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Efficacy of wastewater treatment on *Arcobacter butzleri* density and strain diversity

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ABSTRACT

Arcobacter butzleri is a suspected waterborne enteric pathogen that is ubiquitous in the environment, but the degree to which wastewater treatment prevents entry of *A. butzleri* into environmental waters and the risks posed are not well established. Untreated and treated wastewater samples (n = 260) were collected weekly from the Lethbridge and Fort Macleod wastewater treatment facilities (the two major municipal inputs in southwestern Alberta, Canada) from May 2008 to April 2009. Untreated wastewaters contained high densities of *A. butzleri* and fecal coliform indicators, and densities at Lethbridge were typically higher than at Fort Macleod. Data indicated that *A. butzleri* and fecal coliform densities in wastewater were greatest in autumn and lowest in winter. Mechanical and biological treatment of wastewaters reduced but did not eliminate fecal coliform indicators or *A. butzleri*. At Lethbridge, UVB irradiation of mechanically and biologically treated wastewater further reduced densities of fecal coliform indicators. There was high *A. butzleri* genotype diversity in all sample sources, and survival during treatment was not strain-dependent. No genotype was dominant in any sample source, but 8.9% of genotypes were recurrent over time, and 4.4% of genotypes were detected at both wastewater treatment facilities. The current study demonstrates that viable *A. butzleri* are able to survive wastewater treatment, including UVB irradiation, which may lead to increased density and genetic diversity of this suspected pathogen in environmental waters via wastewater effluent discharge.

1. Introduction

Arcobacter butzleri is the fourth most commonly detected Campylobacteraceae bacterium in human beings with enteric disease (Vandenberg et al., 2004), but key reservoirs and mechanisms of transmission have yet to be determined. The presence of *A. butzleri* in drinking water has been linked to multiple enteric disease outbreaks (Fong et al., 2007; Rice et al., 1999), and this bacterium possesses many genetic traits characteristic of waterborne free-living pathogens (Miller et al., 2007). *Arcobacter butzleri* grows in aerobic, anoxic, and anaerobic environments (Miller et al., 2007; Vandamme et al., 1991), at temperatures as low as 10 °C (Kjeldgaard et al., 2009; Vandamme and De Ley, 1991), and in the presence of a wide range of antimicrobial agents (Atabay and Aydin, 2001; Fera et al., 2003). It has also been detected in human stools and livestock waste, and the presence of *A. butzleri* in surface waters has been linked to fecal contamination (Collado et al., 2008, 2010).

Recent studies indicate that *A. butzleri* in urban wastewaters survive treatment and are discharged into environmental waters (Collado et al., 2008, 2010). Wastewater treatment plants (WWTPs) utilize a combination of mechanical (i.e. screens and sedimentation), biological (i.e. activated sludge and bioreactors), and enhanced (i.e. nutrient removal, chlorine, and UVB irradiation) processes to remove enteric pathogens prior to discharge of effluent into environmental waters (Koivunen et al., 2003; Zhang and Farahbakhsh, 2007). These methods limit the number of fecal coliforms that are released into environmental waters, but their effects on *A. butzleri* cell density, viability, and genetic diversity have not been documented. Considering that *A. butzleri* is a potential pathogen that displays greater survival capacity in water

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containing organic material (Van Driessche and Houf, 2008), it is likely that environmental waters contaminated with *A. butzleri* serve as reservoirs of human-infectious cells for this enteric pathogen.

The purpose of the current study was to determine the efficacy of standard wastewater treatments on the viability and genetic diversity of *A. butzleri* at two WWTPs that discharge treated municipal wastewater into the Oldman River in southwestern Alberta, Canada. We hypothesized that wastewater treatment would reduce the number of viable *A. butzleri* cells entering the Oldman River as effluent. We further hypothesized that the genetic diversity of *A. butzleri* in municipal wastewater would not be affected by wastewater treatment. Primary objectives were to: (i) utilize quantitative PCR to measure total and viable densities of *A. butzleri* in Lethbridge and Fort Macleod wastewater (the two major municipal inputs in southwestern Alberta, which utilize different treatment processes); (ii) comprehensively isolate and genotype *A. butzleri* from Lethbridge and Fort Macleod wastewaters; and (iii) comparatively examine the population structure of *A. butzleri* in municipal wastewaters at various stages of treatment.

2. Materials and methods

2.1. Wastewater sample collection

Wastewater samples were collected weekly from the Fort Macleod and Lethbridge WWTPs in southwestern Alberta from May 2008 to April 2009. At the Lethbridge WWTP the treatment process consists of a mechanical bar screen, grit removal, primary clarifiers, anaerobic, anoxic, and aerobic digesters, secondary clarification, removal of activated sludge, UVB irradiation of liquid effluent, and treated effluent release to the Oldman River via a 1 km-long outfall line. Samples were collected immediately after mechanical bar screening ($n = 52$), after mechanical/biological treatment prior to UVB irradiation ($n = 52$) and after UVB irradiation ($n = 52$) prior to effluent release into the Oldman River. At the time of the study the Fort Macleod treatment process consisted of a mechanical bar screen, grit removal, RBC activated sludge contact tank, secondary clarification, solids removal to a digester and/or recirculated to front of contact tank, and treated effluent release to the Oldman River via a 3 km-long outfall line. Samples were collected immediately after mechanical bar screening ($n = 52$) and at the end of the treatment process ($n = 52$) immediately prior to effluent release into the Oldman River. Samples were maintained on ice and processed within 6 h of collection.

2.2. Wastewater sample processing

A total of 100 ml of each sample was filtered through a 150 mm pre-filter (#1001-150, Whatman International Ltd., Maidstone England) and a GMF grade 0.2 μm filter (#1842-090, Whatman). Both filters were vortexed (high setting) in 10 ml of phosphate buffered saline (PBS; pH 7. X) to release particulates from the filters. The filters were removed, and the suspension was centrifuged at 14 900 × g for 10 min. All but 3.0 ml of supernatant was removed by aspiration. The pellet was suspended by vortexing (high setting), and the suspension was used for DNA extraction, enumeration of fecal coliforms, and isolation of *A. butzleri*.

2.3. Wastewater sample total DNA extraction

For DNA extraction, 200 μl of the suspension was placed in two 2-ml tubes. One of each pair of samples was treated with ethidium monoazide (EMA, Invitrogen Canada Inc., Burlington, ON, Canada) to a final concentration of 100 μg ml^{-1} as previously described (Inglis et al., 2010; Rudi et al., 2005), and Optima water was added to the other sample. Tubes were placed in the dark for 5 min, lids were opened, and all tubes were exposed to light emitted from a 500-W halogen light bulb for 1 min on ice; the light source was situated 10 cm from the samples. Opaque untreated wastewater samples were diluted prior to the addition of EMA in order to facilitate photo-deactivation of any EMA that had not bound to DNA. An internal amplification control (IAC) was also added to each sample to a final concentration of 1.0×10^4 copies μl^{-1} as previously described (Webb et al., 2016). Briefly, the IAC was a synthesized gene designed from a 268-bp sequence encoding a putative carbohydrate kinase (PfkB family; GenBank accession number AEH23732.1) within the genome of *Pyrococcus yayanosii*, a bacterium that is an obligate piezophilic hyperthermophilic archaeon isolated from deep-sea hydrothermal sites. Extraction of total DNA from processed samples was performed using the Powerlyzer Powersoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad CA) according to the manufacturer's recommendations.

2.4. Quantitative PCR of wastewater sample total DNA

Absence of DNA amplification inhibition during PCR was confirmed by quantitative PCR (qPCR) with primers targeting the IAC (Webb et al., 2016). Briefly, amplification was performed with a Stratagene Mx3005P qPCR system (Agilent Technologies, Santa Clara CA) using the following final concentrations of reagents: 1X Quantitect SYBR Green (Qiagen Inc.), 100 μg ml^{-1} UltraPure BSA (Ambion, Life Technologies Inc., Burlington ON), 0.5 μM primer IAC-f (3′-GGTATGCTAGCCCCGCTTAGGGT-5′), 0.5 μM primer IAC-r (3′-TGCTCCAGAAAAGATGTCCAGCGG-5′), 10^{-1}X DNA template, and Nuclease-Free Water (Qiagen Inc.). Samples were quantitated in duplicate reactions. The amplification conditions were one cycle at 95 °C for 15 min, followed by 40 cycles of 15 s at 94 °C, 30 s at 64 °C, and 30 s at 72 °C for data acquisition.

Detection and enumeration of *A. butzleri* DNA was performed using primers targeting a single-copy gene sequence unique to *A. butzleri* (Webb et al., 2016). Briefly, amplification was performed with a Stratagene Mx3005P qPCR system (Agilent Technologies) using the following final concentrations of reagents: 1X Quantitect SYBR Green mastermix (Qiagen Inc.), 100 μg ml^{-1} UltraPure BSA (Ambion), 0.5 μM ddAbutzF (5′-AGTGATGGTGGAGTTGCTAGTC-3′), 0.5 μM ddAbutzR (5′-GTTGCAGGAGCTTTTTCACTCC-3′), 10^{-1}X DNA template, and Nuclease-Free Water (Qiagen Inc.). Samples were quantitated in duplicate reactions, and *A. butzleri* DNA extracted from pure reference strain cultures was used as a positive control. The amplification conditions were one cycle at 95 °C for 15 min, followed by 40 cycles of 30 s at 94 °C, 90 s at 65 °C, and 60 s at 72 °C for data acquisition. At the end of amplification, melt curve analysis was conducted. The data were analysed using MxPro (Version 4.10, Agilent Technologies Inc.). The limit of quantitation of *A. butzleri* DNA was previously determined to be 1.1 genome copies per PCR reaction (Webb et al., 2016), which equates to 1.2 $\log_{10}$ copies ml^{-1} of unprocessed wastewater.

2.5. Enumeration of fecal coliform indicators

To enumerate fecal coliforms, 1.0 ml from each processed wastewater sample was diluted in a ten-fold dilution series in PBS, and 100 μl of each dilution was spread on mFC Agar (Sigma-Aldrich) in duplicate. Cultures were incubated aerobically for 24 h at 45 °C, and blue colonies were enumerated at the dilution yielding 30–300 colony forming units (CFU) per dish. The mean of the two duplicate cultures was calculated.

2.6. Isolation of A. butzleri from wastewaters

Media for isolation of *A. butzleri* were Columbia agar (DF0944-17-0; Difco) containing 10% sheep blood (CBA), Karmali agar (CM0935, Oxoid) with Karmali supplement (KS; SR0167, Oxoid), Karmali agar (CM0935, Oxoid) with Bolton supplement (KB; SR0183E, Oxoid), *Arcobacter* Selection and Isolation Agar (ASIA) (Van Driessche et al., 2003), and Johnson Murano Agar (JMA) (Johnson and Murano, 1999). The isolation method varied by medium: membrane filtration (Engberg et al., 2000) was used for CBA; direct plating of 100 µl of the processed sample was used for KS, KB, and ASIA; and Bolton broth (CM0983, Oxoid) with Bolton supplement (SR0183E, Oxoid) (BBS) was used for enrichment culture with subsequent isolation on KS, KB, ASIA, and JMA. The CBA cultures were incubated at 37 °C for up to 10 days, and all other agar media were incubated at both 30 °C and 37 °C for 72 h. All cultures were maintained in a high hydrogen microaerobic atmosphere (i.e. 5% O_2, 30% H_2, 10% CO_2, and 55% N_2). For enrichment cultures, 25 µl of each sample was added to 2.0 ml of BBS and incubated at both 30 °C and 37 °C. At 24 and 48 h, 10 µl of the enrichment was streaked on KS, KB, ASIA, and JMA.

Two presumptive *A. butzleri* colonies (i.e. by morphology) per medium per sample were collected, streaked for purity on CBA in a microaerobic atmosphere, and examined microscopically for cell size, shape, and motility. Genomic DNA was extracted from isolated *A. butzleri* colonies using the DNeasy blood and tissue kit (Qiagen Inc.) using an automated system (model 740; Autogen, Holliston, MA) according to the manufacturer's specifications. Extracted DNA was identified as *A. butzleri* by endpoint PCR as previously described (Webb et al., 2016). Briefly, amplification reactions consisted of the following reagents: 1X PCR Buffer containing $MgCl_2$ (Qiagen Inc.), 100 µg ml^{-1} UltraPure BSA (Ambion), 0.2 mM dNTP mix (Bio Basic Canada Inc., Markham, ON), HotStar Taq Plus DNA Polymerase (10 units/400 µl; Qiagen Inc.), 0.5 µM ddAbutzF (5′-AGTGATGGTGGAGTTGCTAGTC-3′), 0.5 µM ddAbutzR (5′-GTTGCAGGAGCTTTTTCACTCC-3′), DNA template, and Nuclease-Free Water (Qiagen Inc.). The PCR reaction consisted of activation at 95 °C for 5.0 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 90 s and elongation at 72 °C for 60 s, followed by a final elongation at 72 °C for 5 min and storage at 4 °C. Amplicons were visualized on a QIAxcel capillary electrophoresis machine (Qiagen Inc.) using the AM320 separation and resolution method, with 15–3000 bp alignment marker and 100–2500 bp size marker.

2.7. Subtyping of A. butzleri isolates

Arcobacter butzleri isolates from wastewater and diarrheic human stool samples were subtyped using a previously developed high-throughput and high-resolution CGF genotyping method (Webb et al., 2015). Briefly, a set of 40 accessory genes representative of whole genome single nucleotide polymorphism phylogeny were identified via comparative whole genome sequence analysis, primers were designed and validated, and multiplex end-point PCR was completed with capillary electrophoresis to generate a 40-digit binary (i.e. present or absent) profile for each isolate.

2.8. Comparative genomic analysis

At least one isolate per site per week was arbitrarily selected for CGF genotyping. Three reference strains (RM4018 - PRJNA58557, ED1 - PRJNA158699, JV22 - PRJNA61483) were genotyped *in silico*. Isolates were clustered at 95% fingerprint similarity (i.e. less than two locus mismatches) using simple matching with UPGMA in Bionumerics (version 6.6, Applied Maths, Austin, TX). Genetic diversity (Simpson 1-D) and genotype dominance were calculated using all *A. butzleri* isolates, and genotype recurrence was calculated using one isolate per genotype per week per WWTP (i.e. after clones were removed).

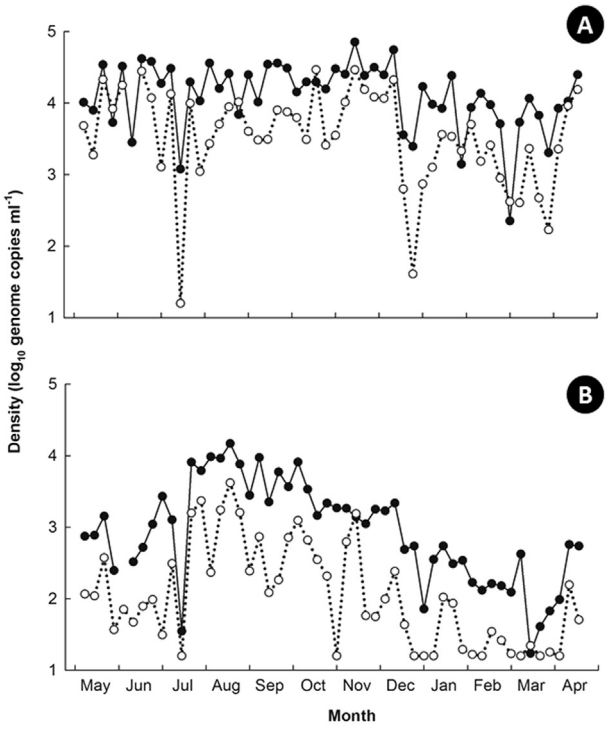

Fig. 1. Quantitation of *A. butzleri* DNA from total (solid lines) and viable (dotted lines) cells in untreated wastewater at the Lethbridge (A) and Fort Macleod (B) WWTPs from May 2008 to April 2009. Values presented as 1.2 $\log_{10}$ genome copies ml^{-1} represent results below the limit of quantitation for *A. butzleri* DNA.

3. Results and discussion

3.1. Detection of viable A. butzleri in untreated wastewater

Arcobacter butzleri DNA was consistently detected in untreated wastewater at the Lethbridge (Fig. 1A) and Fort Macleod (Fig. 1B) WWTPs, although the proportion of *A. butzleri* genome copies from viable cells varied by sample. In previous studies, Collado et al (Collado et al., 2008). detected *A. butzleri* in 100% of sewage and in 96.3% of sludge samples. In addition, Stampi et al (Stampi et al., 1999). found *A. butzleri* to be viable at all stages of solid waste treatment (i.e. primary clarification, activated sludge, thickened sludge, and anaerobically-digested sludge). To assess viability, we treated samples with EMA prior to DNA extraction. Although others have utilized EMA for live/dead cell quantitation of *Campylobacter* (Inglis et al., 2010), *Helicobacter* (De Cooman et al., 2013; Kaebisch et al., 2014), and *Salmonella* species (Chen et al., 2011), our results are likely a conservative estimate of the density of viable *A. butzleri* cells because EMA can penetrate cells possessing an intact cell membrane (i.e. viable cells) (Nocker and Camper, 2006; Nocker et al., 2006).

Untreated wastewater at Lethbridge typically contained greater densities of both *A. butzleri* (Fig. 2A) and fecal coliform indicators (Fig. 2B) than at Fort Macleod, and the density of *A. butzleri* and fecal coliform indicators at both WWTPs tended to be lowest between December and March. The greater densities of *A. butzleri* and fecal coliform indicators at Lethbridge may be attributed to its larger population size and/or greater variety of input sources. At the

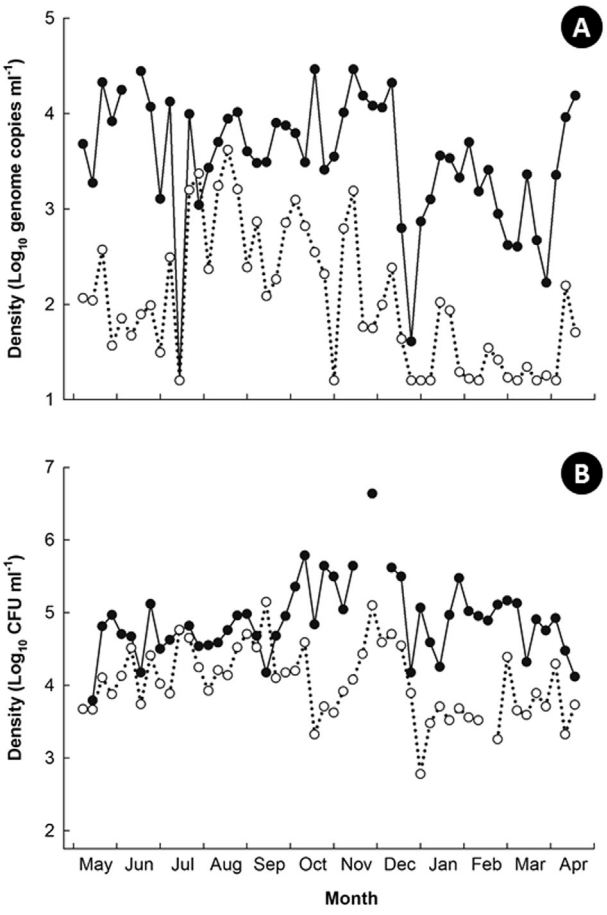

Fig. 2. Quantitation of viable *A. butzleri* DNA (A) and fecal coliform indicators (B) in untreated wastewater at the Lethbridge (solid lines) and Fort Macleod (dotted lines) WWTPs from May 2008 to April 2009. In Figure 2A, values presented as 1.2 $\log_{10}$ genome copies ml^{-1} represent results below the limit of quantitation for *A. butzleri* DNA.

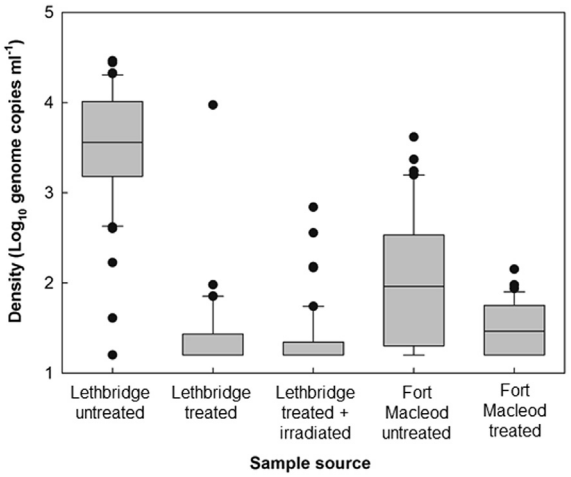

Fig. 3. Quantitation of *A. butzleri* in wastewaters from the Lethbridge WWTP (A) and the Fort Macleod WWTP (B) from May 2008 to April 2009. A total of 52 samples were processed per sample source. Values presented as 1.2 $\log_{10}$ genome copies ml^{-1} represent results below the limit of quantitation for *A. butzleri* DNA. Samples were collected at each WWTP immediately prior to treatment (untreated) and after mechanical/biological treatment (treated), and also at Lethbridge after UVB irradiation (treated + irradiated). There is no median line for Lethbridge treated, and Lethbridge treated and irradiated samples because the medians were below the limit of quantitation.

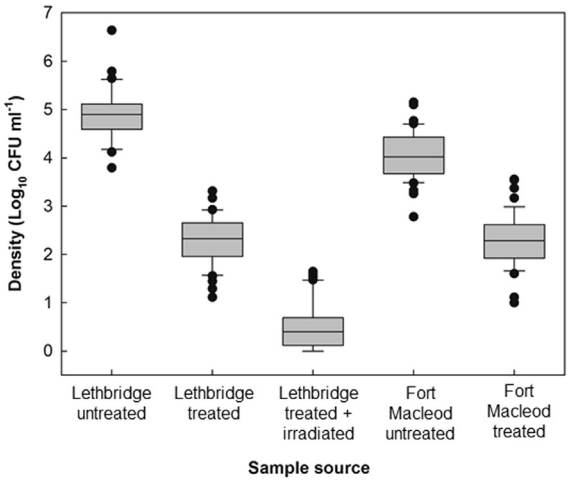

Fig. 4. Quantitation of fecal coliform indicators in wastewaters from the Lethbridge WWTP (A) and the Fort Macleod WWTP (B) from May 2008 to April 2009. A total of 52 samples were processed per sample source. Samples were collected at each WWTP immediately prior to treatment (untreated), and after mechanical/biological treatment (treated), and also at Lethbridge after UVB irradiation (treated + irradiated).

time of sampling for the current study, the Lethbridge WWTP handled 36.0 million liters day^{-1} of input from household wastewater as well as pork, chicken, and cheese processing facilities (Doug Kaupp, City of Lethbridge, personal communication). In comparison, the Fort Macleod WWTP handled 1.5 million liters day^{-1} of input, consisting primarily of household wastewater (Dan Segboer, Town of Fort Macleod, personal communication). The additional load and diversity of inputs at Lethbridge may contribute to increased densities of *A. butzleri* in its wastewater because animal product processing facilities are likely reservoirs for *A. butzleri* (De Smet et al., 2011; Houf et al., 2002). The lower densities of *A. butzleri* and fecal coliform indicators observed between December and March are similar to seasonal trends for enteric pathogens in temperate regions (Hörman et al., 2004; Wilkes et al., 2009). Decreased density during winter months may be attributed in part to cooler wastewaters entering the WWTPs as a result of subzero atmospheric temperatures, or to reduced input from water runoff as a result of precipitation taking the form of snow as opposed to rain.

3.2. Efficacy of wastewater treatment

Mechanical and biological treatment reduced the density of viable *A. butzleri* in wastewater by up to 4.5 $\log_{10}$ genome copies ml^{-1} at the Lethbridge WWTP and up to 3.6 $\log_{10}$ genome copies ml^{-1} at the Fort Macleod WWTP (Fig. 3). UVB irradiation further reduced the density of viable *A. butzleri* at Lethbridge by up to 0.7 $\log_{10}$ genome copies ml^{-1}, however quantitation of DNA in samples treated with EMA may not be a reliable means of determining the effects of UVB irradiation on viable cell density because UV irradiation disrupts the replicative ability of bacterial cells without compromising membrane integrity (Hijnen et al., 2006; Maranger et al., 2002). Fecal coliform indicators in wastewaters after mechanical and biological treatment were reduced by up to 6.6 $\log_{10}$ CFU ml^{-1} at the Lethbridge WWTP, and by up to 5.1 $\log_{10}$ CFU ml^{-1} at the Fort Macleod WWTP (Fig. 4). Although densities of fecal coliforms in treated wastewater after UVB irradiation were further reduced by up to 3.3 $\log_{10}$ CFU ml^{-1} at the Lethbridge

Table 1
Genetic diversity of *A. butzleri* in municipal WWTPs.

Sample source	Isolates	Genotypes[a]	Simpson (1-D)[b]	CI (95%)	CINA (95%)
Lethbridge untreated	128	106	0.996	0.994–0.999	0.993–1.000
Lethbridge biological	158	118	0.995	0.992–0.997	0.991–0.998
Lethbridge effluent	58	49	0.993	0.987–0.999	0.984–1.000
Macleod untreated	162	93	0.984	0.978–0.991	0.977–0.991
Macleod effluent	127	72	0.985	0.979–0.991	0.979–0.992

[a] Partitions were denoted at the 95% similarity level, which was calculated using the simple matching coefficient in BioNumerics (version 6.6, Applied Maths, Austin, TX).
[b] Simpson 1-D, confidence interval (CI), and non-approximated confidence interval (CINA) were calculated using the online tool of the Comparing Partitions Website (http://darwin.phyloviz.net/ComparingPartitions/index.php?link=Tool).

WWTP, these results may overestimate the efficacy of UVB deactivation of cells because the employed culture-based enumeration method may not account for photo-reactivation of UV-treated cells (Guo et al., 2009b; Hallmich and Gehr, 2010).

Although the density of viable *A. butzleri* cells in wastewater was greatly reduced during treatment, viable *A. butzleri* cells were not eliminated, as evidenced by continued detection in wastewater effluent at both WWTPs by qPCR and culture-based isolation. At the time of sampling, wastewater treatment at Fort Macleod consisted of RBC-activated sludge removal and primary clarification, aerobic digestion and secondary clarification. In comparison, Lethbridge wastewater treatment consisted of RBC-activated sludge removal and primary clarification, anaerobic digestion, anoxic digestion, aerobic digestion, secondary clarification, and UVB irradiation. Previous studies demonstrated that mechanical and biological treatment reduces bacterial pathogen densities in municipal wastewaters (Shannon et al., 2007), and that additional UVB irradiation leads to greater reduction in bacterial pathogen viability (Guo et al., 2009a; Hallmich and Gehr, 2010). However, Stampi et al (Stampi et al., 1999). found that *A. butzleri* are less susceptible to anaerobic digestion compared to the enteric pathogens *C. jejuni* and *C. coli*, and Miller et al. (Miller et al., 2007) identified *A. butzleri* genes for mitigation of DNA damage induced by UV irradiation and for increased survival in surface waters. Thus, current wastewater treatment processes may be less effective at reducing the density of viable *A. butzleri* cells compared to other enteric pathogens.

3.3. Comparative genomic analysis

Due to the limitations inherent to culture methods, the total number of *A. butzleri* isolates recovered varied by week and site. CGF genotyping (Webb et al., 2015) was performed on 636 *A. butzleri* isolates, and 338 genotypes were identified. Overall, the genetic diversity of *A. butzleri* in Lethbridge and Fort Macleod wastewaters remained high after mechanical/biological treatment and after UVB irradiation (Table 1). In a previous study (Collado et al., 2010), high *A. butzleri* genetic diversity was detected in wastewater effluent and in surface waters that were contaminated with effluent runoff, but a maximum of 40 *Arcobacter* isolates per sample site were genotyped and no isolates were collected from untreated wastewaters. Similarly to previous studies (Collado et al., 2010; González et al., 2009), no *A. butzleri* genotype dominated any sample source. A total of 30 genotypes (8.9%) were detected at either the Lethbridge or the Fort Macleod WWTP during more than one sample period, and 15 genotypes (4.4%) were detected at both the Lethbridge and Fort Macleod WWTPs. These findings contrast with those of previous studies (Collado et al., 2010; González et al., 2009), which did not detect genotype recurrence. Whether these recurrence events are indicative of conserved and/or similar sources of contamination, or of *A. butzleri* colonization of WWTPs requires further study.

4. Conclusions

Our findings indicated that mechanical and biological wastewater treatments reduce the density of viable *A. butzleri* and fecal coliforms in wastewater effluent, and that UVB irradiation further reduces cell viability. However, a proportion of *A. butzleri* and fecal coliforms survived wastewater treatment, and the genetic diversity of *A. butzleri* was not affected by mechanical and biological treatment, or by UVB irradiation. The survival of genetically diverse *A. butzleri* in municipal wastewaters and their subsequent discharge into surface waters may contribute to increased density and genetic diversity of this suspected pathogen in the environment.

5. Funding information

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