Characterising the microbial communities associated with the water distribution system of a broiler farm and their role in *Campylobacter* infection

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Submitted in partial fulfilment of the requirements of the Degree of Doctor of Philosophy, May 2017

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List of contents

List of figures

List of tables

List of abbreviations

ABS	Acrylonitrile butadiene styrene
AhpC	Alkyl hydroperoxide reductase
AI	Autoinducer
ANOSIM	Analysis of Similarities
BHI	Brain Heart Infusion
BPW	Buffered peptone water
CC	Clonal complex
CDT	Cytolethal distending toxin
CEB	*Campylobacter* enrichment broth
CFS	Cell-free supernatant
CSM	*Campylobacter* selective media
CGH	Comparative genomic hybridization
CiaB	*Campylobacter* invasion antigen B
CprRS	*Campylobacter* planktonic growth regulation system
DWS	Drinking water system
DWDS	Drinking water distribution system
FLA	Free-living amoebae
FLP	Free-living protozoa
FSA	Food Standards Agency
GBS	Guillain-Barre syndrome
GI	Gastrointestinal

LB	Luria-Bertani
LOS	Lipooligosaccharide
MHB	Mueller-Hinton broth
MLST	Multilocus sequence typing
N	Nipple drinkers
Ne	Nipple drinker external
NGS	Next-generation sequencing
Ni	Nipple drinker internal
nMDS	Non-metric multidimensional scaling
OD	Optical density
OUT	Operational taxonomic unit
PAS	Page's amoebae saline
PBS	Phosphate-buffer saline
PFGE	Pulsed-field gel electrophoresis
PPG	proteose-peptone-glucose
PVC	Polyvinyl chloride
RAPD	Random amplified polymorphic DNA
RDP	Ribosomal Database Project
ROI	Rate of intenalization
SFP	Small family poultry
SSU	Small subunit
ST	Sequence type
STE	Sodium Chloride-Tris-EDTA

VBNC	Viable but nonculturable state
WGS	Whole genome sequencing
16S	16S rRNA
18S	18S rRNA

Acknowledgements

First and foremost I would like to express my sincere gratitude to my supervisor, Dr. Chloe James, for her encouragement, guidance, support and trust throughout the project. It has been challenging but also inspiring to work in an ambitious project in a new research group and I am very grateful that you gave me the chance to develop my confidence and abilities during the past three and a half years. I would also like to thank my second supervisor, Prof. Richard Birtles, for providing invaluable support and guidance when needed. I would additionally like to thank Dr. Ian Goodhead for kindly guiding me through the library preparation, sequencing and sequencing analysis. It would have been extremely difficult if not impossible to have done this without your assistance.

I would like to extend my gratitude to my colleagues in the University of Salford for their help inside and outside of the laboratory, especially to Jess Hall for being so helpful with all the day to day problems, to Raed Alnama for being my Campy friend and to Laura Brettell for endless conversations about qPCR and sequencing analysis. I feel very lucky to have been surrounded by the wonderful people in office 217 that have kept me sane and fed through the tough times and to have been part of the research community in Salford University. I would also like to thank all the students that have helped with the collection of samples and have worked along the project through the years, especially to Belen, Davide, Monika, Daniel, Komla, Andrea, Gavin and Luís. My special thanks to the technicians, especially to Sally Shepherd and Andrew Fairclough for their advice and help providing emergency reagents. I am also very grateful to all the people that have provided strains used in this study.

Many thanks to the farm company and farmer for their interest in the project and for allowing us to collect the samples. Also to Prof. Louise Ackers for organising the trip to Uganda where I collected some of the samples.

Last but not least I would like to thank my family, especially my parents Alicia Bou Albalate and Juan Aranega Martínez for fighting for my future and believing in me unconditionally.

Abstract

Campylobacters are zoonotic pathogens with extensive animal and environmental reservoirs in spite of their fastidious nature. *Campylobacter jejuni* is the main bacterial cause of human gastroenteritis worldwide. Epidemiological studies have identified chicken meat as the main vehicle for human infection in industrialised countries. In some countries in the developing world, however, there is little information about *Campylobacter* prevalence in poultry and poultry meat. The dynamics of *Campylobacter* colonisation of chickens remain poorly understood. It is agreed that in most cases *Campylobacter* is transmitted to chickens horizontally from the farm environment. Different sources have been recognised, but their actual contribution to *Campylobacter* epidemiology remains a matter of debate. Water has rarely been identified as a possible source of *Campylobacter* contamination of chickens, but represents an understudied source. Research suggests that natural microbial communities might promote the survival of *Campylobacter* in environmental reservoirs by enabling incorporation into biofilms or interaction with protozoa. The aim of this study was to improve our understanding of *Campylobacter* ecology and epidemiology at the chicken farm level. The main objectives were to unravel the microbial communities associated with the drinking water system of broiler farms and their dynamics through the rearing cycle; to investigate the aerobic survival of the pathogen in co-culture with *Pseudomonas* environmental isolates and *Acanthamoeba polyphaga;* and to study *Campylobacter* prevalence in chicken farms in Uganda.

A longitudinal study was conducted in a commercial broiler farm in the UK during a whole rearing cycle. The purpose of this study was to gain an understanding about the microbial communities that inhabit the drinking water system of the broiler farm, and their

implication in *Campylobacter* ecology in the chicken farm. 16S and 18S rRNA profiling of bulk water and biofilm samples were carried out over a seven-week production cycle period. The same samples were screened for the presence of *Campylobacter* by culture-dependent methods and molecular techniques. Analysis of 16S and 18S rRNA profiles suggested that microbial communities in the water distribution system are niche specific. Biofilm and bulk water samples harboured distinct communities. Moreover, bulk water communities inside the broiler house were significantly different from those sampled from the source water ($R=0.88$, $p<0.05$). Inside the broiler house, microbial communities were found to vary across the rearing cycle. Similar changes in the dynamics of prokaryotic and eukaryotic communities were observed. Bacterial communities were dominated by the phylum Proteobacteria with a shift to Firmicutes towards the end of the cycle, mainly due to an increase in relative abundance of the genera *Staphylococcus* and *Lactobacillus*. Eukaryotic communities included a diversity of yeasts, fungi and protozoa.

Campylobacter spp. were not detected on the UK broiler farm by culture-dependent techniques. However, the emerging gastrointestinal pathogen *Helicobacter pullorum* was isolated by culture towards the end of the rearing cycle. Another emerging pathogen *C. ureolyticus* was detected by both 16S rRNA amplicon sequencing and PCR in water samples and chicken faeces. This is the first report of *C. ureolyticus* in association with poultry. 16S rRNA reads belonging to the genus *Campylobacter* were also found in the sequencing data towards the end of the rearing cycle suggesting that the pathogen could be in a viable but non-culturable state in the water distribution system.

Little information is available on the epidemiology of campylobacteriosis in Africa. In Uganda *Campylobacter* infections have been reported in children, and gorillas have been

identified as carriers but no information is available on *Campylobacter* prevalence in chickens. Studies in neighbouring countries, however, have shown high *Campylobacter* prevalence in poultry. A pilot study, visiting three small-scale farms around Fort Portal, was conducted to investigate the prevalence of *Campylobacter* in chickens in W. Uganda. Through a combination of culture-dependent and culture-independent techniques *Campylobacter* was detected in all three farms with high prevalence. Mixed presence of *C. jejuni* and *C. coli* in the same farm were observed, suggesting that chickens and chicken meat can be a source of human infection in Uganda.

Pseudomonas spp. and protozoa of the genus *Acanthamoeba* are ubiquitous organisms that commonly co-localise in water and in the farm environment with *Campylobacter*. *In vitro* assays were performed to investigate whether they could enhance *C. jejuni* survival under atmospheric conditions. Co-culture assays indicated that certain *Pseudomonas* ssp. and strains were able to enhance *Campylobacter* survival with an increase in viable cell recovery ranging from 0.8 LOG to 4.5 LOG depending on the strain after 24 hours of aerobic incubation, while others showed no interactive effect or even reduced *Campylobacter* survival. *A. polyphaga* was shown to internalize *C. jejuni,* protecting it from the oxygen in the atmosphere and thus enhancing its survival with an increase in viable cell recovery of 2 LOG.

These studies have further shown the importance of chicken farms in *Campylobacter* ecology. It has been demonstrated that microorganisms that are capable of enhancing *Campylobacter* survival *in vitro* are found in the water distribution system of the farm studied in UK. The characterisation of these communities for a whole rearing cycle has led

to the detection of emerging human pathogens in broiler chicken production showing how doing exploratory work targeting whole microbial communities can inform epidemiology.

Chapter 1

General introduction

1.1 The genus *Campylobacter*

Campylobacter belong to the epsilon class of proteobacteria in the order *Campylobacteriales* and the family Campylobacteraceae and are closely related to *Arcobacter*, *Helicobacter* and *Wolinella* (Young *et al.* 2007, Silva *et al.* 2011). The genus *Campylobacter* was initially proposed by Sebald and Veron (1963) and at present it contains 27 species and 8 subspecies (Ngulukun 2017). *Campylobacter* are small (0.2-0.9µm wide and 0.2-5µm long), curved, spiral or rod shaped, non-spore forming, Gram negative bacteria (Epps *et al.* 2013). Most species show a corkscrew-like motility by means of a single polar flagellum or bipolar flagella, with the only exceptions of the non-motile *Campylobacter gracilis* and *Campylobacter showae,* which has multiple flagella (Silva *et al.* 2011). Bacteria in this genus are chemoorganotrophs which use amino acids and tricarboxylic acid cycle intermediates as energy sources (Kaakoush *et al.* 2015). They are nutritionally fastidious and require complex nutritional environments including strictly anaerobic or microaerobic conditions (Man 2011).

Most *Campylobacter* species cause veterinary and/or human infections but they are mainly known for the burden thermophilic species cause as zoonotic pathogens (Humphrey *et al.* 2007). The most important pathogenic species is *Campylobacter jejuni* followed by *Campylobacter coli* (Moore *et al.* 2005). They are classed as thermophilic campylobacters because they can only grow at the narrow temperature range between

30 °C and 46 °C (Humphrey *et al.* 2007). These two species are microaerophilic with a respiratory type metabolism growing best in an atmospheric composition of 5 to 10% oxygen and 5 to 13% carbon dioxide (Jackson *et al.* 2007).

Recent improvements in epidemiological surveillance, including the incorporation of molecular methods, have led to isolation of at least 10 different *Campylobacter* spp., other than *C. jejuni* and *C. coli,* from patients with gastroenteritis; These have been classed as emerging *Campylobacter* pathogens. Among them, *Campylobacter concisus* and *Campylobacter upsaliensis* have shown the highest prevalence (Man 2011). Moreover, *Campylobacter ureolyticus* appears to surpass *C. coli* as the second most common causative agent of campylobacteriosis in samples collected from southern Ireland (Bullman *et al.* 2011a, O'Donovan *et al.* 2014).

1.2 Epidemiology of campylobacteriosis in humans

1.2.1 Prevalence and disease trends

Campylobacter infection is the most frequent bacterial cause of gastroenteritis worldwide and a major public health issue (Kaakoush *et al.* 2015). During the last decade there has been a rise in the incidence of campylobacteriosis in North America, Europe and Australia and data from Africa, Asia and the Middle East suggest that *Campylobacter* infection is endemic in these regions (Kaakoush *et al.* 2015). Furthermore, the reported cases are likely to be an underestimation of the real disease burden owing to underreporting (Wagenaar *et al.* 2013). Diagnosis of bacterial gastroenteritis is largely done by routine stool culture (Humphries and Linscott 2015). However, culture methodologies used in clinical and diagnostic laboratories are not suitable for the

recovery of fastidious emerging *Campylobacter* species, probably underestimating their contribution towards campylobacteriosis (Man 2011). Importantly a high proportion of gastroenteritis cases reported have an unknown etiology both in developed (e.g. 48.6 % reported in UK by (Tam *et al.* 2012a)) and developing countries (e. g. 68.3% reported in rural Western Kenya by (Brooks *et al.* 2006)).

C. jejuni has a very low infective dose. The ingestion of doses as low as 500 and 800 cells has led to human infection in controlled experiments (Robinson 1981, Black *et al.* 1988). Most cases of *Campylobacter* enteritis present as sporadic but outbreaks also take place and are frequently related to consumption of contaminated food (mainly unpasteurized milk and poultry products) or water (drinking and recreational) and to a lesser extent to animal contact (Little *et al.* 2010, Taylor *et al.* 2013). In temperate regions sporadic cases have a distinct seasonal pattern with a peak in the summer months (Ekdahl and Andersson 2004, Samuel *et al.* 2004, Louis *et al.* 2005, Nichols *et al.* 2012).

In developing countries campylobacteriosis is often a pediatric disease (Coker *et al.* 2002, Brooks *et al.* 2006) and *Campylobacter* is the most commonly isolated pathogen in children less than 2 years old with diarrhoea (Deogratias *et al.* 2014, Tafa *et al.* 2014). This is believed to be because of high levels of exposure which result in acquired immunity (Blaser *et al.* 1985, Blaser *et al.* 1986) and asymptomatic infections (Lee *et al.* 2013). Poor hygiene and sanitation and increased contact with animals have been identified as risk factors (Rao *et al.* 2001) and are believed to contribute to easy and frequent acquisition of enteric pathogens such as *Campylobacter* (Coker *et al.* 2002). HIV/AIDS patients also show higher incidence of clinical manifestations associated with substantial mortality and morbidity (Sorvillo *et al.* 1991, Molina *et al.* 1995). In developed

countries people of all ages are affected but incidence is greater in children under 4 years of age (149.2/100,000) than the general population (40-60/100,000) in urban areas (Lévesque *et al.* 2013). A higher incidence in rural settings has been reported in children under 5 years old (234/100,000 vs. 151/100,000) (Strachan *et al.* 2009) and in young adults aged 15-34 (186.8/100,00 vs. 40-60/100,000) and elderly people aged ≥ 75 (120/100,000 vs. 40-60/100,000) when compared to urban areas (Lévesque *et al.* 2013).

The UK's *Campylobacter* notification rate was 104 for every 100,000 inhabitants in 2013 which is among the highest in the developed world (Skarp *et al.* 2016). However the Second Study of Infectious Intestinal Disease in the Community (IID2), including a community cohort and healthcare presentation study, concluded that national statistics underestimate the incidence and reported 1,100 for every 100,000 person-years in the community and 100 GP consultations per 100,000 person-years (Tam *et al.* 2012b). *Campylobacter* was identified as the most common bacterial cause of infectious intestinal disease (Figure 1.1) both in the community and in GP presentation cases (Tam *et al.* 2012a).

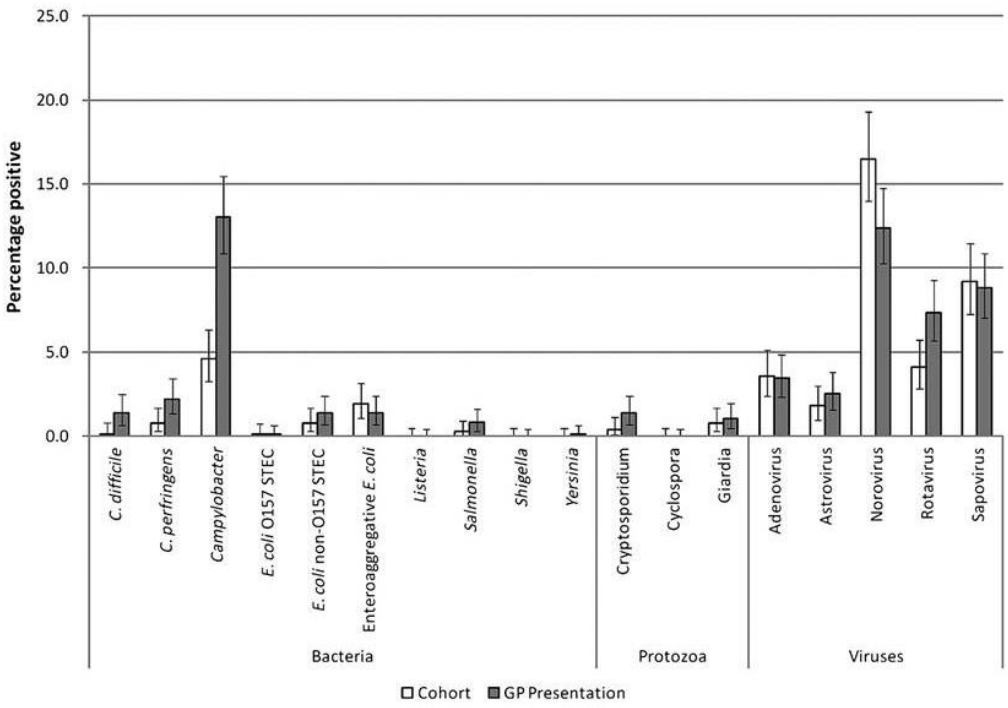


Figure 1.1. Microbiological findings in cohort and general practice presentation cases in the second Study of Infectious Intestinal Disease in the Community. The graph shows the high incidence of *Campylobacter* among the bacterial pathogens. Abbreviations: GP, general practice; STEC, Shiga toxin-producing *Escherichia coli* (Tam *et al.* 2012a).

1.2.2 Symptoms and treatment

Campylobacteriosis symptoms include diarrhoea (frequently bloody but occasionally watery), abdominal pain, fever, nausea and malaise whereas vomiting is rare (Moore *et al.* 2005). Symptoms occur 1 to 10 days after exposure and illness usually resolves itself within 2 to 5 days although it may last up to several weeks (Galanis 2007). In the UK it has been reported that only one in seven patients with campylobacteriosis consult their GP (Tam *et al.* 2012b).

Antibiotic treatment is only required for immunocompromised patients and for those showing severe symptoms (Wieczorek and Osek 2013). Fluoroquinolones such as ciprofloxacin are used as an empirical treatment for diarrhoea (Casburn-Jones and

Farthing 2004) but resistance to these antibiotics is widespread in *Campylobacter* so macrolides such as erythromycin have become the treatment choice for campylobacteriosis (Iovine 2013). Emergence of antibiotic resistance in *Campylobacter* is a cause of concern and it has been related to antibiotic use in food animals (Luangtongkum *et al.* 2009).

Complications of campylobacteriosis are rare and include reactive arthritis and irritable bowel syndrome (Humphrey *et al.* 2007). The most concerning is Guillain-Barre syndrome (GBS) which causes ascending paralysis resulting from the demyelination of the periphereal neurons due to cross-reaction with anti *C. jejuni* antibodies (Nyati and Nyati 2013). Serological studies have shown that 14-38% of GBS patients had evidence of previous *Campylobacter* infection and culture studies have isolated *Campylobacter* from 8-50% of GBS patient's stools after the onset of neurological symptoms (Allos 1998). In a recent systematic review it was reported that the proportion of confirmed cases of *Campylobacter* developing GBS ranged from 0% to 2.08% resulted in a global estimate of 0.07% (Keithlin *et al.* 2014).

1.2.3 *C. jejuni* pathogenesis

C. jejuni causes significant inflammation and enteritis in humans but mostly establishes benign colonization in chickens (Young *et al.* 2007). As most cases of campylobacteriosis result from infection with *C. jejuni* the mechanisms of pathogenicity have been studied mostly in this species (Zilbauer *et al.* 2008). In order to establish an infection in humans it must circumvent the mucus layer of the gastrointestinal (GI) epithelium and interact with the intestinal epithelial cells (Young *et al.* 2007). Mutational studies have shown that several structures and mechanisms are involved in adherence, including surface

molecules such as Lipooligosaccharide (LOS) and the capsule, several adhesins (such as CadF, JlpA and Peb1) and protein post-translational glycosylation systems (both O-linked and N-linked glycosylation) (Young *et al.* 2007). *C. jejuni* is almost unique in expressing two protein glycosylation systems: a general N-linked glycosylation pathway (responsible for post-translational modification of at least 30 proteins) and an O-linked glycosylation (responsible for modification of flagellin proteins) (Zilbauer *et al.* 2008).

C. jejuni are also able to invade the intestinal epithelial cells of the human host, which contrasts with the situation in chickens where *C. jejuni* primarily resides in the mucosal layer in the intestines (Lee and Newell 2006), suggesting that entrance into epithelial cells in the GI may be critical for the development of disease in humans (Ó Cróinín and Backert 2012). Many of the bacterial factors involved in adherence are also implicated in host cell invasion including the capsule, LOS and protein post-translational glycosylation (Ó Cróinín and Backert 2012). A functional flagellar apparatus is also required. The flagellum is used as a secretory device for invasion-associated effector molecules, including *Campylobacter* invasion antigen B (CiaB), other Cia proteins and FlaC (Ó Cróinín and Backert 2012).

C. jejuni produces cytolethal distending toxin (CDT) which is also produced by other bacterial species including *Escherichia coli* and *Helibobacter hepaticus* (Young *et al.* 2007). The toxin causes eukaryotic cells to arrest in the G2/M phase of the cell cycle, preventing transition to mitosis and leading to cell death. CDT consists of three subunits CdtA, CdtB and CdtC. CdtB is the toxic component sharing homology with the mammalian DNase I. CdtB localizes to the nucleus while CdtA and CdtC mediate binding to cholesterol-rich microdomains on the cytoplasmatic membrane of host cells and deliver CdtB. The exact function of CDT in *C. jejuni* pathogenesis remains to be fully elucidated (Lai *et al.* 2016).

Differences in body temperature (41-45 °C in chickens vs. 37 °C in humans) might also influence the different onset of infection in humans and chickens through altered transcription profiles (Young *et al.* 2007).

1.2.4 Attribution of human campylobacteriosis

One of the major challenges in epidemiological studies of *Campylobacter* infection is how to clearly characterise and distinguish different strains and elucidate the complex transmission cycles involving multiple transmission pathways and reservoirs and their relative contribution to human campylobacteriosis (Figure 1.2). A combination of microbiological and other epidemiological approaches such as case-control and source attribution studies are needed to fully elucidate the disease ecology (Wagenaar *et al.* 2013).

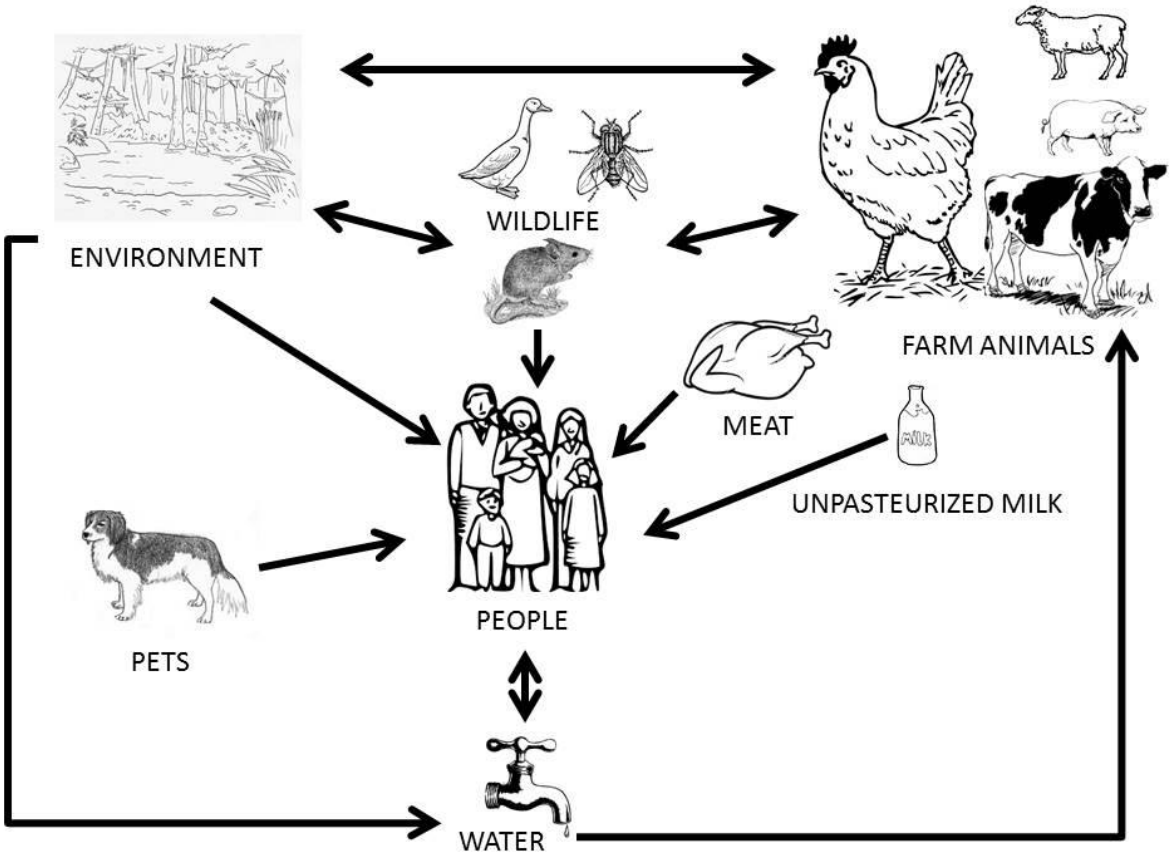


Figure 1.2. *Campylobacter* reservoirs and sources of human infection. The figure summarises the main reservoirs for *Campylobacter* spp. and identified sources of campylobacteriosis and their connection.

The isolation, identification and typing of *Campylobacter* spp. is essential to enable tracking of the pathogen from environmental sources and across the food system. Typing systems can use phenotypic and/or genotypic methods. Phenotypic methods include biotyping, serotyping and phage typing (Eberle and Kiess 2012). Biotyping techniques are used for *Campylobacter* identification to the species level through the expression of metabolic activities and include the use of selective media, Gram staining and biochemical tests (such as catalase and oxidase tests and hippurate hydrolysis) among other strategies (Hansson *et al.* 2004). Serotyping is based on the fact that different

strains have different antigens on their surfaces which can be detected by antibodies and antisera (Wiedmann 2002). The major serotyping scheme for *Campylobacter* spp. is that of Penner and Henesssy (Penner and Hennessy 1980) which identifies heat stable or O antigens and distinguishes 66 different serotypes (McKay *et al.* 2001). An alternative serotyping scheme was developed by Lior *et al.* (1982) which identifies heat labile antigens and can recognize more than 100 serogroups (Serichantalergs *et al.* 2010). These two serotyping techniques have been used independently or in combination for *Campylobacter* spp. surveillance (Woodward and Rodgers 2002). To further subtype within the predominant serotypes phage typing was proposed, giving finer discrimination and increasing the number of typeable strains (Grajewski *et al.* 1985, Frost *et al.* 1999).

Genotyping methods have higher discriminatory power, ease of standardization and reproducibility (Nielsen *et al.* 2000, Schouls *et al.* 2003). A number of genetic approaches have been developed, including pulsed-field gel electrophoresis (PFGE) (Yan *et al.* 1991), flagellin genotyping (Alm *et al.* 1993), random amplified polymorphic DNA (Fujimoto *et al.* 1997), ribotyping (Fayos *et al.* 1992) and multilocus sequence typing (MLST) (Dingle *et al.* 2001). Among them, MLST is the most widely used and has brought major advances in the study of *C. jejuni* population diversity and attribution of infections to sources (Colles and Maiden 2012, On 2013). The key to its success is the generation of unequivocal easy to interpret data (nucleotide sequences) that can be directly compared among different laboratories (Dingle *et al.* 2001).

The initial MLST scheme for *C. jejuni* included seven housekeeping genes (*aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *tkt*, and *uncA*) which allows categorizing strains as sequence types (STs) and grouping related STs into clonal complexes (CC) (Dingle *et al.* 2001). The scheme has

subsequently been extended and modified to be used with other *Campylobacter* species (Miller *et al.* 2005, Dingle *et al.* 2008). However other techniques such as comparative genomic hybridization (CGH), allowing much wider genomic comparisons than MLST, have also been used to study *Campylobacter* population structure with more detail (Champion *et al.* 2005, Hepworth *et al.* 2011, Stabler *et al.* 2013). Most recently, whole genome sequencing (WGS) has been used in epidemiological outbreak investigations (Fernandes *et al.* 2015) and characterisation of sporadic clinical isolates (Cody *et al.* 2013). However WGS data has not yet been applied to source attribution models of campylobacteriosis cases (Llarena *et al.* 2017).

Campylobacter populations are regarded as genetically highly diverse (Stabler *et al.* 2013). This extensive genetic variation has arisen from intragenomic mechanisms such as phase variation and genetic exchange between strains (Young *et al.* 2007). Many CCs are over-represented in particular hosts, for example ST-45 and ST-247 CCs are common amongst broilers (Colles *et al.* 2003, de Haan *et al.* 2010, Griekspoor *et al.* 2010, Jorgensen *et al.* 2011, Colles and Maiden 2012) whilst ST-61 and ST-42 CCs are more often isolated from cattle and sheep (Colles *et al.* 2003, Kwan *et al.* 2008). Other CCs such as ST-21 have been defined as "multihost" and have been isolated from a wide variety of sources (Colles *et al.* 2003). A water/wildlife-associated *C. jejuni* group with novel STs has also been identified and shown to diverge from common CCs associated with agricultural or human sources through CGH (Hepworth *et al.* 2011). Source attribution approaches estimate the contribution of different sources and transmission pathways to campylobacteriosis by comparing *Campylobacter* genotypes from humans with those from potential sources (Golz *et al.* 2014). By estimating the extent of subtype sharing by

human and reservoir isolates MLST has identified potential infection reservoirs which include poultry, cattle, sheep, wildlife and environmental sources such water (Sheppard *et al.* 2009, Strachan *et al.* 2009, Cody *et al.* 2015). Source attribution studies have implicated contaminated poultry meat as the main source of clinical infection (Skarp *et al.* 2016). In parallel, case-control studies have also been used to understand the sources and pathways of *Campylobacter* infection. These retrospective studies are used to determine if an exposure is associated with an outcome (Lewallen and Courtright 1998). Campylobacteriosis case-control studies have identified common risk factors that are associated with the disease such as barbecuing, consumption of meat, swimming, contact with animals, drinking unpasteurized milk or untreated water and recent use of proton-pump inhibitors (Studahl and Andersson 2000, Kapperud *et al.* 2003, Mughini Gras *et al.* 2012, Ravel *et al.* 2016). Foreign travel also remains an important risk factor and the level of risk appears to be associated with travel destinations within South and Southeast Asia, Africa and Latin America being higher risk destinations compared to western European countries (Mughini-Gras *et al.* 2014). Using a case-case study Lévesque *et al.* (2013) reported that occupational exposure to animals and consumption of private well water are specific risk factors for rural areas. In line with source attribution analyses, case-control studies have also concluded that consumption of chicken is a significant risk factor (Wingstrand *et al.* 2006, Tam *et al.* 2009).

1.2.4.1 Farm and domestic animals as a reservoir

Campylobacter spp. are carried by poultry, cattle, sheep and pigs (Humphrey *et al.* 2007). Avian hosts including chickens, turkeys, ducks and geese among others are frequently colonized in the intestinal tract with high numbers of *C. jejuni* and *C. coli* generally with

no overt disease (Sahin *et al.* 2015). In chickens, reports range from about 10^4 to 10^8 CFU per gram of caecal content (Beery *et al.* 1988, Van Deun *et al.* 2008). *Campylobacter* prevalence rates in chickens vary but reach as high as 100% on some farms at slaughter age and chickens are considered the main *Campylobacter* reservoir, particularly for *C. jejuni* (Sahin *et al.* 2015). Thermophilic campylobacters have also been isolated from the intestine and rumen of healthy ruminants with varying overall prevalence typically ranging from 0.8 % to 46.7% in adult cattle (Stanley and Jones 2003, Tyson *et al.* 2016). Similarly, lambs show variable colonization prevalence typically varying from 0.6% to 59% (Stanley and Jones 2003, Yang *et al.* 2014). *Campylobacter* species but particularly *C. coli* are also prevalent in pigs with a prevalence of 66.8% before transport to the abbatoir (Alter *et al.* 2005).

A combination of MLST and case-control studies in Scotland, The Netherlands and urban and rural Canda has concluded that the majority of human *C. jejuni* infections originate from chicken (64.5%-78%) followed by cattle (21.2%-28%) with sheep (2.4%) and pigs (0.01%) contributing to a smaller extent (Sheppard *et al.* 2009, Mughini Gras *et al.* 2012, Lévesque *et al.* 2013). Similarly, the majority of *C. coli* human gastroenteritis originate from chicken (56%-69.6%), sheep (5%-40%), cattle (2%-12.2%) and pigs (<1%-4.9%) (Sheppard *et al.* 2009, Mughini Gras *et al.* 2012, Lévesque *et al.* 2013). A study that focused on children <5 years old found an increased number of cases originating from cattle (42% vs. 35%) and a reduced number of cases originating from chicken (19% vs. 43%) in rural areas compared to urban areas (Strachan *et al.* 2009). In this study 12% of cases in rural areas and 15% of cases in urban areas originated from sheep and <1.4% in both areas originated from pigs (Strachan *et al.* 2009). Therefore, although chickens are

the most important reservoir for *C. jejuni* and *C. coli*, the role of other livestock should not be overlooked.

Farm animals also pose a risk through the contamination of milk. A recent meta-analysis reported a weighted mean *Campylobacter* prevalence value of 1.18% in unpasteurized milk (Christidis *et al.* 2016). Consumption of raw milk has been associated with campylobacteriosis through epidemiological investigations, including genotyping techniques (Heuvelink *et al.* 2009).

Domestic dogs and cats have been shown to be *Campylobacter* carriers (Baker *et al.* 1999, Wieland *et al.* 2005) along with animals in petting zoos such as rabbits, goats, sheep etc. (Evers *et al.* 2014). Genetic association between strains infecting human and their pets has been found but to date it is still unclear if transmission occurs from pets to humans, vice versa or as a result of a third common source of infection (Gras *et al.* 2013).

1.2.4.2 The environment and wildlife as a reservoir

Wildlife also represents a reservoir for *Campylobacter* spp. Several groups of wild birds carry *Campylobacter* (Hughes *et al.* 2009, Cody *et al.* 2015) as well as mammals including rodents (Meerburg and Kijlstra 2007), primates (Misawa *et al.* 2000), hares (Rosef *et al.* 1983), deer, hedgehogs, squirrels, foxes and badgers (Petersen *et al.* 2001). Insects, such as flies (Rosef *et al.* 1985, Shane *et al.* 1985), darkling beetles (Jacobs-Reitsma *et al.* 1995) and cockroaches (Umunnabuike and Irokanulo 1986) have been reported as *Campylobacter* carriers and might also contribute to its spread.

In spite of its fastidious nature *Campylobacter* are able to survive and persist in several environmental niches. *Campylobacter* are frequently isolated from aquatic environments

such as surface waters (rivers, sea and lakes) used for recreational purposes (Savill *et al.* 2001, Vereen *et al.* 2007, Hokajarvi *et al.* 2013). *Campylobacter* has also been found in biofilms formed in sediments, rocks and woods associated with an agricultural watershed (Maal-Bared *et al.* 2012) and in soil around farms (Jensen *et al.* 2006). It has been argued that these *Campylobacter* spp. were shed by wild animals or originated from sewage discharges or agricultural runoff (Pitkanen 2013). However further characterisation of isolates collected from environmental and wildlife reservoirs has identified a distinct group of *C. jejuni* showing signs of niche adaptation and limited overlap with livestock associated strains(Champion *et al.* 2005, Hepworth *et al.* 2011, Stabler *et al.* 2013).

Although campylobacteriosis is primarily a foodborne disease in the developed world, in developing countries environmental contamination is also believed to be an important source, although epidemiological investigations are lacking (Coker *et al.* 2002). In developed countries epidemiological studies have found that small percentages of human isolates are attributable to wildlife and the environment, including waterborne transmission (Sheppard *et al.* 2009, Mughini Gras *et al.* 2012, Ravel *et al.* 2016). Source attribution studies have found that between 4% and 10.1% of human cases can be attributed to environmental reservoirs including water, sand and wild birds (Sheppard *et al.* 2009, Mughini Gras *et al.* 2012). When wild birds have been considered as a separate reservoir they have been reported to account for 2.1% to 3.5% of campylobacteriosis cases (Lévesque *et al.* 2013, Cody *et al.* 2015). Strachan *et al.* (2009) found that in children aged <5 years old non-chicken avian sources, including unidentified birds, ducks, geese, gulls and feral pigeons accounted for 19% of cases in rural areas and 6% of cases in urban areas. In another source attribution study 7.4% of the human cases could be

attributed to river and stream water (Lévesque *et al.* 2013). Consumption of contaminated water either from private wells with no disinfection or as a result of water treatment breaks has been linked to *Campylobacter* outbreaks (Pitkanen 2013). It has been suggested that environmental reservoirs such as water also contribute to the burden of human infection indirectly by contributing to the continual contamination of livestock and poultry (Pearson and Healing 1992).

1.2.5 *Campylobacter* along the food chain

Campylobacter are typically considered foodborne zoonotic pathogens with poultry being the main vehicle of infection and other foodborne pathways being red meat, milk, fruits and vegetables (Whiley *et al.* 2013).

Stages of poultry meat food chain include i) primary production at the farms, ii) transport to slaughter, iii)slaughter and subsequent processing, iv) selling the products at the retail level and v) handling of the products at home or public places such as restaurants (Skarp *et al.* 2016).

At the farm level poultry flocks are highly colonized by *Campylobacter* spp. with prevalences ranging from 30 to 100 % (Golz *et al.* 2014). Broiler chickens present the highest risk due to the high levels of consumption (Humphrey *et al.* 2007). Broiler chickens colonized by *Campylobater* harbour very high numbers of bacteria in their intestines, primarily in the ceca, large intestine and cloaca (Musgrove *et al.* 2001) and shed the bacteria in their faeces until the time of slaughter (Achen *et al.* 1998, Garner *et al.* 2008). Chickens at slaughter can be colonized with up to 10^9 CFU/gram of caecal

content (Cawthraw *et al.* 1996) representing the primary source for carcass contamination (Hutchison *et al.* 2017).

During food processing and storage *Campylobacter* spp. are subjected to a variety of stresses including extreme temperatures, low pH and desiccation (Humphrey *et al.* 2007). Their fastidious growth requirements mean that unlike other bacterial foodborne pathogens *Campylobacter* ssp. are not normally capable of multiplication in food during processing or storage (Park 2002). However, they are able to survive and a high proportion of global poultry produce is contaminated by campylobacters, as reflected by the high contamination incidence at retail around the world ranging from 8.1% to 92.9% with an average of 58% (Suzuki and Yamamoto 2008). In the UK the Food Standards Agency (FSA) reported 55.8% of supermarket chicken as being contaminated with *Campylobacter* from August to December 2016 (FSA, 2016).

Food of non-animal origin can become a source of infection through cross-contamination along the food chain and in the kitchen and this is known to play an important role in the transmission of foodborne illness (Kapperud *et al.* 2003, de Jong *et al.* 2008, Verhoeff-Bakkenes *et al.* 2011). Verhoeff-Bakkenes *et al.* (2011) reported a prevalence of 0.23% in fresh vegetables and fruits contaminated with *Campylobacter* at retail which resulted in an estimated $5.3x10^5$ cases of infection with *Campylobacter* per year in the Netherlands and therefore concluded that consumption of fruit and vegetables represent a risk factor. Hands and cutting boards were identified as the main cross-contamination routes in the kitchen (de Jong *et al.* 2008) and it has been suggested that mild and sporadic infections are more likely to occur through cross-contamination from inadequate kitchen practices (Rodrigues *et al.* 2001). Kapperud *et al.* (2003)supported this hypothesis by showing that

consumption of poultry brought raw into the house and preparation of raw poultry meat were associated with an increased risk whereas consumption of undercooked poultry was not.

FSA has set targets to reduce levels of chicken meat contamination at retail (FSA, 2010). Although there is possibility for intervention at several stages, it has been concluded that changes in farming practices have greater potential for reducing carcass contamination than other interventions along the food chain (Hutchison *et al.* 2017). Controlling *Campylobacter* on-farm will likely also reduce transmission through environmental pathways and therefore have a higher impact in the global *Campylobacter* epidemiology than other interventions later on the food chain (Golz *et al.* 2014). Others however have estimated that on farm interventions are more costly and it is most cost-effective to implement measures during processing, such as improvements in equipment and hygiene practices in processing facilities (Lake *et al.* 2013).

Given the complex ecology and epidemiology of this pathogen, it has been suggested that an interdisciplinary One Health approach involving all stakeholders (veterinary authorities, public health authorities, scientists, police makers, industry etc.) and combining intervention methods at different stages of the food chain is necessary to ultimately reduce the burden of human campylobacteriosis (Golz *et al.* 2014).

1.3 Campylobacter ecology in broiler chickens

Broiler chickens are the main source of human infection by *Campylobacter* (Humphrey *et al.* 2007). The world's estimated stock of chickens for 2014 was over 26 billion, according to statistics from the UN's Food and Agriculture Organization (FAOSTAT, 2014). However,

the true scale of global chicken production is not easy to measure as the number of small scale farms and chickens raised for domestic consumption is very difficult to estimate.

The UK is the second largest EU chicken meat-producing country, with almost 1,5 million tonnes of broiler meat produced in 2015 (AHDB, 2016).

Broiler production is a large scale operation in many countries undertaken by large integrated companies that own a mixture of farms managed by contract farmers (Vidal *et al.* 2014). The commercial production of broilers is diverse. Most broilers are raised in closed systems (intensive or conventional flocks) but in other cases they have access to the external environment (free-range). There is also organic production where there is a lower stock density and slower growing birds (Vidal *et al.* 2014).

In Africa however small family poultry (SFP) which are raised extensively or semi-intensively in relatively small numbers (usually less than 100 birds per flock) make up 80 percent of the total poultry production (Sonaiya 2007). A large proportion of poultry production in these countries often consists of free indigenous unselected breed of various ages (Conan *et al.* 2012). In developing countries SFP has economic and social significance, particularly in the rural areas where they provide a means of income to disadvantaged people (women, people with HIV/AIDS, orphans etc.). Poultry present several advantages as they require little land, labour and other production factors and are accepted in virtually all cultures contributing to food security and economic empowerment (Sonaiya 2007).

1.3.1 Prevalence and trends of *Campylobacter* colonisation of chickens

The high *Campylobacter* prevalence in chickens suggests that these bacteria have evolved to adapt to the different environments of broiler production and readily colonize the avian gut (Lee and Newell 2006). *C. jejuni* is the most commonly reported species associated with chickens, followed by *C. coli* and to lesser extent *C. lari* (van de Giessen *et al.* 1998, Hald *et al.* 2001, Lawes *et al.* 2012, Marotta *et al.* 2015). Mixed infections of *C. jejuni* and *C. coli* have been detected in the same flock (Gibbens *et al.* 2001) and different strains of the same species have also been observed circulating in the same flock, with the diversity of strains present increasing with time (Colles *et al.* 2008, Messens *et al.* 2009). Individual birds can also be colonized by multiple *Campylobacter* strains, including both *C. jejuni* and *C. coli* (De Cesare *et al.* 2008).

After colonization with the bacteria chickens typically remain asymptomatic and as a consequence *Campylobacter* spp. are often described as commensals in poultry (Dhillon *et al.* 2006). However, although a causal relationship to disease has not been fully proved, the dynamics of the relationship between *Campylobacter* and broilers suggests conditional commensalism (Jennings *et al.* 2011). It has been shown that flocks with compromised health or subjected to stress are more likely to be campylobacter-positive (Humphrey 2006, Bull *et al.* 2008). *Campylobacter* has also been quantitatively associated with vibrionic hepatitis (Jennings *et al.* 2011) and might indirectly cause hock lesions and pododermatitis by increasing the wetness of litter by loosening faeces (Williams *et al.* 2013).

A strong seasonal effect, with incidence peaks in summer and autumn under European conditions, has been extensively observed (Russa *et al.* 2005, Ellis-Iversen *et al.* 2009,

Nather *et al.* 2009, Smith *et al.* 2016). For example, in a large study conducted in Great Britain involving 797 flocks, the prevalence was higher in July (54%), August (55%) and September (60%) than during the rest of the year, ranging from 14% to 48% (Jorgensen *et al.* 2011). In a study in the Netherlands including 1737 flocks 43% prevalence was found in winter, 55% in spring, 48% in summer and 53% in autumn (Russa *et al.* 2005). In the UK, organic and free-range flocks show a higher prevalence at slaughter than conventional flocks (Allen *et al.* 2011, Lawes *et al.* 2012). Lawes *et al.* (2012) reported *Campylobacter* prevalence at slaughter of 78.5% in conventional flocks, 94.1% in organic flocks and 90.9% in free-range flocks in a study in the UK which included 1174 slaughter batches. Nather *et al.* (2009) identified as a risk factor the fact of flock originating from organic or free-range farms.

1.3.2 Colonization and transmission

When experimentally infected, chickens as young as one and two days old can become colonised by *Campylobacter* (Cawthraw *et al.* 1996, Ringoir *et al.* 2007). Flagella, flagellar motility and the chemotactic machinery are required for chicken colonization, along with adherence and invasion factors (such as cadF and ciaB), the N-glycosylation machinery and two-component regulatory systems (RacRS and DccRS) (Young *et al.* 2007). The infectious dose required varies significantly among different strains ($1x10^4$-$1x10^6$) (Ringoir and Korolik 2003)and is significantly lower ($1x10^3$-$1x10^4$) decrease in the needed inoculum) after the strain is subjected to *in vivo* passage through chickens (Cawthraw *et al.* 1996, Ringoir and Korolik 2003) suggesting adaptation to the gut environment. In most studies of broiler farms there is a delay of two to three weeks after the arrival of new hatchings from the farm before *Campylobacter* is detected in a flock (Torralbo *et al.* 2014,

Battersby *et al.* 2016). The mechanisms underlying the delay in the onset of colonization could be related to changes in gut microbiome, lack of exposure to the pathogen and maternally conferred immunity (Sparks 2009, Cawthraw and Newell 2010). Cawthraw and Newell (2010) observed that day of hatch chickens were susceptible to colonization but this was followed by increased resistance on day 8 that subsequently declined, in parallel with maternally derived antibodies, by 21 days of age, suggesting that maternally derived antibodies can be protective against *C. jejuni* challenge. An age dependence of transmissibility between birds rather than actual susceptibility to colonization has also been suggested as an explanation (Conlan *et al.* 2011).

Once *Campylobacter* is introduced into the flock and established within an individual bird or group of birds the infection spreads rapidly through the flock within a few days (Battersby *et al.* 2016). *Campylobacter* shedding by colonized birds leads to contamination of the broiler house environment and soon this is sufficient to surpass a threshold that causes extensive colonization in birds (Shreeve *et al.* 2000). Mathematical models have been used to estimate that one colonized bird could, on average, infect 2.37 birds per day (van Gerwe *et al.* 2009). Transmission is believed to occur mainly through the faecal-oral route enhanced by the coprophagic activity of broilers (Newell and Fearnley 2003), although other routes such as the drinking water system (DWS) can also spread the infection (Pearson *et al.* 1993, Messens *et al.* 2009, Cokal *et al.* 2011).

1.3.3 Sources of infection

The initial source or sources of *Campylobacter* infection in broilers remain poorly characterised. The difficulty in the recovery and culturing of the organism, its genetic instability and its complex epidemiology make it difficult to identify routes of colonization

of broilers and other livestock (Young *et al.* 2007, Sparks 2009). Identified sources of colonization are summarised in figure 1.3.

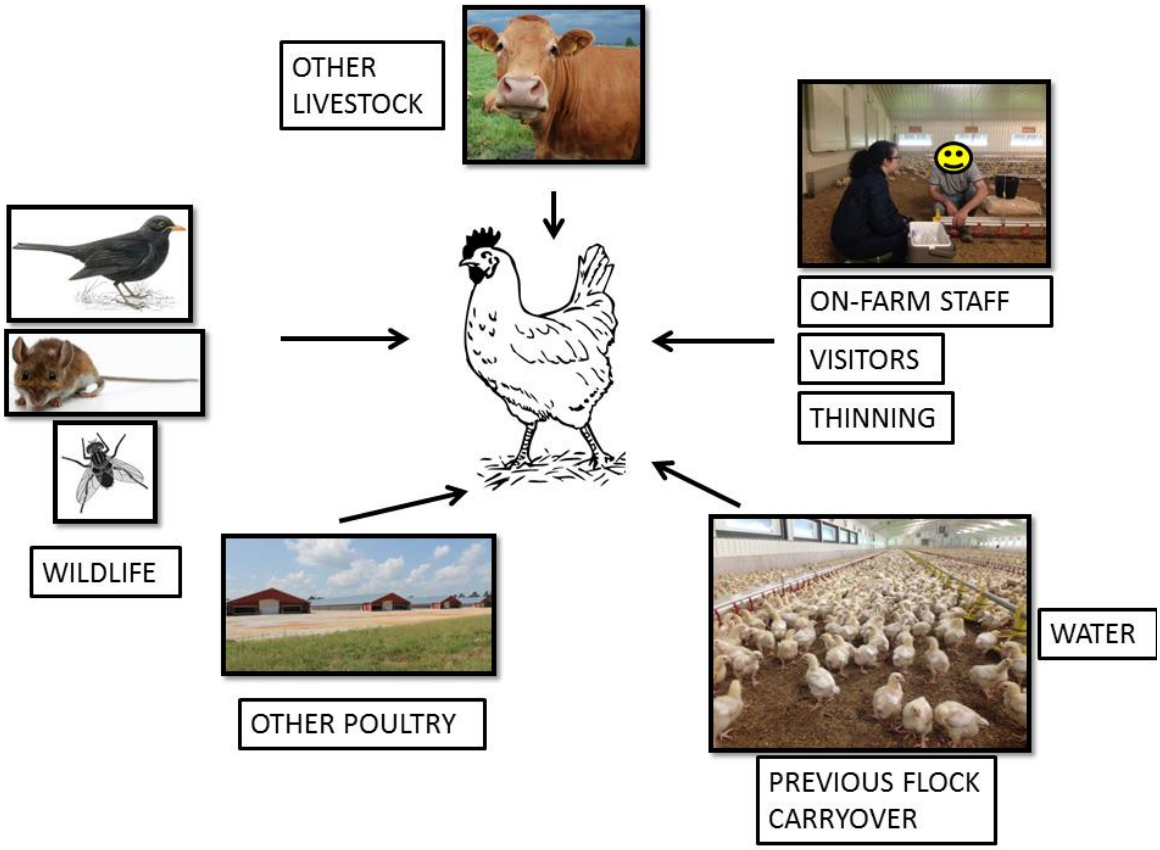


Figure 1.3. Possible sources of *Campylobacter* colonization. Summary of different transmission routes that have been identified in the literature.

The relevance of vertical transmission, either through a transovarian route before completion of shell deposition or by faecal contamination of the egg shell is still debated (Cox *et al.* 2012). However, the majority of evidence accumulated to date suggests that horizontal transmission from the farm environment account for most *Campylobacer* colonization events (Newell *et al.* 2011). In a systematic review of the literature 14 sources of on-farm contamination and 37 contributing factors were identified and ranked according to their relevance for broiler production in UK. The highest ranked sources

associated with *Campylobacter* infection were a depopulation event (thinning), cross-house transfer, on-farm staff and the presence of other livestock while the most important contributing factors were a depopulation schedule, the absence of a hygiene barrier and the presence of multiple broiler houses on-farm (Adkin 2006).

Thinning or partial depopulation applies to a situation in which a proportion of the birds of a flock is removed for slaughter, leaving the remaining birds to grow to normal clearance age. The practice, which allows a larger number of chickens to be placed initially and market flexibility is used by the majority of large-scale producers (Allen *et al.* 2008). A number of studies have identified thinning as an important risk factor for *Campylobacter* infection (Hald *et al.* 2000, Hald *et al.* 2001, Allen *et al.* 2008, Ridley *et al.* 2011, Lawes *et al.* 2012, Smith *et al.* 2016). This activity can imply catching teams visiting multiple farms on the same day and repeatedly entering broiler houses without consistent adherence to good hygiene practice, using difficult to disinfect equipment such as forklifts, trucks and catching crates (Smith *et al.* 2016). Although it has been suggested that the association might actually be due to the confounding effects of age and seasonality trends (Russa *et al.* 2005) others have found a significant effect even when adjusting for age (Lawes *et al.* 2012). More importantly there is epidemiological evidence (obtained by typing) of an association between flock infection and previous contamination of equipment, vehicles and personnel involved in thinning (Allen *et al.* 2008, Ridley *et al.* 2011). Transport crates seem to be a particular cause of concern as they can be contaminated with *Campylobacter* even after cleaning and disinfection and the strains recovered have included genotypes subsequently found in the relevant flocks at final clearance (Allen *et al.* 2008, Ridley *et al.* 2011) or following transport to the

slaughterhouse (Hansson *et al.* 2005). Another factor possibly playing a role is the increased level of stress as a result of the disturbance and feed and water deprivation caused by thinning which might make the remaining birds in the flock more susceptible to *Campylobacter* infection (Humphrey 2006, Bull *et al.* 2008). Higher *Campylobacter* concentrations in positive caecal samples at slaughter at final thin compared with first thin have been recorded (Smith *et al.* 2016). Although thinning is a significant risk factor it occurs towards the end of the rearing cycle and it cannot explain early events of colonization. For example, Allen *et al.* (2008) found that on 21 out of 51 farms studied the target flocks were already colonized at the time of thinning while and additional 37 flocks became colonized 2 to 6 days after thinning.

Not only the thinning crew but any visitors and on-farm staff present a major hazard for the introduction of *Campylobacter* into broiler houses. Battersby *et al.* (2016) showed that preventing direct contact between farm staff and broilers enhances biosecurity and prevents *Campylobacter* infection. Good biosecurity and hygienic measures have been related with a reduction in contamination rate but do not prevent flock colonization (van de Giessen *et al.* 1998, Gibbens *et al.* 2001, Allen *et al.* 2008, Ridley *et al.* 2011, Smith *et al.* 2016) which might be why others have not found them to influence *Campylobacter* status (Nather *et al.* 2009). Biosecurity measures vary on different farms. The Food Standards Agency (FSA) defines biosecurity as "the actions taken to both reduce the risk of infection entering the farm and to remove infection from the farm". FSA recommends i) implementing good hygiene routines for staff, equipment, areas around the poultry building and the actual poultry house previous to restocking; ii) restricting and controlling visits and vehicles; iii) taking measures for keeping away pets, other livestock and wild

birds and iv) controlling pests and insects. Of particular importance are the biosecurity measures related to footwear, either by using dedicated footwear for the poultry house or disinfecting footwear (i.e. boot-dips), in combination with a hygiene barrier that should be located immediately by the pedestrian door into the poultry house and separate it from the "dirty" outside environment (FSA 2006). The presence of a hygiene barrier has been identified as an effective measure for production of *Campylobacter*-free broilers (van de Giessen *et al.* 1998, Evans and Sayers 2000, Gibbens *et al.* 2001, Hald *et al.* 2001).

Poultry itself represents an infection risk for other poultry, as they shed large numbers of campylobacters in their faeces (caecal levels of contamination ranging from 10^4 to 10^7 cfu/g) (Cawthraw *et al.* 1996). Whyte *et al.* (2001) reported levels around 10^6 cfu/g of faeces at farm level by the end of the rearing cycle. Studies have reported extensive *Campylobacter* contamination on the farm surrounding when a flock is colonized by the pathogen (Battersby *et al.* 2016). Therefore is not surprising that an increasing number of broiler houses in a farm has also been identified as a risk factor (Guerin *et al.* 2007, McDowell *et al.* 2008). Other livestock, particularly cattle, also pose a higher risk of infection for the broilers (van de Giessen *et al.* 1998, Hald *et al.* 2000). In a systematic review, on-farm or adjacent to the farm cattle was the most frequently non-broiler animal identified with broiler-flock matching isolates, although directionality was not clear (Agunos *et al.* 2014). It has been suggested that flies could act as vectors and transmit *C. jejuni* from outside reservoirs to the inside of the broiler house (Hald *et al.* 2004). Hald *et al.* (2008) found low *Campylobacter* spp. carriage in flies but concluded that it may nevertheless constitute a risk given the large influx of flies into the broiler

houses observed in their study especially during the summer months, when the positive flies were found, and in environments with livestock faeces where the flies could pick up *Campylobacter*. The same group showed in another study that the use of fly screens in the ventilation vents of broiler houses caused a drop in prevalence of Campylobacter spp.-positive broiler flocks during summer and autumn from 51.4% in control houses to 15.4% in case houses (Hald *et al.* 2007).

Campylobacter spp. genotypes matching those found in the flock have been also reported in association with the DWS of broiler farms but on most occasions this is thought to follow rather than precede colonization of the flock (Gregory *et al.* 1997, Newell and Fearnley 2003, Agunos *et al.* 2014). However, there is evidence of strain carryover in subsequent flocks through *Campylobacter* persistence on the farm water supply (Pearson *et al.* 1993, Cokal *et al.* 2011). Messens *et al.* (2009) isolated the same genotype from the ditch water shortly before it was detected in the broilers. These studies indicate that the water supply might also represent a source of colonization.

The body of research on potential sources of chicken colonization has failed to identify one prominent source but rather seems to point towards a range of different possible sources co-existing as has been recognised by several authors, with horizontal transmission from the environment being the most prominent route (Conlan *et al.* 2007, Cox *et al.* 2012).

Research in microbial interactions has revealed that *C. jejuni* might benefit from permissive conditions within biofilms and by interacting with protozoa associated with the farm DWS (Trachoo *et al.* 2002, Snelling *et al.* 2005). However, the farm DWS has

remained an understudied source of colonization (Adkin *et al.* 2006). This will be investigated as a major aim of this thesis.

1.4 Survival strategies of *Campylobacter* spp. in the environment

Given the importance of horizontal transmission to chickens from the environment a key control strategy to reduce colonization is to interfere with this route. First, it is crucial to understand how and where in the environment *Campylobacter* survive. The bird, animal and human guts all pose challenges for *Campylobacter* spp. but it is believed that survival in the environment between hosts presents the greatest. The ubiquity of *Campylobacter* in spite of its fastidious nature indicates that this organism must have efficient survival mechanisms to overcome the adverse conditions encountered (Murphy *et al.* 2006). However, *C. jejuni* lack many classic stress response mechanisms found in other bacteria (Kassem and Rajashekara 2011) such as type III secretion systems (Young *et al.* 2007) and an RNA polymerase σ factor-mediated global stress response mechanism, mediated by RpoS in other Gram-negative bacteria such as *Salmonella*, *Escherichia coli* and *Vibrio* (Begley and Hill 2015). The following sections will explore different survival strategies that these bacteria use to be able to persist in the environment including entering a viable but nonculturable (VBNC) state, biofilm formation and interaction with other microorganism in mixed popultations.

1.4.1 Viable but nonculturable (VBNC) state

Campylobacter spp. can enter a viable but nonculturable (VBNC) state in response to unfavourable conditions (Murphy *et al.* 2006). This includes oxidative stress (Rollins and Colwell 1986, Klančnik *et al.* 2006) and low nutrient environments (Rollins and Colwell 1986, Pearson *et al.* 1993, Thomas *et al.* 2002) which are usually found in the

environment. High temperatures have also been related with a faster loss of culturability (Rollins and Colwell 1986, Thomas *et al.* 2002). When exposed to stress conditions cognate of those of natural aquatic environments it was observed that decline rates at 20 °C were between 2.2 and 18 times greater than those at 10 °C (Thomas *et al.* 2002). In another study, filter sterilized stream water held at 4 °C sustained populations of > 10^4 CFU/mL for up to 4 months while populations at 25 °C and 37 °C rapidly lost culturability within 28 and 10 days respectively (Rollins and Colwell 1986). A gradual change in morphology from spiral to coccoid cells has been associated with the transition to the VBNC state (Rollins and Colwell 1986, Lazaro *et al.* 1999, Thomas *et al.* 2002).

Different species and strains show different dynamics (decay rates and extent of population degeneration) regarding entering the VBNC state under the same conditions (Thomas *et al.* 2002) or even whether they enter it or not (Tholozan *et al.* 1999) in simulated aquatic environments. It has been suggested that this could explain why certain *C. jejuni* subtypes are more commonly associated with environmental reservoirs (Bronowski *et al.* 2014). Lazaro *et al.* (1999) showed that a human isolate of *C. jejuni* could survive in the VBNC state for up to 7 months in phosphate-buffer saline (PBS) based on several signs of viability (respiratory activity, DNA integrity, cellular integrity and two-dimensional protein profile), implicating the VBNC state in long-term survival in oligotrophic conditions.

In the VBNC state bacteria retain metabolic activity but are not capable of replication and cannot be cultured. Some studies have shown that VBNC forms of *C. jejuni* remain pathogenic and can be recovered by passage through animal hosts but not all the authors have consistently been able to replicate the results (Moore 2001). Given the

contradictory results about resuscitation, the epidemiological relevance of the VBNC state of *C. jejuni* cells is still a matter of controversy. However, observations of persistence of VBNC *C. jejuni* in water (Lazaro *et al.* 1999, Tholozan *et al.* 1999), ability to adhere to chicken carcasses at different temperatures (Jang *et al.* 2007), capability of chicken colonization (Stern *et al.* 1994) and adhesion to human intestinal cells (Patrone *et al.* 2013) suggest a role in the complex epidemiology of this microorganism.

1.4.2 Biofilm formation

A biofilm is a community of microorganisms attached to a biotic or abiotic substratum and encased in an extracellular organic polymeric matrix (Dunne 2002). The vast majority of bacteria in natural environments live in multi-species biofilm communities (Stoodley *et al.* 2002).

Several species of *Campylobacter* have been reported to form monospecies biofilms *in vitro* (Gunther and Chen 2009). Research has uncovered high inter and intraspecies diversity in the *Campylobacter* genus regarding attachment and biofilm formation on inert surfaces (Gunther and Chen 2009, Sulaeman *et al.* 2010, Nguyen *et al.* 2011). However, our knowledge is still very limited as most studies have focused on *C. jejuni* and mostly used static models (Joshua *et al.* 2006, Teh *et al.* 2010). Intraspecific variability in terms of adhesion capabilities ranging from no adhesion to strong adhesion has been reported in the attachment of 22 *C. jejuni* strains to polystyrene (Sulaeman *et al.* 2010) and 13 *C. jejuni* strains to stainless steel and glass (Nguyen *et al.* 2011). Interestingly in these same studies, *C. coli* strains were found to adhere significantly less than *C. jejuni* strains to polystyrene (Sulaeman *et al.* 2010) but more efficiently to stainless steel and glass (Nguyen *et al.* 2011). As the strains used in the two studies were mostly different,

the results could be explained by both interspecies and intraspecies variability and by the effect of the surface materials and illustrate the multifactorial nature of biofilm formation in *Campylobacter*. When the biofilm formation potential of other *Campylobacter* species has been evaluated, anaerobic strains (e.g. *C. concisus*) were better biofilm formers than microaerobic strains (e.g. *C. upsaliensis*) on glass, stainless steel and plastic (Gunther and Chen 2009).

Research suggests that biofilm formation might be promoted in *Campylobacter* in response to particular environmental conditions. It has been demonstrated that increase of temperature (from 4 °C to 55 °C) and contact time (from 1 minute to 240 minutes) result in an increment in the number of cells attached and the strength of attachment to stainless steel for a range of *C. jejuni* and *C. coli* strains (Nguyen *et al.* 2010). In another study Reeser *et al.* (2007) showed that biofilm formation by *C. jejuni* was higher at 37 °C than at 25 °C using a crystal violet biofilm assay. Nutrient availability also affects biofilm formation. Reeser *et al.* (2007) reported decreased biofilm formation in nutrient-rich media and Svensson *et al.* (2009) found that fumarate suppressed biofilm formation while doxycholate increased it. More importantly *C. jejuni* biofilm formation has been reported to be enhanced in aerobic conditions as a response to oxidative stress (Asakura *et al.* 2007, Reuter *et al.* 2010, Turonova *et al.* 2015). Other authors however have found that biofilm formation is enhanced in microaerobic conditions (Reeser *et al.* 2007).

The extracellular polymeric matrix of *C. jejuni* biofilms contains polysaccharides (McLennan *et al.* 2008) and DNA (Svensson *et al.* 2009, Brown *et al.* 2015a, Brown *et al.* 2015b). Svensson *et al.* (2014) suggested that the extracellular DNA is required for biofilm maturation but not for the initial attachment. It has been shown that the physiological

differences between biofilm and planktonic cells can impact virulence and colonization potential. Using growth in agar as a biofilm model, *C. jejuni* biofilm phenotype bacteria had altered attachment and invasion abilities *in vitro* using human epithelial cells (INT 407) and chicken embryo fibroblasts (DF1) and reduced colonization potential in young chickens (Hanning *et al.* 2009). It has been suggested that a biofilms may encompass different physiological stages, as cells with different degrees of motility seem to coexist in biofilms (Turonova *et al.* 2015). Spiral and coccoid cells have been observed to coexist in *C. jejuni* and *C. coli* monospecies biofilms (Nguyen *et al.* 2011). It has also been reported that *C. jejuni* biofilm cells transitioned to the VBNC state more quickly than planktonic cells (Magajna and Schraft 2015).

Molecular mechanisms underlying biofilm formation in *C. jejuni* have been studied. Transcriptomic and proteomic experiments have shown differences in expression patterns associated with immobilized growth of *C. jejuni* (Dykes *et al.* 2003, Kalmokoff *et al.* 2006, Sampathkumar *et al.* 2006). These studies have also indicated an upregulation of pathways involved in management of oxidative stress, also iron acquisition, membrane transport, catabolic functions and energy generation (Kalmokoff *et al.* 2006, Sampathkumar *et al.* 2006). Mutational studies have revealed a role for flagella and motility. Aflagellate mutants have consistently been reported to fail in biofilm formation (Joshua *et al.* 2006, Kalmokoff *et al.* 2006, Reeser *et al.* 2007, Reuter *et al.* 2010, Svensson *et al.* 2014). Moreover, a non-motile *C. jejuni* derivative also showed reduced biofilm formation but the biofilm phenotype could be partially rescued under aerobic conditions (Reuter *et al.* 2010). This is supported by the result of a proteomic study that found that the largest group of proteins with enhanced expression in biofilms was related to the

motility complex including several structural proteins and a chemotactic protein (Kalmokoff *et al.* 2006).

Mutational studies have also provided information about the regulatory networks that control biofilm formation and other shifts in physiology required to adaptation to the different environmental and host niches. The knock out mutant of the posttranscriptional regulator CsrA (involved in numerous processes including virulence, iron acquisition and quorum sensing) formed a very sparse biofilm, showed reduced motility and increased oxygen sensitivity (Fields and Thompson 2008). The authors suggested that it could act as an activator of biofilm formation via the regulation of motility and oxidative stress responses. The two-component regulatory system CprRS (*Campylobacter* planktonic growth regulation system) has also been involved in regulation of the biofilm-planktonic shift. The knock out mutant of the sensor kinase component of the system showed enhanced and accelerated biofilm formation and enhanced expression of oxidative stress tolerance proteins and a flagellar component (Svensson *et al.* 2009). Involvement of two-component regulatory systems in *C. jejuni* biofilm formation had been previously suggested by observations of defective biofilm formation upon inactivation of a putative phosphate transferase (Joshua *et al.* 2006) and overexpression of two-component regulators in a proteomics study (Kalmokoff *et al.* 2006). In another study it was found that a mutant defective in the stringent response, another survival mechanism important for virulence-related phenotypes and transmission-related phenotypes including aerobic survival, showed enhanced biofilm formation (McLennan *et al.* 2008). On the other hand, mutations affecting peptidoglycan structure are defective for motility and biofilm formation (French *et al.* 2005, Frirdich *et al.* 2012).

The implication of oxidative stress resistance regulators in biofilm formation have been debated. Alkyl hydroperoxide reductase AhpC was found to be upregulated in a proteomic study (Kalmokoff *et al.* 2006). However, a knock out mutant of this protein showed enhanced biofilm formation (Oh and Jeon 2014). The authors suggested that the phenotype might be mediated by accumulation of reactive oxygen species. Cj1556, another transcriptional regulator involved in the oxidative stress response has been linked to biofilm formation through the CprRS regulatory framework (Gundogdu *et al.* 2011). Peb4 is a virulence factor implicated in adhesion, invasion and colonization. The deletion of *Peb4* also impairs biofilm formation and represses flagellin components and transporter uptake system proteins (Asakura *et al.* 2007, Oh and Jeon 2014). In line with this Peb4 protein was found to be overexpressed in biofilms (Kalmokoff *et al.* 2006). The quorum sensing system is a population-dependent regulatory mechanism modulating both intra- and interspecies cell to cell communication and involving synthesis, secretion and detection of signalling molecules called autoinducers (AI) (Bassler 1999). *C. jejuni* possess a LuxS/autoinducer-2 (AI-2) system involved in motility, CDT expression, flagellar expression, oxidative stress response and animal colonization (Plummer 2012). Reeser *et al.* (2007) demonstrated that a *luxS* mutant strain had significantly reduced biofilm formation as measured with crystal violet staining. It is unclear if the defects in biofilm formation are a direct consequence of the lost of a functional quorum sensing system or rather a result of altered transcription of flaA (Plummer 2012).

It can be argued that our understanding of the process of biofilm formation at the molecular level is still in its infancy but research suggests that it is a tightly regulated process involving a number of different but interconnected pathways. In the same way

not a lot is known about the actual role of biofilm formation in *Campylobacter* survival in different environments. *C. jejuni* can form monospecies biofilms in various surfaces including some widely used in plumbing and food production. This includes stainless steel (Sanders *et al.* 2007, Gunther and Chen 2009), glass (Dykes *et al.* 2003, Joshua *et al.* 2006), nitrocellulose membranes (Kalmokoff *et al.* 2006) and a variety of plastic surfaces (Asakura *et al.* 2007, Reeser *et al.* 2007, Fields and Thompson 2008, Hanning *et al.* 2008). A role for biofilm formation in the survival of *Campylobacter* in food production and processing environments has been suggested as chicken meat exudate dramatically increased attachment and biofilm formation (Brown *et al.* 2014). However most studies proving biofilm formation have been conducted under conditions that do not realistically reflect those of food-related environments and this needs to be addressed before a role of biofilm formation in survival in these particular environments can be recognised (Teh *et al.* 2014).

There has been a suggestion that *C. jejuni* might survive within biofilms of drinking water systems (DWS) and this might act as a source for initial colonisation of chickens in commercial broiler houses (Pearson *et al.* 1993). Reeser *et al.* (2007) showed biofilm formation by *C. jejuni* in surfaces found in DWS including the hydrophobic plastics acrylonitrile butadiene styrene (ABS) and polyvinyl chloride (PVC) and to a lesser extent copper. Experiments conducted using static models do not reflect the hydrodynamics of real DWS. When biofilms were grown under moderate shaking (80 to 100 rpm) or under flow rates (10 to 300 10 mL h^{-1}) in a modified Robbins device, *C. jejuni* NCTC11168 could not form a biofilm (Joshua *et al.* 2006). In a different study Ica *et al.* (2012) found that pre-formed monoculture *C. jejuni* NCTC11168 biofilms could persist under low flow rates

(45 mL h^{-1}) but were unable to persist at higher flow rates (60 to 150 mL h^{-1}). Recently Culotti and Packman (2015) showed that *C. jejuni* RM 1121 could persist attached to a coverslip but not form a biofilm in a flow cell under oxic oligotrophic conditions under a low flow rate (12 mL h^{-1}). These results suggest that the ability of *C. jejuni* to form biofilms in DWS is dependent on flow rate but might represent an important survival mechanism.

1.4.3 Interactions with other microorganisms

A common strategy for bacterial survival in the environment is through interactions with other microorganisms. It has long been known that the presence of natural microbial communities affect *C. jejuni*'s ability to cope with aerobic oligotrophic environments (Pearson and Healing 1992, Buswell *et al.* 1998b). Nevertheless the specific interactions that take place are poorly characterised.

1.4.3.1 Interactions of *Campylobacter* with other bacteria

Multispecies biofilms are predominant in habitats with medical, industrial and ecological significance and the interactions in these communities can result in emergent functions and capabilities, such as increased tolerance to antimicrobial agents, compared to monocultures (Røder *et al.* 2016). A multi-species biofilm is an ecological niche that encompasses highly heterogeneous local microenvironments which can provide relatively localized homeostatic conditions (Hall-Stoodley and Stoodley 2005). For example, gradients of nutrients and oxygen, formed within a biofilm structure are formed encouraging spatially organised metabolic diversity (Kolenbrander *et al.* 2010). Importantly bacteria in biofilms show greater resistance to harsh conditions such as antimicrobial agents (Lewis 2001). Inter- and intraspecies interactions in biofilms can be

specific and the adhesion of one species to the surface can encourage or inhibit the incorporation of other species (Garrett *et al.* 2008).

C. jejuni encodes multiple transport systems and few biosynthetic pathways suggesting reliance on the uptake of external resources for survival (Bronowski *et al.* 2014). It has been suggested that *C. jejuni* are more likely secondary colonizers of biofilms in poultry farm environments (Hanning *et al.* 2008) and other food-related environments along the food chain (Teh *et al.* 2014). However, there have only been limited studies of the interaction of *Campylobacter* spp. with other species in polymicrobial conditions. Some authors have investigated whether *C. jejuni* is able to survive within multispecies biofilms in poultry related environments. When a *C. jejuni* chicken carcass isolate was incorporated into pre-formed biofilms by Gram-positive isolates from chicken farm nipple drinkers, 2 out of 3 resulted in enhanced *C. jejuni* attachment and survival compared to monoculture (Trachoo and Frank 2002, Trachoo *et al.* 2002). Hanning *et al.* (2008) inoculated *C. jejuni* 43431 into different biofilm populations isolated from poultry environments at different temperatures and demonstrated that *C. jejuni* could incorporate in some combinations resulting in an extended survival but not in others. In the study, *Pseudomonas* spp., *Straphylococcus* spp., *E. coli*, *Bacillus* spp., and *Flavobacterium* spp were identified as putative primary colonizers that might be able to support *C. jejuni* survival. In another study, *C. jeuni* RM1221 was also shown to attach to pre-formed biofilms from a mixed population of bacteria from a chicken carcass rinse remaining in a culturable state at lower temperatures (13 °C and 25 °C) and in a VBNC state at higher ones (37 °C and 42 °C) (Sanders *et al.* 2008).

Other authors have investigated multispecies interactions in biofilms in controlled mixed-microbial populations. Strong biofilm forming capabilities have been reported for combinations of *C. jejuni* with *Enterococcus faecalis* and/or *Staphylococcus simulans*, both originating from poultry (Teh *et al.* 2010). Ica *et al.* (2012) and Culotti and Packman (2015) demonstrated that *P. aeruginosa* and *C. jejuni* formed multispecies biofilms under flow conditions and that these biofilms consume most of the dissolved oxygen creating a localized microaerophilic environment. It has also been demonstrated that this allowed *C. jejuni* to remain in a culturable physiological state (Ica *et al.* 2012) and the multispecies biofilm environment appears to promote both persistence and growth of *C. jejuni* (Culotti and Packman 2015).

Campylobacter have also been detected within polymicrobial biofilms from aquatic environments. In one study *C. jejuni* was recovered from biofilms in sediments and on rocks in higher numbers than in the water column (Maal-Bared *et al.* 2012). Several *Campylobacter* species and strains showed approximately double survival time in tap water in the presence of the background autochthonous water microflora biofilms (Buswell *et al.* 1998a, Buswell *et al.* 1998b). In a broiler house, the biofilm formed on the surface of nipple drinkers contained *Campylobacter* (Zimmer *et al.* 2003). *C. jejuni* has also been reported to persist in biofilms formed with chlorinated water under laboratory conditions (Lehtola *et al.* 2006).

In some of the studies individual members of polymicrobial biofilm communities that supported *Campylobacter* were not identified. A number of studies however have identified *Pseudomonas* spp. in sampled polymicrobial communities (Sanders *et al.* 2007, Hanning *et al.* 2008, Sanders *et al.* 2008) and others have used them in experiments with

controlled communities in the laboratory (Trachoo and Frank 2002, Trachoo *et al.* 2002, Teh *et al.* 2010, Ica *et al.* 2012, Culotti and Packman 2015). Except for the study of Teh *et al.* (2010) which used a *P. aeruginosa* strain that was not a good biofilm former all the studies have found that *C. jejuni* survive better in the presence of a *Pseudomonas* spp. biofilm. Another study found that cell-free culture supernatants of *P. aeruginosa* and *P. fluorescens* increased biofilm formation in *C. jejuni* (Reeser *et al.* 2007), further supporting a role of *Pseudomonas* for enhancing *Campylobacter* survival. Hilbert *et al.* (2010) reported that *C. jejuni* has a longer survival time, despite oxygen stress, when cocultured with *P. fluorescens*, *P. putida*, *P. choloraphis* and *P. fragi* than when cocultured with type strains of meat-spoiling bacteria including *Proteus mirabilis*, *Citrobacter freundii*, *Micrococcus luteus* and *Enterococcus fecalis*. *Pseudomonas* are ubiquitous in the environment and therefore this interactions are likely to contribute to *Campylobacter* survival outside the host (Bronowski *et al.* 2014). However, research has suggested that interactions with other bacteria might also contribute, including unidentified Gram positive isolates (Trachoo and Frank 2002, Trachoo *et al.* 2002), *E. fecalis* (Sanders *et al.* 2007, Sanders *et al.* 2008, Teh *et al.* 2010), *Staphylococcus* spp. (Hanning *et al.* 2008, Teh *et al.* 2010), *Escherichia* spp. (Sanders *et al.* 2007, Hanning *et al.* 2008, Sanders *et al.* 2008), *Bacillus* (Sanders *et al.* 2007, Hanning *et al.* 2008), *Flavobacterium* (Hanning *et al.* 2008) and *Arcanobacterium pyogenes* (Reeser *et al.* 2007)among others.

Although bacteria have been the main focus of most biofilm research protozoa and fungi are also found along with bacteria in polymicrobial biofilms (Kinner and Curds 1987, Greub and Raoult 2004, Harriott and Noverr 2010).

1.4.3.2 Interactions with eukaryotic organisms

Zooplankton organisms including protozoa and certain benthic invertebrates can interact through symbiotic or parasitic associations with pathogenic bacteria representing a protection mechanism against hostile environmental conditions to the bacteria (Greub and Raoult 2004, Bichai *et al.* 2008). Although the evidence is still limited these organisms have the potential to protect pathogenic bacteria against water treatment in WDS (Bichai *et al.* 2008).

The exoskeleton surface of zooplankton provides a microhabitat that encourages bacterial attachment and colonization (Maugeri *et al.* 2004, Bichai *et al.* 2008). *Campylobacter* spp. have been found in association with large plankton (>200 µm) more frequently than free-living in the water column in seawater samples (Maugeri *et al.* 2004). However others have not found any internalized *Campylobacter* in zooplankton in samples collected in water treatment plants in the Netherlands and estimated that the occurrence of internalized *C. jejuni* was lower than one internalized bacterium in 10^5 zooplankton organisms (Bichai *et al.* 2011). These findings might reflect different concentrations of *C. jejuni* in the different habitats (sea vs. water treatment plants) or might just be due to the low number of samples in studies carried out. Grazing by the freshwater crustacean *Daphina carinata* strongly reduced the abundance of *C. jejuni* in natural water simulated environmental conditions (Schallenberg *et al.* 2005). Not only members of the zooplankton community but also the algae *Dianobryon sertularia* and *Euglena gracilis* have been observed to prolong *Campylobacter* spp. survival when in coculture at 10 °C (Axelsson-Olsson *et al.* 2010a).

Studies of the interactions of *Campylobacter* and eukaryotic microorganisms have been mostly carried out using free-living protozoa (FLP), particularly members of the *Acanthamoeba* genus (Vieira *et al.* 2015). FLP comprise heterotrophic protists which do not have an obligate parasitic life cycle (Vaerewijck *et al.* 2014). They are ubiquitous in aquatic and terrestrial ecosystems and also anthropogenic environments, including poultry farms and food-processing plants and there is a growing concern they might contribute to the maintenance of bacterial pathogens including *Campylobacter* spp. (Vaerewijck *et al.* 2014).

In an early study elimination of eukaryotic microorganisms such as protozoa from autochthonous aquatic communities through filtration resulted in higher recovery of culturable *C. jejuni* (Thomas *et al.* 1998). However further research has shown that in addition to the antagonistic role of grazing, protozoa can also benefit *Campylobacter* spp. There is a wealth of evidence that amoebae of the genus *Acanthamoeba* including *A. polyphaga*, *A. castellanii* and *A. rhysodes* support enhanced *Campylobacter* survival under aerobic conditions (Axelsson-Olsson *et al.* 2005, Snelling *et al.* 2005, Axelsson-Olsson *et al.* 2007, Snelling *et al.* 2008, Axelsson-Olsson *et al.* 2010a, Bare *et al.* 2010, Bui *et al.* 2012b, Olofsson *et al.* 2013). There have been some studies suggesting intracellular survival (Snelling *et al.* 2005, Snelling *et al.* 2008, Olofsson *et al.* 2013) while others have reported intracellular bacterial replication when using peptone-yeast-glucose medium and an incubation temperature of 37 °C (Axelsson-Olsson *et al.* 2005, Axelsson-Olsson *et al.* 2007, Axelsson-Olsson *et al.* 2010a). Axelsson-Olsson *et al.* (2007) reported that coculture with *A. polyphaga* could be used to enrich low concentrations of *Campylobacter* spp. In a follow-up study they were able to detect viable Campylobacters

using this method with higher sensitivity than by conventional culture on blood agar (Axelsson-Olsson *et al.* 2010a). However others have observed rapid degradation of intracellular *C. jejuni* and have suggested that amoebae enhances growth and survival of *C. jejuni* through depletion of dissolved oxygen in the coculture (Bui *et al.* 2012b). Dirks and Quinlan (2014) reported internalization but this did not always result in better recovery when compared to the *C. jejuni* controls in the absence of amoebae.

Hartmanella vermiformis and *Tetrahymena pyriformis* are other amoebae that have been shown to prolong *Campylobacter* survival under aerobic conditions (Snelling *et al.* 2005, Axelsson-Olsson *et al.* 2010a). If internalised bacteria are able to survive, amoebae not only protect *Campylobacter* from oxidative stress but also provide increased resistance to chlorine and to the disinfectant Virudine compared to extracellular bacteria (King *et al.* 1988, Snelling *et al.* 2005). In another study it was shown that *C. jejuni* internalized by *A. polyphaga* had increased tolerance to acidic environments compared to planktonic *C. jejuni* (Axelsson-Olsson *et al.* 2010b). Interestingly pre-exposure to environmental stresses (heat, starvation and osmotic stress) significantly reduced *C. jejuni* survival within *A. castellanii* while pre-exposure to oxidative stress had no effect on posterior survival (Bui *et al.* 2012a) suggesting that stress hampers the ability of *C. jejuni* to survive within protozoa.

Environmental conditions such as oxygen concentration, as well as temperature and species and strain of protozoa and *Campylobacter* play a crucial role in the interactions (Axelsson-Olsson *et al.* 2010a, Bare *et al.* 2010). Therefore the different experimental set-up might explain the discrepancies regarding replication and intracellular and extracellular survival reported by different studies.

Very little is known about interactions with other protozoa. In one study using *Colpoda* sp. approximately 8 *C. jejuni* cells per ciliate remained undigested after 5 h with some of the bacteria remaining viable and capable of regrowth, proving that the rate of ingestion exceeded the digestion rate (First *et al.* 2012). In spite of the scarcity of studies research suggests that interactions with eukaryotic microorganisms might prolong *Campylobacter* survival in environmental niches and be a factor behind its persistence.

1.5 Key questions and general hypotheses

In spite of the large body of research built around *Campylobacter* in the last decades a number of questions about their epidemiology remain unanswered. This study has worked under the hypothesis that a deeper understanding of the microorganisms that co-exist with *Campylobacter* in the environment and how they influence its survival is needed to tackle the unanswered epidemiological questions and control the spread of the pathogen.

A key epidemiological question is the source of on-farm chicken colonization. It has been hypothesised that the DWS might act as a reservoir for *Campylobacter* and be a source of colonization (Pearson *et al.* 1993). However the protozoan and bacterial communities in the water distribution system of commercial farms remain poorly characterised (Snelling *et al.* 2006, Hanning *et al.* 2008). The first aim of this study was to profile the microbial communities in a commercial broiler farm and their changes across the rearing cycle in parallel to the detection of *Campylobacter* spp.

Campylobacteriosis is a global public health concern but in developing countries its epidemiology remains poorly understood and in most areas the levels of chicken

colonization remain to be assessed (Coker *et al.* 2002). A second aim of the study was to investigate the presence of *Campylobacter* spp. in semi-intensive chicken farms in W. Uganda.

Little is known about how these fastidious bacteria survive outside their hosts. It has been suggested that *Campylobacter* survive in the environment by interacting with other members of the microbial communities (Buswell *et al.* 1998b). *Pseudomonas* spp. and *Acanthamoeba* have been shown to promote their survival and protect them from a range of stressful conditions (Axelsson-Olsson *et al.* 2010b, Hilbert *et al.* 2010). The final aim of this study was to build on previous knowledge by investigating the effects of a broad range of *Pseudomonas* spp. and strains in *C. jejuni* persistence and assessing whether *C. jejuni* can be internalized in *A. polyphaga*.

Chapter 2

Materials and methods

2.1 Strains and culture conditions

2.1.1 Bacterial culture

All bacterial strains used in this study are listed in table 2.1. Pure cultures of *Campylobacter jejuni* (genome sequenced strains 11168 and 81176) and *H. pullorum* isolates were grown on Columbia agar (Oxoid) supplemented with 5% vol/vol horse blood (TCS Biosciences) or *Campylobacter* Blood-free selective agar base (Oxoid) containing CCDA selective supplement SR 155E (Oxoid) under microaerobic conditions (10% vol/vol CO_2; 5% vol/vol O_2; 85% vol/vol N_2) using a Modular Atmosphere Controlled System (Don Whitley Scientific) at 37 or 42 ˚C. Pure cultures of *C. jejuni* were kindly provided by Craig Winstanley- University of Liverpool and Dennis Linton- University of Manchester.

Pseudomonas aeruginosa PAO1 was grown using Columbia agar base at 25 or 37 ˚C. Other *Pseudomonas* spp. isolates and *Pseudomonas* ssp. strains were grown on Columbia agar, Mueller-Hinton Broth (MHB-Oxoid) or Brain Heart Infusion (BHI-Oxoid) at 25 ˚C. *Pseudomonas* ssp. strains were kindly provided by Craig Winstanley, University of Liverpool and Rob Jackson, University of Reading. *Escherichia coli* K-12 JM103 was grown in Luria-Bertani (LB) broth (LabM) at 37 ˚C with shaking. The strain was kindly provided by the University of Liverpool.

Table 2.1 Bacterial strains.

Species	Strains	Source/Reference
C. jejuni	11168	Parkhill *et al.* (2000)
	81176	Korlath *et al.* (1985)
H. pullorum	1, 2	This study
P. aeruginosa	PAO1	Holloway (1955)
P. fluorescens	WCS 365	University of Reading
	113	
	ATCC 17400	
	Pf-5	
	Pf0-1	
	1-7	This study
P. cichori	907	University of Reading
P. avellanaev	48	University of Reading
P. syringae	pv. *tomato* DC300	University of Reading
	pv. *phaseolicola* 1448A	
	pv. *coriandricola*	
	pv. *glycinea* 49	
	pv. *glycinea* 4180	
	pv. *antirrini* 152E	
	pv. *Lachrymans*	
P. putida	K72440	University of Reading
P. entomophila	L48	University of Reading
P. marginalis	247	University of Reading
P. cornigata	2445	University of Reading
P. tolaasii	2192T	University of Reading
Pseudomonas spp.	Week 1-7	This study
E. coli	K-12 JM103	Hanahan (1983)

2.1.2. Amoebae culture

Acanthamoeba polyphaga was provided in proteose-peptone-glucose medium (PPG) (Glucose 100 mM, NaCl 2.1 mM, $MgSO_4 7H_2O$ 16 µM, $CaCl_2$ 27 µM, KH_2PO_4 100 µM, $Na_2HPO_4 2H_2O$ 1.3 mM and Proteose Peptone (BD) 15g/L) from The University of Strathclyde. Fresh PPG was prepared and amoebae were maintained in 25 mL tissue culture flasks (Fisher Scientific) at 30 °C and sub-cultured when confluent growth was observed at 10X magnification with an Olympus CK Tokyo inverted microscope.

2.1.3. Enrichment and Selective media for isolation of microorganisms.

Table 2.2 details the enrichment and selective media used to isolate *Campylobacter; Pseudomonas* and amoeba species from farm and water samples. Putative positive colonies were picked and sub-cultured onto fresh selective agar then confirmed using standard Gram staining (section 2.3.6) and PCR methods (section 2.4.2).

Table 2.2: Enrichment and Selective media for microbial isolation.

Species	Media composition
***Campylobacter* spp.**	***Campylobacter* enrichment broth (CEB):** Brain Heart Infusion (BHI) broth (Oxoid) supplemented with 5% vol/vol lysed horse blood (TCS Biosciences); *Campylobacter* growth supplement X115 (Lab M Ltd) and Modified Preston *Campylobacter* supplement X114 (Lab M Ltd). ***Campylobacter* selective media (CSM):** *Campylobacter* Blood-free selective agar base (Oxoid) plus CCDA selective supplement SR 155E (Oxoid).
Pseudomonas* spp.** ***Pseudomonas aeruginosa	**Non-selective enrichment:** Buffered peptone water (BPW) (Oxoid). ***Pseudomonas* Selective Agar:** *Pseudomonas* Agar Base (Oxoid) supplemented with modified CFC (Cephalothin, Fucidin, Cetrimide) supplement X108 (Lab M Ltd). ***P. aeruginosa* Selective Agar:** *Pseudomonas* Agar Base (Oxoid) containing CN Supplement (SR0102) (Oxoid).
Amoeba	Non-nutrient agar using 1.5% wt/vol bacteriological agar (Oxoid).

All media were prepared following the manufacturer's instructions.

2.2. Sample sites and sample collection

2.2.1. Large-scale model drinking water distribution system (DWDS)

Polyethylene coupons (20 x 5.5 mm) colonised by biofilms from a pipe water system simulator were kindly provided by Dr. Isabel Douterelo from the University of Sheffield. The large-scale model DWDS was designed to assess the effect of flow rates and temperature on the formation of biofilms within drinking water pipe networks that supply large urban areas (Douterelo *et al.* 2013). Briefly, the system consisted of three recirculating loops of polyethylene pipe (200 m-long with a total volume of 4.5m^3). The system was fed by the local mains water network and was designed to incorporate many coupons, which formed removable elements of the pipes themselves. The coupons were designed for close alignment with the internal pipe surface to minimize turbulence (Deines *et al.* 2010). Coupons were collected, by Dr Douterelo at different times during the running of her DWDS model experiments, at The University of Sheffield. Each coupon was suspended in water from the system (5 ml) and transported to the University of Salford, to be stored at 4°C until processing.

2.2.2. Commercial broiler farm in the UK

2.2.2.1. Farm description

A large commercial broiler farm in UK was studied intensively. The farm drinking water system (DWS) comprised two mains water storage tanks made of plastic holding 10,000 L each. Water came from the mains DWDS to the first tank (1) and from this to the second tank (2) (Figure 2.1 A). The tanks supplied five different broiler houses (approximately 1,206 m^2 each) through a pipe that was fitted with a UV-CS110 light treatment machine (Silverline UK limited) with capacity to process up to 72 L/min (103,680 L in a day) which

was over the peak daily consumption (30,000 L per day). This study focused on broiler house number 5 that typically housed around 24,000 chickens. The main supply pipe connected to an anteroom in each broiler house; where there was a tap for sampling (Figure 2.1 B). Before the anteroom there was a disinfection bath for boots. The anteroom housed the individual broiler house control unit (temperature; light period; bird weight; water consumption). A biosecurity barrier was in place for boot changes for the farmer or boot socks placement for visitors. Other biosecurity measures for visitors included dedicated single use overclothing. Inside broiler house 5, the main water pipe divided into six 64 m blind-ended pipelines (Figure 2.1 D 1-6), each with a tap at the far end for flushing out pipes at the end of each production cycle (Figure 2.1 D). Stainless steel nipple drinkers were installed along the length of each line (Figure 2.1 E). The poultry farm was visited once per week in seven consecutive weeks covering a whole rearing cycle for a broiler chicken flock.

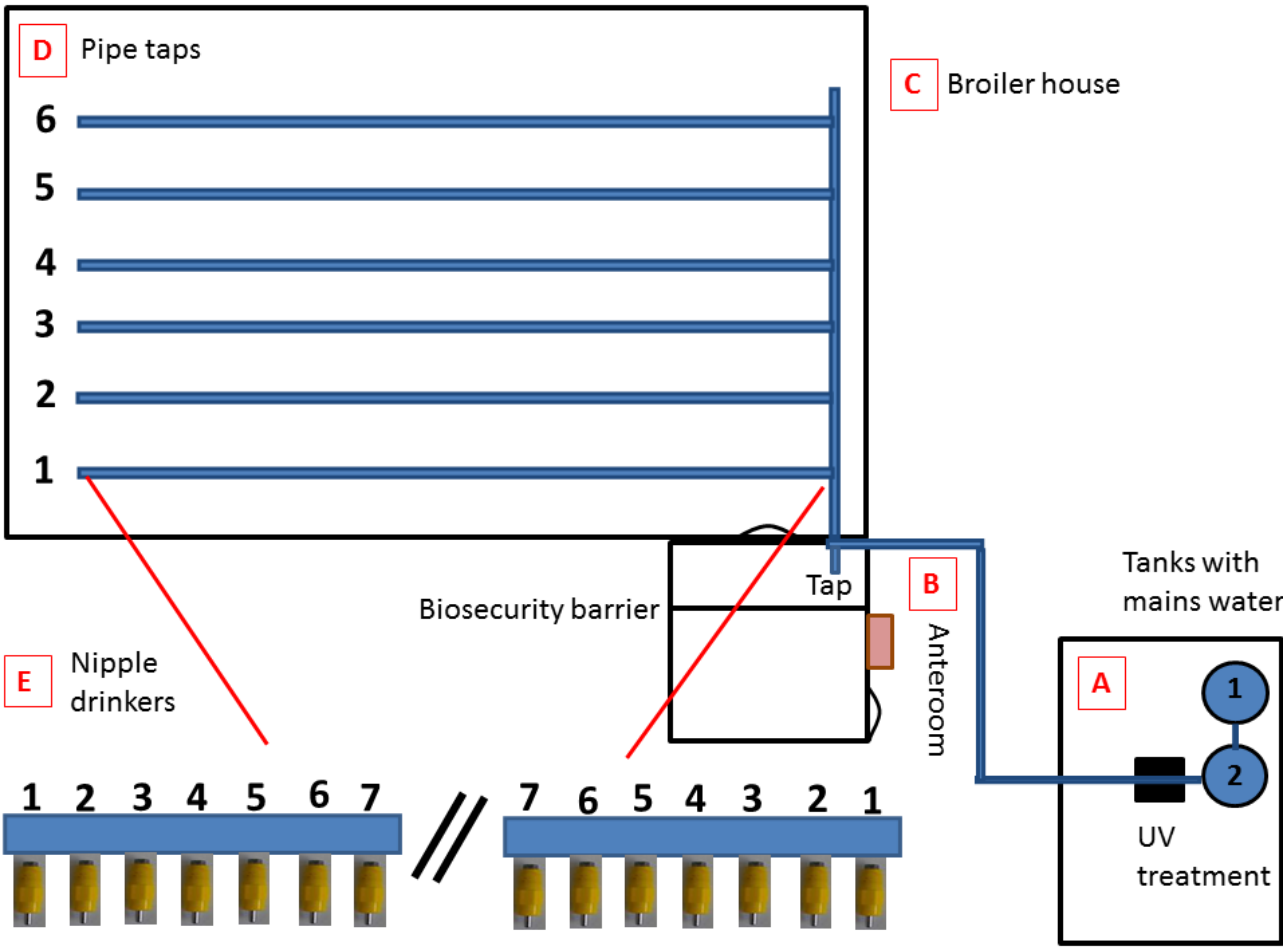


Figure 2.1 Schematic representation of the farm. A) Mains water tanks showing UV treatment installed along the pipe. B) Anteroom showing the disinfection tank for boots, the biosecurity barrier and the tap where the anteroom bulk water was sampled. C) Broiler house showing the six different pipes. D) Pipe taps at the end of each pipeline where the shed bulk water was sampled. E) Amplification of the beginning and end of pipe one showing the nipple drinkers. The numbers indicate the week in which each one was sampled.

2.2.2.2. Biofilm and nipple drinker sampling

Weekly sampling of the biofilms associated with the drinking water system was carried out in the same broiler-house pipeline (line 1 in figure 2.1 D). Biofilms were sampled from 1) nipple drinkers and 2) the inside of the pipeline.

1) Two nipple drinkers (N), one from each end of line 1 (Figure 2.1 E), were collected in Sodium Chloride-Tris-EDTA (STE) 1X pH8 buffer (Fisher Scientific) each week of a 7-week production cycle. On removal, each N was immediately replaced with a new one. Seven adjacent Ns were used to avoid sampling of those that had already been replaced. The external (Ne) and internal (Ni) components of each nipple drinker were collected separately (Fig 2.2). Ne components were suspended in 3.5 mL of STE buffer in a sterile 7 mL Bijou container (StarLab). Ni components were suspended in 900 µL of STE buffer in 2 mL "Crystal Clear" microcentrifuge tubes (StarLab). Samples were transported to the laboratory and processed within 24 h (section 2.4.1.2.1).

2) The second method of sampling biofilms was to swab inside the pipeline through the hole left upon removal of the nipple drinker, using a sterile CLASSIC Q Swab (COPAN). The swab was placed in a 2 mL "Crystal Clear" microcentrifuge tubes (StarLab) containing 270 µL of ATL buffer and 30 µL of Proteinase K from the DNeasy Blood and Tissue kit (250) (Qiagen). Samples were transported to the laboratory and kept at room temperature until DNA extraction (section 2.4.1.2.3).

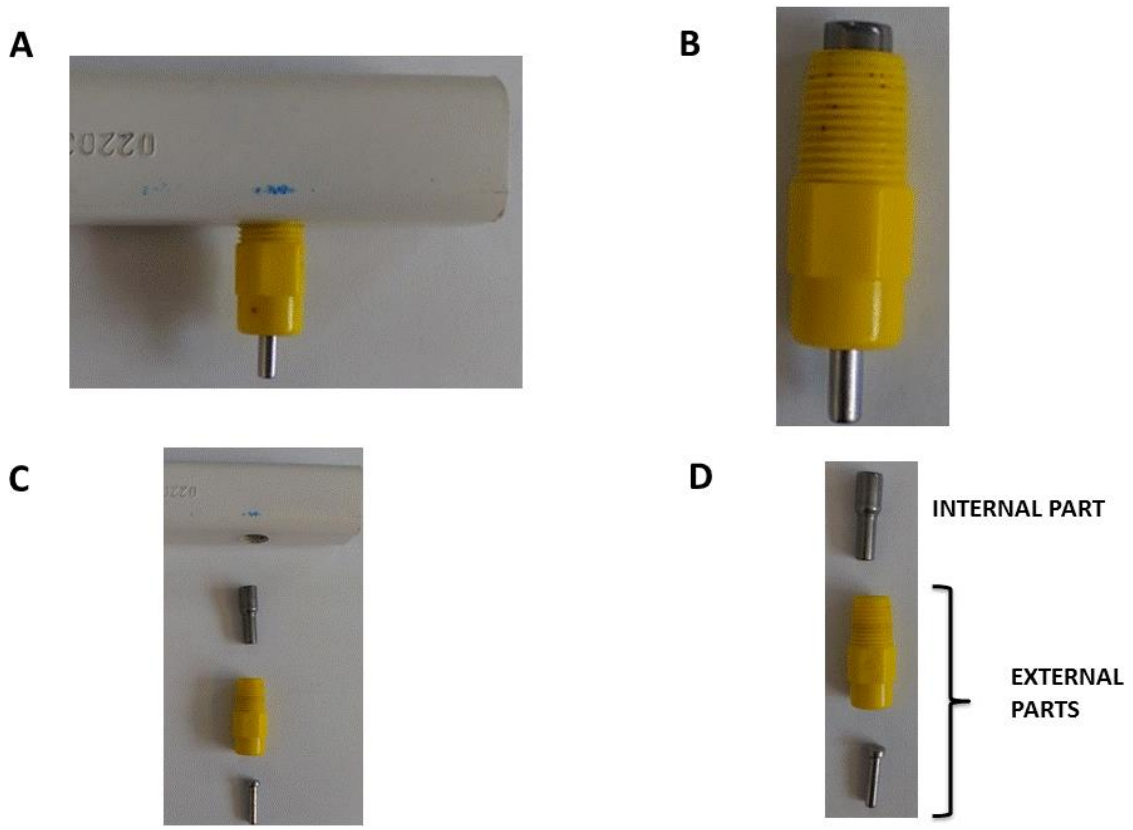


Figure 2.2 Nipple drinker composition. (A) indicating position on the water pipe; (B) showing whole removed nipple drinker; (C) detailing the different parts of the removed nipple drinker ; (D) indicating internal and external parts.

2.2.2.3. Bulk water sampling

One litre Nalgene bottles (Fisher Scientific) were rinsed thoroughly with distilled water and allowed to dry (2 h) then wrapped in foil and sterilised by autoclaving before each sampling. Five litres of water were collected in the Nalgene bottles each week from i) the tap in the anteroom before the pipeline enters the broiler house (Fig 1 B); ii) The tap at the far end of five pipelines inside broiler house 5 (Fig 1 D pipes 1-5). Samples were transported to the laboratory and stored at 4 °C until processing as soon as possible and

always within 24 h. The rest of the water samples (1 L) were used to measure pH using a calibrated pH meter (Hanna) and for *Pseudomonas* spp. isolation (section 2.3.5).

2.2.2.4. Sample collection for *Campylobacter* isolation

A range of other samples were collected from broiler house 5; including faecal samples, swabs from the baskets underneath the nipple drinkers sampled (x2) and boot socks (over-shoes worn as a biosecurity measure during sampling visits). Faecal samples were added to *Campylobacter* enrichment broth (CEB) (Table 2) in sterile 50 mL falcon tubes (Scientific Laboratory Supplies). Swabs from the baskets underneath the nipple drinkers were immediately suspended in 5 mL CEB. Boot socks (x4) were removed on leaving the broiler house and sealed in individual zip-lock plastic bags (ASDA).

2.2.3. Small-scale semi-intensive chicken farms in Uganda

Three different farms, around the Kabarole district of Western Uganda were visited on the same day in May 2016. Faecal samples were collected in 20 mL universal tubes (Starlab), transported to the local laboratory in Mountains of the Moon University, Fort Portal, and processed within the same day for isolation of *Campylobacter* spp. by culture (section 2.3.3). A total of 50 samples were collected. Before transporting the samples to the UK for further molecular analysis, chicken faeces samples were stored in ethanol acquired locally for inactivation of infectious material. Once in the laboratory in the UK, samples were stored at 4 °C until DNA extraction (section 2.4.1.4). Farmers were also informally interviewed on several aspects of management practices, number of chickens and current health status.

2.3 Culture-based screening and isolation of microorganisms from water and farm samples

2.3.1 Screening for the presence of viable *Campylobacter* species in the large model DWDS

To detect the presence of any viable *Campylobacter spp* in the large model DWDS, coupons were placed in BPW at 25 ˚C for 3 and 8 days, then added (2 ml) to CEB (15 ml) and incubated at 42 ˚C under microaerobic conditions. After 2, 4 and 6 days of enrichment 100 µL were plated on CSM and incubated microaerobically at 42^{o}C for 48 h.

2.3.2 Screening for the presence of viable *Campylobacter* species on the commercial broiler farm in UK

Faecal material, swabs of the nipple drinker baskets, boot socks and bulk water samples from the UK commercial broiler study farm, were screened for the presence of viable culturable *Campylobacter* spp. using *Campylobacter* enrichment broth (CEB) and *Campylobacter* selective media (CSM). Negative controls for each different sample type were included and all samples were incubated at 42 °C under microaerobic conditions (10% CO_2; 5% O_2; 85% N_2) using a Modular Atmosphere Controlled System (Don Whitley Scientific) for 24-72 h. Enriched samples were streaked on to CSM and incubated further under the same conditions. Plates were examined daily for growth. Putative *Campylobacter* colonies, isolated on CSM, were picked and propagated on Columbia blood agar plates and confirmed using standard Grams staining (section 2.3.6) and species or strain-specific PCR (section 2.4.2). Approximately 1 mL of each enrichment culture was centrifuged at 14,000 rpm for 1 minute using an accuSpin Micro 17 (Fisher

Scientific) and the pellet was stored at -20 ºC for DNA extraction (section 2.4.1.3) and qPCR (section 2.4.3).

2.3.3 Screening for the presence of viable *Campylobacter* species on the small-scale semi-intensive chicken farms in Uganda

Faecal samples collected from Ugandan chicken farms were processed in the laboratory facilities at Mountains of the Moon University, Fort Portal. A loopful of each faecal sample was plated in *Campylobacter* selective media. Samples 31 to 34 (Farm 2 shed 3) and 44 to 50 (Farm 3 shed 2) were combined independently and plated in one plate for each group as high and low *Campylobacter* load respectively was expected. Plates were incubated under microaerobic conditions using 2.5 L anaerobic jars (Anaerocult) with the CampyGen gas generating system (Oxoid) at 37 °C to 42 °C, 48h. Presumptive *Campylobacter* colonies (small, grey-ish) were sub-cultured and boil-preps were prepared for transport and identity confirmation through PCR at The University of Salford (section 2.4.2). All the colonies from the same plate were pooled in the same tube for boil prep preparation.

2.3.4. Isolation of *Pseudomonas* species from the large model DWDS

Non-selective enrichment was performed to isolate *Pseudomonas spp* from the large model DWDS. A coupon removed from the system 84 days after the simulation started was incubated in 30 mL of BPW at 25˚C for 3, 4 and 9 days. Three-day enriched suspensions were spread-inoculated (100 µl) onto *Pseudomonas* Selective Agar

supplemented with CN Supplement (SR0102) selective for *P. aeruginosa* (Table 2.2). Four and nine-day enriched suspensions were spread-inoculated (100 µl) into *Pseudomonas* Selective Agar containing X108 selective supplement selective for *Pseudomonas* spp. (Table 2.2). Plates were incubated at 25 ^{o}C aerobically for 48 hours. Samples were streak-plated to isolate single colonies that were sub-cultured for confirmation. Stocks were prepared as described in section 2.3.7. *Pseudomonas spp.* identity was confirmed using genus-specific PCR (section 2.4.2) and partial 16S rDNA sequencing (section 2.4.5). *Pseudomonas* isolates were typed by random amplified polymorphic DNA (RAPD) PCR using primer 272 (section 2.4.2).

2.3.5. Isolation of *Pseudomonas* species from the DWDS of a large commercial broiler farm

Pseudomonas spp. were isolated from farm samples using *Pseudomonas* selective media (Table 2.2) Water (150 ml) from the farm pipelines inside and outside of broiler house 5 were filtered as described in section 2.2.2.3. Half of the filter was placed in *Pseudomonas* selective media. Plates were incubated at 25 °C for up to three days. When growth was observed, culture material was streak-plated on *Pseudomonas* selective media or Columbia Agar to obtain single colonies. Stocks were prepared as described in section 2.3.7.

2.3.6 Gram Staining

Gram staining protocol was adapted from the original method ((Gram 1884; cited in Bartholomew and Mittwer (1952)). A heat-fixed smear was prepared by placing a loopful

of bacteria on a drop of water on a glass microscope slide and passing the slide through the flame of a Bunsen burner. The slide was flooded with crystal violet (Pro-Lab Diagnostics) for 30 seconds, rinsed with water and then flooded with Gram's iodine (Pro-Lab Diagnostics) for 30 seconds. Decolourization was performed by flooding the slide with ethanol (--90%, BDH) for 10 seconds and washing thoroughly with water. Safranin (Pro-Lab Diagnostics) was used as a counter-stain and was left for 1 minute for *Pseudomonas* and for up to 15 minutes for *Campylobacter*. Colony morphologies were observed using a microscope (Leica DM500) under oil immersion at x100 magnification.

2.3.7 Preparation of stocks

Stocks for presumptive *Campylobacter* isolates were prepared by scraping as much culture material as possible from a fresh blood agar plate (24h-48h) and inoculating 1 mL of BHI broth (Oxoid) supplemented with 20% vol/vol glycerol (VWR chemicals) into a cryovial (Nunc).

Freezer stocks from presumptive *Pseudomonas* isolates were prepared by inoculating 2-3 bacterial colonies into MHB (Oxoid). After overnight incubation at 25 °C, 200 µL were transferred into 5 mL of MHB for few hours until the culture was in mid exponential phase (Optical density (OD) at 600 nm= 0.5) as measured with a spectrophotometer (Campsec m330). Cultures were then centrifuged (15 minutes, 700xg) using an accuSpin Micro17 (Fisher Scientific) centrifuge. The supernatant was discarded and the pellet re-suspended in 1 mL of LB supplemented with 20% vol/vol glycerol into a cryovial (Nunc).

Freezer stocks were stored at -80°C.

2.3.8 Detection of Amoebae from a large DWDS model

Amoebae were detected by placing coupons from the DWDS model onto a lawn of killed *E. coli* and observing amoeba proliferation and motility. *E. coli* K-12 strain JM103 was grown in LB at 37˚C, with shaking, (18-24 h) and spread-inoculated onto non-nutrient agar plates (Table 2.2). *E. coli* cells were killed by exposing to UV light (ʎ=365 nm) for 30 min using a transilluminator (Uvitec Cambridge). A non-exposed *E. coli* lawn was used as a control. To verify complete killing, a loopful of UV-exposed and control *E. coli* cultures was transferred to LB and incubated at 37 °C, with shaking overnight. 84- days old coupons were then placed onto the UV-killed bacteria plate and incubated at 30˚C. An inverted microscope (Olympus CK Tokyo) was used to detect the presence of amoebae around the edges of the coupon daily. When amoebae were observed pieces of agar were cut and placed in PPG or PPG supplemented with antibiotics (gentamicin 200 µg/mL or gentamicin 50 µg/mL, penicillin 60 µg/mL and Streptomycin 100 µg/mL) to purify the amoebae. All antibiotics were purchased from Sigma.

2.4. Molecular methods for detection / identification

2.4.1. DNA extractions

2.4.1.1. DNA extraction from bacterial isolates

DNA was extracted from 1 mL overnight broth cultures (*Pseudomonas* spp.) or Columbia blood agar plates (*Campylobacter* and *H. pullorum*) using the Isolate II Genomic DNA kit (Bioline) following the manufacture instructions.

2.4.1.2. DNA extraction from farm samples for 16S and 18S community profiling

DNA was extracted from a range of samples collected from broiler farms. The nature of different sample types dictated some variation in the methods used to process samples and extract DNA.

2.4.1.2.1. Nipple drinker processing

Biofilms were detached from individual nipple-drinker components by subjecting each sample to 7 minutes of ultrasonication (amplitude 80%; cycle 0.9) using an UP50H ultrasonic processor (Hielscher) in a laminar flow cabinet (Envair UK Ltd.). Samples were kept on ice during sonication. Negative controls (bijou and microcentrifuge tube containing buffer alone) were included and processed before each sample. The sonicator probe was cleaned with 100% ethanol before each treatment. After sonication duplicate samples were pooled together and suspensions were filtered using a disposable 150 mL Bottle Top Filter unit, containing nitrocellulose filter membrane with 0.22 µm pore size (Corning, Fisher Scientific) and a benchtop vacuum tap. Following filtration the filter (dimensions 7cm x 7cm) was cut and separated from the plastic filter unit and divided in two parts using a disposable sterile scalpel (Swann-Morton) and sterile disposable forceps (VWR International) under aseptic conditions. Both half of the filters were placed in a 2 mL “Crystal Clear” microcentrifuge tubes (StarLab) containing 270 µL of ATL buffer and 30 µL of Proteinase K from the DNeasy Blood and Tissue kit (250) (Qiagen).

2.4.1.2.2. Bulk water processing

Water samples (4 L) were filtered using a disposable 150 mL Bottle Top Filter unit, containing nitrocellulose filter membrane with 0.22 µm pore size (Corning, Fisher Scientific) and a benchtop vacuum tap. Water was poured in the filter unit in a laminar flow cabinet (Envair UK Ltd.). Following filtration the filter (dimensions 7cm x 7cm) was cut and separated from the plastic filter unit and divided in two parts using a disposable sterile scalpel (Swann-Morton) and sterile disposable forceps (VWR International) under aseptic conditions. Half of the filter was placed in 30 mL universal container with 5 mL CEB for *Campylobacter* isolation (section 2.3.2) and the other half was placed in a 2 mL "Crystal Clear" microcentrifuge tubes (StarLab) containing 270 µL of ATL buffer and 30 µL of Proteinase K from the DNeasy Blood and Tissue kit (250) (Qiagen).

2.4.1.2.3. DNA extraction

Filter and swab samples were stored in ATL buffer with Proteinase K from the DNeasy Blood and Tissue kit (Qiagen). Remaining biomass was removed mechanically by sonication on ice (10 min, 50/60 Hz) using a sonication bath (Sonicor). Then they were kept at room temperature until all the samples were collected and all the DNA extractions from the different weeks were carried out at the same time in a category two hood C-Floow 2002 (Envair (UK) Ltd.). Five negative controls were prepared after the last week of sampling and include 1) a sterile swab; 2) a sterile half a filter; 3) the negative controls for sonication in microfuge from the seven weeks tubes pooled together; 4) the negative controls for sonication in sterile polypropylene Bijou containers pooled together; and 5) a negative control just for the reagents in the kit.

Samples were vortexed for 20 seconds and incubated at 56 ˚C on a water bath for 24 h. DNA extraction was then performed using the manufacturer's instructions for the DNeasy Blood & Tissue Kit (Qiagen) with the following modifications: incubation at 70 ˚C instead of 56 ˚C after addition of AL buffer and elution with 50 µL of AE buffer pre-heated at 70 ˚C. A second elution took place leaving the AE buffer for 30 minutes before the centrifugation.

2.4.1.3. DNA extraction from frozen *Campylobacter* enrichment samples

DNA was extracted from frozen enrichment samples using the FastDNA SPIN Kit for Soil (MP Biomedical). Swab samples (x2 for each week) were pooled and extracted together and when there was a lot of material rather than a pellet 0.5 g were taken for the extractions. DNA was extracted following manufacturer instructions with minor modifications. The amount of Sodium Phosphate Buffer was reduced to avoid overfilling the Lysis Matrix. The TissueLyser II(Qiagen) (30 Hz for 1 minute) was used instead of the FastPrep Homogenizer. In step 5 from the manufacturer's protocol the centrifugation was extended to 15 minutes. Before elution, samples were incubated for 5 min at 55 °C in a water bath to improve yield following the manual suggestions. Elution was carried out with 100 µL of DES water. The rest of the samples were extracted following the same protocol with the only exception of pooling the 6 other samples from water outside the shed in the same column after lysis. Negative controls from each sample group were separately pooled and extracted. A negative control for the kit was included in both DNA extraction rounds.

2.4.1.4. DNA extraction from faeces

DNA was extracted from faecal samples (500 mg), collected from a UK commercial broiler farm, using the FastDNA Spin Kit for Faeces (MP Biomedical). The manufacturer's instructions were followed with minor modifications. The TissueLyser II (Qiagen) was used for 2 x 1 min at 30 Hz to lyse microbial cells.

Faecal samples, collected from Ugandan chicken farms (500 mg), were stored in 70% vol/vol ethanol to inactivate any pathogenic agent before transport. DNA was extracted from ~150g faeces using BashingBead Lysis Tubes (Bioline). Cells were lysed using a Tissue Lyser II (Qiagen) for 5 min at 27 KHz then DNA was extracted using the Isolate Faecal DNA Kit (Bioline).

2.4.1.5 DNA Quality Analysis

The purity and efficiency of DNA isolation was assessed using a NanoDrop 2000 Spectrophotometer (Thermo Scientific) or by agarose gel electrophoresis (section 2.4.4).

2.4.2. PCR

2.4.2.1. Preparation of Boil preps

Crude DNA templates for PCR were obtained by suspending a bacterial colony in sterile distilled (sd) H_2O (50 µl) and heating (100 ˚C) for 10 minutes. These boil preps were used immediately or stored at -20 ˚C.

2.4.2.2. PCR primers and conditions

Standard PCR was used to detect the presence, or confirm the identity, of several key bacterial species in a range of samples. The assays were carried out using MyTaq^{TM} Red Mix (1x) in a final volume of 25 µL containing 0.25 µM of each primer and 1 µL of template DNA when template was from pure DNA and either 1 µL or 5 µL when working with boil preps. Random Amplified Polymorphic DNA (RAPD) PCR was carried out to determine relatedness of different *Pseudomonas* isolates (Mahenthiralingam *et al.* 1996). Each RAPD PCR reactions contained 1.6 µM of primer 272 and 2 µL of template DNA. All primers are detailed in table 2.3 and the PCR conditions for each one in table 2.4. PCR conditions for the RAPD PCR were as follows: 4 cycles each consisting of 5 minutes at 94 ˚C, 5 minutes at 36 ˚C and 5 minutes at 72 ˚C followed by 30 cycles consisting of 1 minute at 94 ˚C, 1 minute at 56 ˚C and 2 minutes at 72 ˚C and a final extension step of 10 minutes at 72 ˚C.

Table 2.3 Primer sequences used.

Primers	Target	Reference
PAL1 5'-ATGGAAATGCTGAAATTCGGC-3' PAL2 5'-CTTCTTCAGCTCGACGCGACG-3'	*Pseudomonas aeruginosa*	De Vos *et al.* (1997)
16SPSEfluF 5'-TGCATTCAAAACTGACTG-3' 16SPSEfluR 5'-AATCACACCGTGGTAACCG-3'	*Pseudomonas fluorescens*	Scarpellini *et al.* (2004)
PS-F 5'-GGTCTGAGAGGATGATCAGT-3' PS-R 5'-TTAGCTCCACCTCGCGGC-3'	Genus *Pseudomonas*	Widmer *et al.* (1998)
RAPD 272 5'-AGCGGGCCAA-3'	Genus *Pseudomonas*	Mahenthiralingam *et al.* (1996)
MD16S1 5'-ATCTAATGGCTTAACCATTAAAC-3' MD16S2 5'-GGACGGTAACTAGTTTAGTATT-3'	*Campylobacter jejuni* and *Campylobacter coli*	Denis *et al.* (1999)
341F 5'-CCTACGGGAGGCAGCAG-3' 518R 5'-CCGTCAATTCMTTTGAGTTT-3'	Eubacteria	Muyzer *et al.* (1993)
eub F530 5'-CAGCAGCCGCGGTAATAC-3' eub R790 5'-CTACCAGGGTATCTA AT-3'	Eubacteria	Gee *et al.* (2003)
VS15 5'-GAATGAAATTTTAGAATGGGG-3' VS16 5'-GATATGTATGATTTTATCCTGC-3'	*Campylobacter jejuni*	Yang *et al.* (2003)
hipO F 5'-TGCACCAGTGACTATGAATAACGA-3' hipO R 5'-TCCAAAATCCTCACTTGCCATT-3'	*Campylobacter jejuni*	Vondrakova *et al.* (2014)
glyA F 5'-CATATTGTAAAACCAAAGCTTATCGTG-3' glyA R 5'-AGTCCAGCAATGTGTGCAATG-3'	*Campylobacter coli*	Vondrakova *et al.* (2014)
campF2 5'-CACGTGCTACAATGGCA TAT-3' campR2 5'-GGCTTCATGCTCTCGAGTT-3'	*C. jejuni, C. coli, C. lari*, and *C. upsaliensis* (and possibly other *Campylobacter* ssp.)	Lund *et al.* (2004)
CU-HSP60 F 5'-GAAGTAAAAAGAGGAATGGATAAAGAAGC-3' CU-HSP60 R 5'-CTTCACCTTCAATATCCTCAGCATAATTAAAAGA-3'	*Campylobacter ureolyticus*	Bullman *et al.* (2011a)
cdtBF1 5'-GTCTTTTGAGTGGATTGGATTCT-3' cdtBR2 5'- CACTCCGGGTGCTTGTGTAT-3'	*Helicobacter pullorum*	Rocha *et al.* (2005)
818-839 5'- ATGAATGCTAGTTGTTGTCAG-3' 1265-1247 5'-GATTGGCTCCACTTCACA-3'	*Helicobacter pullorum*	Stanley *et al.* (1994)

Table 2.4 PCR conditions.

Primers	Initial denaturation	Denaturation	Annealing	Extension	N° cycles	Final extension
PAL1 PAL2	95°C 5 min	95°C 30 sec	57°C 30 sec	72°C 1 min	30	72°C 7min
16SPSEflu F 16SPSEflu R	95°C 5 min	95°C 1 min	57°C 30 sec	72°C 1 min	30	72°C 10 min
PS-F PS-R	95°C 5 min	95°C 30 sec	62°C 30 sec	72°C 1 min	35	72°C 7min
MD16S1 MD16S2	95°C 10 min	95°C 30 sec	53°C 30 sec	72°C 1 min	30	72°C 10 min
341F 518R	95°C 5 min	95°C 1 min	53°C 1 min	72°C 1 min	30	72°C 10 min
eub F530 eub R790	96°C 3 min	96°C 10 sec	55°C 10 sec	72°C 50 sec	30	72°C 5 min
VS15 VS16	95°C 5 min	95°C 15 sec	56°C 15 sec	72°C 20 sec	30	72°C 10 min
hipO F hipO R	95°C 5 min	95°C 15 sec	60°C 15 sec	72°C 20 sec	30	72°C 10 min
glyA F glyA R	95°C 5 min	95°C 15 sec	60°C 15 sec	72°C 20 sec	30	72°C 10 min
CampF2 CampR2	95°C 5 min	95°C 15 sec	58°C 15 sec	72°C 20 sec	30	72°C 10 min
CU-HSP60 F CU-HSP60 R	95°C 5 min	95°C 30 sec	58°C 1 min	72°C 1 min	35	72°C 10 min
cdtBF1 cdtBR2	95°C 5 min	95°C 15 sec	60°C 15 sec	72°C 20 sec	30	72°C 10 min

2.4.3. Quantitative (q)PCR

Quantitative (q)PCR reactions were carried out as a more sensitive technique for detecting *Campylobacter* targets. Q-PCR reactions were prepared using SensiFAST^{TM} SYBR & Fluorescein Kit (Bioline) in 20 µL final volume containing 0.2 µM of each primer and 2 µL of DNA template in a Rotor-Gene Q (Qiagen). Primers and conditions used are specified in tables 2.3 and 2.5. A melting curve analysis with default parameters was performed after each qPCR to check the specificity of the reaction. When results were inconclusive an agarose gel was run to check the product size and the presence of non-specific bands (section 2.4.4).

Table 2.5. QPCR conditions.

Primers	Initial denaturation	N° of cycles	Cycling conditions		N° of cycles
			Denaturation	Annealing/Extension	
VS15 VS16	95°C 5 min	1	95°C 5 sec	60°C 10 sec 72°C 20 sec	45
CampF2 CampR2	95°C 10 min	1	95°C 15 sec	66°C 40 sec	40

2.4.4 Gel electrophoresis

PCR products were separated by electrophoresis in 1.5% wt/vol agarose (Bioline) gels containing 1X gel red (Cambridge Biosciences) with 1X Tris/Borate/EDTA running buffer (Severn Biotech) at 70V (30-70 mL) or 110V(100-150 mL) for approximately 1 hour. Molecular size standards were included in all the gels (Hyperladder 1kb/50 bp (Bioline)). DNA was visualised using a G: Box transilluminator (Syngene) with GeneSnap version

7.12.06 (Syngene). QPCR products were occasionally visualized following this same procedure with 2% wt/vol agarose gels.

2.4.5 Strain sequencing

Bacterial isolates were characterised by partial 16S rDNA sequencing PCR which was performed using the primers eub F530 and eub R790 (Table 2.3) using MyTaqTM Red Mix (Bioline) in a final volume of 50 µL containing 0.4 µM of each primer and 2 µL of DNA template. PCR conditions are indicated in table 2.4. The PCR post-reaction amplicon was purified using the Nucleo spin Gel and PCR Clean-up kit (Machery-Nagel) following the manufacturers instructions and eluting with 15 µL nuclease-free sdH_2O (VWR International).Sequencing of amplicons was performed commercially (Source Bioscience). Both strands of each amplicon were sequenced using the same primers as used for their initial amplification. Sequence data from each strand were verified then combined with one another using Chromas Pro (Technelysium Ltd) or FinchTV. Genetic comparisons were done using the BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

2.4.6 Whole-genome sequencing of *H. pullorum*

2.4.6.1 Library preparation and sequencing

Whole genome-sequencing of a single *H. pullorum* isolate was performed as a part of a collaborative strain sequencing project in the University of Salford using the Nextera XT

DNA Library Kit (Illumina) following the manufacturer's instructions. Before sequencing phiX was spiked at 1% vol/vol. Sequencing was carried out in a Miseq in the University of Salford.

2.4.6.2 Primer cross-reactivity with the *H. pullorum* genome

Using the genome information from the sequenced *H. pullorum* isolate, a BLAST database was created by Dr. Ian Goodhead and cross-reactivity with selected primers was carried out using BLAST in the command line.

2.5 Microbial community profiling

2.5.1 16S and 18S library preparation

Amplicon libraries were prepared following the illumina guidelines for 16S rRNA libraries (Illumina 2013). Universal primer sequences (Table 2.6) for 16S and 18S rRNA amplification were chosen from the Earth Microbiome Project (http://www.earthmicrobiome.org/) and modified with the edition of Illumina adapter overhang nucleotide sequences (Illumina 2013). Cycling conditions are specified in table 2.7. The 16S rRNA primers were developed against the V4 region of the 16SrRNA by Caporaso *et al.* (2011) and were subsequently modified to remove primer biases (Apprill *et al.* 2015, Parada *et al.* 2016). The 18S rRNA primers target the V9 region and were designed by Amaral-Zettler *et al.* (2009).

Table 2.6. Primer sequences for 16S and 18S library preparation.

Primers	Target
V4_515F 5'- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGYCAGCMGCCGCGGTAA-3' V4_806R 5'- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACNVGGGTWTCTAAT-3'	16S rRNA V4
V9_Euk1391F 5'- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTACACACCGCCCGTC-3' V9_EukBR 5'- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGATCCTTCTGCAGGTTCACCTAC-3'	18S rRNA V9

Gene-specific sequences are shown in red and adapter sequences are shown in blue.

Table 2.7. PCR cycling conditions for first PCR in library preparation.

Primers	Initial	Cycling conditions				Final
	denaturation	Denaturation	Annealing	Extension	N°cycles	extension
16S	95°C 3 min	95°C 30 sec	50°C 30 sec	72°C 30 sec	30/35	72°C 5 min
18S	95°C 3 min	95°C 30 sec	57°C 30 sec	72°C 30 sec	30/35	72°C 5 min

Amplicon library PCRs (25 µL) were carried out using KAPA HiFi HotStart ReadyMix PCR Kit (KAPA Biosystems) in a Veriti 96 well Thermal Cycler (Applied Biosystem). Table 2.8 details the different conditions used for each sample type. A no template PCR control was included for each reaction.

Table 2.8. PCR conditions used to prepare amplicon libraries from different sample types.

PCR reaction volumes	N° Cycles	16S	18S
DNA – 2.5 µL Primers- 2 µM/each	30	Shed bulk water	Shed bulk water and External nipple drinker
DNA- 5 µL Primers-0.2 µM/each	35	Anteroom bulk water, Biofilm (nipple drinker), Biofilm (swab), External nipple drinker and negative controls.	Anteroom bulk water, Biofilm (nipple drinker), Biofilm (swab) and negative controls.

Extended number of cycles and increased amount of DNA were used for samples where a low DNA yield was expected from previous protocol optimisation steps.

PCR reactions were purified using AMPure XP beads (Agencourt). Beads were brought to room temperature, vortexed and 40 µL were added to each reaction. Thorough mixing was ensured by vortexing each suspension for 20 seconds before incubation at room temperature (5 min), and pulsed centrifuged DNA-bound beads were recovered by placing each reaction in a magnetic stand (Dynamag-2 magnet) until supernatant cleared. The supernatant was discarded and remaining bound DNA was washed twice with freshly prepared 80% vol/vol molecular grade ethanol (Fisher Scientific). Excess ethanol was removed using 10 µL fine tips. Beads were kept on the magnetic stand to air dry for approximately 3-4 minutes with the lids opened and monitored visually to avoid over-

drying. Reactions were removed from the magnetic stand and beads were re-suspended in nuclease-free sdH_2O (VWR International) (52.5 µL for samples in row 1 in table 5 or 27 µL for samples in row 2 in table 5). Tubes were gently agitated, by tapping and incubated at room temperature (2 min) before pulse centrifugation, and placed back in the magnetic stand to recover beads (2 min). Approximately 50 µL and 25 µL of purified samples were recovered (for 16S rRNA and 18S rRNA amplicons respectively) and transferred to a 96-well plate. DNA quality control was performed for selected representative samples representing each sample type using a Tapestation 2200 (Algilent Technologies) using a High Sensitivity D1000 kit (Algilent Technologies) according to the manufacturer instructions.

A second round (indexing) PCR was performed to add Illumina indexes to amplicons in each purified sample using KAPA HiFi HotStart ReadyMix PCR Kit (KAPA Biosystems) and the Nextera XT Index Kit (Illumina) in a Veriti 96 well Thermal Cycler (Applied Biosystem). Details of PCR reaction volumes are given in table 2.9. PCR cycling conditions included an initial denaturation step (95 °C, 3 min) followed by 8 cycles of denaturation (95 °C, 30 s), annealing (55 °C, 30 s) and extension (72 °C, 30 s) and a final extension step (72 °C, 5 min).

Table 2.9. Index PCR reaction volumes.

PCR reaction volumes	16S	18S
1st PCR product – 2.5 µL Primers- 2.5 µL/each Total volume - 25 µL	**Shed bulk water, External nipple drinker and Biofilm (swab).**	**Shed bulk water, Anteroom bulk water, Biofilm (swab), and External nipple drinker.**
1st PCR product -1 5 µL Primers-5 µL/each Total volume - 50 µL	**Anteroom bulk water, Biofilm (nipple drinker) and negative controls.**	**Biofilm (nipple drinker) and negative controls.**

Increased amount of 1st PCR product and increased total volume were used for samples where the Tapestation quantification showed poor DNA yield.

Indexed amplicons were purified using Ampure beads, as described for the clean-up of the first round PCR, with 30 µL of beads for 25 µL reactions and 56 µL of beads for 50 µL reactions. All PCR reactions were re-suspended in 27.5 µL of nuclease-free sdH_2O (VWR International) in the final step and approximately 25 µL were recovered from the tube. For quality control, all samples were analysed on the Tape-Station (Agilent, 2200) with high-sensitivity screen tape (Agilent) in accordance with the manufacturer's instructions, to confirm correct size ranges. Accurate quantification was also performed in duplicate using the Qubit 3.0 Fluorometer (Thermo Fisher Scientific) with Quant-iT dsDNA Assay Kit, High Sensitivity (Invitrogen) and the average concentration measurement was used for normalisation by the size of the amplicon. Samples were artificially given a concentration of 1 ng/µL when the concentration in the Qubit was below that value. Samples were pooled with a final volume of 80 µL with a mean size of 295.3 and 408.0 bp for 16S rRNA and 18S rRNA pools respectively. Pools were further subjected to an extra clean-up with Ampure beads (56 µL) and re-suspended in 85 µL of uclease-free sdH_2O (VWR

International). Approximately 82 µL of each purified pooled library was recovered. Libraries were analysed before and after purification using the TapeStation to confirm that the bead clean-ups had effectively removed primer dimers. 16S rRNA and 18S rRNA pooled libraries were quantified by qPCR using the NEB Next Library Quant Kit for Illumina (New England Biolabs). Two different dilutions (1:10,000 and 1:100,000) were run in triplicate along with the standards. The concentration of the pooled libraries was calculated using the online NEBioCalculator v1.3.12 (nebiocalculator.neb.com). Libraries were then denatured using freshly prepared 2N NaOH and diluted to 10 pM using HT1 Buffer (Illumina). PhiX control was also denatured with 2N NaOH, diluted to 10 pM and spiked-in to the sequencing run to represent 15% vol/vol of the template. 16S and 18S amplicon libraries were combined to equally represent 42.5% vol/vol of the run each. The final pool of 600 µL was incubated at 96 °C for 2 minutes in a heat-block and then placed in ice for 5 minutes before loading into the MiSeq Reagent Kit V2 (500 cycles) (Illumina).

2.5.2 Bioinformatics

Raw sequences passing the quality filter were demultiplexed to different samples using the "Generate FASTQ Workflow" with default parameters in Illumina Basespace. Downstream analyses were performed using Qiime (Caporaso *et al.* 2010). Firstly, additional quality filtering was carried out for read 1 using the script "split_libraries_fastq.py" with a quality threshold of 19. Then chimeric sequences were identified using USEARCH (Edgar 2010) and filtered. Sequences were assigned to operational taxanomic units (OTU) using the Closed-reference method and the SILVA databases (Quast *et al.* 2013). For the 16S sequences OTUs were clustered according to 97% identity using uclust (Edgar 2010) and the 18S sequences were clustered according

to 99% identity, using USEARCH (usearch61) (Edgar 2010). A representative set of sequences was picked from the OTUs for taxonomy assignment using the RDP classifier (Wang *et al.* 2007) for the 16S OTUs and Blast (Altschul *et al.* 1990) the 18S OTUs. After the taxonomy assignment an OTU table with taxonomy was created and split at different taxonomic levels.

2.5.3 Statistical analyses

Statistical analyses for the sequencing data were performed using a non-parametric multivariate framework in the statistical software PRIMER 7 (Clark and Gorley, 2015). OTU table entries were standardised and square and fourth root transformations were used as a data pre-treatment step. Spearman rank correlations were used to determine the similarities among different analysis. The Bray-Curtis coefficient was used to analyse similarities in community structure which were visualized with non-parametric multidimensional scaling plots. Statistical differences among samples were investigated using two-way and one-way ANOSIM (analysis of similarities) (Clarke 1993). Taxonomic compositions were visualized using shade plots.

2.6. Interaction assays

2.6.1 Co-culture bacterial growth assays

Pseudomonas spp. starter cultures were prepared by inoculating a few colonies into MHB and grown overnight at 25 ˚C, then sub-cultured (100 µL) into fresh MHB (5 mL). *C. jejuni* strains 11168 and 81-176 were grown overnight on CBA and then sub-cultured in MHB

and grown again overnight at 42 ˚C under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2).

Cell-free supernatant were prepared by centrifugating the overnight culture at 5,000xg for 5 minutes and filtering 1 mL of the supernatant using a 0.22 µm sterile syringe filter (Fisher) into 1 mL of fresh MHB.

Pseudomonas ssp. (and *Pseudomonas* spp.CFS) and *C. jejuni* strains were co-cultured at a ratio of 1:1 vol/vol in 96-well sterile microtitre plates (Corning). Controls replacing *Pseudomonas* ssp. culture with MHB and blank controls (MHB only) were included. Viable *C. jejuni* cell counts were determined by serial dilution in Phosphate Buffered Saline (PBS) (Amresco) and plating on CBA using the Miles Misra technique (3 x 10 µL spots of each dilution) (Miles *et al.* 1938). An aliquot of the *Pseudomonas* spp. suspensions was placed on *Campylobacter* selective media to verify absence of growth. Co-culture plates were incubated at 25 ˚C under aerobic or microaerobic conditions for 24 h. Following incubation absorbance at 570 nm was recorded using the Multiskan FC plate reader (Thermo Scientific) and viable cell counts were determined by serial dilutions and plating on *Campylobacter* selective agar. Significant differences between the initial and final counts in each co-cultured were determined with a paired t-test. Significant differences between final counts in different co-cultures were determined using a one-way ANOVA (Turkey's method). Significant factors in the experiment comparing microaerobic and aerobic conditions were determined using a two-way ANOVA. A p-value <0.05 was determined statistically significant. Statistical analyses were performed in MINITAB.

2.6.2 Gentamicin protection assay

A gentamicin protection assay was used to determine interactions between *C. jejuni* strain 81-176 and *A. polyphaga.* The protocol was adapted from Dirks and Quinlan (2014). *A. polyphaga* cultures were prepared in PPG in T25 vented tissue culture flasks (Fisher) to a density of almost confluent trophozite coverage, as determined by visualisation using a Olympus CK Tokyo inverted microscope at 10X magnification. Before the experiment the media in two flasks was removed and replaced with 2.5 mL of Page's amoebae saline solution (PAS) per flask. The amoebae were then detached from the surface by incubation at -20 ˚C for 10 min and by pipetting up and down the liquid to the surface of the tissue culture flask. The amoebae were harvested by centrifugation in sterile microfuge tubes (1.5 ml) (VWR International) at 2500 x g for 5 min at 4 ˚C followed by three washes with PAS using the same centrifugation conditions. After the final wash the amoebae were pooled together and re-suspended in a final volume of 1 mL of PAS and a 10 µL aliquot mixed 1:1 with trypan blue (Sigma) and counted using a Neubauer Improved hematocytometer (Marienfeld).

C. jejuni strain 81-176 was plated on CBA from frozen stocks 48 h before the experiment and subcultured in fresh CBA 24 h before the experiment and was grown in microaerobic conditions. A thick suspension of *C. jejuni* culture was prepared in PPG and the colony forming units were calculated by serial dilution and plating on CBA

The rate of internalization of *C. jejuni* by *A. polyphaga* was assessed by co-culturing 100 µL of *A. polyphaga* suspension in PAS with 1 mL of *C. jejuni* 81-176 suspension in PPG. Controls with PPG instead of *C. jejuni* suspension and PAS instead of *A. polyphaga* suspension were included. Samples were incubated at 25 ˚C for 2 h, then washed 3 times in PAS by centrifugation at 2500 g for 5 min at room temperature. Samples were then

incubated for 1 h in PPG (1 mL) containing 200 µg/mL gentamicin (Alfa Aeasar) and washed as indicated above to remove gentamycin and un-bound *C. jejuni* cells. Washed co-culture samples were re-suspended in PPG (1 mL) containing 0.3 % vol/vol Triton X-100 (Scientific Laboratory Supplies) and incubated at 25 ˚with shaking (200 rpm) for 15 min to lyse amoeba and release intracellular *C. jejuni*. Samples were serially diluted and plated on CBA to quantify *C. jejuni* cells that had been internalised and were therefore protected from gentamycin killing. Replica samples were set up for the quantification of amoebae after the experiment. These were re-suspended in PAS (50µL) and trypan blue (50 µL) and counted with the hematocytometer. The rate of internalization (ROI) was calculated using the following formula:

$$\mathrm{ROI} = \frac{[\mathrm{CFU}\ C.\ jejuni\ \mathrm{with}\ A.\ polyphaga] - [\mathrm{CFU}\ C.jejuni\ \mathrm{in\ PAS}]}{[\mathrm{amoebae\ recovered}]}$$

An independent t-test was performed to determine significance in recovery between co-cultivation and control using PAS in MINITAB. A p-value <0.05 was determined statistically significant.

Chapter 3

Longitudinal and spatial microbial community profiling of the drinking water system (DWS) of a commercial broiler farm during a rearing cycle

3.1 Introduction

Advances in next-generation sequencing technologies (NGS) or high-throughput sequencing have revolutionized the field of microbial ecology during the last 10 years (Escobar-Zepeda *et al.* 2015). This cutting-edge technology has led to the establishment of modern metagenomics, a thriving area of research used for functional and sequence-based analysis of the collective microbial genomes contained within an environmental sample by direct genetic analysis (Oulas *et al.* 2015). Metagenetics (amplicon profiling) is the study of all members in a microbial community based on PCR amplification or sequencing of one evolutionarily conserved gene marker for taxonomy or phylogenetic purposes (Escobar-Zepeda *et al.* 2015). Gene marker studies with 16S rDNA, 18S rDNA, ITS, CO1 and others have become the most prevalent NGS approach in ecology (Creer *et al.* 2016). Amplicon profiling of the small subunit rRNA gene (16S rRNA gene in Bacteria and Archaea and 18S rRNA gene in Eukarya), is a widely applied approach to study the taxonomic composition and spatiotemporal patterns of microbial communities, due to its

ubiquity across all domains of life (Sinclair *et al.* 2015). Using this approach microbial life has been found in virtually any ecosystem studied to date (Gilbert *et al.* 2014).

Drinking water distribution systems (DWDS) are extreme oligotrophic environments but not surprisingly they harbour microbial communities (Douterelo *et al.* 2013). These diverse communities include bacteria, archaea, eukaryotes and viruses (Gomez-Alvarez *et al.* 2012). Water utilities most often add free chlorine or monochloramine at the end of the water treatment process to maintain a sufficient disinfectant residual in the DWDS network to supress microbial growth (Williams *et al.* 2004, Henne *et al.* 2012, Hwang *et al.* 2012). Distribution of water without a disinfectant residual also occurs, sometimes with a previous UV disinfection step (Valster *et al.* 2011, van der Wielen and van der Kooij 2013). After disinfected water leaves the treatment plant, regardless of the presence of a disinfection residual, microbial biomass and diversity increase along DWDS (Shaw *et al.* 2014, El-Chakhtoura *et al.* 2015). Accordingly, treatment plant and distribution network samples show different bacterial community structure although a core microbiome can be shared (El-Chakhtoura *et al.* 2015). DWDS microbial communities are shaped by a range of parameters such as plumbing material (Yu *et al.* 2010, Buse *et al.* 2014a), corrosion scale on metal pipes (Sun *et al.* 2014), disinfection procedure (Gomez-Alvarez *et al.* 2012) and hydraulic conditions (Douterelo *et al.* 2013). Water quality parameters have been found to impact microbial communities in DWDS including organic matter content (Sun *et al.* 2014), pH (Pinto *et al.* 2012), temperature (Pinto *et al.* 2014) and conductivity (Pinto *et al.* 2014). The source water has also been shown to influence DWDSs microbial populations. Origin of raw water (surface vs. ground water) (Pinto *et al.* 2014, Sun *et al.* 2014) and upstream microbial composition along the water treatment

plant and DWDS impacts the communities found downstream (Pinto *et al.* 2012, Pinto *et al.* 2014).

Biofilms develop in all surfaces in contact with non-sterile water and therefore are ubiquitous to DWDS where they grow attached to the internal surface of the pipes (Wingender and Flemming 2011). Biofilm formation in DWDS has been described as a sequence of events starting with attachment of single cells scattered on the surface. This is followed by microcolony development which initiates the maturation phase. In this phase increased biomass and biofilm thickness is observed along with almost complete surface coverage and formation of new ecological niches (Martiny *et al.* 2003). As well as increased coverage, a gradual rise in species richness and diversity over time takes place during the early stages of biofilm formation (up to 28 days in the experiment) (Douterelo *et al.* 2014). Fast colonization on stainless steel and other plumbing materials has been reported with up to 10^7 cells/cm^2 after 30 days (Morvay *et al.* 2011). However Martiny *et al.* (2003) could only detect microcolonies in their experimental setting after 3 months. While some species dominate at certain stages, most biofilm studies in DWDS suggest a wide variety of species in low-abundance (Hong *et al.* 2010, Henne *et al.* 2012, Revetta *et al.* 2013). With a common source water, Shaw *et al.* (2014) demonstrated that a common biofilm community structure is reached in DWDS regardless of the treatment method applied. Along these lines, Revetta *et al.* (2013) did not find significant differences in community structure between biofilm devices or surface material but they observed that all biofilms underwent similar succession of taxonomic groups over the 8 months of their experiment. However it is questionable if a real steady state can ever be reached due to

constant variations in hydraulic regimes and changes in the introduction of nutrients, new microorganisms and disinfectants (Batté *et al.* 2003, Martiny *et al.* 2003).

It has been estimated that bulk water bacteria only account for 2% of total bacteria present in a DWDS (Liu 2014). Moreover, biofilm and bulk water microbial composition is very different with biofilms showing higher spatial heterogeneity (Emtiazi *et al.* 2004, Henne *et al.* 2012). Biofilms provide substantial ecological benefits for their inhabitants such as facilitated intercellular communication facilitating synergetic behaviour and horizontal gene transfer (Wingender and Flemming 2011). Microorganisms within biofilms are more resistant to disinfection and other kinds of environmental stress than their planktonic counterparts (Stoodley *et al.* 2002, Emtiazi *et al.* 2004). They provide an intrinsic resistance to disinfectant transfer to deep zones and therefore bacteria in these areas are exposed to sub-lethal doses of the biocides possibly inducing resistance (Batté *et al.* 2003) as has been observed for *E. coli* response to chlorine (Saby *et al.* 1999).

Biofilms within DWDS provide microenvironments for opportunistic pathogens to grow and could be a source of bulk water contamination. Bacterial opportunistic pathogens inhabiting DWDS biofilms include *Legionella pneumophila*, *Mycobacterium* spp., *Ralstonia pickettii* and *Pseudomonas aeruginosa* (Emtiazi *et al.* 2004, Revetta *et al.* 2013, Zeng *et al.* 2013, Sun *et al.* 2014). *Helicobacter*, a genus closely related to *Campylobacter*, has also been detected in a biofilm sampled from a DWDS (Park *et al.* 2001). Some *Campylobacter jejuni* strains have been reported to persist in biofilms formed with oligotrophic surface water (Buswell *et al.* 1998a, Buswell *et al.* 1998b) and chlorinated potable water (Lehtola *et al.* 2006) under laboratory conditions. Potentially pathogenic

fungi also benefit from being established in biofilms in water systems (Hageskal *et al.* 2009, Siqueira *et al.* 2011).

Protozoa like *Acanthamoebae* inhabit drinking water biofilms (Buse *et al.* 2014a). *Acantahmoebae* along with other free-living protozoa (FLP) can proliferate in DWDS where they may protect pathogenic bacteria from unfavourable environmental conditions, enhance multiplication and/or serve as transmission vectors (Valster *et al.* 2009, Buse *et al.* 2014b). *Legionella* ssp. in particular have been observed to benefit from interacting with certain FLP (Buse *et al.* 2014b) and other pathogenic bacteria including *Campylobacter* ssp. may also do so as they have been found to interact with FLP (particularly the genus *Acanthamoebae*) *in vitro* (Snelling *et al.* 2005, Axelsson-Olsson *et al.* 2010a) (see chapter 5). Fifty four bacterial genera were identified inside amoebae in a metagenomics analysis of cultured free-living amoebae (FLA) from drinking water suggesting that the extent of FLA-bacteria interactions might have been underestimated (Delafont *et al.* 2013).

Drinking water has been implicated in sporadic cases and more commonly in outbreaks of campylobacteriosis (Frost 2001) but they have commonly been associated with cross-connections (flow of contaminants into the potable water) and water-treatment breaks as a result of heavy rainfall or sewage contamination (Pitkanen 2013). *Campylobacter* is therefore considered a waterborne pathogen and not part of the endogenous microbial communities in water distribution systems (Westrell *et al.* 2003) but its known to be capable of survival in water, particularly at low temperatures (Cools *et al.* 2003).

It was hypothesised that the drinking water system (DWS) of broiler farms could act as a source or a vehicle of *Campylobacter* colonization and transmission by permitting

Campylobacter survival in low abundance, either via protozoa or permissive physiological conditions within the biofilm, which could offer protection from stresses such as O_2 and disinfectants. Previous investigations of broiler house DWS have focused on the detection of the pathogen or isolation or a few microorganisms and have not fully considered the dynamics of biofilm microbial communities in the pipes and how fluctuations in physiological conditions throughout the rearing cycle might affect potential interactions with *Campylobacter*. In particular, temperature and flow rate are very different in the broiler house system, compared to mains DWDS. The aim of this study was to characterise the spatiotemporal patterns of the microbial communities inhabiting the DWS of a conventional commercial broiler farm during a whole rearing cycle. DNA was extracted from biofilm and bulk water samples across the 7-week rearing period from before the chickens were placed on the farm (week 1) to after the totality of the flock had left for slaughter (week 7). A metagenetics approach was chosen and ribosomal gene markers (16S and 18S rRNA) were amplified and sequenced using an Illumina Miseq.

3.2 Changes in temperature and flow rate and administration of prophylaxis and vaccinations occur on the farm DWS during a typical rearing cycle

The DWS on the farm differs from typical DWDS where other microbial ecology studies have been performed. For example, environmental parameters such as temperature and duration of light period are tightly controlled inside the broiler house during the rearing cycle. Environmental parameters of our study farm were recorded during the study period. Temperature was high (>30 °C) at the beginning of the rearing cycle and was

deliberately decreased by the farmer to 18 °C-25 °C as the chickens grew older (figure 3.1 A). During a typical rearing cycle, the duration of the light period started at 23 h during the first 3 days and dropped one degree at a time down to 18 h by day 7 to then increase again to 21 h on day 31, 22 h on day 32 and 23 h until the end of the rearing cycle on day 39. The average weight of the chickens and water consumption were carefully monitored and recorded. Using the readings of daily water consumption recorded on sampling day, the average flow rate was calculated. Conversely to the trends in temperature, the average water flow rate increased as chickens grew larger and demanded more water (figure 3.1 B and C). pH was measured in the anteroom and shed bulk water and it remained stable and close to neutrality across the seven sampling weeks ranging from pH 6.7 to 7.2.

Another unique feature of the commercial broiler farm DWS is its use for administration of antibiotics and vaccines to the chickens. During this study, Lincospectin which is a combination of the antibiotics Lincomycin and Spectinomycin was administered as a prophylactic treatment during the first 3 days of the rearing cycle which corresponds to the period between weeks 1 and 2 in the sampling scheme. Further on, the vaccine Cevac IBD, protecting from Infectious Bursal Disease virus, was also fed to the chickens through the DWS on day 19 which corresponds to the period between weeks 3 and 4.

The farm comprised 5 broiler houses. This study was allowed access to sampling of 1. Approximately 23,400 chickens were placed on the broiler house sampled. During a standard rearing cycle this number decreases due to chicken mortality, with higher mortality rates at the beginning of the cycle that reduced as the chickens grew larger. A total number of 1,102 chickens died during the rearing cycle studied. Another cause for reduction of chicken numbers is thinning which in this cycle took place on day 32 and

removed 6864 chickens from the broiler house between sampling week 5 and 6. This represents a key risk point for *Campylobacter* contamination and might trigger changes in microbial community profiles due to the introduction of outsiders and external equipment (Allen *et al.* 2008, Smith *et al.* 2016).

Another key difference of the DWS on the farm is the disinfection procedures. After the whole chicken flock leaves for slaughter the pipes are flushed at high pressure and filled with 2% peroxigen which is left overnight and then flushed again at high pressure in the morning. Just before the chickens arrive to the farm the DWS is flushed again to get rid of stagnant water.

It was hypothesised that the changes in environmental parameters, particularly temperature, flow rate and administration of vaccines and antibiotics would result in changes in the microbial communities that inhabit the broiler house DWS.

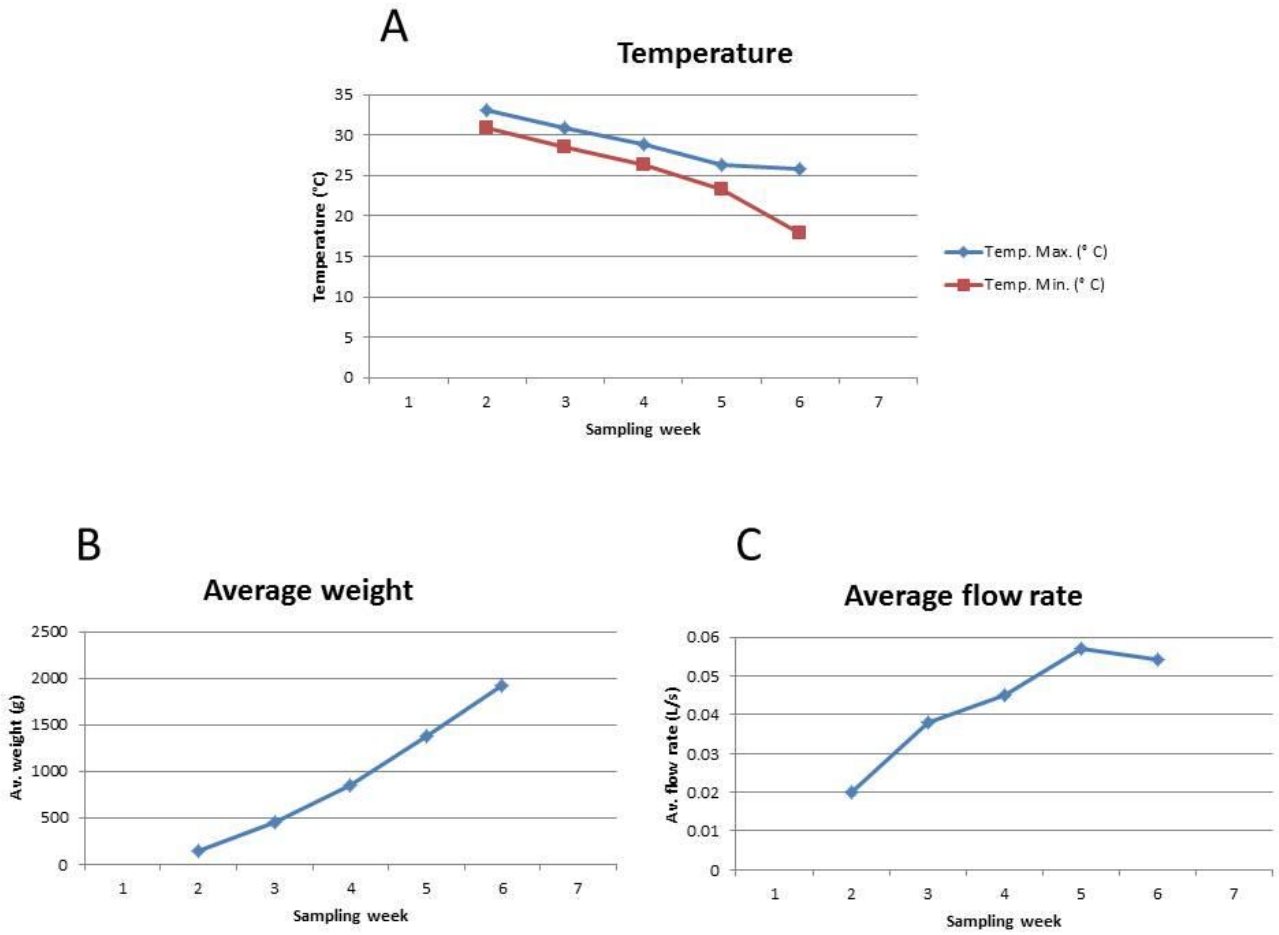


Figure 3.1. Variation of key parameters during the rearing cycle. (A) Minimum and maximum temperature. (B) Average weight of the chickens. (C) Average flow rate.

3.3 DNA extraction and library preparation efficiency differ for samples from different environmental niches

DNA was extracted from several different water and biofilm samples from the broiler house DWS. The “Shed bulk water” (taken from the blind end of water pipes within the broiler house), were compared to “Anteroom bulk water” (taken from the tap immediately before water entered the broiler house). The communities in these samples were compared to those in biofilms collected from the external and internal parts of the nipple drinkers, from which chickens obtain water, and from inside the water pipes. Preliminary work optimizing DNA extraction methods indicated that biofilm DNA extraction from the nipple drinker was challenging probably due to the small amount of starting material. The approximate area of the internal part of the nipple drinker is 3.55 cm^2 which is less than most authors have used in their studies. For example Liu *et al.* (2014) were sampling biofilms by cutting 30-40 cm of pipe out of the distribution system and Revetta *et al.* (2013)used 17.5 g of 2.5 mm diameter glass beads. Nevertheless others have used coupons of only 1.27 cm^2 although they reported higher biomass on unplasticized polyvinyl chloride compared to the metal copper (Buse *et al.* 2014a). Difficulties extracting DNA from DWDS biofilm formed on coupons have also been reported (Douterelo *et al.* 2014). It is therefore known that DNA extraction from DWDS biofilms can be challenging, especially when the starting material is metal and the surface area is small.

Sonication was chosen as a strategy for biofilm removal as it has been used in other biofilm studies (Maal-Bared *et al.* 2012, Mathieu *et al.* 2014) and it was found to be superior to scraping for retrieval of bacteria in biofilms on metal surfaces (Bjerkan *et al.*

2009). Other alternatives such as cell scraping (Roeder *et al.* 2010, Isabel Douterelo *et al.* 2014, Shaw *et al.* 2014) or mechanical disruption (Lu *et al.* 2014) were also considered but were not seen as feasible due to the nipple drinker size and shape.

DNA extracted from the nipple drinker consistently showed a concentration less than 10 ng/µL as measured with Nanodrop and could not be seen on an agarose gel but showed amplification by 16S rRNA primers. The same quality and quantity of DNA were obtained from negative controls processed alongside these samples. In spite of the technical difficulties with the DNA extraction from these samples, nipple drinkers were chosen to sample biofilms because they are easily removed and replaced from the broiler house DWS and thus provide a non-invasive method for biofilm sampling. Sampling of cut-outs of pipes, although desirable from a biological and methodological point of view, is expensive, labour-intensive and in most cases destructive (Isabel Douterelo *et al.* 2014). In our case it was simply not an option as the study site was a working broiler farm.

The internal and external part of the nipple drinkers (see methods chapter figure 2.2) were separated as the external part was in contact with the farm environment and accessible to the chickens while the internal part was in contact with the interior of the pipe and remains more isolated from the farm environment. To complement this sample from the internal part of the nipple drinker biofilm a second method of biofilm sampling was used to attempt a better biofilm recovery from the pipes. A sterile cotton swab was introduced through the hole left when removing the nipple drinker to sample the biofilm in the interior of the pipe, a technique that has also been used before (Hong *et al.* 2010, Luo *et al.* 2013).

DNA extraction from bulk water was more successful and after protocol optimization concentrations greater than 20 ng/µL were obtained from filters on which biomass was collected from 4 L water samples, and good quality DNA could be observed in agarose gels from bulk water samples from inside the chicken shed (data not shown). A 3-fold improvement in DNA yield in these samples was obtained after including a filter sonicating step following filtration (Kesberg and Schleheck 2013). All the samples for which processing involved filtering and recovering DNA from the filter, were subsequently subjected to sonication (see methods chapter section 2.4.1.2.3).

The paramount importance of including negative "blank" controls particularly when working with low biomass samples has been noted in several studies (Charlson *et al.* 2011, Bittinger *et al.* 2014) although in general too little attention seems to be paid to the issue (Glassing *et al.* 2016). Others have observed that including no-sample controls is particularly important when sampling oligotrophic environments, such as drinking water, as oligotrophs often dominate sequences found in clean-room laboratories (Tanner *et al.* 1998, Barton *et al.* 2006). In this study, a different negative control was included for every variation in the processing of the different sample types. To control for the biofilm communities from nipple drinkers (detached from the internal and external parts by sonication), two negative controls (one in a bijou tube for the external part and one in a microcentrifuge tube for the internal part) were processed alongside the samples each week. These controls were then pooled according to their tube type. A sterile cotton swab was used as a negative control for the biofilm samples collected by swabbing. A sterile filter was also included as a control for the filtration process which applied to all

samples except the biofilm collected by swabbing. Finally a blank control with the DNA extraction kit reagents was also included.

The only group of samples that consistently gave DNA concentrations greater than 10 ng/µL (measured on the second DNA elution) was the Shed bulk water samples (table 3.1). This contrasts with all but one of the Anteroom bulk water samples having concentration under 10 ng/µL in spite of the same volume (4L) being filtered in both cases. There is some evidence pointing to the recovery of biofilm by swabbing being more efficient than by sonicating the internal part of the nipple drinker as two biofilm swab samples also show concentrations DNA yield recovered from greater than 10 ng/µL. In the same way there was one sample from the external part from the nipple drinker with a concentration greater than 10 ng/µL. All samples from the internal part of the nipple drinker and negative controls yielded concentrations under 10 ng/µL.

Table 3.1. DNA concentration of the samples included in the amplicon profiling experiment of the broiler farm after DNA extraction and after library preparation.

Week	Sample Type	Nanodrop (DNA extraction second elution) Conc. (ng/µL)	Qubit (Library quantification) Conc. 16S (ng/µL)	Conc. 18S (ng/µL)
1	Shed bulk water	12.2	0.301	22.2
1	Anteroom bulk water	6.8	4.4	20.35
1	Biofilm (nipple drinker)	6.6	2.075	90.9
1	External nipple drinker	14.8	12.55	10.01
1	Biofilm (swab)	17.9	3.43	17.6
2	Shed bulk water	30.6	4.25	23.9
2	Anteroom bulk water	22.9	5.74	25.6
2	Biofilm (nipple drinker)	5.0	1.471	44.3
2	External nipple drinker	4.8	14.9	51.6
2	Biofilm (swab)	8.0	3.76	25.9
3	Shed bulk water	188	8.37	22.15
3	Anteroom bulk water	8.1	54	32.6
3	Biofilm (nipple drinker)	7.2	0.872	20.3
3	External nipple drinker	4.5	25.8	7.28
3	Biofilm (swab)	19.9	0.433	8.44
4	Shed bulk water	165.9	6.77	19.8
4	Anteroom bulk water	3.9	48.2	23.55
4	Biofilm (nipple drinker)	2.5	5.84	8.01
4	External nipple drinker	4.1	32.2	5.54
4	Biofilm (swab)	6.3	2.75	9.14
5	Shed bulk water	38.3	3.45	22.25
5	Anteroom bulk water	3.4	1.3	18.9
5	Biofilm (nipple drinker)	3.4	1.44	8.73
5	External nipple drinker	6.9	47.6	11.9
5	Biofilm (swab)	8.4	16.05	41.1
6	Shed bulk water	23.6	2.11	18.1
6	Anteroom bulk water	2.2	1.325	11.43
6	Biofilm (nipple drinker)	2.3	2.06	20.9

6	**External nipple drinker**	**3.0**	**54.9**	**21.4**
6	**Biofilm (swab)**	**6.3**	**19.15**	**27.2**
7	**Shed bulk water**	**19.1**	**3.25**	**20.85**
7	**Anteroom bulk water**	**6.3**	**58.2**	**23.3**
7	**Biofilm (nipple drinker)**	**1.9**	**2.62**	**48.9**
7	**External nipple drinker**	**4.5**	**47.3**	**13.9**
7	**Biofilm (swab)**	**5.3**	**1.74**	**13.6**
-	**Bijou tube sonication control**	**1.6**	**11.25**	**18.05**
-	**Microcentrifuge tube sonication control**	**2.9**	**2.92**	**13.35**
-	**Filter control**	**2.1**	**0.717**	**5.96**
-	**Swab control**	**8.7**	**2.265**	**80.4**
-	**DNA extraction kit control**	**7.4**	**3.84**	**13.1**

DNA concentration of the second elution was measured using Nanodrop to be used as a proxy of the concentration in the first elution used for library preparation. After library preparation DNA concentration was measured using Qubit.

After the first PCR step for 16S and 18S rRNA library preparation, selected representative samples were analysed on the tapestation and no PCR bands were observed for low yield samples. An increase of the DNA input and PCR cycles for selected low yield sample types was used to solve the problem. Although polymerase errors increase in later cycles of PCR and result in a higher number of artifacts, which in turn increase singletons and diversity estimates, they are confined to small copy numbers and have been suggested to have little impact on the overall sequence representation of different taxonomic groups (Acinas *et al.* 2005, Kebschull and Zador 2015). PCR stochasticity during the first cycles seems to be the major force skewing sequence representation as suggested by Acinas *et al.* (2005) and confirmed by Kebschull and Zador (2015). Regarding the impact of extended cycling on the amplification of contaminants their domination in low-input

biomass samples seems to be relatively independent from the number of cycles when comparing 20 to 40 (Salter *et al.* 2014). A moderate increase in number of cycles to 35 which has been used in other biofilm studies (Schmeisser *et al.* 2003) should not negatively impact the results in excess.

In spite of these modifications, tapestation analyses and qubit quantification showed that some of the libraries were of low concentrations, particularly 16S rDNA libraries (Table 3.1). Primer dimers also presented a problem in some libraries so an extra bead clean-up step was carried out after the separate 16S rDNA and 18S rDNA pools were generated (figure 3.2) to ensure that the final pool would not have primer dimers which could take over the sequencing run. Negative controls showed qubit concentrations in the range of the samples and therefore were treated as any other sample for the pooling process. Concentrations below 1 ng/µL were converted to 1 ng/µL for ease of pooling and all samples were included in the final pool and sequenced.

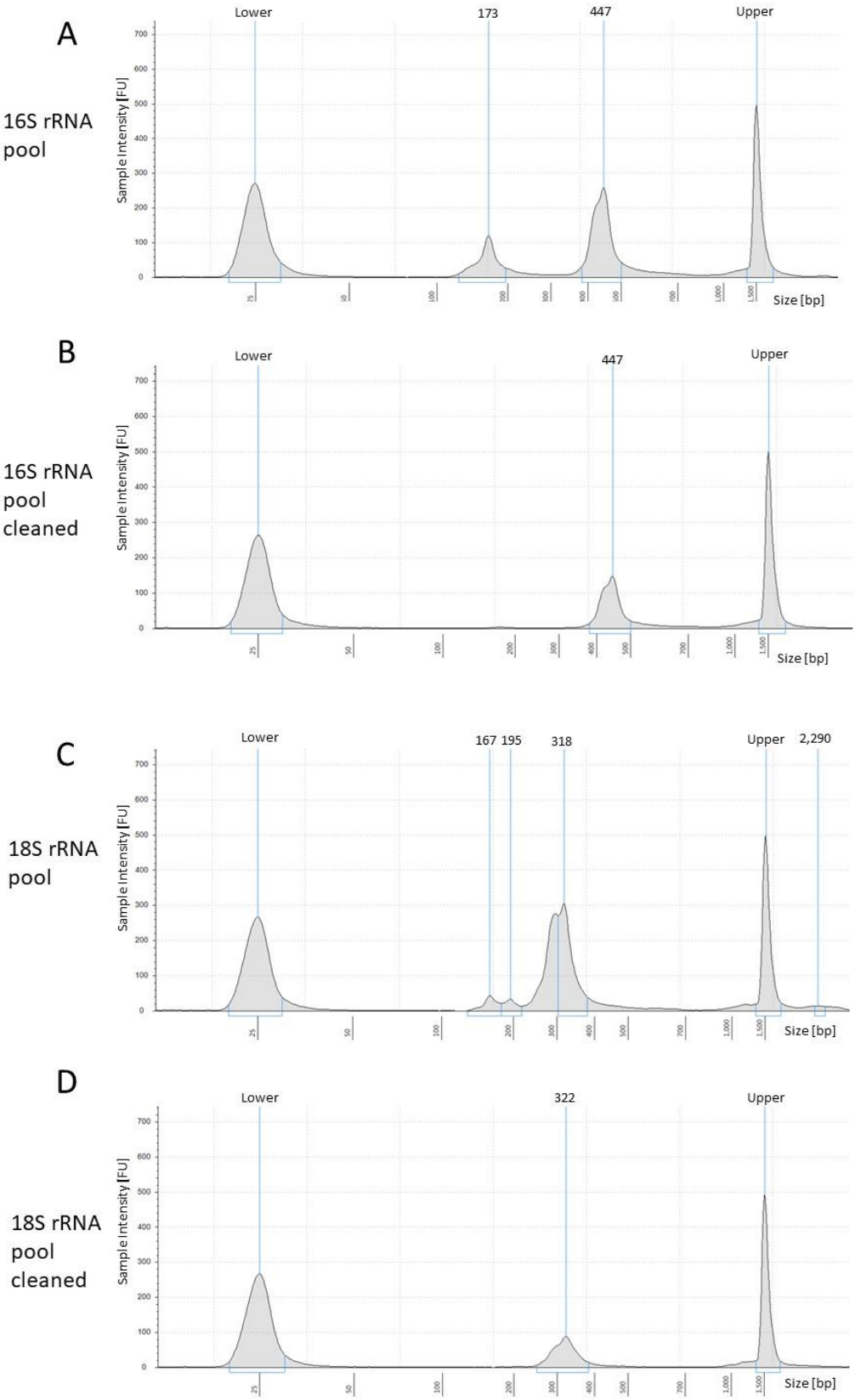


Figure 3.2. Tapestation quantification before and after pooled libraries were cleaned with Ampure Beads. (A) 16S rRNA pool before cleaning. (B) 16S rRNA pool after cleaning. (C) 18S rRNA pool before cleaning. (D) 18S rRNA pool after cleaning. The Upper and Lower markers and the size of the bands are indicated. 16S rRNA PCR product is 447 bp and 18S rRNA PCR product is around 320 bp. The figure shows how unspecific bands that appear on the first pool were eliminated after the clean-up.

3.4 Sequencing results represent all samples but show differing sequencing depths

Amplicon sequencing on the Miseq platform yielded a cluster density of 728±23 K/MM². A total number of 15,378,196 reads were generated of which 13,478,243 passed the quality filter in the automatic Miseq workflow. 78 % of these were assigned to an index with a coefficient of variation for the number of counts across all indices of 0.65, the lowest representation being 0.049 and the highest 3.412(Figure 3.3).

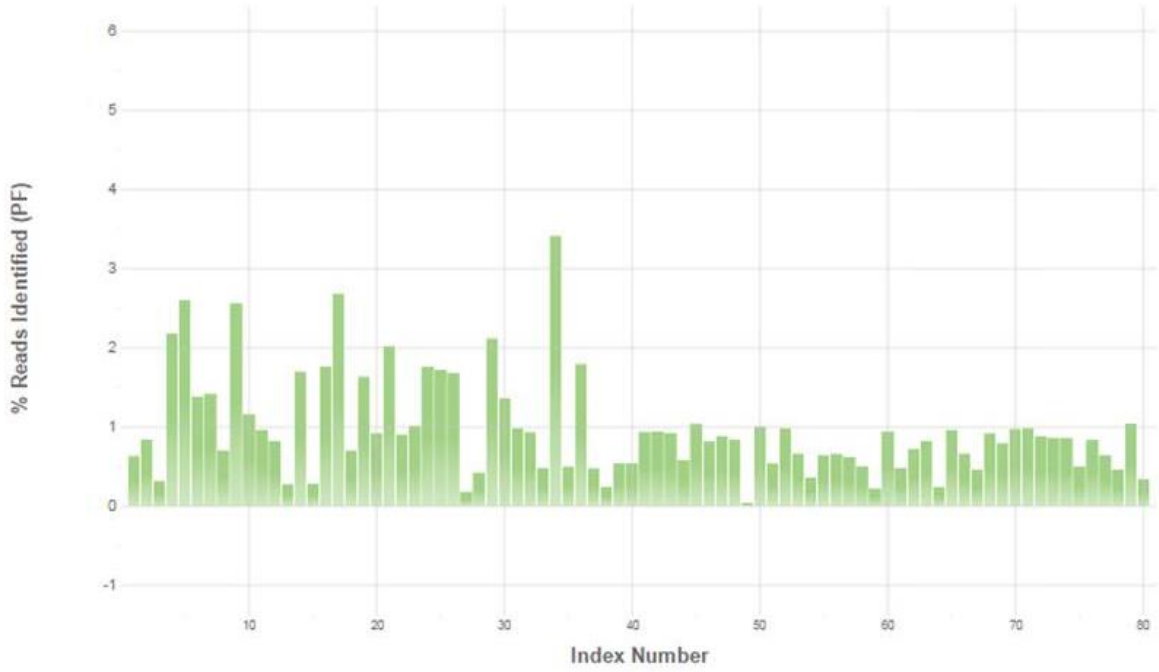


Figure 3.3: Percentage of reads passing quality filter assigned to each sample. The graph was automatically generated after the sequencing run. Index number represents a unique number assigned to each index combination (1-40 correspond to 16S libraries, 41-80 correspond to 18S libraries).

Further sequence analysis were performed using QIIME (Caporaso *et al.* 2010). Quality filtering has been shown to improve downstream analysis (Bokulich *et al.* 2013). With this purpose sequences were passed through the script "split_libraries_fastq.py" modifying the minimum Phred quality score "q" to the value of 19, following the Earth Microbiome Project recommendations, and leaving all the other parameters with default values (maximum number of ambiguous bases "n"= 0, maximum number of consecutive low quality base calls "r"= 3 and percentage of consecutive high quality base calls = 75% (Bokulich *et al.* 2013)) resulting in 5,556,254 16S sequences and 3,139,800 18S sequences. Chimera checking was carried out using USEARCH (Edgar 2010) and an additional 7970 and 1178 sequences were removed from the 16S and 18S datasets respectively leaving a final number of 5,548,284 sequences in the 16S dataset and 3,138,622 sequences in the 18S dataset.

The next step was to cluster the sequences into operative taxonomic units (OTUs) based on sequence identity at a certain threshold. For 16S data 97% was selected as a threshold as it is conventionally assumed to be representative of bacterial species (Drancourt *et al.* 2000). For 18S data 99% was selected instead following common practice in other studies in the scientific community (Valster *et al.* 2009, Valster *et al.* 2011). In both cases the closed-reference approach, which matches sequences to an existing database of reference sequences, was used. Although a substantial amount of good reads might be excluded by using closed-reference OTU picking it represents a screen for contaminants and strict quality filter (Bik *et al.* 2012).

The database chosen was SILVA (Quast *et al.* 2013), which presents the advantage of including all three domains of life (*Eukaryota*, *Archea* and *Bacteria*) for small subunit

rRNA gene (SSU) sequences. Similar domain-specific databases such as Greengenes and Ribosomal Database Project (RDP-II) only cover *Achaea* and *Bacteria* and are thus not fit to process 18S sequences (Quast *et al.* 2013). The SILVA databases are made available as releases and the release 123 was used as it was the most recent release that had been formatted to be used in Qiime at the time the analysis was performed. 5,269,737 16S reads were clustered and assigned to 8,338 OTUs. 2,458,748 18S reads were clustered and assigned to 1,999 OTUs. The average sequence count per sample was 131,743±99,212 for the 16S dataset and 61,468±29,560 for the 18S dataset and the sequence count for each sample is shown in table 3.2. The relatively large standard deviations reflect the variability in sequencing depth in the samples, particularly regarding the 16S dataset.

Table 3.2: Sequence count for each sample in the 16S and 18S datasets after OTU picking.

Week	Sample type	16S sequence count	18S sequence count
1	Shed bulk water	68,304.0	106,182.0
1	Anteroom bulk water	88,420.0	81,174.0
1	Biofilm (nipple drinker)	29,295.0	104,676.0
1	External nipple drinker	259,022.0	62,882.0
1	Biofilm (swab)	303,984.0	97,783.0
2	Shed bulk water	157,671.0	51,314.0
2	Anteroom bulk water	143,062.0	94,129.0
2	Biofilm (nipple drinker)	48,252.0	71,781.0
2	External nipple drinker	309,186.0	3,990.0
2	Biofilm (swab)	127,714.0	98,053.0
3	Shed bulk water	119,140.0	53,179.0
3	Anteroom bulk water	90,184.0	93,994.0
3	Biofilm (nipple drinker)	17,589.0	50,898.0
3	External nipple drinker	197,736.0	26,098.0
3	Biofilm (swab)	29,550.0	59,582.0
4	Shed bulk water	221,505.0	50,848.0
4	Anteroom bulk water	275,260.0	48,657.0
4	Biofilm (nipple drinker)	47,473.0	27,632.0
4	External nipple drinker	187,159.0	24,260.0
4	Biofilm (swab)	110,335.0	100,963.0
5	Shed bulk water	235,728.0	58,183.0
5	Anteroom bulk water	59,592.0	49,286.0
5	Biofilm (nipple drinker)	109,148.0	60,946.0
5	External nipple drinker	208,021.0	27,482.0
5	Biofilm (swab)	207,077.0	20,624.0
6	Shed bulk water	192,547.0	79,153.0
6	Anteroom bulk water	7,107.0	38,602.0
6	Biofilm (nipple drinker)	43,359.0	78,952.0
6	External nipple drinker	251,123.0	90,558.0
6	Biofilm (swab)	155,577.0	84,418.0
7	Shed bulk water	112,040.0	117,740.0
7	Anteroom bulk water	105,904.0	53,694.0
7	Biofilm (nipple drinker)	33,026.0	72,078.0
7	External nipple drinker	407,651.0	90,361.0
7	Biofilm (swab)	47,980.0	53,087.0
-	Bijou tube sonication control	201,818.0	16,901.0
-	Microcentrifuge tube sonication control	24,872.0	46,910.0
-	Filter control	10,213.0	18,900.0
-	Swab control	19,669.0	81,003.0
-	DNA extraction kit control	6,444.0	11,795.0

3.5 Use of square root vs. fourth root transformation for downstream analysis have little effect on the overall microbial community landscape of the broiler house DWS

A non-parametric multivariate framework (Clarke 1993) was used to analyse temporal and spatial differences/similarities in microbial community structure using Primer 7 software (Clarke and Gorley, 2015). Number of OTU reads were divided by total number of reads in each sample to obtain relative abundance profiles in order to make the sample comparison fairer for samples obtained in different ways and with different sequencing depth. Then the OTU relative counts were either square-root or fourth-root transformed to allow greater contribution of the less abundant species to the among-sample resemblances (Clarke 1993, Clarke *et al.* 2014). The Bray-Curtis coefficient (Bray and Curtis 1957) was chosen as a similarity measure and its relative levels (ranks) were used to construct graphical representations in the form of non-metric multidimensional scaling (nMDS) plots (Clarke 1993).

Figure 3.4 shows nMDS plots for the prokaryotic and eukaryotic communities, constructed separately with the 16S and 18S sequences for each of the two data transformations. The intermediate levels of stress (0.17-0.2) indicate that the plot is a relatively good representation of the overall structure of the samples although care should be taken not to over interpret local structures (Clarke 1993). For the prokaryotic community it can be seen that for both transformations (square root and fourth root) samples seem to cluster mainly according to their sample type, with most biofilm and nipple drinker associated samples forming one cluster. In this case, the use of a more severe transformation (fourth root) which more strongly down-weights high abundance species did not seem to have a major effect on the overall sample structure with the

exception of anteroom bulk water samples in weeks 5 and 6. The rest of the anteroom bulk water samples consistently clustered separately from the samples collected inside the broiler house. Another feature worth noticing is that in both plots the samples that stood out from the biofilm/nipple drinker associated cluster were the biofilm from inside the pipes collected with the swab in weeks 2 and 7 and the biofilm collected from the internal part of the nipple drinker in week 2.

The effects of the different transformations seemed to influence the eukaryotic community more than the prokaryotic community. The nMDS constructed from square-root transformed data in figure 3.4 illustrates that the different sample types (except for anteroom bulk water) split in two groups, one with the early samples (weeks 1-2 for the biofilm from the nipple drinker, 1-3 for the biofilm from the pipe collected with the swab and the external part of the nipple drinker and 1-4 for the shed bulk water) and another one with the samples collected towards the end of the rearing cycle. The bulk water samples in weeks 1 to 4 cluster together with biofilm samples from week 2 but there is a separation in the samples towards the end of the cycle between bulk water samples and biofilm/nipple drinker associated samples.

Under a more severe transformation (fourth root) the main clusters according to sample types were maintained but the two group structure was less apparent, especially for the shed bulk water samples which now formed a smaller cluster with samples closer to each other. Biofilm samples from week 2 remained distinct from their sample type cluster but they were less similar to the early shed bulk water clusters. In both transformations, samples from the anteroom bulk water clustered separately from the samples collected inside the boiler house with the exceptions of weeks 1 and 6.

Spearman rank correlation was calculated to determine the effect of transformation on the resulting ranks in the resemblance matrices. As expected from the nMDS plots, the two resemblance matrices deriving from the two different transformations showed a Spearman correlation of 0.92 for the eukaryotic community and 0.95 for the prokaryotic community indicating that the two transformations have little effect on the community structure landscape. The use of a heavier transformation was motivated by the interest in detecting changes in a wider range of species maybe at risk of allowing a greater contribution of the low abundance sequences that are more likely to be erratic. However, based on the high correlations in the resemblance matrices under the different transformations it was deemed appropriate to carry out further analyses using square root transformed data.

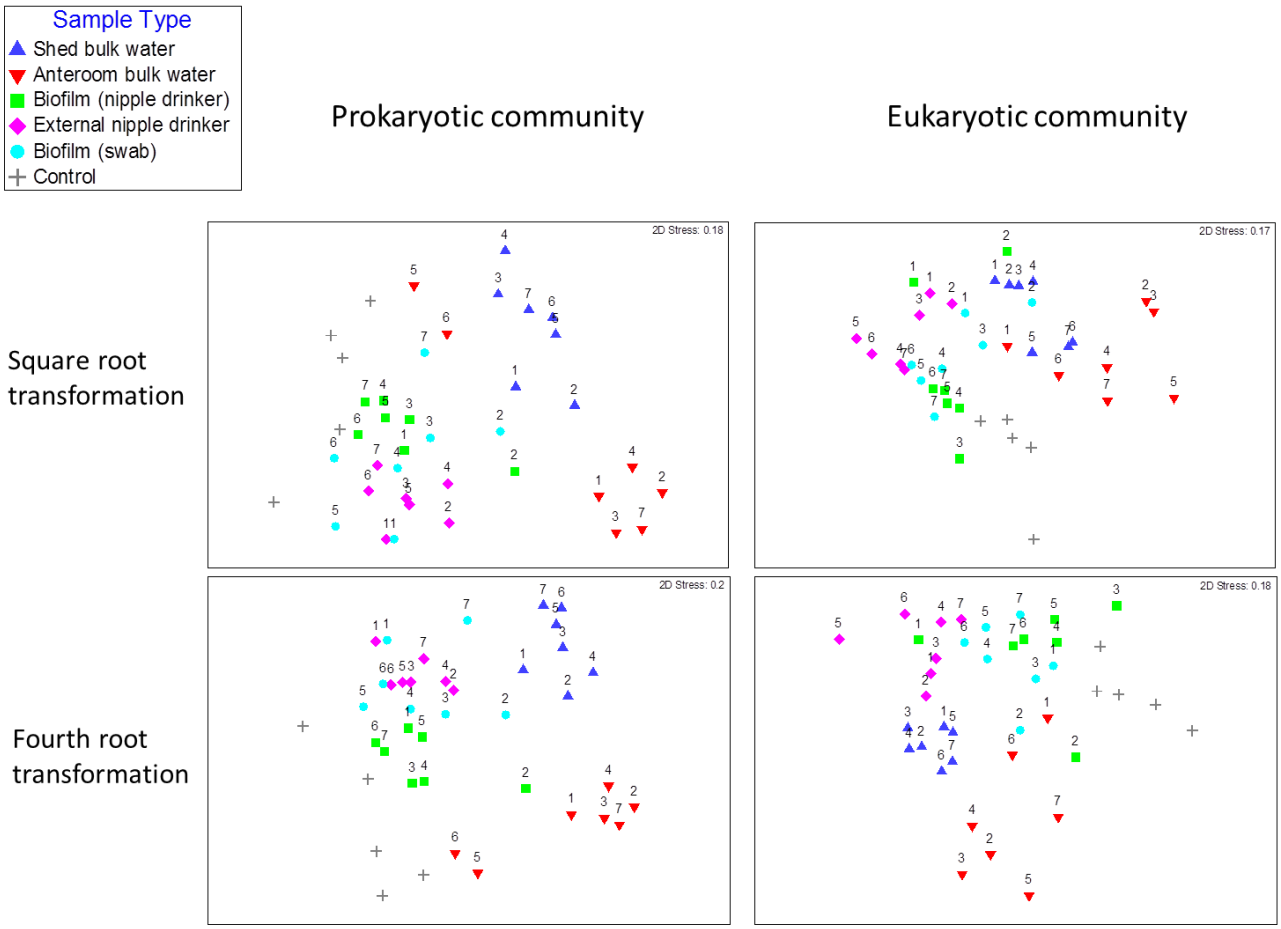


Figure 3.4: Non-metric two-dimensional scaling plots for the prokaryotic (16S) and eukaryotic (18S) communities associated with the DWS of the farm. Bray-Curtis similarities of the relative abundance were calculated after square root or fourth root transformation and used to generate the plots using Primer 7 (Clarke and Gorley, 2015). Symbols represent individual samples and are coloured based on sample type. Sampling week is indicated by a number next to each sample.

3.6 Microbial communities in the DWS of the broiler house are niche-specific and vary across the rearing cycle

Previous studies have found that biofilm and bulk water samples harbour distinct communities (Emtiazi *et al.* 2004, Henne *et al.* 2012). Physical parameters such as temperature (Pinto *et al.* 2014) and flow rate (Douterelo *et al.* 2013) have also been reported to affect community structure. In our study farm these parameters changed across the rearing cycle and antibiotics and vaccinations were administered through the DWS at different time points. Therefore it was hypothesized that microbial communities would vary across the rearing cycle and that different environmental niches would harbour different microbial communities as suggested in the nMDS plots. Formal statistical testing for these hypotheses was conducted through analysis of similarities (ANOSIM) tests, a range of Mantel-type randomization or permutation procedures making no distributional assumptions and depending only on rank similarities among the samples (Clarke and Green 1988, Clarke 1993). This test is commonly used to identify statistical differences between microbial communities in DWDS (Douterelo *et al.* 2013, Revetta *et al.* 2013, Buse *et al.* 2014a).

A first approach to test for global community differences among all the farm samples is through a two-way crossed ANOSIM in the special case of no replication (Clarke and Warwick 1994). There are two factors in the experimental design: the sample type and the sampling week and thus two tests of null hypothesis (Ho) (Ho1: there are no differences in communities in the different environmental niches (allowing for differences across the rearing cycle) and Ho2: there are no differences in communities across the rearing cycle (allowing for differences in the environmental niches)). A two-

way crossed ANOSIM test allows for differences among one factor to be detected independently from the variation due to the other factor through constrained randomisation. The Spearman correlation coefficient (rho) statistic was used to reflect the agreement of pattern among the groups defined by one factor across the separate levels defined by the other factor (Clarke and Warwick 1994).

When the two-way crossed ANOSIM test was applied to test for the null hypothesis that different environmental niches did not harbour distinct communities, allowing for the fact that there may have been temporal variation resulted in a significant rho_{av} value of 0.513 (p=0.00001, 99999 permutations) for the prokaryotic community and 0.696 (p=0.00001, 99999 permutations) for the eukaryotic community. Ho1 was thus rejected at virtually any significance level.

Anteroom bulk water samples were eliminated from the dataset before testing Ho2 as the source water collected in the anteroom was not subjected to the environmental changes related to the rearing cycle. Ho2 can be tested against an ordered alternative which might be more appropriate when a serial change in community composition is suspected as it restricts the alternative to a smaller set of possibilities and increases the chances of accurately detecting and measuring its magnitude (Somerfield *et al.* 2002). The statistic used in this case was R° single, as there was no replication, and the test is based on whether there is a serial trend in the multivariate pattern of the samples across the seven weeks. The ordered test for the null hypothesis that there were no differences across weeks allowing for the fact that there may have been different microbial communities in the different environmental niches was also significant with an average R° of 0.329 (p=0.002, 99999 permutations) for the prokaryotic community and 0.668

(p=0.00001, 99999 permutations) for the eukaryotic community. In this case, the non-ordered test for the same hypothesis was again significant with a rhoav of 0.415 (p=0.002, 99999 permutations) for prokaryotic communities and 0.671 (p=0.00001, 99999 permutations) for eukaryotic communities.

The two-way ANOSIM in the special case of no replication has enough sensitivity to detect the effects of both factors, as illustrated by the low significance levels, due to reasonable levels of overall replication to generate sufficient permutations. The results indicate that there are clear differences in the microbial communities that inhabit the different environmental niches sampled (the bulk water and different biofilms), reflecting a similarity in pattern for each sample type across the 7-week rearing cycle. This trend is clearly seen from the nMDS plots where samples cluster mainly according to their sample type (Figure 3.4).

The statistical tests also support the notion of a dynamic structure, with clear temporal shifts observed. Although less apparent than the clustering for the different sample types the ordination also suggests a common weekly pattern across the different sample types, more easily seen when the anteroom bulk water and control samples were not included (figure 3.5). A consistent pattern of temporal variation across the rearing cycle was observed for biofilm and external nipple drinker samples, while shifts in bulk water communities exhibited a different trajectory especially regarding the eukaryotic communities. It is easy to infer from figure 3.4 that these patterns were not shared by the source water communities.

The interpretation of both ordered and non-ordered tests for the temporal community changes indicated that part but not all the difference in community patterns across the rearing cycle could be explained by serial change during the seven weeks.

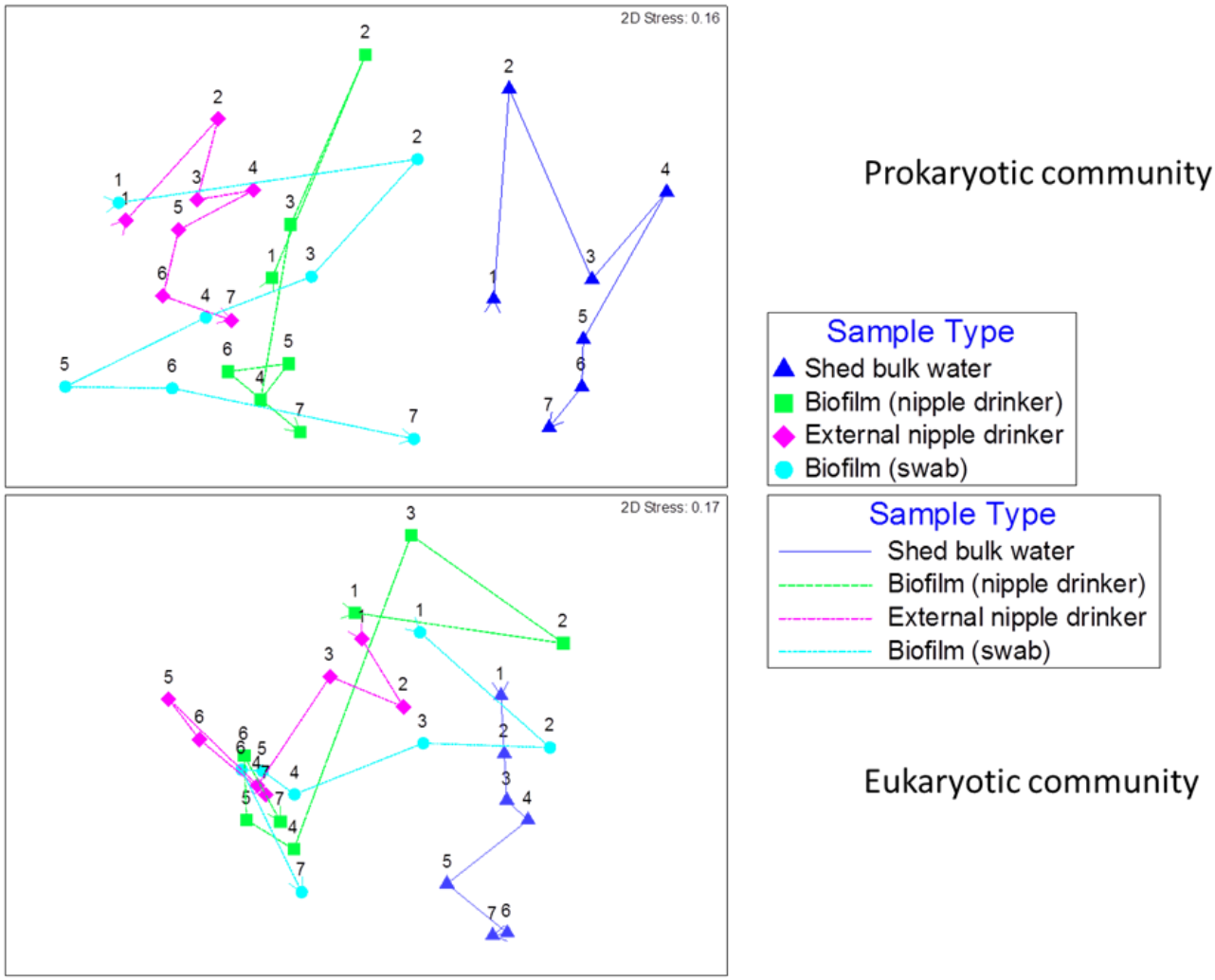


Figure 3.5: Non-metric two-dimensional scaling plots for the prokaryotic (16S) and eukaryotic (18S) communities inside the broiler house. Bray-Curtis similarities of the relative abundance were calculated after square root transformation and used to generate the plots using Primer 7 (Clarke and Gorley, 2015). Symbols represent individual samples and are coloured based on sample type. Sampling week is indicated by a number next to each sample and the trajectory from week 1 to 7 was drawn separately for each sample type. Anteroom bulk water samples and sample controls are not shown.

3.7 Microbial communities from the DWS differ from the sample controls

An alternative way of testing for the variation across the different sample types is to treat the different weeks as replicates and carry out a one-way ANOSIM. This test calculates the value of the statistic R for the data after permuting the labels to then compare the real value of the statistic to the permutation distribution under the null hypotheses of no differences in community composition among samples (Clarke and Green 1988). In this way the global test for the null hypotheses “no differences in microbial communities across sample types” could be rejected at the 0.01% significance level with 99999 permutations as it generated values for the R statistic of 0.615 and 0.57 for the prokaryotic and eukaryotic communities respectively.

The advantage of this approach is that it allows pairwise tests to be performed after obtaining a significant result on the global test (Clarke and Warwick 1994). Table 3.3 shows the R values obtained for each pairwise test comparing different sample types from the DWS. The value of the R statistic is a comparative measure of the degree of separation of the communities being compared (Clarke 1993). Clarke and Gorley (2015) argue that the value of the R statistic is at least as important as its statistical significance especially when a large amount of pairwise tests has been carried out. The R value itself is not unduly affected by the number of replicates included in the comparison, in sharp contrast with the statistical significance, which depends upon the replication levels in the study. This, in turn, determines the number of possible permutations (35 permutations are needed for a standard 5% level test ($p < 0.05$) (Clarke 1993)). After a statistically significant result in the global test the best tool for examining between-group variation is

an examination of the R value for each pairwise comparison. Large values close to 1 indicate complete separation and the smaller the value the less variation among communities.

In this study the considerations discussed above regarding p-values are especially important when considering the pairwise tests involving controls because there are reduced numbers of replicates in the tests. Each pairwise comparison with any sample type and the control group only includes those controls that are relevant for each particular sample type. Table 3.3 shows that the R statistic values resulting from comparisons with the control group were large and significant in most cases, indicating a high variation in community structure between groups. The only exception was observed when comparing the biofilm collected from the internal part of the nipple drinker and its controls. A modest degree of separation can be inferred for the prokaryotic communities (R=0.389, p=0.05). However an R value of 0.071 observed for the eukaryotic communities strongly suggests that communities in these two sample types were not significantly different.

3.8 The microbial communities from the source water differ from the microbial communities inside the broiler house

Comparisons between the rest of the groups confirmed that the microbial communities from the source water collected from the anteroom before water entered the broiler house differed from all environmental niches inside the broiler house. This suggested that distinct microbial communities develop inside the DWS on the broiler farm. Perhaps not surprisingly the highest differences were observed among the anteroom bulk water and the external part of the nipple drinkers (R=0.859 for 16S and R=0.928 for 18S) which are

in direct contact with the farm environment and the chickens themselves. However the source water communities also differed consistently from the two types of DWS biofilm samples (R=0.736/0.737 for 16S and R= 0.719/0.778 for 18S) and from the shed bulk water (R=0.777 for 16S and R=0.634 for 18S).

3.9 Biofilm associated microbial community composition is statistically indistinguishable in two DWS sample types and significantly different from bulk water

Considerable variation was observed between different environmental niches sampled within the broiler house. Perhaps the most striking observation was that the biofilm communities obtained from inside the DWS with different methods were not statistically different from each other (R=0.013 for 16S and R= -0.055 for 18S). This is in spite of the different surface materials (stainless steel nipple drinker and plastic pipe) and DNA extraction methods employed. Interestingly the external nipple drinker communities also showed little separation in community structure from the DWS biofilm communities, particularly with those collected by swabbing (R=0.135 for 16S and R=0.129 for 18S) and to a lesser extent to those from the internal part of the nipple drinker (R=0.484 for 16S and R=0.201 for 18S). This may reflect transfer of microorganisms from chickens when drinking from the nipple drinkers that may enter the DWS and vice versa.

The shed bulk water samples from inside the broiler house were confirmed to differ from the DWS biofilm associated samples. The highest differences were observed between shed bulk water and the external part of the nipple drinkers (R= 0.966 for the 16S and R=0.797 for the 18S), followed by the comparison with the internal part of the nipple

drinker (R=0.845 for the 16S and R=0.742 for the 18S) and the biofilm samples collected by swabbing (R=0.567 for 16S and R=0.605 for 18S).

Table 3.3. Pairwise comparisons of one-way ANOSIM comparing the different sample types collected on the farm DWS.

B Eukaryotic communities

SAMPLE	Shed bulk water	Anteroom bulk water	Biofilm (nipple drinker)	External nipple drinker	Biofilm (swab)	Control
Shed bulk Water	0 0	**0.63** 0.0006 [1716]	**0.74** 0.0006 [1716]	**0.8** 0.0006 [1716]	**0.61** 0.0006 [1716]	**1** 0.028 [36]
Anteroom bulk water	**0.78** 0.001 [1716]	0 0	**0.72** 0.001 [1716]	**0.93** 0.001 [1716]	**0.78** 0.0006 [1716]	**0.71** 0.028 [36]
Biofilm (nipple drinker)	**0.85** 0.0006 [1716]	**0.74** 0.002 [1716]	0 0	**0.2** 0.033 [1716]	**-0.06** 0.712 [1716]	**0.07** 0.3 [120]
External nipple drinker	**0.97** 0.0006 [1716]	**0.86** 0.0006 [1716]	**0.48** 0.0006 [1716]	0 0	**0.13** 0.115 [1716]	**1** 0.008 [120]
Biofilm (swab)	**0.57** 0.0006 [1716]	**0.74** 0.001 [1716]	**0.01** 0.4 [1716]	**0.14** 0.044 [1716]	0 0	**0.92** 0.028 [36]
Control	**0.92** 0.028 [36]	**0.72** 0.028 [36]	**0.39** 0.05 [120]	**0.86** 0.008 [120]	**0.7** 0.028 [36]	NA NA

1 0.9 0.8 0.7 0.6 0.5 0.4 0.3 0.2 0.1 0 -0.1 -0.2

A Prokaryotic communities

Bray-Curtis similarities of the relative abundance were calculated after square root transformation and used for the analysis. For the comparison of each sample type with the controls a subset including only the relevant controls were selected before carrying out the test. (A) 16S amplicon data (B) 18S amplicon data. The number in bold corresponds to the R value, followed by the p-value and the number in brackets is the number of permutations carried out for the test.

3.10 Microbial community temporal variation is greater at the beginning of the cycle

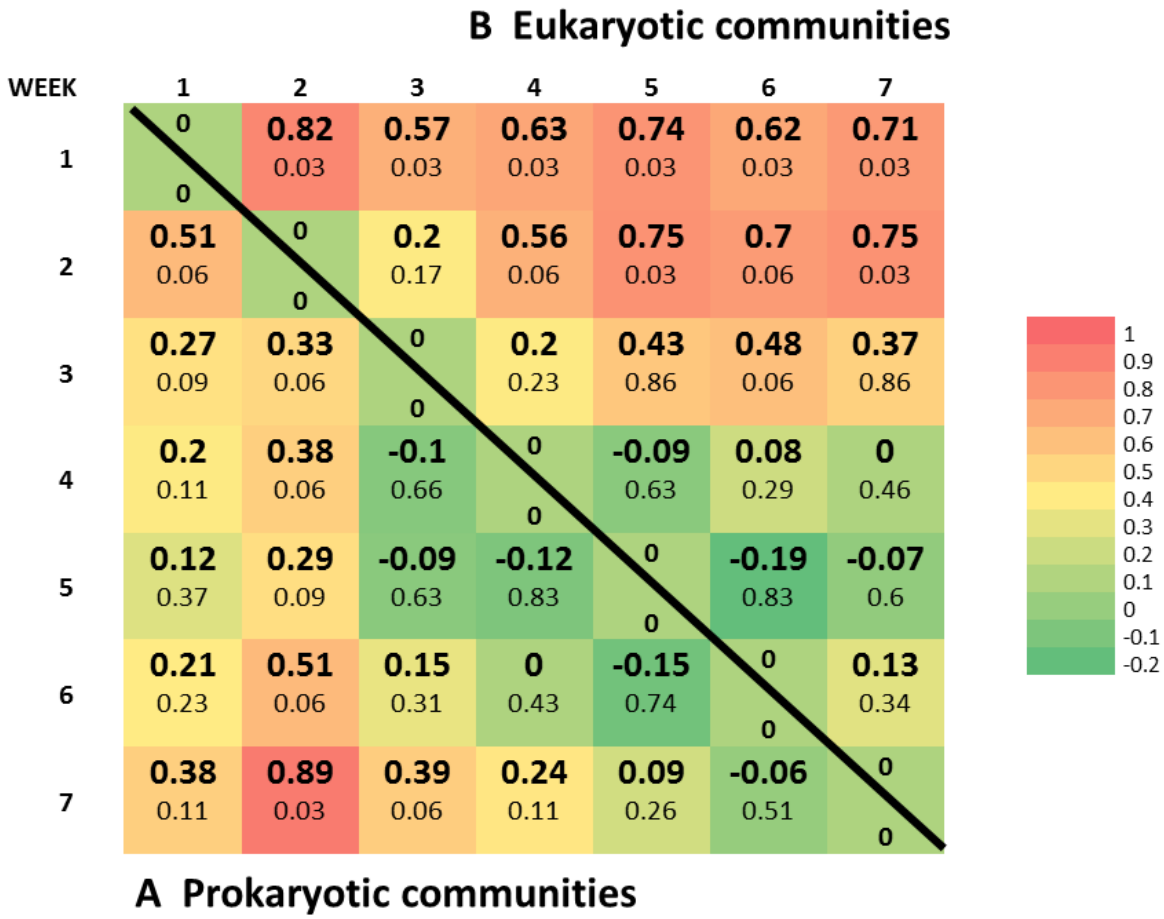
Variation from week to week during the rearing cycle was also analysed using a one-way ANOSIM with the null hypothesis of "no changes in microbial communities across the rearing cycle". Both ordered and non-ordered tests produced virtually identical results. Only the non-ordered test is reported here. Again Anteroom bulk water samples were eliminated as they were not subjected to the change of conditions in the shed during the rearing cycle. In this case the global test gives a sample statistic R of 0.188 (p=0.02, 99999 permutations) and 0.373 (p=0.00009, 99999 permutations) for the prokaryotic and eukaryotic communities. Table 3.4 shows the results of the pairwise tests. These results have to be considered taking into account the relative merits of the absolute value of R and the significance level as in this case only 35 permutations are possible.

The bacterial community structures observed in week 2 seemed most different to those observed on any of the other weeks (R= 0.29-0.89). Eukaryotic communities were most divergent in samples collected at week one compared to other weeks during the rearing cycle (R=0.57-0.82). In general, more divergence was observed at the beginning of the rearing cycle (weeks 1 and 2) than towards the end. A big shift in community structure occurred between week 1 (before chickens were placed on the farm) and week 2 (R=0.51 for 16S and R=0.82 for 18S). Interestingly the R values for this community shift were higher than the comparisons from week 1 and any of the other weeks across the rearing cycle (table 3.4). R values were consistently higher in the eukaryotic community suggesting more marked temporal changes.

Regarding the prokaryotic communities, there was not statistical evidence to distinguish communities in weeks 3, 4 and 5 (3 vs. 4 R=-0.10, 3 vs. 5 R=-0.09, 4 vs. 5 R=-0.12). In the same way weeks 4, 5 and 6 could not be separated (4 vs. 6 R=0, 5 vs. 6 R=-0.15) nor could weeks 5, 6 and 7 (5 vs. 7 R= 0.09, 6 vs. 7 R= -0.06). However the comparison between weeks 3 and 7 (R=0.39) and weeks 4 and 7 (R=0.24) suggests differences in community structure between these weeks. These findings support the idea of a change in community structure along the rearing cycle.

A slightly different temporal variation pattern was observed in the eukaryotic communities. Weeks 4-7 formed a group that shared highly similar microbial communities with R values between -0.19 and 0.13 (table 3.4) and weeks 6 and 7 harbouring the most different communities inside the group. Eukaryotic communities in week 3 differed significantly from those in the weeks 4-7 (R=0.2-0.48) although a lower R value was observed for the comparison between weeks 3 and 4. Community structure observed in week 2 differed slightly from week 3 (R= 0.2) and considerably from weeks 4-7 (R= 0.56-0.75). The difference in communities between weeks 3 and 4 is of the same magnitude of that of weeks 2 and 3 (R=0.198). The results support the idea of a change in community structure along the rearing cycle with a more prominent shift during weeks 1 and 2.

Table 3.4. Pairwise comparisons of one-way ANOSIM comparing the different sampling weeks.



Bray-Curtis similarities of the relative abundance were calculated after square root transformation and used for the analysis. Samples included in the analysis are Shed bulk water, Biofilm (nipple drinker), External nipple drinker and Biofilm (swab). (A) 16S amplicon data (B) 18S amplicon data. The number in bold corresponds to the R value followed by the p-value. 35 permutations were carried out in all the cases.

3.11 Key shifts in phylum abundance occurred during the rearing cycle

Phylum level analysis of the prokaryotic (figure 3.7) and eukaryotic (figure 3.8) community structure was performed by constructing shade plots with untransformed data. Proteobacteria was the most abundant prokaryotic phylum identified through all the samples, including the contols, followed by Bacteroidetes, Firmicutes and Actinobacteria. Interestingly Bacteroidetes was present in higher relative abundance in biofilm samples compared to bulk water samples, particularly in weeks 3, 4 and 6. Firmicutes were present in relative low abundance compared to Proteobacteria and Bacteroidetes. Remarkably they were detected in a high relative abundance in shed bulk water samples from weeks 5, 6 and 7 and in the biofilm sample collected by swabbing in week 7. At these points Proteobacteria was observed to lose its dominance and Firmicutes became the most abundant phylum. This data shows that a shift towards Firmicutes dominance occurs in the bulk water and later, on the biofilm inside the broiler house towards the end of the rearing cycle. A similar phenomenon was observed for the less dominant Actinobacteria which also showed increased relative abundance in the same samples. Although these four phyla dominated the dataset there were another 31 bacterial phyla and 2 archaeal phyla identified in the samples.

There were distinct differences in pattern of eukaryotic phyla present on different sample types (figure 3.7). Opisthokonta which includes the traditional kingdoms Fungi and Metazoa was the group with highest relative abundance across the samples. The subgroup Nucletmycea, which also includes Fungi showed a high relative abundance in week 1 and also in biofilm samples across the rearing cycle. Conversely the subgroup Holozoa, which includes Metazoa, showed a higher relative abundance in bulk water

samples both in the shed and anteroom. Chloropastida, which includes green algae and land plants, was less dominant across the rearing cycle but was present with a higher relative abundance in the biofilm and nipple drinker associated samples in week 2. Rhizaria, which contains many species of amoeba, appeared to be more abundant in the anteroom bulk water and also in the biofilm collected by swabbing in week 2. Discoba which includes Euglenozoans and others was more abundant in week 2 and the biofilm collected by swabbing in week 7. Discosea which is a group inside Amebozoa seem abundant in some anteroom bulk water samples and in the shed bulk water in week 2. Overall week 2 showed the greatest diversity at this level. The most common groups observed in the samples were also present in the controls. The groups Alveolata and Stramenopiles were more abundant in the controls than in the samples. Although with lower relative abundance several Amebozoan groups and other protists were also detected in the samples.

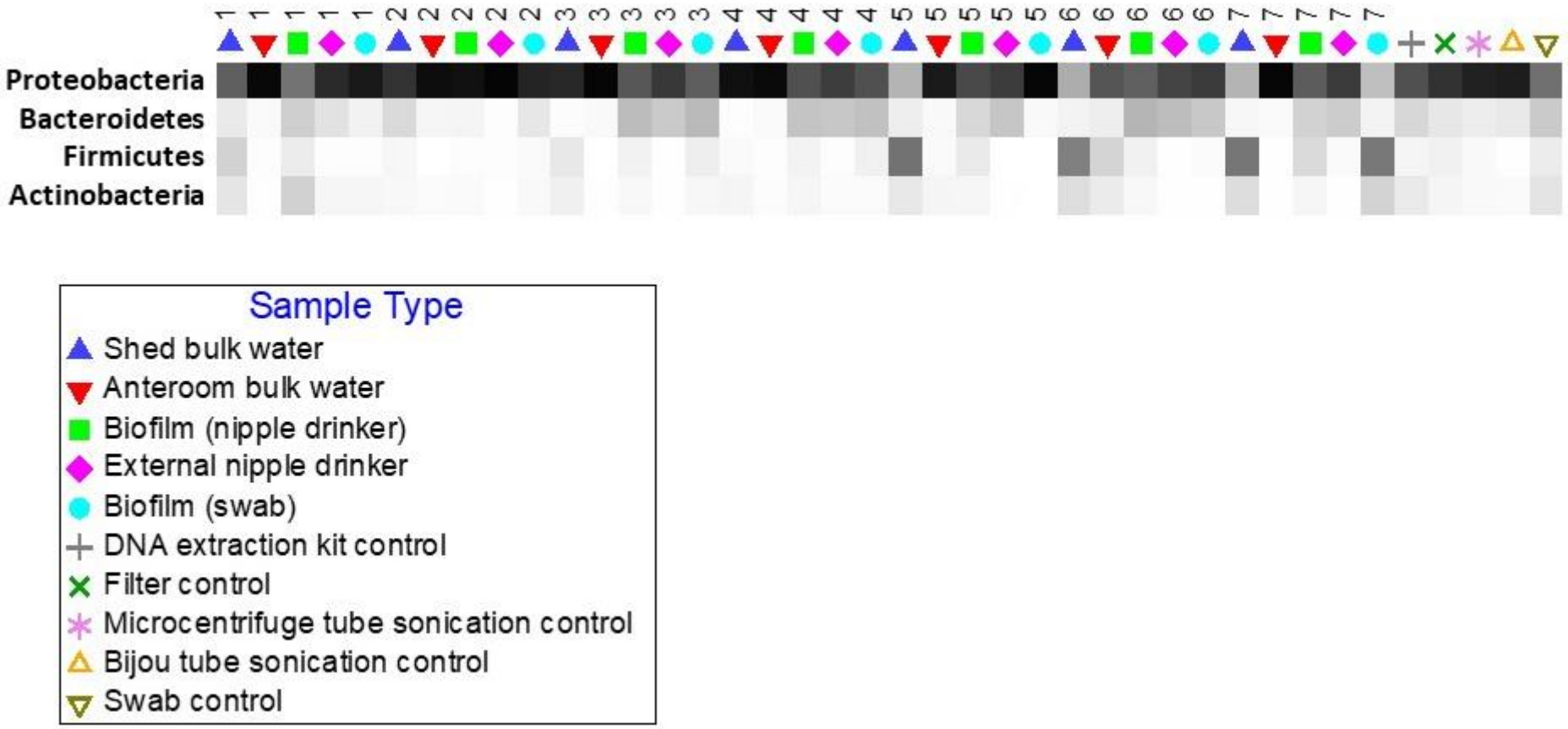


Figure 3.6. Comparison of the relative abundance of the major prokaryotic phyla found on the broiler house DWS. Untransformed relative abundance data was used to generate the shade plot using Primer 7 (Clarke and Gorley, 2015). Symbols represent individual samples and are coloured based on sample type. Sampling week is indicated by a number next to each sample. The colour intensity of scale indicates relative abundance of each phylum. 31 other bacterial phyla and 2 other archaeal phyla were detected but at a level too low to be represented on the shade plot.

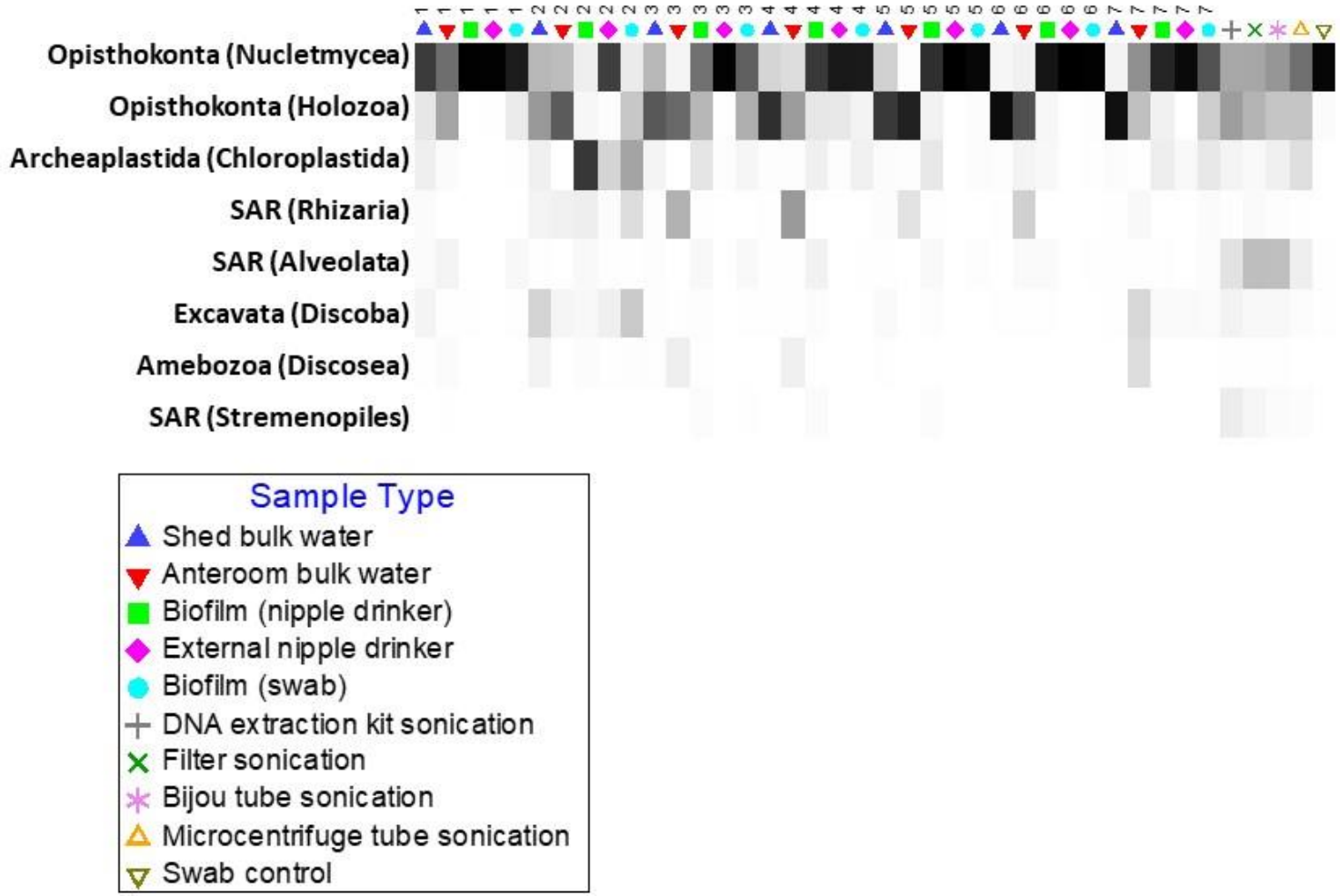


Figure 3.7. Comparison of the relative abundance of the major eukaryotic groups found on the broiler house DWS. Untransformed relative abundance data was used to generate the shade plot using Primer 7 (Clarke and Gorley, 2015). Symbols represent individual samples and are coloured based on sample type. Sampling week is indicated by a number next to each sample. The colour intensity of scale indicates relative abundance of each phylum (white denotes absence and full black represents maximum abundance). 10 other groups were detected but at a level too low to be represented on the shade plot.

3.12 Eukaryotic and prokaryotic composition of the negative controls

The taxonomic composition of the samples at lower taxonomic levels (order, family, genus and species) was also analysed through the construction of shade plots. The greatest strength of these plots is that they accurately represent the dominance patterns of OTUs across all the samples (Clarke and Garley, 2015). The shade plots are thus key to understanding which particular OTUs have contributed to which degree to the multivariate sample analyses. Plots in this study were constructed from a subset of the original OTU table, meaning that different OTUs are identified to different taxonomic levels and some shared taxonomy.

A range of negative control samples were used for this study to control for the different processes that were required to retrieve the biomass from different sample types. Figure 3.8 and 3.9 show shade plots for the control samples in the 16S and 18S dataset. The OTUs present in the DNA extraction kit were also in all the rest of controls although with variable abundances. The most abundant bacterial OTUs in the DNA extraction kit control were *Klebsiella*, *Propinobacterium*, *Acinetobacter*, *Chryseobactreium* and *Pseudomonas* (figure 3.8). Some of them can be found in human skin such as *Klebsiella* (Conlan *et al.* 2012), *Propinobacterium* (Grice *et al.* 2009) and *Acinetobacter* (Costello *et al.* 2009). *Propinobacterium* has been reported as one of the most common DNA extraction kit contaminants in several kits from different companies, including Qiagen (Lusk 2014, Salter *et al.* 2014, Glassing *et al.* 2016). Other bacterial OTUs found in the DNA isolation kit in this study belong to genera that have been previously reported in DNA extraction blank controls are *Klebsiella*, *Acinetobacter*, *Flavobacterium*, *Pseudomonas, Pedobacter*,

Chryseobacterium and *Paracoccus* (Salter *et al.* 2014, Glassing *et al.* 2016). Others have been reported as contaminants in *de novo* assembled genomes, such as *Sphingomonas*, *Pseudomonas*, *Flavobacterium* and *Agrobacterium* (Laurence *et al.* 2014). Some of these such as *Flavobacterium* and *Pseudomonas* have also been reported as laboratory contaminants in ultrapure water systems and PCR reagents (Grahn *et al.* 2003, Laurence *et al.* 2014). To the best of my knowledge, other OTUs reported here such as *Pseudarcicella*, *Moraxella*, Candidatus *Rhodoluna*, *Mycobacterium* and *Albidiferax* have not been commonly reported in blank controls (Salter *et al.* 2014, Glassing *et al.* 2016). Some OTUs showed a higher relative abundance in the filter control such as some of the *Pseudomonas* OTUs and particularly *P. putida*, *Phreatobacter* and the families *Lachnospiraceae* and *Rikenellaceae*. Members of both families have been reported previously in blank controls (Salter *et al.* 2014, Glassing *et al.* 2016) but not *Phretobacter*. Analysis of the shade plots (Figures 3.8 and 3.9) suggested that sonication may have introduced contaminants to the samples, as shown towards the bottom of the matrices. The most relative abundant OTUs in this group were identified as *Escherichia-Shigella,* also identified as potential contaminants before (Glassing *et al.* 2016). Other bacterial taxa that have previously been related to contamination and are associated with the biofilm detachment by sonication are *Rothia, Deinococcus* and *Escherichia coli* and an OTU only classified at family level as a member of *Comomandaceae* (Salter *et al.* 2014, Glassing *et al.* 2016). *Deinococcus,* one of the OTUs identified as *Flavobacterium* and *Rothia nasimurium* seem to be particularly associated with the bijou tube used to sonicate the external part of the nipple drinker. Interestingly *Chlorobiales* was detected in higher relative abundance in both the microcentrifuge tube sonication control used to sonicate the internal part of the nipple drinker and the swab control.

Several of the genera reported here are frequently reported in DWDS studies. For example *Acinetobacter* and *Propinobacterium* which were detected in the controls in this study and others have been reported as abundant genera in biofilms of DWDS (Sun *et al.* 2014). Shaw *et al.* (2014) also detected *Propinobacterium* in a DWDS in spite of stringent measures to reduce laboratory contamination including subjecting the PCR mastermix to UV radiation. On the other hand, genus such as *Pseudomonas* have not only been detected, but are commonly isolated from DWDS (Williams *et al.* 2004, Berthiaume *et al.* 2014). Therefore it can be difficult to discriminate between genuine taxa and contaminants even when negative controls have been processed and care has been taken to minimise contamination.

Figure 3.9 shows the eukaryotic OTUs identified in the different controls. Taxa identified include members of every major group: fungi, protists, plants and animals. Relative to the 16S rRNA sequences identified in the controls, a higher difference in 18S rRNA sample community composition was observed. It is worth to note that some of the fungal taxa identified here have been isolated from drinking water in the past including members of the genus *Phoma*, *Exophiala* and *Penicillum* (Kinsey *et al.* 1998, Doggett 2000, Pereira *et al.* 2009, Oliveira *et al.* 2013). Very little is known about eukaryotic contaminants in sequencing kits, the environment and laboratory reagents.

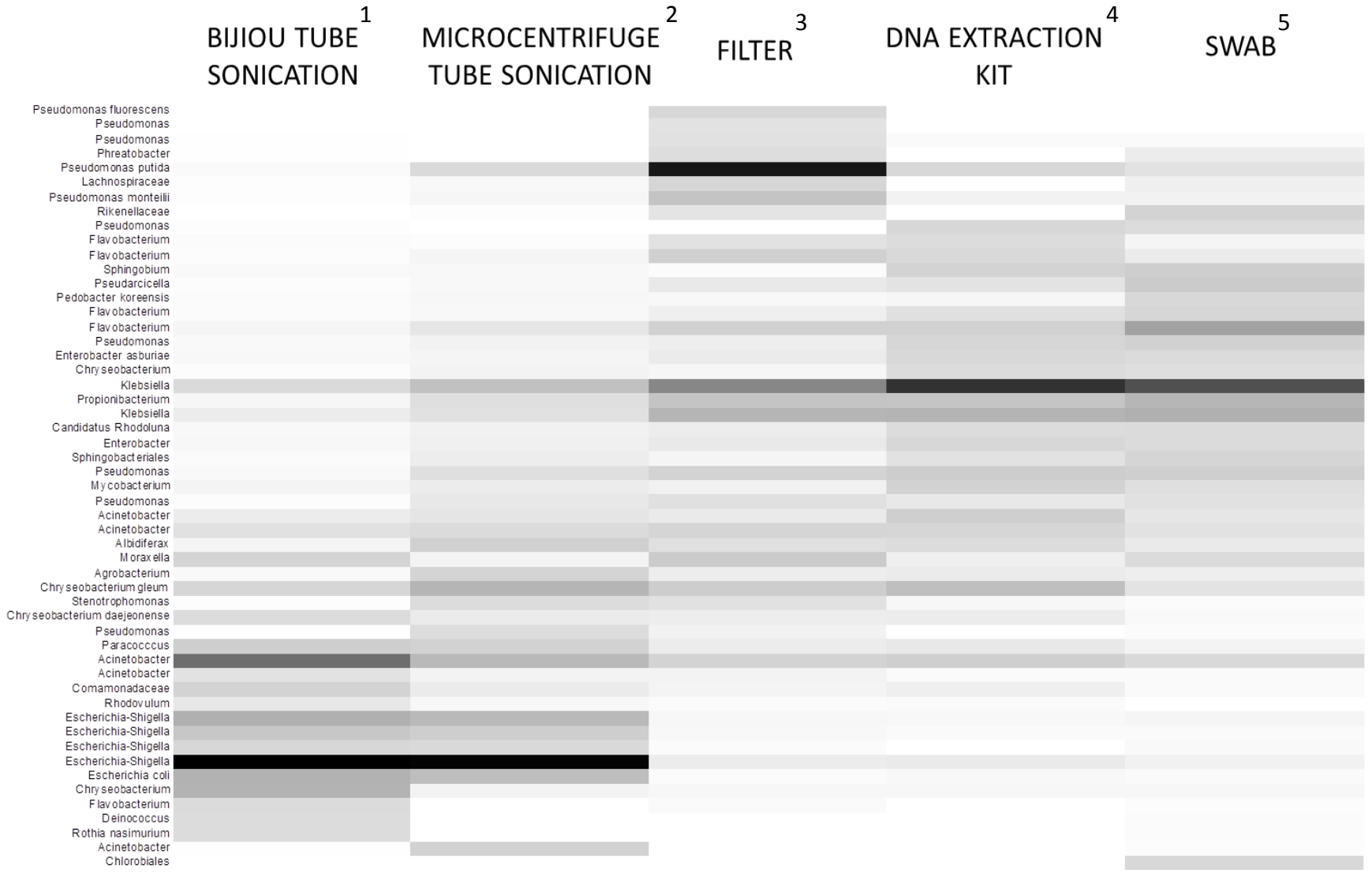


Figure 3.8. Comparison of the relative abundance of the major prokaryotic OTUs found in the controls. Square transformed relative abundance of OTUs accounting at least 0.6% in a sample after transformation was used to generate the shade plot in Primer 7 (Clarke and Gorley, 2015). Both the columns (samples) and rows (OTUs) have been reordered to maximise serial change and aid visualisation and interpretation. The colour intensity of scale indicates relative abundance of each OTU within the subset of the dataset (white denotes absence and full black represents maximum abundance). 1,2 Biofilm biomass was removed from external (bijou) and internal (microcentrifuge tube) nipple drinker components by sonication. Sonication of buffer without the nipple drinker components was used as a control. 3 Biomass from all the water samples and sonicated biofilm samples was collected on filters. The control used a clean filter. 4. Standard DNA extraction kit control. 5. Sterile swabs were used to collect biomass from inside pipes. A sterile swab in the collection buffer was used as a control.

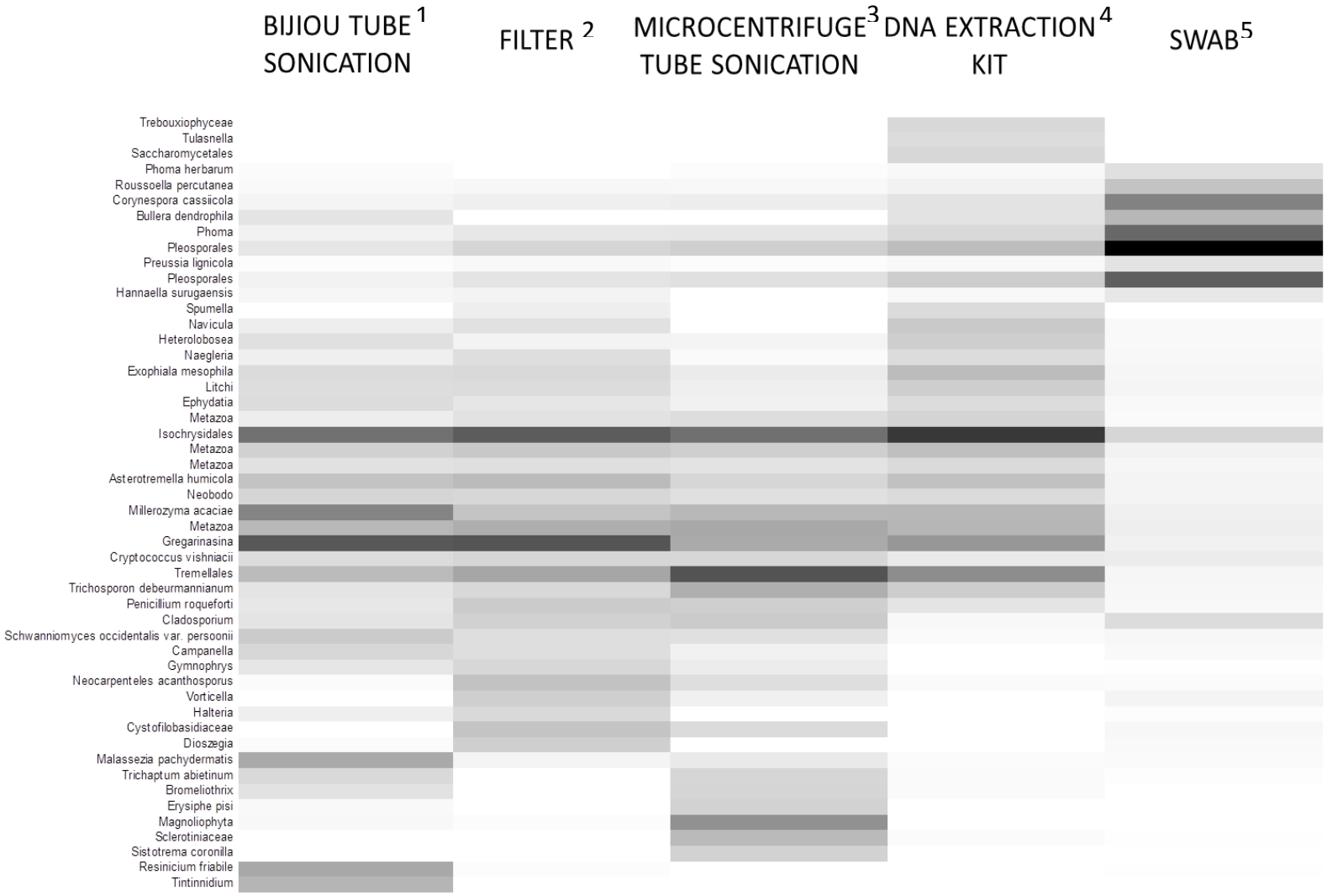


Figure 3.9. Comparison of the relative abundance of the major eukaryotic OTUs found in the controls. Square transformed relative abundance of the 50 most important OTUs after transformation was used to generate the shade plot in Primer 7 (Clarke and Gorley, 2015). Both the columns (samples) and rows (OTUs) have been reordered to maximise serial change and aid visualisation and interpretation. The colour intensity of scale indicates relative abundance of each OTU within the subset of the dataset (white denotes absence and full black represents maximum abundance). 1,3 Biofilm biomass was removed from external (bijou) and internal (microcentrifuge tube) nipple drinker components by sonication. Sonication of buffer without the nipple drinker components was used as a control.2 Biomass from all the water samples and sonicated biofilm samples was collected on filters. The control used a clean filter. 4. Standard DNA extraction kit control. 5. Sterile swabs were used to collect biomass from inside pipes. A sterile swab in the collection buffer was used as a control.

3.13 Taxonomic composition of bulk water communities inside (shed) and outside (anteroom) the broiler house

Community profiles from different niches of the DWS were compared: i) bulk water inside vs. outside the broiler house and ii) bulk water vs. bulk water communities from inside the broiler house.

Firstly the phylogenetic composition of the most relative abundant OTUs in bulk water samples from the anteroom and the shed was compared. The 50 most abundant OTUs in this subset of samples were selected from both the prokaryotic and eukaryotic datasets (figures 3.10 and 3.11). The OTUs that were also identified on the control shade plots are indicated.

Both plots show that, although there was some overlap, bulk water microbial communities inside the chicken shed diverge considerably from those in the source water in the anteroom. Moreover, the community structure inside the broiler house (shed bulk water) shifted across the rearing cycle. Some of the OTUs identified as abundant in the negative controls were also present in bulk water communities (10 out of 50 for the 16S dataset and 14 out of 50 for the 18S dataset).

Figure 3.10 shows the patterns in prokaryotic communities. The anteroom bulk water prokaryotic community was characterized by a higher relative abundance of genera such as *Delftia*, *Pseudomonas* and *Sphingobium*. Moreover, with the exception of weeks 5 and 6 the bacterial composition in this group of samples remained quite uniform. This contrasts with the samples from inside the broiler house where a change in community structure was observed across the rearing cycle. There was a shift in 16S rRNA profile from week 1 to week 2 with some OTUs decreasing in relative abundance, such as

Staphlylococcus, *Lactobacillus*, *Burkholderia*, *Luteibacter* and *Cupriavidus* while others increased including *Aquabacterium*, *Sphingobium*, *Acidovorax* and *Caulobacter*. The profiles recorded for samples collected during weeks 3 and 4 were very similar to each other; characterised by a dominance of *Klebsiella* and *Enterobacter*. Finally profiles observed during weeks 5-7 were also very similar to each other and featured a decrease in *Klebsiella* and *Enterobacter* that dominated bacterial communities in the previous weeks and an increase in *Lactobacillus* and *Staphylococcus*. *Mycobacterium* was detected in constant relative abundance throughout the seven weeks.

Some of the most abundant prokaryotic taxa detected on the farm samples (figure 3.10) were also present in the negative controls. These include *Mycobacterium*, *Enterobacter*, *Klebsiella*, *Sphingobium* and *Pseudomonas*. The distribution of these taxa was not uniform across the samples, but high numbers were observed in particular sample groups. Therefore, it could be argued that these taxa were not introduced as contaminants and were indeed present in the bulk water of the farm DWS.

Figure 3.11 shows the 50 most abundant eukaryotic OTUs detected in the bulk water samples. Although a difference between the community profiles of the source water (anteroom) and the shed bulk water (inside the broiler house) was still observed there was greater overlap between these niches compared to the prokaryotic profiles. However this overlap was mainly found to be due to the presence of OTUs detected in the negative controls and this uniform distribution across samples raised concerns about the possibility that they might have been introduced during processing. If these OTUs are ignored source water communities again shared a fairly homogeneous community structure across the weeks. A high number of OTUs could not be classified beyond Metazoa. However protist taxa related to amoeba were represented by the genera

Acanthamoeba, *Cryptodifflugia* and *Assulina* and the order Euglyphida. *Phialophora*, a member of the fungal phylum Ascomycota, was also abundant in the source water samples collected in the anteroom. In the shed bulk water communities, however, several yeast species belonging to the genera *Candida*, *Nakazaweae* and *Debaryomyces* were among the most abundant eukaryotes. Taxa related to amoeba were represented by the genera *Acanthamoeba* and *Vannella*. In weeks 3 and 4 an increase was observed in relative abundance of the yeast *Candida*. An increase in the relative abundance of an OTU identified as an algae belonging to the order Isochrysidales was observed in samples collected during weeks 5-7.

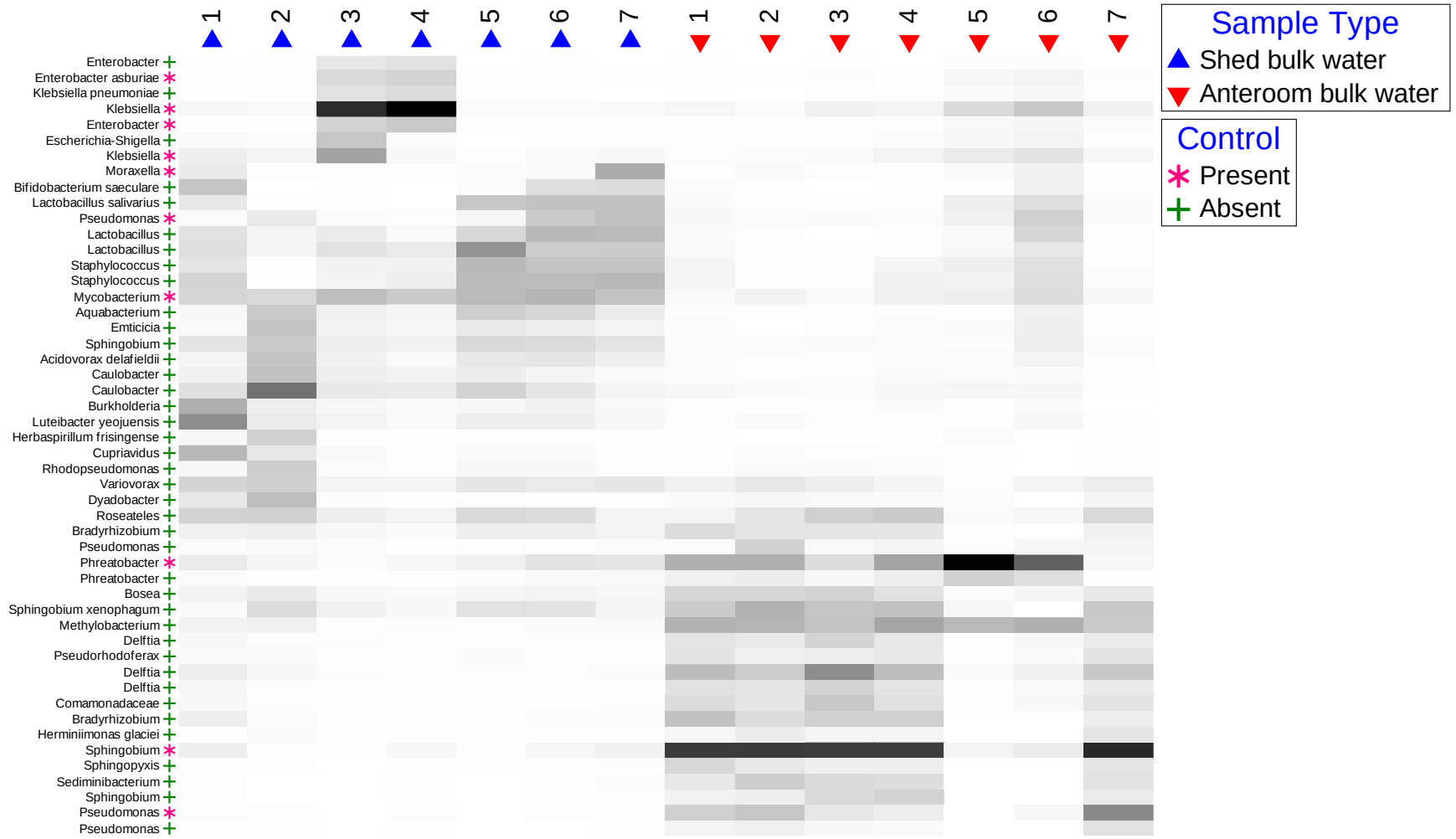


Figure 3.10. Comparison of the relative abundance of the major prokaryotic OTUs found in the bulk water. Square transformed relative abundance of the 50 most important OTUs after transformation was used to generate the shade plot in Primer 7 (Clarke and Gorley, 2015). OTUs were re-ordered to maximize serial variation, but samples were ordered temporally. Triangles and inverted triangles represent individual samples and are coloured based on sample type. Sampling week is indicated by a number next to each sample. The presence of OTUs included in the shade plot of the control samples is indicated with a star. The colour intensity of scale indicates relative abundance of each OTU within the subset of the dataset (white denotes absence and full black represents maximum abundance).

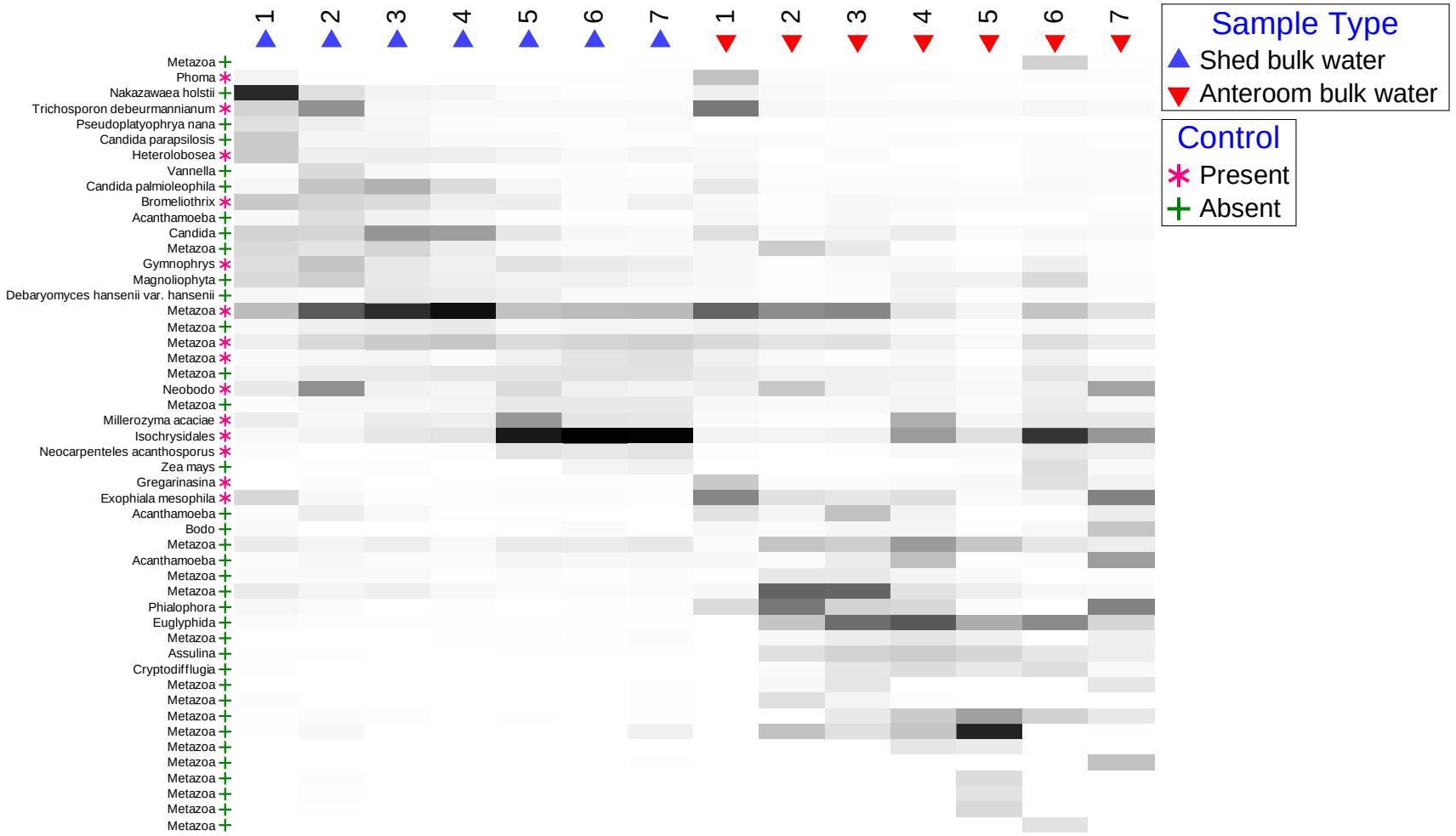


Figure 3.11. Comparison of the relative abundance of the major eukaryotic OTUs found in the bulk water. Square transformed relative abundance of the 50 most important OTUs after transformation was used to generate the shade plot in Primer 7 (Clarke and Gorley, 2015). OTUs were re-ordered to maximize serial variation, but samples were ordered temporally. Triangles and inverted triangles represent individual samples and are coloured based on sample type. Sampling week is indicated by a number next to each sample. The presence of OTUs included in the shade plot of the control samples is indicated with a star. The colour intensity of scale indicates relative abundance of each OTU within the subset of the dataset (white denotes absence and full black represents maximum abundance).

3.14 Comparison of taxonomic composition profiles of biofilm vs. bulk water communities within the broiler house

ANOSIM test analysis showed that biofilm communities in the internal part of the nipple drinker were very similar to those collected from the inside of the water pipes by swabbing. Figures 3.12 and 3.13 show the 50 more abundant OTUs detected in biofilm pipe communities and shed bulk water.

For the prokaryotic communities 34% (17/50) of the OTUs were also present in the control samples in sufficient abundance to be included in the control shade plot (figure 3.12) and similarly for the eukaryotic communities the percentage was 36% (18/50) (figure 3.13).

Figure 3.12 shows the profiles of the prokaryotic communities. Before the chickens were placed into the farm (week 1), the most abundant bacterial genera detected in biofilms were *Devosia*, *Delftia*, *Pseudorhodoferax* and *Brevundimonas*. Bulk water communities however were dominated by *Burkholderia*, *Luteibacter* and *Cupriavidus*. As suspected from the nMDS plots (figure 3.4), bacterial communities had a higher similarity in week 2 with *Aquabacterium*, *Acidovorax*, *Caulobacter* and *Rhodopseudomonas* being the most abundant genera at this stage of the rearing cycle (seven days after the hatchlings had been placed). The greatest dissimilarities between biofilm and bulk water communities were observed in samples collected during weeks 3 and 4. The biofilm communities were dominated by *Pseudomonas*, *Brevundimonas* and *Delftia* and bulk water samples by *Klebsiella* and *Enterobacter*. Bulk water bacterial community profiles were very similar in weeks 5-7. These communities were dominated by *Staphylococcus* and *Lactobacillus,* but genera such as *Aquabacterium*, *Caulobacter* and *Roseateles* which were abundant in

weeks 1 and/or 2, also showed increased abundance towards the end of the cycle. Interestingly, following the increase in *Klebsiella* and *Enterobacter* in bulk water samples in weeks 3 and 4, biofilm communities in weeks 5 and 6 are dominated by *Klebsiella* and those collected by swabbing also by *Enterobacter*. Biofilms in week 7, however, were more similar to the bulk water community collected at the same time and also showed a high relative abundance of *Lactobacillus* and *Staphylococcus* genera, suggesting that microrganisms in the bulk water were incorporated into the biofilm to sufficient levels for detection by week 7. *Sphingobacterium* was more abundant in biofilm samples towards the end of the rearing cycle, particularly in weeks 6 and 7.

Some taxa were particularly associated with a certain environmental niche. For example, *Mycobacterium* was associated with the shed bulk water and *Brevundimonas* with the biofilms throughout the seven weeks. Others, however, were more abundant at certain time points irrespective of the environmental niche such as *Geobacillus* which was associated with bulk water and biofilm samples in weeks 6 and 7. Finally, some of the taxa did not show a clear pattern such as *Sphingobium* which was associated with bulk water samples in every week but weeks 3 and 4 and *Eschirichia-Shigella* which had a high relative abundance in bulk water in week 3 and in the biofilm collected by swabbing in weeks 5 and 6.

It is interesting to note how most OTUs that were also detected in the controls were found in biofilm samples, which had a lower biomass, confirming for this dataset that low biomass samples are more vulnerable to the effects of contamination (Salter *et al.* 2014). Although it is difficult to discriminate when OTUs genuinely belong to the samples, OTUs that are particularly enriched in few samples and accompanied by others with similar taxonomic assignments have been considered as part of the community. Conversely,

OTUs that were distributed in a uniform fashion, only across low biomass samples were more likely to be contaminants.

Figure 3.13 shows the shade plot for the eukaryotic communities in biofilm and shed bulk water samples. Before the placement of the chickens on the farm (week 1) the yeast *Nakazawaea holstii* showed the highest relative abundance. Other yeast genera including *Candida*, *Exophila*, *Debaryomyces* and *Yamadazyma* were also abundant in week 1, particularly in the biofilms. The bulk water in week 1 also contained high relative abundance of the protist genera *Bromeliothrix* and *Gymnophrys* and the class Heterolobosea which, along with the genera *Vannella* and *Neobodo*, were also the most abundant OTUs in bulk water and biofilm communities in week 2. In week 3 the bulk water eukaryotic community profile was still pretty similar to that in week 2. The biofilm profiles, however, showed high relative abundance of other fungal genera such as *Saccharomyces* and *Mucor*. In bulk water communities from week 4 -7 the relative abundance of the OTUs that peaked in week 2 progressively dropped while other taxa increased, most notably the algae order Isochrysidales, some OTUs assigned as Metazoa and the fungal genera *Millerozyma, Aspergillus* and *Neocarpenteles*. In the biofilms a number of fungal OTUs were particularly enriched from weeks 4 to 7 including the fungal genera *Millerozyma*, , *Aspergillus* and *Schwannimyces,* the order Tremellales and the phylum Basidiomycota. Candida was the most abundant genus detected throughout the sampling period. *Penicillum* and *Trichosporon* were abundant at the beginning of the cycle in bulk water and biofilms and subsequently only in biofilms. The yeast *Starmella bombicola* and two Metazoa OTUs particularly showed an increased relative abundance in the biofilm in week 7.

Six different plant taxa were also identified in the 18SrRNA analysis (*Brassica napus*, *Quina pteridophylla*, *Zea mays*, *Magniolophyta*, Apiales and *Oryza meyeriana*). Similar observations have been made in other studies where these OTUs were not considered indigenous community members but “contaminants” within the distribution system(Buse *et al.* 2014a).

A number of OTUs that were present in the negative control shade plot were enriched in a particular group of samples, either at the beginning or at the end of the cycle or in the bulk water samples. Arguably this is not a distribution to be expected from contaminant taxa and those that followed the described patterns have been included in the description.

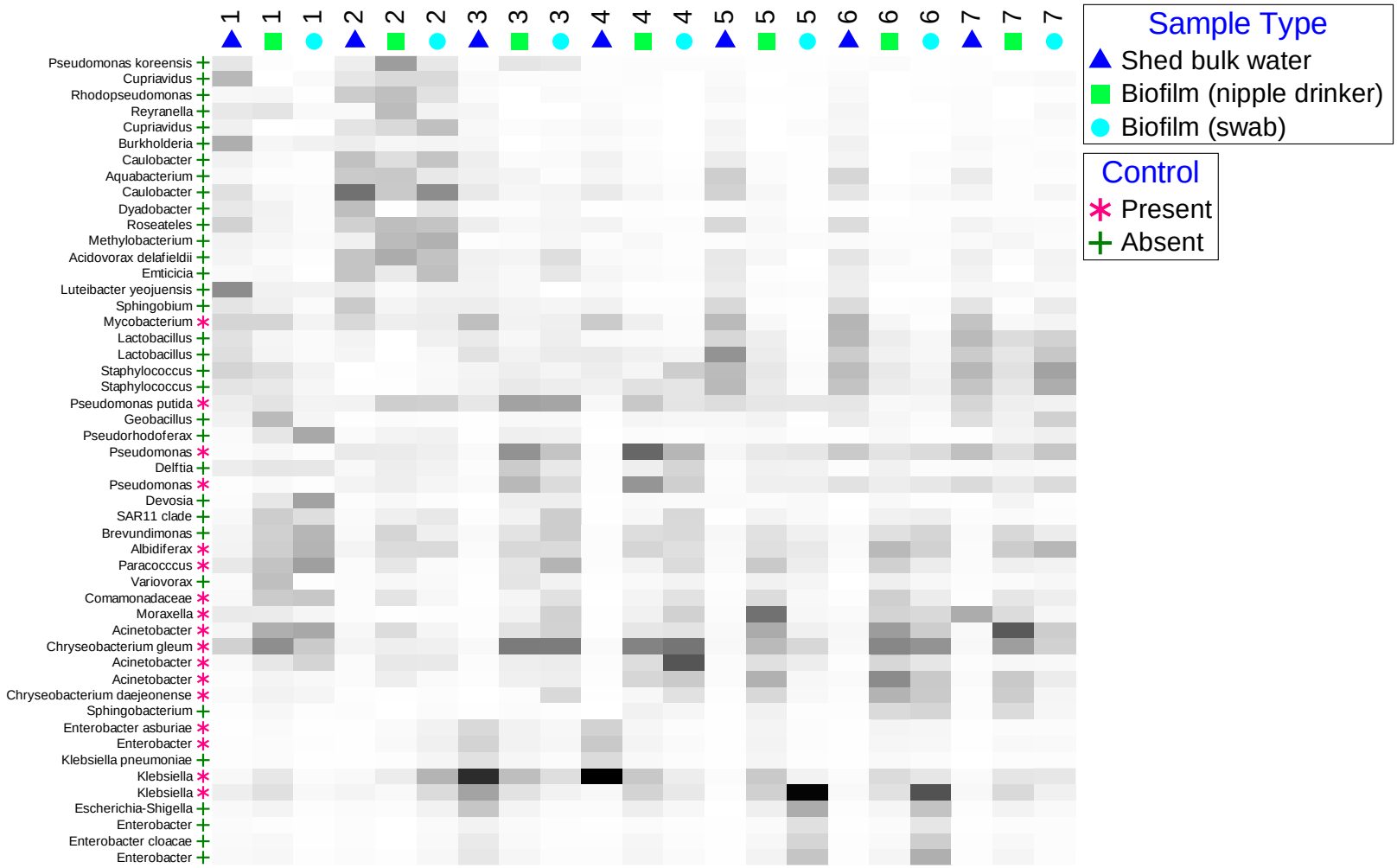


Figure 3.12. Comparison of the relative abundance of the major prokaryotic OTUs found in the bulk water and biofilm inside the broiler house. Square transformed relative abundance of the 50 most important OTUs after transformation was used to generate the shade plot in Primer 7 (Clarke and Gorley, 2015). OTUs were ordered to maximise serial change but samples were ordered weekly. Triangles, squares and circles are representing individual samples and are coloured based on sample type. Sampling week is indicated by a number next to each sample. The presence of OTUs included in the shade plot of the control samples is indicated with a star. The colour intensity of scale indicates relative abundance of each OTU within the subset of the dataset (white denotes absence and full black represents maximum abundance).

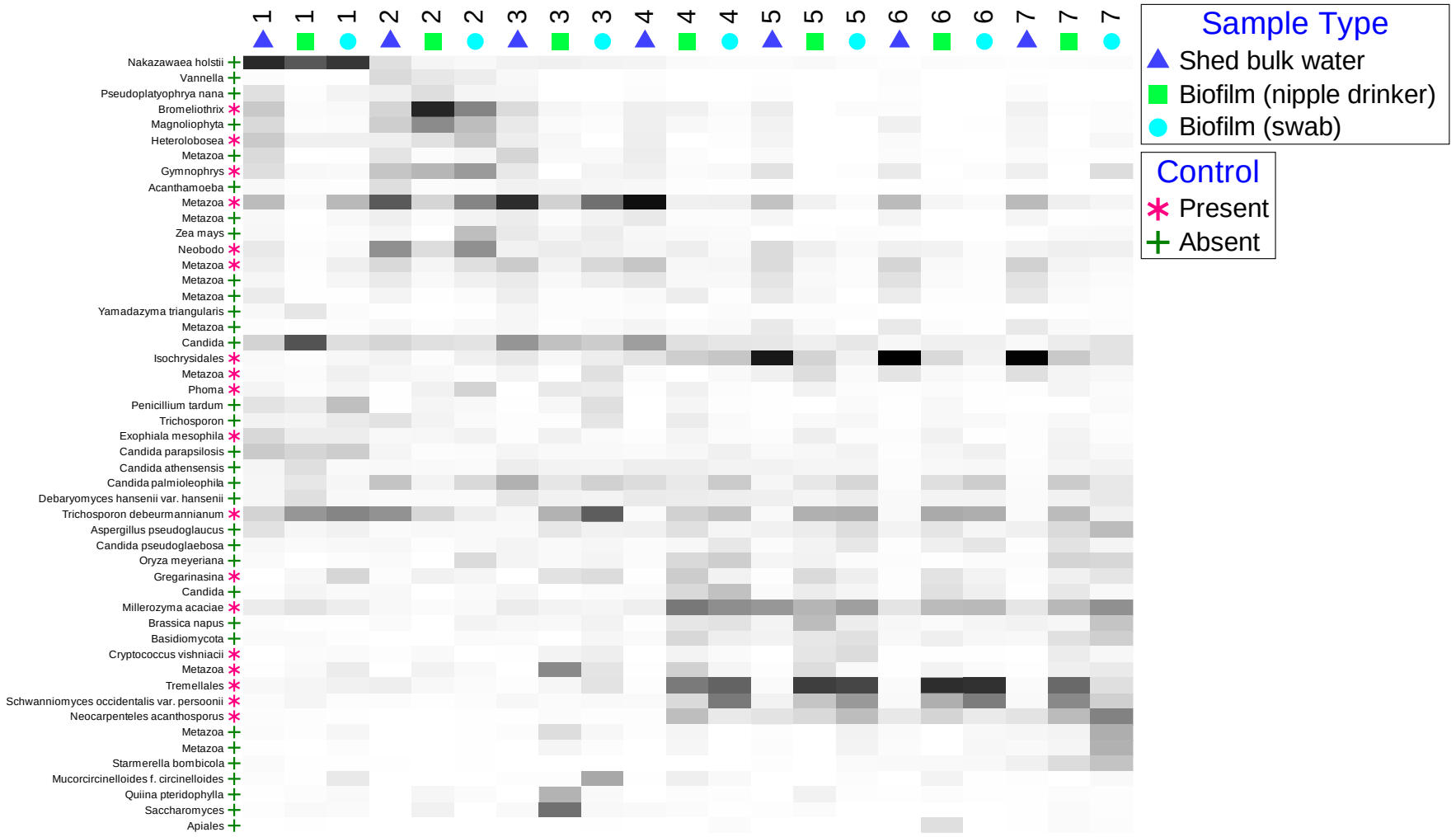


Figure 3.13. Comparison of the relative abundance of the major eukaryotic OTUs found in the bulk water and biofilm inside the broiler house. Square transformed relative abundance of the 50 most important OTUs after transformation was used to generate the shade plot in Primer 7 (Clarke and Gorley, 2015). OTUs were ordered to maximise serial change but samples were ordered weekly. Triangles, squares and circles are representing individual samples and are coloured based on sample type. Sampling week is indicated by a number next to each sample. The presence of OTUs included in the shade plot of the control samples plot is indicated with a star. The colour intensity of scale indicates relative abundance of each OTU within the subset of the dataset (white denotes absence and full black represents maximum abundance).

3.15 Major microbial community shifts correlated with temperature, flow rate and chicken weight

The first and last sampling points in this study were carried out before and after the chickens were on the farm respectively and therefore data of the conditions inside the broiler house was not available. Data from weeks 2 to 6 was used to calculate Pearson correlations between the environmental conditions and the community profiles. The maximum temperature in the shed was positively correlated to minimum temperature ($r=0.935$, $p=0.02$) and negatively correlated to average flow rate ($r=-0.971$, $p=0.006$) and average weight ($r=-0.940$, $p=0.017$). So the maximum temperature in the shed could be used to represent all four variables in correlation analysis.

The most remarkable shift in community structure across the rearing cycle was the increase in Firmicutes towards the end of the cycle, particularly in shed bulk water. This shift does not seem to correlate with the administration of Lincospectin (between weeks 1 and 2), the vaccination (between weeks 4 and 5) or thinning (between weeks 5 and 6) but there was a weak correlaion with the more gradual changes in temperature and flow rate or chicken weight. Indeed the maximum temperature was negatively correlated with Firmicute relative abundance in shed bulk water samples but the association did not reach statistical significance ($r=-0.856$, $p=0.064$). The genera *Staphylococcus* and *Lactobacillus* were identified as the main genera responsible for the increase in Firmicutes. In the shed bulk water samples, a significant correlation was found between the relative read counts of *Staphylococcus* and the maximum temperature ($r=-0.883$, $p=0.047$). In the case of *Lactobacillus* again an association was found that did not reach statistical significance ($r=-0.810$, $p=0.097$).

3.16 *Campylobacter* was detected on the 16S rRNA data on the farm DWS

The community analysis described so far has largely focused on the 50 more abundant taxa. However, it is often important to consider the less abundant members of microbial communities that can have key roles in community function or in development or transmission of disease. One of the major objectives of this thesis was to determine whether microbial communities of the DWS on a broiler farm could support the survival of *Campylobacter* species as this would inform potential strategies to reduce transmission to humans.

The Illumina 16S Metagenomic workflow performs taxonomic classification of 16S rRNA targeted amplicon reads using an Illumina-curated version of the GreenGenes (McDonald *et al.* 2012) May 2013 version database. This provides an aggregate summary report at different taxonomic levels so that researchers can compare the similarities and differences among samples (Illumina 2014).

16S rRNA reads assigned to *Campylobacter* ssp. OTUs were detected in four samples above the 10 read threshold in the water distribution system of the broiler farm in the Basespace 16S Metagenomics report (table 3.5). The highest number of reads was detected in the biofilm samples from the internal part of nipple drinkers in week 5 (before thinning) and in the shed bulk water in week 7. A lower number of *Campylobacter* spp. reads were also detected in the biofilm collected by swabbing in week 5 and the shed bulk water in week 6.

These data were further examined using two alternative platforms: One Codex (Minot *et al.* 2015) and Qiime using the SILVA database (Caporaso *et al.* 2010, Quast *et al.* 2013).

The One Codex data platform is a user-friendly web based tool that allows the analysis of microbial data against a reference database of bacterial, viral, fungal and protozoan genomes (Minot *et al.* 2015). For the two samples with a higher number of reads, the results first seen in the Illumina 16S Metagenomic app were corroborated in the two other platforms (table 3.5). One Codex reported a higher number of hits while QIIME with SILVA reported slightly less reads compared to the results from the Illumina 16S Metagenomic app.

However, no hits were identified that could be assigned to *Campylobacter* OTUs in the samples from week 5 biofilm swabs and the week 6 bulk water when data was analysed using these alternative pipelines. In One Codex a small number of hits were reported for these samples but not higher than which could be considered a background level of misidentification that was commonly observed in several samples when using this platform.

Interestingly virtually all sequences from the biofilm in the nipple drinker in week 5 were identified as *Campylobacter ureolyticus* in all three platforms. *C. ureolyticus* was recently reclassified in the genus *Campylobacter* from its previous *Bacteroides ureolyticus* nominations on the basis of 16S rRNA sequence similarity among other traits (Vandamme *et al.* 2010). Soon after this re-classification it was identified as an emerging gastrointestinal bacterial pathogen (Bullman *et al.* 2011a). Although very little is known about its epidemiology, *C. ureolyticus* has been associated with cattle but not with poultry (Koziel *et al.* 2012).

These results suggest that *Campylobacter* ssp. were present in the water distribution system of the broiler farm in low numbers and open the possibility that it might have

been present in other samples below the limits of detection. Chapter 4 further investigates the results of this study using culture and PCR-based techniques.

Table 3.5. Distribution of reads assigned to the genus *Campylobacter*.

WEEK	SAMPLE	GENUS/SPECIES	BASESPACE HITS	ONE CODEX HITS	QIIME (SILVA) HITS
5	Biofilm (nipple drinker)	*Campylobacter*	4	22	0
		C. ureolyticus	97	194	90
		TOTAL	101	216	90
5	Biofilm (swab)	*Campylobacter*	10	12	0
6	Shed bulk water	*Campylobacter*	16	7	0
7	Shed bulk water	*Campylobacter*	92	178	83

Number of OTUs that match *Campylobacter* from Basespace with Greengenes database, One Codex and Qiime with SILVA database are shown.

3.17 Discussion

This is the first study to simultaneously characterise dynamic prokaryotic and eukaryotic communities associated with the DWS of a broiler farm using high-throughput amplicon sequencing throughout a complete rearing cycle. It has demonstrated that the microbial communities are highly heterogeneous both spatially and temporally and might represent an environmental reservoir for *Campylobacter* spp.

Amplicon sequencing is a widely applied approach to investigate questions related to microbial ecology, such as composition, organization and spatiotemporal patterns of microbial communities (Sinclair *et al.* 2015). However, compared to the number of studies on DWDS using 16S rDNA amplicon profiling little data is available on the eukaryotic diversity in DWDS. In this study both the prokaryotic and eukaryotic communities were studied through 16S and 18S rDNA amplicon profiling. High-throughput sequencing with barcoded samples allowed for the investigation of different environmental niches associated with the DWS of a farm across a whole rearing cycle. It is estimated that only 1-5 % of the bulk water bacterial population in DWDS is culturable (Ultee *et al.* 2004). Bacteria in DWDS biofilms also frequently show low culturability (Martiny *et al.* 2003, Wingender and Flemming 2004) making culture-independent techniques a better approach to unravel their identity.

Culture-independent techniques are also desirable to detect pathogens that persist in drinking water biofilms (Camper *et al.* 1999). Many species of bacteria including *C. jejuni*, *C. coli* and *C. lari* enter a VBNC state as a response to stressful conditions such as starvation and therefore cannot be detected by culture (Li *et al.* 2014). 16S and 18S rDNA amplicon sequencing was therefore a good technique for both main aims of this study:

characterising the microbial communities and investigating *Campylobacter* presence in the WDS.

Proteobacteria were found to dominate the bacterial communities in the source water collected in the anteroom and in the biofilm and shed bulk water communities from inside the broiler house during the first weeks of the rearing cycle. Proteobacteria have been found to dominate DWDS in a wide range of different drinking water ecosystems covering different pipe materials, disinfection procedures and water sources (Martiny *et al.* 2005, I. Douterelo *et al.* 2014, Liu *et al.* 2014, Pinto *et al.* 2014). Interestingly on the farm WDS a shift towards Firmicutes dominance was observed mainly due to an increase in the genera *Staphylococcus*, *Lactobacillus* and to a lesser extent *Geobacillus* towards the end of the cycle. Firmicutes have been previously reported in bulk water and biofilms in DWDS, but commonly represent a low percentage of the reads (Hong *et al.* 2010, Pinto *et al.* 2012, Liu *et al.* 2014, Shaw *et al.* 2014). Sun *et al.* (2014), however also reported a relatively high proportion of Firmicutes in biofilm pipe communities supplied by surface water, and Luo *et al.* (2013) also reported a higher proportion of Firmicutes in one of their biofilm samples, associated with a higher concentration of nitrate and a lower concentration of dissolved oxygen. In this study resources were not available to measure dissolved oxygen levels but it is tempting to speculate that the increase in Firmicutes towards the end of the rearing cycle in the DWS might have been linked to reduced dissolved oxygen levels creating a more suitable microenvironment for *Campylobacter* spp. which were detected following the increased abundance of Firmicutes. It has previously been reported that some microorganisms including *Pseudomonas* spp. (Hilbert *et al.* 2010) and *Acanthamoeba* (Bui *et al.* 2012b) support *Campylobacter* survival under

atmospheric oxygen tension by depleting dissolved oxygen. In this study Bacteoidetes showed a higher relative abundance in biofilm samples, which does not seem to be a common feature in other DWDS studies where bulk water and biofilm communities have been compared (Henne *et al.* 2012, Liu *et al.* 2014). Firmicutes and to a lesser extent Bacteroidetes have been found to dominate the chicken caeca microbiome in several studies (Oakley *et al.* 2014) suggesting that the chickens influence the microbial communities found on the farm DWS. Eukaryotic sequences were identified as being related to several major microbial eukaryotic super groups, namely, protozoa, algae, fungi and metazoa.

Dealing with large OTU datasets is challenging. Summarising OTU data at high taxonomic level allowed for the identification of general patterns in the samples. However it has been observed that even class-level reports might be misleading and do not reflect the variety of behaviours of their individual components (Pinto *et al.* 2014). In this study the discussion at high taxonomic level was followed by insights into the patterns using the original OTU table, allowing for OTUs to be identified to the lowest taxonomic level possible in each case.

The inclusion of negative controls treated identically to other samples through processing and library preparation is indispensable in NGS studies (Nguyen *et al.* 2015). This is particularly important in metagenomics and rRNA amplicon sequencing and when working with environmental samples such as water which is likely to harbour taxa that are indistinguishable from those commonly identified as kit contaminants (Salter *et al.* 2014). In this study 5 different negative controls were included to account for the

different processing steps for different types of samples. There is currently no consensus on how to handle the sequences found in the negative controls (Nguyen *et al.* 2015). One approach is to delete any OTUs that appear in negative controls across all samples. In this study taking this approach with the 16S dataset resulted in a decrease in OTU number from 8,338 to 7,745 and in total count from 5,269,737 to 345,785. Similarly other researchers have also found a dramatic drop in total sequence count indicating that the most abundant OTUs in the experimental samples have been deleted (Bittinger *et al.* 2014, Nguyen *et al.* 2015). Therefore it was decided to include the negative controls and discuss them along with the samples. ANOSIM demonstrated that, in most cases, sample groups and their associated controls harboured distinct microbial communities. The exception was the communities from the internal part of the nipple drinker which did not show a high degree of community separation with their controls particularly for the eukaryotic communities. This sample type had the lowest biomass and therefore was the most vulnerable to the effects of contaminants.

It is not surprising that some of the bacterial genera identified in negative controls in this study had not been reported before. Salter *et al.* (2014) demonstrated that the contamination in different DNA extraction kits and kit batches is not constant or predictable and that there is a variation in contaminant content between laboratories. In this same study they found that a Qiagen kit had the most complex mix of bacterial DNA of all the kits tested. In a recent publication Glassing *et al.* (2016) identified an additional 88 bacterial genera as potential contaminants of molecular biology grade reagents bringing the total number to 181 genera. Very little information is available on the identity of eukaryotic contaminants. In this study a variety of OTUs covering all the major

groups found in the samples were also detected in the negative controls. This include OTUs that have been isolated from drinking water before, suggesting that eukaryotic taxa found in oligotrophic environments could also inhabit laboratories and other clean-room environments, as it has been seen for bacteria before (Tanner *et al.* 1998, Barton *et al.* 2006).

It can be concluded that the variability among samples is explained by their spatial location in the DWS in the farm but also from their temporal collection across the rearing cycle. Microbial communities in the source water collected in the anteroom differed from those found in the different environmental niches inside the broiler house. Bacterial communities in the water collected from the anteroom remained relatively stable across the rearing cycle with the exception of weeks 5 and 6 that show different profiles. The most abundant genera in these samples were *Sphingobium*, *Pseudomonas* and *Delftia* all of which have been detected in DWDS bulk water previously (Williams *et al.* 2004, Martiny *et al.* 2005, Shaw *et al.* 2014). Stability in bacterial community structure within DWDS has also been reported before both during short time-scales (minutes, hours and days) (Henne *et al.* 2012, El-Chakhtoura *et al.* 2015) and during similar time-frames to this 7-week study (McCoy and VanBriesen 2014).

Most of the most abundant eukaryotic taxa could not be identified beyond Metazoa, particularly on the source water collected in the anteroom, but taxa related to fungi and FLA were also identified. Buse *et al.* (2013) reported that 2,099 out of 6,238 sequences detected in a DWDS belonged to the metazoan groups Copepods, Nematodes and Rotifers. *Phialophora* was the most abundant fungal taxa and it has been isolated before in DWDS and described as ubiquitous throughout DWDS networks (Göttlich *et al.* 2002, Oliveira *et al.* 2016). The most abundant FLA were taxa in the phylum Cercozoa: the order

Euglyphida and its genus *Assulina* and the genus *Cryptodifflugia*. Members of Cercozoa have been cultured and identified by culture-independent techniques in DWDS in other studies (Valster *et al.* 2009, Valster *et al.* 2011, Sente *et al.* 2016). Interestingly *Cryptodifflugia* amoebae presence was found to be inversely correlated with temperature in a study in Paris DWDS network which could explain why its relative abundance decreases inside of the broiler house (Delafont *et al.* 2016). In a study in commercial poultry houses *Cryptodifflugia* was isolated by culture in the WDS of two different farms (Bare *et al.* 2009). It is perhaps not surprising that the communities diverged inside the broiler house as there were stark differences in temperature. Moreover lincospectin and a vaccination were administered through the DWS and the chickens can introduce microorganisms via the nipple drinkers.

Inside the broiler house biofilm and bulk water communities had different compositions, which has been previously reported in a range of DWDS (Martiny *et al.* 2005, Henne *et al.* 2012, Douterelo *et al.* 2013, Douterelo *et al.* 2014). In spite of the pivotal role of materials in biofilm formation and composition that has been observed in some studies (Yu *et al.* 2010, Buse *et al.* 2014a, I. Douterelo *et al.* 2014), biofilm communities recovered from the stainless steel nipple drinker and by swabbing the plastic pipes in this study were significantly similar. In agreement with the results presented here Revetta *et al.* (2013) did not find differences in biofilm community structure between polycarbonate and glass beads. Henne *et al.* (2012) also found that bacterial biofilm communities sampled at nearby sample points were similar irrespective of support materials and hypothesised that long-term adjacent co-existence might lead to exchange of resident bacteria. Supporting this hypothesis the microbial communities recovered from the external part of the nipple drinker were relatively similar to the internal pipe biofilm

samples. This result was unexpected as the external part of the nipple drinker is in direct contact with the farm environment and the chicken oral microbiome. However, it is also in co-existence with the microbial communities in the interior of the pipes through the chicken's mouths when they drink and microbial exchange could take place during this process. Previous studies have suggested that *Campylobacter* may be introduced in the DWS on the farm by chickens (Ogden *et al.* 2007).

Bacterial community structure was observed to change across the rearing cycle in both biofilm and bulk water samples. Most bacteria found in the DWS of the farm have been previously reported in DWDS. Some genera such as *Delftia*, *Brevundimonas* and *Devosia* were observed to preferentially inhabit biofilms. All three have previously been reported as being associated with DWDS biofilms (Qin *et al.* 2007, Yu *et al.* 2010, Ling *et al.* 2016). Pinto *et al.* (2014) identified *Brevundimonas* as one of the most abundant OTUs within bulk water of a DWDS and observed that it exhibited peak relative abundance in the colder months, when the water temperature was under 10 °C. Williams *et al.* (2004) could isolate *Brevundimonas* from water at around 20 °C in a DWDS simulator. In this study *Brevundimonas* remained abundant in the biofilms throughout the seven sampling weeks, in spite of the sharp changes in temperature, but its relative abundance in bulk water remained low. In week 2, shortly after the arrival of hatchlings, biofilm and bulk water prokaryotic communities were found to be very similar and included *Cupriavidus*, *Aquabacterium*, *Acidovorax*, *Caulobacter* and *Rhodopseudomonas*. *Acidovorax* has been reported in biofilms (Hong *et al.* 2010, Sun *et al.* 2014), loose deposits (Liu *et al.* 2014) and as the dominant genus in chloraminated bulk water (Williams *et al.* 2004, Pinto *et al.* 2012). Pinto *et al.* (2014) reported peak relative abundance in bulk water in the summer when the water temperature was between 20 °C and 26 °C. The temperature increase on

the farm when the chickens are placed into the shed could explain the increase in relative abundance of this genus. *Caulobacter* and *Cupriavidus* have previously been detected in both biofilms and bulk water (Williams *et al.* 2004, Revetta *et al.* 2010, Berthiaume *et al.* 2014, Sun *et al.* 2014). In the same way *Aquabacterium* has been described simultaneously in the water phase and the biofilm (Kalmbach *et al.* 1997, Kalmbach *et al.* 2000) and has been observed independently of season (Berthiaume *et al.* 2014). *Rhosopseudomonas* can be found in fresh water ecosystems and can grow *in vitro* as a biofilm via photoheterotrophic (in the absence of oxygen and presence of light) and heterotrophic (in the presence of oxygen and absence of light) metabolisms (Kernan *et al.* 2015).

Other genera identified such as *Mycobacterium* showed higher relative abundance in the bulk water in spite of being commonly found in DWDS biofilms elsewhere (Yu *et al.* 2010, Luo *et al.* 2013). Interestingly some genera such as *Escherichia-Shigella*, *Klebsiella*, *Enterobacter*, *Staphylococcus* and *Lactobacillus* showed peaks in relative abundance in the bulk water and subsequently in biofilm samples suggesting that they might have been introduced by the chickens via the nipple drinkers and subsequently became established in biofilms. *Lactobacillus* dominates the microbiota of the chicken gastrointestinal tract (Stanley *et al.* 2014) and *Staphlylococcus*, *Enterobacter* and *Escherichia-Shigella* are genera common in faecal, litter and carcass samples (Oakley *et al.* 2014) and therefore it seems sensible to hypothesise that these OTUs might have been introduced into the farm DWS by the chickens.

A number of fungi were identified among the eukaryotic taxa found on the DWS of the farm. Fungi are heterotrophic organisms and many fungal species tolerate oligotrophic environments. They can colonise DWDS after surviving water treatment or entering

during water works (e.g. when pipes are replaced) (Oliveira *et al.* 2016). Biofilms are an important habitat for fungi in drinking water but they can also be found suspended in the water (Siqueira *et al.* 2011). In this study both yeasts and filamentous fungi have been detected in the biofilms and bulk water, although they were indicated at higher relative abundance in biofilms and nipple drinker associated samples. Some of the yeast-like genera such as *Candida*, *Exophiala* and *Cryptococcus* have been previously isolated by culture (Kinsey *et al.* 1998, Doggett 2000). Others such as *Saccharomyces* and *Debaryomyces* as well as the already mentioned *Candida* and *Exophiala* have been detected using 18S rRNA amplicon sequencing previously (Buse *et al.* 2013, Douterelo *et al.* 2016). Some of the taxa identified here such as *Aspergillus*, *Penicillum, Mucor* and *Phoma* are among the most common fungal isolates from drinking water (Doggett 2000, Göttlich *et al.* 2002, Pereira *et al.* 2009, Siqueira *et al.* 2011, Oliveira *et al.* 2013). *Aspergillus*, *Penicillum* and *Phoma* have also been reported in bulk water and biofilms using sequencing (Douterelo *et al.* 2016). Molecular studies of the eukaryotic diversity in the DWS of broiler farms have observed a high proportion of fungal sequences (Snelling *et al.* 2006, Bare *et al.* 2009). Among them, *Penicillum, Thricosporon* and the yeasts *Candida* and *Debaryomyces* were also identified in this study (Snelling *et al.* 2006, Bare *et al.* 2009).

Other fungal genera showing high relative abundance in this study such as *Nakawaea*, *Millerozyma* and others do not seem to have been reported in DWDS previously in the literature. This is not surprising considering that a limited number of studies are available and most of them use culturing methods and therefore introduce bias towards selecting for certain genera (Hageskal *et al.* 2009, Douterelo *et al.* 2016).

Only some of the protozoa identified in this study had been previously reported in DWDS. This includes the genera *Vannella*, *Neobodo* and *Acanthamoeba* (Valster *et al.* 2009, Valster *et al.* 2011, Buse *et al.* 2013, Delafont *et al.* 2013, Delafont *et al.* 2016, Sente *et al.* 2016). Other genera such as *Bromeliothrix*, *Gymnophrys* and *Pseudoplatyophrya* do not seem to have been reported in DWDS previously. Protozoa such as FLA colonise DWDS and are capable of incorporating in biofilms, particularly increasing their abundance in reservoirs with stagnant water. FLA benefit a range of pathogenic microbial species and are therefore increasingly considered a human health risk (Thomas *et al.* 2010, Thomas and Ashbolt 2011). *In vitro* assays have proved that FLA and other protists such as algae and ciliates can prolong *Campylobacter* survival (Axelsson-Olsson *et al.* 2010a, First *et al.* 2012). Previous studies on the diversity of eukaryotes in the water supplies of intensively reared chickens have identified protozoa that have also been identified in this study and also protozoa that have been seen to enhance *Campylobacter* survival *in vitro*. *Vannella* and *Acanthamoeba* which were commonly found in the DWS of the broiler house in this study have been cultured and detected by sequencing in the WDS of other broiler farms (Bare *et al.* 2009, Bare *et al.* 2011).

Bulk water pH remained close to neutrality across the seven weeks for both water inside and outside the broiler house. The temperature however was higher in the broiler house (maximum temperature range from 33 to 25.9 from weeks 2 to 6) compared to ambient temperature in the anteroom as sampling was carried out during winter (November-December).

A temporal trend of increased bacterial abundance in warmer months associated with seasonal shifts in bacterial diversity has been reported (LeChevallier *et al.* 1991, McCoy and VanBriesen 2012, McCoy and VanBriesen 2014). Temperature is believed to be a key

condition that can lead to microbial proliferation in biofilms (Camper *et al.* 1999). Richness has also been shown to be highly correlated to water temperature (Pinto *et al.* 2014). In this study, DNA concentration measurements showed a higher DNA yield in shed bulk water samples than in anteroom bulk water samples, suggesting that more biomass could be collected in the water from inside the broiler house in spite of filtering the same volume. Moreover, a significant association was found between the temperature in the broiler house and the increase in relative abundance of *Staphylococcus* in the shed bulk water. It has been observed that chlorine disinfectant decay rate is faster at higher temperatures (Kiéné *et al.* 1998, Monteiro *et al.* 2015). High temperature therefore might also influence bacterial populations on the farm through its effects on chlorine. In this study, chlorine levels could not be measured so further research is needed to test this hypotheses. These results suggest that temperature influences microbial community composition and biomass in the chicken house but more research is needed to understand its effects.

Hydraulic regimes and water residence times also change across the rearing cycle as older chickens consume more water and the increase in flow rate was also correlated with the increase in *Staphylococcus* relative abundance. Douterelo *et al.* (2013) observed that different hydraulic regimes affected mechanical stability and bacterial community composition biofilm but not bulk water samples. Overnight stagnation has also been related to changes in community composition and microbial growth in water collected form household taps (Lautenschlager *et al.* 2010).

Cleaning and disinfection procedures before the placement of a new flock combine water flushing and disinfection with 2% peroxygen. Hydrodynamic shear stress induces mechanical detachment and modifies biofilm organization and cohesiveness (Mathieu *et*

al. 2014). Moreover, flushing also results in a change of biofilm bacterial community structure (Douterelo *et al.* 2013, Douterelo *et al.* 2014). However flushing alone does not completely remove bacteria from pipe walls and biofilms can rapidly regenerate (Douterelo *et al.* 2013, I. Douterelo *et al.* 2014). In agreement with this observation, in this study biofilm biomass could be recovered after cleaning and disinfecting the broiler house and previously to the placement of the chickens.

Campylobacter 16S rRNA sequences were detected in the WDS in the biofilm of week 5 and the shed bulk water in week 7. It has been previously suggested that *C. jejuni* might be able to integrate and survive in DWDS biofilms based on laboratory experiments where *C. jejuni* were spiked in pre-formed biofilms (Buswell *et al.* 1998a, Buswell *et al.* 1998b, Lehtola *et al.* 2006). In this study the sequences in the biofilm in week 5 were identified to the species level as *C.ureolyticus*. *C. ureolyticus* is an emerging pathogen that appears to have a significant role in human campylobacteriosis (Bullman *et al.* 2011a, O'Donovan *et al.* 2014). Very little is known about its epidemiology but it is suspected to have a zoonotic potential as it has been isolated from domestic animals (dog, cats and pigs) and bovine samples (Koziel *et al.* 2012, Koziel *et al.* 2014). Moreover, strain typing through RAPD showed that the profile of one isolate from a cat was genetically similar to that from one patient presenting with gastroenteritis (Koziel *et al.* 2014). In their study Koziel *et al.* (2012) also tested 40 chicken caeca and 20 chicken wings and they could not detect any *C. ureolyticus*. This study therefore is likely the first one to detect this emerging pathogen in relation to the chicken reservoir. This was further investigated through PCR and the results are reported in chapter 4.

In conclusion this study has characterised the spatio-temporal dynamics of the microbial communities associated with the water distribution system of a broiler farm for a whole

rearing cycle. The microbial communities are niche specific, differ from those of the source water and change across the rearing cycle. *Campylobacter* sequences have been detected in low numbers in one biofilm and one bulk water sample towards the end of the cycle suggesting a possible role of the DWS as a vehicle of *Campylobacter*. However the epidemiological relevance of this observation requires further investigation.

Chapter 4

Detection of *Campylobacter* and *Helicobacter* species on chicken farms in UK and Uganda

4.1 Introduction

As an urgent matter of food security, the FSA has set bold targets jointly with industry aimed at reducing the incidence of *Campylobacter* infection (FSA 2010). Although several sources of infection have been identified, chicken meat is considered the principal source of *Campylobacter* infection in humans in UK (Sheppard *et al.* 2009). 61.3% of supermarket chicken was found to be contaminated with *Campylobacter,* and 11.4% found to be highly contaminated (more than 1000 cfu / g) between July 2015 and March 2016 (PHE 2017). In 2010 the FSA called for a reduction of the levels of the most highly contaminated chickens at the end of the slaughter process numbers to be reduced from a baseline of 27% in 2008 to 10% by 2015 measured post-chill. It was also expected that the least contaminated chickens (i.e. less than 100 cfu per gram), would get no worse or would improve upon the baseline of 42% (determined in 2008 by the EU) by 2015 (FSA 2010). Although the levels of highly contaminated products have been reduced (7%), the percentage of contaminated chickens was still 55.8% between August and December 2016 (FSA 2016); further measures are needed to meet expected targets. All types of poultry (broilers, layers, turkeys, ducks, fowl, quail, ostriches) can become colonised by *Campylobacter* and, although campylobacters can also survive in the environment, warm-

blooded animals are believed to be the amplification vessel (Wagenaar *et al.* 2006). *Campylobacter* accumulates to high numbers in the chicken caecum (10^9 cfu g^{-1} faeces (Ringoir *et al.* 2007)) and it is this early amplification that greatly increases subsequent spread of the pathogen (Humphrey 2006).

The control of *Campylobacter* originating from broiler chickens is probably the most important element of public health strategies to reduce the burden of human campylobacteriosis globally (Vidal *et al.* 2014). All aspects of food processing, transport, storage and hygiene "from farm to fork" have been considered. However, the main source of meat contamination has been shown to be the flock highlighting the importance of on-farm interventions to reduce colonization at farm level (Marotta *et al.* 2015, Wieczorek and Osek 2015). Most chickens in the UK are reared intensively in large commercial broiler farms (Vidal *et al.* 2014). It has been observed that hatchlings do not harbour *Campylobacter* when delivered to farms, but that they often become colonised within the next 2-3 weeks (Torralbo *et al.* 2014, Battersby *et al.* 2016). Once one bird becomes colonised, amplification and spread of *Campylobacter* occurs rapidly throughout the flock (Battersby *et al.* 2016). Much investigation has searched to identify the source of transmission, however, there is insufficient evidence to support any one single route (Conlan *et al.* 2007). It is likely that several environmental sources play a role in transmission. Control measures have focused on on-farm biosecurity (strict disinfection, use of dedicated boots and clothing, step-over barriers, fly screens etc.) but other methods such as competitive exclusion with probiotics, treatment with bacteriophages and the development of vaccines have been suggested (Humphrey *et al.* 2007, Pasquali *et*

al. 2011). However, no marked decrease in the incidence of infection or colonisation of chickens has been achieved so far.

More intensive surveillance of *Campylobacter* on poultry farms would inform further understanding of transmission, however, the tools for wide-scale surveillance need to be optimised.

The landscape of poultry farming in developing countries differs considerably from the mainly large commercial production of broilers that dominates the UK poultry industry.

In these countries, source attribution studies and national surveillance programmes are less common and the relative contribution of food, water and environmental sources to campylobacteriosis is poorly understood (Senok and Botta 2009, Komba *et al.* 2013). In Botswana, a survey identified that small-scale poultry farms mostly using family labour account for almost 80% of poultry operations (Badubi *et al.* 2004). Management of poultry farms is considered less important than cattle management, and is generally the responsibility of women (Badubi *et al.* 2004). A mixture of indigenous and imported breeds is often kept on the same farm (Kazwala *et al.* 1993, Mdegela *et al.* 2006, Brena *et al.* 2016), which typically rear flocks of between 100 and 2000 birds (Badubi *et al.* 2004). Biosecurity levels are known to be low in these settings, and backyard flocks are kept near households, with owners occasionally reported to sleep with their chickens as a measure of security (personal communication). Such practices present a potential risk of cross-contamination between the poultry and the human living environment (Conan *et al.* 2012, Brena *et al.* 2016). Retail poultry meat has been reported to be frequently contaminated with *Campylobacter* in Nairobi (Kenya) (Osano and Arimi 1999). In a study conducted in fast-food restaurants and semi-settled stands selling chicken in Kampala

(Uganda), several risk factors for cross-contamination were identified, including the use of the same cutting boards and knives for preparation of raw and cooked meat, lack of use of disinfectants for washing contact surfaces and deficiencies in hand washing (Wanyenya *et al.* 2004). In Nigeria, strains isolated from humans and chickens have been phenotypically correlated through biotyping and serotyping suggesting that chickens can also be a source of human campylobacteriosis in developing countries (Adegbola *et al.* 1990). These studies suggest that, as in developed countries, the chicken reservoir might be an important source of human campylobacteriosis in developing countries through chicken meat consumption and contact with chickens.

C. jejuni followed by *C. coli* are the most common *Campylobacter* species isolated from chickens in UK (Evans and Sayers 2000) and associated with human infection in UK and Uganda (Mshana *et al.* 2009, Sheppard *et al.* 2009). In recent years, however other emerging *Campylobacter* species such as *C. concisus* and *C. upsaliensis* have been detected in live chickens, on processed chicken meat and in patients with a range of gastrointestinal diseases, suggesting that these species might also be at risk of zoonotic transmission to humans (Lynch *et al.* 2011, Man 2011, Kaakoush *et al.* 2014). Along these lines the closely related emerging pathogen *H. pullorum* has been detected within the microbiota of chickens and on chicken meat at retail and a role for zoonotic transmission has been suggested but not proved yet (Ceelen *et al.* 2006, Borges *et al.* 2015). *Helicobacter pullorum* has been related to gastroenteritis (Burnens *et al.* 1994), although a similar prevalence has been reported in clinically healthy individuals compared to patients with gastroenteritis (Ceelen *et al.* 2005). Associations have also been suggested between this species and Crohn's disease and hepatocellular carcinoma (Rocha *et al.*

2005, Laharie *et al.* 2009). Genotyping techniques for *H. pullorum* epidemiological investigations are available (Gibson *et al.* 1999, Ceelen *et al.* 2006, Manfreda *et al.* 2011) but chicken and human isolates have rarely been compared. Gibson *et al.* (1999) found in their study that human and poultry isolates in widely different geographical locations had different genotypes with most strains presenting a high degree of genetic diversity.

The overarching goal of this thesis was to identify key parameters of the chicken farm DWS that influence the survival and, therefore, transmission of *Campylobacter* species. Chapter 3 describes the changing environmental conditions and dynamic microbial community profiles of a commercial broiler farm DWS. *Campylobacter spp.* were detected in low abundance, late in the rearing cycle. This chapter describes culture and PCR-based approaches used to investigate the presence and identity of *Campylobacter spp.* during the same rearing cycle in more detail.

The primary aim was to match 16S rRNA profiling data with rates of isolation and PCR detection of *Campylobacter* species during the rearing cycle of the large UK commercial broiler farm. The water system was investigated as a potential source of transmission. A secondary aim was to conduct a pilot study to investigate the prevalence and identity of *Campylobacter* species in small-scale farms in Western Uganda.

4.2 Screening of a typical UK commercial broiler for the presence of campylobacter spp. across the rearing cycle

4.2.1 *Campylobacter* ssp. were not isolated by culture and *C. jejuni* was only be detected by qPCR at the end of the rearing cycle

A range of samples were collected from a typical commercial broiler farm during a single rearing cycle. Samples included those collected for the amplicon profiling study (source

and shed bulk water) but also chicken faeces; swabs of drip-trays that were below each nipple drinker; and boot socks, worn over clean footwear as a biosecurity measure within the broiler house. Samples were inoculated into enrichment broth and then subcultured onto selective media designed to promote *Campylobacter* isolation. In spite of the use of selective enrichment broth and selective media, no *Campylobacter* spp. were isolated by culture during the 7-week longitudinal study. However, several other bacteria and fungi from the farm were able to grow on the selective media, resulting in agar plates that were overgrown in most cases, which hindered the identification of *Campylobacter* colonies that might have been present. Putative *Campylobacter* colonies were analysed by Gram stain and PCR. Two Gram stains from week 6 revealed Gram negative curved rods and tested positive by PCR using MD16S1/2 primers. However they were found not to be *Campylobacter* (Section 4.2.2).

C. jejuni is the most common *Campylobacter* species reported in broiler chickens. A qPCR to detect *C. jejuni* was carried out using the primers VS15/16 designed by Yang *et al.* (2003), using DNA extractions from the *Campylobacter* enrichment broths and also the DNA extractions from the farm DWS used for the amplicon sequencing experiment. The shed bulk water sample from week 7 tested positive for the presence of *C. jejuni* in the sample used for the amplicon sequencing experiment but not in the enrichment sample, suggesting that *Campylobacter* might have been outgrown at the enrichment level. In this same sample an OTU identified to genus level as *Campylobacter* was detected from 16S rRNA Miseq read data.

4.2.2 Two putative *Campylobacter* isolates were identified as the emerging pathogen *Helicobacter pullorum*

Campylobacter-like colonies were isolated from a dip-tray and a pair of the boot socks during week 6 of the rearing cycle. The colonies were small and grey and took 48 hours to grow when subcultured on to blood agar. Gram stain also revealed a pale pink spiral morphology compatible with *Campylobacter* spp.

Further confirmation was sought using primers specific for the *Campylobacter* 16S rRNA gene (MD16S1/2), which amplified a product of expected size , suggesting positive identification (Figure 4.1) These primers target the 16SrRNA gene of both *C. jejuni* and *C. coli* (Denis *et al.* 1999). Universal 16SrRNA primers eub F530 and eub R790 were used to distinguish which species the isolates belonged to by 16SrRNA partial sequencing. Using the NCBI BLAST tool both isolates were confirmed not to be *Campylobacter spp.,* but rather *Helicobacter pullorum,* with a 100% identity (Figure 4.2). DNA was extracted from one of the isolates and whole-genome sequencing was carried out using the "Nextera XT" library preparation method in a collaborative project, which further confirmed the isolate identity as *H. pullorum.*

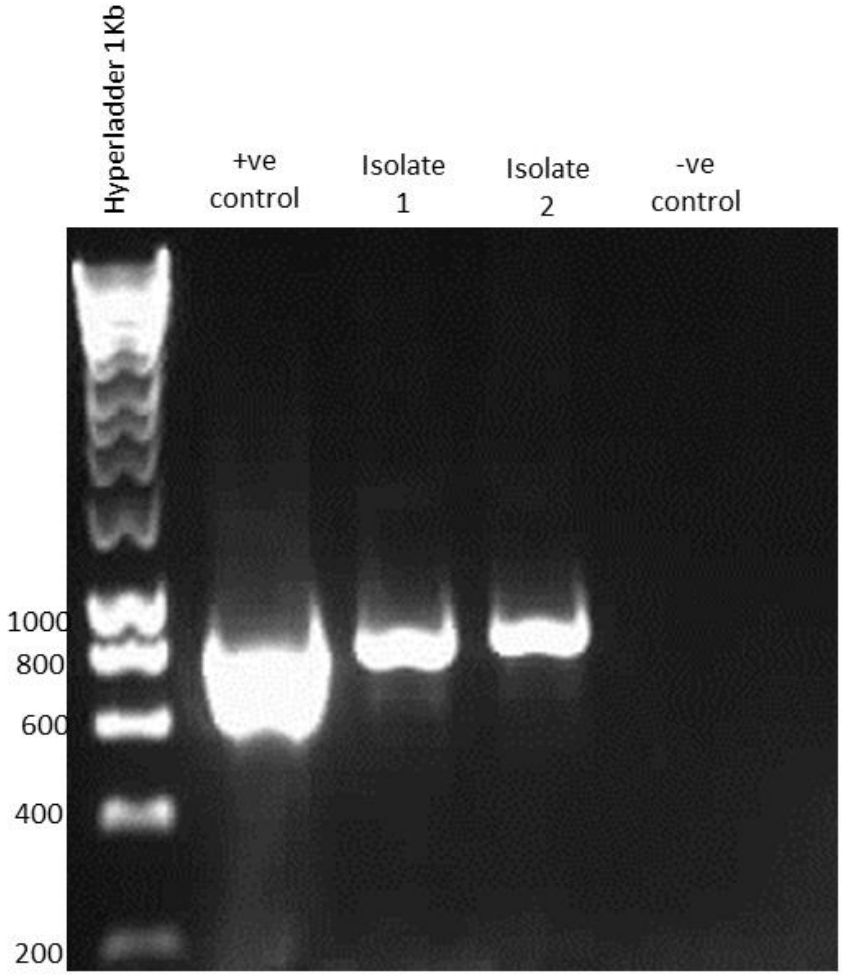


Figure 4.1 1.5% agarose gel showing the result of the PCR for detection of *C. jejuni* and *C. coli* using the primer set MD16S1/MD16S2. Expected product size is 857 bp. DNA was extracted from isolate 1 (dip-tray) and isolate 2 (boot socks) and used for the PCR. A boilprep of C. jejuni 81-176 was used as a positive control. A blank DNA extraction control was used as the negative control. Sizes in base pairs of selected bands in the hyperladder are indicated.

Forward strand

Helicobacter pullorum strain H495 16S ribosomal RNA gene, partial sequence

Sequence ID: KJ534304.1 Length: 1440 Number of Matches: 1

Range 1: 505 to 717 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
394 bits(213)	5e-106	213/213(100%)	0/213(0%)	Plus/Plus

```
Query  1    AGCGCGTAGGCGGGGTAGTAAGTCAGATGTGAAATCCTATGGCTTAACCATAGAACTGCA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  505  AGCGCGTAGGCGGGGTAGTAAGTCAGATGTGAAATCCTATGGCTTAACCATAGAACTGCA  564

Query  61   TTTGAAACTACTACTCTAGAGTATGGGAGAGGTAGGTGGAATTCTTGGTGTAGGGGTAAA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  565  TTTGAAACTACTACTCTAGAGTATGGGAGAGGTAGGTGGAATTCTTGGTGTAGGGGTAAA  624

Query  121  ATCCGTAGAGATCAAGAGGAATACTCATTGCGAAGGCGACCTGCTGGAACATTACTGACG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  625  ATCCGTAGAGATCAAGAGGAATACTCATTGCGAAGGCGACCTGCTGGAACATTACTGACG  684

Query  181  CTGATGCGCGAAAGCGTGGGGAGCAAACAGGAT  213
            |||||||||||||||||||||||||||||||||
Sbjct  685  CTGATGCGCGAAAGCGTGGGGAGCAAACAGGAT  717
```

Reverse strand

Helicobacter pullorum partial 16S rRNA gene, strain 98/58

Sequence ID: AJ876521.1 Length: 966 Number of Matches: 1

Range 1: 479 to 699 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
409 bits(221)	2e-110	221/221(100%)	0/221(0%)	Plus/Minus

```
Query  1    CAGCAGGTCGCCTTCGCAATGAGTATTCCTCTTGATCTCTACGGATTTTACCCCTACACC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  699  CAGCAGGTCGCCTTCGCAATGAGTATTCCTCTTGATCTCTACGGATTTTACCCCTACACC  640

Query  61   AAGAATTCCACCTACCTCTCCCATACTCTAGAGTAGTAGTTTCAAATGCAGTTCTATGGT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  639  AAGAATTCCACCTACCTCTCCCATACTCTAGAGTAGTAGTTTCAAATGCAGTTCTATGGT  580

Query  121  TAAGCCATAGGATTTCACATCTGACTTACTACCCCGCCTACGCGCTCTTTACGCCCAGTG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  579  TAAGCCATAGGATTTCACATCTGACTTACTACCCCGCCTACGCGCTCTTTACGCCCAGTG  520

Query  181  ATTCCGAGTAACGCTTGCACCCTCCGTATTACCGCGGCTGC  221
            |||||||||||||||||||||||||||||||||||||||||
Sbjct  519  ATTCCGAGTAACGCTTGCACCCTCCGTATTACCGCGGCTGC  479
```

Figure 4.2 BLAST alignment of 16S rRNA sequence of farm isolates on Campylobacter selective media. Two isolates from week 6 were subjected to partial 16S rRNA sequencing. Representative BLAST results matching *Helicobacter pullorum* (both isolates gave the same sequence).

A retrospective analysis of all the enrichment samples stored from the longitudinal farm study by PCR using the primers 818-839/1265-1247 designed by Stanley *et al.* (1994) revealed that the bulk water samples collected in weeks 1 and 4; the dip-tray in week 3 and boot socks and faeces in week 5 contained *H. pullorum* DNA (Table 4.1). *H. pullorum* was also detected by PCR in week 6 in the boot socks but not in the dip tray in spite of having been isolated by culture from those two samples. These findings suggest that *H. pullorum,* may be present in the broiler house DWS in low abundance and could be carried over to subsequent flocks.

Table 4.1 Detection of *H. pullorum* during the commercial broiler rearing cycle.

	Week 1	Week 2	Week 3	Week 4	Week 5	*Week 6	Week 7
[1]Bulk water	+ve			+ve			
[2]Drip tray			+ve				
[3]Boot Socks					+ve	+ve	
[4]Faeces					+ve		

DNA was extracted from *Campylobacter* enrichment broths from [1]Bulk water collected from the blind end of the WDS in the broiler house, [2]Two of the drip trays, positioned under each nipple drinker sampled, [2]Pair of boot socks, worn over shoes, whilst within the broiler house, [4]Faeces samples collected from the broiler house floor. **H. pullorum* was isolated by culture in week 6 from the boot socks and the dip tray.

4.2.3 *Campylobacter ureolyticus* was detected by PCR in the drinking water system and in chicken faeces

C. ureolyticus was detected in the 16S amplicon library of biofilm samples from the internal nipple drinker collected in week 5 of the longitudinal farm study (chapter 3). This was an interesting result as *C. ureolyticus* has not been reported in association with poultry before, although epidemiological information about *C. ureolyticus* is very limited (Koziel *et al.* 2012). *C. ureolyticus* optimal growth requires hydrogen-enriched conditions and a temperature range of 35-37 °C (Vandamme *et al.* 2010). These conditions differ from those used in this study (microaerobic conditions and 42°C incubation), which may explain why this species was not isolated. Subsequently the presence of *C. ureolyticus* was investigated in DWS and faecal samples by PCR using the primers CU-HSP60 designed by Bullman *et al.* (2011a). Only a limited number of faecal samples were available as most of the faecal samples (and other farm environmental samples) were processed only to isolate *Campylobacter* spp. by culture. Four faecal samples (2 from week 5 and 2 from week 6) had been kept at -20°C without processing and DNA was extracted. In spite of the small sample size, *C. ureolyticus* was detected in one faecal sample collected in week 6 (Table 4.2). To further investigate the presence of *C. ureolyticus* on the farm environment the DNA samples used for the 16S and 18S amplicon profiling were screened for *C. ureolyticus* by PCR. Unexpectedly the biofilm sample where *C. ureolyticus* was detected by 16S amplicon sequencing was negative by PCR. However, the DNA samples from bulk water in weeks 6 and 7 were positive, possibly suggesting a role in the water distribution system in *C. ureolyticus* transmission on the broiler farm (Table 4.2).

Table 4.2. Screening of samples from the DWS and faeces for *C. ureolyticus* by PCR.

	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
[1]Faeces	NA	NA	NA	NA		+ve	NA
[2]Shed bulk water						+ve	+ve
[3]Anteroom bulk water							
[4]Biofilm (nipple drinker)					*		
[5]Biofilm (swab)							
[6]External nipple drinker							

DNA was extracted from [1]faecal samples collected from the floor and [2-6]different locations on the farm DWS. "NA" indicates that samples were not available for screening. "*" indicates that the sample was positive according to 16S rRNA profiling.

However, during a routine PCR screening for *C. ureolyticus* on farm samples, DNA extracted from pure *H. pullorum* culture also tested positive. This suggested possible cross-reactivity that may affect surveillance results using these primers. A BLAST database was created from the genome sequence of the *H. pullorum* isolate from the farm. Several sets of primers used in this study showed a considerable level of cross-reactivity (Table 4.3). This observation has implications for epidemiological surveillance of *Campylobacter* and *Helicobacter* pathogens.

Table 4.3. Cross-reactivity of primers with *H. pullorum* genome.

Primer Set	Annealing site F Contig, %identitiy, mismatches, nt	Annealing site R Contig, %identitiy, mismatches, nt	Expected size (bp)
CU_HSP60	**Contig_2, 96%, 1, 74460-74484**	**Contig_2, 95%, 1, 74881-74862**	**421**
MD16S	**Contig_9, 100%, 0, 73938-73950**	**Contig_9, 100%, 0, 74785-74767**	**847**

Cross-reactivity of primers specific for *C. jejuni*/*C. coli* (MD16S) and *C. ureolyticus* (CU_HSP60) with the *H. pullorum* genome sequenced in this study. Expected sizes of the amplicons are almost identical to the specific expected sizes for *Campylobacter* spp. (857 bp for MD16S and 429 bp for CU_HSP60).

4.3 Pilot study to assess levels of *Campylobacter* and *H. pullorum* in small holdings in Western Uganda

During a 14 day visit of Fort Portal in Western Uganda, three small holding chicken farms were investigated on the same day. All three farms were small-scale semi-intensive poultry operations in which a small number of birds were produced in complete confinement. Farm 1 had cattle, rabbits, cows, goats and layers in proximity to the chickens. Similarly farm 3 had layers and cattle. A total of 50 faecal samples were collected from the 3 farms (Table 4.4).

Table 4.4: Description of Ugandan Chicken Farms.

Farm	Description	# Faecal Samples
1	**Shed 1: 500 broiler chickens, 3 weeks old.**	**8(1-8)**
	Shed 2: <10 isolated broiler chickens, suspected bronchitis, treated with tetracycline.	**5(9-13)**
		TOTAL: 13
2	**Shed 1: 34 local, mixed sex chickens, 3 months old.**	**7(14-20)**
	Shed 2: 12 local, mixed sex chickens, 1 year old.	**10(21-30)**
	Shed 3: 7 wild, mixed sex chickens.	**4(31-34)**
		TOTAL: 21
3	**Shed 1: 135 kuroilers, mixed sex, 135 birds.**	**9 (35-43)**
	Shed 2: chickens, under 1 week.	**7(44-50)**
		TOTAL: 16

4.3.1 *Campylobacter* ssp. were isolated by culture on all three farms

A total of 50 faecal samples were collected from the three different farms and screened for the presence *Campylobacter* and *H. pullorum* by culture and PCR. *C. jejuni* and/or *C. coli* was isolated from all three farms but not in all the sheds in spite of the presence of other bacteria and fungi that were also able to grow in the selective plates hindering the pathogen isolation. The identity of the colonies was confirmed by PCR using different sets of primers for *Campylobacter* spp. (MD16S1/2 (Denis *et al.* 1999) and CampF2/R2 (Lund *et al.* 2004), *C. jejuni* (VS15/16 (Yang *et al.* 2003) and HipO F/R (Vondrakova *et al.* 2014)), *C. coli* (glyA F/R (Vondrakova *et al.* 2014)) and *H. pullorum* (cdtBF1/R2 (Rocha *et al.* 2005)). Due to extensive overgrowth of background microflora on the plates, the

incidence results are likely to be an underestimation of the real numbers and culture results could only be reported as presence/absence. On farm 1, which contained broilers, *C. coli* was isolated whereas in farm 2, which contained local chickens, *C. jejuni* was isolated. Both *C. jejuni* and *C. coli* isolates were found in farm 3 which contained kuroilers (a mixed race of broiler males and indigenous females originated in India (Khan 2008)). *Campylobacter* ssp. were not isolated from shed 2 in farm 3 which harboured chickens less than one week old (Table 4.5). This is in keeping with a large body of evidence that young chickens are not colonised by *Campylobacter* (Lee and Newell 2006).

4.3.2 *Campylobacter* spp. were detected by qPCR

The culture analysis was complemented with screening by qPCR to study the extent of *Campylobacter* colonization in the different flocks. For several faeces samples, *C. jejuni* and *C. coli* were both detected by culture and therefore a set of primers that could detect both species was desirable for the qPCRs. The primers campF2/campR2 designed by Lund *et al.* (2004) were chosen as they detect *C. jejuni* and *C. coli* and also other *Campylobacter* species (*C. lari*, *C. upsaliensis*, *C. helveticus* and *C. hyoientestinalis*). As expected from previous research qPCR screening detected a high prevalence of *Campylobacter* positive samples. Overall 95% of faecal samples (41/43) were positive for *Campylobacter* (Table 4. 5). There was a 92.3% prevalence detected on farm 1, (8/8 from shed 1 and 4/5 from shed 2). Similarly, there was 95.2% prevalence on farm 2, with 6/7; 10/10 and 4/4 samples testing positive in sheds 1, 2 and 3 respectively. The screening of samples from farm 3 was complicated by an issue with contamination of the negative extraction. Samples from shed 1 all tested positive (9/9) with amplification occurring at least 5 cycles before the negative control amplified, suggesting 100% prevalence. However, samples

collected from shed 2 amplified with similar Ct values to the DNA extraction negative control and therefore it cannot be ruled out that they tested positive as a consequence of contamination (Figure 4.3).

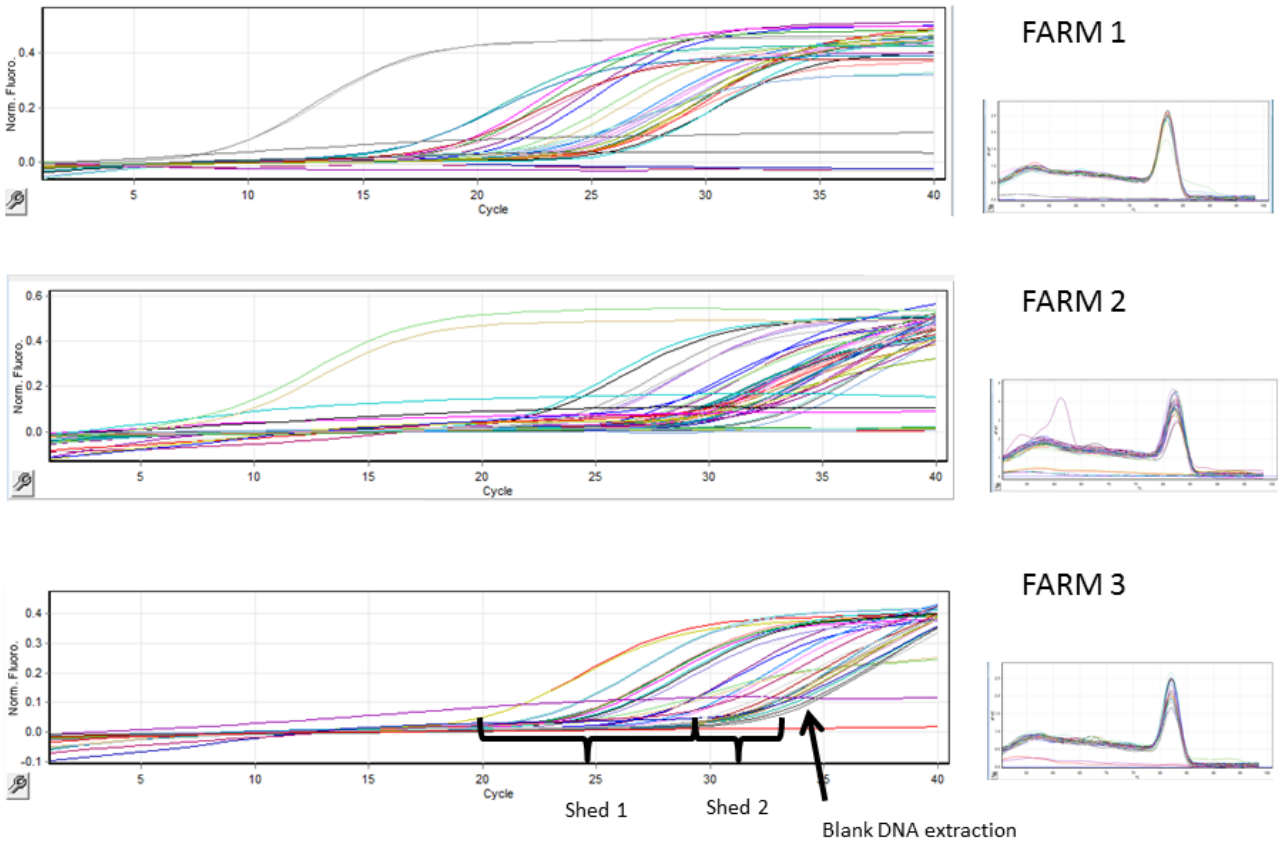


Figure 4.3. QPCR results for detection of *Campylobacter* spp. in faecal samples collected from chicken farms in Western Uganda. The qPCR was carried out using the primers campF2/campR2 designed by Lund *et al.* (2004).

4.3.3 *H. pullorum* were detected by PCR on all three farms

Helicobacter pullorum was detected by PCR on all three farms with an overall prevalence of 14% using an assay designed by Stanley *et al.* (1994) (Table 4.4). The prevalence was higher on farm 1 with 38% samples testing positive than on farms 2 (4.7%) and 3 (6.3%) Only samples from one of the three sheds on farm 2 were positive (Shed 2, 14 %). Similarly on farm 3 only one sample from of the two sheds tested positive (Shed 1, 11%).

Table 4.5 Detection of *Campylobacter* and *H. pullorum* on faecal samples from ugandan chicken farms.

Farm	Shed	[1]Culture	[3]*Campylobacter* spp.	[5]*H. pullorum*
1	**1**	**-ve**	**8/8**	**4/8**
	2	***C. coli***	**4/5**	**1/5**
2	**1**	**-ve**	**6/7**	**1/7**
	2	***C. jejuni***	**10/10**	**0/10**
	3	**-ve**	**4/4**	**0/4**
3	**1**	***C.jejuni & C. coli***	**9/9**	**1/9**
	2	**[2]-ve**	**[4]unconfirmed**	**0/7**

[1]All agar plates were overgrown; but colonies were purified from initial isolation plates and confirmed as *C. jejuni* or *C. coli* by PCR; [2] Faeces samples from shed 2 were pooled for culture. [3] *Campylobacter* spp. screening by qPCR (Lund *et al.* 2004); [4]Detection un-confirmed as amplification was at the same ct as negative control. [5] *H. pullorum* screening by PCR (Stanley *et al.* 1994).

4.4 Discussion

Campylobacter spp. are frequently isolated from chickens worldwide in spite of the different rearing systems used (intensive, semi-intensive and extensive operations) (Sahin *et al.* 2015). In this chapter two small-scale studies in different locations are reported. In the 1st study, a flock was followed for a whole rearing cycle on a commercial broiler farm in the UK to investigate the presence of *Campylobacter* spp. and profile the wider microbial communities in the DWS. The second study involved sampling three small-scale semi-intensive chicken farms in Uganda.

Campylobacter spp. were not isolated by culture during the longitudinal study carried out in the UK. The isolation of *Campylobacter* spp. from the farm environment proved very challenging due to the growth of background bacteria and fungi on the mCCDA plates, particularly from faecal samples and boot socks. The strategy chosen included enrichment in CEB and plating into the mCCDA selective media. CEB contains the antibiotics recommended for the original Preston medium formulation (rifampicin, polymyxin B and trimethoprim) (Bolton and Robertson 1982) but acitione is replaced by amphotericin B. It has been shown that the inclusion of an enrichment step with Preston broth provides more sensitive detection of *Campylobacter* than direct plating from human and animal faeces and water (Bolton and Robertson 1982, Ribeiro and Price 1984). Notably it was demonstrated that it is more successful isolating low counts of *Campylobacter* (Bolton and Robertson 1982). However direct plating has been shown to be more sensitive to detect *Campylobacter* in broiler litter and faeces samples when the contamination is high (Vaz *et al.* 2014). In this longitudinal study low counts of *Campylobacter* were to be expected in the environmental samples, particularly in water,

which led to the choice of including enrichment. It was also anticipated that *Campylobacter* in the DWS may exist in VBNC state that needs to recover before being detected by culture (Cools *et al.* 2003). MCCDA is one of the culture media recommended by the International Organisation for Standardization (ISO) for isolation of *Campylobacter* from a range of samples including environmental and water samples (cited in Smith *et al.* (2015)). The basal media for mCCDA was developed to replace blood with charcoal, ferrous sulphate and sodium pyruvate by Bolton and Coates (1983). In subsequent work cefazolin and sodium deoxycholate were included in the combination as selective agents to use the media, which was named CCDA, for isolation (Bolton *et al.* 1984). In order to improve the selectivity, the mCCDA media was described, where cefazolin was replaced by cefoperazone (Hutchinson and Bolton 1984). Amphotericin B was included to suppress yeast growth at 37 °C (Corry *et al.* 1995).

A comparative study found that mCCDA was more selective than other commonly used media(Cefex, Campy-Line Agar, Campy-CVA agar) for *Campylobacter* spp. detection in chicken faecal samples (Oakley *et al.* 2012). Nonetheless, other studies have reported that faecal contaminants can grow in mCCDA plates (Hutchinson and Bolton 1984, Oakley *et al.* 2012) and other authors have reported failure to detect *Campylobacter* in known positive samples due to microbial growth on antibiotic medium (Steele and McDermott 1984). In other studies it has been recognised that overgrowth of competing flora hampers the recognition of *Campylobacter* colonies (Kiess *et al.* 2010, Vaz *et al.* 2014). Therefore the absence of isolation or detection in the enrichment broth DNA extractions does not necessarily mean that the samples did not contain *Campylobacter* spp. and it remains possible that low concentrations of the bacteria could not thrive due to being

outcompeted by other microorganisms either during enrichment or on the plates. An alternative to the use of antibiotic selective media is filtration through cellulose triacetate membranes directly to the surface of non-selective blood agar plates (Steele and McDermott 1984). Although this method is more labour intensive, a direct comparison of this protocol and the use of selective media based on antibiotics found that filtration resulted in a significant higher prevalence in both human faeces and broiler intestinal samples (Jacob *et al.* 2011). Others however have reported poor performance in terms of both sensitivity and specificity when compared to various selective agars with broiler fresh faecal droppings as samples (Oakley *et al.* 2012). Not only the sample types differ in these two studies but also the filter materials (cellulose-ester (Oakley *et al.* 2012) vs. cellulose acetate (Jacob *et al.* 2011)) and both might explain the discrepancies among the two studies. Conflicting results about the performance of different protocols for *Campylobacter* isolation are common and recovery depends on sample type and state of the cells (Kim *et al.* 2009, Ugarte-Ruiz *et al.* 2012) which makes it difficult to choose the right protocol.

The temperature of incubation (42 °C) might also have affected the specificity of the protocol. Incubation at 42 °C was chosen because that is the approximate body temperature of chickens (Giloh *et al.* 2012), it is among the optimum temperature range of *C. jejuni* (Davis and DiRita 2008) and has been used in the past for *Campylobacter* isolation from water and faeces on chicken farms (Cokal *et al.* 2011, Battersby *et al.* 2016). However Oakley *et al.* (2012) found significantly more non-*Campylobacter* sequence types at 42 °C than 37 °C using mCCDA and other selective agars.

Several qPCR assays have been described to detect *Campylobacter* spp. in different matrices including water and faeces (Lund *et al.* 2004, Vondrakova *et al.* 2014). For the longitudinal farm study, *C. jejuni* specific primers were chosen because *C. jejuni* is the most common species found in chicken flocks in the UK (Evans and Sayers 2000, Jorgensen *et al.* 2011). Using this method *C. jejuni* was detected by qPCR in the shed bulk water in week 7 but not in the same sample that had been enriched in CEB. However, an OTU identified as *Campylobacter* was also found in the 16S rRNA study in this same sample. This result might indicate that low levels of *Campylobacter* spp. were outgrown at the enrichment level, which would explain the lack of isolation and detection by qPCR.

Due to the difficulties with the isolation process it is difficult to assess whether the flock studied was colonized with *C. jejuni*. It has been reported that once *Campylobacter* colonizes the flock it spreads rapidly among the birds and it can be isolated from farm environmental samples (Battersby *et al.* 2016). However, in this study the only samples that were available for screening and had not been processed for culture (and possibly outgrown at enrichment level), were sample from the DWS. In some studies *Campylobacter* has been readily detected in the DWS after the chickens had been colonized with the pathogen (Pearson *et al.* 1993, Ogden *et al.* 2007). However different *C. jejuni* strains are known to differ in their ability to survive in water (Cools *et al.* 2003) and therefore it remains a possibility that the flock was colonized with a strain that survived poorly in the DWS on the farm. Another possibility is that *C. jejuni* was present in low abundance on the farm DWS but the flock was not colonized or only partially colonized and therefore the contamination was not widespread enough for the pathogen

to be isolated or detected. Although *Campylobacter* spp. usually spread rapidly through the flock, partial colonization has also been reported (Allen *et al.* 2007).

In the pilot study conducted in Uganda *C. jejuni* and *C. coli* were isolated by culture in spite of the difficulties with the isolation process due to competing microorganisms. In this study direct plating was used to simplify the processing. *Campylobacter* spp. were isolated on the three farms suggesting extensive chicken colonization in the area. Although to the best of my knowledge this is the first report on chicken colonization in Uganda, studies from small scale farms in Tanzania have isolated *C. jejuni* and *C. coli* from both broilers and local chicken breeds previously (Kazwala *et al.* 1993, Mdegela *et al.* 2006). Using culture techniques, Kazwala *et al.* (1993) reported 26.4% prevalence in broilers and 76.4% prevalence in indigenous poultry while Mdegela *et al.* (2006) reported 69% in broilers and 71% in local chickens. The isolation rate in our study was low. However, qPCR assays detected an overall prevalence of *Campylobacter* spp. of 95%, suggesting that the low rate of isolation was due to the overgrowth of fungi and bacteria on the selective plates.

H. pullorum is an emerging zoonotic pathogen that has been found in various geographical zones and in a wide range of hosts (poultry birds, rodents, rabbits and humans), potentially posing a health risk to humans (Javed *et al.* 2017). Although there is limited information about the occurrence of *H. pullorum* in poultry, variable but often high prevalence rates ranging from 20% to 100% have been reported in chickens in different regions and under different husbandry practices (conventional, free-range and organic farms) in the studies that have addressed the issue (Ceelen *et al.* 2006, Mohamed *et al.* 2010, Manfreda *et al.* 2011, Kaakoush *et al.* 2014). In this study *H. pullorum* was

detected on the farm in UK and also on the three farms in Uganda. The overall prevalence of the farms in W. Uganda was 14%.

In the longitudinal study in the UK *H. pullorum* was isolated on *Campylobacter* selective media (mCCDA). Isolation of *H. pullorum* from fresh chicken abattoir samples (Atabay and Corry 1997, Atabay *et al.* 1998) and from a patient presenting with gastroenteritis (Steinbrueckner *et al.* 1997) in mCCDA has been previously reported, as *H. pullorum* has similar growth requirements to *C. jejuni*/*C. coli* (Microaerobic atmosphere, 37-42 °C temperature, resistant to cefoperazone) (Stanley *et al.* 1994). Using PCR *H. pullorum* was detected in the DWS before it was detected in the chickens, suggesting that the chickens might have become colonized with *H. pullorum* from the DWS. This supports evidence from the microbial community profiling study (chapter 3) showing that chickens may exchange microorganisms with the DWS, which in this case may aid to spread further and maintain *H. pullorum* in-between flocks.

At the moment it is unknown whether *H. pullorum* can be acquired through consumption of contaminated poultry meat, as is the case for *Campylobacter* spp., but given the high prevalence in chickens reported from various regions and the isolation from fresh chicken products at retail it seems likely (Atabay *et al.* 1998, Borges *et al.* 2015, Javed *et al.* 2017). Genotyping studies comparing human and poultry isolates are needed to enable source tracking and infection risk studies (Javed *et al.* 2017).

H. pullorum has been related to gastroenteritis in humans (Burnens *et al.* 1994, Steinbrueckner *et al.* 1997, Ceelen *et al.* 2005) but the accurate identification of the pathogen is challenging due to similarities with closely related *Campylobacter* species (On 1996, Steinbrueckner *et al.* 1997). Primer specificity is a particular cause of concern for

epidemiological surveillance as new taxa are described (On 1996). In this study cross-reactivity has been found for *H. pullorum* with primers specific for *C. jejuni* and *C. coli* (Denis *et al.* 1999) and also for primers specific for *C. ureolyticus* (Bullman *et al.* 2011a). This raises issues regarding the re-evaluation of primer specificity used for epidemiological surveillance. The primers described by Denis *et al.* (1999) have been widely used in the scientific community, with approximately 284 articles citing their work according to Google Scholar. As an example, in a recent study conducted this same year, isolation and the PCR assay described by Denis *et al.* (1999) were used to determine the prevalence of thermotolerant *Campylobacter* spp. in livestock (Rahimi *et al.* 2017). Although the paper by Denis *et al.* (1999) described species specific primers to complement the PCR results of the MD16S primers, and therefore it seems reasonable to conclude that the MD16S primers are unlikely to have been used in important epidemiological studies in isolation, this cross-reactivity is still a cause of concern and illustrates a wider general issue with specificity.

Perhaps more concerning is the cross-reactivity of the CU_HSP 60 primers specific for *C. ureolyticus* with the *H. pullorum* genome of the farm isolate. Using these primers Bullman *et al.* (2011b) detected *C. ureolyticus* in 24.4% of *Campylobacter*-positive faecal samples of patients presenting with gastroenteritis to Cork University Hospital. It was reported as the sole pathogen (without other bacteria and viruses) in the samples of 55 symptomatic patients (16 %) but in 28 patients (8.2%) it was detected in the presence of other *Campylobacter* spp. Based on these numbers the authors have claimed that *C. ureolyticus* appears to surpass *C. coli* as the second most common causative agent of *Campylobacter*-related gastroenteritis in samples collected from southern Ireland and

have suggested that this might represent a national trend in Ireland (Bullman *et al.* 2011a, Bullman *et al.* 2011b, O'Donovan *et al.* 2014). However, as we have shown that these primers cross-react with an *H. pullorum* isolate, there is a risk that these conclusions are an overestimation because some of the positive results might have resulted from cross-reaction with *H. pullorum*. The *Campylobacter*-genus specific primers used in the study by Bullman *et al.* (2011b) do not appear to cross-react with the *H. pullorum* isolate, so the risk of cross-reaction will probably be greater in the samples were *C. ureolyticus* was reported with other *Campylobacter* spp. However it cannot be completely disregarded the possibility that another *Campylobacter* species not tested for other than *C. ureolyticus* had been responsible for the positive result at genus level and the positive result in the *C. ureolyticus* PCR is due to a cross-reaction with *H. pullorum*. The protocol used by this research group includes species-specific PCR assays for *C. jejuni*, *C. coli*, *C. lari*, *C. fetus*, *C. hyointestinalis*, *C. upsaliensis* and *C. ureolyticus* but not *C. concisus* (Bullman *et al.* 2012). This is surprising, as *C. concisus*, along with *C. upsaliensis* has consistently been the predominant emerging *Campylobacter* spp. isolated with patients with diarrhea (Man 2011). In similar studies where both *C. concisus* and *C. ureolyticus* have been considered, *C. concisus* has been found with higher prevalence than *C. ureolyticus*. Collado *et al.* (2013) reported prevalence of 11.4% for *C. concisus* and 3.6% for *C. ureolyticus* in 140 human faecal samples from patients with diarrhoea. Cornelius *et al.* (2012) reported a more dramatic difference with a prevalence of 10.9% for *C. ureolyticus* and 46.9% for *C. concisus* in 128 diarrhoea specimens. Cornelius *et al.* (2012) also associated with gastroenteritis two more *Campylobacter* species not included in the protocol used by Bullman *et al.* (2011b), *C. rectus/showae* (3.9%) and *C. sputorum* (0.78%). On the other hand, they reported a low prevalence of *H. pullorum* (1.6%)

(Cornelius *et al.* 2012). Moreover, in a study conducted in Belgium *H. pullorum* was detected in 4.3% of patients presenting with gastroenteritis (Ceelen *et al.* 2005). Therefore there is a real risk that the use of these primers by Bullman *et al.* (2011b) and others (e.g. Collado *et al.* (2013)) has overestimated the contribution of *C. ureolyticus* to the burden of human campylobacteriosis.

In this study the set of primers were used to investigate the presence of *C. ureolyticus* in chicken faeces and DWS samples after this pathogen was identified in the microbial community profiling experiment. A positive result was found in one chicken faecal samples (week 6) and in the shed bulk water in weeks 6 and 7 but these results are inconclusive due to the cross-reaction of the primers.

Chapter 5

Inter-species interactions enhance survival of *C. jejuni* outside its hosts under aerobic conditions

5.1 Introduction

C. jejuni are fastidious microaerophiles but they are adapted to survive in the environment in spite of low nutrient levels, oxidative stress and other challenges (Murphy *et al.* 2006). The viable but nonculturable (VBNC) state, biofilm formation and interactions with other microorganisms have all been documented as strategies to cope with unfavourable conditions (Bronowski *et al.* 2014).

*Pseudomona*s spp. and free-living protozoa (FLP) are widespread in natural ecosystems such as fresh water and soil (Peix *et al.* 2009, Thomas *et al.* 2010). They frequently colonize drinking water distribution networks regardless of the presence of biocides such as chlorine (Thomas *et al.* 2008, Buse *et al.* 2013, Douterelo *et al.* 2014, Liu *et al.* 2014). They are also part of the microbial communities in broiler farms. FLP are common and widespread in broiler houses in the DWS and to a lesser extent in litter, animal feed and dry areas (Snelling *et al.* 2005, Snelling *et al.* 2006, Bare *et al.* 2009). Importantly they have been shown to persist across rearing cycles in spite of cleaning and disinfection (Bare *et al.* 2011). *Pseudomonas* spp. have also been found in several locations in chicken farms including water drinkers and crates to haul chickens (Hanning *et al.* 2008).

Moreover they are commonly isolated in mixed species communities from broiler chicken carcasses (Arnaut-Rollier *et al.* 1999a, Arnaut-Rollier *et al.* 1999b, Sanders *et al.* 2008).

Some FLP species such as *Acanthamoeba castellani*, *Acanthamoeba polyphaga* and *Tetrahymena pyriformis* can act as hosts of numerous pathogenic foodborne and waterborne bacteria such as *Salmonella*, *Listeria monocytogenes* and *Vibrio* representing a public health risk (Thomas *et al.* 2010, Vaerewijck *et al.* 2014). FLP contribute to the maintenance of pathogens in the environment by prolonging their survival or even promoting their replication, with or without lysis of the protozoan cell (Anacarso *et al.* 2012). When internalized, pathogenic bacteria can be protected from antimicrobial agents (King *et al.* 1988). *Acanthamoeba* represent a hotspot for genetic exchange between eukaryotic hosts, bacteria and viruses and bacterial pathogens that are able to survive FLP digestion might show increased virulence, as FLP share similarities with macrophages (Vaerewijck *et al.* 2014). Fifty-four bacterial genera have been identified inside free-living amoebae (FLA) using a metagenomics approach, suggesting that amoebae can be hosts to large numbers and diversity of bacteria (Delafont *et al.* 2013).

It has been proposed that *C. jejuni* benefits from interacting with FLP, particularly those belonging to the genus *Acanthamoeba* (Vieira *et al.* 2015). Enhanced survival time and/or temperature dependent replication has been observed under aerobic conditions when *C. jejuni* has been co-cultured with *Acanthamoeba* (Axelsson-Olsson *et al.* 2005, Snelling *et al.* 2005, Axelsson-Olsson *et al.* 2010a, Axelsson-Olsson *et al.* 2010b, Bare *et al.* 2010, Bui *et al.* 2012a, Bui *et al.* 2012b). Co-cultivation with *Acanthamoeba* was also able to resuscitate VBNC cells from culture negative samples and enrich very low number of *C. jejuni* (less than 10CFU) (Axelsson-Olsson *et al.* 2005, Axelsson-Olsson *et al.* 2007,

Griekspoor *et al.* 2013). Some authors have suggested that this is a consequence of internalization of the bacteria by the amoebae (Axelsson-Olsson *et al.* 2005, Snelling *et al.* 2005, Axelsson-Olsson *et al.* 2010a, Axelsson-Olsson *et al.* 2010b). Increased tolerance to chlorination and disinfection due to internalization in *Acanthamoeba* has been reported (King *et al.* 1988, Snelling *et al.* 2005, Snelling *et al.* 2008). Others, however, found that a reduction in oxygen concentration in the co-culture was the most likely mechanism for explaining the growth/prolonged survival observed in the co-cultures (Bui *et al.* 2012b).

In addition to the debate on FLP, several studies have demonstrated that bacterial multispecies biofilms can increase *C. jejuni* survival under aerobic conditions (Buswell *et al.* 1998a, Buswell *et al.* 1998b, Zimmer *et al.* 2003, Reeser *et al.* 2007). Multispecies biofilms show high structural heterogeneity with variable oxygen concentration in different areas and the possibility of reaching anaerobic conditions (Costerton *et al.* 1994).

Pseudomonas spp. have been identified as biofilm pioneers providing an environmental refuge for *C. jejuni* (Trachoo *et al.* 2002, Hanning *et al.* 2008, Sanders *et al.* 2008, Ica *et al.* 2012, Culotti and Packman 2015). Hilbert *et al.* (2010) reported survival of over 100 *C. jejuni* field isolates when co-cultured with *P. fluorescens, P.putida* and *P.fragi* isolated from poultry meat. It could be assumed that this enhanced survival rate is due to depletion of oxygen by *Pseudomonas* spp. However, survival was longer in co-cultures with *Pseudomonas* spp. compared to other meat spoiling bacteria, suggesting a more specific interaction. *P. aeruginosa* generates a localized microaerophilic environment that promotes persistence of *C. jejuni* under oxic flow conditions (Ica *et al.* 2012, Culotti and Packman 2015) and in static co-cultures (Trachoo *et al.* 2002). Mixed species biofilm

communities containing an unidentified *Pseudomonas* species and monospecies *P. aeruginosa* biofilm have been shown to promote *C. jejuni* biofilm growth (Sanders *et al.* 2007, Culotti and Packman 2015). *C. jejuni* have been observed to enter the VBNC state when forming part of multispecies biofilms (Buswell *et al.* 1998b). Ica *et al.* (2012) however reported *C. jejuni* entering VBNC state in monospecies biofilms but remaining in a culturable physiological state when in a multispecies biofilm with *P. aeruginosa* which also was more robust and unaffected by higher flow rates.

The relationship between *Campylobacter* and FLP/*Pseudomonas* spp. are species and strain specific (Axelsson-Olsson *et al.* 2010a, Hilbert *et al.* 2010). It seems clear that *Acanthamoeba* has the potential to increase *C. jejuni* survival and to internalize it but the epidemiological relevance of the internalization process remains controversial (Vieira *et al.* 2015). Experimental infection studies have demonstrated colonization of broiler chickens caeca by *C. jejuni* internalized within *A. castellanii* (Snelling *et al.* 2008). Further study of the interactions of *Acanthamoeba* and *C. jejuni* and in particular of the bacterial internalization by the amoebae is warranted. In this chapter a gentamicin protection assay is used to determine the rates of internalization of *C. jejuni* 81-176 by *A. polyphaga*. To build upon evidence of *Pseudomonas-Campylobacter* interactions, a range of environmental *Pseudomonas* isolates (including isolates from DWDS biofilms) were co-cultured with defined *C. jejuni* strains to determine any enhanced survival under aerobic conditions.

5.2 Interactions between *Pseudomonas* and *C. jejuni*

5.2.1 *Campylobacter jejuni* strains 11168 and 81-176 show prolonged aerobic survival when co-cultured with certain *Pseudomonas fluorescens* strains

P. fluorescens is an ubiquitous environmental bacterial species and a well documented inhabitant of DWDS (Schmeisser *et al.* 2003, Douterelo *et al.* 2014). A range of *P. fluorescens* strains were screened for their ability to enhance the survival of *C. jejuni* strains 11168 and 81-176 in aerobic conditions. When co-cultured with *C. jejuni* at 25 °C in MHB, *P. fluorescens* strains grew to optical densities (ODs) between 0.8 and 1.6 (figure 5.1C). Survival of *C. jejuni* was determined after 24h co-culture by measuring CFU/mL on *Campylobacte*r selective media (CSM). *C. jejuni* viable counts decreased significantly in each co-culture after 24h of aerobic incubation at 25 °C in all the co-cultures tested and with both *C. jejuni* strains (*C. jejuni* 11168 p=0.001-0.023, *C. jejuni* 81-176 p=0.000-0.038) with the exception of *C. jejuni* 81-176-*P. fluorescens* Pf0-1 (p=0.061). A one-way ANOVA found statistically significant differences between the final counts recovered with both strains (p=0.000 in both cases). Using the Turkey method as a post-hoc test, three *P. fluorescens* strains (Pf0-1, ATCC 17400 and WCS 365) were grouped together and shown to significantly enhance survival of both 11168 (figure 5.1A) and 81-176 (figure 5.1B) compared to incubation with MHB alone and co-culture with the other strains. However, two strains (Pf-5 and F113) appeared to have an antagonistic effect and reduced *C. jejuni* survival compared to the control. When these *P. fluorescens* strains were incubated with *C. jejuni* 11168 the post hoc test showed no differences between co-incubation with *P.*

flurorescens Pf-5 and F113 and incubation in media (figure 5.1A). However, when the co-cultures were performed with the strain 81-176 the post hoc test showed a statistically significant difference between incubation with *P.fluorescens* F113 and incubation in media. Incubation with *P. fluorescens* Pf-5 was not statistically different to either of these two conditions.

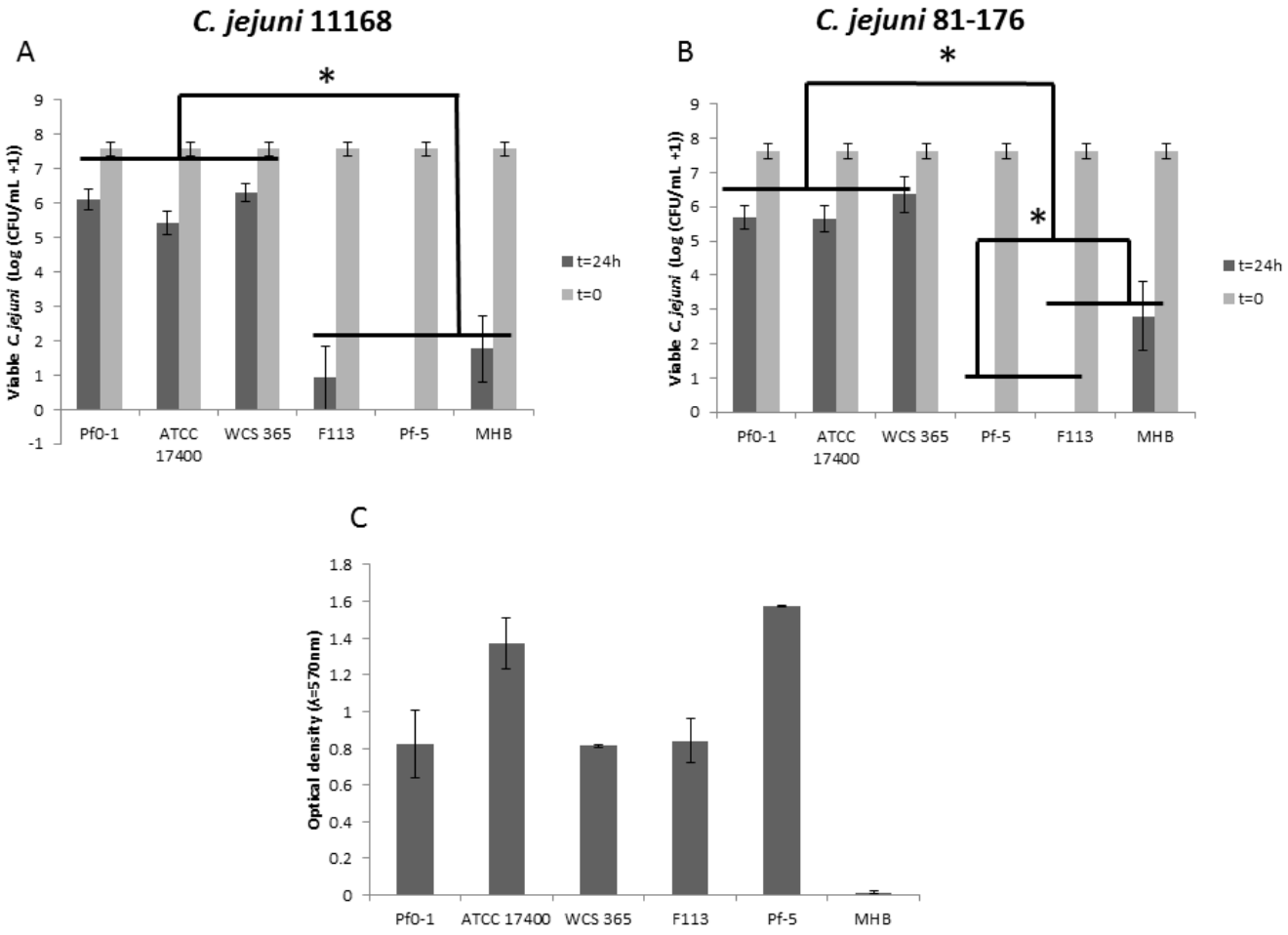


Figure 5.1.Influence of *P. fluorescens* on *C. jejuni* survival is strain specific. (A) Viable cell counts of *C. jejuni* 11168 on CSM co-incubated with five *P. fluorescens* strains under aerobic conditions (0 vs. 24 h). The results shown are the average of three independent experiments and the error bars represent the standard error. (B) Viable cell counts of *C. jejuni* 81-176 co-incubated with five *P. fluorescens* strains under aerobic conditions (0 vs. 24 h). The results shown are the average of four independent experiments (except two independent experiments for Pf-5 and three for Pf0-1) and the error bars represent the standard error. (C) Total bacterial growth in the co-cultures measured as absorbance at λ=570 nm. The results shown are the average of two independent experiments with eight technical replicates using *C. jejuni* 11168. The error bars represent the standard error. (A, B and C) MHB represents *C. jejuni* in monoculture used as a control (no interaction). *Statistically significant results from comparison of viable *C. jejuni* recovered at t=24h (ANOVA with post-hoc Tukey).

5.2.2 *P. fluorescens* cell-free supernatants (CFS) do not enhance *C. jejuni* 81-176 aerobic survival

Since variation was detected in the protective/antagonistic effect of different *P. fluorescens* strains, it was postulated that different secreted factors might influence *C. jejuni* survival. To investigate this, the survival experiments were repeated using cell- free supernatants of *P. fluorescens* rather than live cell suspensions. Figure 5.2 shows that patterns of survival in the presence of live cells were consistent with previous experiments. However, none of the cell-free supernatants conferred any protective effect. A low number of viable *C. jejuni* cells were recovered from 24 h co-culture with ATCC strain 17400 CFS. However this was less than viable cells recovered from MHB alone. Furthermore, variation was observed in viable *C. jejuni* recovery from MHB alone. This is likely to be a result of stochastic variation at low cell counts. Live *P. fluorescens* cells were therefore considered to be required for prolonged *C. jejuni* culturability. However, the inhibitory effect of strains F113 and Pf-5 might be due to a secreted factor.

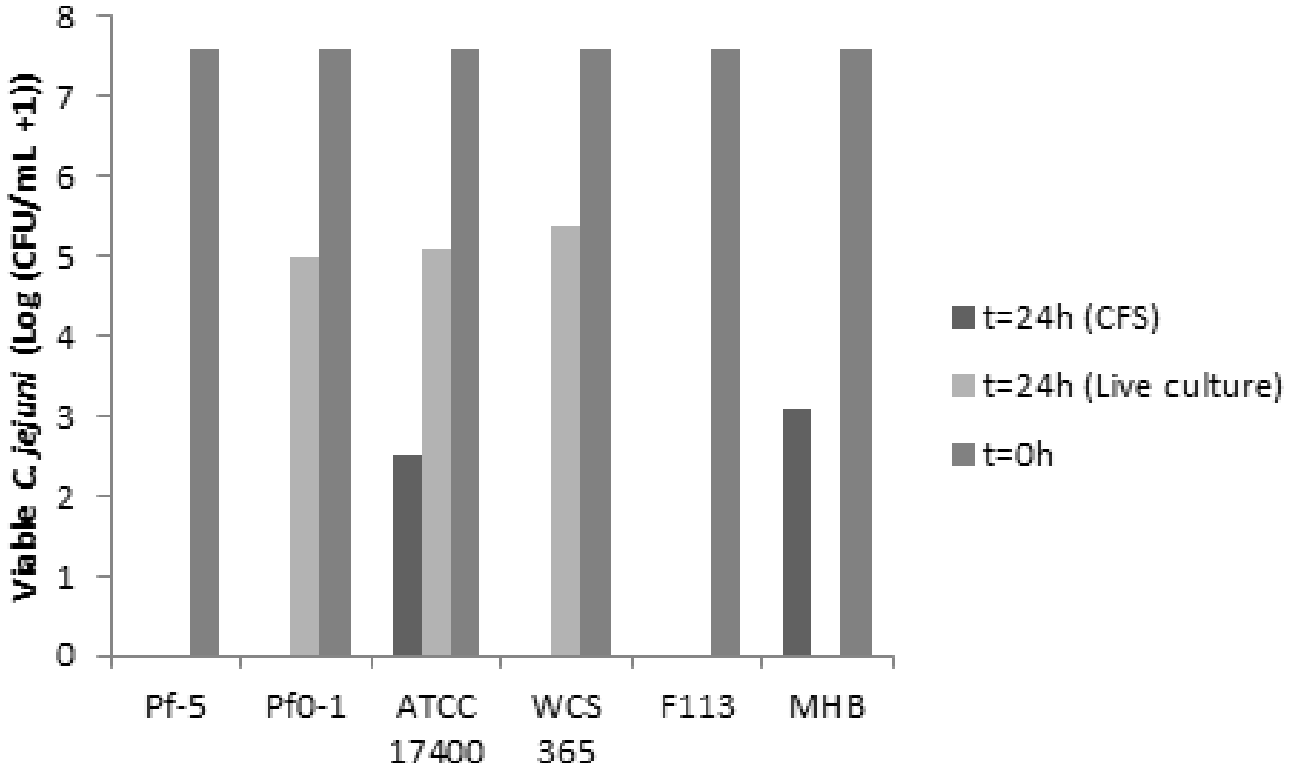


Figure 5.2. Comparison of the effect of live *P. fluorescens* cells vs. CFS on survival of *C. jejuni*. Viable cell counts of *C. jejuni* 81-176 co-incubated with five *P. fluorescens* strains or their CFS under aerobic conditions (0 vs. 24 h). MHB represents *C. jejuni* in monoculture used as a control (no interaction). The graph shows the results of a single experiment.

5.2.3 Enhanced survival of *C. jejuni* 81-176 is promoted by a wide range of environmental isolates of different environmental isolates of different *Pseudomonas* species under aerobic but not microaerobic conditions

The effect of of 14 environmental isolates of *Pseudomonas* on the aerobic survival of *C. jejuni* 81-176 in aerobic conditions was assessed (figure 5. 3A). When co-cultured with *C. jejuni* at 25 °C in MHB, the *Pseudomonas* environmental isolates grew to optical densities (ODs) between 0.2 and 1.6 (figure 5.3C). In these co-cultures, although the number of viable *C. jejuni* 81-176 counts decreased in each co-culture over time after 24h aerobic incubation at 25 °C (from 7 LOG CFU/mL to 5-0 LOG CFU/mL), the difference was not statistically significant for the control with MHB and all the co-cultures ($p>0.05$). This is due to the high variability between the independent assays. However, no viable *C. jejuni* cells could be recovered after co-culture with *P. cornigata* 2445, which like *P. fluorescens* Pf-5 and F113 seemed to enhance *C. jejuni* death. When *C. jejuni* was incubated in MHB it showed a reduced culturability compared to the co-cultures with different *Pseudomonas* isolates, except for *P. cornigata* 2445 (figure 3A). These differences however did not reach a statistically significant level ($p>0.05$).

A small group of *Pseudomonas* environmental isolates were selected to assess whether the protective effect on culturability could also enhance survival in microaerobic conditions. The isolates were able to grow both under aerobic (ODS 0.4-1.4) and microaerobic (ODS 0.1-1.3) conditions but showed a slight decrease in total growth in relation to their growth under aerobic conditions (figure 5.3D). *C. jejuni* 81-176 did not grow in MHB in either aerobic or microaerobic conditions at 25 °C (figure 5.3D).

C. jejuni viable counts decreased in each co-culture after 24h incubation at 25 °C in both aerobic and microaerobic conditions but the differences were not statistically significant

(p>0.05) (figure 5.3B). When the final viable counts in the different co-cultures and the control were compared through a two-way ANOVA both the oxygen level and the co-culture were significant factors (p=0.000 and p=0.004). Using one-way ANOVA it was seen that the three *Pseudomonas* isolates used (*P. avellanaev* 48, *P. larchymans* and *P. marginalis* 247) were able to prolong culturability of *C. jejuni* 81-176 under aerobic conditions (p=0.008) but not under microaerobic conditions (p=0.182) compared to the control (figure 5. 3B).

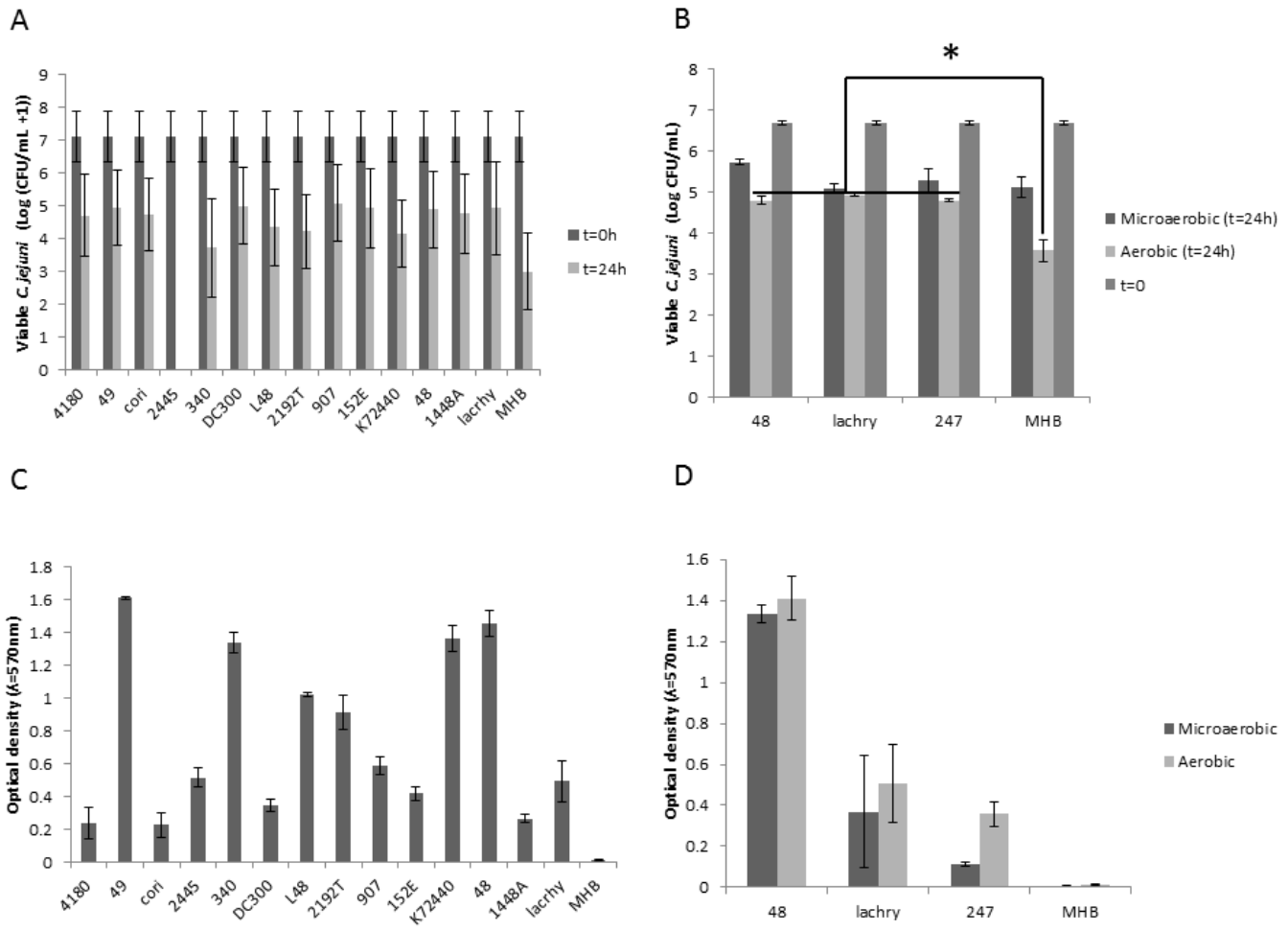


Figure 5.3. *C. jejuni-Pseudomonas* co-cultures under aerobic and microaerobic conditons. 4180 (*P.syringae* pv. *glycinea* 4180); 49 (*P. syringae* pv. *glycinea* 49); cori (*P. syringae* pv. *coriandricola*); 2445 (*P. corrugata* 2445); 340 (*P. putida* 340); DC300 (*P. syringae* pv. *tomato* DC300); L48 (*P. entomophila* L48); 2192T (*P. tolaasii* 2192T); 907 (P. cichorii 907); 152E (*P. syringae* pv. *antirrini* 152E); K72440 (*P. putida* K72240); 48 (*P. avellanae* 48); 1448A (*P. syringae* pv. *phaseolicola* 1448A); lachry (*P. syringae* pv. *lacrhymans*); 247 (*P. marginales* 247). (A) Viable cell counts of *C. jejuni* 81-176 co-incubated with 14 *Pseudomonas* isolates under aerobic conditions (0 vs. 24 h). The results shown are the average of two independent experiments and the error bars represent the standard error. (B) Viable cell counts of *C. jejuni* 81-176 co-incubated with 3 *Pseudomonas* isolates under aerobic and microaerobic conditions (0 vs. 24 h). The results shown are the average of two independent experiments and the error bars represent the standard error. * Statistically significant results from comparison of viable *C. jejuni* recovered at t=24h (ANOVA with post-hoc Tukey). (C and D) Total bacterial growth in the co-cultures measured as absorbance at Λ=570 nm. The results shown are the average of two independent experiments with eight technical replicates. The error bars represent the standard error.

5.2.4 *Pseudomonas fluorescens* can be isolated from biofilms from drinking water distribution systems

Despite evidence that *Pseudomonas* species could enhance *C. jejuni* survival in aerobic conditions from this study and others, the strains and isolates used have been isolated from soil and plant environments (Gross and Loper 2009) or from chicken meat (Hilbert *et al.* 2010). Since the focus of this thesis was on drinking water systems, isolates were sought from this environment for further study. *P. aeruginosa* and *Pseusomonas* spp. selective media were used to isolate *Pseudomonas* spp. from drinking water biofilms. Two putative *P. aeruginosa* and five putative *Pseudomonas* spp. isolates were obtained from one coupon from the large scale DWDS model described by Douterelo *et al.* (2013). The strains were characterized using PCR and partial 16S rRNA sequencing. First they were typed by random amplified polymorphic DNA (RAPD) technique (Mahenthiralingam *et al.* 1996). Figure 5.4 shows that two distinct RAPD profiles were identified, one for the two putative *P. aeruginosa* isolates and a second profile was consistent across all five of the *Pseudomona*s ssp. isolates. Both profiles differed from the profile of *P. aeruginosa* PAO1. According to these results, two isolates for each profile were selected for further characterisation.

PCR, using specific primers for *P.aeruginosa* and *P.fluorescens* positively identified all the putative *Pseudomonas* isolates to be *P. fluorescens*, including those that had been isolated on *P. aeruginosa* selective media. One isolate of each group (putative *P. aeruginosa* and putative *Pseudomonas* spp.) was chosen for partial 16S rDNA sequencing. Using the NCBI BLAST tool both isolates were confirmed as *Pseudomonas* spp. The species could not be confirmed by sequencing as the area sequenced seemed to be

conserved among different *Pseudomonas* including *P. fluorescens* and *P. veronii* for both isolates and also *P. chlororaphis* for isolate P1. All these species showed 100% identity with the sequence obtained and no species belonging to other genera did.

Using a combination of sequencing and PCR it has been established that two different types of isolates belonging to the *Pseudomonas* genus and most likely being *P. fluorescens* were isolated from drinking water biofilms.

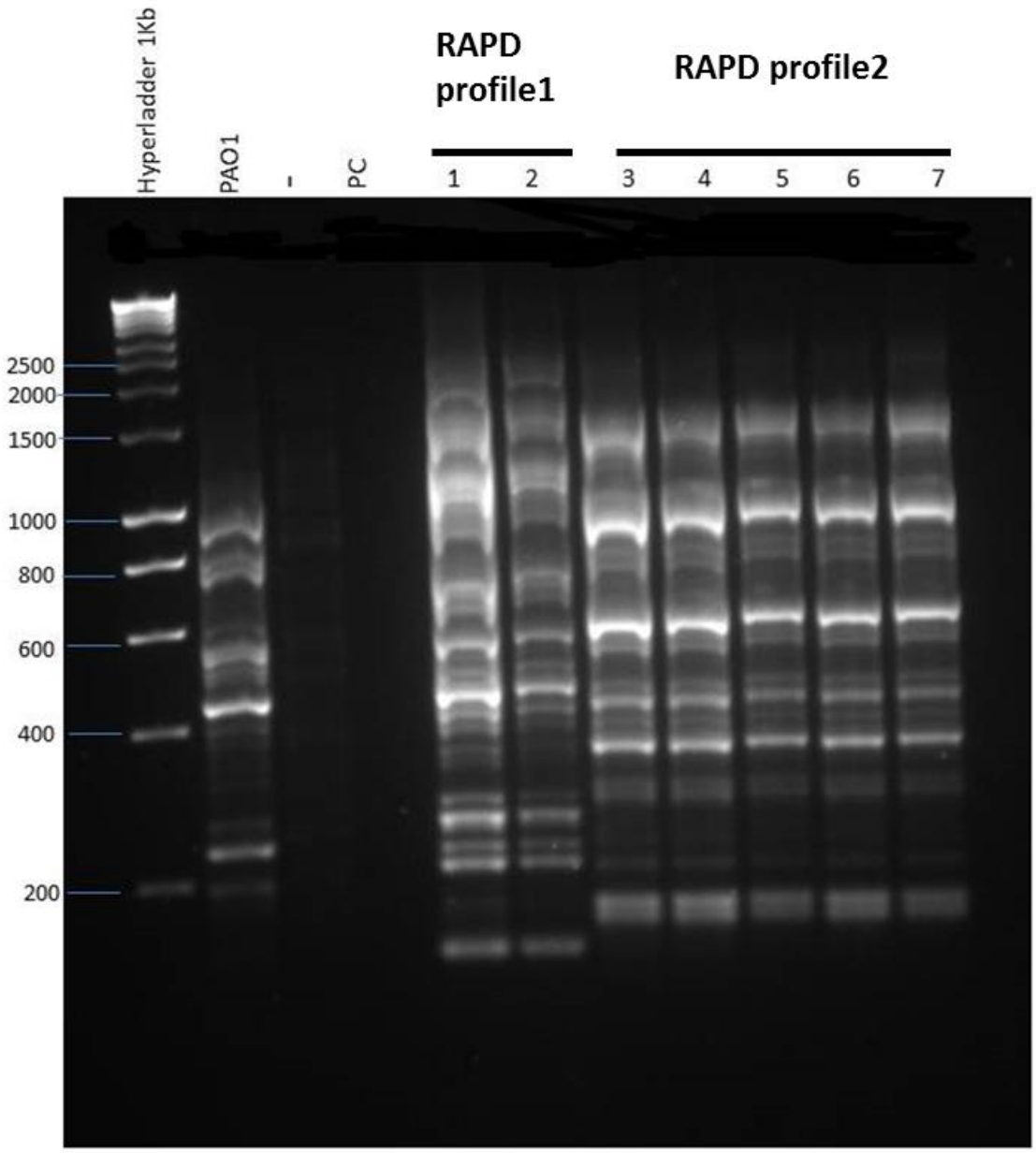


Figure 5.4. 1.5% agarose gel showing the RAPD profiles from *P. fluorescens* isolates from drinking water biofilms. " –"=negative control with water as a template," PC"=primer control, "1-2"=putative *P. aeruginosa* isolates, "3-7"= putative *Pseudomonas* spp. isolates. Sizes in base pairs of selected bands in the hyperladder are indicated.

5.2.5 *Pseudomonas fluorescens* isolated from drinking water biofilms can enhance and reduce *C. jejuni* 81-176 survival under aerobic conditions

Four *P. fluorescens* isolates from biofilms from a DWDS (two from each sequence type) were used in co-culture experiments. *C. jejuni* viable counts significantly decreased in each co-culture after 24h incubation under aerobic conditions in all the co-cultures and the control (p=0.002-0.024). When final viable counts (t=24h) were compared among the different co-cultures and the control with MHB through a one-way ANOVA statistically significant differences were found (p=0.001) (figure 5.5). Results indicated that *C. jejuni* viable counts co-cultured with one sequence type (P1 and P2) were significantly higher than those co-cultured with sequence type (P3 and P4). The viable counts recovered from the control group were not statistically different from either group. These results indicate that from the two sequence types isolated from the drinking water biofilms one has the ability to enhance *C. jejuni* 81-176 survival in a physiological culturable state i*n vitro* while the other has a detrimental effect.

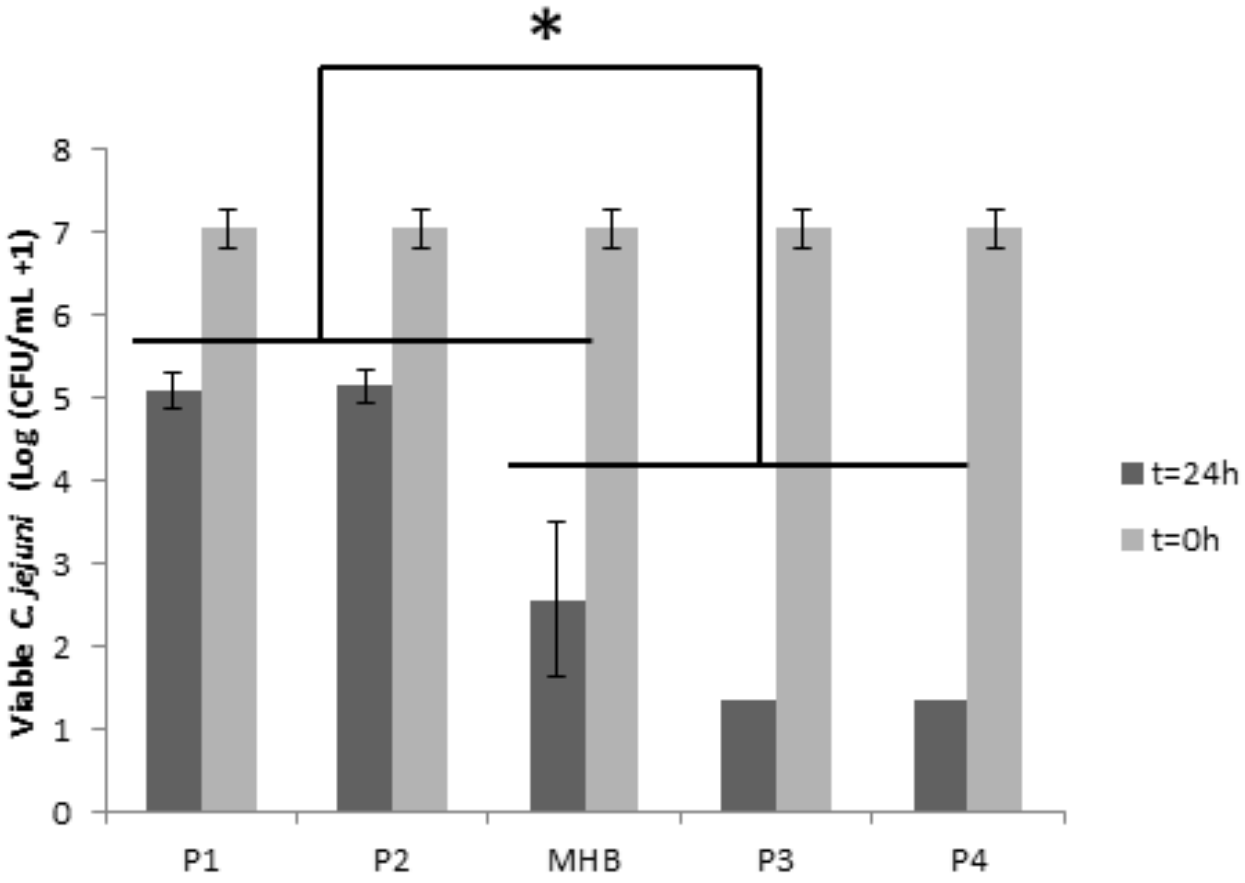


Figure 5.5. *C. jejuni-P. fluorescens* co-cultures using biofilm drinking water isolates. P1 and P2 (*P. fluorescens* RAPD 1); P3 and P4 (*P. fluorescens* RAPD2). Viable cell counts of *C. jejuni* 81-176 co-incubated with 4 *P. fluorescens* isolates under aerobic conditions (0 vs. 24 h). The results shown are the average of four independent experiments and the error bars represent the standard error. * Statistically significant results from comparison of viable *C. jejuni* recovered at t=24h (ANOVA with post-hoc Tukey).

5.2.6 Different *Pseudomonas* isolates were isolated during the rearing cycle from inside the broiler house but not from the anteroom in the longitudinal farm study in the UK

During the longitudinal study in an intensive commercial broiler house in the UK *Pseudomonas* spp. could be isolated from the bulk water collected inside the broiler house but not from the bulk water collected in the anteroom. This was true for all seven weeks, including when the broiler house did not harbour any chickens (on week 1, before the hatchlings got to the farm and on week 7, after the chickens had left for slaughter). In total, 9 different isolates were obtained. In weeks 2 and 4 two different colony morphologies were observed so two different isolates were obtained. All 9 isolates were confirmed to be *Pseudomonas* spp. by PCR. Species determination was also attempted by PCR. All 9 isolates tested negative for *P. fluorescens* and only W4A tested positive for *P. aeruginosa*. Using the RAPD technique it was determined that the isolates showed different profiles (Figure 5.6). This suggests that a heterogeneous population of *P. fluorescens* inhabits the broiler farm water system, with no particular clone dominating during the rearing cycle. *C. jejuni* survival may be enhanced or hindered depending on the dynamics of these *P. fluorescens* populations.

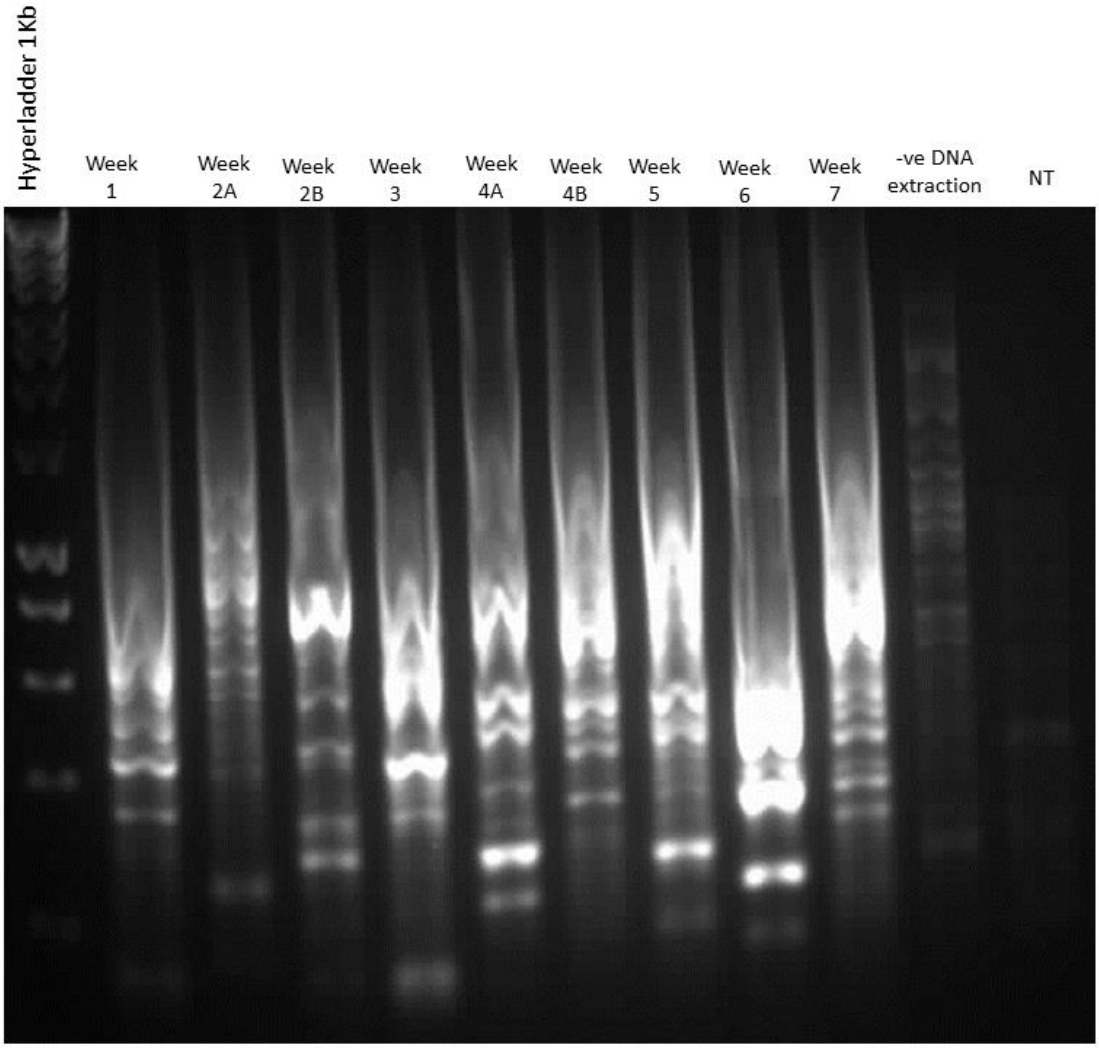


Figure 5.6. 1.5% agarose gel showing the RAPD profiles from *Pseudomonas* spp. isolates from water collected inside of the broiler house. "NT"= negative control with water as a template.

5.3 *Acanthamoebe polyphaga* internalizes *C. jejuni* 81-176

Amoeba of the genus *Acanthamoeba* have been suggested as a nonvertebrate reservoir for *C. jejuni* in the environment (Axelsson-Olsson *et al.* 2005). This is of great ecological and epidemiological relevance as the genus *Acanthamoeba* has been isolated on commercial poultry houses previously (Bare *et al.* 2009) and was detected in the DWS of a broiler house in this study (chapter 3). The majority of research studying the interactions between *Acanthamoeba* and *C. jejuni* suggest intracellular survival but the studies reported high variability in terms of duration and relevance of internal survival (5h-60days) (Axelsson-Olsson *et al.* 2005, Bui *et al.* 2012b). These conflicting results might be due to species and strain variations and different methodologies (Vieira *et al.* 2015).

In this study the ability of *A. polyphaga* to internalize *C. jejuni* 81-176 was evaluated through a gentamicin protection assay. Gentamicin protection assays allow the study of bacterial internalization by eukaryotic cells on the bases that bacteria that have been internalized by the amoebae will be protected while those that remain extracellular will be killed by the gentamicin (Dirks and Quinlan 2014). The results mostly support that *A. polyphaga* is able to internalize *C. jejuni* at very low rate of internalization ranging from 0.00654 to 0.385 in 5 independent experiments where internalization took place. However in one out of six experiments conducted a higher number of *C. jejuni* colonies was recovered from the control with PPG than from the co-culture with *A. polyphaga*, indicating that the bacteria were able to survive the experiment better in the absence of amoebae (Table 5.1).

Overall, in these six experiments significantly higher viable counts were recovered at the end of the experiment when *C. jejuni* were internalized in the amoebae compared to the

control with PPG media (p=0.006) (figure 5.7). In two of them a control co-culture were the amoebae were not lysed with Triton X-100 at the end of the experiment was included. Lower *C. jejuni* viable counts were recovered from this controls (average of $3.91x10^2$) compared to when amoebae were lysed (average of $2.24x10^3$) (Table 5.1). Although the difference was not statistically significant (p=0.378) the lower recovery suggests that *C. jejuni* is internalized by the amoebae.

An average with standard deviation of $5.3x10^3$±9,947 viable *C. jejuni* colonies per mL were recovered from *A. polyphaga*-*C. jejuni* co-cultures and 44±78 viable *C. jejuni* colonies per mL were recovered from *C. jejuni* in PPG (figure 5.7), which represented a $2.43x10^{-4}$ % and $2.01x10^{-6}$% of the initial inocula respectively.

Table 5.1. Details of gentamicin protection assay using *A. polyphaga* and *C. jejuni* 81-176.

ROI	INITIAL CONCENTRATIONS		FINAL CONCENTRATIONS			
	A. polyphaga (trophozites/mL)	*C. jejuni* (CFU/mL)	*A. polyphaga* (trophozites/mL)	*C. jejuni* in co-culture (CFU/mL)	*C. jejuni* in PPG (CFU/mL)	*C. jejuni* in co-culture without amoebae lysis (CFU/mL)
$2.54x10^{-2}$	$1.24x10^6$	$4.30x10^9$	$1.20x10^5$	$3.50x10^3$	0	$7.40x10^2$
$2.38x10^{-2}$	$1.38x10^5$	$5.33x10^9$	$4.00x10^4$	$9.80x10^2$	$3.00x10^1$	$4.20x10^1$
$6.54x10^{-3}$	$2.60x10^5$	$1.33x10^9$	$2.60x10^4$	$1.70x10^2$	0	NA
$3.85x10^{-1}$	$9.80x10^4$	$9.30x10^8$	$6.60x10^4$	$2.55x10^4$	$1.00x10^1$	NA
$6.59x10^{-2}$	$1.38x10^5$	$1.16x10^9$	$3.00x10^4$	$2.00x10^3$	$2.20x10^1$	NA
	$2.18x10^4$	$9.00x10^6$	$2.00x10^4$	$3.20x10^1$	$2.00x10^2$	NA

Each row represents an independent experiment. NA stands for "Not available. ROI stands for "rate of internalization". $\mathrm{ROI} = \frac{[\text{CFU } C.\ jejuni \text{ with } A.\ polyphaga] - [\text{CFU } C.jejuni \text{ in PAS}]}{[\text{amoebae recovered}]}$

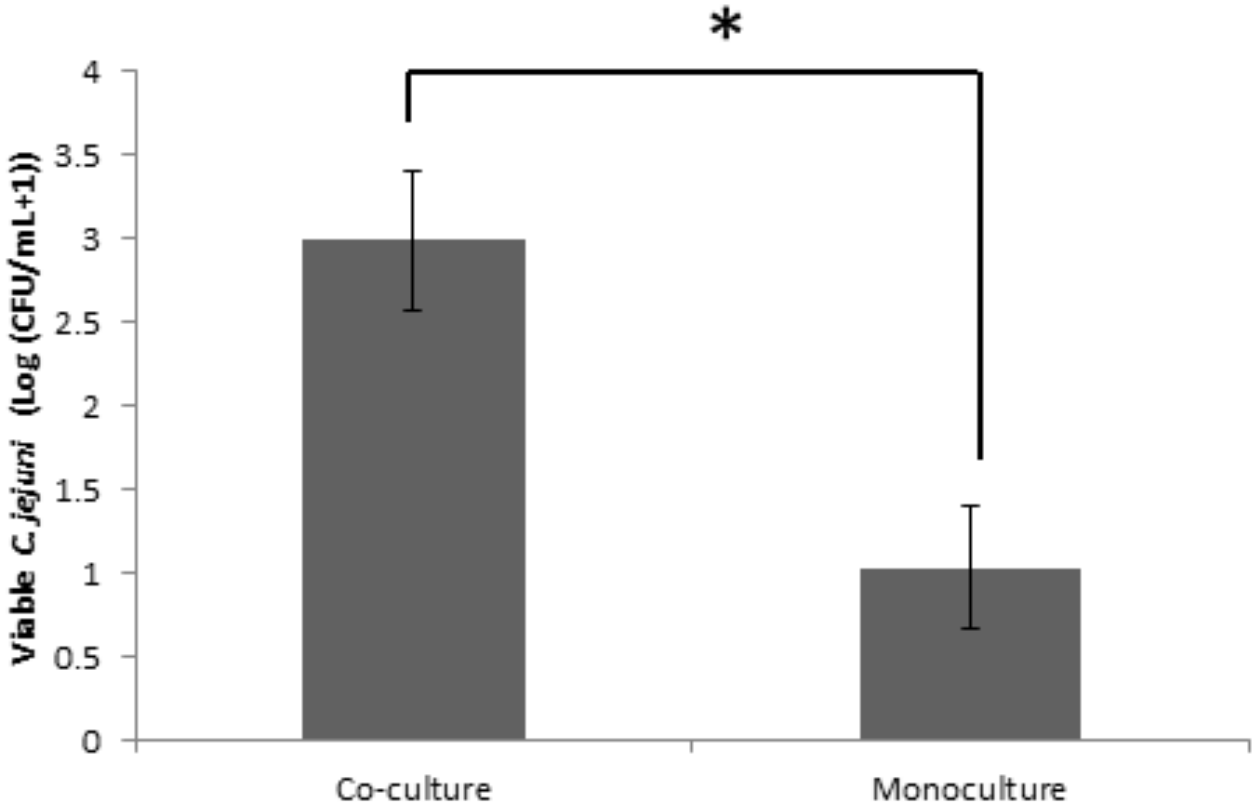


Figure 5.7. Viable cell counts of *C. jejuni* co-coulture with *A. polyphaga*. Viable cell counts of *C. jejuni* 81-176 (co-culture with *A. polyphaga* vs. monoculture in PPG) recovered after gentamicin treatment. The results shown are the average of six independent experiments and the error bars represent the standard error. * Statistically significant results from comparison of viable *C. jejuni* recovered (t-test).

Although isolation was not possible, amoebae could be observed surrounding coupons obtained from the DWDS simulator in Sheffield. This confirms that amoebae are present and viable in drinking water biofilms as was observed on the farm DWS (Chapter 3) and therefore could potentially share a relevant ecological niche with *Campylobacter* spp. and pose a risk for human campylobacteriosis.

5.4 Discussion

The results from this chapter show that certain environmental isolates of *Pseudomonas* and *A. polyphaga* prolong the aerobic survival of *C. jejuni* in a physiologically culturable state. Both *Pseudomonas* spp. and *Acanthamoeba* co-locate with *Campylobacter* in key environmental niches such as water, farms and poultry meat (Bare *et al.* 2009, Buse *et al.* 2014a, Morales *et al.* 2016) and have been found to inhabit the broiler farm DWS at the same time (chapter 3). Therefore these results suggest that these microorganisms might play a role in *Campylobacter* ability to withstand conditions of atmospheric oxygen tension.

More than 200 species are assigned to the *Pseudomonas* genus encompassing medically, agriculturally and biotechnologically relevant bacteria that inhabit a wide range of niches including soil and water environments and have an enormous metabolic diversity (Ozen and Ussery 2012). In this study environmental isolates were used in co-culture experiments to analyse their impact on *C. jejuni* survival. Most isolates were able to

prolong *C. jejuni* culturability under aerobic conditions. These include strains belonging to *P. fluorescens*, *P. syringae*, *P. cichorii*, *P. avellanae* and *P. putida*. Similarly, *P. fluorescens*, *P. fragi* and *P. putida* type strains and chicken meat isolates have been reported previously to show a beneficial effect for the aerobic survival of a range of *C. jejuni* strains (Hilbert *et al.* 2010). In this study, *P. corrugata* 2445 and *P. fluorescens* F113, Pf-5 and P3/4 were detrimental for *C. jejuni* survival in a culturable state in the conditions tested. From all the *P. fluorescens* strains tested, 55% were effective in supporting *C. jejuni* survival under normal atmospheric oxygen tension conditions but 45% were detrimental, showing that the interactions between *C. jejuni* and *Pseudomonas* strains might be strain specific. *P. fluorescens* harbours great genomic diversity which is reminiscent of a species complex rather than a single species (Silby *et al.* 2009) so strain to strain variability is not surprising. Similar results were reported by Hilbert *et al.* (2010)regarding two *P. putida* strains. In this study however both *P. putida* strains tested enhanced *C. jejuni* survival.

Hilbert *et al.* (2010) suggested that *C. jejuni* could whistand high oxygen tension when in co-cultivation with *Pseudomonas* spp. through metabolic commensalism regarding oxygen depletion. In this study when a small subset of *Pseudomonas* environmental strains was co-incubated with *C. jejuni* under microaerobic conditions no differences in final viable counts were observed between the co-cultures and the control with media. Conversely significantly higher *C. jejuni* viable bacteria were recovered from co-cultures carried out in parallel under aerobic conditions when compared to the control with MHB media. These results support the depletion of oxygen as the mechanism *Pseudomonas* spp. use to prolong *C. jejuni* survival. However, absorbance readings indicated that *Pseudomonas* strains that were detrimental or had no effect on *C. jejuni* survival were

also capable of growing under assay conditions. Along these lines Hilbert *et al.* (2010) reported variability among *C. jejuni* strains ability to survive ambient atmospheric oxygen levels, with only some strains being able to multiply in the first hours and differential total survival time. Their observations along with those from this study suggest that there might be a secondary mechanism for the beneficial interaction with *Pseudomonas* spp. beyond the depletion of oxygen. However, this study found no evidence of any secreted factor in CFS that could enhance survival.

Another possibility is that the *Pseudomonas* strains that decreased recovery of *C. jejuni* viable cells produce some kind of secondary metabolite or bacteriocin with anti-*C. jejuni* activity. Environmental and plant-associated *Pseudomonas* spp. show a variety of gene clusters involved in bacteriocin production, which tend to have narrow spectrum and are involved in interference competition between Gram-negative bacteria (Parret and De Mot 2002, Ghequire and De Mot 2014). This genus also produces a remarkable array of secondary metabolites and among them there are some affecting other bacteria (Gross and Loper 2009). Further characterisation of these compounds is needed to determine wether they could inhibit *C. jejuni*. *P. fluorescens* Pf-5 is a commensal rhizosphere bacterium and a well characterized biological control agent that produces a spectrum of antibiotics toxic to plant-pathogenic fungi and oomycetes (Loper *et al.* 2007). *P. fluorescens* F113 also has the ability to inhibit growth of a range of phytopathogens including bacteria, fungi, oomycetes and nematodes and this ability is strongly linked to the production of of a secondary metabolite, 2,4-diacetylphloroglucinol (Redondo-Nieto *et al.* 2013). In a previous report, a bactoriocin produced by *Lactobacillus salivarious* showed *in vitro* cultural inhibition of *C. jejuni* and reduced chicken colonization one

millionfold in chickens (Stern *et al.* 2006). Whether this *P. fluorescens* strains could produce similar bacteriocins that could be applied to reduce chicken colonization remains to be investigated.

Pseudomonas spp. easily form biofilms and take advantage of the increased tolerance to negative environmental influences related to this form of growth, enabling them to inhabit a broad range of niches such as plant roots, soil, water etc. (Ude *et al.* 2006, Masák *et al.* 2014). Environmental isolates have been reported to be better biofilm formers than plant pathogen isolates (Ude *et al.* 2006). Several strains of *P. fluorescens* are good biofilm formers including the strains used in this study *P. fluorescens* WCS 365(Hinsa and O'Toole 2006), *P. fluorescens* Pf0-1(Boyd *et al.* 2014), P. fluorescens Pf-5 (Lee *et al.* 2014), *P. fluorescens* F113 (Barahona *et al.* 2010) and *P. fluorescens* ATCC 17400 (Ude *et al.* 2006). Members of *P. syringae*, *P. putida, P. corrugata*, *P. marginalis*, *P. tolaasii* and *P. cichorii* also form biofilms (Ude *et al.* 2006, Pauwelyn *et al.* 2013). *C. jejuni* has been reported to incorporate as secondary colonizer in biofilms containing *Pseudomonas* spp. (Trachoo *et al.* 2002, Hanning *et al.* 2008, Sanders *et al.* 2008). *P. aeruginosa* promoted not only persistence but growth by generating a microaerophilic environment when growing as a biofilm (Culotti and Packman 2015) and enhanced *C. jejuni* survival in a culturable state (Ica *et al.* 2012). Hilbert *et al.* (2010) also reported *C. jejuni* interacting by fiberlike structures in close contact with *P. putida* in co-cultures where increased aerobic survival and/or growth was observed for different *C. jejuni* strains. In this study only the planktonic survival of *C. jejuni* in co-culture with *Pseudomonas* spp. Further research investigating if biofilms from these environmental

isolates support *C. jejuni* growth/survival under aerobic conditions will aid the understanding of the mechanism or mechanisms behind the enhanced survival.

C. jejuni can also form monospecies biofilms *in vitro* under certain conditions and this has been suggested as a survival strategy in the environment (Joshua *et al.* 2006, Turonova *et al.* 2015). Culture supernatant fluids of *P. aeruginosa* and *P. fluorescens* have been shown to increase biofilm development in *C. jejuni* (Reeser *et al.* 2007). Although biofilm formation was not specifically assessed, in this study no viable *C. jejuni* bacteria could be recovered when live *P. fluorescens* cells were replaced by their CFS, indicating that the enhanced survival in co-cultures is not due to a stable signalling molecule or that the signalling molecule was not produced under the experimental conditions used.

Pseudomonas were isolated from a drinking water biofilm formed in a coupon collected in a large scale DWDS model in the University of Sheffield (Douterelo *et al.* 2013) and from the commercial broiler farm. The isolates from drinking water biofilms had two different profiles determined by RAPD typing (Mahenthiralingam *et al.* 1996). Even though a group of the isolates, identified with a common RAPD profile, were isolated in a medium selective for *P. aeruginosa*, both RAPD groups were identified as *P. fluorescens* through a combination of PCR and partial 16S rDNA sequencing. *P. fluorescens* had been reported before in the test loop facility where the coupon was obtained from (Douterelo *et al.* 2014) and in other studies in DWDS (Schmeisser *et al.* 2003). The isolates from the farm showed different RAPD profiles in each week indicating that there may be a dynamic and heterogeneous population of *P. fluorescens* inhabiting the water system. Since only 1 or 2 colonies were analysed each week, it is not known whether any particular types might dominate at different stages. *Pseudomonas* are common and rapid-colonizing

residents of DWDS that frequently dominate the process of initial surface attachment (Revetta *et al.* 2013, Douterelo *et al.* 2014). In the co-culture experiments performed with the isolates from drinking water biofilms from the facility in Sheffield, isolates belonging to one typing group increased *C. jejuni* viable cell count by 2.5 LOG compared to the control while the isolates belonging to the second typing group were detrimental for *C. jejuni* survival and decreased viable cell count by 1.2 LOG compared to the control. These results confirm what has been discussed previously about the specificity of *Pseudomonas-Campylobacter* interactions. Considering these results, it is unclear whether the *Pseudomonas* populations from the farm WDS would have a net beneficial or detrimental influence on *Campylobacter* survival. It could be speculated that *Pseudomonas* populations, in general, could create niches of reduced oxygen levels within biofilms that build up in the pipes. This could create a permissive environment for *Campylobacter* survival. However, certain *P. fluorescent* types could also inhibit survival. It would be interesting to further co-culture farm isolates of *C. jejuni* with all of the *P. fluorescens* water isolates to evaluate their effects. Particularly, it would be interesting to determine whether certain *C. jejuni* sequence types were able to withstand inhibitory factors and to determine the identity of such factors. Understanding the mechanism behind the inhibition of *C. jejuni* could potentially be applied to the biocontrol of *Campylobacter* spp., as discussed previously in the context of the *P. fluorescens* strains.

Research suggests that *C. jejuni* transmission might also be assisted by *Acanthamoeba* but some researchers remain sceptical about the relevance of an intracellular interaction (Vieira *et al.* 2015). In this study internalization of *C. jejuni* by *A. polyphaga* was assessed using a modified gentamicin protection assay developed by Dirks and Quinlan (2014). The

results mostly suggest that *C. jejuni* was internalized by the protozoa and survived within it for the duration of the experiment, resulting in a 2 LOG increase in viable cell recovery after the experiment when compared to the control with media. However, in one case the co-incubation with *A. polyphaga* resulted in decreased viable cell recovery when compared to the control with media. Dirks and Quinlan (2014) reported similar inconsistent results from their experiments with *A. castellanii* and *C. jejuni* 11168. They hypothesised that this could be due to differences of age and nutrient status of the amoebae culture. However, standardising these factors for amoebae culture is challenging. In this study it was attempted to use amoebae that had reached a confluent state in the flasks but differences in age, nutrient status or other variables such as temperature might account for the conflicting results and the high variability among different co-culture experiments.

Although the rate of internalization reported in this study was low (0.00654-0.385) it resulted in a significantly higher *C. jejuni* recovery at the end of the experiment. It has been reported that *C. jejuni* internalized within *A. castellanii* was able to colonize broilers, suggesting that protozoa internalization might be epidemiologically relevant for on-farm chicken colonization (Snelling *et al.* 2008).

In this chapter a range of *Pseudomonas* environmental isolates and *A. polyphaga* have been shown to be able to enhance *C. jejuni* survival *in vitro*, suggesting a possible role in *C. jejuni* transmission. The results however show that in some cases the interactions with these common members of natural microbial communities have resulted in a lower recovery of *C. jejuni* in a physiologically culturable state. A high specificity and variability has also been observed. Little is known about the interplay of the different survival

strategies of *Campylobacter* such as biofilm formation, VBNC state and interactions with the wider microbial communities and therefore the ecological and epidemiological implications of these *in vitro* assays have to be interpreted with great care. Further research using the relevant field isolates is needed to unravel the complex net of relationships of *Campylobacter* and the wider microbial communities.

Chapter 6

General discussion

Campylobacter infection is a global health issue and the chicken reservoir plays an important role in disease epidemiology (Kaakoush *et al.* 2015). In spite of the large body of research to attempt to control *Campylobacter* in chickens the rate of colonization remains high worldwide, representing a public health risk (Kaakoush *et al.* 2015). Research has failed to identify a sole source of chicken colonization and it is more likely that several horizontal transmission pathways co-exist (Conlan *et al.* 2007, Agunos *et al.* 2014).

This thesis has worked under the hypothesis that microbial interactions are a key factor in *Campylobacter* epidemiology and therefore gaining understanding of the interplay of *Campylobacter* spp. and the microorganisms that co-occur with them in the environment will inform more efficient control strategies. A particular focus has been placed on the microbial communities that inhabit the DWS of a commercial broiler farm and whether they could maintain *Campylobacter* spp. and thus act as source or vehicle of transmission. A range of culture-based and molecular techniques have been used to investigate this hypothesis. Chapter 3 described detailed temporal and spatial 16S rRNA profiling to characterise the microbial communities in the DWS of a broiler farm for a whole rearing cycle. Chapter 4 explained the isolation and detection of *Campylobacter* spp. and the related emerging pathogen *H. pullorum* matching the rearing cycle during which the microbial community profiling was carried out. This chapter also included a pilot study to investigate *Campylobacte*r spp. prevalence in small-scale farms in Uganda. Finally,

Chapter 5 investigated interactions between well characterised strains of *C. jejuni* and common inhabitants of the chicken farm DWS. Data suggested that co-culture with *Pseudomonas* spp. and *A. polyphaga* resulted in prolonged *C. jejuni* aerobic survival in a culturable state.

NGS techniques have revolutionized microbial ecology and epidemiology studies, allowing unprecedented insights into the structure, dynamics and functions of microbial populations (Hepworth *et al.* 2011, Grad and Lipsitch 2014, Escobar-Zepeda *et al.* 2015). Amplicon sequencing is a cost-effective technique that provides insights into microbial community composition (Creer *et al.* 2016). The results from the longitudinal microbial community profiling experiment on the broiler farm DWS, revealed dynamic microbial communities that i) differed from those of the source water, ii) were niche specific and iii) changed across the rearing cycle. However, bacterial communities in DWDS have been suggested to exhibit cyclical seasonal patterns (Pinto et al., 2014). There is a need to repeat this experiment during multiple rearing cycles, on different farms and during different times of the year to fully characterise the microbial dynamics of this environment. Although environmental conditions are tightly controlled in the large commercial broiler house (temperature; light; flock density), certain seasonal changes, such as humidity, are still likely to affect the system. It has been suggested previously that the DWS of the farm could act as reservoir for *Campylobacter* that could subsequently infect the following flocks (Pearson *et al.* 1993, Ogden *et al.* 2007, Cokal *et al.* 2011). This work has provided evidence that microbial exchange between the chickens and the DWS exists and it is extensive, supporting this possibility. Firstly, a shift in microbial communities took place in week 2, which was the first sampling week after the hatchlings arrived to the farm. Secondly, Firmicutes (mainly *Lactobacillus* and *Staphylococcus*)

which dominated bacterial communities in the DWS at certain stages of the rearing cycle are associated with chickens (Oakley *et al.* 2014) and not commonly found in DWDS in the proportions reported here (Liu *et al.* 2014) suggesting that they might have been introduced in the DWS by the chickens. Moreover, microbial communities in the external and internal parts of the nipple drinker and the biofilms collected from the water pipes were remarkably similar across the seven weeks, further suggesting microbial exchange.

Although no *Campylobacter* species were isolated from the UK study farm; a low level of *Campylobacter*-specific DNA was detected towards the end of the rearing cycle, in the DWS, using molecular techniques (amplicon profiling and PCR/qPCR). This suggests that low levels of *Campylobacter* were present in the water system in a VBNC state. Some of these sequences were identified as *C. ureolyticus*, but others were only identified to the level of the *Campylobacter* genus (most likely *C. jejuni*).

The specificity and sensitivity of current detection methods are a known barrier to the study of *Campylobacter* epidemiology; especially as *C. jejuni* has such a low infective dose in chickens and humans (Black *et al.* 1988, Ringoir and Korolik 2003) and can enter a VBNC state (Rollins and Colwell 1986). Therefore, even though *Campylobacter* was rarely detected in the DWS, the low-level prevalence suggests that the DWS might be a reservoir of low concentrations of *Campylobacter* spp., which could potentially pose a risk to chicken colonization. The isolation protocol used in this study lacked the specificity needed to supress the growth of other microorganisms from the farm environment present in the samples that were processed, further complicating the detection of *Campylobacter* spp. Moreover this protocol and others commonly used in routine surveillance are biased towards detection of *C. jejuni* and *C. coli*, and therefore do not

detect emerging *Campylobacter* spp. with more complex growth requirements (Man 2011, Bullman *et al.* 2012). During the UK farm study, the emerging pathogen *H. pullorum* was isolated using methods designed to select for *Campylobacter*. Both *C. jejuni* and *C. coli* were isolated from all Ugandan farms studied, and *Campylobacter* spp. were detected with high prevalence using qPCR. These data suggest extensive colonization of chickens by *Campylobacter* in Uganda. This is, perhaps, not surprising given the lack of bio-security measures and control of environmental parameters on the small holdings. However, *Campylobacter* was not present in all sheds visited, and young chicks did not appear to be colonised.

Both *H. pullorum* and *C. ureolyticus* are emerging gastrointestinal pathogens, which have been proposed to be zoonotic (Koziel *et al.* 2012, Borges *et al.* 2015). Broiler chickens are a known reservoir for *H. pullorum* (Ceelen *et al.* 2006, Manfreda *et al.* 2011) but little is known about the on-farm epidemiology of this pathogen. The results presented here suggest that the DWS might have a role in the colonization and spread of *H. pullorum. C. ureolyticus* had not been related to the chicken reservoir before (Koziel *et al.* 2012). The investigation of the presence of *C. ureolyticus,* in chickens and the farm DWS, highlighted an issue with the specificity of primers designed by Bullman *et al.* (2011a) that were found to cross-react with the *H. pullorum* isolated from the farm. As more genomic information becomes available (Grad and Lipsitch 2014) and more refined techniques lead to identification of more epsilonproteobacteria associated with gastroenteritis (Cornelius *et al.* 2012) a revision of primer sensitivity and specificity is needed to ensure accuracy. This study has raised an issue with some published reports of the prevalence of *C. ureolyticus* in patients presenting with gastroenteritis that have used these primers, potentially overestimating the contribution of *C. ureolyticus* towards campylobacteriosis

(Bullman *et al.* 2011a, Bullman *et al.* 2011b, Collado *et al.* 2013). Nevertheless, *C. ureolyticus* seems to be an emerging gastrointestinal pathogen and it has been detected by sequencing in an unspecified number of samples in patients with gastroenteritis (Bullman *et al.* 2011a) and has also been linked to other pathologies including ulcerative colitis and Crohn disease (O'Donovan *et al.* 2014). One of the advantages of metagenomics and amplicon profiling is that they generate a vast amount of data that can lead to unprecedented results that generate new hypothesis. For instance, the identification of *C. ureolyticus* in the DWS of a broiler farm through 16S rRNA amplicon profiling indicates that further investigation of whether chickens constitute a reservoir for this pathogen (and potentially other emerging *Campylobacter* spp.) is warranted.

Microorganisms do not exist in isolation in the environment but historically research has focused on single isolated strains and only relatively recently has moved towards attempting to consider whole microbial communities aided by the development of experimental and computational technologies such as those associated with rRNA amplicon sequencing (Tan *et al.* 2015). This study has been pioneer in applying these techniques to the DWS of a chicken farm; the results will inform further experiments to identify key interactions that might support *Campylobacter* survival in the farm environment. However, there are important limitations to the use of this technique that need to be considered. An important one is the level of taxonomic discrimination. For the 18S dataset a vast amount of OTUs could only be identified at high taxonomic level. For the 16S dataset most OTUs could be identified to genus level but this is still not enough to tackle certain research questions. Isolation and typing of *Pseudomonas* spp. from the shed bulk water throughout the rearing cycle demonstrated that different strains co-habit and possibly dominate different stages of the rearing cycle, information that cannot

be inferred from the 16S data. The 16S data also indicated that *Pseudomonas* had a higher relative abundance in the anteroom bulk water (source) compared to the shed bulk water (broiler house) but *Pseudomonas* spp. were only isolated from the samples from inside the broiler house as the microbial biomass was greater there. Therefore, further work characterising microbial communities on farm DWS using a combination of culture and molecular based techniques is needed. The *in vitro* co-culture experiments that have been carried out in this and other studies have not always used relevant species or strains (e.g. (Axelsson-Olsson *et al.* 2005, Culotti and Packman 2015)). These studies have shown that *Campylobacter* spp. benefit from interactions with a range of bacteria and protozoa but also that these interactions are species and strain specific. In terms of bacterial interactions, this study has focused on the *Pseudomonas* genus. *Pseudomonas* spp. are well characterised inhabitants of DWDS (Douterelo *et al.* 2013) and were detected and isolated in this study. They have also previously shown to enhance *C. jejuni* survival in aerobic conditions (Hilbert *et al.* 2010). A range of *Psuedomonas* spp. strains and isolates was used in co-culture experiments and although most of them were beneficial for *C. jejuni* survival, four detrimental strains were found. Three of these belonged to the species *P. fluorescens* and included one isolate from DWDS biofilms. Therefore, even better taxonomic resolution to species level is not enough to predict if a particular species might inhibit or enhance *Campylobacte*r survival.

In recent work, investigating the ecological relevance of the internalization of pathogens by protozoa in terms of their spread and survival in the environment was identified as a research priority in microbial ecology (Antwis *et al.* 2017). Research on *C. jejuni*-protozoa interactions suggests that these interactions might allow the pathogen to survive on the farm DWS (Snelling *et al.* 2005) as protozoan communities seem to persist across rearing

cycles (Bare *et al.* 2011) and *C. jejuni* internalized in *Acanthamoeba* have the ability to experimentally colonize chickens (Snelling *et al.* 2008). However, whether protozoa represent a real reservoir or the internalization is a transient stage to digestion remains unclear. This study has contributed to the body of research that has shown that *C. jejuni* can be internalized by *Acanthamoeba* and has identified other protozoa (e.g. *Vannella*) that are also present on the farm DWS and could be of epidemiological importance and can be used to design experiments that mimic real conditions better.

In conclusion, this thesis provides tantalising evidence to support the role of other microorganisms in the ecology of *Campylobacter* and other emerging zoonosis associated with chickens. The broiler farm water system is clearly a unique and dynamic microbial niche that is influenced by changing environmental conditions during the rearing cycle. Key members of the resident communities have the potential to increase or decrease the survival and transmission of *Campylobacter spp.* Further studies are warranted to develop surveillance tools with increased sensitivity and specificity and to elucidate community dynamics over multiple rearing cycles; seasons and different farms. The protective and inhibitory properties of *P. fluorescens* and protozoa need to be elucidated, using field isolates, to identify potential ways to manipulate DWS communities to be less-permissive to *Campylobacter* survival.

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