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ORIGINAL ARTICLE

Natural inactivation of *Escherichia coli* in anaerobic and reduced groundwater

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cell injury, disinfection, *Escherichia coli* (all potential pathogenic types), stress response, water.

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Abstract

Aims: Inactivation rates of *Escherichia coli* in groundwater have most often been determined in aerobic and oxidized systems. This study examined *E. coli* inactivation rates in anaerobic and extremely reduced groundwater systems that have been identified as recharge zones.

Methods and Results: Groundwater from six artesian wells was diverted to above-ground, flow-through mesocosms that contained laboratory grown *E. coli* in diffusion chambers. All groundwater was anaerobic and extremely reduced (ORP < −300 mV). Cells were plated onto mTEC agar during 21-day incubation periods. All data fit a bi-phasic inactivation model, with >95% of the *E. coli* population being inactivated <11·0 h (mean $k = 0{\cdot}488 \pm 0{\cdot}188$ h^{-1}).

Conclusions: The groundwater geochemical conditions enhanced the inactivation of *E. coli* to rates approx. 21-fold greater than previously published inactivation rate in groundwater (mean $k = 0{\cdot}023 \pm 0{\cdot}030$ h^{-1}). Also, mTEC agar inhibits *E. coli* growth following exposure to anaerobic and reduced groundwater.

Significance and Impact of the Study: Aquifer recharge zones with geochemical characteristics observed in this study complement above-ground engineered processes (e.g. filtration, disinfection), while increasing the overall indicator micro-organism log-reduction rate of a facility.

Introduction

The most recent census of groundwater resources in the United States estimates a total of $3{\cdot}1 \times 10^{11}$ l day^{-1} are withdrawn, with $2{\cdot}9 \times 10^{11}$ l day^{-1} being freshwater used for potable and agricultural applications (Maupin *et al.* 2014). These resources are under increasing pressure at a local-to-national scale with regard to quantity and quality due to climate change and significantly increased demand due to population growth.

One technological option for increasing the quantity of groundwater in an aquifer system is the injection of surface water into aquifer zones identified as being unsuitable for potable sources or agricultural uses. For example, the Floridan Aquifer (Miller 1990), from which $1{\cdot}6 \times 10^{10}$ l day^{-1} of freshwater is withdrawn (Maupin *et al.* 2014), has specific zones with moderate-to-high salinity (Miller 1990) and these zones have been identified as acceptable for the injection of a variety of water types. These include the passive recharge of storm water runoff (Bradner 1991), injection for the disposal of treated sewage and industrial wastes (Florida Department of Environmental Protection 2013b), recharge of treated surface water for aquifer storage and recovery (ASR) (Florida Department of Environmental Protection 2013b) and industrial waste streams containing carbon dioxide (Poiencot and Brown 2011; Szulczewski *et al.* 2012).

The retention of any type of recharged or injected product water within an aquifer zone for later extraction is dependent on the stratigraphy, which can be highly variable in the karstic Floridan Aquifer (Renken *et al.* 2005; Reese and Alvarez-Zarikian 2006; Reese and Richardson 2007). Accordingly, water recharged into aquifer zones that may be hydraulically connected to zones used as potable sources must meet or exceed primary and secondary drinking water standards prior to injection

(Florida Department of Environmental Protection 2013a). The monitoring criterion for the microbiological quality of recharge water into these zones in the Floridan Aquifer or other aquifer systems is the presence of the faecal indicator bacteria group, which includes *Escherichia coli* (U.S. Environmental Protection Agency 2006). Therefore, the fate and transport of *E. coli* associated with recharged water in aquifer systems is a significant public health concern. Studies have shown pathogens, including *E. coli*, can be inactivated during storage of the recharged water, although the specific native geochemical conditions which enhanced inactivation were not identified (Page *et al.* 2010a, 2015; Sidhu *et al.* 2010; Toze *et al.* 2010). In this study, *E. coli* inactivation rates were derived from culture-based data following exposure to anaerobic and extremely reduced (< −300 mV) groundwater from six artesian wells in the Floridan Aquifer. Diffusion chambers containing *E. coli* were sampled during a 21 day exposure period from a novel above-ground, flow-through mesocosm that maintained the native aquifer geochemical (except for pressure) and temperature conditions at depth (between 170–540 mbs).

Materials and methods

Site descriptions

Groundwater samples were collected from three artesian monitoring wells in south-central Florida (Fig. 1). Each well collects water from two distinct zones within the Floridan Aquifer system: the Upper Floridan aquifer (UF) and Avon Park Permeable Zone (APPZ) (Table 1). These zones of the Floridan Aquifer extend throughout central to south Florida and have been identified as being acceptable for recharge of treated surface and wastewater.

The Floridan Aquifer in this region of Florida is retained within a karstic stratigraphy, vertically stratified by confining units, maintains potentiometric surface values of 12–14 m (120–140 kPa at sampling depth) and is completely isolated from any other groundwater sources positioned above or below the zones sampled during this study (Miller 1997). Additionally, neither of these zones is impacted by meteoric or surface water as the isotopic age of the groundwater in this region of Florida has been established at approx. $2{\cdot}5 \times 10^4$ years since it was first recharged into the subsurface (Plummer and Sprinkle 2001).

Field data collection

Each well was flushed to waste for a minimum of three casing volumes (Table 1) prior to sample collection and connection of the above-ground mesocosms. During each flushing event, field data were collected from each aquifer zone for temperature (°C), salinity (g l^{-1}), total dissolved solids (TDS; g l^{-1}), dissolved oxygen (mg l^{-1}), pH and oxidation reduction potential (ORP; mV) using a YSI 556 MPS system (YSI Inc., Yellow Springs, OH, USA) attached to a flow cell. The flow cell was attached via polytetrafluoroethylene (PTFE) tubing (OD: 6·0 mm; ID: 3·0 mm) to stainless steel fittings and valves that had been connected to the well heads, establishing a side stream of controlled flow of groundwater while the wells were being flushed. The data collection interval was set at 5 min for the entirety of each flushing event.

Geochemical and nutrient sample collection and analyses

After the minimal flushing volumes had been discharged, samples were collected from the UF and APPZ zones of each well from the PTFE tubing that had been disconnected from the flow cells. All samples for dissolved organic carbon, nutrients, anions and cations were collected into bottles, containing the appropriate preservatives if required, that were provided by the certified commercial laboratory performing the analyses. All sample bottles were immediately stored on ice, in the dark and delivered to a commercial laboratory on the same day as the sample collections.

Bacterial abundances

Samples (50 ml) were collected from each aquifer zone and immediately preserved with filter sterilized formalin to a final concentration of 3% (v/v) and stored on ice. Upon return to the laboratory all samples were stored at 4°C. Within a week of sample collection, all samples were filtered and stained using SYBR Gold (ThermoFisher Scientific, Grand Island, NY, USA) as previously described for the enumeration of bacteria (Lisle and Priscu 2004). All prepared slides were counted using an epifluorescent microscope equipped with a filter cube set specifically designed to optimize the visualization of SYBR Gold.

Diffusion chambers and above-ground mesocosms

The diffusion chambers used in this study are a modification of the design described by McFeters, *et al.* (McFeters and Stuart 1972; McFeters *et al.* 1974; McFeters and Terzieva 1991). The chambers are made of polycarbonate and use polycarbonate membranes (0·2 μm pore size) to retain the bacterial suspensions while allowing dissolved constituents to exchange into and out of the chambers. (See the Supporting Information for a detailed description of the diffusion chamber design.)

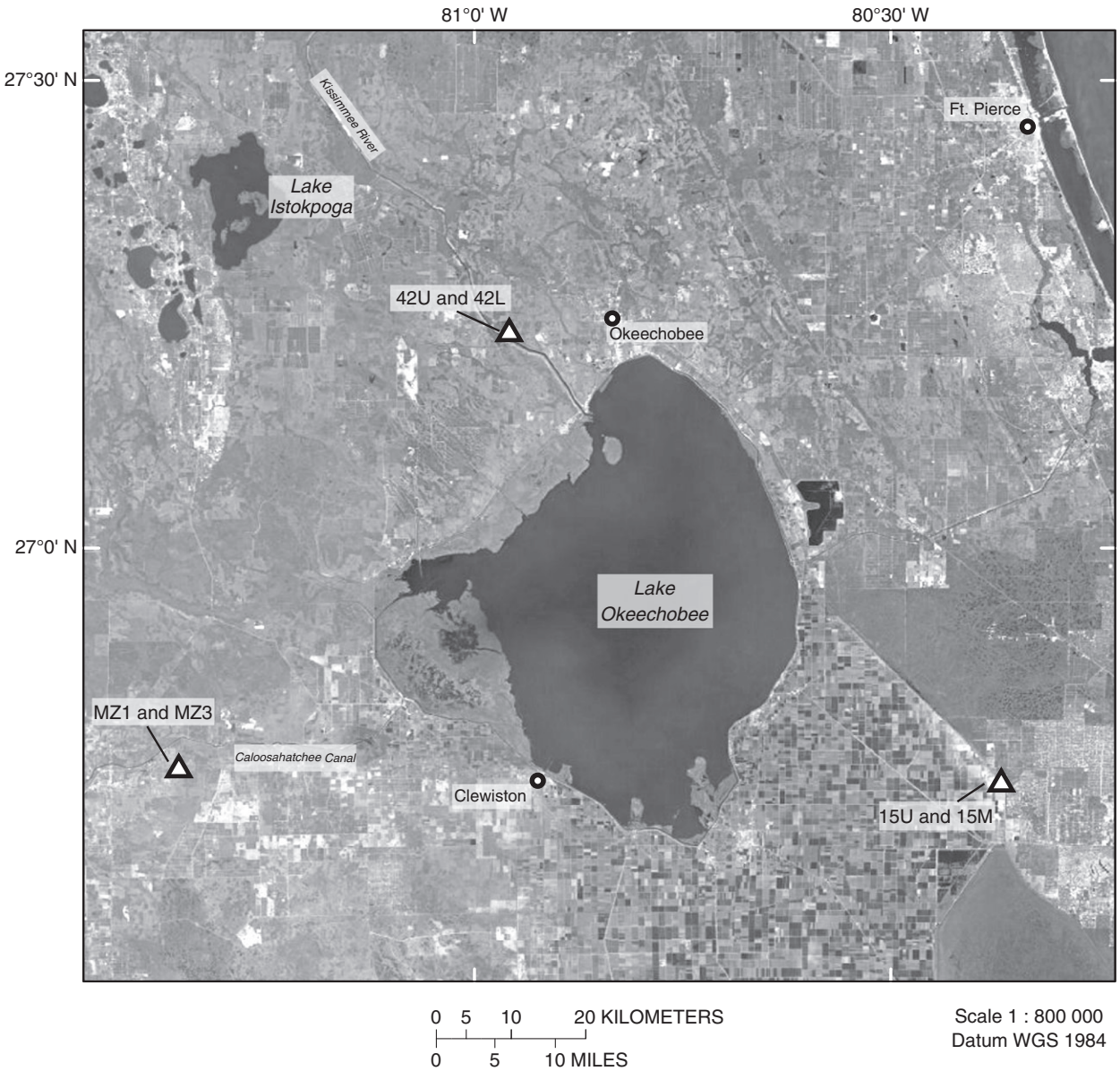

Figure 1 Groundwater sample sites. Each groundwater well (▲) collected water from two distinct zones of the Floridan Aquifer: the Upper Floridan (UF: 42U, 15U, MZ1) and the Avon Park Permeable Zone (APPZ: 42L, 15M, MZ3).

Due to aquifer access constraints which include high artesian pressures, depths of the sampling zones and the multiple time point sampling design of the experiments, down-well deployments of the diffusion chambers were impractical. An above-ground mesocosm system was designed to allow easy access to the diffusion chambers while insulating the chambers from atmospheric oxygen and temperatures and minimizing alterations in the geochemistry of the native groundwater. Hydrostatic pressure at depth (120–140 kPa at sampling depth) was not maintained in the mesocosm (101 kPa). The mesocosm system consists of two compartments; an insulating outer compartment that holds a smaller inner compartment in which the diffusion chambers are suspended.

The outer most compartment is a commercial cooler (95 l) adapted to connect directly to the well head via PTFE tubing (OD: 1·5 cm; ID: 1·3 cm) (Fig. 2). The inner compartment is constructed of stainless steel and has an internal volume of 16·0 l (45·1 cm × 20·3 cm × 17·8 cm) (Fig. 2). The lid of the inner compartment is made of a nontoxic and inert polymer that has been engineered to receive water tight threaded plugs made of the same material. The threaded plugs have attachment points on their undersides for hanging

Table 1 Groundwater well locations and descriptions

Well designation	Location		Aquifer zone	Screen type	Casing diameter (cm)	Production interval (mbls)	Presample flush volume (litres)
	Latitude	Longitude					
42U	27° 13′ 11·16″	−80° 57′ 21·98″	UF	Annular	60·96	170·7–317·0	139 841
42L			APPZ	Open	35·56	399·3–469·4	183 097
15U	26° 44′ 16·08″	−80° 21′ 48·68″	UF	Annular	45·72	298·7–348·7	95 404
15M			APPZ	Annular	30·48	426·7–482·5	93 871
MZ1	26° 45′ 11·42″	−81° 21′ 17·72″	UF	Annular	45·72	204·2–255·1	69 807
MZ3			APPZ	Open	17·78	501·4–536·1	39 929

Mbls, metres below land surface; UF, Upper Floridan aquifer zone; APPZ, Avon Park Permeable Zone.

diffusion chambers that contain the bacterial suspensions. (See the Supplemental Information for a detailed description of the mesocosm design.)

Groundwater flow through the outer compartment was maintained at high enough rates (10 l min^{-1}) to insulate the inner compartment, and thereby the diffusion chambers, from surface temperatures and atmospheric oxygen. The flow rate in the inner compartment was maintained at 150 ml min^{-1} for all experiments. This flow rate established a linear flow velocity of approx. 6·0 m day^{-1} with a residence time of approx. 2·0 h.

The ambient air and groundwater temperatures at the well head and in the outer and inner compartments were monitored throughout each experiment using HOBO Pro v2 temperature loggers (Onset Computer Corp., Inc., Bourne, MA, USA). Prior to each sampling event, separate YSI 556 MPS flow cell systems were connected to each mesocosm to collect field parameter data from the groundwater entering the mesocosm and discharging from the inner compartment.

Bacterial cultures

An environmental strain of *Escherichia coli* (ATCC #BAA-1159), originally isolated from a fresh water source, was used in the inactivation experiments. The *E. coli* strain was grown in Tryptic Soy Broth (BD Diagnostics, Franklin Lakes, NJ, USA) at 37°C with rotational shaking (160 rev min^{-1}) overnight. This culture was processed and diluted with phosphate buffered saline (PBS) (137·0 mmol l^{-1} NaCl; 2·7 mmol l^{-1} KCl; 11·9 mmol l^{-1} PO_4; pH 7·3–7·5) to a final concentration of approx. 5×10^9 CFU ml^{-1}. The diluted suspension was added to filter sterilized groundwater from the respective wells and then used to fill each diffusion chamber at an approximate concentration of 5×10^8 to

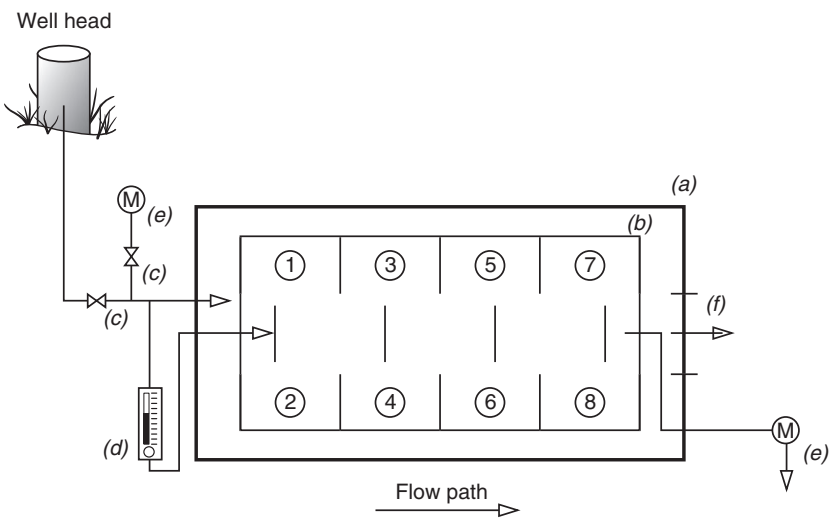

Figure 2 Above-ground mesocosm. The outer compartment (a) holds the stainless steel inner compartment (b) in which individual diffusion chambers (e.g.③) are suspended. The inner compartment is sealed so the groundwater in the outer compartment does not mix with groundwater in the inner compartment. The artesian pressure from each well pushes groundwater to the mesocosm where flow rates are controlled by inline values (c). Groundwater is diverted into the inner compartment through a flow valve (d) that reduces flow rates around the diffusion chambers to those similar in the aquifer. Geochemical parameters are measured in real time using a multi-sensor probe and flow cell (Ⓜ) at the inflow of the mesocosm and the discharge from the inner compartment (e). Groundwater in the outer compartment is discharged through a high capacity opening (f).

1×10^9 CFU chamber^{-1} (approx. 3–7 $\times$ 10^7 CFU ml^{-1}). Dilutions of the cell suspension used to inoculate the diffusion chambers (i.e. time zero data) were processed for cultivability using the media and incubation conditions described below. (See Supplemental Information for a detailed description of culture growth, processing and loading of diffusion chambers.)

After each sampling event diffusion chambers were removed from the inner chamber and *E. coli* suspensions extracted and serially diluted using PBS. Selected dilutions were filtered through membrane filters (47 mm diameter, 0·45 μm pore size), which were placed on modified mTEC agar (BD Diagnostics), hereafter referred to as mTEC agar, incubated at 35°C for 2 h, then transferred to a 44·5°C incubator for an additional 22–24 h (U.S. Environmental Protection Agency 2002). At the end of the incubation period, all filters were counted for the number of CFUs per filter and the final data expressed as CFU ml^{-1} after adjusting for the respective dilution factors. The concentrations of naturally occurring *E. coli* in the groundwater sources were also quantified using the same medium and incubation conditions as described for the laboratory grown strain.

In addition to mTEC agar, the same dilutions were plated onto R2A agar (BD Diagnostics) using a modified drop plate technique (Hoben and Somasegaran 1982). These plates were incubated at room temperature and in the dark for as many days as it took for the CFU values to stabilize, which varied between 10–14 days. All CFU data were adjusted for the respective dilution factors and volumes plated, then expressed as CFU ml^{-1}.

Inactivation data analyses

The recovery data (CFU ml^{-1}) for *E. coli* on mTEC and R2A agar were used to model the inactivation rates in both aquifer zones at each well location. The plate counts from each experiment were first $\log_{10}$-transformed, then analysed with the program GInaFiT (Geeraerd *et al.* 2005) to assist in determining the best fit model for the respective data sets. The best fit model was determined from the root mean squared error (RMSE), which is the standard deviation of the inactivation model prediction error relative to the actual data (Geeraerd *et al.* 2005). The best fit model was determined by that which produced the smallest RMSE for each inactivation data set.

Results

Groundwater and mesocosm geochemistry

The groundwater in all sample sites within the UF and APPZ aquifer zones is anaerobic and significantly reduced (range: −309 to −365 mV), with consistent temperatures (range: 25·9–27·8°C) (Table 2). Dissolved organic carbon concentrations are also relatively consistent within and between the two zones ranging between 1·1–1·9 mg l^{-1} (Table 2). The concentrations of redox-sensitive constituents (i.e. NH_4, SO_4, H_2S, Fe, Mn) are also similar in both aquifer zones, except for sulphate in well MZ3 within the APPZ (i.e. 1800 mg l^{-1}), which was between 3–10 fold higher than that in the other wells (Table 2). In addition to the groundwater sulphate concentration being significantly greater in MZ3, the concentrations of most of the redox-insensitive constituents (i.e. Br, Ca, Cl, Mg, K, Na) were also significantly greater than in the other five wells (Table 2). The relatively elevated concentrations of these dissolved constituents contribute to the high salinity, total dissolved solids and specific conductance values for the groundwater from MZ3, which collectively separates this groundwater source from the other five.

The geochemical parameters measured in the groundwater entering the mesocosm and discharging from the inner compartments (i.e. temperature, salinity, TDS, dissolved oxygen, pH, ORP) were within the standard deviations of the respective parameters in Table 2 (data not shown).

Due to the mesocosms being above ground and in open areas, the maintenance of *in situ* temperatures was a critical parameter to control. The average and range of groundwater temperatures in the outer and inner compartments of the mesocosm and corresponding ambient temperatures are given in Table 3. Collectively, these data confirm the mesocosm design maintains the geochemical character (with the exception of pressure) and temperatures of the native groundwater (Table 2) while insulating the diffusion chambers from ambient temperatures that approached 50°C.

Bacterial abundances in native groundwater samples

The abundance of naturally occurring bacteria in all wells, based on microscopic cell counts, was relatively consistent, ranging from $3{\cdot}92 \times 10^4$ cells ml^{-1} to $8{\cdot}01 \times 10^5$ cells ml^{-1}. All of the groundwater samples were negative for the presence of *E. coli* as no colonies formed on the mTEC agar plates. Additionally, all filters were scanned for presence of protist grazers, which were found to be absent.

Inactivation rates

A biphasic model provided the best fit for all data sets based on the calculated RMSE values for the respective models (Table 4). This biphasic model describes the

Table 2 Groundwater geochemical data

		Well designations					
Parameter	Units	42U (UF)	42L (APPZ)	15U (UF)	15M (APPZ)	MZ1 (UF)	MZ3 (APPZ)
Temperature*	°C	25·9 (1·13)	26·0 (1·29)	26·2 (0·24)	26·1 (0·25)	27·0 (0·19)	27·8 (0·17)
pH*		8·04 (0·36)	7·61 (0·33)	7·6 (0·35)	7·64 (0·32)	8·02 (0·24)	7·38 (0·32)
ORP*	mV	−338 (15)	−351 (32)	−355 (23)	−365 (23)	−312 (25)	−309 (22)
Salinity*	ppt	0·50 (0·01)	3·26 (0·62)	3·17 (0·11)	2·67 (0·04)	1·63 (0·01)	17·03 (1·51)
Total dissolved solids*	g l^{-1}	0·669 (0·02)	3·928 (0·72)	3·819 (0·13)	3·255 (0·04)	2·045 (0·45)	18·19 (5·87)
Specific conductance	mS cm^{-1}	1·029	6·044	5·876	5·009	3·146	27·98
Dissolved organic carbon	mg l^{-1}	1·1	1·2	1·7	1·9	1·2	1·1
Ammonium	mg l^{-1}	0·20	0·26	0·44	0·33	0·19	0·28
Sulphate	mg l^{-1}	180	510	450	370	380	1800
Sulphide	mg l^{-1}	1·4	1·6	3·7	4·2	2·1	1·6
Barium	mg l^{-1}	0·034	0·040	0·015	0·028	0·039	0·035
Bromine	mg l^{-1}	0·03	5·1	4·8	4·3	2·0	34·0
Calcium	mg l^{-1}	44	200	120	110	80	550
Chloride	mg l^{-1}	160	1600	1600	1300	640	9700
Iron (total)	mg l^{-1}	0·12	0·20	0·34	0·40	0·17	0·22
Fluoride	mg l^{-1}	0·57	0·29	0·97	1·10	0·78	0·002
Magnesium	mg l^{-1}	33·0	140·0	130·0	120·0	75·0	650·0
Manganese	mg l^{-1}	0·007	0·006	0·011	0·010	0·013	0·035
Potassium	mg l^{-1}	5·5	40·0	36·0	29·0	24·0	230·0
Silica	mg l^{-1}	14·0	12·0	13·0	13·0	9·8	9·1
Sodium	mg l^{-1}	98	800	890	740	440	4700

UF, Upper Floridan aquifer zone; APPZ, Avon Park Permeable Zone.
*Mean values (± standard deviation) are listed for those parameters measured at each sampling event (n = 10). All other values are the data from one sample collected from the respective wells at the beginning of each experiment.

Table 3 Temperatures (°C) of the groundwater in the mesocosm's two compartments and ambient air

		Inner Compartment		Outer Compartment		Ambient	
Well	Aquifer Zone	Mean (±SD)	Range	Mean (±SD)	Range	Mean (±SD)	Range
42U	UF	25·90 (1·10)	24·22–28·54	25·94 (1·07)	24·29–28·54	27·92 (7·94)	12·17–45·94
42L	APPZ	25·98 (1·36)	23·76–29·41	25·99 (1·33)	23·86–29·32		
15U	UF	26·24 (0·29)	25·84–26·97	26·25 (0·28)	25·89–26·99	30·70 (6·44)	22·99–49·58
15M	APPZ	26·05 (0·22)	25·72–26·50	26·05 (0·22)	25·74–26·50		
MZ1	UF	26·99 (0·17)	26·67–27·33	27·03 (0·16)	26·72–27·41	23·33 (6·82)	9·06–44·50
MZ3	APPZ	27·81 (0·15)	27·46–28·12	27·85 (0·15)	27·48–28·17		

UF, Upper Floridan aquifer zone; APPZ, Avon Park Permeable Zone.

inactivation of bacterial communities where one subpopulation responds to inactivating stressors more rapidly than the second subpopulation (Fig. 3a,b) (Cerf 1977; Crane and Moore 1986; Xiong *et al.* 1999). This type of community structure generates curves with an initial steep and negative slope or inactivation rate for the more sensitive subpopulation, then transitions into a much smaller negative slope that represents the second, more resistant subpopulation's inactivation rate. 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; f and (1-f) are the decimal fractions of the total bacterial abundance in the diffusion chamber that are more and less sensitive to inactivation respectively (Table 4). An estimation of the time required for a 1·0 $\log_{10}$ reduction ($t_{\log 10}$) in CFU ml^{-1} within the two bacterial subpopulations was derived using the relationship:

Table 4 Inactivation rates from a biphasic model for *Escherichia coli* in groundwater

Medium	Variable	Units	42U (UF)	42L (APPZ)	15U (UF)	15M (APPZ)	MZ1 (UF)	MZ3 (APPZ)
mTEC agar	k_1*	h^{-1}	0·295	0·684	0·217	0·540	0·627	0·564
	f †		0·983	0·959	0·998	0·990	0·983	0·988
	t_{log10}‡	h	7·8	3·4	10·6	4·3	3·7	4·1
	k_2§	h^{-1}	0·0088	0·0182	0·0064	0·0135	0·0112	0·0125
	1-f ¶		0·017	0·041	0·002	0·010	0·015	0·012
	t_{log10}	days	11·0	5·3	15·1	7·1	8·6	7·7
	RMSE**		0·126	0·242	0·303	0·379	0·587	0·434
R2A agar	k_1	h^{-1}	0·185	0·172	0·201	0·195	0·606	0·255
	f		0·982	0·996	0·996	0·995	0·995	0·998
	t_{log10}	h	12·5	13·4	11·4	11·8	3·8	9·0
	k_2	h^{-1}	0·0065	0·0003	<0·0001	0·0026	0·0080	0·0035
	1-f		0·018	0·004	0·004	0·005	0·005	0·002
	t_{log10}	days	14·7	>90·0	>90·0	36·6	12·1	27·1
	RMSE		0·201	0·077	0·281	0·286	0·417	0·267

UF, Upper Floridan aquifer zone; APPZ, Avon Park Permeable Zone.

*Inactivation rates derived from the slope of the first phase of the biphasic model.

†The decimal value of the total *E. coli* population inactivated during the first phase of the biphasic inactivation model.

‡Time required for a 1·0 log_{10}-reduction in the *E. coli* population.

§Inactivation rates derived from the slope of the second phase of the biphasic model.

¶The decimal value of the total *E. coli* population inactivated during the second phase of the biphasic inactivation model.

**RMSE = the root mean square error value for the complete biphasic model.

$$t_{\log_{10}} = \frac{2{\cdot}303}{k_n}$$

where, depending on which phase of the inactivation curve is being considered, k_n is either the inactivation rate k_1 or k_2 (Table 4).

The average (± standard deviation) inactivation rate for the first phase of the inactivation model [k_1: [0·380 (±0·218) h^{-1}] was approx. 42-fold greater than the k_2 rate [0·009 (±0·002) h^{-1}] when exposed to groundwater in the UF and recovered on the selective medium mTEC agar (Table 4). Exposure to groundwater in the APPZ inactivated *E. coli* at approx. 1·5-fold greater rates than in the UF. As shown in the UF groundwater, the average k_1 rate [0·596 (±0·077) h^{-1}] in the APPZ groundwater was approx. 40-fold greater than the k_2 rate [0·015 (±0·003) h^{-1}].

When using the nonselective medium R2A agar, the average k_1 [0·331 (±0·239) h^{-1}] and k_2 [0·005 (±0·004) h^{-1}] inactivation rates in UF groundwater were similar to those calculated from the mTEC agar data (Table 4). However, when comparing the average inactivation rates in the APPZ, the average k_1 rate from the R2A agar data [0·207 (±0·043) h^{-1}] was approximately threefold lower than the average rate from the mTEC agar data [0·596 (±0·077) h^{-1}]. Similarly, the average k_2 rate from the R2A agar data [0·002 (±0·002) h^{-1}] was approximately eightfold lower when compared to the average k_2 rate [0·015 (±0·003) h^{-1}] when recovered on mTEC agar. These data indicate R2A agar recovered significantly greater numbers of *E. coli* from the same diffusion chamber samples.

When taken collectively, the k_1 rates predict between 95·9–99·8% of the *E. coli* population present in the recharged water would experience a 1·0-log_{10} reduction in 3·4–13·4 h after contact with the native groundwater in the UF and APPZ zones of the aquifer (Table 4). When comparing inactivation rates between the two aquifer zones, the k_1 and k_2 rates for the APPZ are approx. 1·6-fold greater than the same rates in the UF, when using mTEC agar. However, this relationship inverts for the R2A agar data, with k_1 and k_2 in the UF being approx. 1·6-fold and 2·2-fold greater than in the APPZ respectively (Table 4).

Discussion

Several studies have quantified the inactivation of *E. coli* in groundwater and found their respective data sets fits a linear inactivation model (Table 5) (McFeters *et al.* 1974; Keswick *et al.* 1982; Bitton *et al.* 1983; John 2003; Cook and Bolster 2007; Sidhu and Toze 2012; Page *et al.* 2014; Sidhu *et al.* 2015). Most of these studies were laboratory based or used above-ground and open systems where native groundwater geochemistry conditions were not maintained. Only three studies used an experimental design similar to this study, where *E. coli* within diffusion chambers was in direct contact with native groundwater and geochemical conditions (Sidhu and Toze 2012; Page *et al.* 2014; Sidhu *et al.* 2015). In general, all of these studies were conducted in groundwater that contained

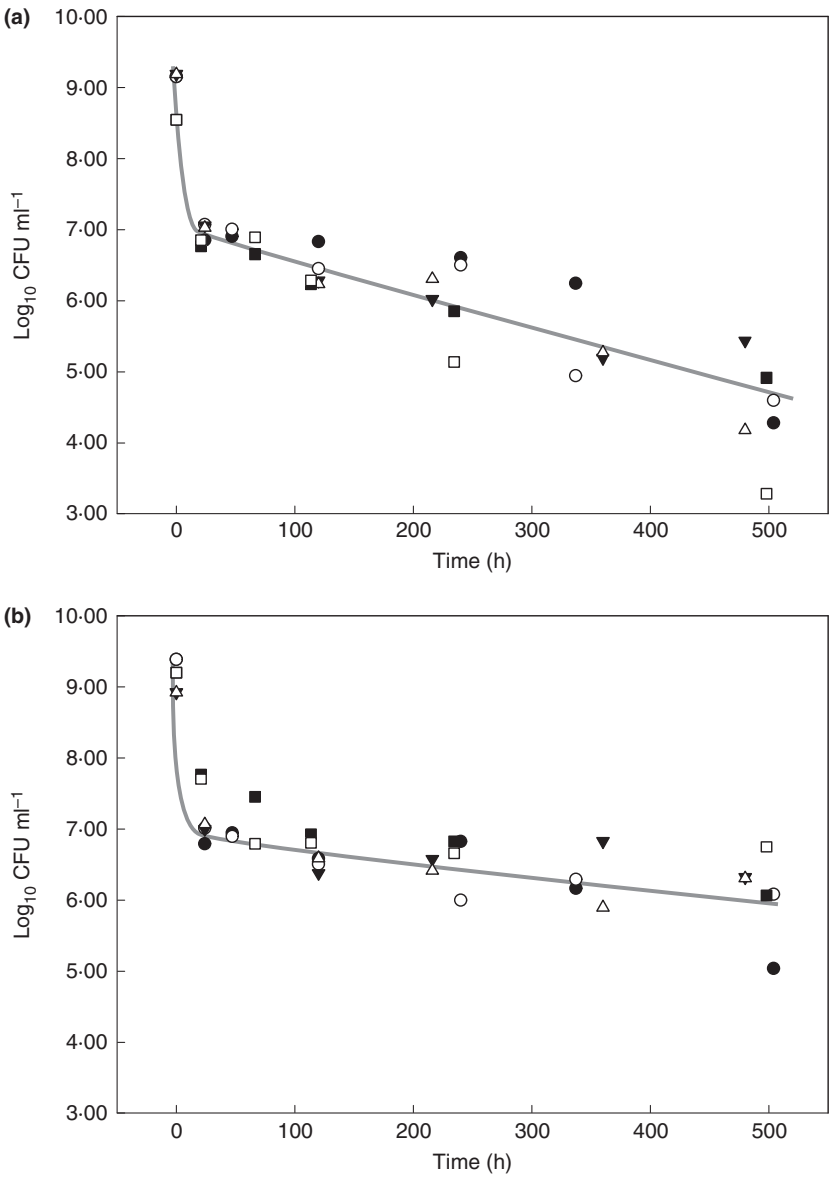

Figure 3 Biphasic inactivation curve data. The *Escherichia coli* inactivation data sets from the respective biphasic models for each well (■ 42U; □ 42L; ▼ 15U; △ 15M; ● MZ1; ○ MZ2) and the general biphasic model trend lines when recovered on mTEC agar (a) and R2A agar (b).

dissolved oxygen (0·1–2·0 mg l^{-1}) with positive or slightly negative ORP values, which are the geochemical variables that differ most between the cited groundwater sources and the UF and APPZ.

The anaerobic and reduced conditions in the UF and APPZ provided an environment in which a significant proportion of an *E. coli* community was inactivated at higher rates than previously published for groundwater systems. However, under the geochemical conditions in these zones of the Floridan Aquifer, the calculated inactivation rates for both subpopulations may be overestimates due to the inhibitory effects of the medium required for regulatory compliance monitoring (i.e. mTEC agar). Ingredients in culture media, similar to

Table 5 *Escherichia coli* inactivation rates in groundwater

Experimental design	Inactivation rate (h^{-1})	Reference
Diffusion chambers (*in situ*)	0·029	Sidhu and Toze (2012)
	0·018	Page *et al.* (2014)
	0·096	Sidhu *et al.* (2015)
Diffusion chambers (above-ground & open systems)	0·015	McFeters *et al.* (1974)
	0·013	Keswick *et al.* (1982)
Bench top beakers	0·007	Bitton *et al.* (1983)
	0·007	John (2003)
	0·002	Cook and Bolster (2007)

those in mTEC agar (Zimbro *et al.* 2009), have been shown to inhibit the growth and overall recovery of indicator bacteria, including *E. coli*, (Bissonnette *et al.* 1975; LeChevallier *et al.* 1983; McFeters 1990; Lisle *et al.* 1998; McFeters and LeChevalier 2000). To provide some insight into the extent that recovery media inhibited the growth of *E. coli* following exposure to groundwater from the UF and APPZ, data from mTEC agar were compared to data from the nonselective medium R2A agar. R2A agar's formulation and lower incubation temperature enhances the repair and recovery of physiologically stressed or injured bacteria from water (Reasoner and Geldreich 1985). Additionally, it contains no selective or differential ingredients as in mTEC agar. The general trend is the k_1 and k_2 inactivation rates derived from colony counts on mTEC agar are systematically greater than those from the R2A agar data (Fig. 4a,b). The difference between the inactivation rates from the two media indicate there is a bias towards lower recovery rates (i.e. CFU ml^{-1}) on mTEC agar, thereby increasing the probability of false negatives and artificially increasing the respective inactivation rates.

The recovery biases associated with using mTEC agar do not, however, explain the most striking difference between the *E. coli* inactivation rates in this study and those from the cited studies: biphasic *vs* linear inactivation models. The biphasic model assumes the presence of two subpopulations in the bacterial community, with one subpopulation being more physiologically susceptible to inactivation than the other (Cerf 1977). However, the cultures used in this study and those cited (Sidhu and Toze 2012; Page *et al.* 2014; Sidhu *et al.* 2015) were all monocultures that had been grown under laboratory conditions. It is presumed that all bacterial cells in these types of cultures are physiologically homogeneous and equally susceptible to inactivation, thereby leading to the

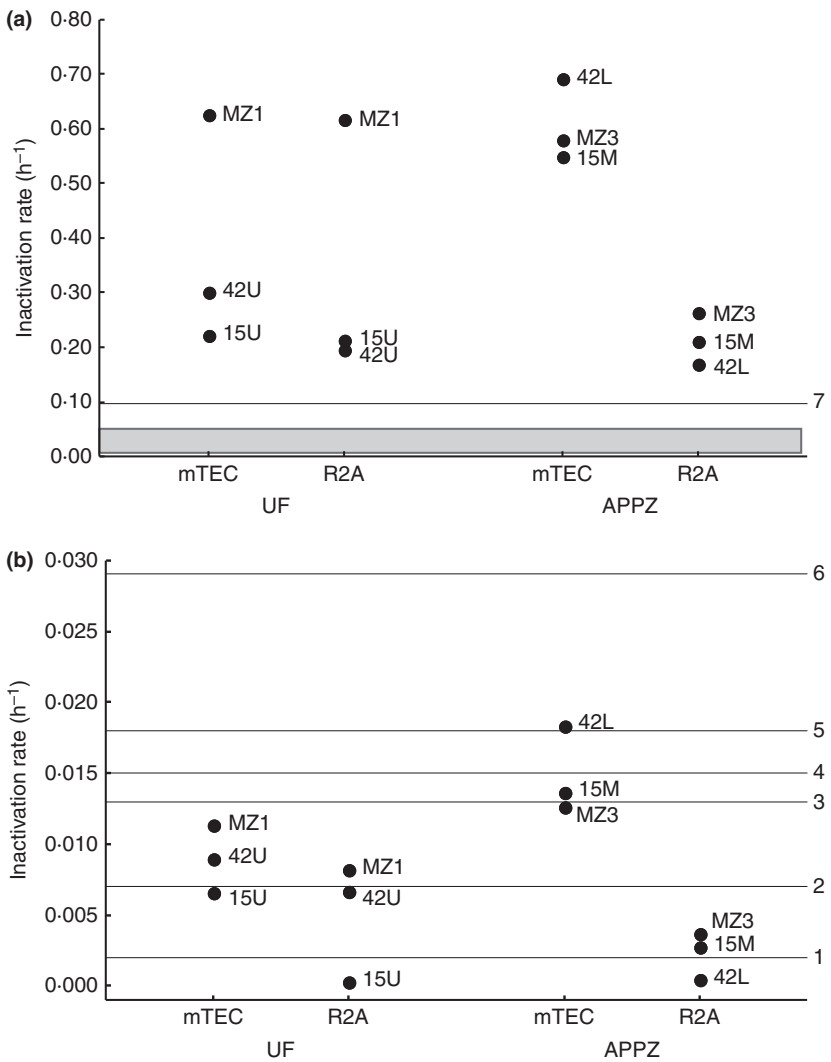

Figure 4 *Escherichia coli* inactivation rates in groundwater. The k_1 inactivation rates (a) for *E. coli* in both aquifer zones and the respective recovery media were significantly faster than the respective k_2 rates (b). The k_2 inactivation rate interval in (a) (▭) is expanded in (b). Only the k_2 inactivation rates were similar to previously published inactivation rates for *E. coli* in groundwater (b), where: 1 = 0·002 h^{-1} (Cook and Bolster 2007); 2 = 0·007 h^{-1} (Bitton *et al.* 1983; John 2003); 3 = 0·013 h^{-1} (Keswick *et al.* 1982); 4 = 0·015 h^{-1} (McFeters *et al.* 1974); 5 = 0·018 h^{-1} (Page *et al.* 2014); 6 = 0·029 h^{-1}(Sidhu and Toze 2012); 7 = 0·096 h^{-1} (Sidhu *et al.* 2015).

application of linear models to describe the inactivation rates. Recently, it has been demonstrated that intracellular stochastic processes in laboratory cultures and environmental populations of *E. coli* promote the development of phenotypically heterogeneous populations, with one of the subpopulations (i.e. persisters) being more resistant to a range of environmental stresses and having reduced growth rates relative to the other subpopulation (Balaban *et al.* 2004; Maisonneuve and Gerdes 2014; Norman *et al.* 2015). I propose a similar process occurred in the *E. coli* cultures in this and the cited studies, whereby within each population retained in a diffusion chamber there existed a subpopulation that was more susceptible to inactivation. The growth rates in this bi-population structure would fit a bi-phasic inactivation model, with the most sensitive subpopulation being inactivated first (k_1 rates) and the slower growing, more resistant subpopulation persisting for longer periods of time (k_2 rates), if a stressor to which the cells were sensitive was present.

Anaerobic conditions and ORP as low as those in the UF and APPZ have been shown to cause cellular damage and reduction in growth rates in *E. coli* (Riondet *et al.* 1999, 2000; Lee *et al.* 2001) and other bacteria included in the bacterial indicator group (Zhu *et al.* 2014). I propose these conditions collectively promote similar types of physiological damage, to which the more sensitive subpopulation (i.e. *f* subpopulation) responds by losing cultivability or being inactivated at a faster rate (k_1 rate) (Table 4).

Regardless of a pre-recharge disinfection step being required or not, microbial indicators will be in the injected water. Admittedly, bacterial indicators have been shown to be inactivated in groundwater at significantly greater rates than pathogens thereby minimizing their ability to predict the inactivation of enteroviruses and protozoa (Page *et al.* 2010b; Sidhu *et al.* 2010, 2015; Toze *et al.* 2010). However, regulatory maximum contaminant levels for monitoring the microbiological quality of water intended for injection are based on the occurrence of *E. coli* (U.S. Environmental Protection Agency 2006). Accordingly, the fate and transport of these micro-organisms in groundwater systems into which they are injected are of public health and regulatory interest.

This study demonstrates anaerobic and reduced groundwater, like that in the UF and APPZ zones of the Floridan Aquifer can enhance the natural inactivation of *E. coli* at rates significantly greater than those previously published from other groundwater systems. However, current regulatory statutes do not recognize reductions of *E. coli* or pathogens during storage of recharged water in the subsurface as a treatment step that may compliment engineered processes (e.g. filtration, disinfection), thereby increasing the overall log-removal rate of a treatment facility.

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Conflict of Interest

The author declared no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Diffusion chamber components.

Figure S2 The outer chamber of the above ground mesocosm.

Figure S3 Inner chamber of the above ground mesocosm.