Journal of Visualized Experiments

Membrane Diffusion Chambers and Above Ground Mesocosms for the Study of Microbial Responses to Groundwater Geochemistry

--Manuscript Draft--

Manuscript Number:	JoVE55151R4
Full Title:	Membrane Diffusion Chambers and Above Ground Mesocosms for the Study of Microbial Responses to Groundwater Geochemistry
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	diffusion chamber, mesocosm, bacteria, viruses, groundwater, inactivation rate
Manuscript Classifications:	2.3: Bacteria; 2.3.120: Bacteria, Aerobic; 2.3.130: Bacteria, Anaerobic; 2.3.440: Gram-Negative Bacteria; 95.51.16: environmental effects (biological, animal and plant); 95.51.26: microbiology
Corresponding Author:	John Lisle US Geological Survey St. Petersburg, FL UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	jlisle@usgs.gov
Corresponding Author's Institution:	US Geological Survey
Corresponding Author's Secondary Institution:	
First Author:	John Lisle
First Author Secondary Information:	
Order of Authors Secondary Information:	
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's 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 strictly anaerobic, extremely reduced (< -365 mV) and approximately 400 m below land surface, an above ground, flow through mesocosm was designed to retain the diffusion chambers while maintaining the at depth geochemical conditions at the surface. This system ensured the E. coli cells were experiencing 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 9-30 fold faster than previously published rates for E. coli in groundwater. The diffusion chambers and above ground mesocosm provided system to study the response of microorganisms to geochemical variable in groundwater that was previously inaccessible.
Author Comments:	
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a	

certain date, please indicate the date below and explain in your cover letter.	

Journal of Visualized Experiments May 31, 2016
One Alewife Center
Suite 200
Cambridge, MA 02140

Dear Editor,

Please find attached the manuscript *Membrane Diffusion Chambers and Above Ground Mesocosms for the Study of Microbial Responses to Groundwater Geochemistry*. I am submitting this manuscript for publication consideration in the Journal of Visualized Experiments.

I was originally contacted by Ms. Alison Hamlin after the publication of a manuscript in which I described the design and construction of a novel above ground mesocosm and diffusion chambers that permit the study of the effects of geochemical variables in groundwater from depths reaching 200 meter below surface on physiological responses of microorganisms. The submitted manuscript describes, in detail, the design and construction of the above ground mesocosm and diffusion chambers and how these were used to determine inactivation rates of E. coli when exposed to anaerobic and extremely reduced groundwater.

I look forward to interacting with Ms. Hamlin and your staff as this manuscript progresses through the review and video process. However, I do realize that the final decision for publication and video production is dependent upon the peer review process.

Sincerely,

John Lisle, PhD
US Geological Survey
Center for Coastal & Marine Research
St. Petersburg, FL 33701

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

AUTHOR:
Lisle, John T.
U.S. Geological Survey
St. Petersburg Coastal & Marine Science Center
St. Petersburg, FL USA
jlisle@usgs.gov

CORRESPONDING AUTHOR: John T. Lisle

KEYWORDS:
Diffusion chamber, mesocosm, bacteria, viruses, groundwater, inactivation rate

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 chamber[6-8,13,16]. 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 PO_4; 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 5×10^9 cells mL^{-1}, based on a previously established standard growth curve[5].

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 5×10^8 and 1×10^9 cells (approximately $3\text{-}7\times10^7$ 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 agar[14] 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 characteristics[14,17] 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; $\log_{10}(x+1)$, where x = CFU mL^{-1} [3]. Analyze the transformed data using the open source software program GInaFiT[3].

NOTE: This program analyzes $\log_{10}$-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 $\log_{10}$-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 model[1,2,15]. 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:

$$\frac{N_t}{N_0} = fe^{-k_1 t} + (1-f)e^{-k_2 t}$$

where N_0 and N_t are the $\log_{10}$-transformed colony counts at time zero and elapsed time t (h); k_1 and k_2 are the inactivation rate constants ($\log_{10}$ 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 $\log_{10}$-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 use[10]. 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.*, O_2, CO_2, 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.

ACKNOWLEDGMENTS:
The author acknowledges South Florida Water Management District (SFWMD) and U.S. Geological Survey's Water Resources Cooperative Water Program and Coastal Marine Program for funding support; Montana State University and Dr. Gordon McFeters for permission to modify their diffusion chamber design; and Dr. June Mirecki (U.S. Army Corps of Engineers) and Robert Verrastro (SFWMD) for constructive conversations and guidance.

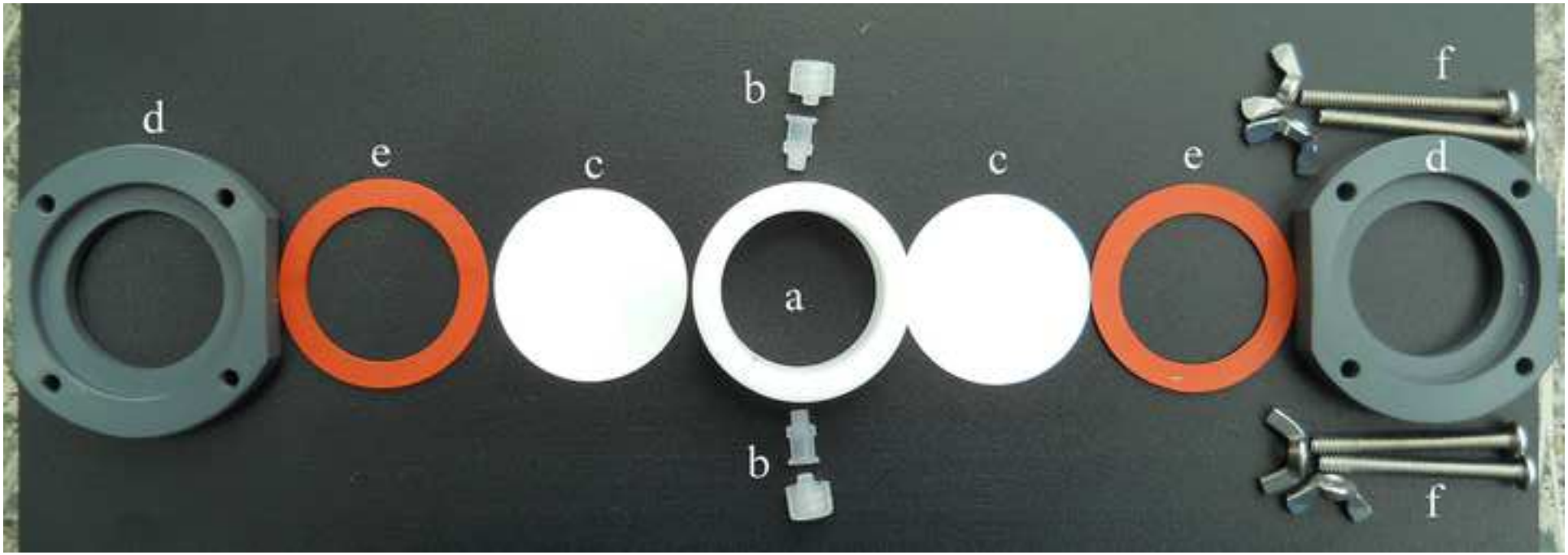
DISCLOSURES:
Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

REFERENCES:
1. Cerf, O. A review: tailing of survival curves of bacterial spores. *J. Appl. Microb.* **42** (1), 1-19, (1977). (DOI: 10.1111/j.1365-2672.1977.tb00665.x)
2. Crane, S. & Moore, J. Modeling enteric bacterial die-off: a review. *Water Air Soil Pollut.* **27** (3-4), 411-439, (1986). DOI: 10.1007/BF00649422

3. Geeraerd, A., Valdramidis, V., & Van Impe, J. GInaFiT, a freeware tool to assess non-log-linear microbial survivor curves. *Int..J..Food Microb.* **102** (1), 95-105, (2005). (DOI: 10.1016/j.ijfoodmicro.2004.11.038)
4. Keswick, B., Gerba, C., Secor, S., & Cech, I. Survival of enteric viruses and indicator bacteria in groundwater. *J. Environ. Sci..Hlth, Part A: Environ. Sci.Engin.* **17** (6), 903-912, (1982). (DOI: 10.1080/10934528209375085)
5. Koch, A. In: *Methods for General and Molecular Bacteriology.* Gerhardt, P., Murray, R., Wood, W., & Krieg, N. eds., Ch. 11, American Society for Microbiology, Washington, DC, 248-277 (1994). (DOI: 10.1002/food.19960400226)
6. McFeters, G., Bissonnette, G., Jezeski, J., Thomson, C., & Stuart, D. Comparative survival of indicator bacteria and enteric pathogens in well water. *Appl. Microb.* **27** (5), 823-829, (1974).
7. McFeters, G. & Stuart, D. Survival of coliform bacteria in natural waters: field and laboratory studies with membrane-filter chambers. *Appl..Microb.* **24** (5), 805-811, (1972).
8. McFeters, G. & Terzieva, S. Survival of *Escherichia coli* and *Yersinia enterocolitica* in stream water: comparison and field and laboratory exposure. *Microb. Ecol.*. **22** 65-74, (1991).(DOI: 10.1007/BF02540213)
9. Page, D., Miotlinski, K., Toze, S., & Barron, O. Human health risks of untreated groundwater third pipe supplies for non-potable domestic applications. *Urban Water J.* **11** (6), 461-466, (2014). (DOI: 10.1080/1573062X.2013.831912)
10. Pyne, R. *Aquifer Storage Recovery: A Guide to Groundwater Recharge Through Wells*. 2nd edn., ASR Systems, Gainesville, FL, (2005).
11. Sidhu, J. & Toze, S. Assessment of pathogen survival potential during managed aquifer recharge with diffusion chambers. *J..Appl.Microb.* **113** (3), 693-700, (2012). (DOI: 10.1111/j.1365-2672.2012.05360)
12. Sidhu, J. *et al.* Pathogen decay during managed aquifer recharge at four sites with different geochemical characteristics and recharge water sources. *J. Environ. Qual.* **44** 1402-1412, (2015). (DOI:10.2134/jeq2015.03.0118)
13. Terzieva, S. & McFeters, G. Survival and injury of *Escherichia coli*, *Campylobacter jejuni*, and *Yersinia enterocolitica* in stream water. *Can. J..Microb.* **37** 785-790, (1991). (DOI: 10.1139/m91-135)
14. U.S.Environmental Protection Agency. Method 1603: *Escherichia coli* (*E. coli*) in water by membrane filtration using modified membrane-thermotolerant *Escherichia coli* agar (modified mTEC). Report No. EPA-821-R-09-007, U.S. Environmental Protection Agency, Office of Water, Washington, DC, 1-24 (2009).
15. Xiong, R., Xie, G., Edmondson, A., & Sheard, M. A mathematical model for bacterial inactivation. *Intern..J. Food Microb*, **46** (1), 45-55, (1999). (DOI: 10.1016/S0168-1605(98)00172-X)
16. Zaske, S., Dockins, W., & McFeters, G. Cell envelope damage in *Escherichia coli* caused by short-term stress in water. *Appl, Environ, Microb,* **40** (2), 386-390, (1980).
17. Zimbro, M., Power, D., Miller, S., Wilson, G., & Johnson, J. *Difco & BBL Manual: Manual of Microbiological Culture Media*. 2nd edn., BD Diagnostics, Sparks, MD, (2009).

Figure

Click here to download Figure Figure 1_1. DiffusionChamber ExplodedView.jpg



Figure

Click here to download Figure Figure 1_2. DiffsuionChamber Complete FrontView.jpg

Figure

Click here to download Figure Figure 1_3. DiffusionChamber Complete SideView.jpg

Figure

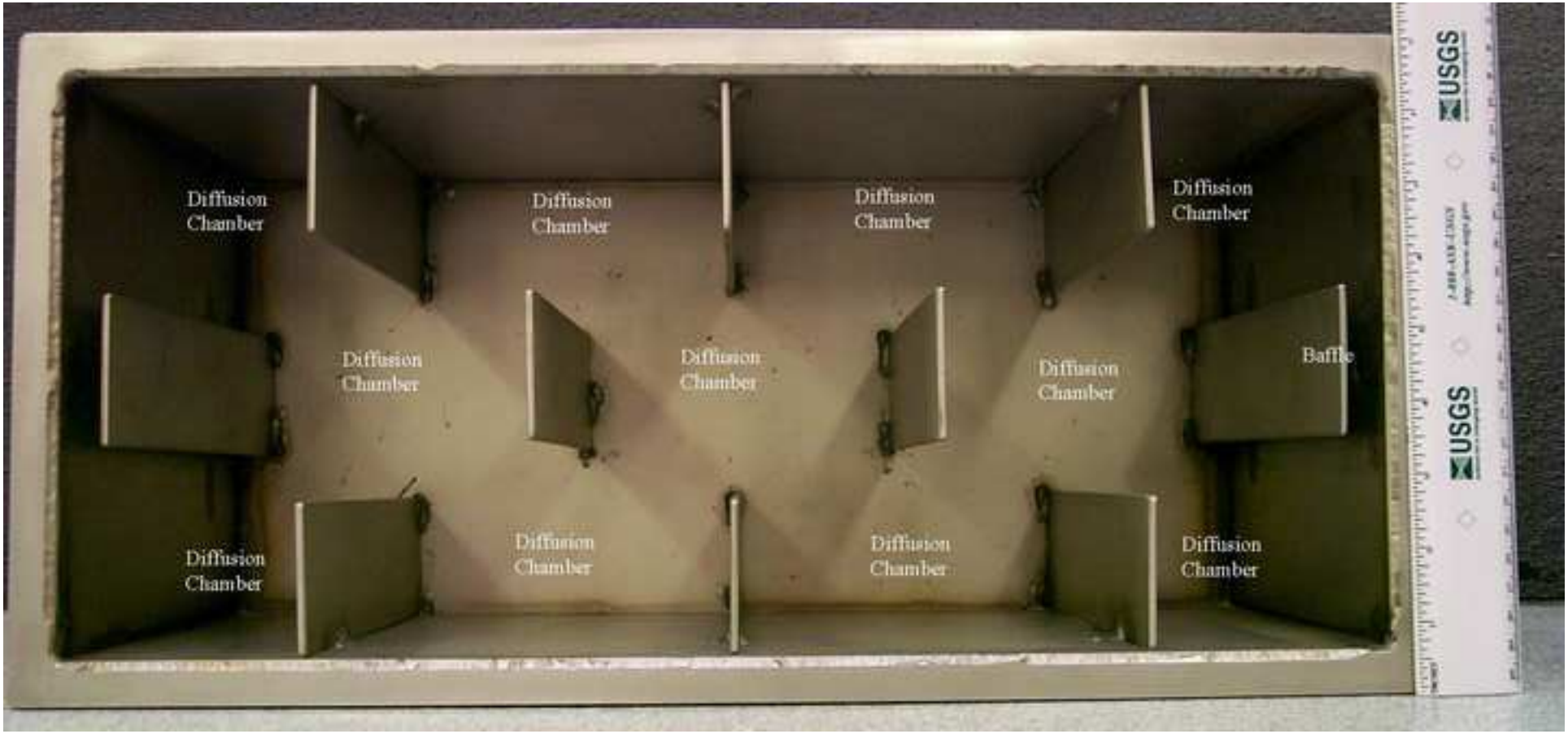
Click here to download Figure Figure 2 Mesocosm Complete.jpg

Figure

Click here to download Figure Figure 3_1. Inner Chamber SideView.jpg

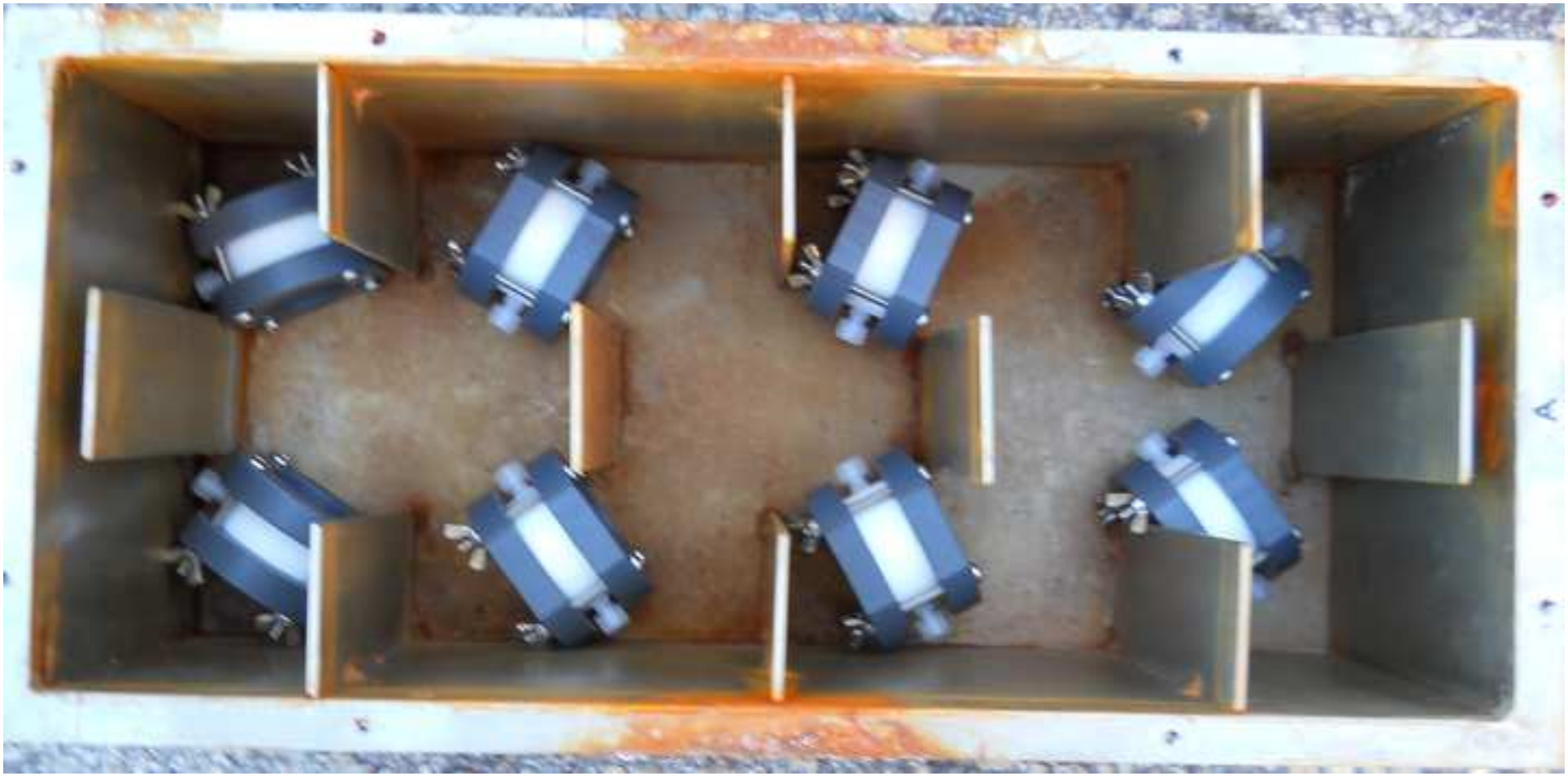
Figure

Click here to download Figure Figure 3_2. Inner Chamber TopView.jpg



Figure

Click here to download Figure JoVE 55151 Figure 3_3. Inner Chamber with Diffusion Chambers.jpg



Figure

Click here to download Figure Figure 4_1. Flow Control System FrontView.jpg

Figure

Click here to download Figure Figure 4_2. Flow Control System BackView.jpg

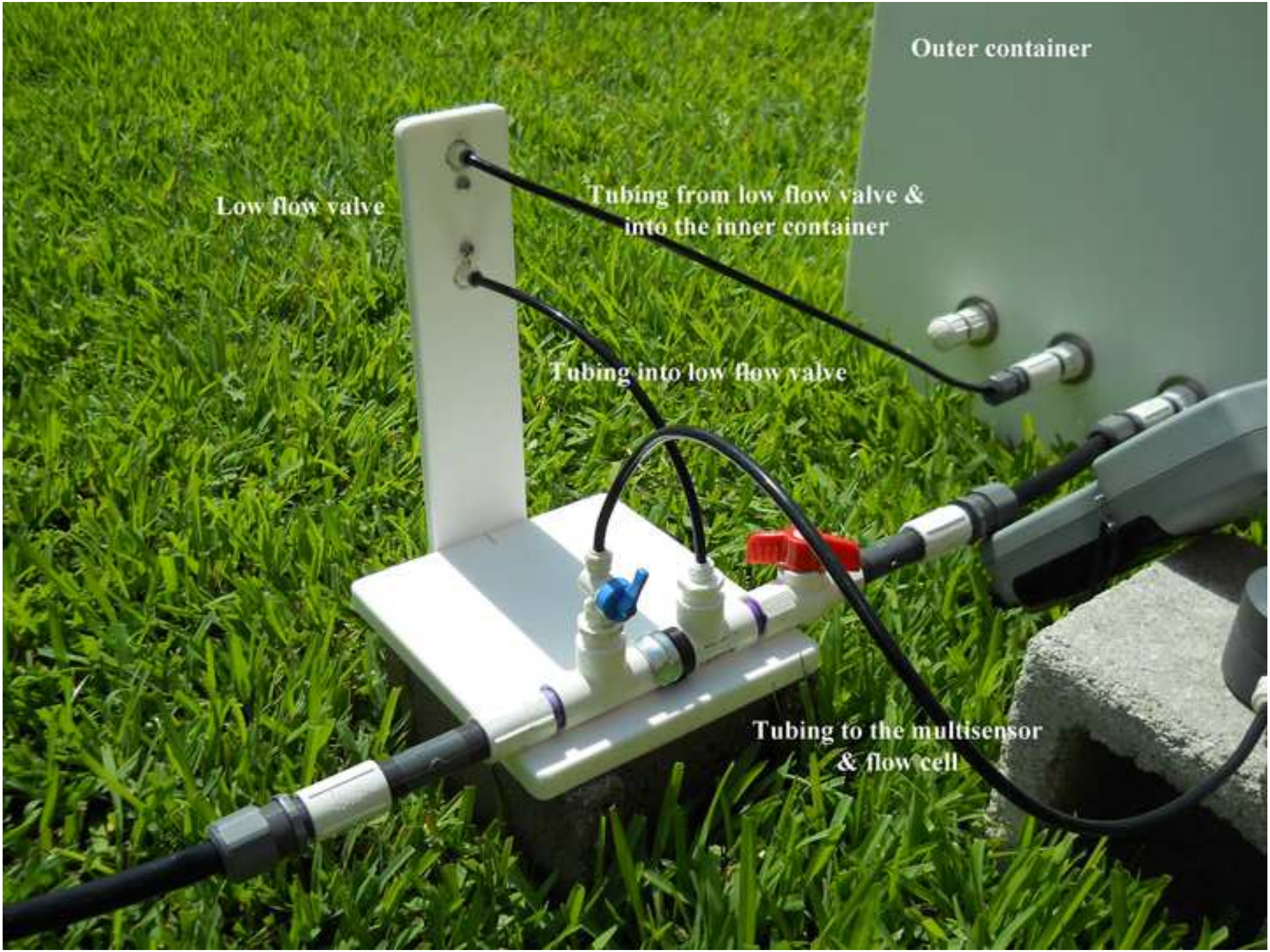


Figure 5

Click here to download Figure JoVE 55151 Figure 5.JPG

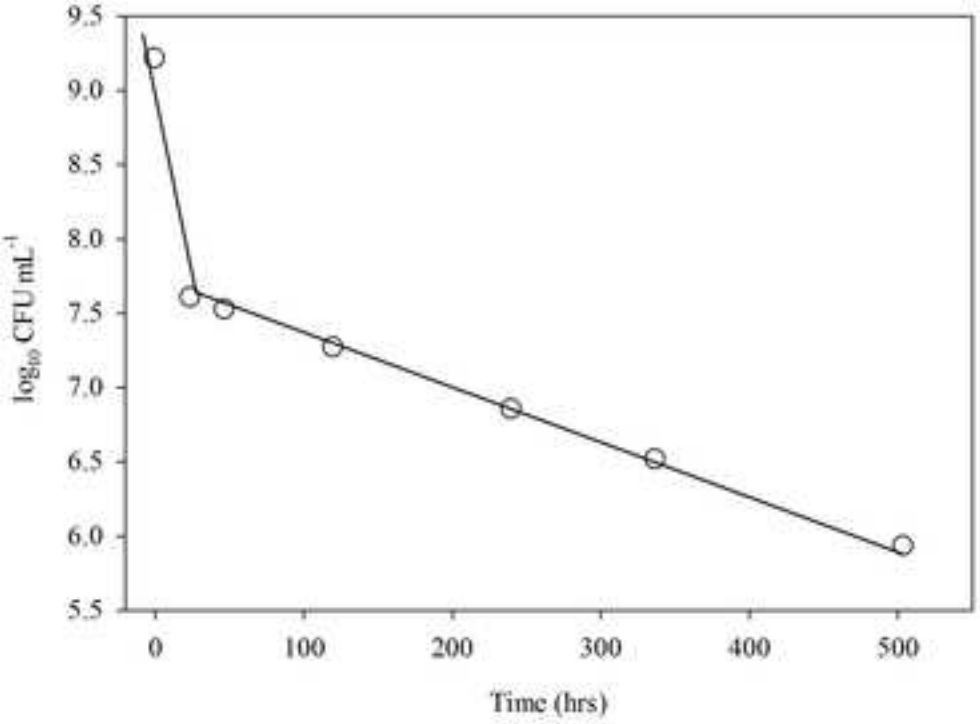


TABLE 1. Geochemical data for water entering and discharging from the mesocosm

Water Source	**Temperature**	**Specific Conductance**	**Salinity**	**pH**	**Dissolved Oxygen**
	°C	mS cm^{-1}	ppt		mg L^{-1}
Groundwater Source	26.1 (0.25)[1]	5.009 (0.284)	2.67 (0.04)	7.64 (0.32)	ND[2]
Inner Container	26.0 (0.14)	5.000 (0.253)	2.65 (0.05)	7.61 (0.28)	ND

[1] Values are expressed as mean (± standard deviation)
[2] ND: not detected

ORP
mV
-365 (23)
-371 (18)

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

Inner Container		**Outer Container**		**Ambient**	
Mean	Range	Mean	Range	Mean	Range
26.24 (0.29)[1]	25.84 - 26.97	26.25 (0.28)	25.89 - 26.99	30.70 (6.44)	22.99 - 49.58

[1] Values are expressed as mean (± standard deviation)

TABLE 3. Inactivation rate data

Time hrs	**Plate Counts** $\log_{10}$ CFU mL^{-1}
0	9.2122
24	7.6034
47	7.5218
120	7.2676
240	6.8499
337	6.5122
504	5.9309

Excel Spreadsheet- Table of Materials/Equipment

Click here to download Excel Spreadsheet- Table of Materials/Equipment JoVE Manuscript Equipment List.xlsx

Name of Reagent/ Equipment	Company
Membrane filters (polycarbonate)	Fisher Scientific
PTFE tube for diffusion chambers	McMaster-Carr
PVC sheet for diffusion chambers	McMaster-Carr
Luer lock female threaded fitting	Cole Parmer
Luer lock syringe fitting caps	Cole Parmer
Silicone gasket material (sheet)	McMaster-Carr
Stainless steel bolt with wingnuts	Home Depot
HDPE for outer container outer walls	McMaster-Carr
Polypropylene tank	US Plastic Corp.
Insulation (foam sheet)	Home Depot
Bulkhead tank fitting	W.W. Grainger, Inc.
Arlington liquid tight cord connectors	W.W. Grainger, Inc.
Arlington liquid tight cord connectors	W.W. Grainger, Inc.
Low flow control meter valve	Brooks Instrument
Tubing push fittings for small tubing and flow control system	Cole Parmer
Tubing push fitting valve for flow control system	Cole Parmer
Tubing push fittings for inner container inflow and outflow	Cole Parmer
PVC pipe and fittings	Home Depot
Stainless steel inner container	McMaster-Carr
PTFE tubing for inner container	Cole Parmer
PTFE tubing for outer container	Cole Parmer
ProPlus Lab Field Kit (4 parameters)	YSI Incorporated
Flow cell	YSI Incorporated
E. coli culture	ATCC
Tryptic soy broth	Fisher Scientific
Phosphate buffered saline (PBS)	Fisher Scientific
20 mL disposable syringe	Fisher Scientific
mTEC agar	Fisher Scientific
Disposable petri plates	Fisher Scientific
Disposable test tubes	Fisher Scientific
Field incubator	EMD Millipore Corp

Catalog Number	Comments/Description
GTTP04700	0.47mm diameter; 0.2 um pore size
8547K21	OD: 2", ID: 1.5" (various lengths available)
8747K185	1/2" thick; 36" x 36" sheet
WU-45502-60	50 pack
WU-30800-30	25 pack
5787T17	1/16" thick; 24" x 24" sheet
	#10, 2"
8619K477	1/2" thick; 48" x 48" sheet
9093	(LxWxH) 36" x 24" x 24"
296960	1" x 8' sheet
3CEC4	1/2", FNPTxFNPT
4JWL9	1/2", MNPT
4JWN2	1/2", MNPT
2L19VVT	0.4-5 GPH
WU-34007-43	1/4" x 1/4" MNPT
WU-01379-61	1/4" x 1/4" MNPT
EW-34040-52	1/4" x 1/4" FNPT
	all are 1/2", Schedule 20
88885K15	7 Guage, 316, 0.075" thickness
WU-06407-41	OD: 1/16", ID: 1/32"
WU-06605-44	OD: 9/16", ID: 1/2"
603223	
606160	
BAA-1159	Dehydrated cell concentrate
DF0370-17-3	500 grams
10-010-023	500 mL
14-823-16J	Luer-Lok tips without needles
B14884	100 grams
FB0875713A	Case of 500 plates
05-538-59A	Case of 500 tubes
XX631K230	

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Membrane Diffusion Chambers and Above Ground Mesocosms for the Study of Microbial Responses to Groundwater Geochemistry

Author(s): John T. Lisle

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via: ☑ Standard Access ☐ Open Access

Item 2 (check one box):

☐ The Author is NOT a United States government employee.

☑ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name:	John T. Lisle
Department:	
Institution:	USGS, Coastal & Marine Science Center
Article Title:	Membrane Diffusion Chambers and Above Ground Mesocosms for the Study of Microbial Responses to Groundwater Chemistry
Signature:	John T. Lisle
Date:	May 31, 2016

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

July 20, 2016

Dear Editor:

As requested in your email from July 1, 2016 I've reviewed manuscript JoVE55151 (*Membrane Diffusion Chambers and Above Ground Mesocosms for the Study of Microbial Responses to Groundwater Geochemistry*) and addressed each of the reviewer's/editor's comments as listed in email. There were a few comments which I felt were too ambiguous but tried to address what I thought the deficiency(ies) the comment was addressing. If I misinterpreted any of these comments, I would like the opportunity to properly address those comments with additional guidance from the editorial staff.

The changes to the revised manuscript are recorded within the manuscript using the Track Changes option in MS Word. Hopefully, these changes have made the manuscript a better product while bringing the format more in line with the publication requirements of JoVE.

My responses to each of the reviewer's/editor's comments as listed in the email are included in this document.

Thanks and I look forward to hearing back from you and your staff,

John Lisle, PhD
US Geologic Survey
St. Petersburg Center for Coastal & Marine Research
600 Fourth Street-South
St. Petersburg, FL 33701

Author's Responses to Editorial Comments

JoVE55151

Membrane Diffusion Chambers and Above Ground Mesocosms for the Study of Microbial Responses to Groundwater Geochemistry (Author: John Lisle)

1. Please re-write steps of your protocol section in imperative tense, as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.). Please try to avoid usage of phrases such as "should be", "could be", "would be" and write in the active/imperative style.

Author's Response: The manuscript was reviewed and changes were made as suggested.

2. Please simplify steps of your protocol section so that individual steps contain only 2-3 actions per step. As written currently, the steps are too long to review in-depth and comment succinctly. (See sections 5, for example).

Author's Response: A complete review of the manuscript was conducted and several of the steps in the protocol which could be sub-divided into smaller steps are now listed as sub-steps. These changes have each step and sub-step containing 2-3 separate actions as requested.

3. JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Please remove all commercial sounding language from your manuscript and replace it with a more generic term as much as possible throughout the entire manuscript. All commercial products should be sufficiently referenced in the table of materials/reagents. Examples of commercial sounding language in your manuscript are "ATCC #BAA–1159", etc.

Author's Response: The reference to the commercial source of the bacterial culture has been deleted. It's not clear how you would prefer the names of media, which are proprietary products with unique names (e.g., mTEC agar), described in the protocol. Are you OK with the product name being listed in the protocol as long as the company name is not included?

4. Step 4.1.9.1 should be a NOTE.

Author's Response: "NOTE" has been added to this step.

5. Please provide a reference for step 7.1.

Author's Response: The appropriate citation has been added to step 7.1.

6. In the representative results section please remove the numbering. Representative results should not be written like a protocol it should discuss specific results.

Author's Response: All numbering has been removed from the Representative Results section.

7. Please remove the embedded Table from the manuscript. All tables should be uploaded to the Editorial Manager site in the form of Excel files. A description of the table should be included with the Figure legends.

Author's Response: The tables have been removed from the body of the manuscript, as requested, and place holder notation added. The table titles have been added to the Figure Legends file.

8. Please remove the embedded figures from the manuscript. Figure legends, however, should remain within the manuscript text, directly below the Representative Results text.

Author's Response: Figure 5, the only figure embedded in the manuscript version I have, has been deleted. You requested this figure legend be left in place, but this was not required for the other figures. The legend for this figure is also listed in the Figure Legends file.

9. Please expand your representative results in the context of the technique you describe; i.e. how do these results show the technique, suggestions about how to analyze the outcome etc. This text should be written in paragraph form under a "Representative Results" heading and should refer to all of the results figures. You may include the figure captions under this heading but the captions and figure text must be separate entities.

Author's Response: Based on the example papers sent and the description of what is required for writing this section of a JoVE paper on your website, I think the current format of the Representative Results meets those criteria and the general ones mentioned in Comment #9. I did remove the numbering within this section as requested in Comment #6. If there is a specific issue with the way I've written this section I'll be more than willing to make the changes but please be more specific with identifying the deficiencies.

10. Each figure or data table must have an accompanying legend including a short title, followed by a short description of each panel and/or a general description. All figures showing data must include measurement definitions and error bars (if applicable). Please include the figure legends as part of the manuscript text (not part of the figure file) directly below the representative results text.

Author's Response: The figure legends have been inserted into the manuscript, in addition to being listed in the Figure Legends file. The table titles have also been listed in the Figure Legends file (see Editor's Comment #8 and author's response). Are you also asking for a detailed legend for the tables as well? Tables are normally constructed in a way that the title and column and row labels provide the information necessary for the reader to interpret the data so a detailed legend is not required.

11. If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as "Re-print with permission from (reference#)" or "Modified from.." etc. And please send a copy of the re-print permission for JoVE's record keeping purposes.

Author's Response: All figures, tables and data sets in this manuscript have not been previously published.

12. Please make sure that the "Discussion" is written under the following sections.
a. Critical steps within the protocol.
b. Modifications and troubleshooting.
c. Limitations of the technique.
d. Significance of the technique with respect to existing/alternative methods.
e. Future applications or directions after mastering this technique.

Author's Response: It's not clear to me if you have cited issues for each of these sections within the manuscript or is this just a list of items to check during my final edits and you have no specific comments. If there are specific issues or deficiencies please provide the line numbers for those passages. In case you are expecting a direct response to each item in this comment:

(a) Critical steps within the protocol: The critical steps in the protocol have been identified by the formatting and additional comments that in my opinion tell the reader these steps are critical for successfully completing the task.

(b) Modifications and troubleshooting: Throughout the manuscript I have added comments or steps and sub-steps that briefly describe possible modifications and identify steps and sub-steps which would be a problem if not properly executed. Are you asking for a section within the Discussion that re-lists and discusses these items?

(c) Limitations of the technique: Considering the focus of the manuscript is the design and construction of a type of mesocosm to study microbial responses under natural conditions, I don't think there is a limitation. This is because if you've the funds, you can make any and all of the items described in this protocol from materials that can withstand the most extreme geochemical conditions that a human can access.

(d) Significance of the technique with respect to existing/alternative methods: There are no alternative methods to the one described in this manuscript. There are alternative designs for the diffusion chambers but these are differences in sizes, shapes and materials but these are not applicable to the environmental conditions described for my systems.

(e) Future applications or directions after mastering this technique: There are several other applications for the diffusion chambers and the above ground mesocosm, which I've noted in the manuscript (e.g., lines 667-682). I don't think there is a higher level of expertise or skill that can be developed on the experience gained from successfully fabricating and deploying the mesocosm and diffusion chambers as described in this manuscript.

13. IMPORTANT: Please copy-edit the entire manuscript for any grammatical errors you may find. The text should be in American-English only. This editing should be performed by a native English speaker (or professional copyediting services) and is essential for clarity of the protocol and the manuscript. Please thoroughly review the language and grammar prior to resubmission. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

Author's Response: I'm not sure if this is a general comment added to all of the Editor's Comment documents or a specific comment on the writing style and structure of this manuscript. If there's a major issue with the English style and structure used in this manuscript please be more specific with examples, denoted by line numbers.

AUTHOR'S RESPONSES TO REVIEWER'S COMMENTS

Manuscript title: Membrane Diffusion Chambers and Above Ground Mesocosms for the Study of Microbial Responses to Groundwater Geochemistry (John Lisle)

Manuscript ID: JoVE55151R1

1. Please re-write steps of your protocol section in imperative tense, as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.). Please try to avoid usage of phrases such as "should be", "could be", "would be" and write in the active/imperative style. As currently written, the protocol section is not in imperative tense.

Author's response: See the tracked changes in the revised manuscript.

2. Additionally, the protocol section, and the steps contain descriptions which are not action items. Please be succinct in explaining what the protocol/technique is and remove all the descriptive text and place them as a NOTE under the protocol step if necessary.

Author's response: See the tracked changes in the revised manuscript.

3. Please simplify steps of your protocol section so that individual steps contain only 2-3 actions per step.

Author's response: See the tracked changes in the revised manuscript.

4. After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10 page limit for the protocol text, but there is a 2.75 pages limit for filmable content. If your protocol is longer than 3 pages, please highlight (in yellow) 2.75 pages (or less) of text to identify which portions of the protocol are most important to include in the video; i.e. which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVEs instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

Author's response: See the yellow highlighted passages in the revised manuscript.

5. Figure Legends section is missing. Please place the "Figure Legends" section before the "Discussion" section after the "Representative results section". Figure Legends should not appear in the protocol section.

Author's response: The legends have been moved to cited location in the revised manuscript.

JoVE5515R3 Author's Responses (John Lisle, USGS)

Editorial comments:

1. Line 34 "Because the groundwater source strictly anaerobic, extremely reduced (< -365 mV) and approximately 400 m below land surface…" – fragmented sentence please revise.

Author response: Completed

2. Line 70 "…land surface where day time temperatures can reach 50°C" – daytime

Author response: Completed

3. Line 77 "For this protocol, use a the diffusion chamber is an alternative design…" fragmented sentence, please revise

Author response: Completed

4. Line 81 "This type of diffusion chambers is used…"

Author response: Completed

5. Note Line 81-84 please break-up or edit for sentence flow.

Author response: Completed

6. Line 89 "choose an appropriate sized membrane" appropriately

Author response: Completed

7. Line 130 "Obtain the inner walls of the outer container that is commercially available polypropylene tank".

Author response: Need clarification on the specific issue with this sentence.

8. Results: Figure 3: please provide a photographic image showing the diffusion chambers (some or all of them) in place in the inner container.

Author response: Completed

9. References: Please use abbreviated journal titles and provide DOI for references where possible.

Author response: Journal abbreviations inserted and DOI information entered for all of the references except #6, 7, 10, 14, 16 and 17. DOI numbers are not available for those publications.

Reviewer #1:

1. What is the expected "life-time" of these chambers? Is it possible to estimate the turnover time for their use? What materials are most likely to fail and/or need replacing?

Author response: I did not perform any type of tolerance experiments for any of the materials described in this protocol, so providing an estimation of the “failure” rate for these materials is not possible. I think it’s apparent in the way the protocol was written that the researcher should select materials for the fabrication of the diffusion chambers and the inner and outer containers that’s resistant to the geochemical conditions of the water which will be in contact with those materials for the expected exposure time.

2. Although the materials appear to be from accessible sources (scientific supply companies and retail stores found in the United States for example), sourcing the construction materials may be somewhat prohibitive to groups from developing countries. But, this in no way invalidates the novelty and utility of the instrument developed and presented.

Author response: Agreed.

Reviewer #2:

1. The experimental protocol language is confusing and long. It can be made shorter.

Author response: I disagree.

2. The experimental protocol should be reported in past tense

Author response: This comment is the opposite of previous editorial comments made by the JoVE staff.

3. At some places in the manuscript the sentence making is improper and it does not make any sense.

Author response: Please provide specific examples.

4. The Sampling for the colony forming units (CFU) was it done in sets? Was the standard deviation and Standard Error accounted for in the samples of CFU?

Author response: The data and interpretations are included as an example of how the diffusion chambers and above ground mesocosm can be used to answer a specific research question. Accordingly, only a single sample was collected for each time point and no experimental repetition was attempted.

5. Why was there an initial rapid inactivation rate followed by a much slower rate? How long was the sampling done? Explanation can be added in the manuscript

Author response: The manuscript is supposed to focus on, in this case, the fabrication of diffusion chambers and an above ground mesocosm, not on describing or defending trends in the representative data. I disagree that an explanation of what might be causing the data to follow a biphasic inactivation model would add substantially to the length of the paper and, in my opinion, be outside the original objective of the manuscript submission.

6. The name of Agent/Equipment and catalog number can be added as supplementary material in the manuscript.

Author response: Perhaps I’m misinterpreting the comment, but per the JoVE guidelines the used of “…agent/equipment and catalog number(s)…” is not permitted.

7. The field application of this research should be elaborated in the discussion.

Author response: I think the field applications for the diffusion chambers and above ground mesocosm are adequately described for the JoVE format.

8. Were other variables like dissolved oxygen,reduction potential etc. monitored for the whole experimental period? If yes then it can be reported as a table.

Author response: Please see the author's response to Comment #5 from Reviewer #2.

9. The authors can add some literature review in the introduction of what previous studies have been attempted

Author response: When considering the objective of the manuscript, I think the "literature review" of previous studies is adequate. I do not agree that increasing the introduction or discussion of previous studies on the inactivation of *E. coli* in groundwater will increase the value of the manuscript.

10. Line 77. For this protocol,use "of a" diffusion chamber

Author response: Completed

11. Line 78. 'Membranes used were....

Author response: Completed

12.Line 149 'Drill a hole of same diameter'

Author response: Completed

13.Line 180. 'Width of the gasket should be equal to the thickness'

Author response: Completed

14.Line 324. As in step 4.1.12 pr 4.1.2?

Author response: Corrected

15. Line 326 needs to be reframed

Author response: Please provide more detail on what needs to be "reframed".

16. Line 360 'Research questions being asked'

Author response: Corrected

Reviewer #3:

1. Introduction has no references to previous studies? Or other approaches that have been adopted to measure microbial decay rates?

Authors response: The focus of the manuscript is the construction and application of diffusion chambers and an above ground mesocosm, not microbial decay rates. The data presented in the Representative Results section was included just as an example. The diffusion chambers and mesocosm can be used to study a variety of microbial responses to almost any environmental stimulus.

2. Suggest include a literature summary of previous decay rates and methods, thereby outlining the gap that this article tries to fill.

Authors response: See author's response to Comment #1 for Reviewer #3.

3. Also why is it important to measure the decay rates? E.g. for drinking water and water recycling via aquifer risk assessment, such as: *Toze, S., Bekele, E., Page, D, Sidhu, J., Shackelton, M. (2010) Use of static Quantitative Microbial Risk Assessment to determine pathogen risks in an unconfined carbonate aquifer used for Managed Aquifer Recharge, Water Research, 44, 1038-1049. *Page, D., Dillon, P., Toze, S., Bixio, D., Genthe, B., Jiménez Cisneros, B., E. and Wintgens, T. (2010) Valuing the subsurface pathogen treatment barrier in water recycling via aquifers for drinking supplies, Water Research, 44, 1841-1852. *Page, D., Vanderzalm, J., Barry, K., Levett, K., Sidhu, J., Toze, S., Kremer, S. and Dillon, P. (2010) Managed Aquifer Recharge (MAR) staged risk assessment framework applied to the Parafield Aquifer Storage Transfer Recovery system to assess human and environmental risks, Journal of Environmental Quality, 39, 6, 2029-2039.

Author response: This is a very relevant collection of publications from a very productive research group, all of which do describe bacterial inactivation rates in a variety of aquatic systems. However, as previously stated, the selection of inactivation data was just to serve as a representative data set which could be collected from the diffusion chambers and mesocosm. Inactivation rates were not the focus of the manuscript and dedicating text to this topic would detract from that describing the diffusion chambers and mesocosm.

4. The protocol seems very involved. It could be better describv3ed by referencing a similar design e.g. Sidhu et al (2015) - already cited and outlining differences.

Author response: I admit the protocol is involved and painstakingly broken down into as many simple steps as possible. This format follows that outlined by the JoVE editorial staff.

5. The biphasic model has also been referred to as the broken stick model by other researchers.

Author response: I agree but would prefer to us a more technical description.

6. Line 542: Sentences such as "treated waters must be free of E. coli prior to recharging and during recovery" is simply untrue. Water is commonly recharged with E. coli present and the aquifer can have a treatment effect on the water quality, e.g. *Page, D.W., Vanderzalm, J.L., Barry, K.E., Torkzaban, S., Gonzalez, D. and Dillon, P. J. (2015) E. coli and turbidity attenuation during urban stormwater recycling via Aquifer Storage and Recovery in a brackish limestone aquifer, Ecological Engineering 84, 427-434

Author response: I disagree but recognize that the differences in opinion may be due to differences in regulatory statutes between the reviewer's and author's state and possibly country. The state of Florida regulations that govern the microbiological quality of water injected into groundwater systems like those described in this manuscript clearly state the concentration of E. coli should be zero.

7. Line 547 use SI units.

Author response: Corrected

8. Line 548 and elsewhere, need to quantify these statements such as "extremely reduced geochemical conditions"

Author response: There are five references within the current version of the manuscript that define the reduced conditions for the example aquifer as < -300 mV. I don't agree that this value has to be inserted after each reference to the reduced conditions within the example aquifer as this is the only water source described in the manuscript.

9. Line 543, why is this important. How is this an improvement over current down hole methods? Given that groundwater chemistry should be quite stable, why would you need to take so many measurements?

Author response: First, I think it's obvious that the time required to open up and extract a set of diffusion chambers in the described above ground mesocosm would be significantly less than the time required to retrieve the same diffusion chambers from 1000's of feet below surface (i.e., few minutes vs. hours) using some type of cable retrieval system. Secondly, there are several scenarios where the geochemistry of the groundwater would change (e.g., contamination plume, recharged surface water with relatively elevated concentrations of dissolved oxygen and nutrients) and the several collection events for samples and diffusion chambers would be necessary to characterize changes in the geochemistry.

10. Line 551 No discuyssion of why the decay is biphasic.

Author response: See author's response to Comment #5 from Reviewer #2.

11. How do these results compare to other E. coli decay rates? A comparison table would be useful here.

Author response: See author's response to Comment #5 from Reviewer #2.