# Interactions between *Arcobacter butzleri* and free-living protozoa in the context of sewage & wastewater treatment

by

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**Abstract**

Water reuse is increasingly becoming implemented as a sustainable water management strategy in areas around the world facing freshwater shortages and nutrient discharge limits. However, there are a host of biological hazards that must be assessed prior to and following the introduction of water reuse schemes. Members of the genus *Arcobacter* are close relatives to the well-known foodborne campylobacter pathogens and are increasingly being recognized as emerging human pathogens of concern. Arcobacters are prevalent in numerous water environments due to their ability to survive in a wide range of conditions. They are particularly abundant in raw sewage and are able to survive wastewater treatment and disinfection processes, which marks this genus as a potential pathogen of concern for water quality. Because the low levels of *Arcobacter* excreted by humans do not correlate with the high levels of *Arcobacter* spp. present in raw sewage, it was hypothesised that other microorganisms in sewage may amplify the growth of *Arcobacter* species. There is evidence that *Arcobacter* spp. survive both within and on the surface of free-living protozoa (FLP). As such, this thesis investigated the idea that *Arcobacter* spp. may be growing within free-living protozoa also prevalent in raw sewage and providing them with protection during treatment and disinfection processes. As *Arcobacter* spp. have been detected in discharged wastewater effluents disinfected by ultraviolet (UV) irradiation, this thesis investigated the potential for free-living protozoa to enhance growth or protect *Arcobacter* spp. from UV disinfection, a process considered highly effective for wastewater treatment and increasingly implemented worldwide.

While wastewater-derived *A. butzleri* showed no change in CFUs when grown with or without free-living amoebae (FLA), there was a significant decrease when in the presence of the ciliate *Tetrahymena pyriformis*. Hence, *A. butzleri* does not appear to use FLP as a vessel for

replication. Herein the first UV dose-response work for *A. butzleri* in water is reported and at the usual wastewater dosages, up to 40 mJ/cm$^2$, a 6-log$_{10}$ reduction occurred. The uncorrected UV doses required to achieve certain log$_{10}$ reductions of *A. butzleri* were greater than the uncorrected UV doses required to achieve the same log$_{10}$ reductions of *E. coli*, a traditional water quality indicator, and *C. jejuni*, a commonly used bacterial reference pathogen in quantitative microbial risk assessments for water reuse schemes. There was no significant difference in log$_{10}$ reductions when *A. butzleri* was associated with FLA. However, there was a significant decrease in log$_{10}$ reduction when *A. butzleri* was associated with *T. pyriformis*, which suggests that free-swimming ciliates provide *Arcobacter* spp. with protection from UV disinfection. These findings point to the importance of considering the ecology of bacterial pathogens during wastewater treatment rather than strictly focusing on CFU counts as an indicator of water quality. *Campylobacter* is the current surrogate for *Arcobacter* treatment efficacy. As *Arcobacter* spp. are much less fastidious and much more abundant in raw sewage than species of *Campylobacter*, this reference pathogen may need to be reconsidered.

## Preface

This thesis is an original work by Mariem Joan Wasan Oloroso. No part of this thesis has been previously published.

To Mrs. Sherry Matthews, for showing me how exciting science can be.
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Educators who care truly do make the world go round.

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## List of Abbreviations & Acronyms

| | |
|---|---|
| *A* | Absorbance |
| AMR | Antimicrobial resistance |
| ANOVA | Analysis of variance |
| ARG | Antimicrobial resistance gene |
| ATCC | American Type Culture Collection |
| BLAST | Basic Local Alignment Search Tool |
| °C | Degrees Celsius |
| CFU | Colony-forming units |
| cm | Centimetre |
| CPD | Cyclobutane pyrimidine dimer |
| CSO | Combined sewer overflow |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxynucleic acid |
| dsDNA | Double-stranded deoxynucleic acid |
| DVC | Direct viable count |
| $E_0$ | Incident intensity |
| $E_{ave}$ | Delivered/effective intensity |
| ECV | Extracellular vesicle |
| EtBr | Ethidium bromide |
| FBS | Fetal bovine serum |
| FIB | Faecal indicator bacteria |
| FISH | Fluorescence *in situ* hybridization |
| FLA | Free-living amoebae |
| FLP | Free-living protozoa |
| *g* | Gravitational unit |
| G | Gauge |
| GPA | Gentamicin protection assay |
| HGT | Horizontal gene transfer |
| *hsp60* | 60 kDa heat shock protein gene |

| | |
|---|---|
| IU | International unit |
| $KH_2PO_4$ | Monopotassium phosphate |
| $K_2HPO_4$ | Dipotassium phosphate |
| L | Litre |
| *L* | Length |
| LED | Light-emitting diode |
| MDR | Multi-drug resistance |
| mg | Milligram |
| $MgSO_4$ | Magnesium sulfate |
| min | Minute |
| $mJ/cm^2$ | Millijoules per square centimetre |
| mL | Millilitre |
| MLB | Multi-lamellar body |
| mm | Millimetre |
| NaCl | Sodium chloride |
| $Na_2HPO_4$ | Disodium phosphate |
| NCBI | National Center for Biotechnology Information |
| nm | Nanometre |
| NNA | Non-nutrient agar |
| NSF | National Science Foundation |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| pen-strep | Penicillin-streptomycin |
| PMA-qPCR | Propidium monoazide quantitative polymerase chain reaction |
| QMRA | Quantitative microbial risk assessment |
| qPCR | Quantitative polymerase chain reaction |
| rpm | Revolutions per minute |
| rRNA | Ribosomal ribonucleic acid |
| $R^2$ | Coefficient of determination |
| s | Second |
| SCGYEM | Serum-casein-glucose-yeast extract medium |

| | |
|---|---|
| SEM | Standard error of the mean |
| *t* | Time |
| TM | *Tetrahymena* medium |
| μL | Microlitre |
| μm | Micrometre |
| UV | Ultraviolet |
| UV-B | Type B ultraviolet |
| UV-C | Type C ultraviolet |
| VBNC | Viable but non-culturable |
| WWTP | Wastewater treatment plant |

# Chapter 1: General introduction

## *1.1 Background on municipal wastewater*

Globally, it is estimated that approximately 80% of wastewater is discharged to the environment without proper treatment (WWAP (United Nations World Water Assessment Programme) 2017). These untreated wastewaters not only serve as a source of pollution but may have specific adverse effects on both the environment and public health (Singh *et al.* 2004, Akpor and Muchie 2011, Lam *et al.* 2015). There is also an increasing demand to reuse wastewater as an alternative source water, and therefore, as a resource in the circular economy (Salgot and Folch 2018, Voulvoulis 2018). When treated fit-for-purpose, there are myriad possibilities for the utilisation of wastewater including:

- An alternative source of water that can be used to address water scarcity;
- A source of energy, nutrients, and other wastewater by-products; and
- A chance to generate new business, research and development opportunities in the ever-growing "green industry" (WWAP (United Nations World Water Assessment Programme) 2017).

Despite wastewater's potential to generate enormous benefits with regard to human health, socioeconomic development, and environmental sustainability, there are however, numerous risks surrounding wastewater reuse that need to be addressed (Salgot *et al.* 2006). For the purpose of this thesis research, pathogen risks associated with wastewater to both humans and animals are further discussed here; and are a critical aspect in all public health guidance on wastewater (World Health Organization 2006).

In order to maximize the utilisation of wastewater and address pathogen risks, it is imperative to understand the microbial ecology of wastewater throughout the wastewater treatment train and how this may impact downstream processes, whether natural or anthropologic (Daims *et al.* 2006). Figure 1.1 illustrates the pathways that wastewater may take with the domestic household as a starting point. Further, wastewater's microbial ecology can be used to inform quantitative microbial risk assessments (QMRAs) which are conducted for water safety management plans and are essential if wastewater reuse is to become a safe, effective, and commonplace practice (World Health Organization 2006, Petterson and Ashbolt 2016).

The examination of wastewater is often separated into biological and physicochemical components. The microbiology of wastewater and wastewater treatment processes is well-documented, particularly in light of recent molecular methods (De Vrieze *et al.* 2020). Bacteria are responsible for many key processes that facilitate secondary wastewater treatment such as ammonium and phosphate reduction (Daims *et al.* 2006). For over one hundred years the activated sludge process has been used for secondary treatment of wastewaters, a process that utilises active bacteria and flocs with free-living protozoa. These microbial flocs facilitate the degradation of organic matter, nitrify ammonium and denitrify nitrogen oxides to nitrogen gas, and form aggregates of microorganisms that can easily settle out, some wasted (for phosphorous removal) and some returned to the bioreactors to maintain activity (Salehiziri *et al.* 2020). The combination of these processes leads to a "higher quality" wastewater that can more easily be disinfected (Chahal *et al.* 2016).

### *1.2 Microbial water quality*

For over 130 years, faecal indicator bacteria (FIB) have been used to assess the microbial safety of water (Ashbolt *et al.* 2001). These FIB suggest faecal contamination, and therefore the potential presence of enteric pathogens. Of particular concern are the pathogens present in raw sewage that often find their way to surface waters through various means such as illegal dumping, combined sewer overflows (CSOs), flooding, and limited capacity to treat raw sewage (Casal-Campos *et al.* 2018) (Figure 1.1). As humans rely on surface waters for purposes such as drinking water and recreation, it is desirable to reduce sewage contamination and/or limit exposure to pathogens within sewage (Schoen *et al.* 2011). Starting with John Snow's discovery of cholera as a waterborne disease, society has increasingly been made aware of the risks posed by faecally contaminated waters. For the most part, faecal contamination of water is generally controlled by wastewater disinfection and drinking water disinfection (Ashbolt 2015a). As such, this presents us with the opportunity to look beyond traditional faecal indicator bacteria to those microorganisms that are opportunistic or grow in the environment as these may pose risks to public health that may not yet be recognised or characterised (Ashbolt 2015a) (Table 1.1).

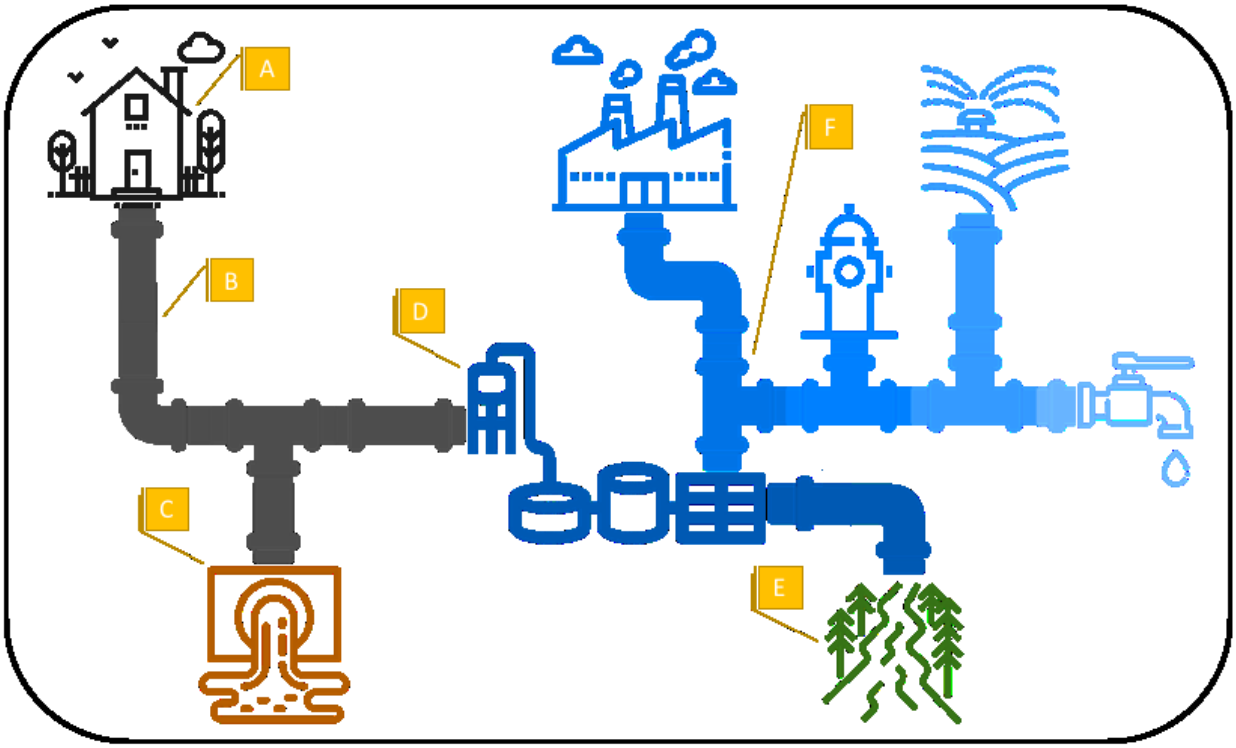


**Figure 1.1.** Wastewater treatment train schematic. **(A)** Wastewater leaves the domestic household and **(B)** travels through sewage pipes. **(C)** During heavy weather events, combined sewage overflows occur and untreated sewage is released into waterways. **(D)** Most of the time, raw sewage is processed at a wastewater treatment plant and is **(E)** discharged into receiving waters. **(F)** In a water reuse scenario, wastewaters are treated to various standards depending on the final end use (e.g., for industrial use, municipal use such as firefighting, agricultural use, and to meet potable water needs).

**Table 1.1.** Lists of the most common human pathogens found in wastewater, the most common indicator organisms and reference pathogens for water, and potentially overlooked saprozoic pathogens in engineered water systems. See Appendix A for definitions of indicator organisms and reference pathogens.

| **Most common pathogens found in wastewater**[a] | **Most common indicator organisms & reference pathogens**[b,c] | **Potentially overlooked saprozoic pathogens**[d] |
|---|---|---|
| Bacteria:<br>• *Salmonella*<br>• *Shigella*<br>• *Vibrio cholerae*<br>• Pathogenic *Escherichia coli*<br>• *Yersinia*<br>• *Campylobacter*<br>• *Leptospira*<br>• *Legionella*<br>• *Staphylococcus*<br>• *Pseudomonas*<br>• *Helicobacter*<br><br>Protozoa:<br>• *Giardia*<br>• *Cryptosporidium*<br>• *Cyclospora*<br>• *Entamoeba histolytica*<br>• *Naegleria*<br>• *Toxoplasma*<br><br>Viruses:<br>• Enteroviruses<br>• Hepatitis viruses<br>• Adenoviruses<br>• Rotaviruses<br>• Noroviruses<br>• Coronaviruses | **Indicator organisms**<br>• Total coliforms<br>• Faecal coliforms<br>• *E. coli*<br>• *Enterococci*<br>• Coliphages<br>• *Clostridium perfringens*<br><br>**Reference pathogens**<br>Bacteria:<br>• *E. coli* O157:H7<br>• *Campylobacter jejuni*<br>• *Shigella sonnei*<br>• *Salmonella enterica*<br><br>Protozoa:<br>• *Cryptosporidium parvum*<br>• *Giardia lamblia*<br><br>Viruses:<br>• Rotaviruses<br>• Noroviruses<br>• Adenoviruses | Bacteria:<br>• *Acinetobacter baumannii*<br>• *Aeromonas hydrophila*<br>• ***Arcobacter butzleri***<br>• *Chlamydiales*<br>• *Legionella longbeacheae*<br>• *L. pneumophila*<br>• Non-tuberculous mycobacteria (NTM)<br>• *Pseudomonas aeruginosa*<br><br>Protozoa:<br>• *Acanthamoeba* spp.<br>• *Balamuthia mandrillaris*<br>• *Vermamoeba* spp.<br>• *Vahlkampfia*<br>• *Naegleria fowleri*<br><br>Protozoan Viruses:<br>• *Mamavirus*<br>• *Mimivirus* |

[a]Bitton 2011
[b]US EPA 2012; Ashbolt *et al.* 2001
[c]NRMMC 2006, 2009; Schoen *et al.* 2017
[d]Ashbolt 2015b; McLellan and Roguet 2019

Numerous studies have shown that one of the most common bacteria present in high concentrations in sewage are the emerging human pathogens in the genus *Arcobacter* (Collado *et*

*al.* 2008; Lu *et al.* 2015; Chen *et al.* 2019; McLellan and Roguet 2019; Kristensen *et al.* 2020). Among others, Ashbolt (2015b) lists *Arcobacter butzleri* as a potentially overlooked saprozoic pathogen (Table 1.1). In addition to the emergence of saprozoic pathogens in the context of water and health, it is increasingly being brought to attention how free-living protozoa (FLP) play a role in the proliferation, persistence, and protection of human pathogenic bacteria within water systems.

## *1.3 General overview of Arcobacter*

*Arcobacter* was first isolated from aborted bovine fetuses in Belfast, Ireland in 1977 (Ellis *et al.* 1977) and were later classified as "aerotolerant campylobacters" (Neill *et al.* 1979). In 1991, the *Arcobacter* genus was proposed to differentiate species of this genus from that of the closely related *Campylobacter* genus (Vandamme *et al.* 1991). Like *Campylobacter* spp., *Arcobacter* spp. are spiral-shaped, Gram-negative bacteria that can grow in microaerophilic conditions (Lehner *et al.* 2005). Unlike *Campylobacter* spp., members of the *Arcobacter* genus display aerotolerance and are able to grow in a wider range of temperatures (15-42°C) (Collado and Figueras 2011). Members of this genus have been isolated from a wide range of environments and there are currently 29 known species of *Arcobacter* (Brückner *et al.* 2020).

### 1.3.1 *Arcobacter* and its public health relevance

Since the creation of this genus, *Arcobacter* spp. are being increasingly known as emerging human pathogens, as well as potential zoonotic agents. Arcobacters have been isolated from foods of animal origin such as poultry, pork, beef, shellfish (clams and mussels), and milk (Ramees *et al.* 2017). Infection with *Arcobacter* spp. have been associated with gastrointestinal symptoms such as abdominal cramping, diarrhoea, and gastroenteritis. Cases of bacteraemia have also been associated with *Arcobacter* spp. infections (Collado and Figueras 2011). Known *Arcobacter* spp. infections are often treated with antibiotics. However, there has been an increasing prevalence of *Arcobacter* spp. resistant to antibiotics (Ferreira *et al.* 2019). One of the most clinically relevant *Arcobacter* species, *Arcobacter butzleri*, is known to express numerous antibiotic resistance genes (ARGs) (Miller *et al.* 2007). Furthermore, there are reports of multidrug-resistant (MDR) strains of *Arcobacter* spp., with a higher prevalence of resistance occurring in human isolates compared to environmental isolates (Ferreira *et al.* 2019). In

addition to evidence of *Arcobacter* spp. being capable of expressing antimicrobial resistance (AMR), species of the genus have been detected in a wide range of environments. The ability to persist in these various habitats, and to withstand harsh conditions, has particular implications when it comes to arcobacters commonly found in water environments (Van Driessche and Houf 2008).

### 1.3.2 Water as a route of transmission for *Arcobacter* spp.

Water seems to play an important role in the transmission of pathogenic arcobacters, given their isolation from numerous natural and artificial aquatic environments, including rivers, lakes, beaches, groundwater, seawater, and wastewater (Ramees *et al.* 2017). Since 1983, at least five outbreaks associated with *Arcobacter* spp. have occurred (Vandamme *et al*. 1992, Rice *et al*. 1999, Fong *et al*. 2007, Lappi *et al*. 2013, Jalava *et al*. 2014). Of these five outbreaks, three were determined to be waterborne and two associated with faecal contamination (Rice *et al.* 1999, Fong *et al.* 2007, Jalava *et al.* 2014). *Arcobacter* species readily form biofilms in water (Houf *et al.* 2002, Ferreira *et al.* 2013), and are considered free-living in the environment (Miller *et al.* 2007). However, it is unclear across the various species and genogroups of *Arcobacter* which may be reliant upon growth within an animal host (Gilbert *et al.* 2019). Specifically, interactions with free-living protozoa, such as amoebae, have only been described for a marine symbiont (Hamann *et al.* 2016). Although *Arcobacter* spp. are ubiquitous in numerous water and terrestrial environments, they often do not dominate in these environments. However, their abundance in sewage is increasingly being brought to the forefront.

### 1.3.3 *Arcobacter* spp. in sewage

*Arcobacter* spp. are consistently present in municipal sewage systems (Chen *et al.* 2019; Collado *et al.* 2008; Fisher *et al.* 2014; Lu *et al.* 2015; McLellan and Roguet 2019; Stampi *et al.* 1999). Furthermore, there is evidence that arcobacters become enriched in the sewage environment (McLellan and Roguet 2019). In wastewater treatment systems, *Arcobacter* spp. make up a large proportion of the bacterial community. Several studies have described the genus *Arcobacter* as one of the most dominant genera of total bacteria in raw sewage at abundance percentages of up to 23% according to sequencing methods (Fisher *et al.* 2014; Kristensen *et al.* 2020; Lu *et al.* 2015; McLellan *et al.* 2010). Further, of the potential pathogens present in raw

sewage, it has been reported that up to 96% of this population consists of *Arcobacter* spp. (Lu *et al.* 2015). Additionally, *A. butzleri* concentrations in sewage may exceed $10^5$ CFU/100 mL (Banting *et al.* 2016). Of particular concern, *Arcobacter* spp. seem to survive the entire wastewater treatment process, including changes in pH, biological activity, and disinfection such as UV-B irradiation (Webb *et al.* 2016b). *Arcobacter* spp. are often associated with raw sewage as evidenced by its presence in faecally contaminated waters (Fisher *et al.* 2014). Additionally, the dominant *Arcobacter* phylotype in humans is also the dominant phylotype in sewage (McLellan *et al.* 2010). As such, the proliferation and subsequent release of these phylotypes may have implications for public health.

### *1.4 Free-living protozoa in the environment*

Free-living protozoa are single-celled, generally non-parasitic, eukaryotic microorganisms with phagocytic capabilities. FLP are generally divided into three groups: amoebae, ciliates, and flagellates (Warren *et al.* 2016). These groupings are based on morphology and locomotion rather than phylogeny (with the exception of ciliates) for practical reasons (i.e., for the ease of isolation, identification, and cultivation methods). This thesis work focuses only on the amoebae and ciliates (Figure 1.2). FLP are generally aquatic organisms and are ubiquitous in natural and man-made water environments (Warren *et al.* 2016). They have been isolated from rivers, lakes, oceans, cooling towers, hospital pipes, tap water, and wastewaters (Thomas *et al.* 2010, Madoni 2011, Scheid 2019a). FLP are both planktonic and live within biofilms depending on the organism's morphology and/or life stage (Rodríguez-Zaragoza 1994, Thomas *et al.* 2010, Bitton 2011). They feed on bacteria and other microorganisms smaller than themselves and are known to control bacterial populations in different environments, particularly within wastewater treatment systems (Bitton 2011, Madoni 2011).

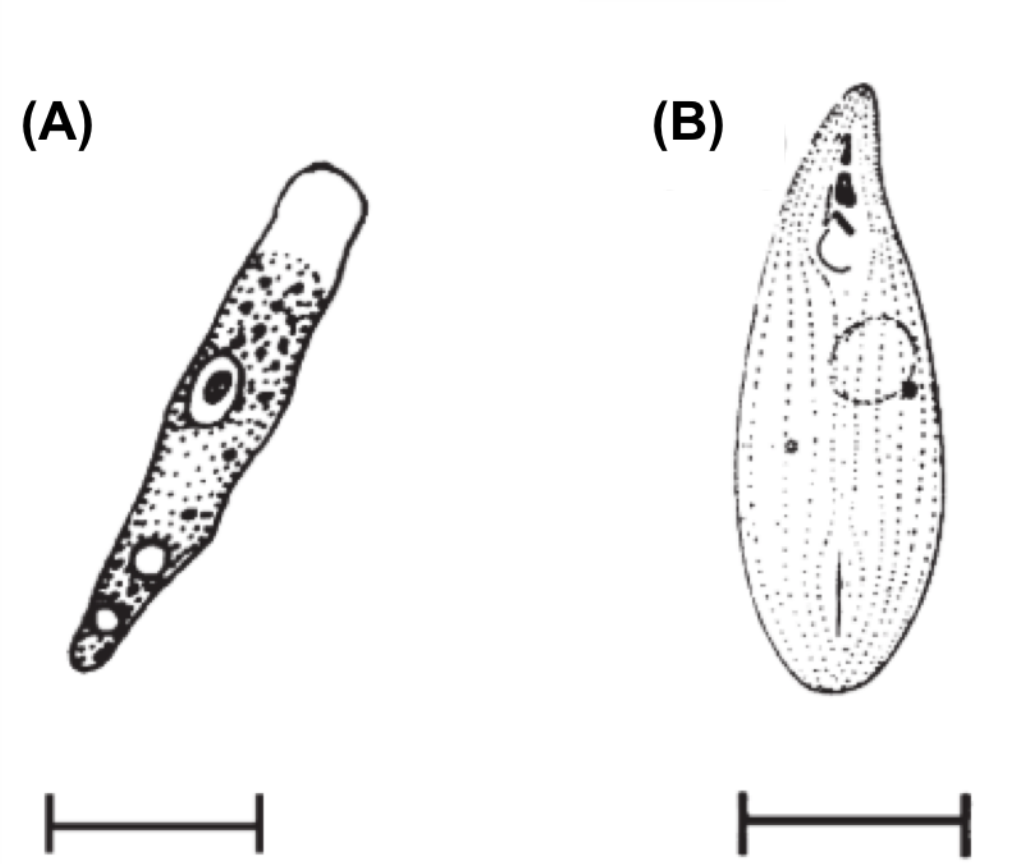


**Figure 1.2.** Examples of two of the three the main groups of FLP, amoebae and ciliates. **(A)** The amoeba *Vermamoeba vermiformis* (scale bar = 10 μm) and **(B)** the ciliate *Philasterides armata* (scale bar = 25 μm). Adapted from Warren, Esteban, and Finlay (2016).

### 1.4.1 Free-living amoebae

Free-living amoebae (FLA) are characterised by their pseudopodia. Through the extension and retraction of these pseudopods, FLA are able to adhere to and move across surfaces. Pseudopods also provide FLA with feeding capabilities through phagocytosis (Warren *et al.* 2016). FLA reproduce by binary fission and may have different stages throughout their life cycle. When FLA are in suitable conditions, they exist in an active trophozoite form that allows them to feed and replicate. When adverse conditions are encountered (e.g., low nutrient conditions), a trophozoite may transform into an extremely resilient cyst. Cysts are often highly resistant to higher temperatures, starvation conditions, and desiccation and allow FLA to "hibernate" until conditions allow for excystation. As such, FLA may be able to persist for long periods of time in various environments despite unfavourable surroundings (Thomas *et al.* 2010,

Scheid 2019a). The following FLA were chosen for examination in this thesis research for the main reason that they are representative of the three most common genera of FLA identified in the general environment and, more specifically, in raw sewage and wastewater treatment systems: *Acanthamoeba, Vermamoeba,* and *Naegleria* (Rodríguez-Zaragoza 1994, García *et al.* 2011).

*1.4.1.1 Acanthamoeba spp.*

*Acanthamoeba* is the most abundant genus of FLA in the environment (Khan 2006). During their life cycle, *Acanthamoeba* exist in two forms: an active trophozoite and a dormant cyst. In general, *Acanthamoeba* trophozoites range from 12-35 µm in diameter. Trophozoites of this genus are characterised by spiny-looking structures protruding from the surface of the trophozoite, which are known as acanthopodia. When the trophozoites encounter harsh conditions, they undergo a transformation into cysts. These cysts range in size from 5-20 µm and are double-walled, with the inner wall being distinctly star-shaped (Khan 2006).

*Acanthamoeba* have the ability to survive in a wide range of environments. As such, they have been isolated from seawater, salt water lakes, freshwater lakes, pond water, river water, stagnant water, sewage, beaches, soil, sediments, compost, bottled water, distilled water bottles, swimming pools, public water supplies, ventilation ducts, air-conditioning units, surgical instruments, contact lenses and their cases, the air-water interface, and from the atmosphere (Khan 2006, Muchesa *et al.* 2014, Rodríguez-Zaragoza 1994, Scheid 2019a). Species of this genus have also been isolated from eye wash stations, dialysis units, and hospitals.

Possibly due to their abundance in the environment, *Acanthamoeba* spp. have been implicated in more infections than any other FLA. They are the causes of diseases such as the usually fatal granulomatous amoebic encephalitis and *Acanthamoeba* keratitis. The ability for *Acanthamoeba* cysts to remain viable while maintaining pathogenicity for several years has thus implicated the cyst's role in the transmission of *Acanthamoeba* infections (Mazur *et al.* 1995). Since the discovery of *Acanthamoeba*'s role in Legionnaires' disease in 1980 (Rowbotham 1980), *Acanthamoeba* has been and continues to be the most studied genus of FLA.

#### *1.4.1.2 Vermamoeba vermiformis*

Like *Acanthamoeba*, species of the *Vermamoeba* genus have two life stages, the trophozoite and the cyst. *Vermamoeba* trophozoites are generally longer than they are wide, causing them to look slug-like. The trophozoites may range in size from 22-42 µm long (Scheid 2019b). When moving *V. vermiformis* trophozoites are usually monopodial ("mono-" = one, "-podial" = foot), but may become multipodial during direction changes. Cysts of the species are small and spherical, around 6 µm in diameter (Delafont *et al.* 2018). These cysts often cluster, forming aggregates. The cysts are composed of proteins, glucose polymers, and a double-layered wall with a thin endocyst and a thicker exocyst. Due to the different cyst compositions, *V. vermiformis* cysts are more sensitive to disinfection treatments (e.g., chlorine, heat shock, UV) than *Acanthamoeba* cysts (Delafont *et al.* 2018).

Similar to *Acanthamoeba*, *V. vermiformis* (formerly *Hartmanella vermiformis*) are also found in diverse environments but are more common in aquatic environments. They are particularly able to thrive in engineered water systems. *V. vermiformis* has frequently been isolated from water treatment and distribution systems, as well as tap water installations, fountains, and swimming pools (Delafont *et al.* 2018, Scheid 2019b). *Vermamoeba* are frequently present in hospital and dental environments, more so than *Acanthamoeba.* They are particularly abundant in hospital hot water systems. As *V. vermiformis* is known to be more thermotolerant than other FLA species, this may explain its prevalence in engineered water systems which are more likely to contain and cycle hot water compared to natural water systems (Rohr *et al.* 1998, Rhoads *et al.* 2015).

Although there have been reports of corneal damage and keratitis associated with *V. vermiformis* infections (Scheid 2019b), there have generally been no known reports of serious infections directly caused by the species.

#### *1.4.1.3 Willaertia magna*

Another thermotolerant FLA species, *W. magna* is closely related to the pathogenic *Naegleria fowleri*, which is also known as the "brain-eating amoeba" (Robinson *et al.* 1989). Although *Willaertia* sp. has been associated with gastric infection in a dog (Steele *et al*. 1997), to date, there is no literature that suggests *W. magna* to be pathogenic to humans. The largest of the three amoebae described above, *W. magna* trophozoites vary in size from 50-100 µm in length.

Their cysts are 18-21 µm in diameter (Hasni *et al.* 2019). Unlike *Acanthamoeba* and *V. vermiformis*, *W. magna* may also assume a flagellate stage, possessing four flagella (Robinson *et al.* 1989, Hasni *et al.* 2019). Flagellated *W. magna* have the ability to divide and can pass flagella onto the daughter cells (Robinson *et al.* 1989).

Since its discovery in 1984 (de Jonckheere *et al*. 1984), *W. magna* has not been as well-studied as *Acanthamoeba* sp. and *V. vermiformis*. *Willaertia* spp. have been isolated from freshwater sediments in geographically diverse locations (e.g., Australia, France, Japan, Indonesia, Iran, Madagascar, USA) (de Jonckheere *et al*. 1984, Dobson *et al*. 1993, Niyyati *et al*. 2009). More specifically, *W. magna* have been isolated from bovine faeces, soil, thermal waters, and canals (de Jonckheere *et al*. 1984, 2007).

### 1.4.2 Free-swimming ciliates

Ciliates have elongated, ovoid bodies and are characterised for their possession of cilia. These are short hairs that are distributed over the surface of ciliate bodies in structured rows called kineties (Warren *et al.* 2016). The rapid movement of these cilia allow for the quick movement of ciliates through liquid media. Cilia also play a role in feeding and will sweep smaller microorganisms and other detritus into the oral groove of a ciliate (Warren *et al.* 2016). Ciliates in general appear to be the most abundant protozoan group in activated sludge, while free-swimming ciliates are commonly found in the liquor phase of activated sludge in wastewater treatment plants (WWTPs) (Madoni 2011). Further, they are usually associated with high bacterial concentrations as it is during the early stages of wastewater treatment that free-swimming ciliates have the biggest impact on the reduction of bacteria (Bitton 2011, Madoni 2011).

#### *1.4.2.1 Tetrahymena pyriformis*

*Tetrahymena pyriformis* is a freshwater ciliate that is generally 60-100 µm long and 30 µm wide. It is characterised by its pear-like shape and 17-23 longitudinal kineties on its cell surface (Elliott 1973). *T. pyriformis* is one of the most commonly used ciliated models in laboratory studies due to its short life cycle and ease of cultivation (Sauvant *et al.* 1999). They are commonly found in streams, rivers, ponds, and lakes (Elliott 1970). *T. pyriformis* is not a

known human pathogen, but is well-known to be a bacterivore which has implications on the transmission of human pathogenic bacteria (Elliott 1970, Madoni 2011).

### 1.4.3 FLP as an environmental reservoir and protective agent for human pathogenic bacteria

Free-living protozoa are known to act as environmental reservoirs for a number of human pathogenic bacteria. As such, FLP are well-known to be the 'Trojan horses' of the microbial world, especially with regard to some bacteria which are resistant to their digestion (Greub and Raoult 2004, Matz and Kjelleberg 2005). Bacteria that have developed mechanisms to avoid digestion by FLP have gone on to form endosymbiotic relationships with FLP acting as hosts. FLP house these digestion-resistant bacteria and transport them from one location to another. As a result of being internalised by FLP, bacteria can further exploit their hosts and benefit from them through two different means: as a vessel in which they can replicate, and as protection from stressors in the environment that can lead to their increased persistence.

Perhaps the most well-known instance of a bacteria-FLP interaction that results in the replication of internalised bacteria is that of *Acanthamoeba* and *Legionella pneumophila* (Rowbotham 1980). In this landmark study, Rowbotham reported on the replication of *L. pneumophila* in vacuoles of *Acanthamoeba* and further suggested that the cause of Legionnaires' disease is due to the inhalation of packaged *L. pneumophila* in trophozoites or released vesicles rather than the inhalation of free *L. pneumophila.*

In addition to providing a conducive environment in which to reproduce, FLP have been shown to provide protection to bacteria from harsh external processes such as disinfection. Human pathogenic bacteria such as *E. coli*, *C. jejuni*, *Klebsiella pneumoniae*, and *L. pneumophila* have been shown to not just survive chlorination and ultraviolet (UV) irradiation when ingested by FLP, but have also been shown to display increased tolerance of free chlorine and UV (King *et al.* 1988, Cervero-Aragó *et al.* 2014). As such, the ability for bacteria to resist digestion by protozoan predators is often seen as a precursor to both the development of a bacterial species' pathogenicity to humans and its ability to successfully persist in a wide range of environments (Greub and Raoult 2004, Thomas *et al.* 2010). The co-evolutionary relationship between bacteria and FLP can have wide-ranging effects on both public health and the environment. Following this, it is especially important to focus on these relationships in the

context of water, not just at the drinking water stage, but also during and after the wastewater treatment stages.

### *1.5 Wastewater treatment*

To control pollution and prevent the spread of waterborne diseases, wastewaters must first be treated before being discharged into the environment or used in water reuse schemes (Figure 1.1). In general, the wastewater treatment process involves the removal of contaminants and suspended solids to such a degree that the water is "clean" enough to be returned to the environment. Wastewater treatment systems have evolved from basic sedimentation to complex systems that consist of several main treatment processes (Hammer and Hammer 2012a). Today, biological, chemical, and physical processes are all involved throughout the different stages of wastewater treatment. Table 1.2 describes the wastewater treatment stages in Edmonton, Alberta.

**Table 1.2.** The main stages of the wastewater treatment process at the Gold Bar Wastewater Treatment Plant in Edmonton, Alberta. [a,b]

| Treatment Stage | Processes | Resulting Effluent |
|---|---|---|
| **Primary** | **Physical removal of solids:**<br>• Heavier solids (sludge) settle to the bottom of clarifier tank and are scraped off<br>• Lighter solids (scum) float to the top of the tank and are skimmed off | **Primary effluent:**<br>• Still contains dissolved solids<br>• Moves on to secondary treatment stage |
| **Secondary** | **Biological conversion of organic material:**<br>• Microorganisms break down dissolved organic matter; remove phosphorus and ammonia<br>**Settling out of microorganisms:**<br>• Microorganisms form "flocs" that settle out of the liquid and are removed and re-directed | **Secondary effluent:**<br>• Almost all organic impurities removed<br>• Moves on to tertiary treatment stage |
| **Tertiary** | **Disinfection:**<br>• Secondary effluent is treated with high-intensity ultraviolet light<br>**Polishing:**<br>• Secondary effluent is passed through membrane filters | **UV disinfected:**<br>• Discharged into the North Saskatchewan River<br>**Membrane-filtered:**<br>• Re-directed to industrial plants |

[a]EPCOR 2020
[b]Hammer and Hammer 2012b

### 1.5.1 UV disinfection of wastewater

Ultraviolet irradiation is increasingly becoming the most common form of wastewater disinfection prior to the discharge of treated wastewater into receiving bodies. UV irradiation is known for being effective in inactivating pathogens, particularly the chlorine-tolerant parasitic protozoa and enteric viruses (Adeyemo *et al.* 2019). UV irradiation's disinfection properties lie in its ability to:

- Damage double stranded DNA (dsDNA) and create free radicals or reactive oxygen species that can further damage cells (Rastogi *et al.* 2010);
- Create dimers between nucleotides which leads to the inhibition of replication and transcription processes (Coohill and Sagripanti 2008); and
- Induce photochemical reactions in cellular enzymes and proteins (Rastogi *et al.* 2010).

UV disinfection is an attractive approach to wastewater disinfection because it does not involve the addition of chemicals nor does it lead to the production of disinfection residuals (Hassen *et al.* 2000). Despite the effectiveness at which UV disinfection acts against bacteria (Hijnen *et al.* 2006), there is evidence that *A. butzleri* survives various stages of wastewater treatment, including UV disinfection (Webb *et al.* 2016b, Kristensen *et al.* 2020). At the time of writing, there is currently no published data on the direct effect of UV irradiation on *A. butzleri* in water. Given potential UV resistance of environmental arcobacters, combined with no reported specific UV dose-response studies, UV disinfection, along with potential FLP interactions were the focus of this thesis research.

## *1.6 Project Scope & Objectives*

This thesis was conducted with the aim of exploring the small world of *Arcobacter* within the larger universe of wastewater and wastewater treatment. Concentrations of *A. butzleri* in sewage are greater than that of *Campylobacter* spp. by up to 4 orders of magnitude (Banting *et al.* 2016). The key significance here is that campylobacters are used as the reference bacterial pathogen group to define $\log_{10}$-reductions necessary for safe exposures (Schoen *et al.* 2017) (Table 1.1), and hence treatment needs may be severely underestimated if arcobacters represent a waterborne infection risk. Based on epidemiology, infections by campylobacters and *Salmonella enterica* are thought to be the most important waterborne bacterial pathogens and hence expected to occur in sewage at the highest concentrations. Given that humans (both symptomatic and non-

symptomatic) only shed up to 1.5 $\log_{10} \cdot mg^{-1}$ of *A. butzleri* in their faeces (Webb *et al.* 2016a), higher levels of *Arcobacter* in sewage compared to those in human faeces imply growth of *Arcobacter* spp. in sewage and/or within wastewater treatment systems. However, it is unknown where such growth may be occurring, although *Arcobacter* spp. have been reported to persist within *Acanthamoeba* trophozoites and live as epibionts on the marine flagellate *Lenisia limosa* (Hamann *et al.* 2016, Villanueva *et al.* 2016).

Nonetheless, it is unknown whether arcobacters are able to replicate within wastewater protozoa. However, a relatively closely related species *Campylobacter jejuni*, has been reported to replicate within FLP and be released in extracellular vesicles (ECVs) by both amoebae and ciliates (Axelsson-Olsson *et al.* 2007, Trigui and Charette 2016). Additionally, given its likely growth in the environment, the public health implications of *Arcobacter*'s survival through the wastewater treatment process could make this group an important consideration in future risk assessments. Therefore, it was hypothesised that free-living protozoa may play a role in the replication of *A. butzleri* in sewage as well as protect *A. butzleri* from UV disinfection. Following this, the overall objectives of this thesis were as follows:

1. Elucidate and characterize the interactions between *Arcobacter butzleri* and commonly occurring free-living protozoa in raw sewage (Chapter 2); and
2. Examine the efficacy of UV disinfection on *Arcobacter butzleri* alone and when associated with free-living protozoa (Chapter 3).

# Chapter 2: Interactions between *Arcobacter butzleri* and free-living protozoa

## *2.1 Introduction*

The genus *Arcobacter* consists of emerging pathogens that are increasingly being associated with faecal contamination (Collado *et al.* 2008). *Arcobacter* spp. have been reported to thrive in sewer systems, are present throughout the entire wastewater treatment train, and have been detected in treated wastewater effluent that is discharged into the environment (Stampi *et al.* 1999, Fisher *et al.* 2014, Webb *et al.* 2016b, Kristensen *et al.* 2020). Of the 29 species that comprise the genus, three species in particular have emerged as potential human pathogens: *A. butzleri*, *A. cryaerophilus,* and *A. skirrowii* (Collado and Figueras 2011). The dominant *Arcobacter* species in sewage seems dependent on the detection method used. Isolation studies have found *A. butzleri* to be the most commonly found *Arcobacter* species in sewage (Collado *et al.* 2008, 2010). However, according to metagenomic studies, *A. cryaerophilus* appears to be the dominant species (Fisher *et al.* 2014). Although arcobacters only comprise <0.001% of the human gut microbial community, they consistently make up a large portion of the bacterial community in sewage (Shanks *et al.* 2013, Fisher *et al.* 2014, Cai *et al.* 2014, Lu *et al.* 2015).

Also found in sewage environments are single celled free-living protozoa. Within sewage environments, protozoa play an important role by controlling bacterial populations through predation. The relationship between free-living protozoa and bacteria has been extensively studied with various FLP acting as environmental reservoirs for numerous bacterial species. There have been a couple of studies regarding the interactions of *Arcobacter* spp. with FLP. Hamann *et al.* (2016) reported an episymbiotic relationship between *Arcobacter* sp. and the free-living protist *Lenisia limosa* in marine waters. Additionally, *A. butzleri* has been shown to survive in the presence of the free-living amoeba *A. castellanii* for up to 10 days (Villanueva *et al.* 2016). As such, it is plausible that *Arcobacter*'s abundance in sewage may be due to interactions with FLP that allow for the replication of the bacteria.

Hence, the objective of this chapter was to determine the interactions between *A. butzleri*, the most clinically relevant and commonly isolated *Arcobacter* species, with various FLP commonly found in sewage and water environments in general. Co-cultures of *A. butzleri* with free-living amoebae and the ciliate *Tetrahymena pyriformis* were completed to determine the kinetics of growth of *A. butzleri* when associated with FLP. Various imaging methods were then

carried out to determine whether *A. butzleri* was internalised and subsequently digested or released by the FLP.

## *2.2 Materials & Methods*

### 2.2.1 Isolation, cultivation, & maintenance of organisms

#### *2.2.1.1 Isolation & cultivation of A. butzleri*

The *A. butzleri* isolate used in this thesis was isolated from sewage samples collected from the Pine Creek Wastewater Treatment Plant in Calgary, Alberta, Canada in 2015. Samples were collected from the raw, post-grit screened influent, shipped to the Alberta Provincial Laboratory in Edmonton, and processed within 24 hours of sample collection (Banting *et al.* 2016). Processing was as follows: 300 mL of sterile buffered water was added to 100 mL of wastewater, after which the sample was centrifuged at 10,000 × *g* for 20 min at 20°C. Samples were resuspended in Bolton broth with either selective supplement or rifampin (10 mg/L) and polymyxin B (5,000 IU/L). Isolates were kept in skim milk stocks at a 20% volume concentration. Cultures were inoculated into Bolton broth and incubated at 30°C in microaerophilic conditions (CN0025A; CampyGen™, Oxoid, Basingstoke, United Kingdom) when needed for experiments.

#### *2.2.1.2 Cultivation & maintenance of free-living amoebae*

Strains of *Acanthamoeba polyphaga* (ATCC 30461), *Vermamoeba vermiformis* (ATCC 50237), and *Willaertia magna* (ATCC 50035) were maintained in an amoebae-specific nutrient-rich broth (modified ATCC medium 1021) described in Table 2.1. Amoebae were maintained in 25 $cm^2$ non-vented cell culture flasks in 5 mL of serum-casein-glucose-yeast extract medium (SCGYEM) at 25°C. The media were changed weekly, and cultures were sub-cultured once a month. This was done by washing the monolayer with fresh media and transferring half of the suspension to a new flask.

**Table 2.1**. Media ingredients for the growth and maintenance of free-living amoebae (SCGYEM) and *T. pyriformis* (TM).

| **SCGYEM (ATCC medium 1021)[1]** | | **TM (ATCC medium 357)[2]** | |
|---|---|---|---|
| Casein | 10.0 g | Proteose Peptone | 5.0 g |
| Glucose | 2.5 g | Tryptone | 5.0 g |
| Yeast Extract | 5.0 g | $K_2HPO_4$ | 0.2 g |
| $Na_2HPO_4$ | 1.32 g | Distilled Water | 1.0 L |
| $KH_2PO_4$ | 0.8 g | | |
| Distilled Water | 900 mL | | |
| Foetal Bovine Serum (FBS) | 20 mL | | |

[1]All components except for the FBS were combined, autoclaved at 121°C for 20 min. Prior to use, the medium was filter-sterilised using a 0.22-micron Stericup® vacuum filtration unit (S2GPU05RE; MilliporeSigma, Burlington, MA, USA). Following cooling, the FBS was aseptically added to a final concentration of 10%. Media were stored at 4°C when not in use.
[2]All components were combined and autoclaved at 121°C for 15 minutes.

*2.2.1.3 Cultivation & maintenance of T. pyriformis*

The media used to grow and maintain *T. pyriformis* was ATCC medium 357 *Tetrahymena* medium (TM) (Table 2.1). A live stock of *Tetrahymena pyriformis* (ATCC 30203) was stored in 11% dimethyl sulfoxide (DMSO) at -80°C. When needed, stocks were revived by the addition of TM and incubated in 25 $cm^2$ cell culture flasks at 25°C. Stocks were sub-cultured bi-weekly by transferring 100 µL of the culture to a new flask with 7 mL of fresh medium. As per the suggestion by Berk and Garduño (2013), sub-culturing was completed up to a maximum of a year before a new frozen stock was revived.

*2.2.1.4 Wastewater FLA isolates*

Wastewater was collected from the Gold Bar Wastewater Treatment Plant in Edmonton, Alberta, Canada on March 19, 2019. Samples were taken from 5 stages of the treatment train: post-grit, primary effluent, aerated sludge, post-membrane, and final effluent (post-UV). From each sample, 100 mL was vacuum-filtered through a 0.22 µm membrane filter. The filters were then placed upside-down onto non-nutrient agar (NNA) plates (0.062 g $MgSO_4$, 0.400 g $KH_2PO_4$, 0.662 g $Na_2HPO_4$, 15 g agar [Select Agar™, Invitrogen™, Waltham, MA, USA], 1 L distilled water) seeded with *Escherichia coli* (ATTC 25922). The plates were incubated at 30°C

and observed daily under an inverted light microscope (CKX41; Olympus Life Science) for amoebal growth.

Sections of the agar with different morphological growth were excised and placed upside-down onto freshly seeded NNA plates. Isolates were sub-cultured in this way until pure cultures were obtained. Following this, amoebae were scraped off the agar and inoculated into 5 mL of SCGYEM and 50 uL of penicillin-streptomycin (pen-strep) at a working concentration of 100 ug/mL (HyClone™, GE Life Sciences, Marlborough, MA, USA) in 25 $cm^2$ cell culture flasks and incubated at 25°C. The cultures were observed daily for growth and contamination. Media was changed daily with progressively lower volumes of pen-strep added until a majority of *E. coli* were eliminated. Isolates continued to be maintained in SCGYEM at 25°C and were microscopically identified based on morphology.

Once isolates were grown to confluence, 2 mL of freely suspended amoebae culture were washed (4000 × *g* for 5 minutes) in phosphate buffered saline (PBS) (HyClone™, GE Life Sciences, Marlborough, MA, USA) and were lysed by boiling at 95°C for 10 minutes in a heating block. DNA extractions were then completed using the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Extracts were then amplified using the following 18S rRNA primers (Wang *et al*. 2014):

- EUK370F: 5'-AGGGTTYGAYTCCGGAGAGG-3'
- EUK1642R: 5'-CCTTTGTACACACCGCCCG-3'

The above primers were verified to work when they were tested with the ATCC strains of amoebae mentioned earlier. PCR conditions were optimised for amplification with an initial denaturation at 95°C for 5 minutes, followed by 35 cycles at 95°C for 15 seconds, 58°C for 30 seconds, and 72°C for 1 minute. The PCR product was run on 1% agarose gel with EtBr and the target DNA fragment was visualised with a UV transilluminator. Target DNA extraction was completed with the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) and sent for Sanger sequencing. To confirm the morphological identification of the wastewater isolates, sequences were compared to those in NCBI's rRNA/ITS database using the nucleotide-nucleotide Basic Local Alignment Search Tool (BLAST).

### 2.2.2 Co-culture methods

#### *2.2.2.1 Co-culture of A. butzleri & free-living amoebae*

After amoebae were grown to confluence, they were scraped from the bottom of the cell culture flask with a cell scraper. The entire contents of the flask were transferred to a 15 mL conical Falcon tube and centrifuged at 4000 × *g* for 5 min. The supernatant was discarded, and the pellet was resuspended in 3 mL of autoclaved river water which was collected from the North Saskatchewan River in Edmonton, Alberta. After this washing process was repeated 3 times, 10 µL aliquots were taken from each sample to be counted on a haemocytometer (Hausser Scientific Bright-Line™, Fisher Scientific) at 100x magnification on an inverted microscope (CKX41; Olympus, Center Valley, PA, USA). Based on these counts, the final volumes to be used were adjusted so that co-cultures would have a final amoebae concentration of $10^5$ cells/mL.

To prepare bacteria for the co-culture, 1 mL of *A. butzleri* culture was plated onto Bolton agar (CM0983; Oxoid, Basingstoke, United Kingdom) and incubated at 30°C in microaerophilic conditions for 48 h. The bacteria were scraped off the agar and suspended in PBS to an optical density of 1.0 at 600 nm which had an approximate concentration of $10^9$ cells/mL. This suspension was diluted tenfold for a final working concentration of $10^8$ cells/mL.

In 15 mL conical tubes, the following were added: 3 mL river water, appropriately calculated volume of FLA, and 150 µL *A. butzleri*. Tubes were centrifuged at 1000 × *g* for 1 min to facilitate contact between the FLA and bacteria before briefly vortexing. Tubes were incubated at 30°C at an inclined position under static conditions to maximise the surface area to which the FLA could attach.

At the designated time points, tubes were placed on ice for 2 min to facilitate detachment of the FLA. Tubes were then repeatedly tapped on a solid surface to dislodge the amoeba and vortexed. The co-cultures were then passed through a 3 mL syringe (309657; BD, Franklin Lake, NJ, USA) attached to a 20 G needle (305176; BD) 10 times to lyse the amoebae. The resulting lysate was serially diluted and plated onto Bolton agar before being incubated at the appropriate conditions for *A. butzleri*. After 48 h, CFUs were counted.

*2.2.2.2 Co-culture of A. butzleri and T. pyriformis*

The co-culture of *A. butzleri* and *T. pyriformis* is adapted from the co-culture protocol of Berk and Garduño (2013). To prepare *T. pyriformis* for co-culture, 250 µL of culture was sub-cultured into 7 mL of TM and allowed to grow for 3-4 days in a cell culture flask. Following this, the ciliates were transferred to a 15 mL conical tube and centrifuged at 600 × *g* for 3 min. Half of the supernatant was quickly removed before adding 8 mL of autoclaved river water. Ciliates were allowed to adjust to the osmolarity for 10 min before being centrifuged again. The resulting pellet was quickly transferred to another conical tube to which river water was added to a final volume of around 10-12 mL.

For the enumeration of the ciliates, a 10 µL aliquot of the prepared suspension and 1 µL of 4% formalin were mixed on a glass slide. The ciliates were allowed to settle for 5 min before the entire drop was manually microscopically scanned and counted. Bacteria were prepared in the same way as described above for the co-culture with FLA. To 2 mL of environmental water, ciliates and bacteria were added so that the resulting ratio was around $10^4$ ciliates/mL to $5\times10^7$ bacteria/mL (Berk and Garduño 2013). Co-cultures were carried out in 12-well plates and incubated at 25°C.

At the designated time points, co-cultures were transferred from the wells to 15 mL conical tubes and lysed with a 21 G needle. However, after observation, it was found that this method was insufficient to lyse the ciliates. Instead, the co-cultures were sonicated in a sonicator bath (Bioruptor® Plus, Diagenode, Denville, NJ, USA) at high intensity for 1 min (Pushkareva and Ermolaeva 2010). Three cycles of 20 s were found to adequately lyse most, if not all, of the ciliates in the sample, without killing the bacteria (Appendix B). From the resulting lysate, 500 µL was set aside for further molecular work and 100 µL was serially diluted and plated onto Bolton agar before being incubated at the appropriate conditions.

*2.2.2.3 Transwell co-cultures*

Suspensions of *A. butzleri* and FLA were prepared as described above. In a 12-well Transwell® plate (C3401; Corning Incorporated, Corning, NY, USA), 150 µL bacterial suspension was placed in the basal chamber of each well, after which enough river water was added to a total of 2 mL. The appropriately calculated volumes of FLA were then added to the apical chambers, after which river water was added to a total volume of 1 mL. Chambers were

separated by a 0.45 μm membrane. At the designated time points, 100 μL of bacterial suspension was removed from the basal chambers of each FLA type, serially diluted, and plated onto Bolton agar. The plated cultures were incubated and enumerated as described above.

### 2.2.3 Imaging methods

#### *2.2.3.1 Transmission electron microscopy*

At the designated time points, co-cultures were centrifuged for 5 min at 4000 × *g*. The supernatant was discarded, and the pellet was resuspended in 1 mL fixative (2.5% glutaraldehyde, 2% paraformaldehyde in 0.1M phosphate). Samples were then processed at the Microscope Facility at the University of Alberta's Biological Sciences Department. Briefly, the co-cultures were fixed for 2 hours at room temperature. The co-cultures were then post-fixed with 1% osmium tetroxide for 1 h before being resuspended and cured in 100% SPURR resin. Sectioning was done by an ultramicrotome (Ultracut E Reichert-Jung) and stained with uranyl acetate and lead citrate. Imaging was completed with a Philips/FEI (Morgagni) Transmission Electron Microscope and a Gatan Digital Camera. Approximately 20 fields of view were examined for each co-culture.

#### *2.2.3.2 Imaging flow cytometry*

A bacterial suspension was prepared as previously described to a working concentration of $10^7$ cells/mL. To the suspension, 5 μL of acridine orange was added and the suspension was incubated at 37°C for 1 h. The suspension was then washed (13.3 rpm for 3-4 min) before being resuspended in sterile water and co-cultured with the protozoa. The co-cultures were subsequently run on the Amnis® ImageStream®$^X$ Mk II Imaging Flow Cytometer (Luminex Corp., Seattle, WA, USA).

#### *2.2.3.3 Fluorescence microscopy*

A bacterial suspension was prepared to a working concentration of $10^7$ cells/mL. The LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to stain the suspension according to the manufacturer's instructions. After a 15-minute incubation in the dark, the suspension was centrifuged (13.3 rpm for 3-4 min) and resuspended in a solution of 0.85% NaCl. The stained bacteria were then co-cultured with

amoebae that were isolated from sewage and *T. pyriformis*. After 2 h of incubation, the co-cultures were imaged on a fluorescence microscope (EVOS® Cell Imaging System, Thermo Fisher Scientific, Waltham, MA, USA).

### 2.2.4 Molecular methods

#### *2.2.4.1 DNA extraction*

Samples were extracted using the DNeasy Blood and Tissue Kit according to the manufacturer's instructions with minor modifications. Prior to elution, the samples were incubated at room temperature for 10 min in elution buffer that was warmed up to ~42°C. Resulting extracts were eluted at 200 µL and stored at -20°C until needed.

#### *2.2.4.2 Quantification of A. butzleri by qPCR*

For further confirmation of quantification, qPCR experiments were performed using the PrimeTime® Gene Expression Master Mix Kit (1055772; IDT, Coralville, IA, USA). The primers used were specific to *A. butzleri* and targeted the *hsp60* gene. The primers and probe, previously described by de Boer *et al.* (2013), were as follows:

- Abutz-F: 5'-CTC TTC ATT AAA AGA GAT GTT ACC AAT TTT-3'
- Abutz-R: 5'-CAC CAT CTA CAT CTT CWG CAA TAA TTA CT-3'
- Abutz-PMGB: FAM-CTT CCT GAT TGA TTT ACT GAT T-MFQMGB

The forward and reverse primers were used at a concentration of 0.3 µM, while the probe was used at a concentration of 0.1 µM. Bovine serum albumin at a concentration of 200 µg/mL (B6917; Sigma-Aldrich, Oakville, ON, Canada) was included in the reaction mix. Reactions were run according to the following conditions: 95°C for 3 min, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. The quantification of the target was based on a standard curve. Plasmids had a starting concentration of 500,000 copies/5 µl and were serially diluted to create a standard curve consisting of five different concentrations. With the exception of the standard curve reactions, which were run in triplicate, all reactions were performed in duplicate. Amplification and quantification were completed on the Rotor-Gene Q™ 6,000 qRT cycler system (QIAGEN, Hilden, Germany).

### 2.2.5 Statistical analysis

All statistical analyses were performed on GraphPad Prism (Version 8.0, GraphPad Software, LLC, San Diego, USA). Growth curves for all FLP co-cultures, except for co-cultures completed in Transwell® plates, were analysed for statistical differences between treated groups among all time points. For these groups, *p*-values are the result of unpaired, two-tailed Student's t-tests with $\alpha = 0.05$. Growth curves of Transwell assay co-cultures were analysed for statistical differences between all groups with ANOVA.

## *2.3 Results & Discussion*

### 2.3.1 Co-cultures of *A. butzleri* with FLA in river water

The cells of the *A. butzleri* isolate used in these experiments were pictured to be rod-shaped cells about 1-2 µm in length and 0.5 µm in diameter (Figure 2.1). These dimensions are consistent with what has been reported in literature for *A. butzleri* (Vandamme *et al.* 1991).

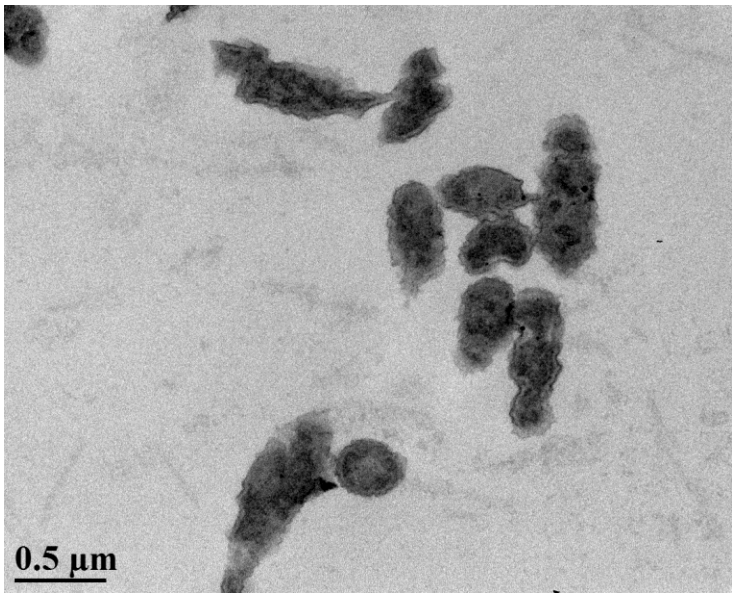


**Figure 2.1.** Electron transmission micrograph of *A. butzleri* cells.

Three species of FLA that are representative of the most common genera of FLA found in the environment, along with an environmental wastewater FLA isolate, were used for co-culture with a wastewater isolate of *A. butzleri*. Based on its spindle-like morphology, the environmental FLA isolate appeared to be a species of *Acanthamoeba*. Sequence results based on

the 18S rRNA gene showed that the closest phylogenetic species to the environmental isolate was that of *Acanthamoeba lenticulata* PD2S with 99.39% homology.

In river water, *A. butzleri* experienced an overall decrease in cell numbers over time. When *A. butzleri* was co-cultured with FLA, there was also an overall decrease in cell numbers with all four FLA (*A. lenticulata, A. polyphaga, V. vermiformis, W. magna)*, which suggests a lack of replication in association with these particular FLA. When co-cultured with *A. polyphaga* (Figure 2.2a) and *A. lenticulata* (Figure 2.2d), the growth curve of *A. butzleri* was not significantly different from that of the controls at all time points ($p > 0.05$*)*. This suggests that *A. butzleri* concentrations did not increase in the presence of *A. polyphaga* and *A. lenticulata*.

*Acanthamoeba* is widely distributed in the environment and is well-known for the ability of its species to act as hosts for a wide range of human pathogenic bacteria (Khan 2006, Visvesvara *et al.* 2007). *A. polyphaga* was used for this study in particular as the species can be used to isolate and enrich bacteria that are difficult to culture (Rowbotham 1980, La Scola *et al.* 2001, Adékambi *et al.* 2004). Further, to date, interactions of *A. butzleri* with FLA have only been studied with *Acanthamoeba castellanii* (Villanueva *et al.* 2016). In this study, the authors also saw a steady decrease in CFU numbers of control bacteria and bacteria in co-culture with amoebae. In contrast to the approximate 1.5-$\log_{10}$ decrease of *A. butzleri* in both the presence and absence of *A. polyphaga* and *A. lenticulata* over a period of four days, Villanueva *et al*. (2016) showed an approximate 0.3-$\log_{10}$ decrease over a period of ten days when *A. butzleri* was co-cultured with *A. castellanii* compared to when the bacteria were on their own. One distinction to note is that the authors used amoebae growth medium as their culture medium, while the work described in this thesis used autoclaved river water, a nutrient-poor medium. These differences in media may account for the differences between study results as the growth medium used in the Villanueva study may have been providing an environment that better facilitated the survival of *A. butzleri* and *A. castellanii*.

Similar to the results obtained with *A. polyphaga*, *A. butzleri* co-cultured with *V. vermiformis* showed no significant difference between the control and the co-culture ($p > 0.05$) (Figure 2.2b). There was, however, a significant increase in *A. butzleri* numbers on day 3 ($p = 0.0197$). However, as CFUs quickly decreased back to the same levels as day 2, this spike may have been an anomaly. As of writing, there have been no studies regarding interactions of

*A. butzleri* with *V. vermiformis.* However, the interactions between *V. vermiformis* and *Campylobacter* have been studied. Axelsson-Olsson *et al.* (2010) showed that the three most common *Campylobacter* species responsible for causing gastroenteritis were capable of surviving up to 23 days when co-cultured with *V. vermiformis*.

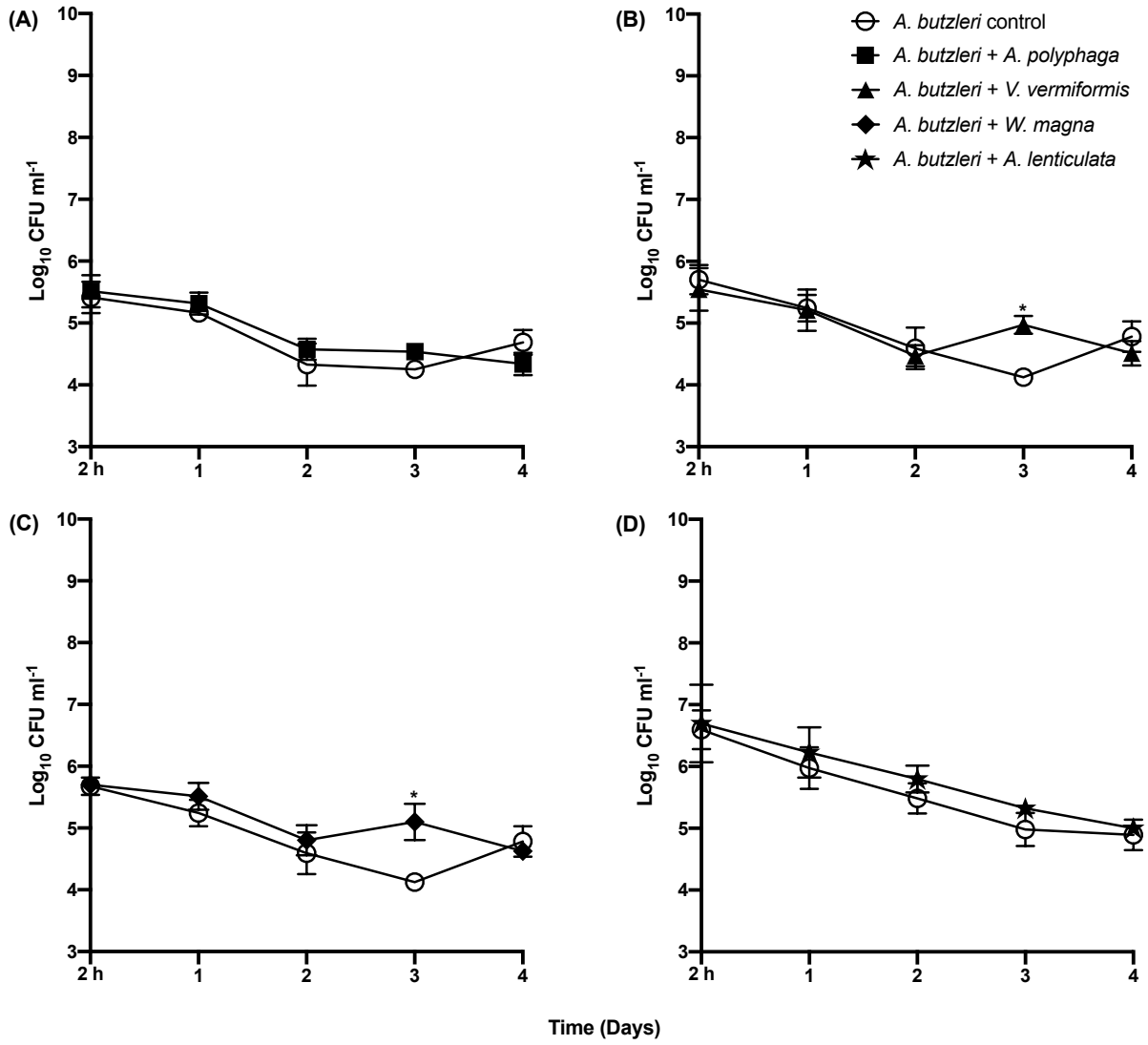


**Figure 2.2.** Kinetic growth of *A. butzleri* in co-culture with different free-living amoebae in river water at 30°C. Enumeration was done through CFU counts. Co-cultures were carried out with **(A)** *A. polyphaga* (N = 9), **(B)** *V. vermiformis* (N = 5), **(C)** *W. magna* (N = 5), and **(D)** *A. lenticulata* (N = 3). Error bars represent ± SEM and asterisk indicates significance ($p < 0.05$).

Initially, experiments with *W. magna* were only completed over a period of four days (Figure 2.2c). Similar to the first three FLA discussed, there was an overall decrease in

*A. butzleri* over four days when cultured with *W. magna*. While CFUs appeared to increase from days 2 to 3, there were no significant differences between the control and co-cultures among all time points, except for at day 3 ($p < 0.05$). As was the case with the first three FLA, this also suggests that *A. butzleri* may not be replicating within trophozoites of *W. magna*.

One thing to note is that compared to the other FLA used in this study, *W. magna* takes a longer time to encyst (Shaheen *et al.* 2019). As such, a second set of experiments were completed which tracked the growth of *A. butzleri* with and without *W. magna* over a longer period of seven days, the data of which are shown in Table 2.2. In this set of experiments, counts were only completed on the first, fifth, and seventh days of co-culture.

**Table 2.2.** Average CFU counts of *A. butzleri* when cultured in the presence and absence of *W. magna* in river water at 30°C over a period of seven days. Means are the average of four independent experiments. *P*-values are the results of unpaired, two-tailed t-tests ($\alpha = 0.05$).

| | **Day 0** | | | **Day 5** | | | **Day 7** | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean | SEM | *p*-value | Mean | SEM | *p*-value | Mean | SEM | *p*-value |
| ***A. butzleri* control** | 5.64 | 0.16 | **0.72** | 4.69 | 0.08 | **< 0.05** | 4.94 | 0.19 | **0.71** |
| ***A. butzleri* + *W. magna*** | 5.53 | 0.22 | | 5.33 | 0.07 | | 5.03 | 0.09 | |

When co-cultures between *A. butzleri* and *W. magna* were repeated over a longer time period, there was a significant difference between numbers on day 5 ($p < 0.05$). However, overall, *A. butzleri* also decreased regardless of the presence of *W. magna*. It would be worth repeating these experiments over the longer time period with sampling taking place at each day to get a more cohesive picture of how *A. butzleri* may be behaving.

There are currently no studies regarding the relationship between *Arcobacter* spp. and *W. magna*. However, compared to *A. polyphaga* and *V. vermiformis*, which are susceptible to infection by *L. pneumophila*, *W. magna* (strain c2c) has been shown to resist infection by *L. pneumophila*, Paris (Dey *et al.* 2009). In this study, CFUs of *L. pneumophila* stayed constant over 4 days of co-culture, while CFUs increased when associated with *Acanthamoeba* and *Vermamoeba* (*Hartmanella*).

As was presented here, studies involving the interactions of *A. butzleri* and closely-related species *C. jejuni* with FLP have generally involved the traditional use of culture methods to enumerate and track the growth kinetics of the bacteria over time (Axelsson-Olsson *et al.* 2010, Olofsson *et al.* 2013, Villanueva *et al.* 2016). In this thesis, the interactions of *A. butzleri* with various FLP were additionally quantified with qPCR (Figure 2.3). Using qPCR to detect the *hsp60* gene in *A. butzleri* allowed for the determination of whether there is either an increase, decrease, or no change in gene copy numbers. The change, or lack thereof, in total gene copy numbers would then indicate whether *A. butzleri* was 1) replicating, 2) being digested by the FLP, or 3) unaffected by the presence of FLP.

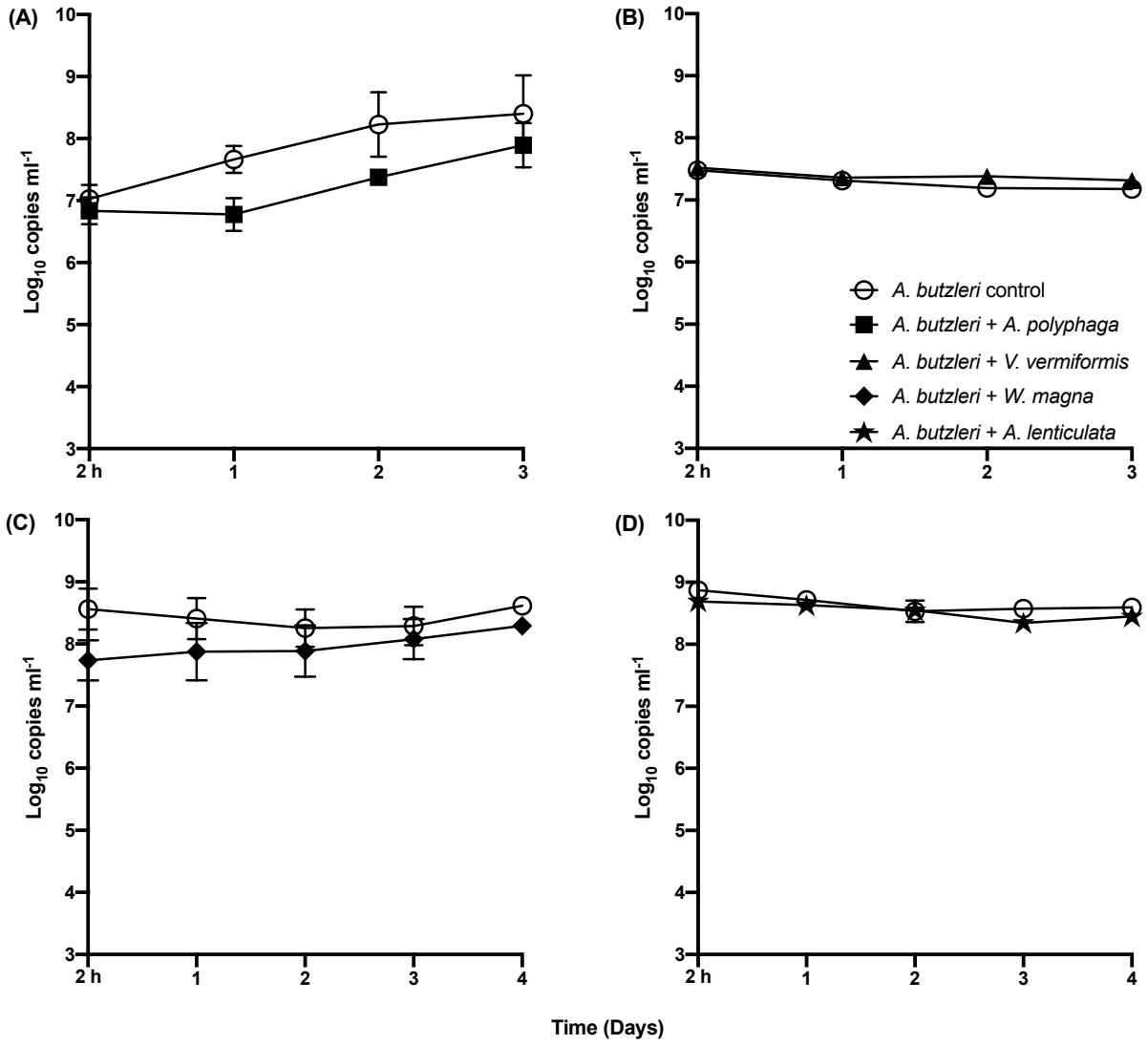


**Figure 2.3.** Total gene copy numbers of *A. butzleri* in co-culture with different free-living amoebae in river water at 30°C. Enumeration was completed via qPCR with *hsp60*, a single-copy gene, as the gene target. Co-cultures were done with **(A)** *A. polyphaga* (N = 3), **(B)** *V. vermiformis* (N = 3), **(C)** *W. magna* (N = 3), **(D)** *A. lenticulata* (N = 3). Error bars represent ± SEM.

In contrast to the culture data, qPCR data indicate that *A. butzleri* copy numbers appeared to increase over a period of three days in both the control and in *A. polyphaga* co-culture (Figure 2.3a). This increase in total copy numbers would suggest that bacterial replication occurred regardless of the presence of *A. polyphaga.* However, similar to the culture results, there were no significant differences between *A. butzleri* alone and in co-culture with *A. polyphaga* at all time points ($p > 0.05$). Further, copy numbers of *A. butzleri* on day 3 were not significantly different

from those on day 1. Copies of *A. butzleri* remained steady over time when they were also associated with *V. vermiformis, W. magna,* and *A. lenticulata* with no significant differences among all time points compared to the control (Figure 2.3b-2.3d). The relatively stable gene copy numbers over time may suggest three things: 1) that *A. butzleri* may not have been replicating, 2) nor were they digested by the amoeba, or 3) the rate at which the amoebae were consuming *A. butzleri* was equal to that of their planktonic growth.

When *A. butzleri* was co-cultured with *W. magna* for a longer period of time (i.e., up to day 7), there were statistically significant differences between *A. butzleri* alone and with *W. magna* (Table 2.3). Gene copy numbers in the control were significantly higher than those associated with *W. magna*, which suggests that *A. butzleri* may get digested by *W. magna* at later time points when any nutrients in an already nutrient-poor environment may be even more limiting. Low copy numbers associated with *W. magna* may also suggest that as time goes on, the rate at which *A. butzleri* is being consumed outpaces the rate at which it grows.

**Table 2.3.** Average *hsp60* gene copy numbers of *A. butzleri* when cultured in the presence and absence of *W. magna* in river water at 30°C over a period of seven days. Means are the average of three independent experiments. *P*-values are the results of unpaired, two-tailed t-tests ($\alpha$ = 0.05).

| | **Day 0** | | | **Day 5** | | | **Day 7** | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean | SEM | *p*-value | Mean | SEM | *p*-value | Mean | SEM | *p*-value |
| ***A. butzleri* control** | 8.89 | 0.03 | **< 0.01** | 8.61 | 0.03 | **< 0.01** | 9.20 | 0.24 | **0.03** |
| ***A. butzleri* + *W. magna*** | 8.72 | 0.01 | | 8.31 | 0.02 | | 8.40 | 0.02 | |

Finally, as the qPCR data showed a steady number of *hsp60* gene copies over time (Figure 2.3b), the idea that the CFU spike on day 3 of the *V. vermiformis* co-culture (Figure 2.2b) suggests that this may be an anomaly associated with the culturability of *A. butzleri* at that time point.

The gentamicin protection assay (GPA) is a method that is commonly used to determine the internalisation of bacteria into protozoan cells (Dirks and Quinlan 2014, Kim *et al.* 2019).

The GPA involves treating the co-culture with the antibiotic gentamicin for a period of time in order to eliminate any bacteria external to the protozoan cell. Since external bacteria are eliminated, it is assumed that any bacteria enumerated after lysing of the cell were those that were internalised and survived digestion. In this thesis, the GPA was not completed. Rather, the total bacteria (internal and external) were enumerated for two reasons. Firstly, preliminary experiments indicated no growth of *A. butzleri* over time, which meant that intracellular growth of the bacteria was likely not occurring within the protozoa. Secondly, during a test run, the gentamicin dose as used by Villanueva *et al.* (2016) ended up killing all culturable cells. As there was no apparent replication of *A. butzleri* and the use of GPAs have been shown to kill intracellular bacteria (Kim *et al.* 2019), it was decided that it was more prudent to enumerate the total number of bacteria in order to obtain a bigger picture of the interactions between the bacteria and the FLP.

Since the GPA was not used in these experiments, it had to be determined whether *A. butzleri* actually does get ingested by FLA as FLA are sometimes resistant to bacterial infection. Figure 2.4 to Figure 2.6 illustrate the internalisation of *A. butzleri* in all four FLA. A commonality seen between all four FLA was the tendency for *A. butzleri* to cluster both within the trophozoites and within vesicles released by the amoebae. This clustering behaviour may have consequences when it comes to the bacterial acquisition of antimicrobial resistance genes through horizontal gene transfer (HGT) (Von Wintersdorff *et al.* 2016).

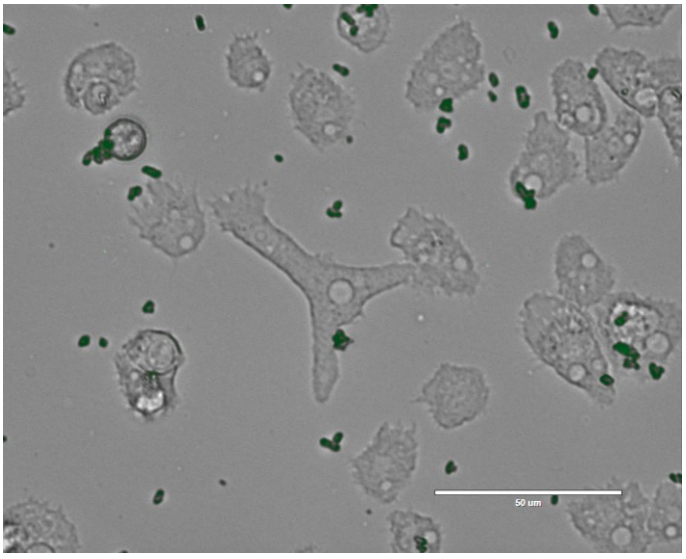


**Figure 2.4.** Fluorescence micrograph of *A. butzleri* (green) clustered around and/or within trophozoites of *A. lenticulata*, the wastewater FLA isolate. Scale bar represents 50 µm.

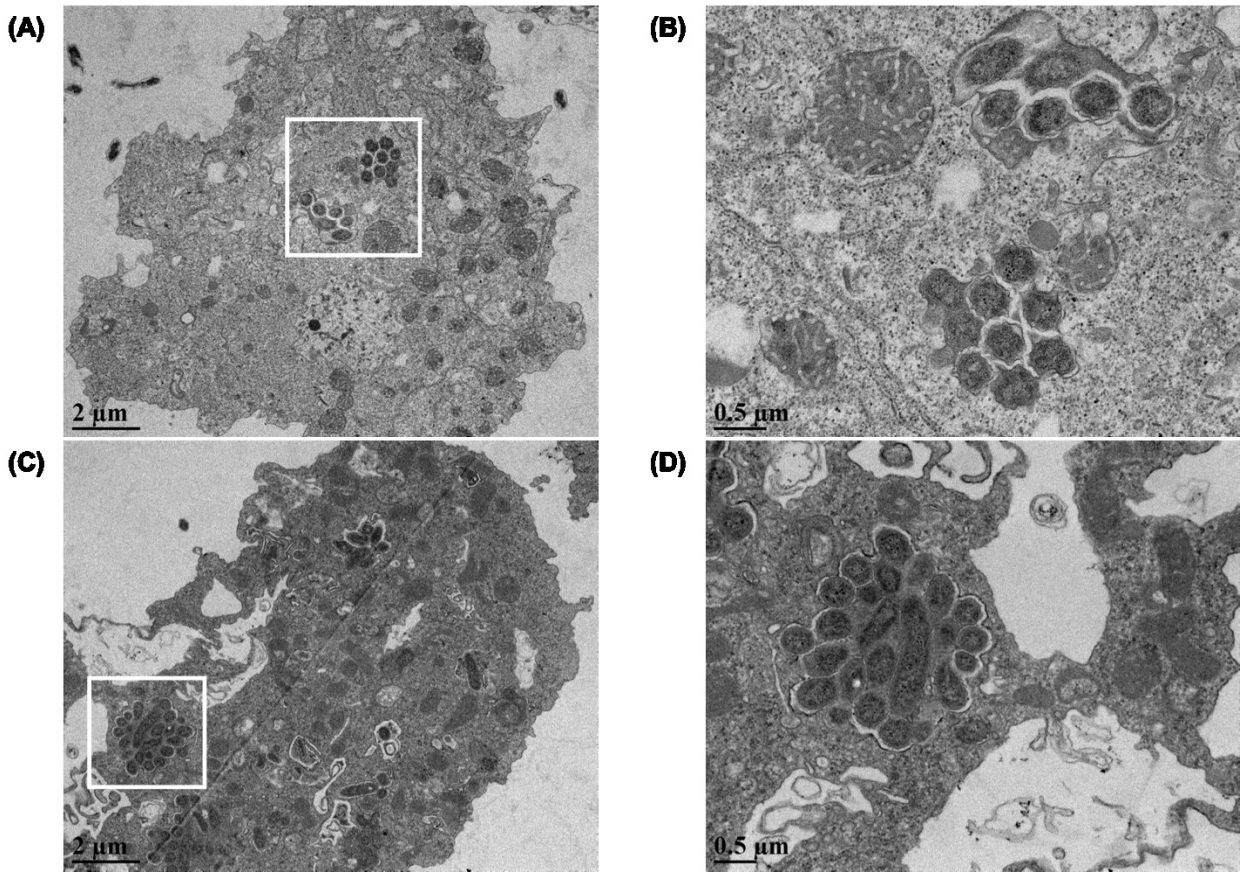


**Figure 2.5.** Transmission electron micrographs of *A. butzleri* contained within **(A)** *A. polyphaga* and **(C)** *W. magna* phagosomes after 24 h of co-cultures. The white squares indicate areas of the photo that have been zoomed in for **(B)** *A. polyphaga* and **(D)** *W. magna.*

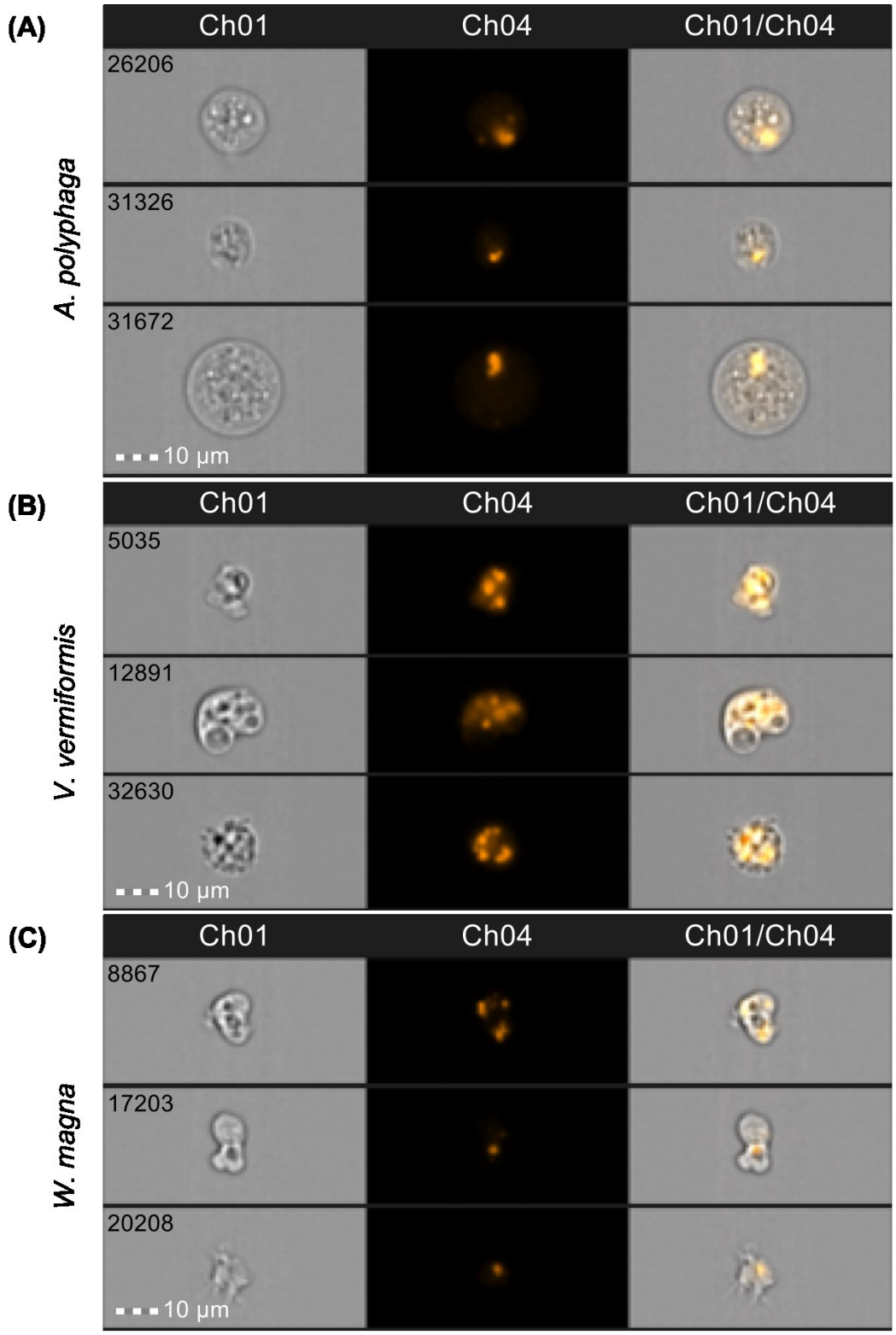


**Figure 2.6.** Images showing the internalisation of *A. butzleri* (orange) within **(A)** *A. polyphaga*, **(B)** *V. vermiformis*, and **(C)** *W. magna*. Bacterial cells were stained with acridine orange and imaged on an imaging flow cytometer.

To determine whether increases in *A. butzleri* numbers alone and in co-culture were real, co-cultures were also performed in Transwell® plates (Figure 2.7). Here, the bacteria and amoebae were physically separated by a semi-permeable membrane with the idea that *A. butzleri* may be able to grow extracellularly, based on increased growth seen in the original co-cultures (culture and qPCR-based). When cultured alone in river water, *A. butzleri* steadily decreased over time by approximately 3-$\log_{10}$ (Figure 2.8a), which contradicts the qPCR results of the *A. polyphaga* and *W. magna* co-cultures. However, when *A. butzleri* was in the presence of, but physically separated from all 4 FLA, the bacteria experienced increased persistence compared to the control. However, this increase was only significant with *A. polyphaga* ($p = 0.0020$), *V. vermiformis* ($p = 0.0490$), and *W. magna* ($p = 0.0002$). This suggests that these amoebae may be releasing factors that allow the bacteria to survive for longer periods of time in culture. Total numbers of bacteria remained constant over time in all treatments and there were no differences between the bacteria control and bacteria associated with FLA over time among all time points ($p = 0.2776$) (Figure 2.8b).

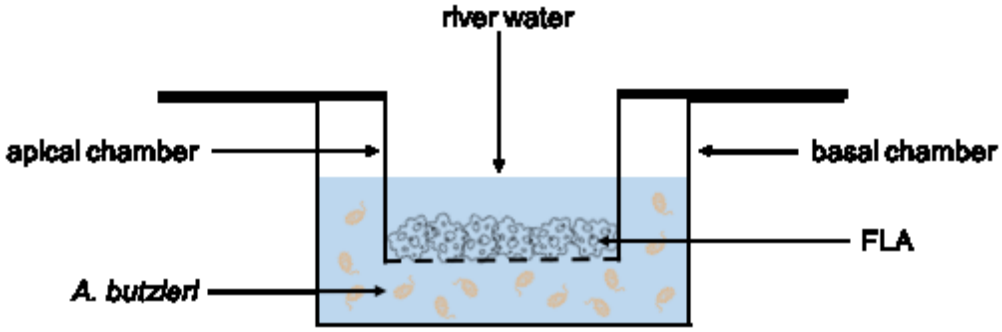


**Figure 2.7.** Schematic of a Transwell assay with *A. butzleri* (orange) in the basal chamber and FLA (black) in the apical chamber.

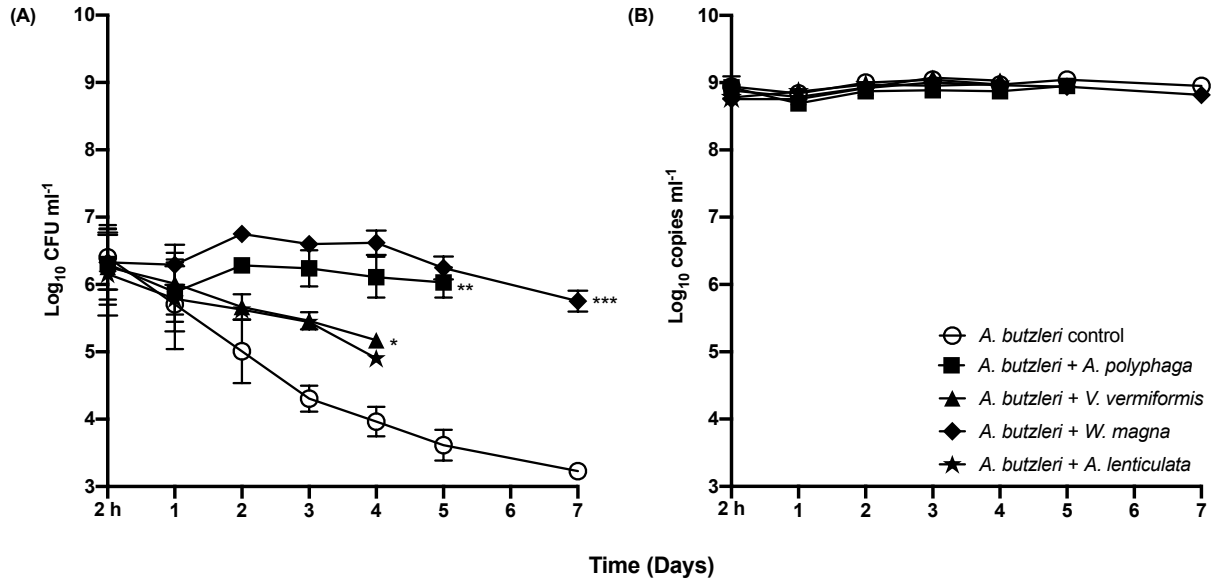


**Figure 2.8.** Comparison of the kinetics of growth of *A. butzleri* alone and in co-culture with different amoebae over time in river water at 30°C in Transwell® plates where the amoebae and *A. butzleri* were physically separated. Time periods differ due to the different encystment times of each amoeba type. **(A)** $Log_{10}$ CFU $mL^{-1}$ of *A. butzleri* over 4, 5, or 7 days depending on amoebae type (N = 5). **(B)** $Log_{10}$ *hsp60* copies $mL^{-1}$ of *A. butzleri* (N = 3). Error bars represent ± SEM and asterisks indicate significance ($p < 0.05$).

### 2.3.2 Co-cultures of the ciliate *Tetrahymena pyriformis* in river water and wastewater

Ciliates are regular predators of bacteria in the wastewater treatment system. While there is currently no research on interactions between ciliates and *Arcobacter* spp., it has been reported that the ciliate *T. pyriformis* increases the persistence of *C. jejuni* in the environment (Trigui and Charette 2016).

To determine whether *A. butzleri* could survive interactions with ciliates in the environment, it was co-cultured with *T. pyriformis*, a model ciliate, in both river water and wastewater for up to 5 days. Just as was done with the FLA co-cultures, estimates were made by CFU counts and through qPCR. In river water, numbers of total bacteria decreased by 1-$log_{10}$ over time. This number increased to 1.5-$log_{10}$ when bacteria were co-cultured with *T. pyriformis*, which suggests a loss of cells that may be due to digestion by the ciliates (Figure 2.9a). Although there was no significant difference between CFUs of *A. butzleri* with or without *T. pyriformis* present, the idea that the bacteria are being digested by the ciliates was confirmed by qPCR

which showed a constant level of bacteria in the control over time, but an approximate 1-$\log_{10}$ decrease at day one when associated with *T. pyriformis* (Figure 2.9b).

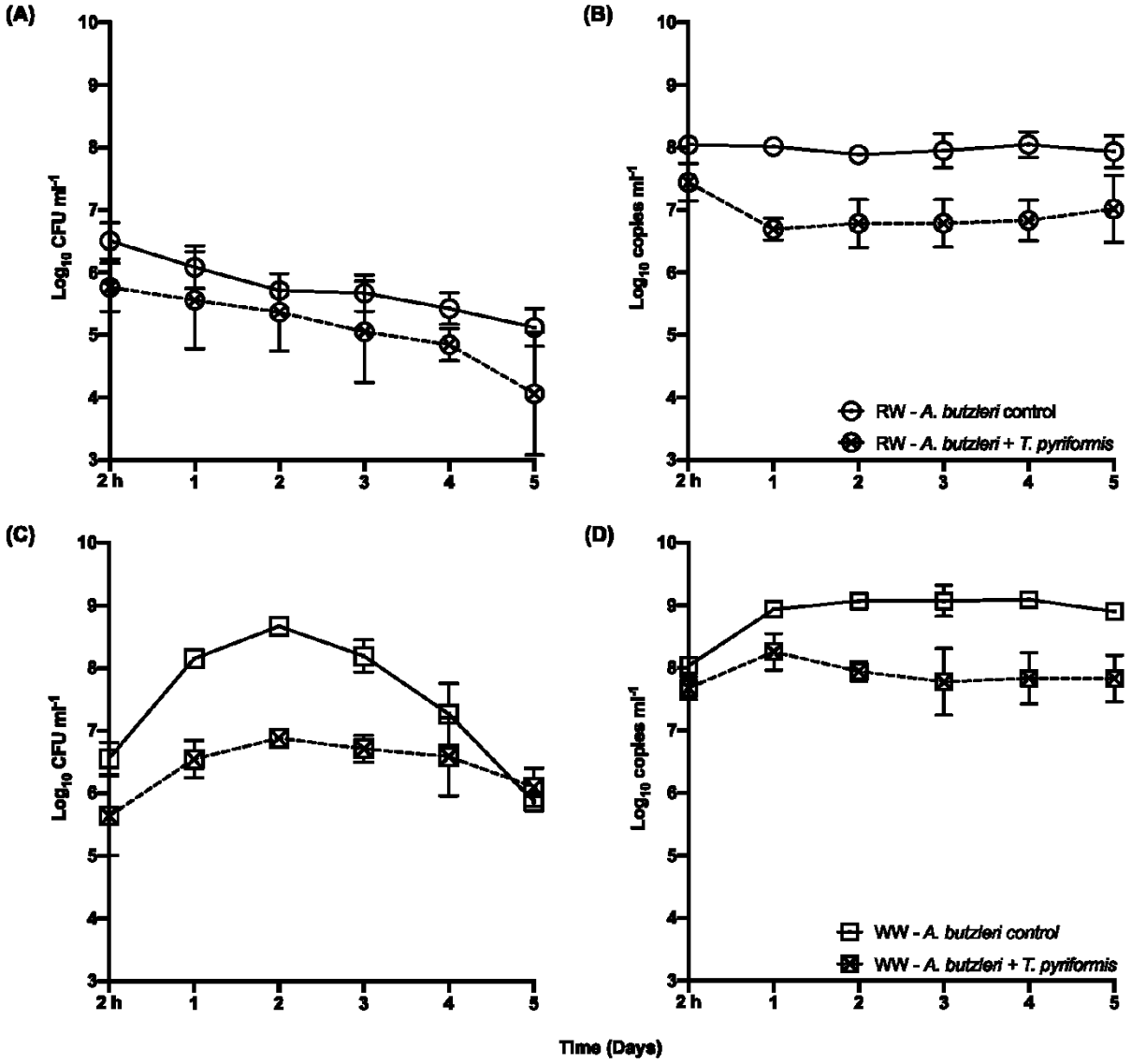


**Figure 2.9.** Kinetic growth of A. *butzleri* in co-culture with *T. pyriformis* over 5 days in environmental waters at 25°C. Co-cultures were performed in river water (circles) and enumerated via **(A)** CFU counts (N = 5) and **(B)** qPCR (N = 3). Co-cultures were also completed in wastewater (squares) and enumerated via **(C)** CFU counts (N = 3) and **(D)** qPCR of *hsp60* gene copies (N = 3). Error bars represent ± SEM and asterisks indicate significance ($p < 0.05$).

In comparison, when *A. butzleri* was cultured in wastewater, culturable cells increased approximately 2-$\log_{10}$, reaching a peak on day 2 (Figure 2.9c). When co-cultured with

*T. pyriformis*, the bacteria also experienced an increase of 1.5-$\log_{10}$. From days 1 to 3, there was a significant difference between the *A. butzleri* control compared to the co-culture ($p < 0.05$). As with the culturable cells, qPCR also showed an increase in total genomic copies over time, which contrasted with levels seen in river water (Figure 2.9d). In wastewater, copies of *hsp60* increased over 24 h, before either staying constant over time (*A. butzleri* control) or decreasing in the case of the co-culture.

Lower levels of *A. butzleri* in association with *T. pyriformis* compared to that of the bacteria alone in both culture and qPCR show that total numbers of *A. butzleri* decrease over time, which indicates the possibility that *A. butzleri* are being digested by the ciliates. However, qPCR results also show that once *hsp60* copies decrease, they tended to stay relatively constant for the remainder of the time period. Hence, there is a possibility that the feeding of *T. pyriformis* on *A. butzleri* reaches a point at which saturation occurs, whereby *T. pyriformis* no longer internalises any bacteria and those that are internalised get released in vesicles until numbers of intracellular *A. butzleri* reach levels that are once again acceptable to the ciliate. Thurman *et al.* (2010) suggest that *T. pyriformis* egest bacterial cells when concentrations of bacteria reach certain thresholds within vacuoles of the ciliate. They observed that when food vacuoles contained approximately six bacterial cells, complete digestion of those cells occurred. However, higher levels of bacteria in vacuoles resulted in partial digestion of vacuole contents along with the release of undigested bacteria from ciliate cells. Steady copy numbers may also suggest that *A. butzleri* may be growing at a rate that is equal to that at which the ciliates consume them. Monitoring numbers of ciliates at the same time as bacteria could be beneficial when it comes to further exploring the interactions between *A. butzleri* and *T. pyriformis*. Tracking the population of ciliates over time would give a better idea as to the extent to which *T. pyriformis* consider *A. butzleri* to be a nutrition source.

It is worth noting that the enumeration of *A. butzleri* from all co-cultures involved all those that were both intracellular and extracellular (planktonic and in vesicles). However, it might be prudent to further focus on the vesicles released by FLP that may contain viable bacteria. The viability of *C. jejuni* packaged in multilamellar bodies (MLBs) released from *T. pyriformis* was demonstrated by Trigui and Charette (2016), revealing that *C. jejuni* packaged and released in MLBs survived 24 hours longer than bacteria cultured without *T. pyriformis*. The

researchers also examined this phenomenon in *A. castellanii* but found that the amoeba did not release any vesicles containing *C. jejuni*.

As shown with these experiments, *A. butzleri* does not appear to use FLP as a vessel for replication. This may be due to several reasons. First, the FLP used may not be the ideal hosts for *A. butzleri*. In these experiments, ATCC strains of FLP were used, all of which were not isolated from sewage. Further, of the amoebae that were isolated from wastewater samples, only one was used due to its ability to grow well in culture. Second, the isolate of *A. butzleri* used may not be dependent on FLP for survival or replication. Following this, it may be that *A. butzleri* in general does not depend on FLP for replication at all as it is considered to be free-living (Miller *et al.* 2007, Collado and Figueras 2011).

When *A. butzleri* was cultured in wastewater, there was an increase in both culturable bacteria and detectable gene copies even when ciliates were not present. From this, it may be inferred that the wastewater itself enriches the growth of *A. butzleri* which does not seem to be the case for other enteric bacteria in sewage such as *C. jejuni* (Banting *et al.* 2016). Furthermore, Kristensen *et al.* (2020) reported *Arcobacter* to be more abundant in sludge supernatant than activated sludge, which supports this work's findings of increased CFUs and gene copies when *A. butzleri* was cultured in wastewater. As FLP are likely to be found in higher numbers in activated sludge due to their larger size, the idea that *A. butzleri* does not depend on FLP for replication is consistent with the idea that sludge supernatant enriches free-living *A. butzleri*.

The two enumeration methods used in this chapter, plate counts and qPCR, were meant to complement each other and give a better understanding as to what interactions were taking place between *A. butzleri* and the various FLP. Over all the co-culture experiments, qPCR results yielded higher gene copies than CFU counts. There may be a few reasons why these differences were observed. First, a single CFU does not necessarily mean that a single bacterial cell was initially deposited onto agar medium (Hazan *et al*. 2012). The earlier co-culture images showed clusters of *A. butzleri* both within and outside of the amoebae (Figure 2.4 – Figure **2.6**). These clusters, when plated, may have been resulting in a single CFU, which would have led to an underestimation of viable, culturable cells. Secondly, qPCR does not discriminate between viable and non-viable cells, and while this discrepancy was expected, it is also well known that culture methods do not necessarily capture all viable cells (Li *et al*. 2014). Further, Fera *et al*. (2008) demonstrated the ability for *A. butzleri* to enter a viable but non-culturable (VBNC) state in

nutrient-limiting seawater. Hence, the culture results presented in this chapter may not have been reflective of the total number of viable cells and may have been excluding VBNC cells, which may also account for the higher gene copy numbers seen in qPCR.

Following this, it would be worth looking into determining the viability of *A. butzleri* before, during, and after co-culture with different FLP and their vesicles. The viability of *Helicobacter pylori* within trophozoites of *A. castellanii* has been demonstrated by (Moreno-Mesonero *et al*. 2016) with the use of fluorescence in-situ hybridization (FISH) in combination with direct viable counts (DVC-FISH) as well as the use of propidium monoazide with qPCR (PMA-qPCR). These methods would be worth applying in future work to ascertain the extent to which *A. butzleri* stay viable or become non-viable in association with FLP.

Finally, the culture bias towards *A. butzleri* may be excluding species of *Arcobacter* that may replicate in association with FLP. Based on metagenomic analyses, *A. cryaerophilus* is the most abundant species of *Arcobacter* in sewage (Kristensen *et al.* 2020). However, studies reporting *A. butzleri* as the most common *Arcobacter* species in human stools, sewage, or faecally impacted waters utilised culture methods targeted towards the detection of *A. butzleri* (Collado *et al.* 2008, 2010, Van den Abeele *et al.* 2014, Levican *et al.* 2016, Talay *et al.* 2016, Brückner *et al.* 2020). As such, it might be prudent to repeat these experiments with *A. cryaerophilus* to provide a more comprehensive understanding of *Arcobacter* in sewage.

# Chapter 3: UV disinfection of *Arcobacter butzleri*

## *3.1 Introduction*

Soon after the germicidal effect of ultraviolet (UV) light was established in the late 19th century, the first application of UV as a water disinfection treatment was reported in Marseille, France (Henry *et al.* 1910, as cited in Hijnen *et al.* 2006). UV gained enhance usage as an effective water disinfection treatment when it was shown to be effective against chlorine-resistant *Cryptosporidium* oocysts and *Giardia* cysts in Europe and the US (Campbell *et al.* 1995, Clancy *et al.* 1998). Today, UV light is well-known to be highly effective at inactivating a broad range of common waterborne pathogens, including bacteria, protozoa, and viruses (Tomb *et al.* 2018).

The ability of UV light to inactivate microorganisms lies in its capacity to target the building blocks of the cell, nucleic acids and proteins (Beck *et al.* 2018). UV light exposure generates reactive oxygen intermediates which interact with nucleic acids and proteins, leading to the oxidation of nucleic acids and proteins. As a result of oxidation, a number of things (among others) occur:

- The formation of cyclobutane pyrimidine dimers (CPDs), which are the major photoproducts that occur when DNA is exposed to UV light (Rastogi *et al*. 2010);
- The formation of pyrimidine (6-4) pyrimidone adducts, which have been reported to be more mutagenic than CPDs (Rastogi *et al*. 2010, Yokoyama and Mizutani 2014);
- Adjacent cytosine to thymine (CC → TT) tandem mutations (Ravanat *et al*. 2001); and
- Oxidation of the aromatic residues of tryptophan, tyrosine, and histidine and the disulfide residues of cysteine (Santos *et al*. 2013).

These damages to the DNA structure and other cell components lead to the inhibition of DNA/RNA replication and transcription, which ultimately lead to cell death (Coohill and Sagripanti 2008, Rastogi *et al.* 2010, Hammer and Hammer 2012b).

Traditionally, faecal coliforms are the bacterial reference organism for the effectiveness of wastewater treatment and disinfection (Ashbolt *et al.* 2001). However, it has been argued that treatment efficacy should not be measured by the presence/absence of faecal coliforms as these are the most susceptible to the disinfection processes and are not always directly associated with health risk (Bitton 2011, Levy *et al.* 2012, Rodrigues and Cunha 2017). The absence of faecal

coliforms may not be an accurate representation of what has survived the various treatment processes and is still left viable in the water. Further, the presence of faecal indicators does not necessarily confirm the presence or survival of bacterial pathogens (Ashbolt *et al.* 2001).

In southern Alberta, viable *Arcobacter* spp. have been detected in treated wastewater effluent that has been disinfected with UV light (Banting *et al*. 2016, Webb *et al.* 2016b). More recently, *Arcobacter* was found to be the most abundant bacterial genus detected in treated wastewater effluent discharged from 14 different WWTPs in Denmark (Kristensen *et al.* 2020). Further, the authors report *Arcobacter* spp. in effluents at levels similar to those detected in the influent. *Arcobacter* spp. are known to be more suited to surviving in the environment than members of the closely related genus *Campylobacter* (Van Driessche and Houf 2008, Collado and Figueras 2011, Brückner *et al.* 2020). Thus, the detection of viable arcobacters in wastewater effluents may present downstream public health concerns and may require the re-evaluation of which bacteria are used as bacterial reference pathogens or surrogates in wastewater treatment.

Currently, there is no UV dose-response data for *Arcobacter* spp. in water. Additionally, it is unknown how the ecology of *Arcobacter* spp. affects UV disinfection. As such, the objectives of this chapter were to: 1) establish a UV dose-response curve for *A. butzleri*; and 2) determine how relationships with FLP may affect the UV disinfection of *A. butzleri* in water.

## *3.2 Materials & Methods*

### 3.2.1 Creating a UV dose-response curve for *A. butzleri*

UV disinfection was carried out with an AquaSense Pearl Beam UV-C LED collimated beam system (Florence, KY, USA) with the set-up as pictured in Figure 3.1. Prior to beginning irradiation of the samples, the system's intensity ($E_0$) was measured by a NSF-certified UVX-25 radiometer (UVP, Upland, CA, USA). In a 60 mm petri dish, 210 µL of *A. butzleri* culture was suspended in 22 mL of autoclaved, filter-sterilised river water, resulting in a diluted culture that had a height ($L$) of 1 cm. A 2 x 5 mm magnetic stir bar was placed in the dish and placed on a stir plate at a rotation speed of 400 rpm. The suspension was allowed to mix for 1 min before three 1 mL aliquots were taken for absorbance measurements ($A$). These absorbance measurements, along with the other measured variables were used to calculate the delivered or effective intensity ($E_{ave}$) using Equation 3.1 (NSF International 2014):

$$E_{ave} = 0.98\left[\frac{E_0}{L}\left(\frac{(1-A)^L - 1}{\ln(1-A)}\right)\right] \quad \textbf{Equation 3.1}$$

The effective intensity was then used to calculate the exposure times ($t$), in seconds, required to reach different UV doses:

$$t = \left(\frac{1000 \times dose}{E_{ave}}\right) \quad \textbf{Equation 3.2}$$

Prior to carrying out UV irradiation, a control experiment was completed to ensure that there was no impact of the experimental set-up on bacterial viability. A 500 µL aliquot was taken at a dosage of 0 mJ/cm$^2$. The culture was then irradiated at a wavelength of 255 nm before aliquots were taken at the following doses: 5, 10, 15, 20, 25, 30, 40 mJ/cm$^2$. The aliquots were serially diluted in PBS before being plated onto Bolton agar and incubated at the appropriate conditions. Following incubation, CFUs were enumerated and $\log_{10}$ reductions for each dose were calculated with the following equation:

$$Log_{10}\,Reduction = Log_{10}\left[\frac{\left(\frac{CFU\ control}{100\ mL}\right)}{\left(\frac{CFU\ dose}{100\ mL}\right)}\right] \quad \textbf{Equation 3.3}$$

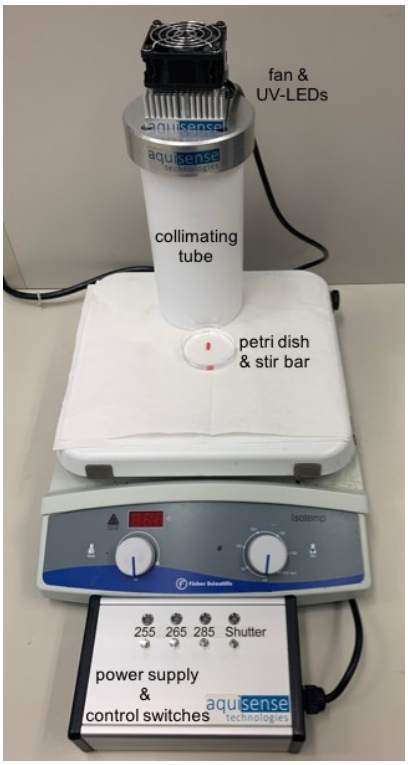


**Figure 3.1.** UV-C LED collimated beam system set-up.

### 3.2.2 UV disinfection of protozoan co-cultures

The bacteria, FLA, and ciliates used in this chapter were cultivated and maintained as mentioned in Chapter 2. Co-cultures of *A. butzleri* with the four different FLA (*A. lenticulata*, *A. polyphaga, V. vermiformis, W. magna*), and ciliate (*T. pyriformis*) were set up similar to how co-cultures were prepared in Chapter 2, with the following differences:

- Once *A. butzleri* were grown in liquid culture for 48 h, the culture was spun down at 4000 × *g* for 30 min before being re-suspended in autoclaved, filter-sterilised river water and adjusted to an OD of 1; and
- The ratio of amoebae to bacteria used was the same as in Chapter 2, however, the volumes used were increased relative to the size of the Petri dish.

After 2 h the co-cultures were transferred to a Petri dish before being irradiated according to the conditions described above. After irradiation, co-cultures were syringe-lysed with a 1 mL syringe (309659; BD, Franklin Lakes, NJ, USA) attached to a 27 G needle (305109; BD, Franklin Lakes, NJ, USA) before being plated on Bolton agar and incubated according to the conditions described above. CFU counts were completed.

### 3.2.3 Statistical analysis

All statistical analyses were performed on GraphPad Prism (Version 8.0, GraphPad Software, LLC, San Diego, USA). Dose-response curves were analysed for statistical differences between treated groups among all time points. For these groups, *p*-values are the result of a one-way ANOVA with $\alpha = 0.05$.

## *3.3 Results & Discussion*

### 3.3.1 UV disinfection of *A. butzleri* in water

The effect of UV on *A. butzleri* has been studied in the context of food safety (Lee and Choi 2012). However, as of writing, there is currently no UV dose-response data for *A. butzleri* in the context of water and wastewater. As such, UV inactivation values for *E. coli* and *C. jejuni* were used to estimate the dose range and dosage points for *A. butzleri* (Butler *et al.* 1987, Wilson *et al.* 1992, Hijnen *et al.* 2006). The data points from each UV experiment with *A. butzleri* alone were plotted to create a dose-response curve for the bacteria when irradiated with 255 nm UV

light (Figure 3.2). From GraphPad Prism, a fourth-order polynomial equation provided the best fit to the dose-response curve ($R^2 = 0.923$). From this model, doses required to achieve specific $log_{10}$ reductions were interpolated.

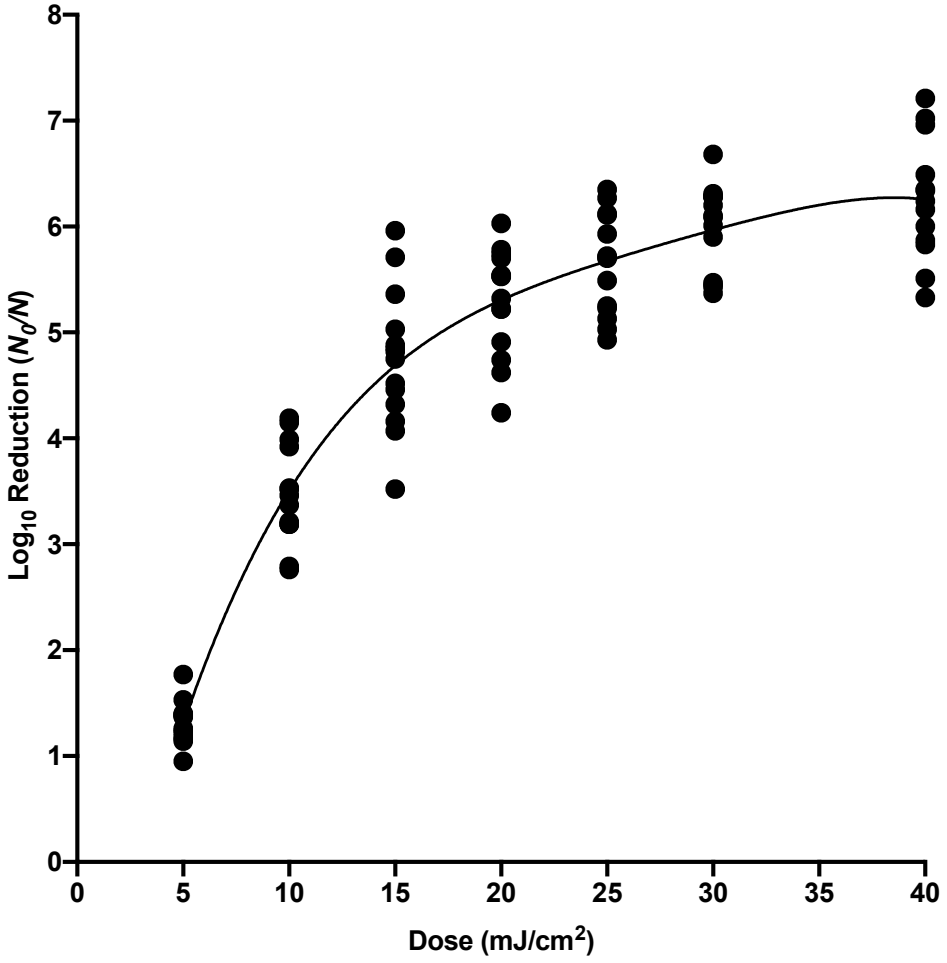


**Figure 3.2.** Dose-response curve of *A. butzleri* when UV irradiated with a wavelength of 255 nm in autoclaved, filter-sterilised river water. Data points are the means of 14 independent experiments, where $N_0$ is the initial concentration of bacteria before UV irradiation and $N$ is the concentration of bacteria after irradiation at a certain dose.

River water (autoclaved and filter-sterilised) inoculated with *A. butzleri* was UV irradiated up to a dose of 40 mJ/cm$^2$. Over this dose range, a 6-$log_{10}$ reduction of *A. butzleri* was achieved. As of December 4, 2019, Edmonton's Gold Bar WWTP has a UV dose setpoint set at 23 mJ/cm$^2$ (EPCOR 2019). Based on our generated UV dose-response curve, this setpoint seems to be adequate to achieve a 5.7-$log_{10}$ reduction for *A. butzleri*, which was interpolated from the fourth-order polynomial regression.

In a study completed by Lee and Choi (2012), *A. butzleri* was inoculated onto a steel surface, and UV-C disinfection doses of 108, 216, and 324 mJ/cm$^2$ achieved average $\log_{10}$ reduction values of 2.1, 4.1, and 5.2 among three different strains of *A. butzleri*. Lee and Choi (2012) also reported that *A. butzleri* was more difficult to control on food compared to when it was on steel. In summary, both the current work and that of Lee and Choi (2012) have implications on water reuse in food production and processing as it is worth noting that treated wastewater meant for water reuse may still contain viable *Arcobacter* cells which could potentially contaminate food products. Additionally, the survival of more UV-resistant strains post-disinfection of wastewater may have implications when these resistant strains make their way into food processing systems that utilise UV disinfection (e.g., wash water).

Because reference dose-response curves were not created in this work, there was no direct basis of comparison for the generated UV dose-response curve for *A. butzleri*. As such, this UV dose-response curve was compared to those that have been published for *C. jejuni* and *E. coli* (Figure 3.3) (Butler *et al*. 1987, Wilson *et al*. 1992, Sommer *et al*. 2000).

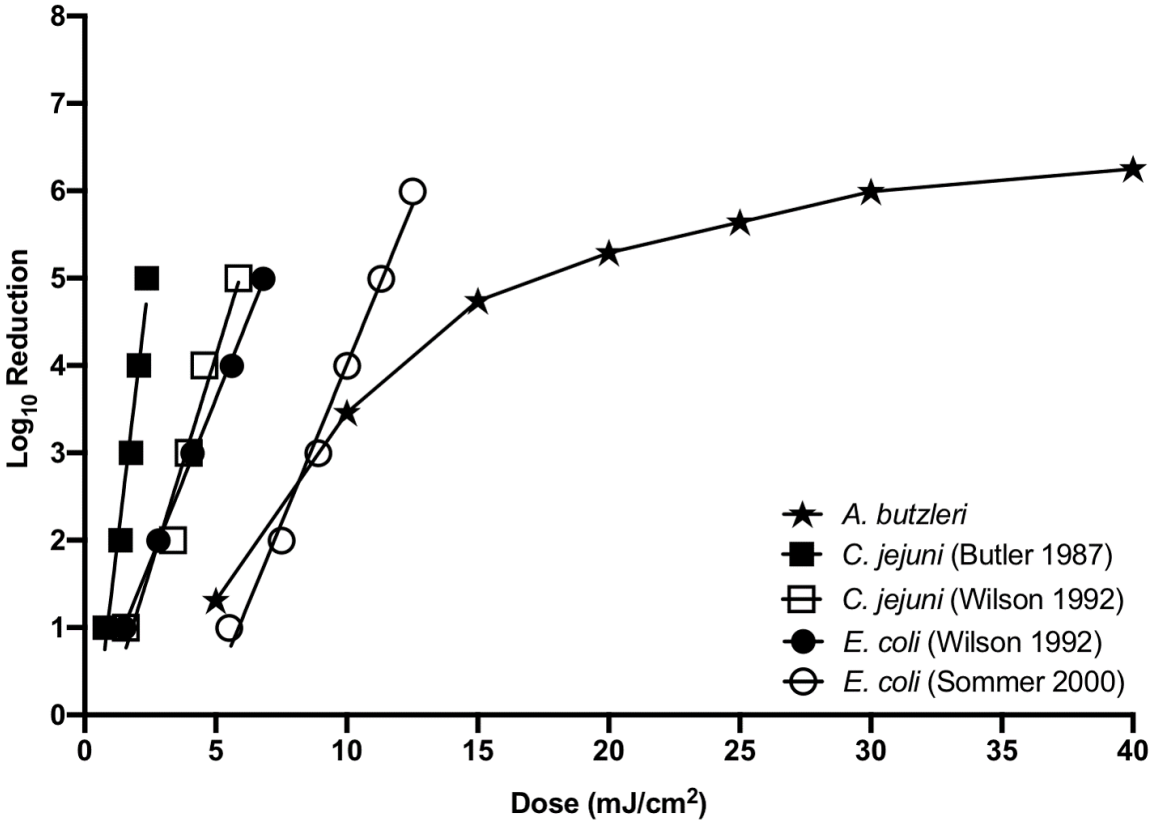


**Figure 3.3.** Comparing the UV dose-response curves for *A. butzleri* (stars) to those published for *C. jejuni* (squares), and *E. coli* O157 (circles) (Butler *et al.* 1987, Wilson *et al.* 1992, Sommer *et al.* 2000).

The bacterial species *C. jejuni* and *E. coli* were chosen for two reasons. The first being *Campylobacter*'s close relation to *Arcobacter* and its use as the reference bacterial pathogen group to define $\log_{10}$-reductions necessary for safe exposures (Vandamme *et al.* 1991, Collado and Figueras 2011, Schoen *et al.* 2017). Secondly, the use of *E. coli* as an indicator organism in wastewater treatment is widely prevalent (Bitton 2011, Hammer and Hammer 2012c, EPCOR 2019) . At the Gold Bar WWTP, for example, *E. coli* counts are used as a measure of water quality throughout the wastewater treatment train (e.g., during discharge and wastewater bypass events, after UV disinfection) (EPCOR 2019).

Previously reported UV doses required to achieve 1 to 5 $\log_{10}$ reductions of *C. jejuni* ranged from 0.8 to 5.9 mJ/cm$^2$ (Butler *et al*. 1987, Wilson *et al*. 1992). These doses are less than those required to reach the same $\log_{10}$ reductions reported earlier for *A. butzleri* (4.2 to 17 mJ/cm$^2$). Dose ranges of 0.2 to 12.5 mJ/cm$^2$ were reported to achieve a 1 to 6 $\log_{10}$ reduction for pathogenic and non-pathogenic *E. coli*, which are also less than those required to achieve the same $\log_{10}$ reductions for *A. butzleri* (Wilson *et al*. 1992, Sommer *et al*. 2000).

It must be noted, however, that the *A. butzleri* dose-response data and those published for *C. jejuni* and *E. coli* cannot be considered to be direct comparisons due to differences in methodology. For example, neither Butler *et al*. (1987) nor Sommer *et al*. (2000) used wastewater isolates. Butler *et al*. (1987) completed UV irradiation experiments with a human clinical isolate of *C. jejuni*, while the isolates used in the study by Sommer *et al*. (2000) were isolated from hamburger and humans, among other environments. Additionally, collimated beam apparatuses and irradiation media differed across all three studies. Other factors to take into consideration when UV dose-response curves are generated and compared to one another include turbidity, the matrix in which irradiation occurs (e.g., drinking water, wastewater, peptone broth), and whether bacteria irradiated are environmental strains or lab-grown strains as environmental strains of the same bacterial species tend to exhibit increased resistance to UV irradiation (Hijnen *et al*. 2006).

The UV dose-response data presented for *A. butzleri* may serve as a starting point for disinfection schemes in QMRAs. Hijnen *et al.* (2006) suggest that the process of photoreactivation within bacterial cells may require increased UV doses to achieve the same levels of $\log_{10}$ reductions compared to when photoreactivation does not occur. For example, Butler *et al*. (1987) reported that the most UV-resistant *E. coli* strain achieved a 6-$\log_{10}$ reduction

at 12.5 mJ/cm$^2$. However, when photoreactivation was taken into account, the same strain required a dose of 30.0 mJ/cm$^2$ to achieve the same $\log_{10}$ reduction. As photoreactivation was not examined in this thesis, this area would be worth exploring in future studies, especially with regard to *A. butzleri* that are released into the environment.

In QMRAs for water, reference pathogens are chosen based on the context of the risk assessment and how relevant they are to exposure pathways (WHO 2016). For example, the reference pathogen for bacteria in QMRAs for Australia's water reuse schemes is *Campylobacter* because it is the leading cause of bacterial gastroenteritis in the country (NRMMC *et al*. 2006). As such, reference pathogens are often associated with waterborne disease and act as representatives for different microbial groups (i.e., *Campylobacter* for bacteria, *Cryptosporidium parvum* for protozoa, adenovirus for viruses) (NRMMC *et al*. 2006, Schoen *et al*. 2017). The ideal reference pathogen would typically have properties of:

- High concentrations in water;
- High pathogenicity;
- Subject to low removal in treatment; and
- Capability of long survival in the environment (NRMMC *et al*. 2006).

The use of reference pathogens in water treatment is necessary as there are numerous pathogens associated with waterborne disease and to quantify them all would be impossible. Reference pathogens fulfill the assumption that if they are under control or are at acceptable levels, then all other pathogens – that are easier to control – should also be under control (NRMMC *et al.* 2006; WHO 2016).

Currently, campylobacters are commonly used as a reference pathogen for bacteria (Schoen *et al.* 2017). However, there is evidence that detections of *Campylobacter* are over-reported and that a fraction of these detections are actually *Arcobacter*. This subsequent under-reporting of *Arcobacter* spp. is due to unspecified testing for arcobacters as well as *Arcobacter* cross-reactivity in tests for *Campylobacter* (Banting *et al.* 2016). In addition to these testing issues, it may be worth evaluating *Arcobacter* as a potential replacement for *Campylobacter* as a reference pathogen as *Arcobacter* spp. have a greater capacity to grow in a wider range of conditions, which allows members of the genus to better survive in the environment.

### 3.3.2 UV disinfection of *A. butzleri* in association with FLP

Shown in Figure 3.4 are the UV dose-response curves of *A. butzleri* when associated with different FLP. When *A. butzleri* were associated with all four types of FLA, there were no significant differences in $\log_{10}$ reduction compared to *A. butzleri* alone ($p > 0.05$). There was, however, a significant $\log_{10}$ reduction difference between *A. butzleri* associated with *A. polyphaga* compared to those associated with *W. magna* ($p = 0.01$). This indicates the possibility that *W. magna* is better able to protect *A. butzleri* from UV light. As *W. magna* are prolific producers of vesicles, the vesicles themselves, once released, may also be providing protection to the bacteria (Berk *et al.* 1998, Brandl *et al.* 2005).

Compared to when they were associated with the FLA, there was a dramatic and significant decrease in $\log_{10}$ reduction when *A. butzleri* were associated with the ciliate *T. pyriformis* ($p = 0.02$). When *A. butzleri* was associated with *T. pyriformis*, they experienced an overall $\log_{10}$ reduction of 1.10 to 3.04 over the UV dose range. This $\log_{10}$ reduction range was much smaller compared to those of all the FLA, which had an average $\log_{10}$ reduction of 1.44 to 6.51 over the same UV dose range. The ability of *T. pyriformis* to better protect *A. butzleri* compared to the FLA may be due to its free-swimming capabilities.

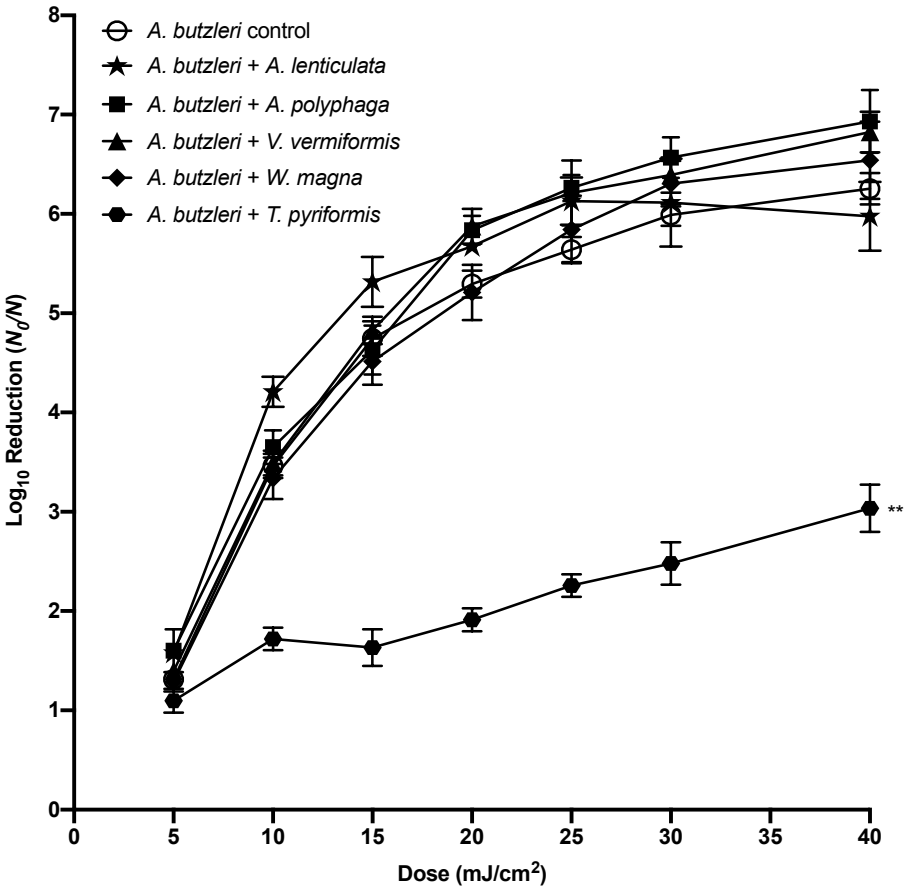


**Figure 3.4.** UV dose-response curves of FLP-associated *A. butzleri* when irradiated with a wavelength of 255 nm. Data are the means of 3-6 independent experiments, where $N_0$ is the initial concentration of bacteria before UV irradiation and $N$ is the concentration of bacteria after irradiation at a certain dose. Error bars represent ± SEM and significance ($p$ <0.05) is denoted with asterisks.

Unlike the FLA used in this experiment, which must be attached to a surface in order to move, *T. pyriformis* are ciliates that are highly motile in liquid media. When *T. pyriformis* cells were observed after being treated to a UV dose of 40 mJ/cm$^2$, the cells were still highly motile and exhibited the same behaviour prior to being exposed to UV. As such, it is likely that part of the ability of *T. pyriformis* to protect *A. butzleri* from UV lies in the likelihood that the ciliate cells directly shielded any *A. butzleri* cells that were not internalised. In a study by King *et al.* (1988), the following bacterial pathogens ingested by *T. pyriformis* experienced a 50-fold increase in resistance to free chlorine: *C. jejuni, E. coli, Klebsiella pneumoniae, Legionella gormanii, Salmonella typhimurium, Shigella sonnei,* and *Yersinia enterolitica.* Resistance was measured as the contact time needed to achieve a 99% reduction in culturable cells. However,

unlike the *A. butzleri* here, King *et al.* (1988) reported that the aforementioned pathogens were resistant to digestion by *T. pyriformis*. Additionally, these bacterial pathogens also experienced increased disinfection resistance when they were ingested by *A. castellanii*.

In Chapter 2, the likelihood of *T. pyriformis* digesting *A. butzleri* after internalisation was reported. As such, the ability of *T. pyriformis* to protect ingested *A. butzleri* from UV can only occur within the time period in which *A. butzleri* are ingested to the time that they are digested or expelled. This is significant as the wastewater treatment process in Edmonton's Gold Bar WWTP lasts around 18 h from the time of wastewater input (EPCOR 2019). Ciliates appear to start digesting *A. butzleri* sometime between 3-24 h after contact as they seem to protect *A. butzleri* from UV disinfection after 2 h of co-culture. As such, it may be that ciliates are more relevant to the ecology of *A. butzleri* in wastewater compared to FLA. This is especially so because of the tendency for *A. butzleri* to be free-living rather than attached to flocs during secondary treatment (Kristensen *et al.* 2020). As free-swimming ciliates are much more motile than FLA, there is a higher chance that ciliates could ingest and transport *A. butzleri* throughout and beyond the treatment process while also providing them with a haven of protection during UV disinfection.

# Chapter 4: Implications & conclusions

Arcobacters have been isolated from wastewaters at high densities from around the world. These places include Canada, China, Denmark, Germany, Italy, Saudi Arabia, South Africa, Spain, Turkey, the United Kingdom, and the United States (Kristensen *et al.* 2020, Shrestha *et al.* 2019 and references within). Of most interest is that the reported numbers of *Arcobacter* spp. in wastewater are significantly higher than those reported in the human gut microbiome, which indicate growth in the environment (Fisher *et al.*, 2014). Further, *Arcobacter* spp. being detected in treated wastewater effluents pose public health risks (Kristensen *et al.* 2020, Lu *et al.* 2015, Shrestha *et al.* 2019, Webb *et al.* 2016). Chapter 2 highlighted the possible relationships between *A. butzleri* and FLP in wastewater, while Chapter 3 examined the effect of UV disinfection on *A. butzleri* and these FLP associations. Taken together, the results of both chapters highlight the importance of understanding microbial ecological dynamics in sewage and during wastewater treatment. This may be especially relevant to future water reuse scenarios where various water treatment processes are required to achieve different bacterial $\log_{10}$ reductions depending on final end use (Schoen *et al.* 2017).

The work in this thesis was completed with a single *Arcobacter* species, *A. butzleri*, an isolate from raw sewage at a WWTP in Calgary, Alberta. Because access to this environmental isolate was readily available, it was decided that this species would be the focus of this thesis. Further, it was also chosen for the reason that the species is the most common *Arcobacter* species isolated from sewage through culture methods (Collado *et al.*, 2008, 2010, González *et al.* 2007). However, researchers have suggested a culture bias in favour of *A. butzleri* (Houf *et al.* 2002, Levican *et al.* 2016), which is apparent in the different *Arcobacter* species recovered based on detection method. *A. cryaerophilus* appears to be the most abundant *Arcobacter* species in wastewater according to sequencing methods and is still the second-most common *Arcobacter* species isolated using traditional culturing methods. In contrast, using the same sequencing methods, *A. butzleri* was only the eleventh most abundant *Arcobacter* species in wastewater (Fisher *et al.* 2014). Hence, further research is needed to determine the ecology of *A. cryaerophilus* and other *Arcobacter* species in the wastewater treatment train. These other species include, in order of abundance based on sequencing, *A. suis, A. ellisii, A. cibarius, A. clocacae,* and *A. defluvii* (Fisher *et al.* 2014).

In this body of work, FLP seemed to play a minimal role in the proliferation of *A. butzleri* in water. Unlike *Legionella, A. butzleri* did not appear to rely on FLP to reproduce, nor did they appear to be amongst the amoeba-resisting bacteria that amplify within amoebae (Thomas *et al.* 2010). However, further research is needed to determine what mechanisms allow *A. butzleri* to evade digestion by FLA. Conversely, Thurman *et al.* (2010), suggest that the inability of protozoa to digest bacteria may have more to do with the abundance of bacteria present rather than the bacteria having mechanisms that allow them to escape digestion. Hence, additional work may be required to determine whether there is a relationship between the density of bacteria and its ability to evade or succumb to digestion by protozoa. Additionally, it was shown that the ciliate *T. pyriformis* may be digesting *A. butzleri*, which may rule out their possibility of acting as a vessel in which *A. butzleri* could replicate. However, the FLA used in this study did readily ingest *A. butzleri* which suggests that FLA may yet act as transport vehicles for *A. butzleri* throughout and beyond the wastewater treatment train.

The wastewater matrix itself seems to play a role in the large numbers of *Arcobacter* spp. detected in sewage. However, the upstream sections of the sewer environment itself are suggested to be responsible for the high numbers of *Arcobacter* spp. in sewage. Although *Arcobacter* spp. are capable of forming and living within biofilms, McLellan and Roguet (2019) reported a low abundance of *Arcobacter* in sewer biofilms compared to sewer sediments. As FLA are mainly found within biofilms, these results seem to validate the current study findings that FLP may not play a large role in the reproduction of *A. butzleri* in sewage. Further research is needed to determine which aspects of the sewer environment are responsible for the prolific growth of *Arcobacter* spp. in sewage.

The current study is the first to report a UV dose-response curve for *A. butzleri* in water. Based on a rough comparison, the UV doses needed to cause certain $\log_{10}$ reductions of *A. butzleri* are greater than the doses needed to cause the same $\log_{10}$ reductions of *E. coli* and *C. jejuni*. As photoreactivation was not taken into account during experiments, further study is needed to determine the extent to which it occurs in *A. butzleri* and how it would affect UV inactivation doses. Additional research may be needed to further develop the dose-response curve of *A. butzleri* to fully understand the effect UV has on arcobacters, especially in the context of wastewater treatment. For example, the calculation of a k-value, a measure of how UV-sensitive or resistant an organism, is essential for translating the results of a bench-scale

study to large-scale application (Hijnen *et al.* 2006). FLA do not seem to play a significant role in protecting *A. butzler*i from UV irradiation. However, *T. pyriformis* played a significant role in protecting *A. butzleri* from UV irradiation. Further research is needed to determine the extent to which protection from the ciliates was a result of internalisation of the bacteria, "shading" of extracellular bacteria, or packaging and release of bacteria within undigested food vacuoles. Taken together, these results may provide greater insight into the detection of *Arcobacter* spp. in treated wastewaters.

An important concept that was not taken into account during experiments were those arcobacters that may have been in a VBNC state, which was discussed in Chapter 2 with regard to FLP interactions. However, VBNC states of *A. butzleri* in UV inactivation is also relevant and it would be worth exploring the extent to which UV disinfection truly inactivates *A. butzleri*. For example, the UV dose-response curve generated for *A. butzleri* in Chapter 3 assumed that any $\log_{10}$ reductions that occurred were due to complete cell death. However, it is possible that UV irradiation may have been inducing a state of VBNC in *A. butzleri* as the ability of UV to induce a VBNC state has been demonstrated for *E. coli* and *P. aeruginosa* (Zhang *et al.* 2015).

Following this, it would be prudent to examine and compare the state of *A. butzleri* when they are in stressed conditions (e.g., in low nutrient conditions such as river water) versus when they are in "ideal" growth environments (e.g., in nutrient-rich media such as wastewater). Carrying out these types of pre-experiments prior to introducing additional stressors may then provide information that is necessary to determine the factors that induce VBNC states in *A. butzleri* and the extent to which they may be induced. By determining the extent to which these conditions induce a state of VBNC, it would be easier to determine whether $\log_{10}$ reductions seen in co-cultures with FLP and UV irradiation were actually due to cell death or whether they were due to VBNC bacteria not showing up in culturable counts. Additionally, further research into whether FLP interactions and/or UV irradiation lead to the resuscitation of VBNC forms of *A. butzleri* would also be relevant to filling in research gaps regarding VBNC *Arcobacter* spp., especially in the context of water treatment. Finally, the use of imaging flow cytometry in the context of examining VBNC states in *A. butzleri* before, during, and after FLP interactions and UV disinfection is a tool worth investigating due to its ability to provide both quantitative and qualitative information.

Antimicrobial resistance is a significant concern in water reuse (Christou *et al.* 2017, Gudda *et al.* 2020). Studies have shown that arcobacters in wastewater carry a wide range of antibiotic resistance gene traits, with *A. cryaerophilus* expressing 25 categories of ARGs (Millar and Raghavan 2017, Hultman *et al.* 2018). The ingestion of *A. butzleri* by FLP in this thesis highlighted two concerns. First, the clustering of *A. butzleri* cells within FLP is highly conducive to the transfer of ARGs through HGT. Second, FLP may act as Trojan horses within which AMR may be transmitted to the environment as wastewaters are hotspots for ARGs. Additional research is required to determine whether FLP have an impact on *Arcobacter's* acquisition and/or expression of AMR as there are reports that associations with FLA lead to ARG upregulation as well as the protection of bacteria from the effects of antimicrobials (Barker *et al.* 1995, Miltner and Bermudez 2000, Vaerewijck *et al.* 2014). Additionally, there is evidence that UV disinfection selects for AMR in bacteria (Meckes 1982, Zhang *et al.* 2009, Kauser *et al.* 2019). Further research needs to be completed to determine whether UV disinfection or other wastewater treatment and disinfection processes affect ARG expression in *Arcobacter* spp.

There is need for better understanding of bacterial pathogens that are not considered traditional indicators of water quality but are still present in wastewaters and are known to pose risks to public health. Researchers have suggested *Arcobacter* as an alternative indicator to traditional FIB as a result of the genus's association with faecally contaminated water (McLellan *et al.* 2010, Newton *et al.* 2013). Currently, *Campylobacter* is used as the reference pathogen for bacteria in Australia and the United States (NRMMC *et al.* 2006, US EPA 2012). The results of this thesis provide additional evidence for the consideration of *Arcobacter* as a reference pathogen in QMRAs for the evaluation of water reuse schemes.

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## Appendix A: Definitions

**Table A.1.** Definitions for index, indicator, and reference pathogens relating to water quality.

| Group | Definition | Example |
|---|---|---|
| **Index organisms**[a] | A group/or species indicative of pathogen presence. | *E. coli* as an index for *Salmonella* |
| **Indicator organisms**[b] | Microorganisms whose presence is indicative of pollution or of more harmful microorganisms. | |
| **Faecal indicators**[a] | A group of organisms that indicates the presence of faecal contamination. Their presence does not necessarily correspond to the presence of pathogens. | Thermotolerant coliforms or *E. coli* |
| **Process indicators (surrogates)**[a,b] | A group of organisms that demonstrates the efficacy of a process and used in place of the direct measurement of hazards. | Total heterotrophic bacteria or total coliforms for chlorine disinfection |
| **Reference pathogens**[b,c] | Classes of pathogens with potential adverse health impacts that are used to determine whether the group of which it represents is largely under control. | *Campylobacter* for bacteria, rotavirus and adenovirus for viruses, and *Cryptosporidium parvum* for protozoa and helminths |

[a]Ashbolt *et al.* 2001
[b]NRMMC 2006
[c]Schoen *et al.* 2017

## Appendix B: Verifying sonication parameters for experiments with *T. pyriformis*

**Table B.1.** Average $\log_{10}$ CFU counts before and after sonication of *A. butzleri* to verify culturability. A one-tailed, unpaired t-test ($\alpha = 0.05$) determined that the sonication parameters did not lead to bacterial loss of culturability.

| Condition | Average $\text{Log}_{10}$ CFU/mL | Standard Deviation | p-value |
|---|---|---|---|
| Non-Sonicated (N = 5) | 6.10 | 0.184 | 0.06 |
| Sonicated (N = 5) | 6.30 | 0.171 | |

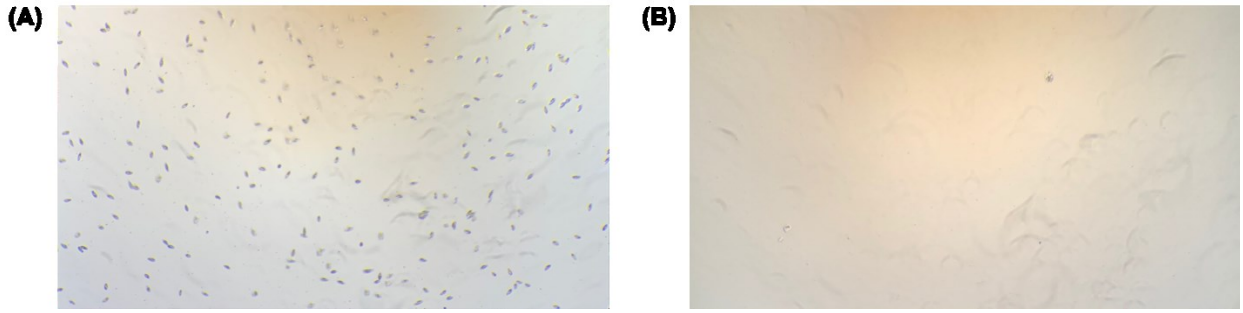


**Figure B.1.** Before **(A)** and after **(B)** sonication of *T. pyriformis* in a sonicator bath at high intensity for 1 min (3 cycles of 20 s). Each image is representative of ten fields of view from three trials completed in duplicate at 40x magnification.