**TITLE:**

**Membrane Diffusion Chambers and Aboveground Mesocosms for the Study of Microbial Responses to Groundwater Geochemistry**

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**SHORT ABSTRACT:**

Membrane diffusion chambers and an aboveground mesocosm are described and used to characterize the influence of groundwater geochemistry on *E. coli* inactivation. The diffusion chambers and mesocosm can be adapted to study the responses of bacterial and viral communities to a wide range of environments and geochemical conditions.

**LONG ABSTRACT:**

Characterizing the responses of microorganisms to environmental stimuli, such as physical and geochemical conditions within an ecosystem, is a critical aspect of understanding how microbes influence their local environments. These types of studies rely on community-level responses, because it is extremely difficult to isolate a single microbial genus or species of interest from the rest of the community. An alternative approach is to isolate the microbe of interest and perform laboratory-based studies, where specific environmental conditions are imposed on the microbial culture. However, these types of studies cannot completely mimic *in situ* conditions and the biological and chemical interactions. Here, we describe the design and fabrication of membrane diffusion chambers that retain a known concentration of a single bacterium (*E. coli*), while allowing these bacterial cells to experience the physical and geochemical conditions in naturally flowing groundwater over extended periods of time. Because the groundwater source is strictly anaerobic, extremely reduced (< -300 mV), and approximately 400 m below the land surface, an aboveground flow-through mesocosm was designed. This mesocosm retains the diffusion chambers while maintaining the at-depth geochemical conditions at the surface. This system ensured that the *E. coli* cells experienced the *in situ* geochemical conditions in the native aquifer. The groundwater geochemistry had a significant negative effect on the survival of *E. coli*. The inactivation kinetics followed a biphasic model, with the initial inactivation rate being 9- to 30-fold faster than previously published rates for *E. coli* in groundwater. The diffusion chambers and aboveground mesocosm provided a system with which to study the response of microorganisms to geochemical variation in groundwater, which was previously inaccessible.

**INTRODUCTION:**

One of the central objectives for studying microbial ecology is assessing how microbes respond to stimuli from the surrounding environment. These types of studies most often assess community-level responses due to an inability to isolate the response of a single genus or species from the rest of the community responses. One alternative is to expose a cultured microbial isolate of interest to simulated environmental conditions and stimuli in laboratory-based studies. However, these controlled conditions seldom truly mimic those of the natural environment of interest.

An option to avoid these limitations is the use of membrane diffusion chambers placed in the ecosystem of interest. These types of diffusion chambers are designed to retain a single strain of the microorganism while allowing the diffusion of gases and dissolved species present in the ecosystem into and from the chamber. The membranes not only retain the microbe of interest but also prevent grazing by and contamination from macro- and microorganisms outside the chamber. This design ensures the recovery of the microbe of interest after exposure to complex environmental conditions and stimuli that cannot be replicated in the laboratory.

The following protocol describes the design and fabrication of one type of membrane diffusion chamber for the retention of *E. coli* during prolonged exposure to a groundwater source. Additionally, because the groundwater source for the research effort described in this protocol was from an artesian aquifer zone located approximately 450 m below the land surface, an aboveground flow-through mesocosm that contains the diffusion chambers is also described. The mesocosm was designed to maintain the critical geochemical conditions of the aquifer zone (*i.e.,* strict anaerobiosis, extremely reduced, and a moderate temperature) at the land surface, where daytime temperatures can reach 50 °C. Collectively, the diffusion chambers and mesocosm provided a system to study the influence of prolonged exposure to native groundwater geochemistry on the inactivation of *E. coli*.

**PROTOCOL:**

**1. Design and construction of diffusion chambers**

1.1) The diffusion chamber described in this protocol is an alternative design to the McFeters diffusion chamber6-8,13,16. Use membranes with 47 mm diameter, 0.2 µm pore-size polycarbonate membrane filters.

NOTE: This type of diffusion chamber is used to retain bacterial suspensions while protecting the bacteria from predation by grazing microorganisms and contamination by native bacteria. However, the source water and its dissolved constituents diffuse into and out of the chambers.

NOTE: The choice of membrane and pore size are dependent upon which microorganism is contained within the diffusion chamber. Since the diffusion chambers are fabricated on site, a decision was made to design the chambers to accept commercially available membrane sizes. For example, if a virus is the microorganism loaded into the diffusion chamber, choose an appropriately sized membrane (*e.g.,* 25 mm diameter and 0.02 µm pore-size). Therefore, the final diffusion chamber design is scaled down relative to the dimensions used in this study to accept a 25 mm-diameter filter. Also, membrane sheets with the appropriate pore size for the respective microorganisms of concern are commercially available and can be manually cut to size.

1.2) Fabricate/construct the diffusion chamber from polytetrafluoroethylene (PTFE) and polyvinylchloride (PVC).

1.2.1) Ensure that each diffusion chamber consists of a cylindrical PTFE central chamber (2.5 cm length, 3.8 cm diameter, and with a wall thickness of 0.7 cm), into which two polypropylene female Luer lock syringe fittings, with water tight caps, are inserted.

1.2.2) Place a polycarbonate membrane filter (47 mm and 0.2 µm pore-size) on either end of the central chamber.

1.2.3) Place silicone gaskets (5.0 cm total diameter, 0.6 cm wide, 2.0 mm thick, and a 3.8 cm-diameter opening) into the 0.7 cm-wide and recessed zones in each PVC end plate (7.0 cm wide, 1.3 cm thick, and a 3.8 cm-diameter opening).

1.2.4) Slide the two end plates, with gaskets, over the ends of the central chamber.

1.2.5) Secure the four stainless steel bolts (#10, 3.8 cm) and wing nuts through the four holes in the PVC end caps to complete the diffusion chamber.

[Place Figures 1-1, 1-2 and 1-3 here]

**2. Design of the aboveground mesocosm for the diffusion chambers**

2.1) Design the aboveground mesocosm, composed of two distinct containers: (1) a large outer container, designed to allow relatively high flow rates of source water through the container, which insulates the inner container and diffusion chambers from elevated ambient temperatures and oxygen and (2) an inner container, in which the diffusion chambers are positioned and the geochemical conditions and source water flow rates closely mimic those of the native source water.

2.2) Fabricate the outer walls of the outer container by cutting 1.3 cm-thick high-density polyethylene (HDPE) sheets into two 106.0 cm × 75.6 cm (sides), two 75.6 cm × 75.6 cm (ends), and two 107.3 cm × 76.9 cm (top and bottom) pieces.

2.2.1) Secure the side, end, and bottom pieces of HDPE using stainless steel screws.

2.3) Obtain the inner walls of the outer container, a commercially available polypropylene tank (91.4 cm × 61.0 cm × 61.0 cm).

2.4) Insert the polypropylene tank into the HDPE box and fill the space between the two boxes with a 2.54 cm-thick sheet of insulation.

2.5) Secure the two boxes using stainless steel bolts and nuts along the top edge of both boxes to complete the outer container structure.

2.6) Drill appropriately sized holes to accommodate bulkhead fittings at approximately 15.0 cm from the bottom of one end of the outer container (inflow) and approximately 40.0 cm from the bottom on the opposite end of the container (outflow).

NOTE: These bulkhead fittings allow the leak-proof insertion of tubing into and from the outer container of the completed mesocosm.

2.7) Drill a high flow rate discharge opening (7.6 cm diameter) at approximately 60.0 cm from the bottom of the discharge end of the outer container. Insert PVC fitting and piping to discharge water from the outer container.

2.8) Drill a hole of the same diameter as those described in step 2.6 at approximately 15.0 cm from the bottom of the outer container and secure a bulkhead fitting; this is the low-flow discharge fitting that connects to the stainless steel inner container

2.9) Place the second 107.3 cm × 76.9 cm piece of HDPE on top of the outer container to act as a protective lid.

[Place Figure 2 here]

NOTE: If the mesocosm is placed where the discharging source water will cause some type of inconvenience or damage (*e.g.*, erosion, staining, flooding, etc.), use a PVC fitting for the high flow rate discharge opening, into which additional PVC pipe and fittings can be attached. This will allow one to direct the discharging water away from the mesocosm to a desired location (*e.g.*, storm sewer, river bank, etc.).

2.10) Weld sheets of 7-gauge 316 stainless steel to form an open container with approximate dimensions of 45.1 × 20.3 × 17.8 cm.

2.11) Weld vertical stainless steel inserts in a pattern similar to that shown in Figure 3 to ensure laminar and plug flow.

2.12) Drill a 1.5-cm hole at the bottom of one end of the inner container (inflow) and a second hole at the top of the other end (outflow).

2.13) Weld threaded stainless steel inserts into both 1.5-cm holes. The threaded inserts should accommodate 6.0 mm-outside-diameter tubing.

2.14) Cut a piece of HDPE with the same dimensions as the opening of the stainless steel inner container.

2.15) Cut a 2.0 mm-thick silicone sheet with the same dimensions as the opening of the stainless steel inner container. The width of the gasket should be equal to the thickness of the inner container wall.

2.16) Secure the lid and silicone gasket using six spring clamps.

[Place Figures 3-1 and 3-2 here]

NOTE: The material used to construct the inner container does not have to be stainless steel. This material was chosen because the geochemistry of some source water targeted for research projects is extremely reduced (< -300 mV), and anaerobic groundwater contains high concentrations of hydrogen sulfide. The critical criteria for the construction of an inner container are: (1) the isolation of the inner volume from the source water in the outer container, (2) a baffle system that prevents hydraulic short-circuiting, (3) water-tight access ports that allow easy access to and retrieval of diffusion chambers, and (4) an inability to float.

NOTE: All of the tubing used in this project was black PTFE. However, the biogeochemical characteristics of the source water may dictate the use of a different tubing material. PTFE tubing was chosen for three reasons: (1) the source water is anaerobic, and the negative influence of oxygen transfer through PTFE is minimal under the flow rates in the mesocosm, (2) the black coloration prevents exposure of the source water to sunlight, and (3) the black causes minimal development of chemical scaling and biofilms.

2.2) Use a flow rate control system to regulate the high flow rates of the source water through the outer container while reducing the flow rates through the inner container (and thereby around the diffusion chambers).

2.2.1) Insert a PVC-threaded nipple (2.5 cm diameter) into one end of a one-way PVC valve that has a threaded female fitting on the opposite end.

2.2.2) Attach a PVC-threaded couple to the nipple and a push tube fitting that accommodates a 1.5 cm-outside-diameter tubing to the couple; this end of the flow control system fits into the mesocosm.

2.2.3) Glue two PVC T-connectors, which have one threaded female opening, in series using the slip fitting openings. Glue these into the slip fitting on the one-way valve.

2.2.4) Insert a threaded push tube fitting, which accommodates 6.0 mm-outside-diameter tubing, into the remaining opening in the T-connector closest to the one-way valve.; the tubing from this fitting will go to the low-flow valve.

2.2.5) Insert a threaded low-flow valve, which accommodates 6.0 mm-outside-diameter tubing, into the other T-connector; the tubing from this fitting will go to the sensor flow-through cell.

2.2.6) Glue the threaded PVC couple to the remaining opening in the last T-connector.

2.2.7) Attached a PVC nipple to the threaded couple and a push tube fitting that accommodates 1.5 cm-outside-diameter tubing; this end of the flow control system connects to the groundwater source.

2.2.8) Connect the low-flow control valve to the open push tube fitting in the flow control system using a length of 6.0 mm-outside-diameter, 0.3 mm-inside-diameter PTFE tubing.

2.2.9) Connect the low-flow control valve to the inner container using a length of PTFE tubing, as previously described, by passing the tubing through the bulkhead fitting in the outer container and into the push tube fitting at the bottom of the end of the inner container.

2.2.10) Connect the flow cell for the multi-sensor system using a length of PTFE tubing, as previously described.

[Place Figures 4-1 and 4-2 here]

NOTE: The flow cells are designed to retain commercially available multi-sensor probes. Flow cells and multi-sensor probes are placed in line with the water flow to monitor and record field data for the source water as it enters the mesocosm and discharges from the inner container after contact with the diffusion chambers.

2.4) Connect the flow cells and multi-sensor probes to both sources prior to each sampling event, allow them to equilibrate during the retrieval of the diffusion chambers from the mesocosm, and record the data after the mesocosm has been returned to normal flow conditions.

NOTE: The variables monitored for this project were temperature (°C), specific conductance (mS cm-1), salinity (ppt), pH, dissolved oxygen (mg L-1), and oxidation-reduction potential (ORP; mV). Comparisons of these water quality variables in the incoming source water and that discharging from the inner container provides a measure of the changes in water quality in the slower-flowing source water.

**3. Aboveground flow-through mesocosm set up**

3.1) Connect a length of PTFE tubing (1.5 cm outside diameter, 1.3 cm inside diameter) to a stainless steel valve on the well head and on the inflow tubing push connector on the flow control system.

3.2) Connect a second length of this same PTFE tubing to the outflow end of the flow control system and an inflow tubing push connector in the outer container .

3.3) Insert the PTFE tubing from the outflow side of the low-flow regulator valve through an inflow tubing push connector in the outer container and into the inflow tubing push connection in the inner container.

NOTE: The length of tubing leading from the low-flow regulator valve has to be long enough to reach from the low-flow control valve, through the wall of the outer container, and to the inner container. Using two or more pieces of tubing with connectors or splices is not recommended when the source water is under pressure, like an artesian aquifer. This is because the gases in these types of source water will degas due to the decreases in pressure as the water moves from its usual depth to the mesocosm. Any irregularities or breaks in the tubing will promote excessive degassing, which can influence the geochemistry of the source water reaching the diffusion chambers.

3.4) Insert a second length of PTFE tubing into the outflow push tube connector, through the outflow push tube connector in the discharge end of the outer container, and into a flow cell with a multi-sensor probe.

NOTE: The flow rates through the outer container were set at approximately 10.0 L min-1 by adjusting the inflow valve of the flow rate control system. The flow rate in the outer container was set to allow a relatively short residence time for the source water, thereby minimizing the exposure of the inner chamber and diffusion chambers to high ambient temperatures and oxygen concentrations.

NOTE: The flow rates through the inner container were set at approximately 150 mL min-1 by adjusting the low-flow regulator valve. The flow rate in the inner chamber was selected based on published groundwater flow rates in the research project area. The 150 mL min-1 rate provided a linear flow velocity of approximately 6.0 m d-1, with a residence time in the inner chamber of approximately 2.0 h.

3.5) Confirm the flow rates manually by using a graduated cylinder and stopwatch during each sampling event.

NOTE: It is recommended to have the complete mesocosm set up and connected to the source water, with the flow rates established, before loading the diffusion chambers with the bacterial suspension. Keep the time interval between adjusting the cell concentrations and loading a completed diffusion chamber to a minimum.

**4. Bacterial culture growth and processing and loading diffusion chambers**

4.1) Inoculate a 5.0-mL primary culture with E. coli in tryptic soy broth and incubate at 37 °C with gentle rotational shaking (160 rpm) overnight.

4.1.1) Transfer a 100-µL sample of the primary culture into a fresh 5.0 mL of the same medium and grow it under the same conditions.

4.1.2) The next day, transfer 1.0 mL of the overnight culture into 15 -mL of the same medium and again grow overnight under the same conditions.

4.1.3) Prior to processing the overnight culture, visit the source water site and collect and filter sterilize approximately 1.0 L of source water; the filter-sterilized source water will be used to fill the diffusion chambers.

NOTE: If the research site is not located relatively close to the laboratory in which the *E. coli* culture is being processed, collect an additional volume of source water in a container with an opening large enough to fit the diffusion chambers, has a watertight closure, and can protect the chambers from direct sunlight.

4.1.3.1) Keep all diffusion chambers completely submerged.

NOTE: The more source water volume between the diffusion chambers and the air space in the container, the better the diffusion chambers are isolated from non-native source water conditions (*e.g.*, oxygen, temperature, and sunlight). There is no need to filter sterilize this volume of source water. Use this container to transport the loaded diffusion chambers (as in step 4.1.3) from the laboratory to the mesocosm at the research site when setting up the mesocosm and after each sampling event. Keep the time any diffusion chamber is exposed to ambient conditions to an absolute minimum.

4.1.4) Remove between 20 and 25 mL of this overnight culture and centrifuge for 5 min at 10,000 × g and 4 °C.

4.1.5) Resuspend the resulting pellet in 20 mL of phosphate-buffered saline (PBS; 137.0 mM NaCl, 2.7 mM KCl, and 11.9 mM PO4; pH 7.3-7.5) and centrifuge again using the same parameters.

4.1.6) Resuspend and dilute the resulting pellet with PBS to a spectrophotometric absorbance value (λ = 420 nm) that represents a cell concentration of 5×109 cells mL–1, based on a previously established standard growth curve5.

4.1.7) Add 198 mL of filter-sterilized source water to a sterile 250-mL flask. Transfer 2.0 mL of the adjusted *E. coli* suspension and gently swirl to mix.

4.1.8) Remove the caps from the syringe fittings in a diffusion chamber.

4.1.9) Collect 15 mL of the adjusted *E. coli* suspension in a sterile syringe, hold the syringe with the opening pointing up, connect it to the syringe fitting in the diffusion chamber, and gently push the cell suspension into the diffusion chamber.

NOTE: For this experimental design, the approximate *E. coli* concentration per diffusion chamber will be between of 5×108 and 1×109 cells (approximately 3-7×107 cells mL–1). However, this final concentration per diffusion chamber can be adjusted to any value for any microorganism.

4.1.10) Hold the syringe pointing upward while it is connected to the diffusion chamber and secure the cap for the open syringe fitting.

4.1.11) Gently flip the diffusion chamber with the syringe still attached, remove the syringe, and secure the cap onto the syringe fitting.

NOTE: With regard to the number of diffusion chambers needed for an experiment, that will be determined by the research questions being ask. Always plan on loading and transporting one diffusion chamber to act as the “time zero” data point. Load, transport, and process this diffusion chamber as planned for the others done at later time points. This ensures that “time zero” data are not biased due to different treatments prior to the recovery of the microorganism from the diffusion chambers. If the researcher is not next to the mesocosm to transfer the diffusion chamber to the inner container, immediately place the loaded diffusion chamber into the portable container that contains the native source water. Secure the closure and transport the diffusion chambers to the mesocosm.

**5. Placement and retrieval of diffusion chambers in the mesocosm**

5.1) Reduce the flow rate into the outer container using the one-way flow valve on the flow rate control system.

5.2) Connect the flow cells and multi-sensor probes to monitor the incoming source water and water discharging from the inner container.

5.2) Remove the mesocosm lid and open the outer container discharge valve to lower the water level to just below the top of the inner container.

NOTE: This will prevent the mixing of the water between the two containers while placing and retrieving diffusion chambers from the inner container.

5.3) Remove the spring clamps from the inner container to release the lid. Gently place the loaded diffusion chambers inside the inner container so the membrane filters are aligned with the flow path. Replace the lid and re-attache the spring clamps.

NOTE: Remember, the “time zero” diffusion chamber is still in the large volume of source water that transports the other diffusion chambers.

5.4) Close the discharge valve on the outer container and return the incoming flow valve to its original position to establish high flow rates through the outer container. Confirm that the water level is rising in the outer container and replace the lid on the mesocosm.

5.5) Record the data from the two multi-probe sensors. Manually check the discharge flow rate from the inner container.

5.6) For each sampling event, follow these same steps to access the inner container and place the retrieved diffusion chambers into a container with enough native source water to completely cover the chambers. Immediately return to the laboratory for the recovery and processing of the cell suspensions from the retrieved diffusion chambers.

**6. Bacterial suspension recovery from diffusion chambers and processing for cultivability**

6.1) Carefully remove a diffusion chamber from the container filled with native source water. Carefully remove the cap from the syringe fitting that is pointing up. Attach a sterile syringe and gently flip the diffusion chamber so that the syringe is below the diffusion chamber.

6.2) Carefully remove the other syringe cap. Slowly draw the cell suspension in the diffusion chamber into the syringe.

NOTE: Do not perform this step too rapidly! Too much inward pressure may rupture one or both of the membrane filters, resulting in a total loss of the cell suspension.

6.3) Immediately transfer the contents in the syringe to a sterile 50-mL tube.

NOTE: If dilutions are required, immediately perform a series of dilutions in sterile 15-mL tubes, using PBS as the diluent.

6.4) Use the standard membrane filtration method for the recovery of *E. coli* from environmental waters, using mTEC agar14 to recover the *E. coli* from the diffusion chamber suspension. Incubate at 35 °C for 2 h and then transfer to a 44.5 °C incubator for an additional 22-24 h.

6.5) Count the number of colony forming units (CFU) per filter based on diagnostic colony characteristics14,17 and normalize these values to the volumes plated; express the final values in units of CFU mL–1.

NOTE: It is recommended that, prior to the initial mesocosm set up event and prior to each diffusion chamber recovery event, all materials and supplies (*e.g.*, media plates, dilution blanks, pipets, membrane filtration method supplies, incubator temperatures set, etc.) be organized and appropriately labeled.

**7. Data analysis**

7.1) Transform all CFU mL-1 data using the formula; log10(*x*+1), where *x* = CFU mL-1 3. Analyze the transformed data using the open source software program GInaFiT3.

NOTE: This program analyzes log10-transformed data using a suite of six equations that have been shown to represent the most common inactivation data distributions for bacteria based on cultivability.

**REPRESENTATIVE RESULTS:**

The objective of the mesocosm design was to isolate the bacteria within the diffusion chambers from the elevated temperatures, sunlight, and oxygen at the surface while maintaining the temperature and geochemical characteristics of the groundwater that is in contact with the diffusion chambers. Additionally, exposing the bacteria to groundwater flow rates representative of those in the subsurface is an important variable to control.

The data in Table 1 show that there are no significant differences between the variables in the groundwater source and the inner container, as the values are within their respective standard deviations.

[Place Table 1 here]

The maintenance of source water temperature around the diffusion chambers and within the inner container is a critical factor in modeling the influence of the source water on the inactivation of *E. coli* (Table 2). The mean temperatures in the outer and inner containers are equivalent, even when ambient temperatures were, on average, approximately 4.0 °C higher. The ability of the mesocosm to insulate the water in the inner container is evidenced by comparing the range of temperatures within the mesocosm to ambient air temperatures, which were as high as approximately 50.0 °C during the summer months.

[Place Table 2 here]

The log10-transformed plate count data from each of the diffusion chamber collection time points are given in Table 3.

[Place Table 3 here]

The best-fit model for the data in Table 3 was determined to be the biphasic inactivation model1,2,15. This biphasic model describes the inactivation of bacterial communities that contain two subpopulations, with one subpopulation being inactivated more rapidly than the second subpopulation. This community structure generates curves with a relatively rapid initial inactivation rate for the more sensitive subpopulation, and then it transitions into a much slower inactivation rate that represents the second, more resistant subpopulation response. The two subpopulations are assumed to be independently and irreversibly inactivated, with both inactivation rates following first-order reaction kinetics.

The equation used to model the biphasic inactivation data is:

where *N0* and *Nt* are the log10-transformed colony counts at time zero and elapsed time *t* (h); *k*1 and *k*2 are the inactivation rate constants (log10 CFU mL-1 h-1) for the more and less sensitive subpopulations, respectively; and *f* and (1-*f*) are the decimal fractions of the total bacterial abundance in the diffusion chamber, which are more and less sensitive to inactivation, respectively.

The data in Table 3 generated a biphasic inactivation curve (Figure 1) with the following outputs: *k*1 = 0.387 h-1, *f* = 0.97; *k*2 = 0.008 h-1, and 1-*f* = 0.03.

[Place Figure 5 here]

**FIGURE LEGENDS:**

**Figure 1. Diffusion chambers.** (1) Each diffusion chamber is composed of a central PTFE chamber (2.5 cm length, 3.8 cm diameter, and a wall thickness of 0.7 cm) (a) into which two polypropylene female Luer lock syringe fittings, with watertight caps, are inserted (b). (c) A polycarbonate membrane filter (47 mm, 0.2 µm pore-size) is placed on either end of the central chamber. (d) The PVC end plates (7.0 cm wide, 1.3 cm thick, and a 3.8 cm-diameter opening) have 0.7 cm-wide recessed zones machined into one side and into which silicone gaskets (5.0 cm total diameter, 0.6 cm wide, 2.0 mm thick, and a 3.8 cm-diameter opening) (e) are inserted. The two end plates, with gaskets, are slid over the ends of the central chamber, and the completed diffusion chamber is held together with four stainless steel bolts (#10, 3.8 cm) and wing nuts (f). (2) Front view of a complete diffusion chamber. (3) Side view of a complete diffusion chamber.

**Figure 2. Outer container of the aboveground flow-through mesocosm.** The outer container contains the inner container. The groundwater source is connected through tubing to the flow control valve system. This system diverts, allowing high groundwater flow rates through the outer container. The valve system diverts some incoming groundwater flow into a flow cell with a multi-sensor installed to record geochemical data on the incoming groundwater. Flow is also diverted to a low-flow valve that establishes the aquifer flow rates in the inner chamber, which contains the diffusion chambers. The high-flow groundwater is discharged through a larger-diameter pipe. The discharge from the inner chamber is directed to a second flow cell and a multisensory system to record the geochemical data in the inner chamber.

**Figure 3. Inner container of the aboveground flow-through mesocosm.** (1) The inner container is constructed of 7-gauge, 316 stainless steel and has an internal volume of approximately 16.0 L (45.1 × 20.3 × 17.8 cm). The lid of the inner container is made of a solid HDPE. A 2.0 mm-thick silicone gasket is cut to fit the top edge of the inner container before the lid is attached. The lid is secured to the inner container, and a water-tight seal is established by the placement of six spring clamps. (2) The internal volume is baffled with vertical stainless steel inserts that ensure laminar and plug flow while allowing the placement of diffusion chambers between the baffles. (3) The inner container with eight diffusion chambers positioned in the groundwater flow path.

**Figure 4. Flow rate control system.** (1) The flow rate control system is directly connected to the groundwater source using 1.5 cm-outside-diameter tubing. Back pressure is established by slightly closing the PVC valve. This back pressure diverts groundwater into the multisensory probe and flow cell to collect geochemical data. Flow is also diverted to the low-flow valve, which controls the flow through the inner container and past the diffusion chambers. (2) Back view of the flow rate control system.

**Figure 5. Inactivation of *E. coli* in anaerobic and extremely reduced groundwater.** The log10-transformed CFU mL-1 data collected at specific time points (n = 7) followed a biphasic inactivation model, with an initial rapid inactivation rate (*k*1 = 0.387 h-1) that was followed by a much slower rate (*k*2 = 0.008 h-1).

**Table 1. Geochemical data for water entering and discharging from the mesocosm.**

**Table 2. Temperatures (°C) in the mesocosm and ambient air.**

**Table 3. Inactivation rate data.**

**DISCUSSION:**

Adequate sources of fresh water for drinking and agriculture are necessary for sustaining and developing communities. One of the technologies for the development of fresh water storage capacity is aquifer recharge, where treated, fresh surface water is injected into an aquifer and later recovered for human use10. These treated waters must be free of *E. coli* prior to recharging and during recovery.

The aboveground mesocosm described in this study allows the characterization of *E. coli* inactivation in groundwater following the injection of this bacterium into an inaccessible aquifer system, which in this case was hundreds of meters below the surface of the Earth. The anaerobic and extremely reduced geochemical conditions (< -300 mV) in the aquifer inactivated *E. coli* cells at a rate (*k*1) that was 9- to 30-fold faster than published inactivation rates for *E. coli* in groundwater that also used diffusion chambers (range: 0.013-0.042 h-1) 4,6,9,11,12. The remaining *E. coli* cells were inactivated at a rate (*k*2) similar to those in the cited studies.

The diffusion chambers and mesocosm design described in this study provided an aboveground system with which one can study the responses of bacterial cells to groundwater geochemical conditions in aquifer systems that are directly inaccessible to humans. The diffusion chamber design and fabrication is flexible and only dependent upon the geochemical conditions of the water source being studied, the microorganism to be retained (*e.g.,* bacterium, virus, or protozoa), and the access to the source water (*e.g.,* shallow private well, surficial aquifer, deep biosphere, or artesian wells). For example, the diffusion chambers material (*i.e.,* PTFE) and membrane (*i.e.,* 0.2 µm pore-size) in this study were chosen because of the chemically aggressive water and the use of a bacterium, respectively. The shape of the diffusion chambers (and the development of an aboveground mesocosm) was decided upon because it was impractical to design a deployment system that would allow the chambers to remain at depth against the artesian pressure in the well.

The mesocosm design can also be used with any type of non-artesian water source, but a pumping system may have to be used to maintain flow through the mesocosm. Also, by adapting the flow control system to include additional ports for inserting tubing, additions to the incoming source water (*e.g.*, O2, CO2, nutrients, and disinfectants) can be introduced into the water flowing into the inner chamber to assess how other variables influence the response of the microorganism in the diffusion chambers. Finally, the diffusion chambers do not have to be deployed within a mesocosm, or any type of container. They can be placed directly into lake and stream waters and sediments, marine water and sediments, sewage streams, soils, beach sands, etc.

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**DISCLOSURES:**

Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

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