. This value for ø lies near the middle of the available soil porosity range, 0.3-0.7, and fits within the sandy loam of the used experimentation soil. A larger soil porosity used in our experiment would correlate to a higher pore space and therefore more area for chemicals to diffuse through gas or liquid existent in the soil body. Concentration throughout the soil would increase with a higher ø and decrease with a lower ø.

Water and Gas Content

The water content of soil is related to its pore space and porosity as discussed. Porosity can be used to help determine the degree of water saturation and the gas content of soil[13]. Chemicals dissolved in water are diffused through the soil along with the water contents. The water content of soil, along with porosity, impact this diffusion of chemicals. Along the same lines, the amount of diffusion of chemical compounds through gas, namely air, relates to the gas content of a soil body. An example of this is methane. CH_4 is commonly measured in soil gas, and so included in this project, because excess amounts can produce unwelcome and potentially dangerous consequences such as a flammable mixture with air that creates an explosion[15]. CH_4 diffusion through soil is impacted by the soil gas along with porosity[15].

Vapor Pressure

Vapor pressure is the pressure of vapor from evaporation of a liquid in a closed container[16]. For the purposes of this experiment, the soil body is considered as a closed container. Vapor pressure increases with an increase in temperature and decreases with a decrease in temperature[16]. Vapor pressure is important to driving soil diffusion. As environmental factors like temperature are kept constant, different resulting concentrations of chemical compounds depends on vapor pressure.

Concentrations and Fluxes

Table 3 lists concentrations of the six chemicals (TNT, ethanol, toluene, methane, benzene, and acetone) in descending order according to the concentration at 2 inches below the soil surface, 37.37 inches above the chemical source.

Table 3. Descending Order of Concentration

Chemical	**Concentration ($\frac{mol}{m^3}$)**
Acetone	0.502
Benzene	0.262
Methane	0.170
Ethanol	0.0889
Toluene	0.0592
TNT	5.53×10^{-7}

Acetone and benzene, with concentrations of 0.502 $\frac{mol}{m^3}$ and 0.262 $\frac{mol}{m^3}$ respectively, are present in the largest concentrations within the unlimited time frame. Total flux over a three day period was determined for each chemical, based on the top surface area of the column. These fluxes are shown in descending order in Table 4. While acetone and benzene have higher concentrations over an unlimited period of time when they end up reaching steady state, methane has a higher flux.

Table 4. Descending Fluxes

Chemical	**Total Flux Over 3 Days (mol)**
Methane	3.14×10^{-2}
Acetone	2.04×10^{-6}
Ethanol	3.86×10^{-7}
Benzene	2.18×10^{-8}
Toluene	7.96×10^{-9}
TNT	1.03×10^{-12}

5. CONCLUSION and RECOMMENDATIONS

Conclusions

The first objective for this project was to design and manufacture a column of appropriate dimensions to study interaction between chemicals and mycorrhizal networks of fungi and bacteria. Steel soil columns were designed and constructed. The second objective for this project was to perform a lab demonstration of the sterilization concept within the designed column. To this end, it was determined if the packed soil column could withstand sterilization within an autoclave. It was also determined if the autoclaved was successful in sterilizing the soil within the soil column itself. The goal of the demonstration was to see if soil and the soil column can be sterilized as a single unit, thus avoiding the chance for accidental contamination when carrying out tests on the soil column. One trial was completed with the use of PVC caps on either end of a steel column, and the other trial was completed with the use of iron caps on either end. The PVC caps, while maintaining their structure and seal, were difficult to remove without additional machinery. The iron caps were able to be removed and replaced, allowing for flexibility in future experiments as columns can be connected. The final design, using iron caps, survived the autoclave. Soil from both sterilized and unsterilized columns was plated to determine if the autoclave could sterilize the soil within the soil column. It was found that the sterilized column soil samples had little to no growth, compared to the growth seen in unsterilized column soil samples. This demonstrated the effectiveness of the autoclave sterilization process.

The third objective for the project was to deduce mass transfer equations relating to the diffusivity of six organic compounds in soil. This objective was accomplished and concentrations of the compounds through a one meter section of soil were graphed. It was determined that methane had the highest total flux over a period of three days.

Recommendations

Improvements

It was found that PVC caps were unsuitable for the desired column performance. A future improvement would be to solely rely on the use of the threaded iron caps for their integrity in the autoclave and ease of use with the column itself.

Due to Covid-19 restrictions, only two trials of column autoclaving were carried out. These trials demonstrated the basic principles of the experiment and showed how the steel columns and then iron and PVC caps performed under experimental conditions. More trials with longer observation periods for plated soil samples would determine if all of the plates eventually would grow amounts of bacteria as was seen in the unsterilized samples.

Future work

Future work on this topic can include a determination of the strains of bacteria that survived the autoclave. The experiment should start with running the process using our methods

to determine if user error could have contributed to the presence of bacterial growth. If growth persists throughout subsequent trials, then work should be made for identifying the microbe. This can be done via serial dilutions, plating, and isolation streaks. Those results can be analyzed for morphology and growth patterns as compared to known literature to identify the microbe. One method to do this is a serial dilution of bacterial material that is plated on a variety of different media. Isolation streaks can then be carried out. The growth patterns and morphology can be referenced with sources to attempt to identify the types of bacteria that are surviving the sterilization process.

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APPENDICES

Appendices

Appendix A: Governing Mass Balance Simplified and Constant Solved

$0 = D_{ab} * \frac{\partial^2 C_a}{\partial z^2}$

First integration: $C_1 = D_{ab} * \frac{\partial\ C_a}{\partial z}$

Second integration: $\frac{C_1}{D_{ab}} \int \partial z = \int \partial\ C_a$

General solution: $C_a = \frac{C_1 z}{D_{ab}} + C_2$

Using the second boundary condition: $C_{a_0} = \frac{C_1 z_0}{D_{ab}} + C_2 = 0.365 \frac{mol}{m^3} = \frac{C_1(0)}{D_{ab}} + C_2$

$C_2 = 0.365 \frac{mol}{m^3}$

Using the first boundary condition: $C_{a_L} = \frac{C_1 L}{D_{ab}} + C_2 = 0 = \frac{C_1(1\,m)}{3.21x10^{-10} \frac{m^2}{s}} + 0.365 \frac{mol}{m^3}$

$C_1 = -\ 1.172x10^{-10} \frac{mol}{m^2 s}$

Specific Solution: $C_a = \frac{-1.172x10^{-10} \frac{mol}{m^2 s} * z}{3.21x10^{-10} \frac{m^2}{s}} + 0.365 \frac{mol}{m^3}$

Appendix B: Determining Flux

$N_a = -D_{AB} \frac{dCa}{dz}$

Example for TNT

$\frac{dCa}{dz} = \frac{-1.96*10^{-15} \frac{mol}{m^2 s}}{1.8*10^{-10} \frac{m^2}{s}} = 1.09 * 10^{-5} \frac{mol}{m^4}$

$N_a = (-\ 1.8 * 10^{-10} \frac{m^2}{s}) * 1.09 * 10^{-5} \frac{mol}{m^4} = 1.96 * 10^{-15} \frac{mol}{m^2 s}$

$W_a = SA * N_a$

$W_a = (0.0254\,m^2)^2 \pi * 1.96 * 10^{-15} \frac{mol}{m^2 s} = 5.82 * 10^{-5} \frac{mol}{s}$

Appendix C: Concentration profiles for chemicals transport through soil

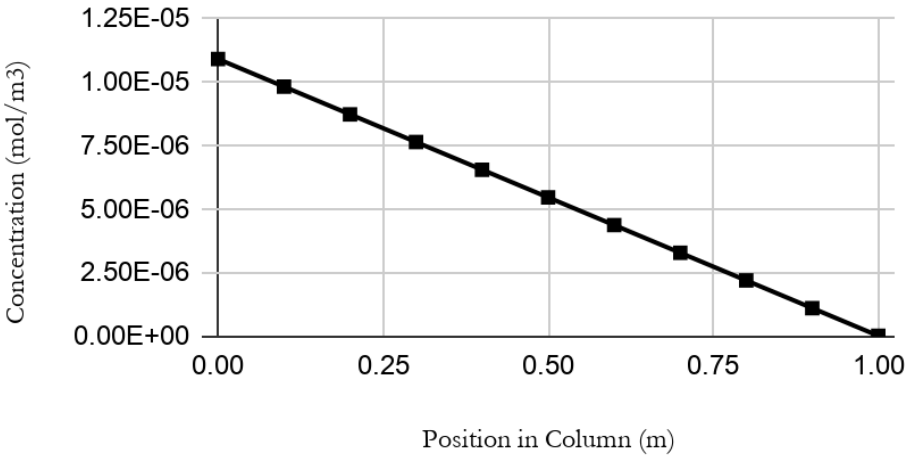

Figure 19: Concentration Profile of TNT in Soil

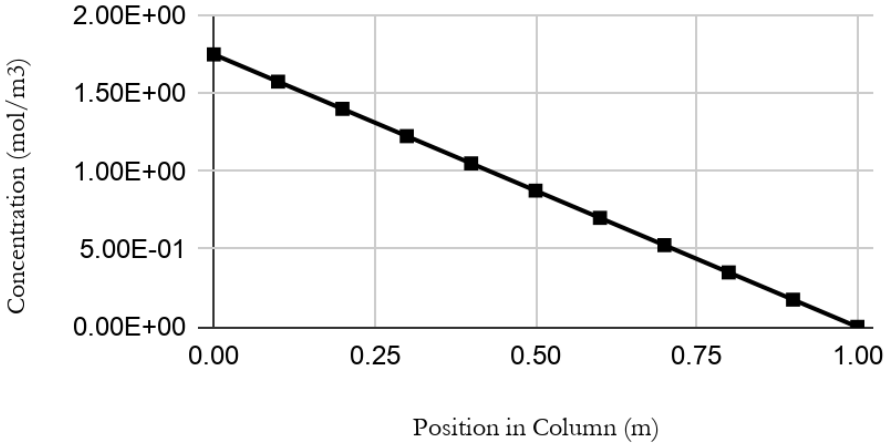

Figure 20: Concentration Profile of Ethanol in Soil

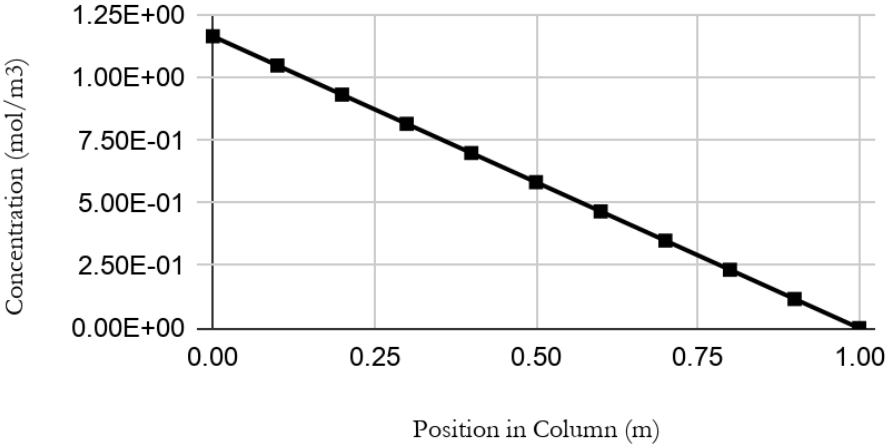

Figure 21: Concentration Profile of Toluene in Soil

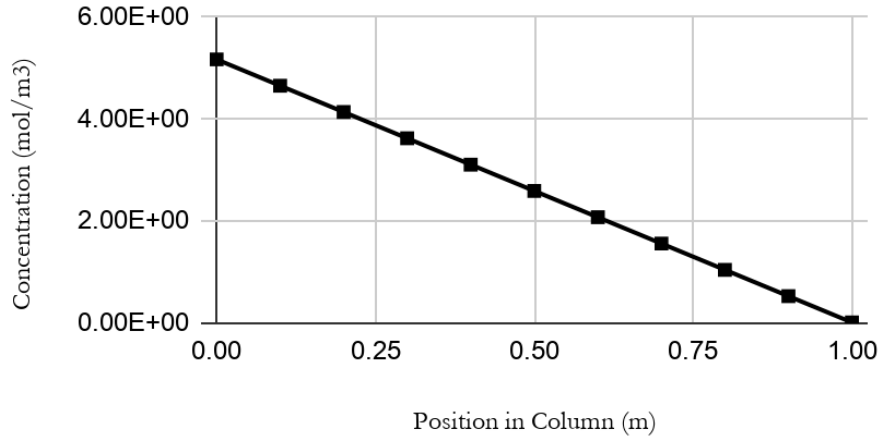

Figure 22: Concentration Profile of Methane in Soil

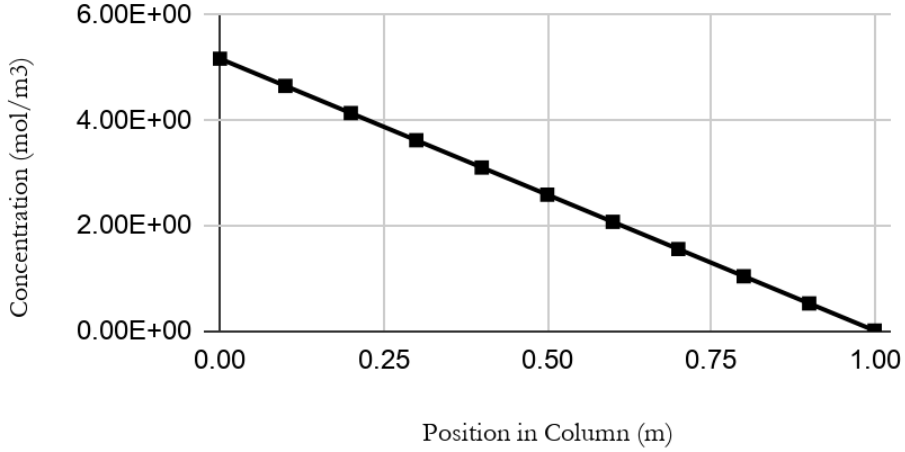

Figure 23: Concentration Profile of Benzene in Soil

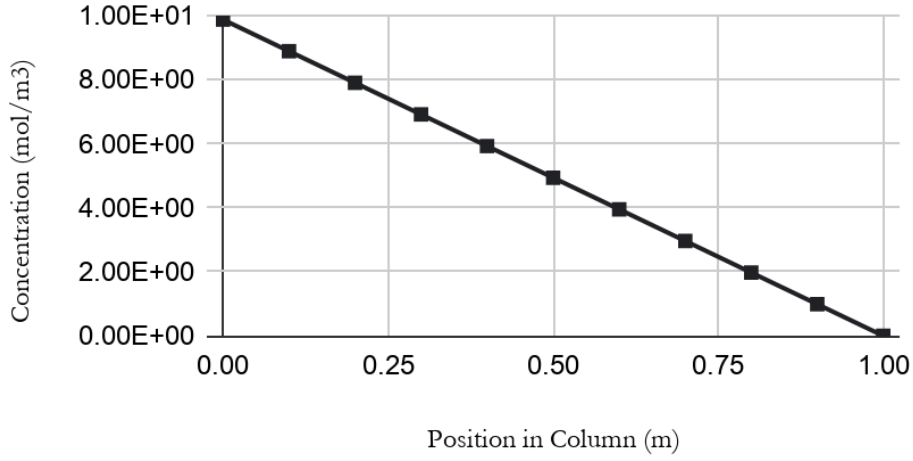

Figure 24: Concentration Profile of Acetone in Soil