Natural Inactivation of MS2, Poliovirus Type 1 and Cryptosporidium parvum in An Anaerobic and Reduced Aquifer

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ORIGINAL ARTICLE

Natural inactivation of MS2, poliovirus type 1 and *Cryptosporidium parvum* in an anaerobic and reduced aquifer

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Abstract

Aims: The study of microbial inactivation rates in aquifer systems has most often been determined in aerobic and oxidized systems. This study examined the inactivation (i.e. loss of infectivity) of MS2, poliovirus type 1 (PV1) and *Cryptosporidium parvum* in an anaerobic and reduced groundwater system that has been identified as storage zones for aquifer storage and recovery (ASR) facilities.

Methods and Results: Anaerobic and reduced (ORP < ⁻250 mV) groundwater from an artesian well was diverted to an above-ground, flow-through mesocosm that contained diffusion chambers filled with MS2, PV1 or *Cryptosporidium parvum*. The respective infectivity assays were performed on microorganisms recovered from the diffusion chambers during 30- to 58-day experiments. The net reduction in infectivity was 5.73 $\log_{10}$ over 30 days for MS2, 5.00 $\log_{10}$ over 58 days for PV1 and 4.07 $\log_{10}$ over 37 days for *C. parvum*. The best fit inactivation model for PV1 was the log-linear model and the Weibull model for MS2 and *C. parvum*, with respective inactivation rates (95% confidence interval) of 0.19 (0.17–0.21) $\log_{10}$ day^{-1}, 0.31 (0.19–0.89) $\log_{10}$ day^{-1} and 0.20 (0.14–0.37) $\log_{10}$ day^{-1}.

Conclusions: The groundwater geochemical conditions in this aquifer enhanced the inactivation of MS2, PV1, and *C. parvum* at rates approximately 2.0–5.3-fold, 1.2–17.0-fold, and 4.5–5.6-fold greater, respectively, than those from published studies that used diffusion chambers in aerobic-to-anoxic groundwater systems, with positive redox potentials.

Significance and Impact of the Study: Geochemical conditions like those in the aquifer zone in this study can naturally and significantly reduce concentrations of microbial indicators and pathogens of human health concern in injected surface water. Appropriate storage times for injected surface water could complement above-ground engineered processes for microorganism removal and inactivation (e.g. filtration, disinfection) by naturally increasing overall microorganism log-inactivation rates of ASR facilities.

KEYWORDS

bacteria, inactivation, protozoa, viruses, water

INTRODUCTION

Climate change and urbanization continue to impact the quality and quantity of water resources along coastal zones worldwide. These processes are most evident in Florida, United States, where continued increases in population and vulnerability of coastal zones to sea-level rise and salt-water intrusion result in increasing demands for high-quality water whilst the sources for this resource are diminishing (Maliva et al., 2021). These demands are currently being met by accessing Florida's groundwater systems, from which approximately 63% of the agricultural and non-municipal users and 85% of the municipal suppliers use them as their sources for water (Maliva et al., 2021).

In addition to conservation, one or more of the technologies collectively known as managed aquifer recharge (MAR) are currently being employed to mitigate reductions in useable surface and groundwater. MAR technologies can be generally described as the treatment and injection of surface water into aquifer zones to either replenish an aquifer zone or later recovered and discharged into surface water systems that are used for potable sources or maintenance of hydrologic conditions within ecosystems (Dillon et al., 2019). The MAR technology of injecting treated surface water into an aquifer storage zone for later recovery for a surface application is categorized as aquifer storage and recovery (ASR; https://www.sfwmd.gov/our-work/alternative-water-supply/asr; Pyne, 2005).

The application of ASR for restoring and maintaining hydrologic flow through an ecosystem is currently being implemented as part of the Comprehensive Everglades Restoration Plan (CERP; https://www.evergladesrestoration.gov/; https://www.sfwmd.gov/our-work/cerp-project-planning). The general concept is water extracted from surface water sources is partially treated and injected into specific zones of the Floridan Aquifer when surface water is plentiful (e.g. rainy season), later recovered during dry periods and discharged into the headwaters of the Everglades when flow decreases below a critical rate. These cycles of injection and recovery of treated surface water artificially augment the volume of water flowing through the Everglades at rates that support the health of the ecosystem.

The treated surface water was injected into the aquifer zones at ASR facilities is monitored prior to injection for specific constituents, which include the microbial contaminants as described in the primary and secondary drinking water standards (i.e. faecal coliforms, *E. coli*; FDEP, 2013a, 2013b; USEPA, 2006). Coliforms and *E. coli* have been shown to survive treatment processes (i.e. rapid sand filtration and UV disinfection) used at an ASR facility in Florida (Mirecki, 2013), thereby placing the ASR facility in violation of the coliform or *E. coli* regulatory standards.

However, faecal coliforms, *E. coli*, bacteriophages (e.g., MS2) and eukaryotic viruses (e.g. adenovirus, rotavirus) and encysted protozoans (e.g. *Cryptosporidium* sp., *Giardia* sp.) have been shown to be inactivated during exposure to native groundwater in aquifer storage zones at MAR facilities (Page et al., 2010, 2015; Sidhu et al., 2010; Toze et al., 2010), including *E. coli* in the Floridan Aquifer (Lisle, 2016). When considering the risk to human health from consumption or exposure to water recovered from storage zones at ASR facilities in Florida, it is important to understand if microorganisms of regulatory interest are inactivated during exposure to geochemical conditions during storage in the aquifer.

In this study, the inactivation rates for a surrogate commonly used to assess the fate and transport of eucaryotic viruses (MS2 bacteriophage) in groundwater systems (Mesquita & Emelko, 2012), and a human enterovirus (poliovirus type 1) and encysted eukaryotic protozoan (*Cryptosporidium parvum*), both of which are of regulatory and public health significance in groundwater, were derived from infectivity-based data following exposure to anaerobic and extremely reduced (<−250 mV) groundwater from the Upper Floridan Aquifer zone of the Floridan Aquifer. Diffusion chambers containing known concentrations of each of the microorganisms were sampled during 30- to 58-day exposure periods using an above-ground, flow-through mesocosm that maintained native aquifer geochemical (except for pressure) and temperature conditions at depth (between 170 and 270 mbs).

MATERIALS AND METHODS

Sample site location and hydrogeology

The artesian groundwater source well (27°09′17.3″; 80°52′27.4″W) is located within the Kissimmee River ASR (KRASR) facility located near the confluence of the Kissimmee River and Lake Okeechobee near Okeechobee, Florida (Mirecki, 2013; Mirecki et al., 2013). This well is 0.254 m diameter steel cased to 174.3 m below land surface (mbls) with a single screened collection zone between 174.3 and 268.2 mbls. The collection zone is within the artesian Upper Floridan Aquifer (UFA) that is characterized as a thick sequence of interlayered marine calcareous and dolomitic limestones of Eocene and Oligocene age, overlain by a confining unit consisting of approximately 122 m of Hawthorn Group interlayered clays, silts and fine sands (Scott, 1988). The lower confining

layer consists of 122–152 m of dolomitic limestone, dolomite, and dolostone (Golder & Associates, 2007; Reese & Richardson, 2007; Waldron & Horvath, 2010). These confining units isolate this zone of the UFA from other groundwater sources positioned above or below (Miller, 1997). Additionally, the collection zone is not impacted by meteoric or surface water as the isotopic age of the groundwater in this region of UFA has been estimated at approximately 2.5×10^4 years since it was first recharged into the subsurface (Plummer & Sprinkle, 2001).

Groundwater chemistry

The general geochemistry and nutrient data (mean ± SD) for the zone of the UFA accessed during this study were taken from monitoring wells associated with the KRASR facility property (Mirecki, 2013; Table 1).

Diffusion chambers

The diffusion chambers used in this study are modifications of the designs described by McFeters and Stuart (1972), McFeters et al. (1974), McFeters and Terzieva (1991) and later by Sidhu and Toze (2012). The central chambers are PTFE pipes (OD: 50.75 mm; ID: 38.00 mm; length: 25.5 mm) with a completed volume of approximately 30.0 ml. Each end of the central chamber is sealed with two polycarbonate end caps into which silicone gaskets, then commercially available membrane filters (47 mm diameter) are placed. The two end cap assemblies are placed on either end of the central chamber and secured in place with four stainless steel bolts and nuts that are tightened to ensure a watertight seal. Two polypropylene female Luer lock adapters, with sealing caps, are attached via threaded openings on opposite sides of the central chamber to facilitate connections with Luer lock syringes (Figure S1). For this study, all diffusion chambers were fitted with 0.02-μm pore size membrane filters (Whatman Anodisc; GE Healthcare Bio-Sciences).

Above ground mesocosm

A two-container system was designed to allow groundwater to flow at high rates through a larger container and around a smaller stainless-steel container that is positioned within the larger container. The high groundwater flow rates insulate the inner chamber and its contents from fluctuations in ambient temperatures and oxygen (Figure 1; Figure S2).

TABLE 1 Upper floridan aquifer geochemical data

Parameter	Units	Mean (±SD)
Temperature	°C	25.56 ± 0.27
pH		7.89 ± 0.21
ORP	mV	−258.4 ± 30.75
Specific conductance	μS cm^{-1}	1269.8 ± 156.32
Turbidity	NTU	0.45 ± 0.36
Colour	PCU	5.85 ± 1.2
Total dissolved solids	mg L^{-1}	727.8 ± 110
Total alkalinity (as $CaCO_3$)	mg L^{-1}	85.2 ± 4.58
Aluminium	μg L^{-1}	5.65 ± 7.99
Barium	μg L^{-1}	29.02 ± 3.16
Boron	μg L^{-1}	82 ± 18.38
Bromide	mg L^{-1}	660 ± 138.2
Calcium	mg L^{-1}	46.42 ± 4.11
Chloride	mg L^{-1}	232.6 ± 50.96
Copper	mg L^{-1}	1.38 ± 0.66
Fluoride	mg L^{-1}	0.53 ± 0.04
Iron	μg L^{-1}	90.17 ± 77.92
Magnesium	mg L^{-1}	36.52 ± 2.72
Manganese	μg L^{-1}	4.45 ± 1.93
Potassium	mg L^{-1}	7.3 ± 1.52
Silica	mg L^{-1}	8.2 ± 5.11
Sodium	mg L^{-1}	137.14 ± 37.33
Sulphate	mg L^{-1}	184.6 ± 12.66
Sulphide	mg L^{-1}	1.07 ± 0.22
Zinc	μg L^{-1}	9.72 ± 11.42
NO_2-N	mg L^{-1}	<0.01[a]
NO_3-N	mg L^{-1}	<0.03[a]
NH_3-N	mg L^{-1}	0.22
Total PO_4-P	mg L^{-1}	0.03
Ortho PO_4-P	mg L^{-1}	<0.01[a]
Total organic carbon	mg L^{-1}	1.7
Dissolved organic carbon	mg L^{-1}	1.40 ± 0.28

[a]Denotes the analytical detection limit.

The groundwater source was connected to the larger outer container (340 L HDPE; Cole-Parmer; Figure 1a) using black PTFE tubing (1.27 cm OD) that attached to the wellhead via a stainless steel valve attached and through a watertight fitting located at the bottom of one end of the container. The groundwater discharged from the outer container through a 5.10-cm diameter opening located at the top and opposite end of the container (Figure 1f). The lid to the outer container was secured using nylon straps. The groundwater flow rate through the outer container was set at approximately 10.0 L min^{-1}.

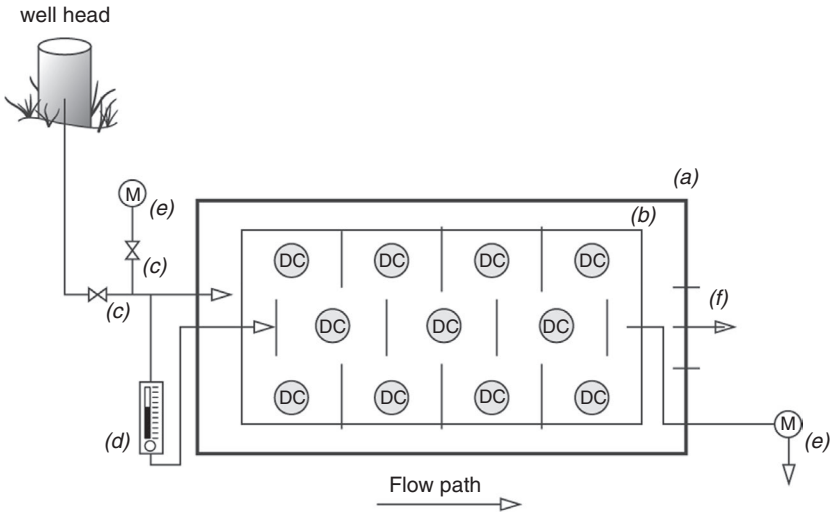

FIGURE 1 Above ground mesocosm. The outermost container (a) contains the inner container (b) in which the diffusion chambers (DC) are placed. The groundwater flows into the outer container through a three-way valve (c) to control the flow into the field variable measurement sondes (e) and a low flow control valve (d) that control the groundwater flow through the inner container. The groundwater moving through the outer container is discharged through the end of the mesocosm (f) and through another sonde (e) from the inner container

The second and inner component of the mesocosm was a stainless steel container (45.1 cm × 20.3 cm × 17.8 cm), with an internal volume of 16.0 L (Figure 1b). This inner container is baffled with vertical stainless steel inserts that ensure laminar flow, whilst allowing the placement of diffusion chambers (DC; Figure 1) between the baffles. The lid of the inner container was HDPE, onto which a silicone gasket had been placed, making for a watertight seal once secured with a series of eight equally positioned stainless steel clamps (Figure S3). Groundwater from the wellhead was diverted into the inner container via a three-way valve that had been inserted into the high flow volume PTFE tube prior to entering the outer container (Figure 1c). A low flow rate control valve (MR3000; Brooks Instrument; Figure 1d) was connected to the three-way valve via black PFTE tubing (0.47 cm OD) with the tubing from the outflow side of the valve traversing the wall of the outer container through a watertight fitting and into one end of the inner container via a push-tube fitting. A longer piece of tubing was connected to the discharge end of the inner container and though a watertight fitting located on the opposite end of the outer container (Figure S2). The flow rate through the inner container was approximately 150.0 ml min^{-1} for a residence time of approximately 2.0 h. The inner and outer containers were allowed to fill completely and discharge to waste for at least two days before inserting the diffusion chambers as described below. Groundwater entering the outer and inner containers was measured for temperature, redox potential, conductivity and dissolved oxygen using YSI 59M systems (M) positioned in flow cells (Figure 1e).

Sources of the microorganisms used in this study

A titered suspension of each microorganism was provided by Biological Consulting Services of North Central Florida, Inc. The bacteriophage MS2 (ATCC #15597-B1) was obtained from American Type Culture Collection (ATCC) and supplied as a suspension in Butterfield's Phosphate Buffer Dilution Water (BPBDW; 0.6 M KH_2PO_4, pH 7.2). The poliovirus-type 1(PV1) isolate (ATCC #VR-1562) was also obtained from ATCC and supplied as a suspension in Dulbecco's Modified Eagle's Medium (DMEM; 15-018-CM; Corning, Inc.). The *Cryptosporidium parvum* oocyst suspension (<4 weeks post shedding) was obtained from Bunchgrass Farms (Deary, Idaho, ID) and supplied as a suspension in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10.0 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4), supplemented with 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin.

Diffusion chamber preparation

Sterile glass collection bottles (1.0 L) were filled with groundwater using sterile silicone tubing connected to the wellhead and inserted to the bottom of each bottle. The groundwater was allowed to flow for at least five bottle volumes before capping the bottle, ensuring there was no head space. An additional volume (~4.0 L) of groundwater was collected into a set of wide mouth HDPE bottles, to

be used for transport of the diffusion chambers from the laboratory to the mesocosm.

Once in the laboratory, the 1.0 L volume was filter-sterilized into a second sterile 1.0 L bottle through a Steritop™ bottle-top 0.22 μm pore-size filtration system (MilliporeSigma) whilst the headspace in the filtration system was under constant N_2 gas flow. For each microorganism, 500 ml of filter-sterilized groundwater was transferred to separate sterile flasks, under constant N_2 gas flow, and a volume of the titered stock suspensions was added to achieve final concentrations of 1.62×10^6 PFU ml^{-1} for MS2, 3.50×10^6 MPN viruses ml^{-1} for PV1 and 2.80×10^4 MPN oocysts ml^{-1} for *C. parvum* in each diffusion chamber for the respective experiments.

Each diffusion chamber was filled with the respective microbe suspensions by first removing the two Luer lock caps. Approximately, 35.0 ml of the microbial suspension was collected into a sterile syringe, which was immediately connected to the open Luer lock fittings on the bottom of the chamber and the microbial suspension was slowly injected. Once the diffusion chamber was filled, the cap was replaced on the open fitting on the top, the diffusion chamber flipped and the syringe removed before replacing the second cap, thereby sealing the diffusion chamber. Each filled diffusion chamber was placed in one of the wide mouth bottles containing groundwater for temporary storage and transport to the mesocosm. For each experiment, 15 diffusion chambers were loaded with a single microorganism and placed into the inner container of the above-ground mesocosm.

Diffusion chamber deployment and the recovery and processing for MS2, PV1 and *C. parvum*

For the deployment of the diffusion chambers, the groundwater level in the outer container of the mesocosm was allowed to drain to a depth just below the top of the inner container. The lid of the inner container was removed and all but one of the diffusion chambers were placed in the container, so the membrane filters were perpendicular to the groundwater flow. The inner container's lid was replaced, the water level and flow in the outer container was returned to the original configuration. The diffusion chamber not placed in the inner chamber was immediately transported to the laboratory (~2.0 h) in a dark and insulated container that maintained a temperature close to that of the native groundwater (~26°C). Once in the laboratory, the total volume in each diffusion chamber was collected into a sterile syringe, then transferred to a sterile 50.0 ml tube for processing and enumeration for MS2, PV1 and *C. parvum* as described below.

A diffusion chamber was retrieved from the inner container on days 1–4, 7, 10, 11, 16, 23, 30 after the initiation of each experiment (i.e. time zero). The PV1 and *C. parvum* experiments were extended for two additional sampling events at 37 and 58 days after the initiation of the respective experiments.

Bacteriophage MS2 enumeration

Water samples recovered from the diffusion chambers were processed for infectivity of an *Escherichia coli* host (ATCC #15597; American Type Culture Collection) using the double agar overlay method, as described in EPA Method 1602, section 11 (USEPA, 2001). Briefly, MS2-containing water from the diffusion chamber was serially diluted in BPBDW. Triplicate 1.0 and 0.1 ml subsamples from each of a range of dilution tubes and 0.2 ml of an overnight culture of *E. coli* host, grown in tryptic soy broth (TSB) at 35°C with low rotational mixing, were transferred to separate tubes of molten TSB with 2% (w/v) agar that had been tempered at 50°C. Each MS2:host tube was immediately but gently mixed then poured onto the surface of a tryptic soy agar (TSA) plate and allowed to harden. All plates were incubated at 36.5 ± 1°C for 18–24 h. The resulting plaques on each plate were counted and the plaque-forming units (PFU) per volume of sample processed were calculated, with the final data reported as PFU ml^{-1} ± standard deviation.

Poliovirus type 1 (PV1) enumeration

Water samples recovered from the diffusion chambers collected at each time point were processed for detection of PV1 infectivity using EPA Method 1615 (Fout et al., 2014). Briefly, each sample was serially diluted in BPBDW. A volume of a selected range of dilutions was transferred to separate T-25 culture flasks, one flask per dilution, in which monolayers of Buffalo Green Monkey (BGM) kidney cells, provided by the U.S. EPA, had been established. All flasks were incubated at 36.5 ± 1°C for up to 14 days in a 5% CO_2 humidified incubator and monitored daily until the number of infectious loci per flask stabilized. Flasks were monitored daily for cytopathic effects (CPE) as infectious foci development throughout the incubation period. Each of the inoculated flasks was scored as positive or negative based on the presence or absence of infectious foci. The MPN of infectious (viable) PV1 (MPN infectious units ml^{-1} ± standard error) was determined using the MPN calculating software MPNCALC (version 1.2.0; https://mpncalc.galaxytrakr.org/).

Cryptosporidium parvum enumeration

Water samples recovered from the diffusion chambers collected at each time point were processed for detection of *C. parvum* infectivity using a cell culture-based assay described by Johnson et al. (2012). Briefly, monolayers of the human ileocecal adenocarcinoma HCT-8 cell line (ATCC #CCL-244) were cultivated in cell culture flasks containing RPMI 1640 cell culture medium with GlutaMAX (Invitrogen) containing 5% heat-inactivated foetal bovine serum (HyClone), penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}), amphotericin B (0.25 μg ml^{-1}), and 20 mM HEPES buffer. All flasks were incubated at 36.5 ± 1°C in a 5% CO_2 humidified incubator.

Pretreatment to induce excystation was performed by incubating in acidified (pH 2.0) Hank's Balanced Salt Solution (HBSS; H9394, Sigma-Aldrich Corp.), containing 1% trypsin (AHBSS/T) for 1 h at 37°C and constant agitation. The excysted oocysts were suspended and diluted in DMEM cell culture media. The undiluted and respective dilutions were inoculated onto the HCT-8 monolayers and incubated at 36.5 ± 1°C for 64 to 72 h in a 5% CO_2 humidified incubator. Monolayers were then fixed and stained using rat anti-*Cryptosporidium* sporozoite antibody (Sporo-Glow, A600FLR-1X; Waterborne, Inc.). Slides were examined using epifluorescence microscopy equipped with a filter cube set optimized for FITC. Each of the inoculated wells was scored as positive or negative based on the presence or absence of infectious foci, respectively. The MPN of infectious (viable) *C. parvum* (MPN infectious foci ml^{-1} ± standard error) was determined using the same MPN calculating software as described for the PV1 data.

Inactivation data analyses

The enumeration data for MS2, PV1 and *C. parvum* at each time point were used to model the respective inactivation rates. All data were first $\log_{10}$-transformed, then analysed to determine the best-fit model using the software program GInaFiT (version 1.7; Geeraerd et al., 2005).

The best fit model for the respective inactivation data sets was determined to be those models with the lowest RMSE values, which measures the variances between the input and modelled data, and the highest $R^2_{adjusted}$, which measures the models' standard errors. If more than one model for the respective data sets produced similar RMSE and $R^2_{adjusted}$ values, the simpler model was selected.

The suite of inactivation models in GInaFiT include deterministic (e.g. log-linear model) and probabilistic (e.g. Weibull model) models, from which the derived inactivation rates are expressed as 1.0 $\log_{10}$ reductions day^{-1} and days 1.0 $\log_{10}$ $reduction^{-1}$ (i.e., T_{90}), respectively. To normalize the inactivation rates for direct comparison, the standard errors (SE) of the model inactivation rates were first used to calculate the respective 95% confidence intervals [±1.96 (SE)] and expressed as lower and upper limits of those inactivation rates. All inactivation rates are expressed, with the lower and upper limits on those estimates, in units of 1.0 $\log_{10}$ reductions $(day)^{-1}$ and days $(1.0\ \log_{10}\ reduction)^{-1}$.

RESULTS

MS2 inactivation

During the 30-day incubation period in native groundwater from the UFA, the infectivity within the initial population of MS2 (1.62×10^6 PFU ml^{-1}) was reduced by 5.73 $\log_{10}$ (Figure 2). The best fit model for the inactivation data (PFU ml^{-1} day^{-1}) was the Weibull frequency distribution model (Mafart, et al., 2002), a probabilistic model,

$$N_t = N_0 - \left(\frac{t}{\delta}\right)^p$$

where N_t is the $\log_{10}$-transformed PFU ml^{-1} data at a specific time during the experiment; N_0 is the modelled $\log_{10}$ transformed concentration of MS2 (PFU ml^{-1}) at the beginning of the experiment; δ is the time required for a 1.0 $\log_{10}$ reduction in the MS2 concentration [days $(\log_{10}\ reduction)^{-1}$]; p is the shape parameter that determines the overall shape of the inactivation curve; t is time (days). The MS2 inactivation rate (T_{90}) was 3.24 (1.12–5.36) days for a 1.0 $\log_{10}$ reduction in the bacteriophage concentration (Table 2).

Poliovirus type 1 inactivation

During the 58-day incubation period for the PV1 experiment, the initial population (3.50×10^6 MPN infectious units ml^{-1}) was reduced by 5.00 $\log_{10}$ (Figure 3). The best fit model for the inactivation data (MPN infectious units ml^{-1} day^{-1}) was the log-linear model (Bigelow & Esty, 1920),

$$N_t = N_0 - \frac{k_{max} * t}{2.303}$$

where N_t are the $\log_{10}$ transformed MPN values at a specific time during the experiment; N_0 is the modelled $\log_{10}$ transformed concentration of PV1(MPN infectious units ml^{-1}) at the beginning of the experiment; k_{max} is the maximum inactivation rate (day^{-1}) and t is time (days). The maximum

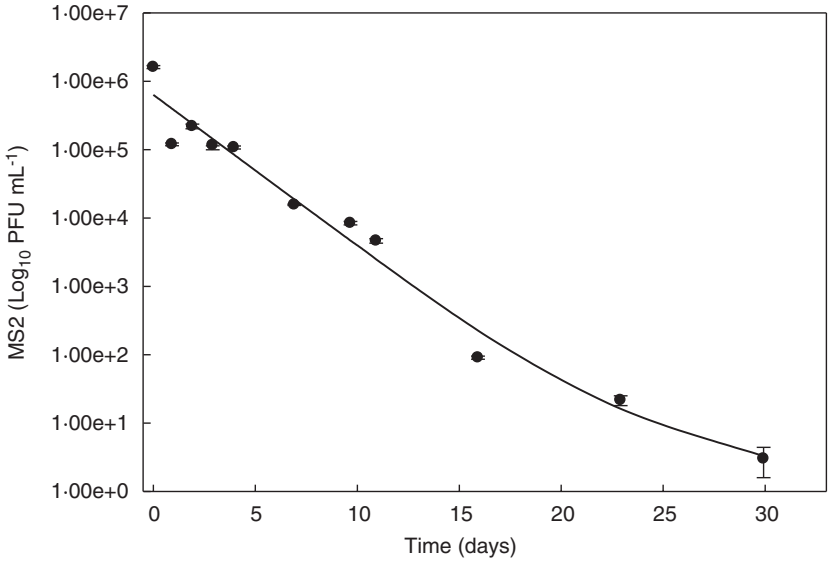

FIGURE 2 MS2 bacteriophage inactivation data. The line represents the biphasic model data. Symmetrical error bars are standard deviations on each mean of the model input data (PFU ml^{-1})

inactivation rate (k_{max}) for PV1 was 0.19 (0.17–0.21) log_{10} in the concentration of PV1 per day (Table 2).

C. parvum inactivation

As with PV1, the *C. parvum* inactivation experiment was extended to 58 days. However, the MPN estimate of *C. parvum* in the last sample included zero. The inclusion of this last data point in the analysis was considered inappropriate, and removed from the modelled data set, as it could not be unequivocally determined if the number of infectious oocysts was reduced to zero during the 21 day period between the sampling events on days 37 and 58. Thereby, the oocysts infection data between and including time zero and day 37 were included in the model analysis.

During the 37-day incubation period, the number of infectious *C. parvum* oocysts declined 4.07 log_{10} from the initial concentration of 2.80×10^4 MPN infectious foci ml^{-1} (Figure 4). The Weibull inactivation model (Mafart, et al., 2002), as described for the MS2 data, was determined to be the best fit model with the exception the N_t and N_0 data are log_{10}-transformed MPN infectious foci ml^{-1} values. The *C. parvum* inactivation rate (T_{90}) was 5.04 (2.69–7.39) days for 1.0 log_{10} reduction in oocyst concentration (Table 2).

DISCUSSION

The literature is replete with studies on the inactivation of MS2, PV1 and *C. parvum* following exposures to a variety of disinfectants. These disinfection-related inactivation rates are not comparable to the in-situ and natural inactivation rates derived during this study. However, there are numerous examples of reductions in the concentrations of MS2 (Bae & Schwab, 2008; Gordon & Toze, 2003; John & Rose, 2005; Ogorzaly et al., 2010; Regnery et al., 2017; Schijven & Hassanizadeh, 2002; Schijven et al., 1999; Toze, 2004; Yang & Griffiths, 2013; Yates et al., 1985) and PV1 (Bae & Schwab, 2008; Charles et al., 2009; Gordon & Toze, 2003; John & Rose, 2005; Mitchell & Akram, 2017; Toze, 2004; Yang & Griffiths, 2013; Yates et al., 1985) in groundwater systems when used as tracers or surrogates for the fate and transport of infectious viruses of human health concern.

Relative to the viruses, there are fewer published data on the inactivation of *C. parvum* in groundwater systems (Bae & Schwab, 2008; Ives et al., 2007; Mitchell & Akram, 2017; Ogorzaly et al., 2010; Page et al., 2010; Regnery et al., 2017; Sidhu & Toze, 2012; Sidhu et al., 2010, 2015; Toze et al., 2010). The variability of the respective inactivation and T_{90} (i.e. length of time for the original microbe concentration to be reduced by 1.0 log_{10}) rates data range over orders of magnitudes (Table 3). The underlying reasons for this variability in the collective data from the cited studies are the differences in experimental conditions under which the studies were conducted (e.g. laboratory-based studies using containers or columns filled with aquifer material, low temperatures, aerobic and oxidized groundwater, differences in the groundwater chemistries and detection and quantification methods, etc.) and how the inactivation data are modelled (e.g. linear versus non-linear models). Thereby, the direct comparison of inactivation data from this study to those from disinfection inactivation studies, laboratory bench-top experiments and field studies that use groundwater not geochemically comparable to those from this study should only be attempted with appropriate caveats.

TABLE 2 Inactivation model parameters and rates

Microorganism	Best fit model	N_0 ($\log_{10}$)	Model parameters			RMSE	R^2_{adj}	Inactivation rates ($\log_{10}$ reduction day^{-1})	T_{90} [days ($1.0\ \log_{10}$ reduction)$^{-1}$]
			k_{max} ($\log_{10}$ day^{-1})	δ (days $\log_{10}^{-1}$)	p				
MS2	Weibull	5.98 ± 0.26		3.24 ± 1.08[a]	0.78 ± 0.11	0.332	0.967	0.31 (0.19–0.89)[b]	3.24 (1.12–5.36)
Poliovirus type 1	Log-linear	6.41 ± 0.13	0.19 ± 0.01			0.344	0.944	0.19 (0.17–0.21)	5.26 (4.76–5.88)
C. parvum	Weibull	4.40 ± 0.15		5.04 ± 1.20	0.69 ± 0.07	0.183	0.980	0.20 (0.14–0.37)	5.04 (2.69–7.39)

[a]All ± are standard errors of the parameter estimates.

[b]Rates in parentheses are calculated in 95% confidence intervals.

There are a limited number of investigations on the inactivation of viruses and *Cryptosporidium* sp. in groundwater, which also used diffusion chambers, which are similar enough to this study for comparison (Page et al., 2014; Sidhu & Toze, 2012; Toze et al., 2010). The inactivation rates (T_{90} values) in groundwater systems with measurable levels of dissolved oxygen (2.0–7.0 mg L^{-1}) and positive redox potentials (~$^{+}$220 mV) ranged from 0.093 to 0.168 $\log_{10}$ day^{-1} (6.0–10.8 days) for MS2, 0.010–0.176 $\log_{10}$ day^{-1} (5.7–100.0 days) for adenovirus, 0.034 $\log_{10}$ day^{-1} (29.4 days) for rotavirus and 0.025–0.082 $\log_{10}$ day^{-1} (12.2–40.0 days) for *Cryptosporidium* sp. Additionally, whilst all of the MS2 inactivation data followed a log-linear model, whilst data for adenovirus and rotavirus (Page et al., 2014) and *Cryptosporidium* sp. (Toze et al., 2010) were best described by non-linear models (i.e. bi-phasic models).

The inactivation rate values from this study, as $\log_{10}$ reductions $(day)^{-1}$ (Table 2), are greater than those discussed in studies that also used diffusion chambers in groundwater (Page et al., 2014; Sidhu & Toze, 2012; Toze et al., 2010) by approximately 2.0–5.3-fold for MS2; approximately equivalent to 17.0-fold greater than adenovirus for PV1; 5.0–6.2-fold greater than rotavirus for PV1; 4.5–5.6-fold greater for *Cryptosporidium* sp.

Since the experimental designs for this study and the studies which also used diffusion chambers are similar, these relatively elevated inactivation rates for MS2, PV1 and *C. parvum* can be attributed to differences in the geochemistry of the groundwater in which these studies were conducted (Page et al., 2014; Sidhu & Toze, 2012; Toze et al., 2010). The two geochemical variables which separate the UFA from the cited groundwater systems are the stable anaerobic state, negative redox potentials and relatively higher concentrations of hydrogen sulphide (Table 1).

We propose these conditions make the aquifer zones in the UFA, a challenge at the physiological level for microorganisms from the surface to survive and persist. A previous study on the inactivation of *E. coli* (i.e. loss of culturability) in diffusion chambers during exposure to groundwater in the UFA and Avon Park Permeable Zone (APPZ) of the Floridan Aquifer, demonstrated the geochemical conditions in both aquifer zones produced inactivation rates 21-fold greater than those derived from studies using oxygenated groundwater with positive redox potentials and no detectable sulphides (Lisle, 2016). These enhanced inactivation rates were proposed to be induced by exposure to the anaerobic and reduced groundwater, which resulted in levels of physiological stress and/or injury from which the *E. coli* cells could not recover. With respect to a mechanism through which the geochemical conditions in the aquifer accessed for this study affects viral infectivity, we propose the low oxidation/reduction

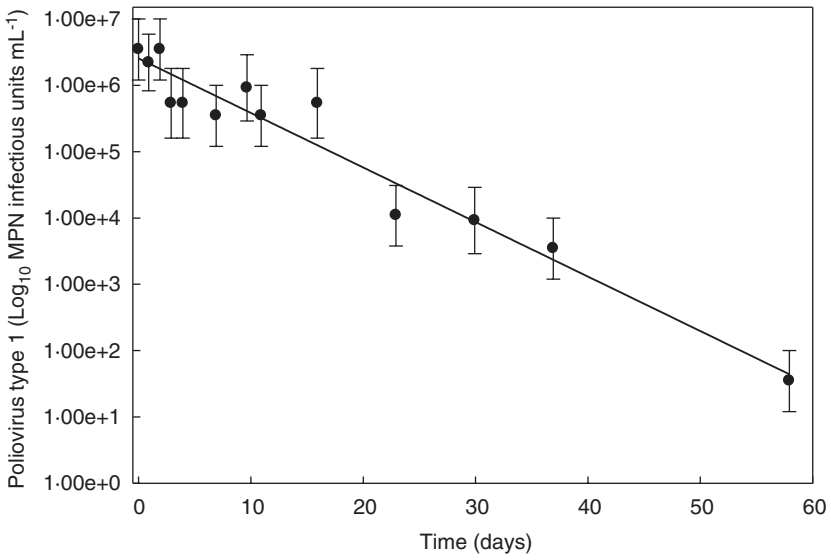

FIGURE 3 Poliovirus type 1 inactivation data. The line represents the log-linear model data. Asymmetrical error bars are 95% confidence intervals on the MPN estimates of the model input data (MPN infectious units ml^{-1})

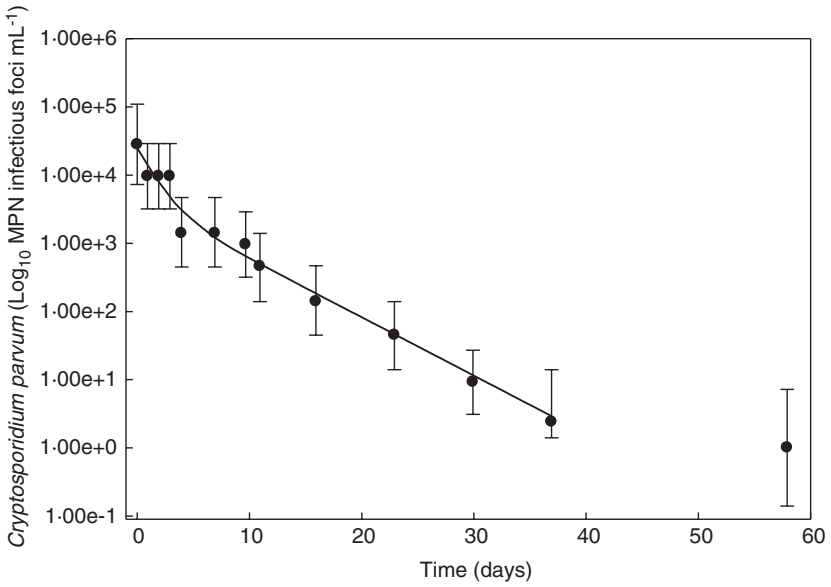

FIGURE 4 *Cryptosporidium parvum* inactivation data. The line represents the biphasic model data. Asymmetrical error bars are 95% confidence intervals on the MPN estimates of the model input data (MPN infectious foci ml^{-1})

TABLE 3 Literature inactivation rates and T_{90} estimates

	Inactivation rates		T_{90}	
Microorganism	**Published rates[a] (log_{10} day^{-1})**	**This study (log_{10} day^{-1})**	**Published rates[a] (days)**	**This study (days)**
MS2	0.0012–2.50	0.16–0.81	0.04–833.3	2.38–4.96
Poliovirus type 1	0.005–10.24	0.17–0.21	0.10–200.0	5.00–5.56
C. parvum	0.0083–1.04	0.14–0.37	0.96–120.5	3.84–6.24

[a]Bae and Schwab (2008); Charles et al. (2009); Gordon and Toze (2003); Ives et al. (2007); John and Rose (2005); Mitchell and Akram (2017); Ogorzaly et al. (2010); Page et al. (2010); Regnery et al. (2017); Schijven et al. (1999); Schijven and Hassanizadeh (2002); Sidhu et al. (2010); Sidhu et al. (2015); Sidhu and Toze (2012); Toze (2004); Toze et al. (2010); Yang and Griffiths (2013); Yates et al. (1985).

potentials (⁻325–⁻200 mV) and the presence of hydrogen sulphide (Table 1), which has been shown to inhibit the infectivity of RNA viruses (Bazhanov et al., 2017), collectively exert a level of injury at the viral surfaces and internally, to nucleic acids and proteins, which prevents successful infection of cell lines. Hydrogen sulphide has also been shown to enhance the inactivation of *E. coli* (Fu et al., 2018).

All cited studies, including this study, which used diffusion chambers, do not quantify the adsorption/desorption of the bacteria, viruses and encysted protozoans to and from the aquifer matrix as would occur during their transport through aquifer systems. Therefore, the derived inactivation rates should be considered conversative estimates for reductions in concentrations of the microorganisms assayed when applied to column and field-scale studies and reactive transport models.

The inactivation rate data from this study show the geochemical conditions of the UFA aquifer zone naturally inactivate viral surrogates (MS2), human enterovirus (PV1) and encysted protozoans (*C. parvum*) of human health and regulatory concern. The $\log_{10}$ inactivation rates for MS2 ($5.73\log_{10}$ 30 days^{-1}), PV1 (5.00 $\log_{10}$ 58 days^{-1}) and *C. parvum* (4.07 $\log_{10}$ 37 days^{-1}) indicate an effective reduction in the concentrations of these microorganisms would occur during typical storage periods (3–6 months) for injected surface water in the UFA. Currently, this $\log_{10}$ reduction or log-removal rates during the storage phase of ASR are not incorporated into relevant regulatory statutes or acknowledged by the regulatory agencies as providing an additional water treatment barrier for the protection of human health.

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CONFLICT OF INTEREST

The authors declare the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest. 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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REFERENCES

Bae, J. & Schwab, K. (2008) Evaluation of murine norovirus, feline calicivirus, poliovirus, and MS2 as surrogates for human norovirus in a model of viral persistence in surface water and groundwater. *Applied and Environmental Microbiology*, 74, 477–484.

Bazhanov, N., Escaffre, O., Freiberg, A., Garofalo, R. & Casola, A. (2017) Broad-range antiviral activity of hydrogen sulfide against highly pathogenic RNA viruses. *Scientific Reports*, 7, 41029.

Bigelow, W. & Esty, J. (1920) The thermal death point in relation to time of typical thermophilic organisms. *Journal of Infectious Diseases*, 27, 602–617.

Charles, K.J., Shore, J., Sellwood, J., Laverick, M., Hart, A. & Pedley, S. (2009) Assessment of the stability of human viruses and coliphage in groundwater by PCR and infectivity methods. *Journal of Applied Microbiology*, 106, 1827–1837.

Dillon, P., Stuyfzand, P., Grischek, T., Lluria, M., Pyne, D., Jain, R. et al. (2019) Sixty years of global progress in managed aquifer recharge. *Hydrogeology Journal*, 27, 1–30.

Florida Department of Environmental Protection (FDEP). (2013a) Drinking water standards, monitoring, and reporting. *Florida Administrative Code* Rule Chapter 62-550, Rules 102-828.

Florida Department of Environmental Protection (FDEP). (2013b) Underground injection control. *Florida Administrative Code* Rules Chapter 62-528, Rules 100-900.

Fout, S., Brinkman, N., Cashdollar, J., Griffin, S., Mcminn, B., Rhodes, E. et al. (2014) *Method 1615 Measurement of Enterovirus and Norovirus Occurrence in Water by Culture and RT-qPCR. EPA/600/R-10/181*, p. 116. Washington, DC: U.S. Environmental Protection Agency.

Fu, L., Wei, Z., Hu, K., Hu, L., Li, Y., Chen, X. et al. (2018) Hydrogen sulfide inhibits the growth of *Escherichia coli* through oxidative damage. *Journal of Microbiology*, 56, 238–245.

Geeraerd, A., Valdramidis, V. & Van Impe, J. (2005) GInaFiT, a freeware tool to assess non-log-linear microbial survivor curves. *International Journal of Food Microbiology*, 102, 95–105.

Golder and Associates. (2007) *Lake Okeechobee aquifer storage and recovery (ASR) regional project site characterization report: Construction of single-zone monitor well MW0010 and groundwater sampling site 2 (Kissimmee River site), Okeechobee County, Florida*. Jacksonville, FL: Golder and Associates.

Gordon, C. & Toze, S. (2003) Influence of groundwater characteristics on the survival of enteric viruses. *Journal of Applied Microbiology*, 95, 536–544.

Ives, R., Kamarainen, A., John, D. & Rose, J. (2007) Use of cell culture to assess *Cryptosporidium parvum* survival rates in natural groundwaters and surface waters. *Applied and Environmental Microbiology*, 73, 5968–5970.

John, D. & Rose, J. (2005) Review of factors affecting microbial survival in groundwater. *Environmental Science & Technology*, 39, 7345–7356.

Johnson, A., Giovanni, G. & Rochelle, P. (2012) Comparison of assays for sensitive and reproducible detection of cell culture-infectious *Cryptosporidium parvum* and *Cryptosporidium hominis* in drinking water. *Applied and Environmental Microbiology*, 78, 156–162.

Lisle, J. (2016) Natural inactivation of *Escherichia coli* in anaerobic and reduced groundwater. *Journal of Applied Microbiology*, 120, 1739–1750.

Mafart, P., Couvert, O., Gaillard, S. & Leguerinel, I. (2002) On calculating sterility in thermal preservation methods: application of the Weibull frequency distribution model. *International Journal of Food Microbiology*, 72, 107–113.

Maliva, R., Manahan, W. & Missimer, T. (2021) Climate change and water supply: governance and adaptation planning in Florida. *Water Policy*, 23, 521–536.

McFeters, G., Bissonnette, G., Jezeski, J., Thomson, C. & Stuart, D. (1974) Comparative survival of indicator bacteria and enteric pathogens in well water. *Applied Microbiology*, 27, 823–829.

McFeters, G. & Stuart, D. (1972) Survival of coliform bacteria in natural waters: field and laboratory studies with membrane-filter chambers. *Applied Microbiology*, 24, 805–811.

McFeters, G. & Terzieva, S. (1991) Survival of *Escherichia coli* and *Yersinia enterocolitica* in stream water: comparison and field and laboratory exposure. *Microbial Ecology*, 22, 65–74.

Mesquita, M. & Emelko, M. (2012) Bacteriophages as surrogates for the fate and transport of pathogens in source water and in drinking water treatment processes. In: Kurtboke, I. (Ed.) *Bacteriophages* (Chp. 4). London, UK: IntechOpen Limited, pp. 256.

Miller, J. (1997) Hydrogeology of Florida. In Randazzo, A. & Jones, D. (Eds.) *The geology of Florida*. Gainesville, FL: University Press of Florida, pp. 69–88.

Mirecki, J. (2013). *Comprehensive Everglades Restoration Plan aquifer storage and recovery pilot project: Final technical data report for the Kissimmee River and Hillsboro ASR systems*. Jacksonville, FL: South Florida Water Management District, West Palm Beach, FL & U.S. Army Corps of Engineers, pp. 370.

Mirecki, J., Bennett, M. & López-Baláez, M. (2013) Arsenic control during aquifer storage recovery cycle tests in the Floridan Aquifer. *Groundwater*, 51, 539–549.

Mitchell, J. & Akram, S. (2017) Pathogen specific persistence modeling data. In Rose, J. & Jimenez-Cisneros, B. (Eds.) *Global water pathogen project*. Lansing, MI: Michigan State University, pp 1–24.

Ogorzaly, L., Bertrand, I., Paris, M., Maul, A. & Gantzer, C. (2010) Occurrence, survival, and persistence of human adenoviruses and F-Specific RNA phages in raw groundwater. *Applied and Environmental Microbiology*, 76, 8019–8025.

Page, D., Dillon, P., Toze, S. & Sidhu, J. (2010) Characterising aquifer treatment for pathogens in managed aquifer recharge. *Water Science & Technology*, 62, 2009–2015.

Page, D., Miotlinski, K., Toze, S. & Barron, O. (2014) Human health risks of untreated groundwater third pipe supplies for non-potable domestic applications. *Urban Water Journal*, 11, 461–466.

Page, D., Vanderzalm, J., Barry, K., Torkzaban, S., Gonzalez, D. & Dillon, P. (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.

Plummer, N. & Sprinkle, C. (2001) Radiocarbon dating of dissolved inorganic carbon in groundwater from confined parts of the Upper Floridan aquifer, Florida, USA. *Hydrogeology Journal*, 9, 127–150.

Pyne, D. (2005) *Aquifer storage recovery: a guide to groundwater recharge through wells*. Gainesville, FL: ASR Systems.

Reese, R. & Richardson, E. (2007) *Synthesis of the hydrogeological framework of the Floridan Aquifer system and delineation of a major Avon Park permeable zone in central and southern Florida*. Report 2007-5270. Reston, VA: USGS Scientific Investigations.

Regnery, J., Gerba, C., Dickenson, E. & Drewes, J. (2017) The importance of key attenuation factors for microbial and chemical contaminants during managed aquifer recharge: a review. *Critical Reviews in Environmental Science and Technology*, 47, 1409–1452.

Schijven, J. & Hassanizadeh, S. (2002) Virus removal by soil passage at field scale and ground-water protection of sandy aquifers. *Water Science and Technology*, 46, 123–129.

Schijven, J., Hoogenboezem, W., Hassanizadeh, M. & Peters, J. (1999) Modeling removal of bacteriophages MS2 and PRD1 by dune recharge at Castricum, Netherlands. *Water Resources Research*, 35, 1101–1111.

Scott, T. (1988) *The lithostratigraphy of the hawthorn group (miocene) of Florida*. Tallahassee, FL: Florida Geological Survey Bulletin 59.

Sidhu, J. & Toze, S. (2012) Assessment of pathogen survival potential during managed aquifer recharge with diffusion chambers. *Journal of Applied Microbiology*, 113, 693–700.

Sidhu, J., Toze, S., Hodgers, L., Barry, K., Page, D., Li, Y. et al. (2015) Pathogen decay during managed aquifer recharge at four sites with different geochemical characteristics and recharge water sources. *Journal of Environmental Quality*, 44, 1402–1412.

Sidhu, J., Toze, S., Hodgers, L., Shackelton, M., Barry, K., Page, D. et al. (2010) Pathogen inactivation during passage of stormwater through a constructed reedbed and aquifer transfer, storage and recovery. *Water Science and Technology*, 62, 1190–1197.

Toze, S. (2004) Pathogen survival in groundwater during artificial recharge. In Steenvoorden, J. & Endreny, T. (Eds.) *Wastewater re-use and groundwater quality*. Wallingford, UK: International Association of Hydrological Sciences, pp 70–84.

Toze, S., Bekele, E., Page, D., Sidhu, J. & Shackleton, 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.

U.S. Environmental Protection Agency (USEPA). (2001) *Method 1602: Male-specific (F^+) and Somatic Coliphage in Water by Single Layer (SAL) Procedure*. Washington, DC: U.S. Environmental Protection Agency.

U.S. Environmental Protection Agency (USEPA). (2006) Ground water rule. *Federal Register*, 71, 65574–65660.

Waldron, M. & Horvath, L. (2010) *Construction of proximal monitor well #18 (MW-18), Kissimmee River ASR Pilot Site, Okeechobee County, Florida*. Fort Myers, FL: Entrix Inc.

Yang, Y. & Griffiths, M. (2013) Comparative persistence of subgroups of F-specific RNA phages in river water. *Applied and Environmental Microbiology*, 79, 4564–4567.

Yates, M., Gerba, C. & Kelley, L. (1985) Virus persistence in groundwater. *Applied and Environmental Microbiology*, 49, 778–781.

SUPPORTING INFORMATION

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Figure S1. Diffusion chambers. (a) A chamber is composed of two polycarbonate end caps (1), into which silicone gaskets (2) are placed, followed by placement of membrane filters (47mm diameter) (3) onto the silicone gaskets. The central chamber (4), which is fabricated from PTFE pipe (OD: 50.75 mm; ID: 38.00 mm; length: 25.5 mm) is sealed by placing the end cap containing silicone gaskets and membrane filters over both end of the chamber. Two polypropylene female luer lock adapters, with sealing caps, (5) are attached via threaded openings on opposite sides of the central chamber. Four stainless steel bolts and nuts (6) are used to tighten the end caps and ensure a watertight seal is made between the central chamber and membrane filters. (b) Completed diffusion chamber.

Figure S2. Above ground mesocosm. (a and b) The groundwater source is connected to the mesocosm using black PTFE tubing (1.27 cm OD) (1) that feeds a set of low flow valves (2) that diverts a proportion of the flow into black PTFE tubes (0.64 cm OD) (3) that connect to the stainless steel inner containers (Figure S3) that hold the diffusion chambers. The majority of the groundwater flow moves to the outer container (6) via PTFE tubing (4). (c) The completed mesocosm with the lid secured.

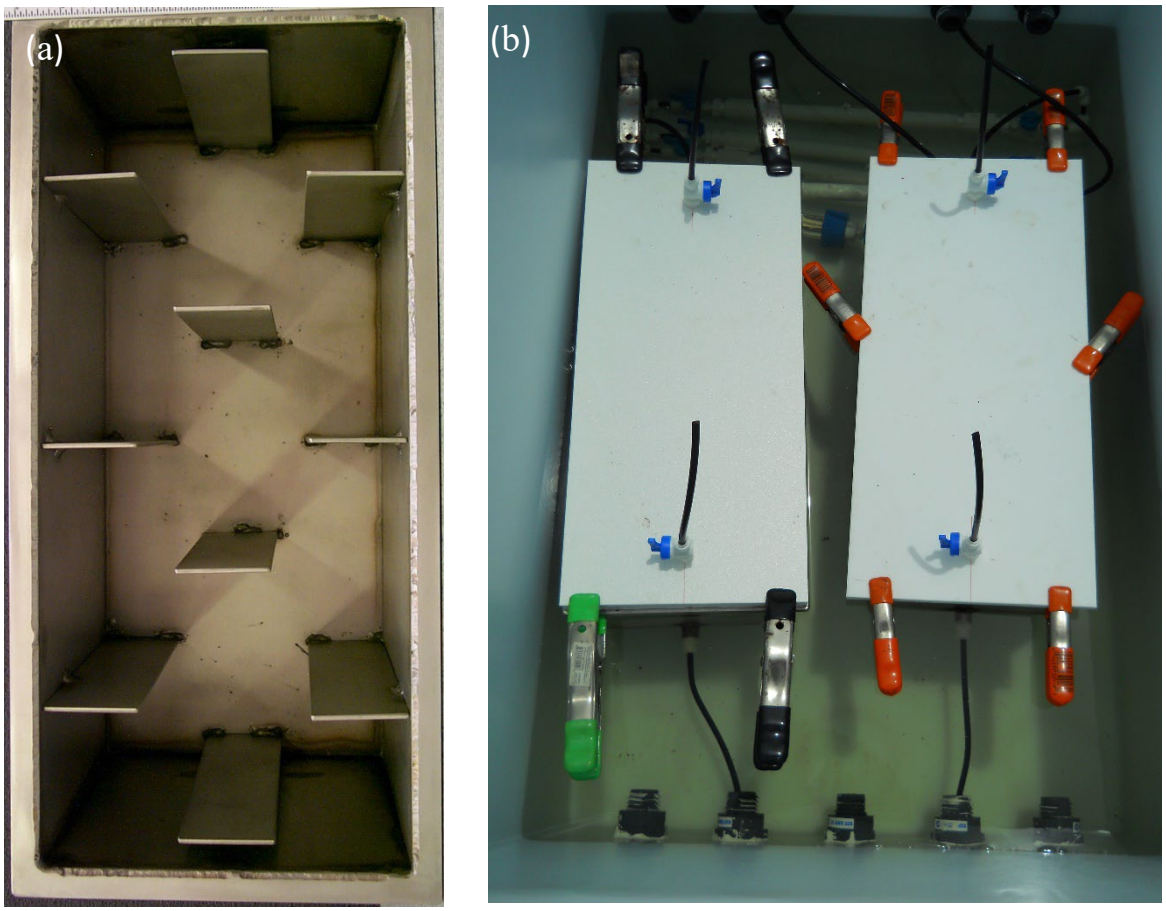

Figure S3. Inner container that holds diffusion chambers. (a) The inner container is stainless-steel (45.1 cm × 20.3 cm × 17.8 cm), with an internal volume of 16.0 L. This inner container is baffled with vertical stainless-steel inserts that ensure laminar flow, while allowing the placement of up to 15 diffusion chambers between the baffles. (b) The HDPE lid to this container has a silicone gasket to ensure a watertight seal is made after the placement of the diffusion chambers and tightening of the lid. The lid is tightened and made watertight by the placement of clamps that compress the lid and silicone gasket into the flat lip at the top of the container.