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Survival of animal and human-associated *Listeria monocytogenes* in drinking water and biofilms

Louise Appel Bjergbæk, Martin Hesselsoe, Sussie Pagh and Peter Roslev

ABSTRACT

Land slugs are occasionally observed as contaminants in groundwater wells and drinking water treatment plants including storage tanks. Slugs may feed on carrion and feces, and they are potential vectors of pathogens such as *Listeria monocytogenes*. We isolated *L. monocytogenes* from the pest slug *Arion vulgaris* and examined the persistence and survival of human- and slug-derived *L. monocytogenes* in groundwater-based drinking water and biofilms. *L. monocytogenes* survival was evaluated using cultivation and cultivation-independent techniques. *L. monocytogenes* remained culturable for 35–47 days in drinking water with first-order decay rates between 0.314 and 0.457 h^{-1} (*T*99 $\geq$10 days). Attachment of *L. monocytogenes* to filter sand delayed washout from drinking water filters and increased persistence 2–3-fold. Indigenous biofilms stimulated initial surface attachment 10–100-fold but *L. monocytogenes* declined more rapidly in drinking water biofilms compared with virgin filters not colonized by microorganisms. Grazing by protozoa likely attenuated *L. monocytogenes* survival in some drinking water biofilms. A comparable survival pattern was observed for *L. monocytogenes* and the fecal indicator bacterium *E. coli*. The study suggests that live *L. monocytogenes* can persist for weeks as sessile organisms in groundwater-based drinking water supplies and may subsequently be released to the drinking water.

Key words | drinking water and biofilms, *Escherichia coli*, *Listeria monocytogenes*, slugs, survival

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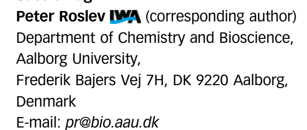

HIGHLIGHTS

- This is likely the first study examining the persistence of *Listeria monocytogenes* from humans and slugs in drinking water and biofilms.
- Land slugs can contaminante groundwater wells, drinking water treatment plants and water storage tanks. Slugs feed on carrion and feces, and are potential vectors of *L. monocytogenes*.
- Live *L. monocytogenes* can survive for weeks in drinking water and filter material may increase persistence.

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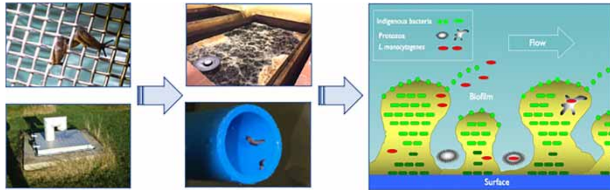
GRAPHICAL ABSTRACT



INTRODUCTION

Contamination of water resources and drinking water with small animals and animal waste may result in transmission of live pathogens (Levy 1990; Bitton 2014; Hynds *et al.* 2014; Stokdyk *et al.* 2020). Gastropods such as land slugs are occasionally observed as contaminants in groundwater wells and drinking water storage tanks and as unwelcome intruders at drinking water treatment plants. Slugs often feed on a variety of organic food items and are also known to ingest feces from animals and humans as part of their diet (coprophiles). Several potential pathogens have been observed in the intestinal tract and on the exterior surface of land slugs including *Escherichia coli, Enterococcus faecalis, Pseudomonas aeruginosa, Clostridium perfringens, Clostridium botulinum* and *Listeria monocytogenes* (Elliot 1970; Sproston *et al.* 2006; Gismervik *et al.* 2014, 2015).

The land slug *Arion vulgaris* (formerly *Arion lusitanicus*) is considered a major pest in many regions of Europe and the population has increased significantly in the last 20 years (Pfenninger *et al.* 2014; Zemanova *et al.* 2016). *A. vulgaris* is active during periods with high humidity and during the night (Cameron 2016). *A. vulgaris* prefers to hide in dark and moist places, and seeks protection in moist and frost-protected places during winter (Cameron 2016). *A. vulgaris can* range in size from >10 cm to <1 mm and this and other land slugs can penetrate protective barriers less than a few mm (Figure 1). As a result, *A. vulgaris* can be observed in many anthropogenic habitats and sometimes as a contaminant in urban and rural water utilities. *A. vulgaris* has been shown to be a potential vector for *L. monocytogenes* and this pathogen has been detected in slug mucus and feces at concentrations of 10–1,205 CFUs per g slug biomass (Gismervik *et al.* 2015). Because *A. vulgaris* is a potential vector for Listeria it is likely that this and other pathogens can be transmitted to drinking water if the water utility is compromised by slugs.

Listeria monocytogenes is a facultative anaerobic Gram-positive, non-spore-forming psychrotrophic bacterium that is relatively resistant to adverse environmental conditions (Ivanek *et al.* 2006; Gandhi & Chikindas 2007). *L. monocytogenes* is widespread in the environment including soil, water and feces, and it has many known animal reservoirs (Ivanek *et al.* 2006; Hellberg & Chu 2016). *L. monocytogenes* is a pathogen and the causative agent of listeriolosis and other illnesses in animals and humans. Listeriolosis is associated with a relatively high case-fatality rate in humans, and because the mortality rate can be high, many countries have a zero tolerance for this bacterium in relation to food and water (Gião & Keevil 2014; Pandove *et al.* 2016). *L. monocytogenes* has multiple environmental transmission routes and the prevention of infection of animals and humans is complex and challenging (Ivanek *et al.* 2006). Interestingly, the environmental occurrence and survival of *L. monocytogenes* may increase with future climate changes (Hellberg & Chu 2016). However, little is known about the persistence of *L. monocytogenes* in many types of drinking water and in drinking water biofilms. Indigenous drinking water biofilms have been implicated as environmental reservoirs or safe havens for intruding pathogens (Keevil 2002; Wingender & Flemming 2011; Bitton 2014).

In the present study, we investigated the persistence of slug- and human-associated *L. monocytogenes* strains in municipal drinking water produced from groundwater. Flow systems were used to examine the fate and survival

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Figure 1 | Examples of juvenile slugs occasionally observed in water supplies: (a) juvenile *Arion vulgaris*, (b) juvenile *Arion distinctus* and *Deroceras reticulatum/agreste*, (c) *Arion* sp. slug crossing a 4 mm mesh barrier, (d) *Deroceras reticulatum/agreste* slugs crossing a 2 mm mesh barrier.

of *L. monocytogenes* in drinking water sand filters with and without biofilms. Radiolabeling of *Listeria* cells was used to study cell turnover, and results for *L. monocytogenes* were compared with results for the traditional fecal pollution indicator *E. coli*.

MATERIALS AND METHODS

Drinking water

Drinking water samples for *Listeria* screening were obtained from five rural Danish drinking water treatment plants that use groundwater as source water (Ingstrup, Mosbjerg, Tolne, Kringelmosevej, Ravnshøj). Grab samples (10 L) of source water were collected before water treatment. The water treatment consisted of aeration and basic sand filtration followed by distribution without disinfection. Aeration followed by filtration in open or closed sand filters without subsequent disinfection represent the standard operating procedure for the vast majority of municipal and private waterworks in Denmark.

For microcosm experiments, municipal drinking water was obtained from Aalborg Municipality (Denmark). The source water was non-disinfected hard groundwater (12–16 °dH) abstracted directly from chalk aquifers. Limited water treatment is employed by this municipality before distribution to the consumers, and this water was selected for the microcosm experiment to mimic groundwater and drinking water with minimum treatment. The drinking water is naturally nutrient-poor (low in total P, N and organic C), with a non-volatile organic carbon concentration (NVOC) of 1.3 mg/L, a chemical oxygen demand (COD) of 0.4 mg/L, an NO_3^- concentration of 4.8 mg/L, and concentrations of NH_4^+, NO_2^-, and total P below 0.1 mg/L. The water temperature, pH and turbidity were 9.8 °C, 7.6 and <0.05 FTU, respectively.

Isolation and growth of bacteria

The *Listeria* strains used in the drinking water experiments are listed in Table 1. Six *Listeria* strains were isolated from the pest slug *A. vulgaris* (Figure 1). *A. vulgaris* was collected in areas with drinking water abstraction and slug samples

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Table 1 | Origin of the *Listeria* strains used in drinking water experiments

Organism	Strain	Origin
L. monocytogenes	Slug1	Slug
L. monocytogenes	Slug2	Slug
L. monocytogenes	Slug3	Slug
L. monocytogenes	Slug4	Slug
L. monocytogenes	DSM 20600	Rabbit
L. monocytogenes	DSM 12464	Poultry
L. monocytogenes	SS-ENK	Human infection
L. monocytogenes	Scott A	Human infection

were subsequently used for enrichment and isolation of *Listeria*. Slug slime and slug feces were serially diluted in Ringer's solution and then streaked onto Brilliance Listeria Agar (Oxoid Ltd, UK). The Listeria agar was supplemented with a selective supplement (Nalidixic acid, Polymyxin B, Ceftazidime, Amphotericin), and a differential supplement (Lecithin) to promote identification of presumptive *L. monocytogenes* (Oxoid Ltd, UK). Plates were incubated for 48 h at 30 °C and then screened for presumptive *L. monocytogenes* (ß-glucosidase positive and Lecithinase positive). Isolated strains were subsequently characterized by the Oxoid Biochemical Identification System (OBIS; Oxoid Ltd, UK) and the PhenePlate system (PhPlate Microplate Techniques AB, Sweden).

The human clinical strain *L. monocytogenes* SS-ENK was kindly provided by Prof. H. Schönheyder, Aalborg University Hospital, Denmark. The clinical reference strain *L. monocytogenes* Scott A was kindly provided by Prof. S. Knøchel, University of Copenhagen, Denmark. *L. monocytogenes* DSM 20600 and *L. monocytogenes* DSM 12464 were obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Germany).

Environmental *Listeria* strains and reference *Listeria* strains were grown for 18 h at 30 °C in Brain Heart Infusion Broth (BHI; LAB M, UK) before being spiked into drinking water microcosms. All strains were centrifuged (10,000 g; 10 min) and washed with filter sterilized drinking water before spiking. *E. coli* ATCC 59222 (American Type Culture Collection, USA) was included in some survival experiments to compare results obtained with *Listeria* with that of a traditional fecal indicator bacterium (FIB). *E. coli* ATCC 25922 was grown for 18 h at 30 °C in Tryptic Soy Broth (TSB; Difco, USA) and then centrifuged and washed as described above for *Listeria*.

Drinking water microcosms

Initial survival experiments with *L. monocytogenes* in drinking water were conducted using glass microcosms as described by Bjergbæk & Roslev (2005). The water used in the microcosms was municipal drinking water as described above. *L. monocytogenes* were inoculated into 100 mL drinking water to obtain a final concentration of approximately 10^6 bacteria mL^{-1}. The microcosms were incubated in the dark at 10 °C on a rotary shaker at 120 rpm. Triplicate microcosms were incubated for each *Listeria* strain.

Experiments focusing on the persistence of *L. monocytogenes* in drinking water filters with and without biofilms were carried out using 50 cm^3 glass columns packed with 50 gram of 2 mm quartz sand (Figure 2). Two systems were constructed containing either sterile quartz sand ($n = 3$) or quartz sand covered with a drinking water biofilm ($n = 3$). The drinking water biofilm had been established over three months in a flow system with a continuous flow of municipal tap water (non-disinfected). Initial drinking water biofilm formation on quartz sand and subsequent survival experiments with *Listeria* were carried out in the dark at 10 °C. Drinking water was pumped through the sand columns at a flow of 8 mL min^{-1}. The water was recycled from a 5 L reservoir and was partly replaced with fresh drinking water every 48 h.

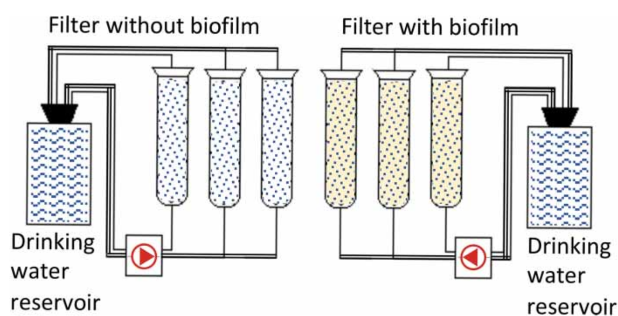

Figure 2 | Drinking water filter system for studying survival and attachment of *L. monocytogenes* and *E. coli* to filter quartz sand with and without an indigenous drinking water biofilm.

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L. monocytogenes were inoculated into the drinking water reservoirs of the flow systems to a final concentration of approximately 10^6 cells mL^{-1} (Figure 2). Separate flow systems were inoculated with *E. coli* to similar densities to compare the persistence of *Listeria* and that of a traditional FIB. Water was sampled from the reservoir every 48 h before the water was replaced. Quartz sand was sampled from the columns at the end of the experiment and transferred to filter sterilized drinking water. Attached bacteria were dispersed by three sequential treatments of 30 sec sonication (47 kHz, Branson Ultrasonics, USA), and 30 sec vortex mixing. Water and biofilm suspensions were analyzed for bacterial content as described below.

Enumeration of bacteria

Culturable *Listeria* in environmental samples were analyzed in triplicates by membrane filtration of 1–4 L groundwater using 0.45 μm mixed cellulose ester filters (Millipore, USA). The filters were transferred to sterile pads (Millipore, USA) soaked with Fraser Broth (Sigma-Aldrich), and resuscitated for 18 h at 30 °C followed by a transfer to Brilliance Listeria agar with selective and differential supplements as described above. *Listeria* plates were incubated at 36 °C and examined after 48 h.

Culturable *L. monocytogenes* in laboratory experiments was detected in water and biofilm suspensions by plating 0.1 mL aliquots of serially diluted samples onto Brilliance Listeria agar with selective and differential supplements as described above. Plates were incubated at 36 °C and examined after 48 h.

Culturable *E. coli* in laboratory experiments was detected in water and biofilm by membrane filtration followed by incubation for 24 h at 36 °C on Chromocult Coliform Agar (CCA; Merck, Germany) as described previously (Roslev *et al.* 2004). Filtration was carried out using 0.45 μm mixed cellulose ester filters (Millipore, USA).

Direct viable count (DVC) combined with fluorescent *in situ* hybridization (FISH) was used to detect viable *L. monocytogenes* using a modified version of the method described by Besnard *et al.* (2000). Samples were filtered onto black polycarbonate membrane filters (0.2 μm pore size; Poretics Products, USA). Filters were placed on pads soaked with Brain Heart Infusion Broth containing 1.0% yeast extract (Difco, USA) and ciprofloxacin at a final concentration of 1.0 μg mL^{-1} (Sigma-Aldrich, Germany). Filters were incubated for 7 h at 30 °C and FISH was then performed on the filters using the 'Lis 637' probe to identify *L. monocytogenes* (Schmid *et al.* 2003). FISH-positive cells were enumerated by epifluorescence microscopy using a Zeiss Axioskop (Zeiss, Germany) at a magnification of 1,000. At least 40 random microscope fields were enumerated for each hybridization. The most accurate cell count was obtained for filters containing 10^5–10^6 cells and was evaluated by comparing measured and nominal concentrations. The sample volume for filtration was then adjusted accordingly. Cells were considered DVC-positive if they were FISH-positive and $\geq$1.5 times the average length of control cells that had not been incubated with ciprofloxacin.

Turnover of ^{14}C-labelled bacteria

L. monocytogenes and *E. coli* were radiolabelled with ^{14}C to study cell turnover in drinking water biofilms. Endogenous bacterial respiration in live cells will produce radiolabelled CO_2 ($^{14}CO_2$) whereas ingestion and degradation of labelled bacteria by other microorganisms (e.g., grazing by protozoa) will further increase $^{14}CO_2$ release (Taylor & Sullivan 1984).

L. monocytogenes DSM 12464 and *E. coli* ATCC 25922 were grown for 18 h at 30 °C in 1 mL 10% Tryptic Soy Broth supplemented with 0.125 mL [^{14}C]glucose (7.4 MBq mL^{-1} stock solution, 11.47 GBq $mmol^{-1}$, D-[U-^{14}C]glucose; Amersham Pharmacia Biotech, UK). Cultures were washed twice with filter sterilized drinking water, and then inoculated into drinking water microcosms containing: (1) filter sterilized drinking water and sterile quartz sand (control bottles), (2) drinking water and sterile quartz sand, or (3) drinking water and sand covered with drinking water biofilm. Filter sand was sterilized by preheating to 450 °C. The microcosms consisted of 60 mL glass bottles with 50 mL drinking water, sealed with butyl rubber stoppers. All microcosms were spiked with either ^{14}C-labelled *L. monocytogenes* (approximately 5×10^5 cells mL^{-1}) or ^{14}C-labelled *E. coli* (approximately 1×10^6 cells mL^{-1}). The total amount of radioactivity was approximately 1.0×10^4 Bq per microcosm with ^{14}C-labelled *L. monocytogenes* and 1.7×10^4 Bq per microcosm inoculated with ^{14}C-labelled *E. coli*. Microcosms were

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incubated in the dark at 120 rpm on a rotary shaker at 10 °C. Gas and liquid sampled from the microcosms were analyzed for $^{14}CO_2$. Gas samples of 1 mL were injected into scintillation vials containing an 8 mL CO_2 trap (ethylene glycol monomethyl ether and ethanolamine 7:1; Merck, Germany). Vials were shaken whereafter a 10 mL scintillation cocktail was added (Hionic Fluor; PerkinElmer®, USA). The $^{14}CO_2$ in the liquid samples was evacuated and trapped after acidification. The radioactivity was quantified using a Packard 1600 Tri-Carb Liquid Scintillation Analyzer (Packard, NL). Counts were corrected for quench using external standards.

Data analysis and statistics

Comparisons of differences in survival between *Listeria* strains and between different detection methods were carried out using the nonparametric Mann–Whitney *U* test (Wilcoxon rank sum test) using KaleidaGraph 4.5.4 (Synergy Software, USA).

RESULTS AND DISCUSSION

Presence and survival of *L. monocytogenes* in drinking water

Source water from five rural drinking water treatment plants was examined for the presence of *Listeria* spp. The water supplies use groundwater as source water and employ conventional water treatment consisting of aeration and sand filtration. Disinfection is not part of the operating procedures at these treatment plants. Presumptive *Listeria* was not detected in water volumes <0.01 L whereas analysis of 1–4 L resulted in detection of presumptive *Listeria* in four out of five rural water supplies (0.3–90 CFU/L). This initial screening was not intended to be exhaustive but it indicated that low concentrations of *Listeria* were detectable and this suggested a potential for occurrence of *Listeria* in rural water supplies. It was not possible to establish the origin of the *Listeria* but it is known that rural groundwater is sometimes prone to contamination with live invertebrates and waste from wildlife and farm animals (Levy 1990; Bitton 2014; Hynds *et al.* 2014; Stokdyk *et al.* 2020).

Survival of *L. monocytogenes* in drinking water was examined by spiking human and animal strains into drinking water microcosms at 10 °C (Figure 3). The drinking water used in the microcosms was municipal drinking water from groundwater and 10 °C is a typical temperature in such systems. The standard operating procedure in this municipality advocates distribution of water to consumers without water treatment or disinfection (non-treated pristine groundwater). The drinking water contained no detectable coliform bacteria or intestinal enterococci (<1 CFU/100 mL), and the heterotrophic plate count was <10 CFU/mL. Background concentrations of *L. monocytogenes* were not detected in the microcosms (<1 CFU/1,000 mL).

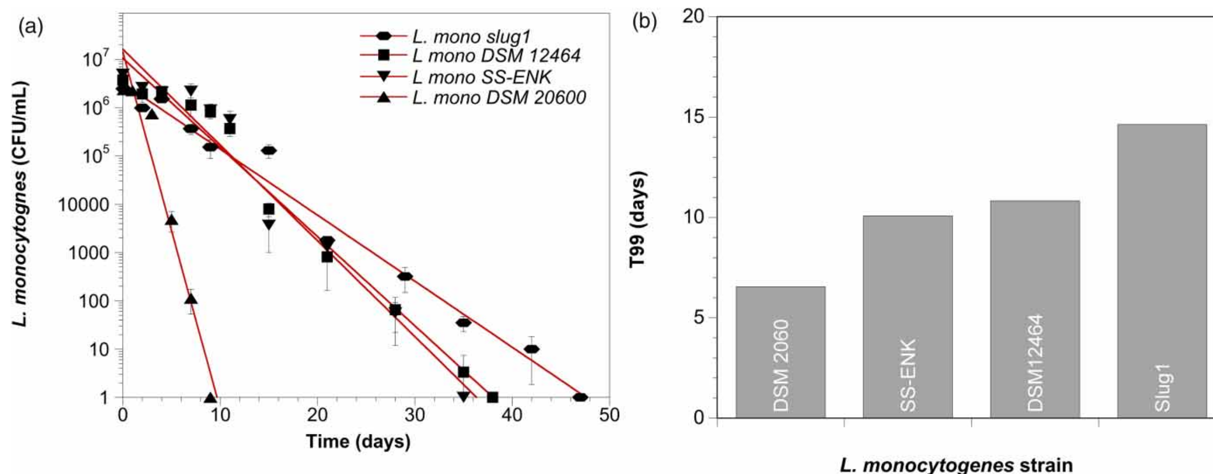

Figure 3 | (a) Survival of different *L. monocytogenes* strains from animals and humans in drinking water at 10 °C and (b) the time estimated for a 99% decrease in CFU (*T*99). See Table 1 for full names and origins of the different *L. monocytogenes* strains. Data represent means of triplicate experiments ± standard deviation.

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Drinking water microcosms were subsequently spiked with *L. monocytogenes* from the pest slug (*Arion vulgaris*), a strain from poultry (DSM 12464), a strain from rabbit (DSM 20600), and a human clinical isolate (SS-ENK).

One of the spiked *L. monocytogenes* strains (DSM 20600) lost culturability after only nine days in drinking water whereas the three other strains remained culturable for up to 35–47 days (Figure 3). *L. monocytogenes* DSM 20600 is a laboratory strain (type strain) originally isolated in 1926, and this bacterium may subsequently have lost some ability for survival under nutrient-poor conditions resulting in relatively poor survival in drinking water. The first-order decay rate for *L. monocytogenes* DSM2060 was 0.703 day^{-1}. In contrast, decay rates for *L. monocytogenes* SS-ENK, *L. monocytogenes* DSM12464 and *L. monocytogenes* slug1 were 0.457, 0.425 and 0.314 day^{-1}, respectively. The decay rates for the latter three *L. monocytogenes* strains correspond to *T*99 values of 10–15 days in drinking water (i.e., the time for a 99% decrease in CFU) (Figure 3).

After 28 days of incubation in drinking water, the survival of *L. monocytogenes* was also evaluated based on DVC-FISH. At this time-point, less than 0.01% of the initial population of *L. monocytogenes* remained culturable but the DVC-FISH method suggested that many *Listeria* were still substrate-responsive (DVC-positive). The number of nonculturable cells with detectable metabolic activity was 10–100-fold higher than the number of culturable cells (Mann–Whitney $p < 0.05$). This suggested that 0.1%–1% of the *L. monocytogenes* cells inoculated into drinking remained metabolically active after 28 days and were potentially viable. The relatively long survival of *L. monocytogenes* in non-disinfected drinking water observed in the present study supports previous studies suggesting that *Listeria* can survive for extended periods in cold and oligotrophic environments (Besnard *et al.* 2002; Ivanek *et al.* 2006; Budzińska *et al.* 2012). Although the conditions do not favor cell proliferation, low temperatures and trace concentrations of organics may support cell metabolism and survival. Besnard *et al.* (2002) reported that *L. monocytogenes* remained culturable in sterile water at 4 °C for up to six weeks, and thereafter maintained cell integrity and metabolic activity for more than ten weeks. Budzińska *et al.* (2012) also observed that environmental survival of *L. monocytogenes* was superior at low temperatures (4 °C) compared with higher temperatures (20 °C). Collectively, these findings emphasize the ability of *L. monocytogenes* to survive well at low temperatures, which is a characteristic that makes the control of this organism a challenge (Gandhi & Chikindas 2007).

Effect of surface attachment and biofilms on survival of *Listeria* in drinking water

Pathogens in drinking water utilities may survive as free-living planktonic cells in the water phase or in sessile communities attached to submerged surfaces colonized by microorganisms indigenous to the environment (Bitton 2014). The effect of surface attachment and biofilms on the survival of *L. monocytogenes* in drinking water was investigated using a laboratory-scale filter system containing either virgin quartz sand or quartz sand colonized by microorganisms indigenous to drinking water (Figure 4). The attachment and survival of *E. coli* was studied in parallel experiments to compare results for *L. monocytogenes* with those of a traditional FIB (Figure 4(b)). The drinking water biofilm in the filter system had been established over three months using a continuous flow of non-disinfected municipal tap water (Figure 2). The drinking water biofilm cell count determined by DAPI staining was $4.75 \times 10^6 \pm 3.98 \times 10^5$ cells/cm^2 at the beginning of the experiment.

Samples from the effluent of the filter columns showed that live *L. monocytogenes* and *E. coli* persisted in the flow systems past the theoretical washout (Figure 4(a) and 4(b)). The theoretical washout was calculated based on no attachment of cells to surfaces and also considered the partial water replacement during the experiment. Culturable cells of *L. monocytogenes* and *E. coli* persisted about three times longer than the projected washout. The theoretical washout corresponded to a dilution rate of 2.3 d^{-1} whereas the measured exponential decline for *L. monocytogenes* and *E. coli* in the absence of biofilms corresponded to a disappearance rate of 0.79 d^{-1} and 0.67 d^{-1}, respectively. Low water-temperatures (10 °C) and low concentrations of bioavailable organic carbon in the drinking water prohibited growth of *E. coli* and *L. monocytogenes* in the experiments, and only survival was observed. The absence of growth has also been observed for other fecal microorganisms in this type of drinking water (Roslev *et al.* 2004). The presence of low

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concentrations of *L. monocytogenes* and *E. coli* in the water phase past the theoretical washout suggested a slow release of cells attached to surface material in the flow systems.

L. monocytogenes and *E. coli* showed a more rapid decline in drinking water flow systems containing biofilm-covered filter material compared with columns with only virgin filter material (Figure 4). The removal rate for *L. monocytogenes* measured in the effluent was 1.37 d^{-1} in the presence of a drinking water biofilm and 0.79 d^{-1} in the absence. This observation was investigated further by targeting bacterial cells attached to filter surfaces with and without an indigenous biofilm. The results confirmed that the presence of a drinking water biofilm affects the surface attachment and persistence of *L. monocytogenes* and *E. coli*. Interestingly, the initial attachment of *L. monocytogenes* to biofilm-covered filter material was 10–100-fold greater compared with virgin surfaces (Figure 5), and the difference was statistically significant (Mann–Whitney, $p < 0.026$). After the initial attachment, there was, however, a rapid decline in the concentration of detectable *L. monocytogenes* on the biofilm-covered material but only a slow decline in concentrations of attached cells on the virgin material (Figure 5). After 14 days, *L. monocytogenes* could no longer be detected on biofilm-covered surfaces,

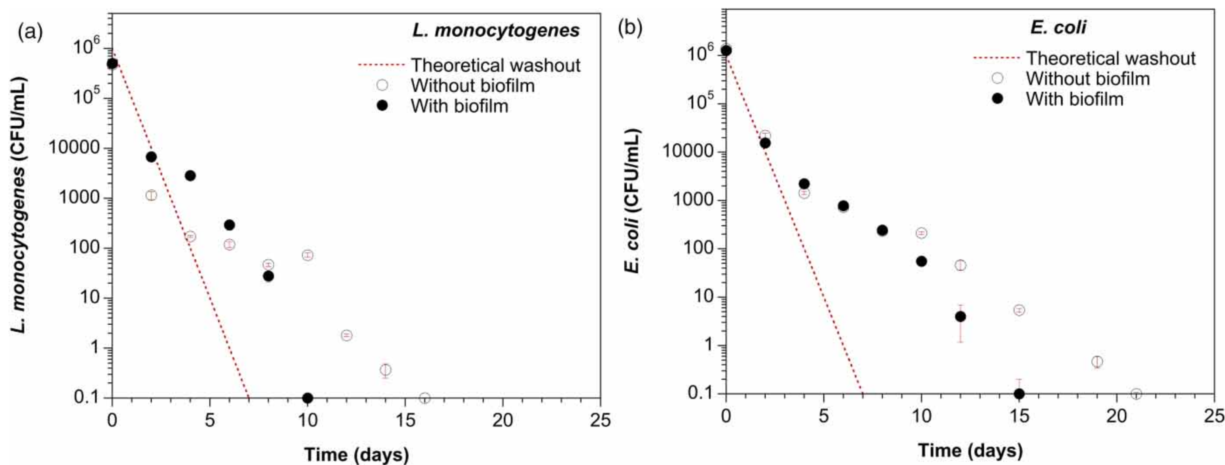

Figure 4 | Concentrations of (a) *L. monocytogenes* DSM 12464 and (b) *E. coli* ATCC 2522 in the effluent from drinking water filters without biofilm (open symbols) and filter material with an established drinking water biofilm (closed symbols). The theoretical washout curve is shown as a hatched line. Data represent means of triplicate experiments ± standard deviation. Some error bars are contained within the symbols.

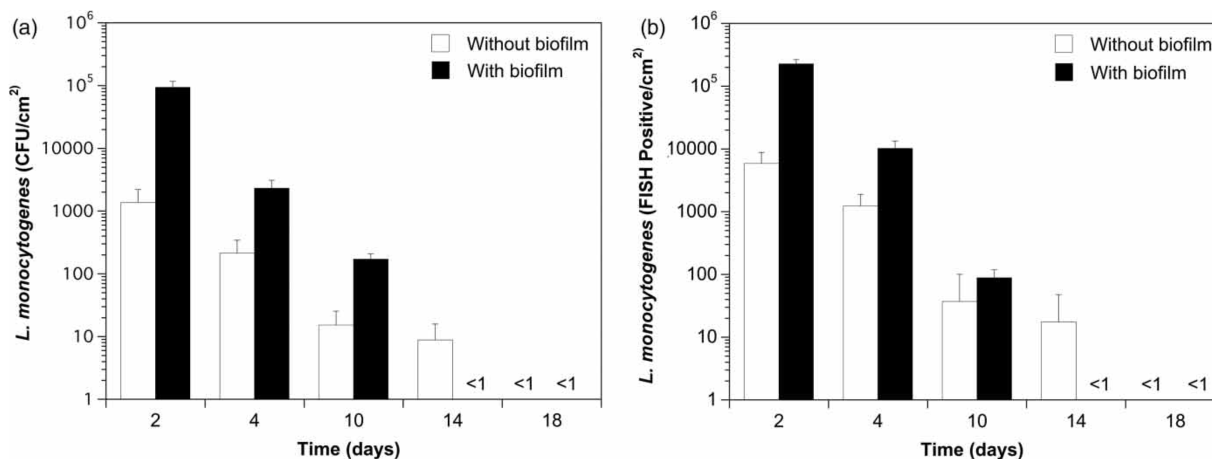

Figure 5 | Recovery of *L. monocytogenes* DSM 12464 from the surface of filter material in drinking water filters without biofilm (white bars) and filter material with an established drinking water biofilm (black bars) using (a) cultivation-based enumeration and (b) FISH-based enumeration. Data represent means of triplicate samples ± standard deviation.

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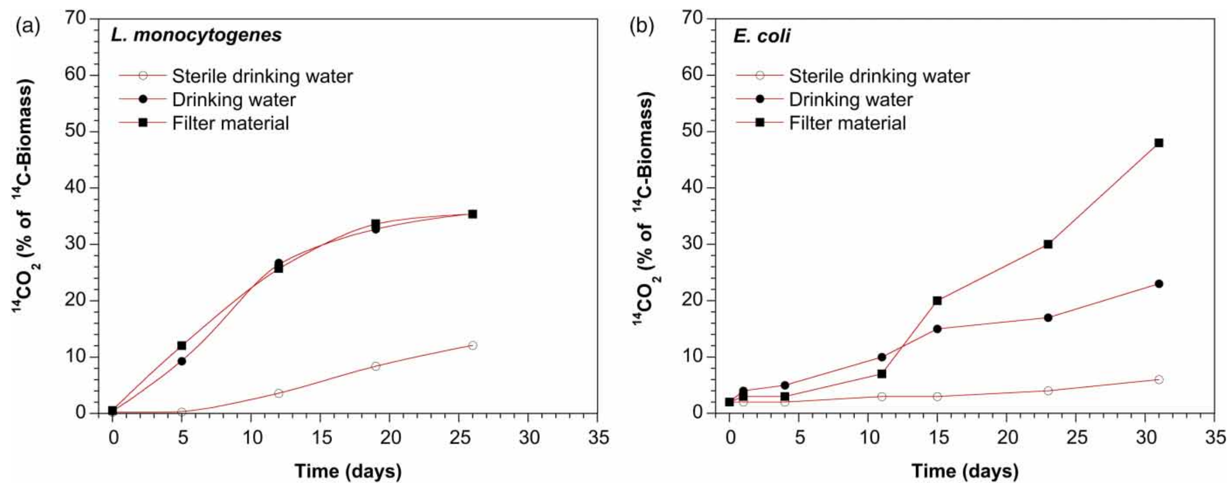

Figure 6 | (a) Fate of ^{14}C-labelled *L. monocytogenes* DSM 12464 and (b) *E. coli* ATCC 25922 inoculated into drinking water microcosms measured as production of $^{14}CO_2$. (a) *L. monocytogenes* in filter sterilized drinking water with sterile filter material (open circles), *L. monocytogenes* in drinking water with indigenous microflora and sterile filter material (filled circles), *L. monocytogenes* in drinking water with indigenous microflora and biofilm-covered filter material (filled squares). (b) *E. coli* in filter sterilized drinking water with sterile filter material (open circles), *E. coli* in drinking water with indigenous microflora and sterile filter material (filled circles), *E. coli* in drinking water with indigenous microflora and biofilm-covered filter material (filled squares). Data represent means of triplicate experiments.

whereas attached *L. monocytogenes* were detectable on surfaces without an initial biofilm (Figure 5). Comparable results were obtained with a traditional cultivation dependent method (CFU) and a FISH staining technique (Figure 5). Use of FISH confirmed that the observed decrease in surface-attached cells was not merely due to loss of culturability but due to a *de facto* decrease in cell numbers. Similar results were obtained in parallel experiments with *E. coli* where cells could be detected for >40 days on virgin filter material without an initial drinking water biofilm (data not shown). Collectively, these findings suggest a potential for extended survival of *L. monocytogenes* on surfaces in drinking water which may be useful information in relation to risk assessment of contaminated water supplies. A potential for extended survival of sessile *L. monocytogenes* in water supply systems may also be relevant for the food industry because tap water is often used for manufacturing and cleaning but sources of *Listeria* contamination are sometimes elusive (Ivanek *et al.* 2006; Gandhi & Chikindas 2007; Gião & Keevil 2014).

Turnover of ^{14}C-labelled *L. monocytogenes* and *E. coli*

Results from experiments with surface attachment of *L. monocytogenes* and *E. coli* to filter material indicated that surfaces covered with drinking water biofilms stimulated initial surface attachment, but also caused decreased survival of attached cells. This phenomenon was investigated further by measuring the fate of ^{14}C-labelled *L. monocytogenes* and *E. coli* inoculated into drinking water microcosms (Figure 6). The turnover of radiolabelled bacterial cells was measured as recovery of $^{14}CO_2$ with and without an indigenous drinking water microflora. Net growth of *L. monocytogenes* and *E. coli* was not observed in our drinking water microcosms (Figures 3–5), and $^{14}CO_2$ released from radiolabelled bacteria likely originated from endogenous metabolism and mineralization of ^{14}C-labelled biomass. Mineralization of ^{14}C-labelled bacterial biomass to $^{14}CO_2$ may then be used as a tracer of starvation metabolism within the labelled bacteria and predation (grazing) by other microorganisms (Taylor & Sullivan 1984). In the present study, control microcosms containing filter sterilized drinking water and sterile virgin filter sand were therefore included to establish release of $^{14}CO_2$ from *L. monocytogenes* and *E. coli* without competition or predation from indigenous microorganisms.

Turnover of ^{14}C-labelled *L. monocytogenes* and *E. coli* was three and ten times greater in microcosms with an indigenous microflora (filter material with biofilm and drinking water) compared with microcosms without native drinking water microorganisms (Figure 6). In experiments with *E. coli*, the turnover of ^{14}C-labelled bacterial cells was also

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more rapid in bottles with biofilm-covered filter material compared with drinking water without filter material (Figure 6). After >20 days, the release of $^{14}CO_2$ from *L. monocytogenes* and *E. coli* in microcosms without indigenous microflora corresponded to 6%–12% of the initial amount of radiolabelled cell material. In contrast, microcosms with native drinking water microorganisms caused a 37%–62% mineralization of radiolabelled *L. monocytogenes* and *E. coli* (Figure 6). After 26 days, approximately 35% of the added ^{14}C-labelled *L. monocytogenes* had been recovered in experiments with *L. monocytogenes* and biofilm-covered filter material, which corresponded to a 30% recovery as $^{14}CO_2$ after 23 days in microcosms containing *E. coli* and biofilm-covered filter material.

Biofilms in drinking water systems are sometimes implicated as environmental reservoirs or safe havens for intruding pathogens (Keevil 2002; Wingender & Flemming 2011; Bitton 2014; Pandove *et al.* 2016). In the present study, surface attachment of *L. monocytogenes* and *E. coli* to biofilm-covered filter material increased the persistence and delayed washout from drinking water flow systems. This is in agreement with previous studies suggesting that *L. monocytogenes* can attach and form biofilms on different surfaces in tap water (Gião & Keevil 2014; Pandove *et al.* 2016). However, both *L. monocytogenes* and *E. coli* disappeared more rapidly from biofilm-covered filter surfaces compared with filter material not colonized by indigenous microorganisms. The results obtained from release of $^{14}CO_2$ from radiolabelled *L. monocytogenes* and *E. coli* suggested that the native microflora in drinking water and filter material negatively affected the survival of the two fecal bacteria. Turnover of *L. monocytogenes* and *E. coli* biomass increased dramatically when the fecal bacteria were incubated with indigenous microorganisms compared with incubations without the native microflora. The increased turnover of cell material may be caused by increased competition from other bacteria and predation (grazing) by drinking water protozoa. Studies have shown relatively high concentrations of protozoa including ciliates, flagellates and amoeba in drinking water utilities (Sibille *et al.* 1998; Artz & Killham 2002; Bitton 2014). Bacterial consumption in drinking water biofilms due to grazing by protozoan amoeba can amount to $>10^4$ cells/cm^2/h (Bitton 2014). Hence, grazing by protozoa has a potential to reduce numbers of fecal contaminants in drinking water. Non-indigenous pathogens such as *L. monocytogenes* are likely more exposed to grazing compared with indigenous bacteria embedded in already established drinking water biofilms, and protozoan grazing is therefore a factor that likely affects the long-term survival of *L. monocytogenes* in non-disinfected drinking water.

CONCLUSIONS

Land slugs often have an omnivorous diet and feed on both live and dead organic material including detritus, carrion and feces. Slugs may penetrate drinking water supply systems and represent potential vectors of human- and animal-associated pathogens such as *Listeria monocytogenes.* Human- and animal-derived *L. monocytogenes* remained culturable for days to weeks in non-disinfected drinking water, and attachment to filter material increased persistence and delayed washout from drinking water flow systems. *L. monocytogenes* declined more rapidly in systems with biofilm-covered filter material compared with systems with virgin filter material not colonized by indigenous drinking water microorganisms. Live and sessile *L. monocytogenes* can likely persist for weeks on surfaces in cold nutrient-poor drinking water and may subsequently be released to the water phase with potential implications for public health. Contamination of water supplies by potential *Listeria* vectors such as land slugs should therefore be prevented.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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