University of Strathclyde

The Ecology of *Legionella* species in Compost

A Thesis presented for the Degree of Doctor of Philosophy

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Declaration of Authenticity

This thesis is the result of the original research conducted by Sandra Currie. Where others have contributed this has been fully acknowledged. All external sources used in this study have been correctly referenced. It has been composed by the author and has not been previously submitted for examination that has led to the award of a degree.

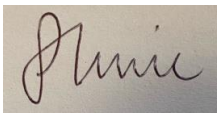
Signed: 

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Table of Contents

List of figures

List of tables

Abstract

Legionella species can cause infection in humans, ranging from mild to life-threatening. These bacteria are commonly associated with water environments, but have also been sporadically linked with composts and associated habitats. Gardening is a popular pastime for millions of people in the UK and further afield, and the presence of human pathogens in the garden environment has the potential to pose a risk to public health. However, there is limited research examining environmental *Legionella* spp in areas other than the water habitat and therefore the level of risk posed is unclear. The key aim of this thesis was therefore to investigate the ecology of *Legionella* spp in compost in order to improve understanding of the potential risk.

Compost samples from different sources were examined and found that *Legionella* spp. are commonly present in products available in the UK: 15/24 (62.5%) samples tested by culture and 22/22 (100%) samples tested by PCR identified *Legionella* spp. Storage conditions were then assessed to determine if greenhouse conditions encourage growth of these organisms further increasing likelihood of exposure. DNA extraction and semi-quantitative PCR methods were developed and applied to compost samples stored in three mock greenhouse systems. The work showed that humidity is likely a critical factor in the maintenance and proliferation of these organisms in compost. In addition, *Legionella* spp. may behave differently in peat-containing composts than in peat-free composts in the greenhouse setting.

To determine where *Legionella* enters the compost environment, and therefore identify opportunities to reduce the presence of these organisms in the compost chain, samples from a green waste processing site were examined. *Legionella* spp were identified using PCR at all stages of the manufacturing process, suggesting they may be present in small numbers throughout.

The work outlined in this thesis has added to the available knowledge on *Legionella* spp. in the compost environment. Further work is required to define and minimise risks posed to workers and members of the public exposed to these organisms in this environment.

1. INTRODUCTION

Legionella species are bacterial respiratory pathogens capable of causing large outbreaks, smaller clusters, and sporadic cases. Infection may be asymptomatic, but can also result in mild respiratory symptoms observed as Pontiac fever or the more serious Legionnaires' disease, which includes symptoms such as severe pneumonia and, in some cases, death. The most common species is *Legionella pneumophila* Sg1, which accounted for >80% (923/1148) of culture-confirmed cases in Europe in 2019 (ECDC, 2021). The picture of infection is somewhat different in the southern hemisphere, where the prevalence of *L. longbeachae* infection in Australia and New Zealand is much higher than in the UK. Infections account for around 40% of the legionellosis cases reported (Li *et al.* 2002, The Institute of Environmental Science and Research Ltd. 2012). Although *Legionella* spp are commonly isolated from water sources (Den Boer *et al.* 2002, Borella *et al.* 2004, Patterson *et al.* 1997), a small number of studies have been published which suggest a link to soil as a source (van Heijnsbergen *et al.* 2014, by Schalk *et al.* 2014, Thacker *et al.* 1978). Potts *et al.* (2013) described eighteen *L. longbeachae* cases notified between 2008 – 2013 in Scotland. In most of these cases, contact with potting compost prior to infection was identified.

Growing media, also known as potting compost or potting soil, is made from degraded organic matter, including composted green waste, wood/bark, coir or peat in varying combinations, and may also contain additives such as vermiculite and perlite (Denny and Waller, 2015). Proportions of components may vary further as the UK moves towards plans to eliminate the unnecessary use of peat by the year 2030 (HM Government, 2011). Concurrently, the EU Landfill Directive 1999/31/EC requires a reduction in landfill waste disposal, which may lead to increased composting of green waste; however, at present, it is unclear what impact Brexit will have on this directive.

Compost can be produced in several different settings: from small-scale production in household environments to medium/large-scale production in reactor vessels, static piles and windrows (Hansen, 1995). During production, the temperature increases as biodegradable compounds are

decomposed until a peak temperature is reached and then begins to fall as the availability of biodegradable compounds decreases (Hoitnick and Grebus, 1997; Füelky and Benedek, 2010). In the UK, the British Standards Institute Publicly Available Specification for Composted Materials (BSI PAS 100) has been developed to provides guidelines on the production of consistent high-quality composts. In Scotland, composts must conform to BSI PAS 100 in order to be sold as a product (WRAP, 2020). One of the requirements in BSI PAS 100 is the inclusion of a high temperature step where the mix must reach ≥65°C for seven non-consecutive days. Part of this process requires investigation of samples for *E. coli* and *Salmonella* spp. but not for *Legionella* spp.

As noted above there has been a wide array of research into the link between water and *Legionella* spp; however, there is less information about the role that a compost habitat may play for these organisms. A simple, rapid search on the literature databases PubMed and Web of Science can be used to highlight this point: searching for "Legionella water" gave 3951 and 3620 results on Web of Science and PubMed respectively. In comparison, a search for "Legionella soil" gave 267 and 192 results, while for "Legionella compost" yielded 41 and 35 results.

The overall aim of this thesis was to investigate the ecology of *Legionella* spp. in compost to improve understanding of these bacteria and their habitat.

1.1. Thesis structure

In this thesis, the term compost will be used broadly to encompass all possible definitions, including growing media, potting soil and posting compost. Where specific media have been used, they will be described in the text, such as composted green waste or shop-bought growing media.

The literature review (Chapter 2) explores the current status of research in this field. It highlights key gaps in the literature, specifically around the small number of studies investigating species other than *L. pneumophila* Sg1 and environmental *Legionella* spp. in areas other than the water habitat. This led to three key objectives being outlined for investigation:

1. To what extent are *Legionella* spp. present in UK composts?
2. Do compost storage conditions impact the survival and proliferation of *Legionella* spp?
3. Are *Legionella* spp. detectable during the manufacture of compost products?

The work in Chapter 3 investigated the first objective: To what extent are *Legionella* spp. present in UK composts? Twenty-four compost samples from different sources were examined for *Legionella* spp. using culture. Samples were also examined for *Legionella* spp and *Acanthamoeba* spp using direct PCR before and after an eight-week enrichment period.

Chapter 4 and 5 investigated the second aim: Do compost storage conditions impact the survival and proliferation of *Legionella* spp? In Chapter 4, several methods were explored in an experimental setup. This included *in silico* analysis of primers and development of standard curve. In addition, denaturing gradient gel electrophoresis (DGGE) and subsequent product sequencing were used to further assess the PCR's suitability and investigate a potential method for distinguishing PCR products easily.

In Chapter 5, compost samples were stored in three mock greenhouse systems before analysis using the DNA extraction and semi-quantitative PCR methods developed in Chapter 4. Samples were spiked and unspiked with *Legionella* spp. and *Acanthamoeba* spp. and were examined to determine situations in which *Legionella* spp. may proliferate and pose a risk to public health

The work in Chapter 6 looked at samples from a green waste processing site at different stages of the composting process to investigate the third objective: Are *Legionella* spp. detectable during the manufacture of compost products?

Finally, Chapter 7 describes the impact of the work in this thesis and explores possibilities for future work.

2. LITERATURE REVIEW

2.1. Legionella

2.1.1 Background

Legionella spp. were first discovered after a large outbreak of respiratory infection occurred at a convention of the American Legion in 1976, which led to the hospitalization of >100 ex-servicemen and subsequently 29 fatalities (Fraser *et al.*, 1977). The causative agent of this outbreak was unknown and due to the fastidious nature of Legionellae, it took almost two years before researchers identified a rod-shaped bacterium was identified in the Gimenez-stained blood and spleen smears of Guinea pigs infected with lung tissue from Legionnaires disease patients (McDade *et al.*, 1977). These Gram-negative organisms were named *Legionella* after the members of the American Legion infected during the outbreak, and *pneumophila*, Greek for "lung-loving", due to the pneumonic illness it caused. *Legionella* spp. are aerobic bacteria that require specific amino acids (e.g., L-cysteine) and high iron concentrations for growth. They range between 0.3-0.9µm wide and 2-6µm long. Table 2.1 highlights further metabolic and morphologic information.

There are over 50 known species of *Legionella,* with 64 serotypes (Hilbi *et al.*, 2010; Adams, 1999); however, most are rarely identified in clinical samples, and others have only been identified once. As well as clinical samples, *Legionella* spp. are often isolated from the environment, where they exist either in a biofilm or as endosymbionts of protozoan hosts. Almost half of the identified species have been cited as causative agents of human disease (Fields *et al.*, 2002); however, only a few species account for most infections: *L. pneumophila*, *L. longbeachae, L. anisa* and *L. bozemanii* (Beauté and Robesyn 2017). Of these, *L. pneumophila* represents the highest burden of disease, causing 96.3% (1106/1148) of culture-confirmed cases in Europe in 2019 (ECDC, 2021).

Infection with *Legionella* spp. may be asymptomatic; hospital patients and healthy individuals have been shown to experience increased antibody titres to *Legionella* antigens without showing clinical

signs of infection (Fukunaga *et al.*, 1990; Rudbeck *et al.*, 2009). Symptomatic infection with this organism may also present as legionellosis in one of two distinct clinical manifestations: Pontiac fever (PF)—a self-limiting influenza-like illness; or Legionnaires disease (LD)—a more serious pneumonia that can be fatal. Both are described in more detail below. There have also been several atypical manifestations of *Legionella* infection reported in the literature, including septic foot infection and endocarditis (Dugar *et al.*, 2009; Leggieri *et al.*, 2012) caused by *L. longbeachae*.

Table 2.1: Detail on *Legionella* species (adapted from Botzenhart *et al.*, 1998).

Component	**Details**
Width	0.3-0.9µm
Length	2-6µm
Cell wall	Gram-negative, branched-chain fatty acids, no capsule
Respiration	Aerobic
Metabolism	Non-fermentative
Cell morphology	Rod-shaped
Colony morphology	Raised, ground glass appearance
Growth requirements	L-cysteine, iron salts
Growth temperature (optimal)	15-43°C (optimal 36°C)
Motility	One or more polar or sub-polar flagella
Generation time	99 minutes (optimal)

Cases of Legionnaires' can be sporadic, or part of a cluster or an outbreak. Numerous outbreaks of LD have been described, with notable incidents including a significant outbreak in Murcia, Spain, which affected >800 people (García-Fulgueiras *et al.*, 2003) and the most significant UK outbreak to date, in Barrow-in-Furness (2002) with 170 confirmed and 498 suspected cases (Telford *et al.*, 2006). Outbreaks of PF have also been described, notably the first case linking the causative agent of LD

and PF (Kaufmann *et al.*, 1981). Due to the number of people affected, many of the cases described in the literature represent outbreaks, including the first description of LD by Fraser *et al.* (1977); however, some cases thought to be sporadic may be linked to the same infection (Bhopal *et al.*, 1992). Den Boer *et al.* (2007) describe the successful implementation of a novel national surveillance system in the Netherlands, set up after an outbreak affecting 188 people, to identify clusters of two or more cases and remove the infection source to prevent infection spread. All of the outbreaks described above were caused by *L. pneumophila;* however, outbreaks of LD have also been reported where *L. longbeachae* is the causative agent, mainly in the southern hemisphere, such as a statewide outbreak in South Australia involving 23 patients (Steele, Lanser and Sangster, 1990a). In addition, cases of PF caused by *L. longbeachae, L. feelei, L. anisa* and *L. micdadei* have also been described (Herwaldt *et al.*, 1984; Goldberg *et al.*, 1989; Fields *et al.*, 1990; Cramp *et al.*, 2010).

L. pneumophila remains the most widely researched species; simple searches using Web of Science and PubMed give 198 hits and 228 hits respectively when searching for "*L. longbeachae*" compared to 8191 hits and 6401 hits respectively when searching for "*L. pneumophila*". The bias is likely due to *L. pneumophila* being the first isolated organism and the most common cause of Legionnaires disease in the Northern Hemisphere. For example, *L. pneumophila* Sg 1 were responsible for 85.6% of culture-confirmed cases of Legionnaires disease reported to the European Legionnaires Disease Surveillance Network (ELDSNet) in 2015 (Beauté and Robesyn 2017). In an international-collaborative study by Yu *et al.*, 2002, the most common causative agents in cases of sporadic community-acquired legionellosis were (in ranked order) *L. pneumophila* (91.5%)*, L. longbeachae* (3.9%)*, L. bozemanii* (2.4%)*, L. micdadei* (0.6%)*, L. dumoffii* (0.6%)*, L. feeleii* (0.4%)*, L. wadsworthii* (0.2%) and *L. anisa* (0.2%); isolates originated from USA (72.2%), Italy (12.6%), Switzerland (6.1%), Australia (4.7%) and New Zealand (4.3%).

2.1.2 Disease Symptoms

Several symptoms associated with LD appear after a 2-10 day incubation period and include malaise, shortness of breath, fever and diarrhoea, representing the most severe form of the disease and is on average, fatal in 10% of cases (Beauté *et al.*, 2014). PF is a less severe manifestation of infection, with flu-like symptoms appearing 1-2 days after exposure and resolving without intervention within a week (Kaufmann *et al.*, 1981).

In a comparison between LD pneumonia, caused by *L. pneumophila* and *L. longbeachae*, there was no significant difference in symptoms between the two species (Amodeo *et al.*, 2010). However, a more recent comparison by Cameron *et al.* (2016) found that patients infected with *L. pneumophila* were significantly more likely to be smokers than patients with *L. longbeachae* infection. Likewise, those infected with *L. pneumophila* were more likely to have a cough, confusion, lethargy or gastrointestinal symptoms. Infection with *L. pneumophila* often presents with diarrhoea, which is unusual for a pulmonary pathogen. Rowbotham (1998) cultured *L. pneumophila* Sg 1 from faeces using amoebic co-culture. The author suggested that diarrhoea could be caused by toxins released from ruptured *Legionellae* and that they likely enter the digestive system inside, and are protected by, swallowed cells. Naik *et al.* (2008) highlighted that cases of Legionnaires disease often include extrapulmonary manifestations, including neurological symptoms in 4-53% of cases. However, this area needs further study as many symptoms are not well defined in the literature.

Several atypical manifestations of *Legionella* infection have also been reported (Lowry *et al.*, 1991; Lowry and Tompkins, 1993; Megarbane *et al.*, 2000; Grimstead *et al.*, 2015), many of which have been included in Table 2.2. Interestingly, many of these unusual manifestations do not follow the pulmonary route thought to be key in LD and PF. Patten *et al.* (2010) and Lowry *et al.* (1991) suggest that some *Legionella* spp., such as *L. pneumophila* and *L. longbeachae*, may enter the body through wounds, accounting for cases of skin infection occasionally reported. In a review of atypical infections, *L. dumoffii* was isolated from a patient's bone sample (sternum), and another patient had

a ‘cyst’ containing *L. dumoffii* and *L. pneumophila*. The primary source of infection appeared to be tap water from the intensive care unit, and neither patient showed evidence of pulmonary disease (Lowry *et al.*, 1991). Megarbane *et al.* (2000) describe three cases of pancreatitis caused by *L. pneumophila* but note that pathophysiology remains undetermined. Symptoms vary between patients, which may be due to both the immune status of the patient and the strain of *Legionella* spp. responsible for the infection. Table 2.2 summarises the symptoms.

The reason why exposure to *Legionella* spp. can result in different clinical manifestations: asymptomatic, PF, LD, remains unclear. There does not appear to be any difference between *Legionella sp*ecies and their ability to cause Pontiac Fever. *L. pneumophila, L. longbeachae,* and *L. micdadei* have all been responsible for outbreaks of this and similar fevers (Fields *et al.*, 1990; Benin *et al.*, 2002; Cramp *et al.*, 2010). For example, *L. micdadei*, found in a leisure complex whirlpool spa, caused Lochgoilhead fever, a Pontiac Fever-like illness (Goldberg *et al.*, 1989). Pontiac Fever is a less severe manifestation of *Legionella* spp. infection than Legionnaires disease, and described symptoms can be seen in Table 2.2. Unlike LD, where both immunosuppression and increased age are risk factors for infection, PF does not appear to discriminate between adults and children, healthy or immunocompromised individuals (Goldberg *et al.*, 1989) and is resolved without medical intervention (Kaufmann *et al.*, 1981). Indeed, exposure to a PF source is more likely to result in illness than exposure to an LD source. Information for clinicians from the Centers for Disease Control and Prevention (CDC) shows that when exposed to the source of LD, <5% of individuals become ill, compared with >90% of those exposed to the source of PF (Fraser *et al.* 1977 and Glick *et al.* 1978). Rowbotham (1980a) suggested that pathogenesis of LD involves the invasion and replication of *Legionella* bacteria within human cells, while PF is due to hypersensitivity caused by an unknown bacterial or amoebal-host component, thus causing a difference between these infection types. This theory is supported by Miller *et al.* (1993). They suggested that biocide-killed *L. pneumophila*, made airborne during the use of a whirlpool spa, were responsible for an associated PF outbreak.

Table 2.2: Symptoms associated with different manifestations of infection with *Legionella* spp.

Manifestation	**Example of described species**	**Signs and symptoms**	**References**
Asymptomatic	*L. pneumophila* Sg5, Sg6	Elevated antibody titre	Fukunaga *et al.*, 1990; Rudbeck *et al.*, 2009
Legionnaires Disease	*L. pneumophila* *L. longbeachae* *L. bozemanii* *L. micdadei*	Fever, confusion, myalgia, diarrhoea, malaise, respiratory symptoms: cough; shortness of breath; pneumonia, confusion, anaemia, renal failure, neurological symptoms	Naik *et al.*, 2008; Amodeo *et al.*, 2010; Beauté J, 2015
Pontiac Fever	*L. pneumophila* *L. anisa* *L. micdadei* *L. feeleii* *L. longbeachae*	Influenza-like symptoms: headache, fever, myalgia, malaise	Glick *et al.*, 1978; Kaufmann *et al.*, 1981; Herwaldt *et al.*, 1984; Goldberg *et al.*, 1989; Fields *et al.*, 1990; Cramp *et al.*, 2010
Atypical	*L. feeleii* *L. pneumophila* Sg8 *L. longbeachae*	Cellulitis	Loridant *et al.*, 2011; Padrnos *et al.*, 2014; Grimstead *et al.*, 2015
	L. longbeachae	Endocarditis	Leggieri *et al.*, 2012
	L. longbeachae *L. pneumophila* Sg1	Pancreatitis	Eitrem *et al.*, 1987; Franchini *et al.*, 2015
	L. pneumophila (non-Sg 1)	Pericarditis	Schaumann *et al.*, 2001
	L. pneumophila Sg1	Pyelonephritis	Delicata and Banerjee, 2015

2.1.3 Diagnosis and Treatment

2.1.3.1 Diagnosis

Numerous clinical diagnostic techniques are available to support the identification of *Legionella* spp. in patient samples, including the urinary antigen test (UAT), serological testing, PCR, culture and MALDI-TOF. Culture on buffered charcoal yeast extract agar (BCYE) remains the "gold standard" in identification of *Legionella* spp, however, colony growth can take 3-10 days which is much slower than other methods and is undesirable in a clinical setting where rapid diagnosis is preferred (Hayden *et al.,* 2001). This is likely one of the reasons why 88.6% (6234/7034) cases in Europe in 2015 were tested by UAT compared with only 12.7% (890/7034) tested by culture, although some samples may have been tested by both methods (Beauté and Robesyn 2017). Kohler *et al.* (1984) developed the UAT and found that antigen excretion begins at the onset of symptoms and persists until treatment. However, they developed the assay using *L. pneumophila* Sg 1, and it remains unclear whether antigen excretion occurs in other *Legionella* infections. As a result, the UAT is specific for *L*. *pneumophila* Sg 1.

In the UK, pneumonia affects up to 11 in 1,000 adults annually and can be caused by several different bacteria, viruses and fungi. The causative agent for community-acquired pneumonia (CAP) is not identified in 59.6% of cases (Arancibia *et al.,* 2014). It is possible that a change in diagnostic practice could lead to the identification of more cases than currently observed. Work in Denmark suggests that patients with reported Legionellosis represent <50% of actual infection (WHO 2007), and the underdiagnosis along with underreporting are thought to occur across Europe (ECDC 2018) and further afield (Stout and Yu, 1997). McNally *et al.* (2000) also highlighted the lack of identification of causative agents in many cases of CAP. They found that 14 of 99 (14%) samples from patients with CAP of unknown aetiology had at least a fourfold rise in antibody titres to the antigens *L. bozemanii*, *L. anisa*, *S. lyticum*, *Legionella*-like Amoeba Pathogen (LLAP)-1, LLAP-6, LLAP-9 or LLAP-10. In addition to incorrect or slow diagnosis of LD, the self-limiting nature of Pontiac Fever means that it is unlikely to be correctly diagnosed unless an outbreak occurs (Nicolay *et al.,* 2010).

Current guidelines from the National Institute for Health and Care Excellence (NICE) recommend that clinicians consider that *Legionella* UAT is requested in patients with moderate to high severity CAP (NICE, 2019). While they also recommend that blood and sputum are taken (NICE, 2019), testing of these samples for *Legionella* spp. must be specifically requested by the clinician (British Thoracic Society, 2009). Low severity CAP cases are unlikely to be tested for *Legionella* spp. except during an outbreak (British Thoracic Society, 2009) and as mentioned above, mild cases of Pontiac fever may be missed entirely. In addition, Thalanayar *et al.* (2014) showed that the urine test is not always accurate; in this instance, they showed negative results when there was a positive serological response to *L. pneumophila* Sg1. To remedy these issues, García *et al.* (2004) advise culture of immunocompromised pneumonia patient samples. Pravinkumar *et al.* (2010) emphasise that samples should "be sent to a national *Legionella* reference laboratory for PCR and culture" in cases of community-acquired pneumonia with unidentified causative agents; however, this is not an official guideline.

It is also important to note that while the UAT is quick and cheap in identifying LD, overreliance on this test may be a contributing factor in underreporting of Legionellosis caused by species other than *L. pneumophila* Sg1 across Europe (Potts *et al.* 2013). Cases *of L. longbeachae* infection have been seen in Australia since 1989 (Lim *et al,* 1989) and it is likely that this species is tested for more widely in Australia and New Zealand than in the Northern Hemisphere, due to increased awareness amongst clinicians. The diagnosis of other species is often overlooked due to a lack of appropriate tests (Luck, 2010; Roig & Rei, 2003), and therefore, *L. longbeachae* and other species may be missed as etiological agents when using conventional diagnostic techniques.

In the summer of 2013, a cluster of *L. longbeachae* infections occurred in Scotland, and PCR initially identified 4/6 of these cases in the NHS Lothian region. This diagnostic lab had implemented *Legionella* spp. PCR testing for all severe CAP patients in 2010 (Potts *et al.,* 2013). Murdoch *et al.* (2013) suggested that PCR diagnosis may be more effective than the preferred culture method.

When comparing data on Legionellosis two years before and two years after the introduction of PCR testing for *Legionella* spp. on all respiratory specimens, they found a fourfold increase in the positive diagnosis of *Legionella* spp. infections. PCR is relatively fast in identifying *Legionella* spp. and does not have the species-specific limitations of the UAT. The British Thoracic Society (2009) recommends using this technique over serological testing where available. The UK Standard for Microbiology Investigation for Identification of *Legionella* spp. (UK SMI ID18, 2015) recommends using PCR testing alongside other diagnostic techniques for the most appropriate public health response and patient treatment. As the use of this method for identification, typing and confirmation increases, faster and more sensitive diagnosis of this disease may occur in the future.

2.1.3.2 Treatment

Asymptomatic infections do not require treatment, and PF is generally self-resolving without treatment. After diagnosis, both Legionnaires' disease and atypical manifestations of *Legionella* infection may be treated using macrolide antibiotics such as Azithromycin (Plouffe *et al.* 2003), Telithromycin (Carbon and Nusrat, 2004), Erythromycin and Clarithromycin (Hamedani *et al.* 1991), as well as the fluoroquinolones Levofloxacin (Yu *et al.* 2004) and Ciprofloxacin (Haranaga *et al.* 2007). Both classes of antibiotics can enter macrophages where they interact with intracellular *Legionella* spp. The macrolides class of antibiotics are bacteriostatic and work by inhibiting bacterial protein synthesis, whereas the fluoroquinolone class are bacteriocidal; they interact with the enzymes DNA gyrase and topoisomerase IV to cleave bacterial DNA (Hooper 1999).

These antibiotics are not often included in the first-line treatment for mild or moderate community-acquired pneumonia unless the clinician suspects Legionellosis (BNF NICE, 2022). Due to the relatively slow nature of some diagnostic methods, administration of the correct antibiotics may be delayed. Optimised processes and rapid diagnoses of *Legionella* spp. infection may be beneficial in replacing the empirical antibiotic regimes with specific, timely antibiotic treatments. The resistance of various bacteria to all classes of antibiotics has been increasing over time. However, to date

limited antimicrobial resistance has been observed in *L. pneumophila*. This is complicated by the lack of gold-standard method for minimum inhibitory concentration (MIC) determination in *L. pneumophila*, which is impacted by the fastidious nature of *Legionella* spp. and the charcoal included in BCYE agar, which binds to antimicrobials. Recent articles present broth dilution as a method that may provide a basis from which any changing susceptibility patterns in *Legionella* spp. can be identified (Isenman *et al.* 2018, Wilson *et al.* 2018, Portal *et al.* 2021).

2.1.4 Source

Hubálek (2003) describes Legionellosis as sapronotic, a human disease transmissible from abiotic environments, including soil, water and decaying plants. *Legionella* spp. do not spread between human hosts and do not seem to have an animal reservoir, but are commonly isolated from both man-made and natural water and soil-based environments, justifying this classification. Free-living amoebae are likely the environmental host for *Legionella* spp, enabling them to survive in harsh conditions and inadvertently training them for survival in human macrophages (Molmeret *et al.* 2005).

Humans are the most commonly described host of *Legionella* spp; they are a terminal host – human to human transmission does not occur. Some animals are also susceptible to disease, including Guinea pigs and mice, commonly used as model organisms (Doyle *et al.* 1998, Brieland *et al.* 1996), and it is likely that animals also have the potential to be terminal hosts. Animals including pigs, sheep, goats, dogs, antelopes, buffaloes, horses and calves but not laboratory rabbits, have all showed increased antibody titres to different species including, but not limited to *L. pneumophila* Sg 1-6*, L. gormanii, L. dumoffii, L. bozemanii* and *L. micdadei* (Barth *et al.* 1983, Phakkey *et al.* 1990, Boldur *et al.* 1987, Cho *et al.* 1984, Collins *et al.* 1982). This list is not definitive as many of these tests were conducted in the early days of *Legionella* research, and cross-reactivity between tests was likely (Barth *et al.* 1983). Research in this area is minimal and not up to date with current identification techniques. In addition, a number of the antibody titres were relatively low. Only one

study attempted isolation of *Legionella* bacteria from animals; Boldur *et al.* (1987) describe the isolation of *L. pneumophila* Sg1 from calf lungs. They found that 17% of lungs (24/139) were positive for *Legionella* by direct immunofluorescence, and organisms were only isolated from two cadavers (1.4%) (Boldur *et al.* 1987). This indicates that increased antibody titre may result from background exposure to *Legionella* spp. and may not indicate illness in all cases. Despite this, it is likely that infection may occur in some instances. After describing a calf's death due to a presumptive *L. pneumophila* Sg1 infection, Fabbi *et al.* (1998) suggest that calves are accidental hosts of *Legionella* spp., much like humans.

A commonly cited source of infection in humans and animals is water. Early work and most modern research have focussed both artificial and naturally occurring water habitats. For example, large outbreaks have been associated with cooling towers at Melbourne Aquarium (Greig *et al.* 2004) and whirlpool spa and sprinklers at a flower show in the Netherlands (Den Boer *et al.* 2002). Other artificial sources, including hot water supplies in homes and healthcare facilities, are linked to outbreaks and sporadic infections. For example, Borella *et al.* (2004) isolated *Legionella* spp. from 22.6% (33 of 146) tap water samples taken in a multi-region study of Italian domestic hot water and in samples taken between 1993 and 1994, *Legionella* spp. were isolated from water supplies in 55% of organ transplant units in the UK (Patterson *et al.* 1997). In the USA, *Legionella* was responsible for 58% of drinking water-associated outbreaks from 2009-2010 (CDC, 2013). More unusual sources include an industrial pressure test pump which creates water aerosols when in use (Euser *et al.* 2014a), a car wash facility (Euser *et al.* 2014b) and car air conditioning systems (Sakamoto *et al.* 2009a).

Recreational water sources are also commonly associated with sporadic cases and outbreaks of *L. pneumophila* and *Legionella* spp, including spa pools, showers and air conditioning facilities in hotels and cruise ships (Fraser *et al.* 1977, Tobin *et al.* 1980, Jernigan *et al.* 1996, Benin *et al.* 2002, Beauté and Robesyn 2017). In the United States 2009-2010, *Legionella* spp. were responsible for 4/57 (7%)

of waterborne disease outbreaks in treated recreational water (CDC, 2014). Natural recreational waters are also sources of infection; hot springs used for bathing in Taiwan contained *Legionella* spp. in 21 of 91 (23%) of sites tested, with the most common species identified as *L. pneumophila* (8/91, 8.8%) (Hsu *et al.* 2006). The isolation of *Legionella* spp. from environmental sources highlights their ability to survive in a variety of different conditions. Sheehan *et al.* (2005) found DNA sequences for four *Legionella* spp., potential host amoebae *Acanthamoeba* spp., *Euglena* spp. and cultured *L. micdadei* from an algal mat (pH 2.9) at three site temperatures: 30°C, 35 and 38°C. However, when authors attempted culture of previously isolated strains of *Legionella* at low pH, they were unsuccessful. They suggested that *Legionella* spp. can survive in a higher pH niche within host organisms. Additionally, there were differences between temperatures and species identified. All seven DNA sequences extracted from the 30°C showed >99% sequence similarity to *L. sainthelensi*, only identified in one other DNA sequence isolated from the 35°C sites. The authors concluded that this strain is rare above 35°C, unlike the sequences with 98% similarity to an unnamed *Legionella*-like amoebal pathogen (LLAP) isolated from 2/3 of the 38°C samples, but not isolated at 30°C. The idea that the distribution of different species is affected by temperature was supported by Borella *et al* (2004) who tested the level of *Legionella* contamination in Italian domestic hot-water systems and found that those with a lower operating temperatue were more likely to be contaminated with *L. pneumophila* Sg 1 as opposed to those with higher operating temperatures which were dominated by *L. pneumophila* Sg2-14. The authors hypothesised that different strains of *Legionella* react differently to environmental pressures, resulting in a preference for different ecological niches.

The source of *Legionella* spp. in recreational and domestic water supplies remains unclear. Interestingly, *Legionella* spp. were not cultured from 23 roof-collected rainwater samples in New Zealand (Simmons *et al.* 2001), but in a study by Ahmed *et al.* (2008), 7 of 27 (26%) roof collected rainwater samples were positive for the *Legionella*-specific *mip* gene, implying that they contained live or dead *Legionella* organisms. A second study by the same author, again examining roof-harvested rainwater from Queensland, Australia, found 12 of 214 (5.6%) samples positive for

Legionella spp. by *mip* gene presence, again implying the presence of live or dead *Legionella* organisms in these samples (Ahmed *et al.* 2010). A Japanese study isolated *Legionella* from 7/18 (38.9%) rainwater puddles on an asphalt road, first by culture and later confirmed using PCR. They did not isolate *Legionella* directly from rainwater by culture but suggested viable but non-culturable (VBNC) organisms may be present after identifying *Legionella* DNA in 1/10 (10%) direct rainwater samples (Sakamoto *et al.* 2009b). A more recent study in the Netherlands isolated *Legionella* from water on roads in 3/77 samples (3.9%) using amoebal coculture (van Heijnsbergen et al 2014). In this study, rainwater was not tested before contact with the ground but soil samples were taken from the side of the road and 6/20 (30%) tested positive for *Legionella*, with the authors suggesting that puddles were possibly contaminated by soil runoff after rainfall. A further study by Schalk *et al.* (2014) examined soil next to an outdoor whirlpool after speculating that this was the source of a *L. pneumophila* ST47 infection in the Netherlands in 2013. While the authors identified *L. pneumophila* ST47 in a soil sample taken next to a fountain, they did not consider the possibility of soil contamination from water leaving the whirlpool in their discussion, resulting in a lack of clarity around the potential source. It should be noted that water from the whirlpool was culture negative for *Legionella* at the time of infection and was not retested in the 2014 study. The distinction between water and soil samples was similarly blurred in a study by Travis *et al.* (2012), who looked at water-saturated soil samples from the homes and workplaces of patients infected with Legionnaires' disease in Sa Kaeo, Thailand. They found that 22/39 (56.4%) samples were positive for *Legionella*, with isolates comprising 12 known species, three novel species, and three untypeable species, suggesting that the growth of *Legionella* spp. was sustained in the water-saturated soils tested.

An outbreak of Legionnaires disease among 81 patients and employees at a hospital in Washington D. C., USA, in July and August 1965 was identified retrospectively through examination of serum samples archived by the CDC. Nineteen out of twenty-six (85%) serum samples had at least a fourfold increase of IFA in titre (≥64) to *L. pneumophila* Sg1 (Thacker *et al* 1978). The source of

infection was thought to be aerosolized particles from a soil excavation site close to the hospital, as the incidence of infection in patients showed a significant geographical link between the location of the excavation site and the location of patient's beds within the hospital. The soil excavation site was active in the weeks leading up to and during the start of the outbreak and had also experienced heavy rain and high winds nine days before the first case of infection. In addition, 74/81(91.4%) cases slept beside an open window or had grounds privileges (Thacker *et al.* 1978). This work was in the early days before a standard profile for these organisms had been established, and these patients were only tested against *L. pneumophila* Sg1 (Thacker *et al.* 1978). While modern investigation centres on the investigation of water systems, work by Thacker et al (1978) represents a time when investigations into the main environmental reservoir for this organism were still underway and pre-conceptions of the course of Legionellosis had not yet been established, thus identifying a potential ink between LD and soil. In Europe in 2013, only 22% (636/2878) cases underwent environmental investigation, with *Legionella* identified in less than half of cases (267/624). In addition, with the source of infection not identified in four of the top ten most significant reported clusters of LD in Europe in 2013 (ECDC, 2015), an environmental source of LD may have been missed during investigations due to an emphasis on water as a source. In the introductory chapter to *Legionella* (Marre *et al.*, ASM Press, 2002), Joseph McDade recollects early work that led to the discovery of *L. pneumophila* after the outbreak of Legionnaires disease associated with the Bellevue-Stratford Hotel Outbreak in 1976. He highlights the importance that recognition and pursuit of anomalies in routine investigation plays in new discoveries, and the potential pitfalls of sticking to a standard diagnostic algorithm. This may well be true in the current system: while the importance of water as a source of infection and *L. pneumophila* Sg1 as an aetiological agent cannot be denied; it is likely that the overreliance on the Urinary Antigen Test and the traditional focus on water as the habitat for these organisms is detrimental to the diagnosis of infection and identification of organisms from other sources, such as soil.

The high microbial diversity in soils means that it is possible that *Legionella* was not isolated from this habitat in the early stages of research due to inhibition by other organisms, plate overgrowth and insufficient agar media, and it may be the case that after the link between water and *Legionella* was established alternative sources were overlooked. Combined with the affinity of *L. pneumophila* to water systems and bias due to species-specific tests such as UAT, this means that *L. pneumophila* has been established as the dominant organism in this field of study. To date, the vast majority of research into *Legionella* spp. has focussed on *L. pneumophila* and their association with water.

Investigations identifying *L. pneumophila* and *Legionella* to genus level from water systems are well documented; however, the link between *L. longbeachae* and water sources is less well established, with only two examples in the literature where this species has been isolated from water. Lau *et al.* (2013) found one isolate of *L. longbeachae* Sg2 after testing 3900 cooling-tower water samples in New Zealand. However, they did not find evidence of *L. longbeachae* Sg 1, which is commonly associated with legionellosis in New Zealand. Consequently, the authors suggested that water was "not a natural habitat for pathogenic *L. longbeachae*." This opinion was contrasted by Thornley *et al.* (2017). They isolated *Legionella longbeachae* Sg1 from cooling tower water and linked this to a confirmed case of LD with serological evidence of a rise in titre to *L. longbeachae* Sg1, to one probable case of LD and three probable cases of Pontiac Fever, although isolates were not obtained from patients for direct comparison with environmental isolates.

A large LD outbreak, where *L. longbeachae* was identified as the causative agent, occurred in Australia in 1989. During the investigation, potting soil was identified as a potential source for *L. longbeachae* (Steele, Lanser and Sangster, 1990a). In later work, *L. pneumophila* and *L. micdadei* were isolated from potting mix (Steele, Moore and Sangster, 1990b). Since then, these species and other human pathogens, such as *L. bozemanii* and *L. anisa,* have been isolated from potting mixes in Japan (Koide *et al.* 2001), Switzerland (Casati *et al.* 2009) and Greece (Velonakis *et al.* 2010) as well as further investigations in Australia (Hughes and Steele, 1994).

L. longbeachae has been associated with potting soil and gardens in several cases where humans have been infected (Patten *et al.* 2010, Pravinkumar *et al.* 2010, deJong and Zucs 2010, Potts *et al.* 2013). In Canada, two cases were attributed to soil in patients without contact with growing media (Picard-Masson, 2016). After *L. pneumophila*, *L. longbeachae* was the second most commonly isolated species from patients with Legionellosis worldwide in 2002 (Yu *et al.* 2002). *L. longbeachae* was first isolated in 1981 from respiratory tract specimens from four patients with pneumonia and similar symptoms to those seen in patients with LD caused by *L. pneumophila* (McKinney *et al.* 1981).

Historically, the incidence of infection with *L. longbeachae* in Europe has been low; however, as noted by Whiley and Bentham (2011), the number of cases of infection appears to be increasing. Lindsay *et al.* (2012) noted that *L. longbeachae* had been cited as the causative agent in only eleven cases of infection in the UK since 1984; seven of these occurred in Scotland. Further work by Potts *et al.* (2013) revealed that between 2008 and 2013, eighteen cases of *L. longbeachae* infection had been detected in Scotland. In most cases, the patient had been in contact with commercially available compost before the onset of symptoms. In addition, isolation of *Legionella* sp. from growing media in Europe is also increasingly common (Casati *et al.* 2009, Velonakis *et al.* 2010); however, source attribution is not always straightforward (Bacigalupe *et al.* 2017).

The incidence of human infection with *L. longbeachae* is much higher in the southern hemisphere than in Europe; infection rates are more equivalent to those for *L. pneumophila.* For example, a review of legionellosis survey data in Australia from 1996 to 2000 reported that 42% of cases were attributable to *L. longbeachae*, compared with 51% of cases where the causative agent was *L. pneumophila* (Li *et al.* 2002). In New Zealand, the Ministry of Health found that, in 2011, *L. longbeachae* was responsible for more cases than *L. pneumophila*, with 42% and 30% of instances of laboratory-reported infection, respectively (The Institute of Environmental Science and Research Ltd. 2012). Human infection with *L. longbeachae* has also been noted in the USA (CDC, 2000), Japan

(Okazaki, 1998) and Thailand, where Phares *et al.* (2007) found that *L. longbeachae* was responsible for 5% of clinically defined cases of pneumonia in a rural district, whereas *L. pneumophila* was not reported. However, Wallis and Robinson (2005) associated a case of *L. pneumophila* infection with soil. As discussed above, an outbreak of LD linked to *L. pneumophila* was also thought to have soil as a source (Thacker *et al.* 1978).

2.1.5 Route of transmission

The route of transmission for Legionnaires' disease is widely regarded as through the inhalation or aspiration of water aerosols contaminated with *L. pneumophila* (Fields *et al.* 2002). For infection linked to compost use, there is slightly more debate. There have been suggestions that *Legionella* spp. may be able to enter the body through open abrasions in the skin (Patten *et al.* 2010 and Lowry *et al.* 1991), while Steele, Lanser and Sangster (1990a) suggested that *L. longbeachae* leaches out of the potting mix after watering and may be present in aerosols formed during the watering process, which the gardener could inhale. Doyle *et al.* (1998) found that an aerosolised Australian clinical isolate of *L. longbeachae* Sg1 was lethal to 3 out of 5 exposed Guinea pigs. Lung tissue showed similar characteristics to infection with *L. pneumophila* Sg1 upon post-mortem examination, suggesting that aerosolization in the manner described by Steele, Lanser and Sangster (1990a) would be a viable route of infection. There is also evidence that *Legionella* spp. were present in water used for plant irrigation (Stojek and Dutkiewicz, 2002) which could infect humans through aerosols and may also be responsible for contamination of composts and potting soils if used during manufacture.

Inhalation or aspiration of contaminated dust or soil particles (de Jong and Zucs 2010, Cameron *et al.* 1991), live bacterial cells, or protozoa containing the bacteria (Atlas, 1999) are also potential routes of infection for this organism. Rowbotham (1990) suggested that vesicles or amoebae could prevent dehydration of Legionellae and provide a large dose of Legionellae to a potential host. The author suggests that this would account for the lack of patient-patient spread in Legionellosis infection. Cabello-Vílchez *et al. (*2014) isolated *Acanthamoeba* spp. from 21 (28.4%) out of 74 nasal swabs

taken from healthy individuals in Peru, which suggests that the inhalation of infected amoebae is possible, and Gilbert *et al.* (2004) found amoebae-resisting bacteria after amoebal co-culture of human nasal swabs (7 out of 444 samples (1.6%)). This work suggests that bacteria present inside amoebae can enter the human respiratory system via the nasal cavity. An alternative mode of infection linked to amoebae was suggested in work by Berk *et al.* (1998), who described the release of respirable vesicles by *Acanthamoeba polyphaga* and *A. castellanii*. The vesicles contained live clusters of *L. pneumophila,* which may be another way in which these bacteria are dispersed. A higher number of vesicles containing *L. pneumophila* were released when amoebae were fed a mixture of *E. coli* and *L. pneumophila* (Berk, 1998), suggesting that a mixed bacterial environment is favourable for the release of vesicles containing *Legionella* spp. Cramp *et al* (2010) described a cluster of Pontiac Fever due to *L. longbeachae,* and suggest that aerosolized potting mix was the responsible agent. Still, while they eliminated water as a potential source of infection, the authors did not distinguish between the contaminated soil particles, dust, vesicles, amoebae and bacteria present within the aerosolised mix. However, this does support the theory that the inhalation of aerosols consisting of contaminated water or compost particles is the most likely route of infection, as does the evidence that this is the method for transmission of *Legionella* spp. found in water. Conza *et al.* (2013) isolated *L. pneumophila* from 10.6% (5/47) and FLA from 19.1% (9/47) of bioaerosol samples collected at composting facilities; however, the authors did not isolate *L. pneumophila* and FLA simultaneously from the same sample, including potential intracellular *Legionella* spp. Further studies have isolated *Legionella* spp. and *L. pneumophila* in compost bioaerosols from both static and agitated samples (Bonifait *et al.* 2017, Nasir *et al.* 2018). It is likely that live *Legionella* spp., contaminating compost particles or water droplets, are dispersed in aerosols when compost is handled or when bags are opened. O'Connor *et al.* 2007 highlighted that the presence of *Legionella* spp. in compost did not necessarily indicate that those handling it would become infected. Education about potential risk factors and hand washing before eating, drinking and smoking decreased the incidence of infection. The effectiveness of hand-washing in reducing

infection may indicate that particles are aspirated from the hands after compost use (O'Connor *et al.* 2007, Picard-Masson 2016). In a case-control study of *L. longbeachae* infection in New Zealand, risk factors for infection included using potting mix, putting unwashed hands near the face after using potting mix, and moving compost e.g. tipping. However, using masks and gloves when handling compost was not protective against infection (Kenagy *et al.* 2017).

2.1.6 Habitat

Many factors contribute to the survival, dissemination, and behaviour of *Legionella* spp. in compost, including temperature, the presence of host species such as free-living amoebae, and biofilm creation. Described below is the key work that has been carried out and how it may relate to the survival of these bacteria in the compost environment.

2.1.6.1 Host species

Some species of Legionellae may have adapted to soil life; for example, Cazalet *et al.* (2010) found sequences homologous to protein from plants and phytopathogenic bacteria in the *L. longbeachae* genome. However, it is also likely that Legionellae survival in compost and the composting process is aided by an association with soil-dwelling host species, which may provide a niche habitat away from the potentially harmful environment. Rowbotham (1980b) first introduced the idea that *Legionella* spp. replicate within free-living amoebae, when he observed *L. pneumophila* Sg1-6 replicating inside *Acanthamoeba castellanii, A. polyphaga,* and other *Acanthamoeba* spp., *Naegleria gruberi* and *N. jadini*. Since then, several organisms have been described as potential hosts for *Legionella* spp., including ciliated protozoa *Tetrahymena pyriformis* (Fields *et al.* 1984), the nematode *Caenorhabditis* spp. (Brassinga *et al.* 2010) and the slime mould *Dictyostelium discoideum* (Hägele *et al.* 2000). It has been suggested that while they can survive outside of host cells, Legionellae are only able to replicate in the presence of protozoa (Solomon and Isberg, 2000, Fields *et al.* 2002, Ewann and Hoffman 2006), meaning that in order for *Legionella* spp. to proliferate in the environment, a host species would also need to be present. It is therefore essential to look for potential hosts in

environmental *Legionella* spp. studies where possible. *Acanthamoeba* spp are the most commonly associated protozoan hosts associated with *Legionella* spp in the literature, however there are a number of other possible hosts. The species of *Legionella* and the species of protozoa impacts the relationship between the two, as does the presence of endosymbionts (Croze *et al.* 2021, König *et al.* 2019). A summary of known species associated with *Legionella* spp. is shown in Table 2.3.

The mechanism for infection of host species by Legionellae has not been described for many of the organisms included in Table 2.3. Still, for some, such as *Acanthamoeba* spp., Legionellae may use a similar mechanism to that described for the infection of human macrophages. For example, *Legionella* spp. use the dot/icm type IV secretion system (T4SS) to infect human macrophages, which is also used by *Legionella* spp. to enter, replicate within and exit from *Acanthamoeba* spp. (Hilbi *et al.* 2011). It is also thought that the ability to replicate within amoebae may be a prerequisite for human infection (Molmeret *et al.* 2005), although this may be species-dependent (Neumeister *et al.* 1997). More recent studies have suggested that virulence of *Legionella* spp increases after they have passaged through a protozoan host, but that the impact on release of different virulence factors varies depending on the species of *Legionella* (Gomes *et al.* 2018).

In addition to acting as a "training ground" for infection of macrophages, host species likely play a vital role in the survival and spread of *Legionella* spp. in the environment (Molmeret *et al.* 2005) and have also been shown to be hosts for other human pathogens, for example, *E. coli* O157 (Barker *et al.* 1999), *Vibrio cholerae* (Abd *et al.* 2005), *Mycobacterium* spp. (Ben Salah & Drancourt, 2010) and *Campylobacter jejuni* (Olofsson *et al.*, 2013). Host species may play additional protective roles in the environment, such as protection from desiccation, death by UV light, and protection from predators. Bryant *et al.* (1982) described changes in the bacterial population caused by fluctuating moisture content in soil inoculated with *Pseudomonas paucimobilis* or with these bacteria plus the free-living amoeba *A. polyphaga*. The authors found that bacteria-only populations were reduced by 60% from

starting numbers after the initial drying period. In contrast, populations with bacteria and amoeba did not significantly change in numbers after drying.

Several studies have demonstrated the ability of *Acanthamoeba* spp. cysts to survive harsh conditions, including survival in UV light. For example, *A. castellanii* needs 15 times more exposure to UV light than *E. coli* before 99.9% deactivation occurs (Chang *et al.* 1985). Amoebal cysts are also known to resist high temperatures (Coulon *et al.* 2010) and chlorine treatments (Kilvington and Price, 1990). It may be the case that amoebae encyst, protecting Legionellae within from high temperatures and disinfection: Kilvington and Price (1990) described the survival of *L. pneumophila* within cysts of *A. polyphaga*, even after exposure to 50mg/l free chlorine. Aksozek *et al.* (2002) suggested that the ability of cysts to survive in harsh environmental conditions may be favourable to their host species, an idea supported by Boratto *et al.* (2013). They tested the amoebal pathogen *Acanthamoeba polyphaga mimivirus* (APMV) for survival both alone and inside a host amoeba under adverse conditions. The authors found that after UV irradiation, temperatures up to 75°C and exposure to chemical biocides including 70% ethanol and active chlorine, the survival rate increased when the virus was inside the amoebal host compared with the virus alone. However, when Greub & Raoult (2003) studied *L. pneumophila* grown within *Hartmannella vermiformis,* they did not find "any bacteria within vacuoles or the cytoplasm of a mature cyst" in 120 cysts examined using electron microscopy. The authors did find *L. pneumophila* in an encysting cell, and in the cell wall of an amoeba, in one instance each respectively.

Table 2.3: A summary of the host species of *Legionella* spp. identified to date

Host Species	**Associated *Legionella* spp. *negatively associated, i.e. bacterial numbers decreased**	**Reference**
Acanthamoeba castellanii	*L. pneumophila* LLAP10 *L. micdadei* *L. anisa* *L.* erythra *L. hackeliae** *L. bozemanii ** *L. oakridgensis**	Rowbotham 1980; Hägele *et al* 2000
Acanthamoeba polyphaga	*L. pneumophila*	Rowbotham 1980
Acanthamoeba spp. (Environmental Isolate)	*L. pneumophila*	Rowbotham 1980
Acanthamoeba palestinensis	*L. pneumophila*	Anand *et al.* 1983
Acanthamoeba culbertsoni	*L. pneumophila*	Miyamoto *et al* 2003
Acanthamoeba royreba	*L. pneumophila*	Tyndall *et al.* 1982
Naegleria lovaniensis	*L. pneumophila*	Tyndall *et al.* 1982
Naegleria gruberi	*L. pneumophila*	Rowbotham 1980
Naegleria jadini	*L. pneumophila*	Rowbotham 1980
Naegleria fowleri	*L. pneumophila*	Newsome *et al.* 1985
Tetrahymena pyriformis	*L. pneumophila*	Fields *et al.* 1984
Tetrahymena thermophila	*L. pneumophila*	Kikuhara *et al.* 1994
Caenorhabditis elegans	*L. pneumophila* *L. longbeachae*	Brassinga *et al.* 2010
Caenorhabditis briggsae	*L. pneumophila* *L. longbeachae*	Brassinga *et al.* 2010
Dictyostelium discoideum	*L. pneumophila* Corby LLAP10 *L. longbeachae* *L. micdadei ** *L. anisa** *L.* erythra* *L. hackeliae** *L. bozemanii ** *L. oakridgensis**	Hägele *et al* 2000; Solomon *et al* 2000
Hartmannella (Vermamoeba) vermiformis	*L. pneumophila* Leeds *L. pneumophila* Lincoln *L. pneumophila* York	Rowbotham 1986; Fields *et al.* 1989
Hartmannella cantabrigiensis	*L. pneumophila* Leeds *L. pneumophila* Lincoln *L. pneumophila* York *L. pneumophila* Sg1	Rowbotham 1986; Fields *et al.* 1989
Balamuthia mandrillaris	*L. pneumophila*	Shadrach *et al* 2005
Echinamoeba exundans	*L. pneumophila* Sg1	Fields *et al.* 1989
Vahlkampfia jugosa	*L. pneumophila* Leeds *L. pneumophila* Lincoln *L. pneumophila* York	Rowbotham 1986

A possible reason for the limited evidence of *Legionella* spp. within amoebal cysts may be the expulsion of vesicles containing *Legionella* from amoebae before encysting. This is thought to be a protective mechanism to prevent amoebae from becoming infected with Legionellae (Rowbotham 1986) and was also demonstrated by Berk *et al.* (1998), who described *A. polyphaga* and *A. castellanii* expelling vesicles containing live *L. pneumophila* after feeding them at 25°C and 30°C. They found that the vesicles were released before encystment and were protective against biocides, freeze-thawing and sonication and suggested that culture may underestimate viable cell counts. Further work by Berk *et al.* (2003) examined *Legionella*-containing vesicles expelled from protozoans isolated from cooling towers. They found that *L. pneumophila* contained in vesicles were protected from drying for ten days, whereas *L. pneumophila* alone did not survive. The collective body of work examining interactions between *Legionella* spp. and host species indicates that this relationship is likely to protect these bacteria in the environment and may well explain their isolation from extreme environments such as compost, which undergoes a natural heating process during production. The production of compost and its microbiological processes are further described in Section 2.2

2.1.6.2 Temperature

Temperature may play a vital role in the survival and proliferation of *Legionella* spp. and their hosts. The maintenance of set temperatures in hot water systems is used as a control measure to prevent the growth of *Legionella* spp. based on the survival limits noted in Table 2.1. HSE guidelines suggest the avoidance of water temperatures between 20-45°C (HSE ACOP, 2013) and recommend that heated storage tanks are heated to 60°C for an hour each day. The distribution temperatures for hot and cold water should be above 50°C and below 20°C, respectively (HSE, 2003). However, despite maintenance of hot water tanks at 60°C and intermittent “heat and flush” treatments (where the temperature of the water is raised to 77°C, and outlets are flushed for 30mins), these bacteria can recolonize water systems (Stout *et al.* 1998). While high temperatures have been shown to kill or prevent the growth of *Legionella* spp., specific temperatures, such as the 20-45°C range identified by the Health and Safety Executive (HSE ACOP, 2013) and environmental factors such as air

temperature, rainfall and humidity may contribute to "ideal" conditions for *Legionella* spp. replication in the environment. In the cluster of cases of *L. longbeachae* infection in Scotland described by Potts *et al.* (2013), all were linked to patient contact with potting soil; although a common source was not identified. The authors suggest that higher than usual temperatures due to warm weather, combined with storage of bags in sheltered, enclosed spaces may have contributed to the growth of *Legionella* spp. resulting in higher than usual concentrations of these bacteria in the compost before use. This is a theory supported by Lindsay *et al.* (2012), who found that storing compost in greenhouse conditions increased *Legionella* numbers by 20% compared with ambient conditions in a small preliminary study. In addition, when examining differences between cases of *L. pneumophila* and *L. longbeachae* infection, Amodeo *et al.* (2010) found a significant difference between the seasons that cases occurred – the number of *L. longbeachae* cases was significantly higher in summer compared with *L. pneumophila*. In contrast, the number of *L. pneumophila* cases was significantly higher in winter compared with *L. longbeachae.* As *L. longbeachae* is the *Legionella* species most associated with compost use (Section 2.1.4 above), these results may indicate that increased temperatures seen in summer result in proliferation of this organism, but may also be linked to increased gardening activity during summer months (Amodeo *et al.*, 2010).

There are several possible reasons for *Legionella* spp. survival at increased temperatures. The bacteria may be tolerant of higher temperatures, protected in biofilms (further described below in Section 2.1.6.3), or harboured in amoebae (as described in Section 2.1.6.1), allowing them to slowly recolonize the system after the heat has reduced. However, the link between FLA and *Legionella* spp. in the environment is unclear, and several studies are contradictory. In a review paper, Hilbi *et al.* (2011) reported that Legionellae in the environment could "persist in association with thermotolerant amoebae". This is supported by Rohr *et al.* (1998) who isolated four strains of *H. vermiformis* and two strains of *Saccamoeba* spp., both with the ability to grow at 53°C, from hospital hot water systems known to be infected with *Legionella* spp. The authors did not look directly for *Legionella* spp. in the water samples.

Similarly, when comparing intracellular counts of *L. pneumophila* Suzuki and *L. pneumophila* Lp01 in *A. castellani,* Ohno *et al.* (2008) found that bacterial numbers significantly decreased at temperatures below 20°C, different to results obtained at 25°C where bacterial growth occurred. The authors also noted a significant increase in the number of amoebae present when incubated with bacteria compared with amoebae incubated alone at 15°C. In contrast, at 35°C, the number of amoebae surviving after incubation with bacteria were significantly lower than amoebae incubated alone (Ohno *et al.* 2008). These results suggest that *A. castellanii* can kill or clear *L. pneumophila* at low temperatures and also that these bacteria may disrupt amoebal cells at higher temperatures. (Ohno *et al.* 2008). This is supported by Berk *et al.* (1998), who noted that when *A. polyphaga* and A. *castellanii* were fed live *L. pneumophila* at 35°C, the amoebae lysed, but that lysis did not occur after feeding at 25°C or 30°C (Berk *et al.* 1998); however, this does not support the theory that amoebae harbour *Legionella* spp. at higher temperatures. In addition, a study by Conza *et al.* (2013), examining compost samples and bioaerosols for the presence of *Legionella* spp. and FLA, found that compost samples that had tested positive for these organisms had statistically significant lower temperatures than the negative controls. Samples positive for *Legionella* spp. had a mean average temperature of 38.69°C, whereas negative samples had a mean temperature of 53.24°C. Similarly, free-living amoebae positive samples had a mean average temperature of 39.10°C compared with 53.18°C for negative samples, suggesting that lower temperatures may favour *Legionella* spp. growth.

A possible explanation for these results is that *Legionella* spp. may selectively infect different amoebal species dependent on temperature. Solomon and Isberg (2000) noted that *L. pneumophila* grows in soil amoebae *Dictyostelium discoideum* at a lower temperature (25.5°C) and slower speed (100-fold growth in three days) than in the traditionally identified hosts of human macrophages and *A. castellanii*, both of which had a growth temperature of 37°C and 1000-10000 fold growth increase after three days. Rohr *et al.* (1998) isolated *Acanthamoeba* spp. from nine hospital water samples but could only culture six of these at 40°C, and only one at 44°C, indicating that some, but not all,

Acanthamoeba species can grow at increased temperatures. The authors isolated *Naegleria* spp. from cool, moist areas within the hospitals tested but could not culture these isolates at 42 or 44°C (Rohr *et al.* 1998). Work carried out to date on *Legionella* and FLA association at different temperatures indicates that slight differences in the habitat of these species, including temperature differences, could affect the outcome of their relationship.

2.1.6.3 Biofilms

The microbial community most likely plays a role in the survival and maintenance of *Legionella* spp. in the compost habitat. Biofilms are ubiquitous in both artificial and environmental habitats, from medical devices to water pipes and from leaf surfaces to river beds. Environmental biofilms consist of complex communities of microorganisms bound within an extracellular matrix and are known to support *Legionella* spp. growth in water systems (Murga *et al.* 2001), as well as the garden environment. Thomas *et al.* (2014) isolated *Legionella* spp. and FLA from biofilm samples in a garden hose, which have the potential to be spread via aerosols created when the hose was in use.

Compared with planktonic cells, disinfection of bacteria within biofilms requires higher concentrations of biocide before treatment is successful (LeChevallier *et al.* 1988), and biofilms also protect *Legionella* spp. from extreme conditions in the environment, such as low pH (Sheehan *et al.* 2005) and high temperatures (Farhat *et al.* 2010). In a review by Declerck (2010), it was suggested that while *Legionella* is capable of forming monospecies biofilms in some cases (Mampel *et al.* 2006), it is likely that in the environment, these bacteria are secondary colonisers; i.e., they attach to biofilms, which have already formed. However, some bacterial strains have been found to inhibit the growth of *Legionella* spp., for example, *Streptococcus* species and *Staphylococcus saprophyticus* KC isolated from human pharyngeal flora (Flesher *et al.* 1980), and Ishida *et al.* (2014) found that an obligate intracellular endosymbiont of *Acanthamoeba* spp. isolated from the environment, *Neochlamydia* S13, protects to the protozoa from infection with *L. pneumophila*. Furthermore, in nutrient-rich conditions, monospecies biofilms formed by some bacterial species such as

Empedobacter breve and *Microbacterium* sp. support attachment of *L. pneumophila*, whereas other bacterial biofilms such as *Pseudomona*s spp., *Corynebacterium glutamicum*, or *Klebsiella pneumoniae* did not (Mampel *et al.* 2006). A possible explanation for this was identified in Kimura *et al.* (2009), who identified that growth and production of a monospecies biofilm by *Legionella* spp. are suppressed by a chemical signalling molecule, or autoinducer, released by *Pseudomonas aeruginosa*. However, Murga *et al.* (2001) found that *L. pneumophila* was able to persist in a mixed biofilm, despite the presence of *P. aeruginosa* and *K. pneumoniae*, but it was unable to replicate without the presence of a host species, in this case, *H. vermiformis*.

Temmerman *et al.* (2006) hypothesised that heat treatment creates an abundance of dead cells which can be used by surviving *Legionella* spp, to feed and ultimately recolonize the environment. They found that *Legionella* spp. can use dead bacteria and other material from biofilms for growth, although the growth rate is much slower than in a host species (Temmerman *et al.* 2006). Bigot *et al.* (2013) suggest that multiplication within amoebae may enhance the ability of *L. pneumophila* to colonize biofilms, and based on their work on the visualisation of *Legionella* spp. within multispecies biofilms, Taylor *et al.* (2013) suggested that after replicating within amoebal hosts, Legionellae may lie dormant and, to a limited extent, replicate within a biofilm when conditions are favourable. Morimatsu *et al.* (2012) examined *Pseudomonas putida* biofilm formation in high- and low-nutrient environments at 5°C, 10°C, 20°C and 30°C. The authors found that biofilm detached at high temperatures under nutrient-rich conditions but remained attached at low temperatures. Under nutrient-poor conditions, biofilm detached regardless of temperature. While it would be unwise to assume the behaviour of two unrelated bacterial species is similar, this work highlights how differences in nutrient availability and temperature can affect the behaviour of organisms within a biofilm. Multispecies biofilms in nutrient-deficient conditions can be colonised by *L. pneumophila* in less than 2 hours, but only after the addition of *A. castellanii* (Declerck 2010). This may be significant in the compost environment, where small numbers of *Legionella* spp. could re-colonise their

environment within a relatively short time after being exposed to the high temperatures involved in the composting process: further discussion of this process can be found in section 2.2.

2.2. Compost

2.2.1 Background

Compost is the product of decomposed organic materials; however, it is often used to describe a wide range of growing media and potting mixes. In “Modern Potting Composts” (1976), Bunt describes compost as a word with several meanings, namely a) decomposed plant material, b) a mixture of animal manure and straw, c) growing media composed of a mixture of minerals and organic materials, all of which can generally be defined under the umbrella term plant growing media. This thesis will use compost in the broadest term to encompass all possible definitions. Where specific media have been used, they will be described in the text, such as composted green waste or shop-bought growing media. Compost is used to support plant growth in domestic settings, as well as for agricultural and municipal purposes: Of 3,876,000m^3compost supplied to the UK market in 2014, 1.4% was exported, 28.4% used by the professional market, and 70.2% used for amateur/retail market (Denny and Waller, 2015). Uses can be as diverse as an amendment in arable farming and agriculture to forming green roofs for modern buildings. Other possible uses include as a fertiliser and soil conditioner in horticulture and landscaping applications, erosion-control, and covering landfill sites (Füleky and Benedek 2010).

2.2.2 Components

This highly variable media can contain numerous components dependent on availability, cost and desired end product. Compost may include any number of components, which are too numerous to list here in full. Common additives include sphagnum moss peat, composted organic material (from household green waste to municipal solid waste, to composted commercial wastes, e.g. bark and wood remnants), coir (coconut husk fibre), loam (mineral soil) and sand, and perlite and vermiculite to enhance drainage properties. A breakdown of components used in growing media production in

the UK in 2014 can be seen in Figure 2.1: Growing media by component in the UK, 2014. Data from Denny and Waller (2015). (based on data from Denny and Waller, 2015). Components may change between manufacturers and even between batches of compost by the same manufacturer, dependent on the market prices of components products. For example, a poor coconut harvest may result in shortages of coir and increased prices, which may lead compost manufacturers to substitute this component with another to keep manufacturing costs down. The availability of components is likely also a factor in their inclusion into growing media mixes. Carlile (2008) highlights that composted bark is the principal component in Australian growing media, whereas, in the UK, bark only made up 4% of growing media components in 2014 (Denny and Waller 2015). The author also notes that where composted bark is used, the species will vary depending on local growth (Carlile, 2008). The use of different components in different regions could contribute to the presence and survival of *Legionella* spp. and its supporting organisms within growing media. In their discussion on using biocontrol agents to prevent the growth of undesired organisms, Hoitnick and Grebus (1997) emphasise the importance of feedstock, composting manufacture, maturing environment, and how compost is used on the ability of microflora to thrive or lay dormant within this media.

Traditionally in the UK, growing media was composed of loam-based mineral soils but moved to peat, perlite, vermiculite and shredded barks in the mid-20th century, as high-quality loam increased in cost and became more difficult to acquire (Bunt 1976). Since then, peat has been the dominant growth medium used in the UK and throughout Europe; however, as plans to eliminate the unnecessary use of peat by the year 2030 are implemented (HM Government, 2011), the market will increasingly move towards the use of peat-free composts. Furthermore, increased composting of green waste resulting from reduced landfill waste disposal, as required by the EU Landfill Directive 1999/31/EC, may be used to provide alternative compost constituents. In addition, home composting is becoming increasingly popular as the general public becomes increasingly aware of waste disposal issues and the reduction in landfill space. The introduction of EU Landfill Directive

1999/31/EC has also led to councils developing waste reduction plans, many of which include mass composting from the municipal green waste collection including the provision of compost bins and kitchen caddies for use within the home and garden.

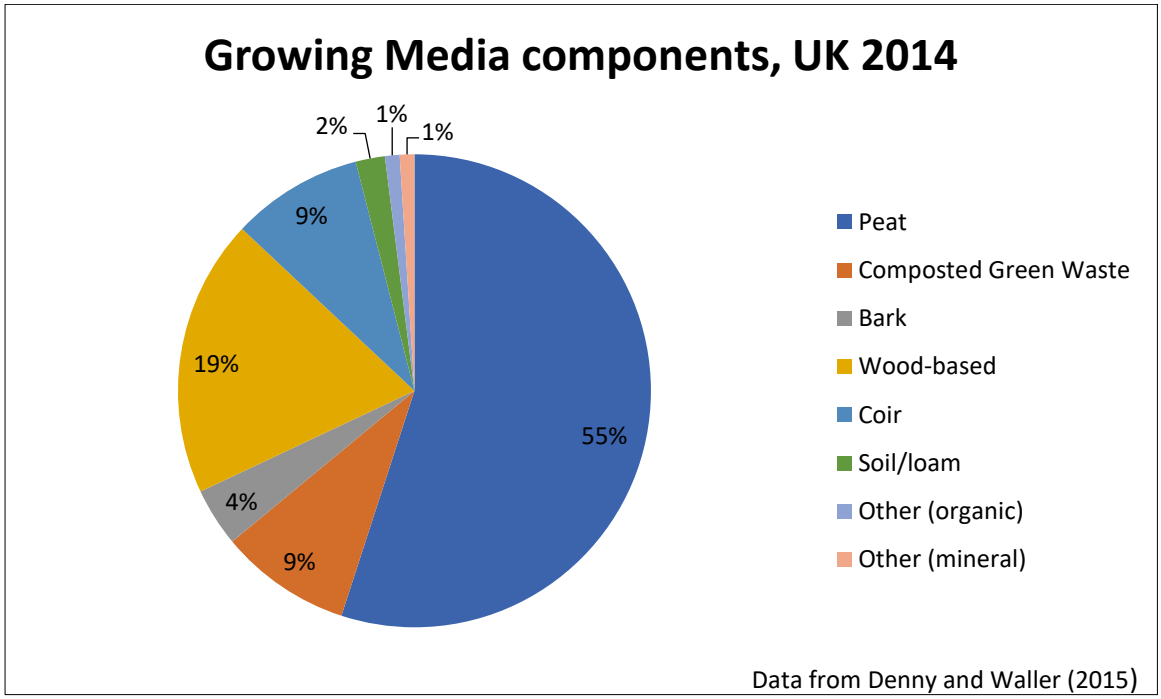


Figure 2.1: Growing media by component in the UK, 2014. Data from Denny and Waller (2015).

2.2.3 Production methods

Compost production consists of three phases, where the temperature increases as highly biodegradable compounds are decomposed by facultative and obligate aerobic bacteria in an exothermic process until a peak temperature is reached, and then begins to fall as the availability of biodegradable compounds decreases (Hoitnick and Grebus, 1997; Füelky and Benedek, 2010). Organic matter degrades at different rates depending on its composition, from the quickly degrading carbohydrates, sugars and proteins, to the less degradable fats and hemicellulose, and finally the very slow decomposers cellulose and lignin (Epstein, 1997). The more easily degraded compounds decompose rapidly at the start of the composting process, increasing the compost pile's temperature as microorganisms multiply and release heat. Hoitnick and Grebus (1997) state, "compost piles must be turned frequently to expose all parts to high temperatures to produce a

homogenous product free of pathogens and weed seeds". Temperatures reached can vary depending on feedstock and composting method used (Epstein, 1997). An overview of the composting process can be seen in Table 2.4.

Table 2.4: An overview of the composting process (Hoitnick and Grebus, 1997; Füelky and Benedek, 2010; Epstein, 1997)

Phase	Temperatures	Process
1	Increasing <40°C	Components are combined, and during the initial 24-48 hours, easily biodegradable compounds are broken down in metabolic processes by facultative and obligate aerobic bacteria, yeasts, fungi, ants, nematodes and oligochaete worms. The reactions release heat and carbon dioxide. Increasing temperature leads to increased microbial activity and increased oxygen demand. During this phase, mesophilic bacteria thrive, and the number of thermophiles begins to increase.
2	High 40-70°C	Temperatures of 40-70°C prevail, and less biodegradable substances, including cellulose and lignin, begin to break down, and ammonia, carbon dioxide, water and heat continue to be released. The heat released decreases the bacterial generational time, resulting in higher numbers of bacteria. During this phase, thermophilic bacteria reach levels of around 10^9 organisms/wet gram compost.
3	Decreasing <70°C	Most microbiological activity occurs within the first seven days due to the decrease in readily available biodegradable components after this time. As a result, oxygen consumption decreases in phase 3 and temperature begins to decrease. Mesophilic organisms recolonize the compost from the outer layers, which have a lower temperature and can reach levels of 10^{11} organisms/wet gram compost. Yeasts, fungi, ants, nematodes and oligochaete worms, absent in phase 2, return in this phase. This maturation stage can take between three weeks and six months and is completed when microbial activity, organic content and temperature have reduced. Compost can be applied to lawns and gardens without detriment to the plants and surrounding soil environment.

Compost can be produced in several different settings: from small-scale production in household environments to medium/large-scale production in reactor vessels, static piles and windrows

(Hansen, 1995). The processes described in Table 2.4 represent active composting, where components are automatically turned in a reactor or manually operated machinery in windrows. Aeration and turning are used to provide heat distribution throughout the sample and optimal conditions for the growth of microorganisms. In some cases, the treatment of components may lead to passive composting, for example, in household settings, where the material is placed into a bin or onto a pile without active turning or monitoring. In this scenario, temperatures reached during the composting process may be much lower, only reaching 30°C, and it may take longer for mature compost to form (Füelky and Benedek 2010).

Pathogenic microorganisms may be present in waste streams, and if temperatures do not reach optimal temperatures, these pathogens may remain in the final compost. In early composts, loam soil was steam treated to kill pathogens; however, steam treatment was not deemed necessary in the UK following a move to peat-based composts (Bunt 1976). Today, the steam treatment of growing media does not occur in the UK, despite moving away from peat and towards composted green waste. Instead, heat production due to metabolic processes which occur during the composting process is relied upon, and guidelines on specific temperatures are detailed in the British Standard BSI PAS 100. To satisfy this standard, the compost mixture must reach a temperature of ≥65°C for seven non-consecutive days and tests for *E. coli* and *Salmonella* spp. For *E. coli*, there must be ≤1000CFU/g fresh mass growing media, and *Salmonella* spp. must be absent from a 25g sample of fresh mass. Free-living amoebae and *Legionella* spp. are not tested for under BSI PAS 100. In three cases of Legionnaires' disease in Scotland linked to compost use, all of the compost used conformed to the PAS 100 Standard (Pravinkumar *et al.* 2010), indicating that the temperatures reached in PAS 100 certification are not sufficient to eliminate *Legionella* spp. from composted material, or that contamination occurs after the pasteurisation step. Similar guidelines in Australia (AS 4454:2012) stipulate that windrows must be turned a minimum of three times, with the core temperature reaching 55°C for a minimum of 3 consecutive days before each turn. As discussed above, cases of *L. longbeachae* appear to be more prevalent in Australia, and the difference in compost production

methods noted here may be responsible, at least in part, for the higher number of cases seen in comparison with Europe.

The organic material used in compost production provides a wide variety of nutrients that support microbial growth. A study by Casati *et al.* (2010) found that several species of *Legionella* were present at composting facilities. They noted that these sites should be considered reservoirs of the bacteria. Samples were taken from sites where waste was actively composted and sites where green waste was stored without intervention (Casati *et al.,* 2010). The source of Legionellae in the composting chain is unclear. Historically *Legionella* spp. have not been found in fresh green waste, or have been found only in minimal numbers (Casati *et al.* 2010, Steele, Moore, Sangster, 1990b) whereas in a more recent study 97/142 (68%) samples from green waste composting facilities in the Netherlands were found to contain *Legionella* DNA by qPCR (Huss *et al.* 2020).More work needs to be done in this area, but there is a suggestion that bacterial contamination of the compost piles occurs via wind and rain (Casati *et al.* 2010). This would not be limited to compost produced outdoors, as end product from in-vessel composting may be left in open windrows during maturation or before being sold. Composts and their components may also provide an environment that supports biofilm formation, especially when left undisturbed. Biofilms are known to support the growth of *Legionella* spp. and their host *Acanthamoeba* spp. in water systems (described above in Section 2.1.6.3), which may also be the case in the compost environment.

A cluster of six *L. longbeachae* infections described by Potts *et al.* (2013) did not identify a common product or manufacturer despite isolating the organism in growing media from 5/6 of the cases indicating that *Legionella* spp. may survive in compost made up of several different components. Devos *et al.* (2005) suggest that the nutritional requirements of *L. pneumophila* can be met in the environment through using excess amino acids produced and released by other microorganisms, or released by dead and decaying matter. Thus, it is possible that nutrients released during the decaying process in composting may support *Legionella* growth. Leaf litter and topsoil from a pine

plantation in Australia were shown to support proteobacteria, the phylum which contains *Legionella* spp. (Zhang *et al.* 2009), and a novel species of *Legionella*, *L. norrlandica*, has been isolated from a wood processing plant in Sweden (Rizzardi *et al.* 2015). Conza *et al.* (2013) examined bioaerosols and compost samples from four green waste processing facilities in southern Switzerland. It showed that *Legionella* spp. can be released in compost bioaerosols. In a study by Chang *et al.* (2008), an essential oil extracted from the evergreen tree *Cinnamomum osmophloem* showed anti-*L. pneumophila* activity at 42°C. However, while direct application of essential oils may be successful in the inhibition of bacterial growth, after 12 weeks, volatile oil contents from some bark samples were shown to have reduced by 90% (Aaron, 1974 as personal communication in Bunt, 1976), suggesting that inhibitory essential oils would not prevent bacterial growth in composted samples.

2.3. Key Research Aims

This literature review highlights numerous questions about *Legionella* spp. in the compost environment. In particular, the two main gaps in current research are:

- The lack of studies completed using species other than *L. pneumophila* Sg1
- The lack of studies examining environmental *Legionella* spp. in areas other than the water habitat.

The use of the urinary antigen test as a primary form of diagnosis and the fastidious nature of *Legionella* spp. in culture may have contributed towards the preference for *L. pneumophila* in research studies, and only further studies using alternative *Legionella* spp. will confirm or deny their relevance to the burden of human infection. It is important to note that while *L. longbeachae* is the species most commonly associated with infections related to compost, *L. pneumophila* infection has been associated with soil in previous cases (Wallis and Robinson 2005, Thacker *et al.* 1978), and *L. longbeachae* was not isolated in samples taken from compost-making facilities and green waste storage plants (Casati *et al.*2010). This thesis intends to examine *Legionella* spp. as a whole, to

prevent bias towards or away from specific species. The research questions chosen aim to increase the available knowledge of *Legionella* spp. rather than specifically *L. pneumophila* Sg1. As the broad scope of this research project is to investigate the ecology of *Legionella* spp. in compost, this thesis aims to also to build on the knowledge of environmental *Legionella* spp. in this habitat.

Many factors contribute to the survival, dissemination, and behaviour of *Legionella* spp. in compost, including temperature, the presence of host species such as free-living amoebae, and biofilm creation. It is beyond the scope of this thesis to investigate all of these factors; therefore, research questions have been devised based on the potential impact on public health. Namely, are *Legionella* spp. present in UK composts; can storage of this media increase the numbers of *Legionella* spp. to infective levels; and are *Legionella* spp. present in green-waste facilities in the UK?

2.3.1 To what extent are Legionella *spp. present in UK composts?*

Over the past five years, there has been an increase in cases of Legionellosis identified where compost has been named a probable source of infection (Pravinkumar *et al.* 2010, Lindsay *et al.* 2012, Potts *et al.,* 2013). *Legionella* spp. have been isolated from growing media associated with specific cases. However, a full-scale investigation of numerous brands has not been completed, and therefore the full scale of compost contamination with *Legionella* spp. in the UK remains unknown. This research aims to complete a survey of multiple brands of commercially available compost to determine the level of *Legionella* spp. contamination in these products in the UK.

2.3.2 Do storage conditions impact Legionella *spp. levels in compost?*

Lindsay *et al.* (2012) suggested that warm, humid conditions provided in a greenhouse environment may increase the numbers of *Legionella* spp. in compost after they performed a preliminary study, in which levels of *Legionella* spp. rose under greenhouse conditions. The limited increase of Legionellae seen in some amoebal enrichment studies (Koide, 2001) also suggests that warm conditions in the presence of an amoebal host are favourable to the multiplication of *Legionella* spp. Furthermore, a cluster of six *L. longbeachae* infections described by Potts *et al.* (2013) did not identify a common

product or manufacturer; however, all of the samples were stored inside cases' house, greenhouse, car, polytunnel, shed or garage (Potts *et al.,* 2013). These environments all have the potential for higher than ambient temperatures, especially during the summer months when the clinical cases occurred. This, combined with the higher than average temperatures seen in Scotland during this cluster occurred leads the authors to suggest that climatic conditions and storage of the growing media may have enabled high levels of growth, leading to an increased risk of human infection. It is, therefore, possible that the way in which compost is stored may impact the level of *Legionella* spp. this media contains, potentially enabling growth to infective levels and impacting human health. However, a full-scale study examining the relationship between temperature and *Legionella* spp. survival in compost has not been undertaken. The second aim of the work outlined in this thesis is, therefore to develop methods suitable for quantitative identification of *Legionella* spp. in compost media and to use these methods to examine the behaviour of *Legionella* spp. under greenhouse storage conditions.

2.3.3 Are Legionella *spp. detectable during compost manufacture?*

As shown in Section 2.2.3, in Phase 3 of production, compost is recolonized with high levels of mesophiles (10^{11} organisms/wet-gram soil), which have survived the potentially lethal high temperatures of Phase 2 on the cooler outer layers of the windrow. This mechanism may be utilised by *Legionella sp*p. Casati *et al.* (2010) sampled products and swabbed machinery from green waste sites in Switzerland and found six of eight sites to be contaminated with *Legionella sp*p. These bacteria may be introduced to compost through the components, through contamination of windrows or another method. Examining samples taken from different points during the composting process for the presence or absence of *Legionella* spp. may aid identification of when these bacteria enter the composting process or when conditions are favourable, and they can replicate to a detectable level. The final aim of this research project is to examine the presence of *Legionella* spp. at different stages in a windrow composting facility to determine the point of introduction and critical stages for survival and growth of this organism during compost production.

3. ANALYSIS OF UK COMPOSTS FOR *LEGIONELLA* SPP. AND *ACANTHAMOEBA* SPP

3.1. Introduction

As discussed in the literature review (Section 2.1.4), cases of infection with *Legionella* spp. have been linked to compost use, and Legionellae have been directly isolated from some soils and composts. For example, Steele *et al.* (1990a) isolated *L. longbeachae* serogroup-1 from potting mixes and soil from potted plants in the homes of four patients while investigating the source of a state-wide outbreak of Legionellosis in South Australia in the late 1980s. In further work, the authors detected *L. longbeachae* and other species of *Legionella* in almost three-quarters of potting soils manufactured in Australia that had been investigated (Steele *et al.* 1990b). Additionally, Cramp *et al.* (2010) first documented *L. longbeachae* serogroup-2, from aerosolised potting-mix, as the causative agent in an outbreak of Pontiac fever that was traced to exposure to aerosolised potting mix in a horticultural nursery.

In addition to studies in Australia (Steele *et al.* 1990b, Hughes and Steele, 1994) and New Zealand (Cramp *et al.*, 2010), pathogenic and non-pathogenic *Legionella* spp. have been isolated from potting soils in Japan (Koide *et al.* 2001), Switzerland (Casati *et al.*, 2009) and Greece (Velonakis *et al.* 2010). Of the species isolated*, L. pneumophila, L. longbeachae, L. bozemanii, L. micdadei,* and *L. anisa* are known to cause disease in humans (Fields *et al.*, 2002). However, except for one case of *L. pneumophila* sg1 (Wallis and Robinson, 2005) and *L. pneumophila* pneumonia reported by Thacker *et al.* (1978), only *L. longbeachae* have been directly linked to compost or potting soils as infection sources (Steele *et al.* 1990a, Cramp *et al.* 2010).

In Scotland, Lindsay *et al.* (2012) and Potts *et al.* (2013) both described cases of infection with *L. longbeachae* linked to potting composts. These cases represent an increased incidence of *L. longbeachae* infection over the past five years, further described in the literature review (Section

2.1.4). While *Legionella* spp. has been isolated from UK composts (Lindsay *et al.*, 2012, McCabe *et al.*, 2011), these studies have only looked at a minimal sample size. To date, the prevalence of *Legionella* in a wide range of UK composts has not been studied. In order to determine the extent of compost contamination in the UK, it is necessary to examine a variety of compost samples in an attempt to address this knowledge gap.

3.2. Methods

3.2.1 Sample processing

Twenty-two brands of composts purchased from retailers in the UK were analysed for the presence of *Legionella* spp. Typical ingredients included a mixture of sphagnum moss peat, composted organic material, sand, vermiculite, perlite, loam and coir. Six of the composts were designated peat-free. In addition to the branded composts tested, one sample of green-waste compost treated to PAS 100 specifications (BSI: PAS 100:2018) and one sample of homemade compost (matured from garden waste, ash, rainwater and limited household waste) were tested. Compost was taken from five distinct areas of each bag via cores (4cm in diameter and 6cm in length) to ensure representative samples. These materials were then well-mixed in sterile containers before analysis. Each of the 24 samples was tested three times.

3.2.2 Culture and enrichment

The test procedures were adapted from those previously published (Lindsay *et al.* 2012, Steele *et al.* 1990a, Casati *et al.,* 2009) and an overview of the steps involved can be seen in Figure 3.1 Briefly, 5g of compost were added to 50ml sterile distilled water (dH_2O) before being shaken for 1hr at 150rpm. After 15 mins of settling, a 200µl aliquot was taken and acid-treated using an equal volume of 0.2M HCl-KCl (pH2.2) for 15minutes. Acid treatment was carried out in an attempt to suppress unwanted compost flora; Legionellae are relatively acid-resistant. The 15-min treatment time was deemed appropriate experimentally by monitoring levels of bacteria and fungi in the compost samples. A ten-fold dilution of the acid-treated sample was prepared in sterile dH_2O, and 50µl were plated onto

Buffered Charcoal Yeast Extract (BCYE) Agar, supplemented with *Legionella* BCYE-α growth supplement (potassium hydroxide buffer, ferric pyrophosphate, L-cysteine HCl and α -Ketoglutarate; Oxoid SR0110) and GVPC (glycine, vancomycin hydrochloride, polymyxin B sulphate, Cycloheximide: Oxoid SR0152). The first two samples were tested at the Scottish Haemophilus Legionella Meningococcus and Pneumococcus Reference Laboratory (SHLMPRL), Glasgow (Samples 1 and 2 in Table 3.2), and a compost sample was not retained for further processing. The remaining samples numbered 3 to 24 were left at 30°C, as described by Koide *et al.* (2001), to allow "enrichment" of *Legionella* by any amoeba that may be present in the sample. After eight weeks, these samples were processed using the methods above, and the remaining samples were frozen at -80°C until needed.

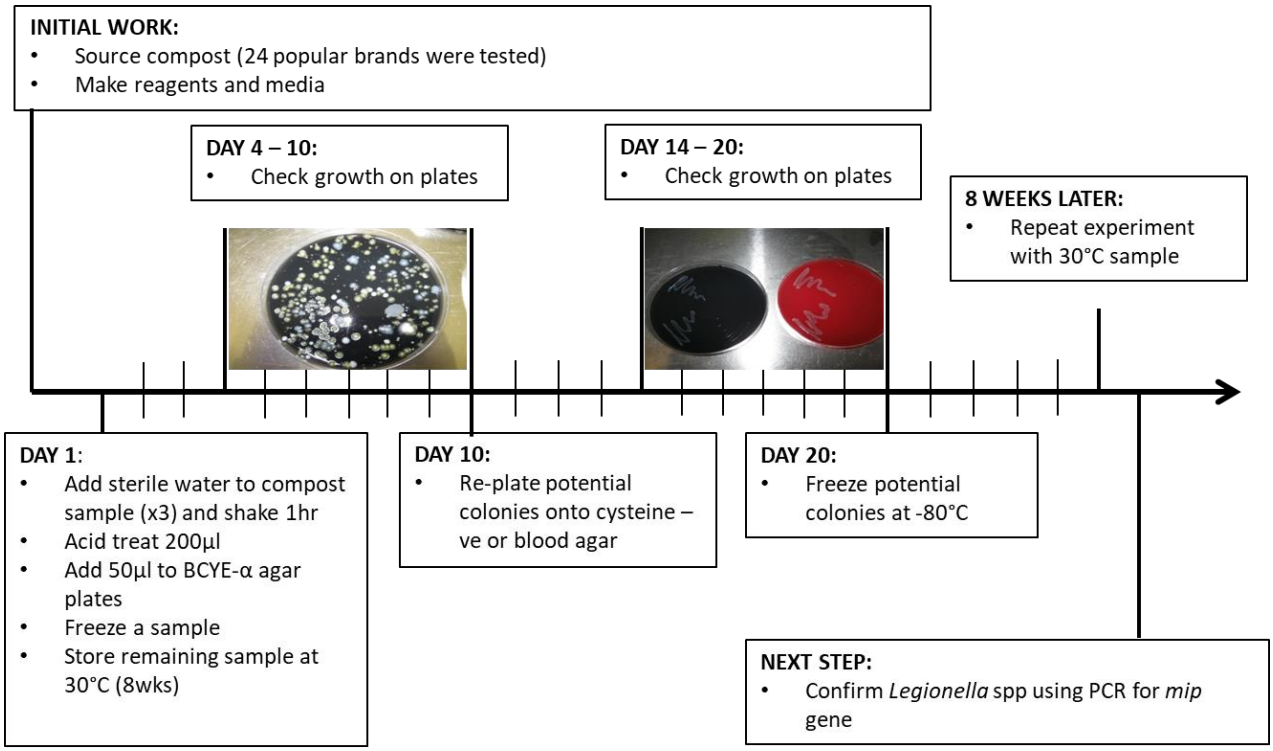


Figure 3.1: Summary of culture methods

BCYE plates were incubated for 3-7 days in humid conditions at 37°C and periodically examined for *Legionella* colonies using a light microscope with a cold light source (Schott KL1500); *Legionella* colonies have a distinctive ground-glass appearance. Due to sample dilution, the detection limit of one colony per plate corresponded to 4000 CFU in 1g compost material. While this is relatively high, it was deemed necessary to prevent inhibition of growth by other soil organisms. Presumptive colonies were sub-cultured onto fresh BCYE-α containing GVPC, and cysteine-negative agar (BCYE-α supplement without L-cysteine, Oxoid: SR0175), as a negative control, as *Legionella* spp. require cysteine to grow. Any colonies that grew on BCYE-α but not on cysteine-negative agar were sub-cultured to ensure purity and stored on Microbank™ beads at -80°C (Pro-lab Diagnostics).

Serotyping of strains was done at The Scottish *Haemophilus*, *Legionella*, *Meningococcus* and *Pneumococcus* Reference Laboratory, at Stobhill Hospital, Glasgow. Latex agglutination for *L. pneumophila* strains was carried out using the *Legionella* Latex Test for *L. pneumophila* sg 1, *L. pneumophila* sg2-14 and *Legionella* spp (*Legionella longbeachae* 1 & 2, *Legionella bozemanii* 1 & 2, *Legionella dumoffii, Legionella gormanii, Legionella jordanis, Legionella micdadei* and *Legionella anisa*). (Oxoid, Basingstoke, UK). This is a non-molecular test which enable rapid identification of specific *Legionella* spp. Latex particles in the kit have been sensitised with antibody against specific cell wall antigens, and agglutinate in the presence of these antigens, forming visible clumps. In addition, indirect immunofluorescent antibody (IFA) testing was performed on strains with more than one serogroup using antisera raised in guinea pigs and fluorescent-tagged anti-guinea pig sera.

3.2.3 PCR and sequencing of bacterial colonies

Following the culture and enrichment of the samples, potential *Legionella* spp. colonies were analysed by PCR with *Legionella*-specific macrophage infectivity potentiator (*mip*) primers (Ratcliff *et al.*, 1998) using the protocol in Table 3.1. Potential *Legionella* spp. colonies isolated from compost were re-grown from Microbank™ beads by running beads across the surface of BCYE-α agar plates before incubation. After 3-7 days in humid conditions at 37°C, DNA were extracted by suspending a

single colony in 500µl of sterile nuclease-free water and heating to 100°C for 10mins in a heat block (Ratcliff, 1998). A 10^{-1} dilution of cell lysate was used as the DNA template in each PCR reaction, including an extract from *L. pneumophila* Sg 1 Philadelphia and *L. longbeachae* Sg 1 clinical isolates (kindly provided by Dr Diane Lindsay at SHLMPRL) as a positive control in each reaction.

PCR was carried out using a 20µl-reaction volumes, containing 10µl PCR Taq MasterMix (MegaMix Blue, Microzone, Haywards Heath, UK), 6µl nuclease-free water, 0.5µl 0.5µM forward primer, 0.5µl 0.5µM reverse primer and 3µl template DNA per well.

Successful PCR products were determined by gel electrophoresis using a 1% agarose gel made with 1% TAE buffer (Tris base, glacial acetic acid, EDTA) and PCR-grade agarose and stained with 2µl ethidium bromide per 100ml of gel. The PCR Mastermix contained a loading buffer, and therefore 5µl PCR product was added directly to each well. For each gel, a single lane was run with a base-pair ladder (3µl, with 3µl of loading buffer) as a reference. Lambda DNA/Hind III digest (125-23130bp) and 50bp ladders (50-1000bp) were used, depending on laboratory stock. Gels were run in 1% TAE buffer at 80V for at least one hour. After electrophoresis, bands of PCR products were visualised in the gels with a UV benchtop transilluminator (2UV, UVP, Upland, CA, United States of America).

Table 3.1: Primers used in PCR reactions

Primer Name	Amplicon size (bp)		Primer sequence	Cycle conditions
mip (*Legionella* macrophage infectivity potentiator gene) (Ratcliff 1998)	661 to 715	Forward primer (Legmip_f)	5'-GGRATTVTTTATGAAGATGARAY TGG-3'	1 x 96°C (3min) 35 x 94°C (1 min), 58°C (2min), 72°C (2min) 1 x 72°C (5min) Hold at 4°C
		Reverse primer (Legmip_r)	5'-TCRTTNGGDCCDATNGG NCCDCC-3'	
		Primer for sequencing: Legmip_fs	5'-TTTATGAAGATGARAYTGGTCR CTGC-3'	N/A
JP (*Legionella* spp. specific 16S gene) (Jonas *et al* 1995)	386	Forward primer (JFP)	5'-AGGGTTGATAGGTTAAGAGC-3'	1 x 95°C (5min) 40 x 94°C (1min), 57°C (1min30s), 72°C (1min) 1 x 72°C (10min)
		Reverse primer (JRP)	5'-CCAACAGCTAGTTGACATCG-3'	
JDP (*Acanthamoeba* spp.) (Scroeder *et al* 2001)	423 to 551	Forward primer	5'-GGCCCAGATCGTTTACCGTGAA-3'	1 x 95°C (7min) 40 x 95°C (30s), 55°C (30s), 72°C (1min) 1 x 72°C (15min)
		Reverse primer	5'-CTCACAAGCTGCTAGGGAGTCA-3'	
Muyzer (16SrRNA general bacterial population) (Muyzer *et al* 1993)	193	Forward primer	5'CCTACGGGAGGCAGCAG-3'	1 x 98°C (3min), 40 x 94°C (15s), 60°C (15s) 55°C (15s) 1 x 72°C (3min)
		Reverse primer	5'-ATTACCGCGGCTGCTGG-3'	

PCR products that showed expected amplicon length on the gels, (as shown in Figure 3.2 were extracted and purified using polyethene glycol (PEG) precipitation. The PEG precipitation is based on methods observed at SHLMPRL. Briefly, 20% PEG/2.5M NaCl solution was prepared with 20g of PEG (8000), 14.6g of NaCl and 100 mL distilled water to a 500mL conical flask. The solution was mixed on

a shaker table at 200rpm until fully dissolved before being filter sterilised through a 0.2µm filter into sterile 50ml centrifuge tubes. To perform PEG precipitation, 30µl of 20% PEG/2.5M NaCl was mixed with each PCR product in a separate sterile 1.5ml tube and incubated at 37°C for 30 minutes. Tubes were centrifuged at 13000rpm for 10 minutes, and then the supernatant was removed with a pipette and discarded without disturbing the pellet. The pellet was washed using 500µl of 70% Ethanol before being centrifuged again at 13000 for 10 minutes. Ethanol was quickly removed from the tubes using a pipette, and the pellet was left to air dry before being resuspended in 20µl nuclease-free water.

The clean DNA samples were quantified using the Epoch Microplate Spectrophotometer (BioTek, Winooski, VT, United States of America), and DNA sequences were determined using the LightRun sequencing service (GATC Biotech). Returned sequences were compared with the Health Protection Agency's (HPA) *Legionella mip* gene sequence database (http://www.hpa-bioinformatics.org.uk/cgi-bin/legionella/mip/mip_id.cgi) in order to identify *Legionella* at the species level.

3.2.4 Direct PCR of post-enrichment compost samples

After all samples had been cultured and sequences of *Legionella* spp. isolates obtained, post-enrichment compost samples were removed from the -80°C freezer and DNA was extracted from a 0.25g sample of compost using the MoBio PowerLyser PowerSoil DNA extraction kit. Compost is high in humic acids, which inhibit PCR reactions if not entirely removed from the sample and so DNA samples were diluted 10^{-1} and 10^{-2} following extraction to minimise the impact of any remaining humic acids on the reaction. As above, *mip* primers (Radcliff, 1998) were initially used. Primers targeting a *Legionella* spp. specific sequence on the 16S-rRNA gene (Jonas *et al.* 1995) were also used in a separate PCR reaction with the extracted DNA; these primers were denoted JFP and JRP (jointly 'JP') to represent Jonas forward primer and Jonas reverse primer, respectively. In addition to this, primers specific to *Acanthamoeba* spp. (Scroeder *et al.* 2001) were used in a further PCR to identify samples that may contain these free-living amoebae. *Acanthamoeba* spp. are known hosts

of *Legionella* spp. in water systems (Section 2.1.6) and may provide a niche in which *Legionella* spp. can replicate during the enrichment period; therefore, samples were tested in retrospect to determine whether any correlation between *Acanthamoeba* spp. presence and increased numbers of *Legionella* spp. exists after enrichment. PCR reactions followed methods as detailed in Section 3.2.3 and protocols as noted in Table 3.1.. Table 3.1 outlines primers used in all experiments throughout the thesis, while the Muyzer primers were not used in this chapter they are used in Chapter 5. As in Section 3.2.3, PCR products were visualised using electrophoresis via an ethidium-bromide-stained gel; bands were compared against a base-pair ladder to ensure correct amplicon length.

3.3. Results

3.3.1 Culture and enrichment

Twenty-four different composts were tested for the presence of *Legionella* spp. by culture on BCYE-α agar and confirming positive colonies using PCR of the *mip* gene. As shown in Table 3.2, 15 of the 24 (62.5%) samples tested contained *Legionella* spp. Ten out of twenty-four samples (41.7%) were positive in the first round of testing. Of the 22 samples, which were re-tested after enrichment at 30°C, 13 (59.1%) tested positive after the eight week incubation period. Owing to sample dilution, the detection limit of one colony per plate corresponded to 4000 CFU in 1g of compost material. Although this is relatively high, it was deemed necessary to prevent inhibition of growth by other soil organisms. Thus, the maximum concentration of *Legionella* spp. found was $4x10^4$ CFU/g.

Table 3.2: Results of initial and post-enrichment culture from twenty-four compost samples, including closest species and percentage identity to this species as determined by *mip* analysis. Sample type is defined by "PF" for peat-free, "PC" for peat-containing, "GW" for green waste, "HM" for homemade

Sample	Sample Type	Isolate name	Initial Culture (number of colonies x % identity)			Post-Enrichment Culture (number of colonies x % identity)		
			1	2	3	1	2	3
1	PF	*L. micdadei*	1 x 100%			NT	NT	NT
2	PF					NT	NT	NT
3	PC	*L. sainthelensi*					8 x 99.83%	
4	GW							
5	PC	*L. longbeachae*	1 x 99.83%					
		L. sainthelensi					1 x 99.48%	
6	PC							
7	PC	*L. micdadei*	1 x 100%					
		L. longbeachae			1 x 100%			2 x 99.83%
		Legionella species K			1 x 99.66%			
8	PC	*L. birminghamensis*		1 x 100%				
		L.pneumophila Sg-1 OLDA		1 x 99.65%				
		L. pneumophila Sg-4 Portland				1 x 99.83%		
9	PC	*Legionella* 99-113	1 x 100%, 1 x 97.64%		1 x 100%	1 x 100%		
		L. sainthelensi						4 x 99.66%, 1 x 99.83%
10	HM	*L. gormanii*				1 x 95.52%		
		L. quateirensis						1 x 84.66%
11	PC	*L. longbeachae*	1 x 99.83%					
		Legionella 99-113			1 x 100%			
12	PC	*L. gormanii*						1 x 95.52%
13	PC							
14	PC	*Legionella* species A IMVS 36	1 x 76.41%					
		Legionella 99-113				2 x 100%		
15	PC	*L. sainthelensi*				3 x 99.83%	4 x 99.83%, 1 x 100%	1 x 98.45%, 1 x 99.83%
16	PC	*Legionella* 99-113	1 x 100%					1 x 100%, 1 x 99.31%, 1 x 97.64%
17	PF							
18	PC							
19	PC							
20	PC	*L. longbeachae*		1 x 99.83%				
		L. sainthelensi					1 x 99.83%	
21	PC							
22	PF	*L. spiritensis*	1 x 99.83%				2 x 99.83%	
		L. feeleii		1 x 87.23%		1 x 87.23%		
23	PC							
24	PC	*Legionella* 99-113				1 x 100%		

3.3.2 PCR and sequencing of bacterial colonies

Twelve species were identified in the compost samples using *mip* gene speciation (Table 3.2). The most commonly isolated *Legionella* species was *L. sainthelensi*, which were present in 5 of the 24 (20.8%) samples, but only isolated after the 8-week enrichment period (Table 3.2). Two isolates of *L. pneumophila* were found in the same compost sample; both tested negative with the rapid latex agglutination kit however the strains were identified as *L. pneumophila* sg4 Portland and *L. pneumophila* sg1 OLDA by indirect immunofluorescent antigen testing. *L. longbeachae* Sg 1 was isolated from four compost samples, making it the second most commonly isolated named organism. Three unnamed species were isolated: *Legionella* 99-113, *Legionella* species K, and *Legionella* species A.

In total, there were eight species with low (<98%) percentage identity matches on the HPA *mip* database: four were isolated from peat-free composts, and four from composts containing peat. A BLAST search showed: samples 9 and 16 had *Legionella* 99-113 with a 100% identity match; sample 22 (initial and after enrichment) had *L. feelei* with a 99% identity match; and samples 10 and 12 had *L. gormanii* with a 99% identity match.

3.3.3 Direct PCR of post-enrichment compost samples

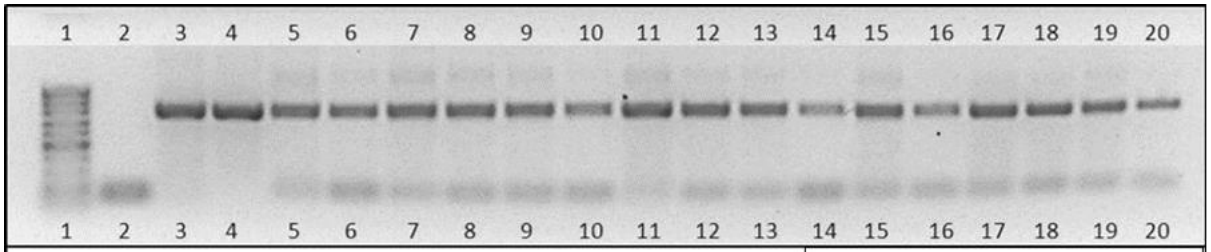
After the initial culture of organisms from compost samples before and after an enrichment period at 30°C, post-enrichment samples were frozen at -80°C. These samples were revisited later in order to compare culture with direct PCR. *Legionella* spp. are fastidious, and it was hypothesised that the number of positive samples would be higher in the direct PCR samples than in the culture samples. It should be noted that the use of PCR only identifies the presumptive presence of specified *Legionella* spp. DNA in the compost samples and does not distinguish between viable/non-viable organisms and remnant DNA.

Initial PCR attempted with *mip* primers was unsuccessful despite numerous modifications made to temperatures and times used in the PCR reaction. Personal communication with Dr Diane Lindsay at

SHLMPRL highlighted the lack of specificity of *mip* primers for species other than *L. pneumophila* when used with mixed samples. The second set of primers, JP primers (Jonas *et al.*, 1995), which amplify a *Legionella*-specific sequence of the 16S-rRNA, were used to identify *Legionella* spp. in direct PCR in DNA extracts from the post-enrichment compost samples.

PCR with the JP primers showed that all samples were positive; therefore, assuming a good specificity of primers to *Legionella* spp., these results suggest that *Legionella* spp. is present or has been present at some point in all of the samples. **Error! Reference source not found.**2 shows that all PCR products, including controls, showed the same size bands.

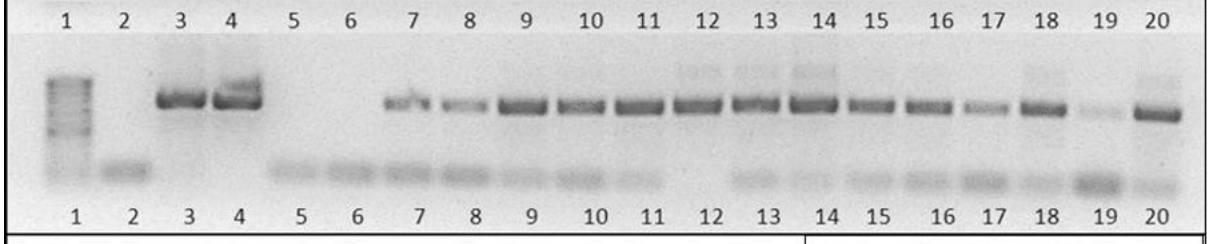
Samples were also tested using JDP primers specific to *Acanthamoeba* spp, a potential host for *Legionella* spp. in compost. Out of 22 post-enrichment samples tested, 15 were positive (68.18%) for *Acanthamoeba* spp. (Table 3.3). Again it should be noted here that PCR does not distinguish between viable/non-viable/dead organisms and remnant DNA; therefore only distinguishes whether samples had any *Acanthamoeba* spp. Table 3.3 summarises all results in this chapter, including cultures and PCR on initial and post-enrichment samples. Four samples (4, 6, 13, 17) were only positive for *Legionella* spp using the JP primers and were not positive for *Legionella* spp by culture or for *Acanthamoeba* spp using JDP primers. Eight samples (4, 6, 13, 17, 18, 19, 21, 23) were only positive using direct PCR, either for *Legionella* spp., *Acanthamoeba* spp. or both. Five samples (5, 7, 8, 9, 20) were positive for all culture and PCR tests.



Key:

Expected Band size: 386bp

1. 50-500bp DNA Ladder
2. **BLANK** Primers and nuclease free water
3. **+VE CONTROL** *L. longbeachae* Sg 1 DNA neat
4. **+VE CONTROL** *L. longbeachae* Sg 1 DNA 10^{-1}
5. Sample 5 DNA neat
6. Sample 5 DNA 10^{-1}
7. Sample 10 DNA neat
8. Sample 10 DNA 10^{-1}
9. Sample 14 DNA neat
10. Sample 14 DNA 10^{-1}
11. Sample 11 DNA neat
12. Sample 11 DNA 10^{-1}
13. Sample 13 DNA neat
14. Sample 13 DNA 10^{-1}
15. Sample 18 DNA neat
16. Sample 18 DNA 10^{-1}
17. Sample 8 DNA neat
18. Sample 8 DNA 10^{-1}
19. Sample 9 DNA neat
20. Sample 9 DNA 10^{-1}



Key:

Expected Band size: 386bp

1. 50-500bp DNA Ladder
2. **BLANK** Primers and nuclease free water
3. **+VE CONTROL** *L. longbeachae* Sg 1 DNA
4. **+VE CONTROL** *L. longbeachae* Sg 1 DNA 10^{-1}
5. **–VE CONTROL** *P. aeruginosa* DNA
6. **–VE CONTROL** *P. aeruginosa* DNA 10^{-1}
7. Sample 21 DNA 10^{-1}
8. Sample 24 DNA 10^{-1}
9. Sample 23 DNA 10^{-1}
10. Sample 22 DNA 10^{-1}
11. Sample 3 DNA 10^{-1}
12. Sample 16 DNA 10^{-1}
13. Sample 12 DNA 10^{-1}
14. Sample 7 DNA 10^{-1}
15. Sample 19 DNA 10^{-1}
16. Sample 15 DNA 10^{-1}
17. Sample 17 DNA 10^{-1}
18. Sample 20 DNA 10^{-1}
19. Sample 6 DNA 10^{-1}
20. Sample 4 DNA 10^{-1}

Figure 3.2: Agarose gel of PCR products from post-enrichment samples. Expected band length 386bp. (Chapter 3, JP primers, Jonas protocol)

Table 3.3: Results from culture and PCR experiments in Chapter 3. Parentheses indicate the organism under investigation in each experiment. "D" represents detected, "ND" not detected and "NT" not tested.

Sample	Initial Culture (*Legionella sp*)	Post-Enrichment Culture (*Legionella sp*)	Direct PCR using JDP Primers (post-enrichment) (*Acanthamoeba sp*)	Direct PCR using JP Primers (post-enrichment) (*Legionella sp*)
1	D	NT	NT	NT
2	ND	NT	NT	NT
3	ND	D	D	D
4	ND	ND	ND	D
5	D	D	D	D
6	ND	ND	ND	D
7	D	D	D	D
8	D	D	D	D
9	D	D	D	D
10	ND	D	D (faint)	D
11	D	ND	D	D
12	ND	D	D	D
13	ND	ND	ND	D
14	D	D	ND	D
15	ND	D	D	D
16	D	D	ND	D
17	ND	ND	ND	D
18	ND	ND	D (faint)	D
19	ND	ND	D	D
20	D	D	D	D
21	ND	ND	D (faint)	D
22	D	D	ND	D
23	ND	ND	D	D
24	ND	D	D	D

3.4. Discussion

Legionella species were detected by culture at levels ranging from 4.0 x 10^3 (limit of detection) to 4.0 x 10^4 cfu/g in 62.5% (15/24) of the composts tested, suggesting that these organisms are common contaminants of UK composts. The contamination frequencies were higher than those in compost surveys in Greece (Velonakis *et al.* 2010) and Switzerland (Casati *et al.* 2009), where *Legionella* spp. were isolated from 27.3% (6/22) and 45.7% (21/46) of compost samples, respectively.

These studies challenge earlier work by Steele *et al.* (1990b). They tested sixty-four compost samples from Australia, Greece, Switzerland and the UK, and found *Legionella* spp. in 73% (33/45) of Australian composts tested but failed to isolate the organism from European composts (14 from the UK, four from Greece, and one from Switzerland). A potential explanation for this was the difference in compost feedstocks, as *Legionella* spp. were found in 80% of composted bark and sawdust samples, the main ingredients in Australian compost. At the same time, they were absent in peat samples, a significant component in European composts (Steele *et al.*, 1990b). In Japan (Koide *et al.* 2001) and Greece (Velonakis *et al.* 2010), all compost samples composed purely of peat were also negative for *Legionella* species. However, samples containing peat mixed with other components contained these bacteria (Velonakis *et al.* 2010). The current study isolated *Legionella* spp. from two-thirds (12/18) of composts containing peat mixed with other components. These results suggest that, although peat alone may not support the survival of *Legionella* spp., its addition to potting mixes does not prevent contamination with Legionellae. Lindsay *et al.* (2012) hypothesised that the increased use of green wastes and the decline of peat in commercial multipurpose composts due to environmental concerns in the UK might partly explain the increased incidence of *L. longbeachae* infection in Scotland.

Traditionally, peat has been the dominant growing media used in the UK and across Europe. However, a White Paper on the Natural Environment, issued by HM Government in the UK (HM Government 2011), lays out plans to eliminate the unnecessary use of peat in England by the year 2030, including a voluntary phase-out target of 2020 for amateur gardeners. The use of peat-free compost is encouraged to prevent the destruction of lowland peat bogs. As a result of policy change in England, the UK composting market will increasingly move towards the use of peat-free composts; the market is currently 57.5% peat-free (HM Government 2011). One possible replacement for peat is green waste. The EU Landfill Directive 1999/31/EC aims to reduce biodegradable waste going to landfill, and the recycling rate of waste, including green waste by composting, however at the present time it is not clear what impact Brexit will have on this directive. Standards are in place to

assure compost quality from green waste, i.e. PAS100 (BSI 2011); however, the only microbial requirement relates to indicator species such as *Escherichia coli* and *Salmonella* spp. *Legionella* spp. were not cultured from the BSI PAS 100:2011 treated sample tested in this experiment (Sample 4); however, it was positive using direct PCR for *Legionella* spp., indicating that these bacteria may well be present in this sample. There is no provision for *Legionella* species in current microbial standards (BSI:PAS 100:2018), despite their isolation from composts and composting facilities in Australia (Hughes and Steele, 1994) and Switzerland (Casati *et al.* 2010); the latter study established green-waste collection and composting sites in Switzerland as an important reservoir for *Legionella* species. As the volume of composted green waste increases and the volume of peat decreases, there may be an increase in *Legionella* spp. isolated from UK compost.

The incidence of *Legionella* infection does not seem to be comparable with the presence of known pathogenic *Legionella* spp. in composts, e.g. almost 17% of composts in this study tested positive for *L. longbeachae*, the causative agent in only 11 cases of infection in the UK since 1984 (Lindsay *et al.* 2012). This discrepancy may be attributable to cases of pneumonia caused by species other than *L. pneumophila* going undetected. Of the 12 different species of *Legionella* isolated in this study, at least eight are known to cause human disease. However, in Europe, the primary diagnostic test used for detecting LD is the urinary antigen assay, used in 88.6% of cases in 2015 (Beauté and Robesyn, 2017), which identifies only *L. pneumophila* Sg 1 with any degree of sensitivity (Potts *et al.* 2013). This issue of under-reporting of cases of legionellosis caused by species other than *L. pneumophila* was highlighted by Whiley and Bentham (2011) and Lindsay *et al.* (2012). Whiley and Bentham (2011) also commented that individuals with Pontiac fever do not generally require hospitalisation, and therefore *Legionella* infection, potentially related to compost, would not be diagnosed. In addition, only one of the 24 compost samples tested was positive for *L. pneumophila,* specifically Serogroup 1 OLDA and Serogroup 4 Portland. Both of these can cause disease, however, both strains were unreactive with the latex agglutination kit for *L. pneumophila* sg 1, *L. pneumophila* sg2-14 and *Legionella* spp., so they may not have been picked up during routine laboratory testing.

Furthermore, the identification of currently unnamed *Legionella* species in this study reflects the diversity of strains in compost. The eight isolates with <98% *mip* speciation may represent new species and a new reservoir for infection. Knowledge on the phylogenetic diversity of clinical and environmental strains of *Legionella* is limited, especially for strains other than *L. pneumophila.* Early work showed a homology of 76% when the *mip* gene of *L.pneumophila* Sg1 and *L. longbeachae* Sg 1 were compared (Doyle *et al.* 1998), while more recent work showed 3% sequence variation in the *mip* gene when 105 clinical and environmental *L. pneumophila* isolates were compared (Gaia *et al.* 2005). In work published after experimental work in this thesis was published, Bacigalupe *et al*. (2017) found that *mip* speciation was not fully discriminatory when used for speciation, when they compared 64 *L. longbeachae* isolates using whole genome sequencing. In five compost samples where more than one isolate of *L. longbeachae* had been isolated, they found that isolates were distributed across the phylogenetic tree indicating a highly diverse population of *L. longbeachae* in compost. In addition they note extensive horizontal gene transfer and recombination occurs between *Legionella* spp and suggest that there may be a shared habitat between to enable this to occur. This supports the findings of multiple species in this chapter but highlights that the species may have been incorrectly designated for some of the samples and is therefore a limitation of this work. Knowledge on the genomic diversity of *Legionella* species is likely to increase as the use of whole genome sequencing becomes more widespread (Hottel 2019).

It is also important to note the relatively high detection limit resulting from the methods used in this study. The high level of dilution and extended acid treatment were deemed to be necessary because of the high levels of bacteria and fungi present in the samples. Although different strains of bacteria will react differently to the acid exposure time, the isolation of 12 species from 62.5% of samples indicates a diverse population of *Legionella* species present in UK composts. Although higher numbers of Legionellae would perhaps have been obtained with a shorter treatment time, detection may have been negated by competing compost microflora. It is also possible that *Legionella* spp may have been contained within a host-species such as *Acanthamoeba* spp and that isolation and culture

of host organisms from the compost samples would have increased *Legionella* spp ascertainment. The methods used mean that it is likely that *Legionella* species have only been isolated using culture in the compost samples containing a higher burden of *Legionella* spp. A negative result does not rule out the presence of *Legionella* species at levels <4000 CFU/g. This is one possible explanation for why all samples were positive for *Legionella* spp. using the JP PCR method; the results are discussed below. Low levels of legionellae in composts may help to explain the low incidence of human infection as compared with the relatively high contamination rate.

Another potential reason for the low incidence of infection compared with the relatively high contamination rate of compost with Legionellae may be that the numbers of bacteria are not high enough to cause infection in humans. Steele *et al.* (1990b) suggested that low numbers of Legionellae in source materials could multiply during the composting process to form populations of detectable size. This concept was reiterated by Whiley and Bentham (2011) who suggested that the composting process is a "catalyst for growth" of *L. longbeachae* as it is commonly found in potting mixes, but rarely in natural soils. However, this theory would suggest that all composts contain *Legionella* spp. at numbers capable of causing infection, which does not appear to be the case due to the low incidence rate. For example, while Casati *et al.* found more *Legionella* spp. using direct PCR on compost samples compared with traditional culture methods, some compost samples remained *Legionella* sp free (Casati *et al.*, 2009). Lindsay *et al.* (2012) isolated *L. longbeachae* from potting compost used by patients with *L. longbeachae* infection that had been stored in greenhouse conditions. The authors also noted that in a preliminary study, compost stored in greenhouse conditions had increased *Legionella* spp. over a three week period (Lindsay *et al.*, 2012). They suggested that a full-scale study into the effect of greenhouse conditions was required to investigate this theory further.

To an extent, the eight-week enrichment period in this study also demonstrated the potential increased temperature and humidity have to enhance *Legionella* spp. numbers. Ten of the 24

compost sample in the current study were positive by culture after initial sampling; this increased to 13 of 24 after enrichment. This technique was first used with compost samples by Koide *et al.* (2001) based on previous work where water samples containing amoebae were incubated to improve Legionellae detection rates (Sanden *et al.*, 1992). Koide *et al.* (2001) directly isolated *Legionella* from 10 of 24 samples taken from composted wood products and potting mixes. Initial samples were suspended in sterile water and incubated at 33°C for 2-3 months, allowing time for any amoebae in the sample to replicate and therefore allowing time for small numbers of intracellular legionellae to replicate to a level above the limit of detection. After the enrichment process, 22 of the 24 samples were positive for Legionellae. Ten of the 24 compost samples were positive upon direct sampling; this increased to 13 of 24 after enrichment. However, as seen in Table 3.2, Legionellae were not detected after enrichment in several samples that were positive upon direct plating, indicating that the technique was not always successful. A similar effect was seen by Koide *et al.* (2001), whereby, upon direct plating, *L. bozemanii* was isolated from two samples of composted wood products, and *L. birminghamensis* was found in a potting mix sample; however, neither was detected after enrichment. In both studies, samples were not inoculated with amoebae; therefore, it is possible that suitable protozoa were not present in all of the samples, or in sufficient numbers to allow the successful replication of *Legionella* species. To build upon this work in future studies, isolation and culture of amoebal hosts may inform the dynamics within each sample, and the likelihood of amoebae aiding growth of *Legionella* spp during the enrichment period. In the current work, Samples 3, 9 and 15 showed the greatest increase in numbers of legionellae after the enrichment period; these three samples all contained *L. sainthelensi*, which was only isolated after the enrichment period. Sheehan *et al.* (2005) took samples at 30°C, 35°C and 38°C from a pH2.7 algal mat community at Yellowstone National Park. The authors found seven sequences in 30°C samples, which all showed >99% similarity to *L. sainthelensi,* but only found this species in one of thirty-one (3.2%) sequences from higher temperature samples. A species of *Legionella*-like amoebal pathogen (LLAP) was the most commonly identified sequence from the algal mat, accounting for around half

of the total sequences, but this sequence was not recovered from any of the samples taken at 30°C. The results suggest that amoebal enrichment may be species and/or temperature-dependent. The presence of amoebae in compost may allow some *Legionella* spp. to multiply to potentially infectious numbers under the right conditions; however, a full-scale study into the effect of greenhouse conditions is needed to investigate this theory further.

Except for Sample 14, all samples which were negative for Legionellae culture before enrichment and positive after enrichment were positive by PCR for *Acanthamoeba* spp. In the post-enrichment samples, 13 samples were positive for *Legionella* spp. by culture, and 15 tested positive for *Acanthamoeba* spp. by PCR. Ten samples tested positive for both *Legionella* spp. and *Acanthamoeba* spp. Thus, of the post-enrichment *Legionella* spp. containing samples, 10/13 (76.9%) were potentially influenced by replication within *Acanthamoebae*. The remaining *Legionella* spp. positive post-enrichment samples may have been amplified by a different host, amplified without a host, already present in the sample and able to maintain the status quo during the enrichment period – perhaps through survival in a biofilm (as described in Section 2.1.6.3). Alternatively, there may not be a link between *Acanthamoeba* spp. and *Legionella* spp. in the samples taken during the current experiment. Unidentified host species or biofilms in the system could provide protection or a niche for multiplication during the enrichment period. These results show that the presence of Acanthamoebae is not a necessary factor for the long-term survival of *Legionella* spp. at 30°C. Conversely, the presence of Legionellae is not a requirement for the long-term survival of *Acanthamoeba* spp. at 30°C.

All of the post-enrichment samples tested directly for *Legionella* spp. using PCR with JP primers were positive for *Legionella* spp, compared with 13/22 (59.1%) of post-enrichment culture samples (Table 3.3). There are several possible explanations for this discrepancy in results. *Legionella* spp. are fastidious, and it is unlikely that all species would grow on artificial media. It is also possible for DNA from non-viable or non-intact cells to be extracted from the soil in some cases (Clark *et al.,* 2008).

The presence of non-viable cells or the high limit of detection of *Legionella* spp. in culture may be the reasons why *Legionella* spp. were picked up by PCR from all samples but not by culture on BCYE-α in all samples. Propidium monoazide has been suggested to enable the differentiation of live cells from dead cells using PCR methods. However, the amount needed increases with the increasing complexity of the sample being tested, resulting in increased toxicity. Taylor *et al.* (2014) suggest that this method is unsuitable for use in environmental samples that are more contaminated than simple waters. This method would, therefore, be unsuitable for use with compost derived samples.

The JP primers used in this have been published several times, initially by Jonas *et al.* (1995) and again in Wellinghausen *et al.* (2001); however, this was for use in water, a considerably less diverse habitat than compost. Sheehan *et al*. (2005) also used the Jonas primers and a separate set of primers to confirm *Legionella* spp. in an algal mat. In work published after completing practical work for this thesis, Marchand *et al.* (2018) used the same Jonas primers to examine soil samples for *Legionella* spp: they also found 100% (n=16) of the samples tested be positive using this method. In the work described in this chapter, bands on the agarose gel for the post-enrichment PCR using JP primers were all the same size, including the positive control (**Error! Reference source not found.**2), indicating reasonable specificity in the PCR. The above result likely indicates the presence of *Legionella* spp. in all of the samples. However, there is a slight possibility that cross-reaction may also have caused positive results due to non-specific primer attachment. In order to confirm that the results obtained in the above work are accurate, further work on the specificity of the JP primers should be carried out.

The current study has shown that UK composts are commonly contaminated with *Legionella* species, many of which have been shown to cause human disease. Cases of non-*L. pneumophila* infection, which are generally increasing globally (Whiley and Bentham, 2011), have been associated with compost use, primarily where the causative agent is *L. longbeachae*. Despite this, care should be taken when defining the source of an infection. In work published after completing the experimental

work for this thesis, Bacigalupe et al (2017) examined 64 clinical and environmental isolates of *Legionella longbeachae* using whole genome sequencing and found that the genome of clinical samples was significantly smaller than the genome of the environmental samples. They suggested that this may be linked to infectivity and highlighted that a genomic epidemiological link has not been identified between environmental and clinical samples to date. They suggested that future investigations should use extensive sampling and whole genome sequencing to ensure specific comparisons can be made.

O'Connor *et al.* (2007) highlighted that the presence of *Legionella* species in compost does not necessarily indicate that those handling it will become infected. During a case-control study examining the association of *L. longbeachae* infection with handling potting mixes, the authors used multivariate analysis to demonstrate that awareness of potential health risks in using compost was protective against infection, although the authors could not demonstrate the effect that this knowledge may have on gardeners. The authors also found that not washing hands after gardening, before eating or drinking, was a risk factor for infection. Australia already has general hygiene warning labels on compost to educate users of potential risks. The work of O'Connor *et al.* (2007) and Kenagy *et al.* (2017) would suggest that this is a beneficial approach to protecting public health. Therefore, it would seem worthwhile considering a similar warning scheme in the UK and other countries to increase public awareness. Such a scheme should highlight the need for good hygiene practices, including hand-washing after compost use, the need to reduce inhalation of compost, and a recommendation to open bags in a well-ventilated area.

Clinicians should be aware of the increased incidence of cases of non-*L. pneumophila* infection, and, to allow for accurate diagnosis, urinary antigen testing should not be the only diagnostic tool used in cases of community-acquired pneumonia, particularly if an association with gardening has been identified.

4. DEVELOPMENT OF A METHOD TO IDENTIFY LEGIONELLA SPP IN COMPOST

4.1. Introduction

As noted in Section 2.2.3, the British Standards Institute Publicly Available Specification for Composted Materials (BSI PAS 100) is the current standard used by environmental and public health agencies to ensure production of composts is consistent and of a high-quality. In Scotland, composts must conform to BSI PAS 100 in order to be sold as a product (WRAP, 2020). However, although part of this process requires investigation of samples for *E. coli* and *Salmonella* spp. investigation for *Legionella* spp is not required.

As described in Chapter 3, fifteen of twenty-four (62.5%) compost samples tested positive for *Legionella* spp. using culture-based methods, and 100% tested positive using PCR; in the latter case, JP primers were used to amplify a *Legionella*-specific sequence of the 16S rRNA gene (Jonas *et al.* 1995). Therefore, it would be advantageous to develop a Real-Time quantitative PCR (qPCR) method, which could be used to quantify *Legionella* spp. DNA in an environmental sample, to further examine the ecology of *Legionella* spp. in compost. Quantitative-PCR can be direct or indirect: direct qPCR method has a fluorescent dye that emits a colour when chelated with double-stranded DNA; the indirect qPCR method uses a labelled probe, which emits light when DNA is synthesised. In both cases, the level of fluorescence is measured by the qPCR equipment at specific points during thermal cycling and is proportional to the amount of DNA in each sample. Such a technique would help establish which environmental conditions facilitate the growth of *Legionella* spp., for example, conditions during compost storage. However, for the development of this method, it was first necessary to establish the suitability of the JP primers for the identification of *Legionella* spp. from mixed environmental samples.

The primers have been tested against a panel of other organisms by several authors, including Wellinghausen *et al.* (2001) and Joly *et al.* (2006). All samples tested in Chapter 3 likely showed a positive result; they all contained *Legionella* DNA. However, there were concerns that the positivity could be due to cross-reaction with other microorganisms present in the compost samples. Compost is a complex media, and when this work was performed, these primers had not been used previously in soil or compost. However, they had been used extensively in clinical samples, domestic and environmental water samples, and biofilm/algal mat samples (Jonas *et al.* 1995, Dobrowsky *et al.* 2014, Edagawa *et al.* 2008, Sheehan *et al.* 2005). They were also used in a qPCR to amplify *Legionella* spp. DNA from hospital water; however, these samples are nutrient-poor and have lower diversity than compost (Wellinghausen *et al.* 2001). Since all samples tested positive for *Legionella* spp. using JP primers in Chapter 3, it was first necessary to assess these primers for use in compost samples before the next experiment to ensure accurate results. In a study published after the work in this chapter was completed, Marchand *et al.* (2018) used the same JP primers to examine soil samples for the presence of *Legionella* spp. Similar to the work in Chapter 3, they found all samples were positive by PCR (n=16), with only one sample positive by culture, further verifying the suitability of these primers (Marchand *et al.* 2018).

It was also necessary to develop a suitable method for DNA extraction, in order to provide representative DNA samples for PCR from each compost sample. The MoBio PowerLyser PowerSoil DNA extraction kit used in Chapter 3 required a 0.25g sample of soil for analysis; this sample was relatively small when compared to the much larger growing-media samples. Therefore, the method would need to enable the extraction of DNA from a sample larger than 0.25g. It was hoped that completing the steps discussed above would enable JP primers to more confidently identify *Legionella* spp. DNA via PCR from DNA-extracted compost samples. Developing a DNA extraction protocol from soil and compost samples larger than those currently used would provide a more representative sample of compost flora than current methods.

Experiments to examine different survival conditions of *Legionella* spp. in compost require appropriate methods to analyse the presence of this organism in suitable sample types. The main aim of this chapter's work was to develop methods that could be used to examine the growth of *Legionella* spp. in compost materials. To achieve this, the JP primers used in Chapter 3 were further investigated to confirm their suitability for identification of *Legionella* spp. by PCR of DNA extracted from compost samples. The primers were also analysed *in-silico* and by denaturing gradient gel electrophoresis (DGGE). Following this, DNA extraction techniques were compared to enable the optimal DNA yield from compost samples. Finally, the methods developed in this chapter were developed to study the behaviour of *Legionella* spp. in different conditions (Chapter 5).

4.2. Methods

4.2.1 Confirmation of primer specificity

4.2.1.1 In Silico analysis

PCR with the JP primers showed that all samples from Chapter 3 were positive. The length of amplicons obtained in PCR with JP primers in Chapter 3 was checked using agarose gel electrophoresis, and all showed bands of similar size (**Error! Reference source not found.**2). As amplification of incorrect sequences may sometimes lead to incorrectly sized PCR products, this is the first step used to demonstrate PCR specificity. But, primers specificity should be checked by the Probe Match tool on the Ribosomal Database Project (RDP) website (http://rdp.cme.msu.edu/probematch/search.jsp) (Cole *et al.* 2014, Greuter *et al*. 2016). This site enables researchers to compare a primer set against a database of bacterial and archaeal 16S rRNA sequences. The primers were also entered into the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome), which searches a comprehensive database for the entered nucleotide sequence to find sequences of >95% sequence identity match (Altschul *et al.* 1990).

4.2.1.2 *Denaturing Gradient Gel Electrophoresis (DGGE)*

Denaturing gradient gel electrophoresis is a method which can be used to identify sequence differences in DNA products. It was used to compare PCR products from culture positive and culture negative samples in Chapter 3, to investigate if this would be a suitable method to distinguish different *Legionella* spp. following molecular identification from a compost sample. Methods used in the preparation and process of DGGE were adapted from Muyzer *et al.* (1993) and are described in full below.

4.2.1.2.1 Preparation of samples

DGGE samples included the eight culture-negative/PCR-positive samples from Chapter 3 (Samples 4, 6, 13, 17, 18, 19, 21, and 23). Additionally, one culture-positive/PCR-positive sample from the compost with the highest amount of *Legionella* spp. from culture (Sample 15), two compost isolates (*L. longbeachae* Sg1 and *L. pneumophila* Sg 1 OLDA from Sample 20 and Sample 8, respectively), a blank (nuclease-free water) and negative control (*Pseudomonas aeruginosa*) were selected for use.

DNA was extracted from cultures of the positive *L. longbeachae* Sg1 and *L. pneumophila* Sg1 OLDA using methods and the selected compost samples from Chapter 3. Extracted DNA was stored at -80°C until needed. For *P. aeruginosa*, 1ml aliquot was taken from a culture in LB broth, once it had reached stationary phase, for DNA extraction. The sample was placed into a 1.5ml tube and centrifuged at 10000g for 5mins; the supernatant was discarded, and the pellet was re-suspended in 1ml sterile PBS. This wash was repeated twice. Then, cells were thermally lysed at 80°C for 10mins to extract the DNA. The sample was left to cool before a 100µl aliquot was diluted 1 in 10 with nuclease-free water. This 10^{-1} dilution was used as the negative control DNA template in further PCR reactions.

A PCR using JP primers was run for each of the eight test samples and positive and negative controls, using protocols and reagents described in Chapter 3. After the PCR, contaminants such as primers, additional nucleotides, polymerase enzymes and salts were removed from the PCR products using

the Qiagen QIAquick PCR purification kit. Next, the clean DNA samples were used as the template DNA in a PCR using JP primers, with a G-C clamp for DGGE added to the 5’ end of the forward primer (JFP).

G-C clamp for DGGE 5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGC-3'

The clamp used, shown above, was first described by Muyzer *et al.* (1993) and is used to prevent the DNA fragments from fully melting and allow all samples to be visualised clearly on the gel. The PCR involved protocols and reagents described in Chapter 3, and products were stored at -20°C.

4.2.1.2.2 Preparation of gel

DGGE involves a gel with a denaturant gradient that changes vertically, from a low concentration (30%) at the top to a high concentration (60%) at the bottom. The denaturants in the gel are urea and formamide; they cause a partial dissociation of the A+T and C+G bonds between double strands of DNA, and eventually prevents the PCR products from migrating through the gel. C+G bonds require a higher amount of denaturant before they dissociate, which is why a GC clamp is used in sample preparation (Myers *et al.* 1987).

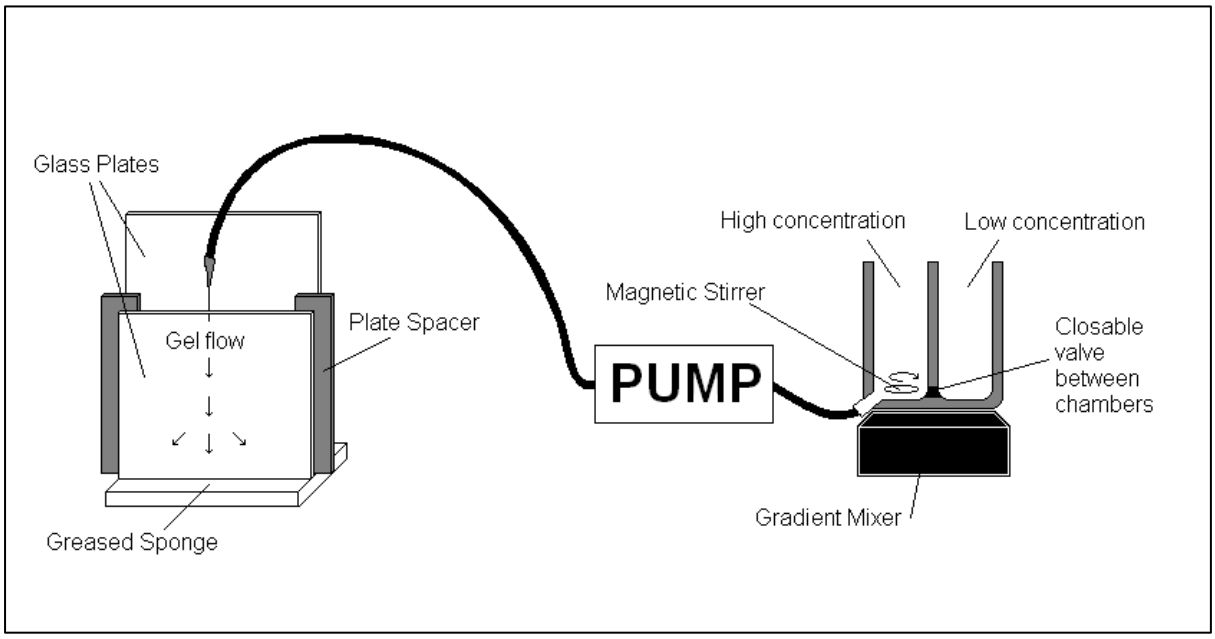


Figure 4.1: A simplified diagram of DGGE gel formation equipment

The gel was prepared using a specially designed apparatus from BioRad, for use with the BioRad D-Code Universal Mutation Detection system. A simplified diagram of the system can be seen in Figure 4.1. Two glass plates were clamped to a stand (not shown), with spacers at the sides to provide a uniform gel width, and a greased sponge at the bottom to prevent leakage of gel from the setup. A needle attached to a gradient forming chamber was placed in between the two glass plates.

Two gel solutions of low and high concentration were prepared in separate glass beakers before being added to separate chambers of the gradient mixer. One contained a magnetic stirrer to allow mixing of the two gels in a gradient before setting. Two solutions with 30% and 60% denaturant strengths were made to prepare the gels; 20 ml of the 30%-denaturant solution was added to one glass beaker, and 20ml of the 60%-denaturant solution was added to another. Next, 266µl of 10% ammonium persulphate solution (APS) were added to each beaker, followed by 26µl of tetramethylethylenediamine (TEMED); both were swirled to mix. Before pumping the solutions between the plates, the valve between the two chambers was opened. The addition of TEMED catalyses gel polymerisation; therefore, the steps above were performed in quick succession to prevent the solidified gel from clogging the gradient mixer or connecting tubes. After the pumping was finished, a comb was added to the top of the newly formed gel to create sample wells. The gel polymerised for two hours at room temperature and then stored overnight at 4°C.

4.2.1.2.3 DGGE procedure

The gel was allowed to reach room temperature, and the greased sponge and comb were removed. The wells created by the comb were cleaned of gel residue by repeatedly pipetting warm 1xTAE buffer into and out of the openings. The gel was then added to the BioRad DCode gel core and placed into the system electrophoresis tank filled with 1xTAE buffer at 65°C. The temperature of the electrophoresis tank was reduced to 60°C, and PCR products (from Section 4.2.1.2.1) were added to the wells. The DGGE was run at 200V for 4h 30m before being removed from the tank and the gel core. Next, the spacers and clamps were removed, and the gel was added to a staining tray

containing 200ml 1xTAE buffer and 20µl ethidium bromide. The tray was covered, protected from light, and placed on a shaker at 150rpm. After 30mins, the gel was removed from the staining tray and examined using a UV transilluminator before being photographed.

4.2.1.2.4 Sequencing of DGGE gel

Numerous bands were identified in the DGGE gel, either representative of different *Legionella* spp. or different species of bacteria being amplified by the JP primers. First, DNA were extracted using methods adapted from Ferris et al. (1996) to determine the nature of the bands. Next, representative bands were excised from the post-DGGE gel using a fresh, sterile scalpel for each band taken. The gel slices were added to a sterile microcentrifuge tube and stored at -80°C overnight. Each gel slab was crushed the following day using a sterile pipette tip, and 150µl nuclease-free water was added to each tube. The tubes were left to incubate at 4°C for a further 24 hours. A 3µl aliquot of the resulting mixture was used as a template in a further PCR reaction using the JP primers and the Jonas protocol described previously (Table 3.1). The resulting PCR products were treated as described in Chapter 3. Briefly, they were run on a standard agarose gel electrophoresis. Positive samples were purified using PEG precipitation before being quantified using the Epoch Microplate Spectrophotometer (BioTek)and sent away for sequencing using the LightRun system at GATC Biotech, Konstanz, Germany.

4.2.2 Preparation of 16S-rRNA Legionella *standard curve for qPCR*

Testing in Section 4.2.1.1 and Section 4.2.1.2.4 above established that the JP primers amplify a *Legionella*-specific sequence of the 16S-rRNA gene via PCR. These primers have already been used in quantitative PCR, identifying *Legionella* spp. in water samples (Wellinghausen *et al.* 2001), but they have not been used with compost samples. To use these primers to quantify *Legionella* spp. DNA in compost samples using qPCR, it was first necessary to form a standard curve. Standards are created using dilutions of a template of a known concentration that can be used as a reference to determine the quantity of target DNA in an unknown sample (BioRad, 2006). DNA from a culture of *L.*

pneumophila Sg1 OLDA (Sample 8 from Chapter 3) was extracted using the methods described in Section 3.2.3. The cell lysate was diluted to 10^{-1} in nuclease-free water and analysed via PCR with JP primers and methods described in Section 3.2.3 before being cleaned using the Qiagen QIAquick PCR Purification Kit. Purified DNA was then quantified in duplicate using the Epoch Microplate Spectrophotometer (BioTek). Mean values for double-stranded DNA (ng/µl) and the amplicon length of 386bp, as described by Jonas *et al.* 1995, were used to determine DNA copy number (http://cels.uri.edu/gsc/resources/cndna.html). The sample was diluted using nuclease-free water to create a dilution series from 10^9 to 10^1 copies of amplicons; this becomes a standard curve for qPCR reactions.

Sso-Advanced SYBR Green Mastermix was used for qPCR. Times and temperatures from the protocol described by Wellinghausen *et al.* (2001) were modified according to manufacturers guidelines resulting in a protocol using one cycle of 98°C for 5min, followed by 40 cycles at 98°C for 15s, 55°C for 15s and 60°C for 30s. Fluorescence was measured after each cycle. In addition, a melt curve was generated at 55-95°C at ΔT = 10°C per minute, which shows the melting point of the PCR products and can, therefore, identify whether more than one target has been amplified, e.g. primer-dimers (BioRad, 2006).

4.2.3 Development of DNA extraction protocol

In Chapter 3 (Section 3.2.4), DNA was extracted from compost samples using the MoBio PowerLyser PowerSoil DNA extraction kit according to the manufacturer's instructions, which required a 0.25g sample of soil for analysis. While samples were mixed thoroughly, it was unlikely to achieve complete homogeneity. Therefore, extracting DNA from a larger sample would more likely capture potential *Legionella* spp. 'hot-spots' and better represent the micro-flora in the compost. To achieve this, *L. longbeachae* Sg1, isolated from compost in Chapter 3 (Sample 20), and *Acanthamoeba castellanii* were used to spike 10g compost samples. In addition, the compost brand that had the

highest level of *Legionella* spp. growth by culture in Chapter 3 (Sample 15) was used, as this brand could support the survival of *Legionella* spp.

L. longbeachae Sg1 were grown on BCYE-α agar at 37°C as described in Section 3.2.2. Using a McFarland standard number 2 (ProLab diagnostics) as a visual representation of $6x10^8$ cfu/ml, colonies of *L. longbeachae* Sg1 were added to 1ml sterile dH_2O. A serial dilution of the *Legionella* solution was created in sterile dH_2O and plated on BCYE-α agar and incubated at 37°C as described above to confirm the McFarland standard. The solution was then diluted in sterile dH_2O and added to each compost sample at a concentration representing $5x10^3$ CFU/g compost. This concentration corresponds to the levels of $4x10^3$ CFU/g to $4x10^4$ CFU/g detected by culture in Chapter 3. *Acanthamoeba castellanii* (CCAP 1501/1A) was grown at room temperature in a tissue flask to a concentration of 10^5cells/ml (determined by cytometer) in 30ml sterile Proteose-Peptone Glucose (PPG) solution. The cells attached to the flask during growth were washed three times in sterile Page's Amoeba Saline (PAS) before being dislodged from the flask and resuspended in 30ml sterile PAS. Fifty microlitres of this solution were added to each 10g compost to represent $5x10^2$ cells/g compost sample, giving a Multiplicity of Infection (MOI) ratio of 10:1 *Legionella:Acanthamoeba* cells. Seven 10g-compost samples were spiked as described above and frozen at -80°C for one week.

Seven methods were tested, as outlined in Table 4.1. These were based on Ernebjerg and Kishony (2012), which involved the dispersion of cells with the detergent Tween 80 (0.05% concentration), with or without the use of 50mM tetrasodium pyrophosphate (TTSP), and a Stomacher Laboratory blender (Seward Ltd, UK), which Smalla *et al.* (2001) also used to remove cells from root samples.

Filter-sterilised Tween-80 solutions were used within 24hours of production, covered in foil to prevent UV degradation and stored at 4°C until needed. In this test, 10g compost samples were mixed with 40ml of 0.05% Tween 80 solution with or without TTSP in a Stomacher bag containing a large filter insert. Samples were left to settle for 5mins before being placed into the Stomacher laboratory blender at High speed for 30s and then 60s on ice; this was repeated twice. The digestate

was either centrifuged or filtered (sterile 0.2µm nitrocellulose filter paper) to separate the dispersed bacterial cells. Centrifugation involved an initial slow step to settle large particles present in the post-stomacher solution, followed by a longer, faster step to pellet bacterial cells from the supernatant. Afterwards, the MoBio PowerLyser PowerSoil kit, according to the manufacturer's instructions; the final DNA was stored at -80°C until needed. Methods can be seen in detail in Table 4.1.

After DNA extraction, a neat, 10^{-1} and 10^{-2} dilution of sample DNA from each extraction method were prepared. Different dilutions were used for the possibility of humic acids remaining in the sample, which could inhibit the reaction. Samples were used in qPCR, with methods and reagents as described in Section 3.2.3. The extraction method with DNA extract resulting in the highest level of fluorescence during qPCR was further tested to determine the most suitable dilution to use as a template in further qPCR experiments. Along with 10µl nuclease-free water samples, 10µl dilutions of DNA from the chosen extraction method from neat to 10^{-1} were UV irradiated (disinfected) in the PCR-preparation cabinet, which dimerises DNA to render it un-replicable. This eliminates the possibility of detecting and quantifying any amount of genes previously in the sample. After UV irradiation, 2µl of a 10^5 stock of *L. longbeachae* DNA was added to each irradiated sample and mixed. This shows which DNA dilution had the least inhibition, and therefore, the dilution of DNA extracts to be used in PCR reactions. These mixes were compared with samples that had not been irradiated in a qPCR reaction using methods and reagents as described in Section 3.2.3.

Table 4.1: Methods used in the extraction of DNA from compost samples.

Sample Number	Tween 80	TTSP	0.2µm vacuum filter	Blender	Kit	Centrifuge	Method
1	☑	X	X	☑	☑	1000g for 5 mins, 10mins at 10000g	Filtrate placed into a centrifuge tube and centrifuged at 1000g for 5 mins. Supernatant placed in a clean centrifuge tube and centrifuged for 10mins at 10000g. c) Pellet used in MoBio kit
2	☑	X	X	☑	☑	5000g for 5 mins, 10mins at 10000g	Filtrate placed into a centrifuge tube and centrifuged at 5000g for 5 mins. Supernatant placed in a clean centrifuge tube and centrifuged for 10mins at 10000g. c) Pellet used in MoBio kit
3	☑	X	☑	X	☑	X	Filtrate placed vacuum filter apparatus and filtered using a 0.2µm nitrocellulose filter paper b) Filter paper folded and added to MoBio kit.
4	☑	☑	X	☑	☑	1000g for 5 mins, 10mins at 10000g	Filtrate placed into a centrifuge tube and centrifuged at 1000g for 5 mins. Supernatant placed in a clean centrifuge tube and centrifuged for 10mins at 10000g. c) Pellet used in MoBio kit
5	☑	☑	X	☑	☑	5000g for 5 mins, 10mins at 10000g	Filtrate placed into a centrifuge tube and centrifuged at 5000g for 5 mins. Supernatant placed in a clean centrifuge tube and centrifuged for 10mins at 10000g. c) Pellet used in MoBio kit
6	☑	☑	☑	X	☑	X	Filtrate placed vacuum filter apparatus and filtered using a 0.2µm nitrocellulose filter paper b) Filter paper folded and added to MoBio kit.
7	X	X	X	X	☑	X	a) This sample was not blended. 0.25g taken from 10g sample and used directly in the kit.

4.3. Results

4.3.1 Confirmation of primer specificity

4.3.1.1 In Silco analysis

The results from the RDP ProbeMatch tool can be seen in Table 4.2. This was performed in 2013 and results may differ if repeated now due to changes to the database over time. The results indicate that the JP forward primer was highly specific, but the reverse primer less so. The results from the nucleotideBLAST search showed a similar pattern, with a 100% match to *L.* for the forward primer, but a match to various bacteria, without a specific match to one species or genus, when the reverse primer was searched against the BLAST database. It is important to note that when primers were combined, the RDP ProbeMatch tool gave 165 positive hits out of 373333 bacterial sequences searched. When the primers were used together, the hits were 100% *Legionella* genus, highlighting that these primers had good specificity to *Legionella* spp.

Table 4.2: ProbeMatch results for JP primers

	Forward Primer	**Reverse Primer**	**Both Primers**
Number of Positive Database Hits	298/373333 bacterial sequences searched (0.08%)	5448/373333 bacterial sequences searched (1.46%)	165/373333 bacterial sequences searched (0.04%)
Percentage Match of positive database hits for *Legionella* spp.	100%	10.4%	100%

4.3.1.2 Denaturing Gradient Gel Electrophoresis (DGGE)

The results from the DGGE (Table 4.2) show a similar banding pattern in all columns except the blank and the negative control (Lanes 1 and 3). This indicates that bands at the bottom of the gel, except

lane 2, were caused by PCR remnants, such as primers and dimers. Lanes 2 and 8 represent the positive controls *L. pneumophila* Sg 1 OLDA and *L. longbeachae* Sg 1, respectively: both had a single clear, strong band, along with other less-clear bands. The DNA used for these samples was extracted from a single colony for each species; therefore, multiple banding was not due to the use of a mixed species template. The solid bands for these two controls were not equal in the DGGE gel, indicating slight differences in the DNA sequence may have been present between samples, thus causing differences in migration of these amplicons.

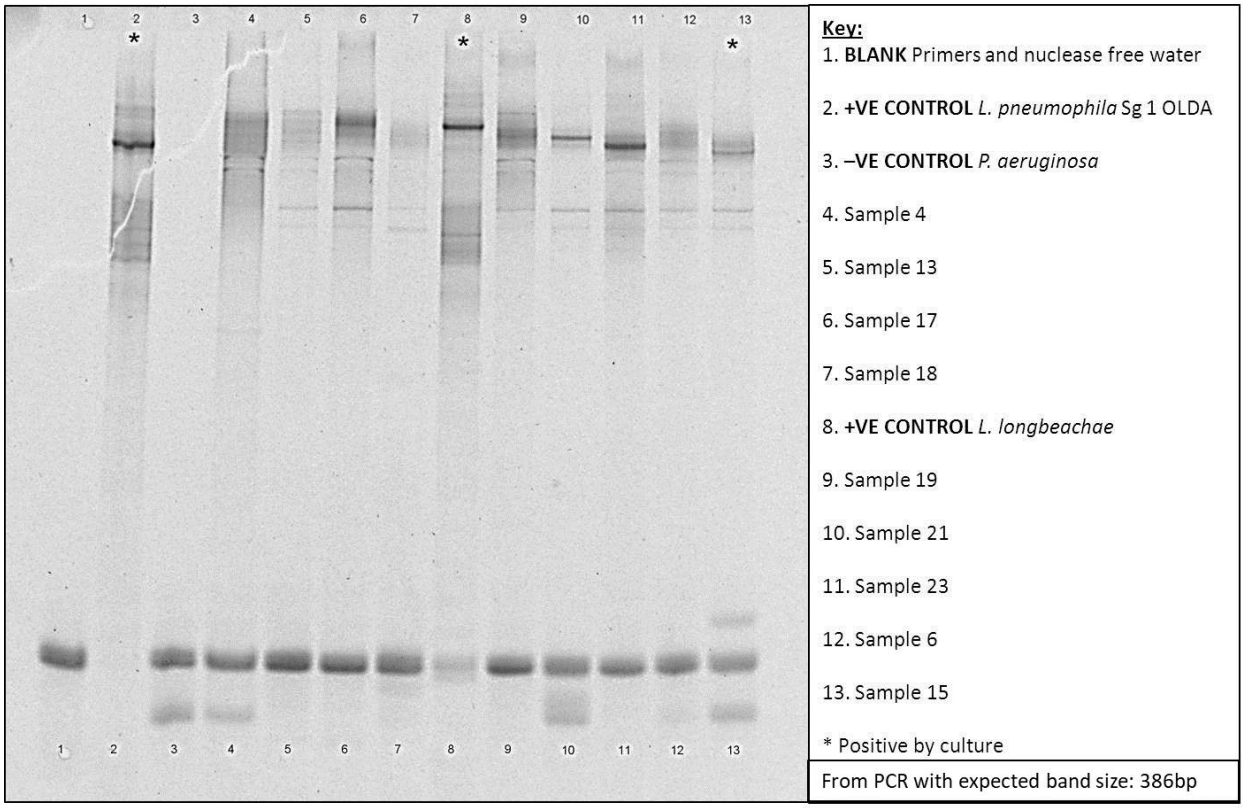


Figure 4.2: DGGE gel with key

4.3.1.3 Sequencing of DGGE gel

After excision from the DGGE gel, the extracted DNA was run in a PCR reaction using JP primers without a GC clamp. As shown in Figure 4.3, all products were positive, except the reactions with blank PCR template (Lane 2), the blank gel template (Lane 4) and the *P. aeruginosa* gel extract (Lane

6). These results indicate that the lowest bands in each lane of Figure 4.2 do not represent a PCR positive sample and should not be considered during the analysis of this gel.

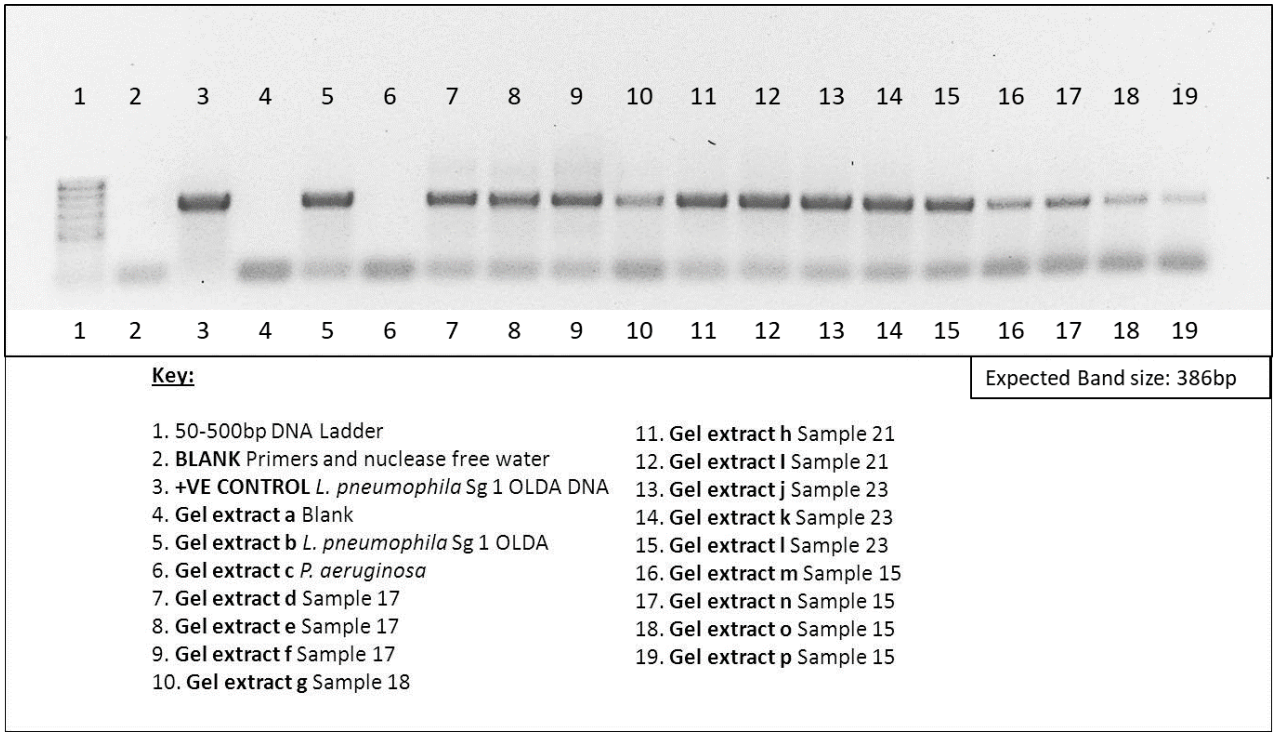


Figure 4.3: Agarose gel of PCR products from PCR with JP primers of DGGE excised bands.

The products from PCR with JP primers or the excised gel DNA extracts that were positive by agarose gel electrophoresis were cleaned using PEG precipitation and quantified using the BioTek micro-spectrophotometer as 10 -50 ng/µl was needed for the sequencing run. All samples were successfully sequenced using the LightRun service at GATC Biotech; amplicon length can be seen in Table 4.3. All sequences showed a good match to *Legionella* spp. using both nucleotideBLAST and the SeqMatch search (at http://rdp.cme.msu.edu), indicating that these primers specifically target *Legionella* spp. in compost.

Table 4.3: Sequencing results from excised DGGE bands (NT represents sample not tested)

Excised band	Length	Matches at *L.* genus level (Seqmatch)	Lane in DGGE	Sample in Chapter 3
A	NT	NT	1	Blank
B	318	100%	2	Positive control
C	NT	NT	3	Negative control
D	348	100%	6	17
E	287	100%	6	17
F	318	100%	6	17
G	266	100%	7	18
H	347	100%	10	21
I	301	100%	10	21
J	344	100%	11	23
K	342	100%	11	23
L	319	100%	11	23
M	338	100%	13	15
N	343	100%	13	15
O	315	100%	13	15
P	85	100%	13	15

4.3.2 Preparation of 16S-rRNA Legionella *standard curve for qPCR*

Based on the results above, it was decided to use the JP primers to develop a method for qPCR of DNA from compost samples.

Table 4.4: Quantification of *L. pneumophila* Sg1 OLDA for the development of a qPCR standard, based on UV micro-spectrometry.

	Sample	*L. pneumophila* Sg1 OLDA
ng/µl	**a**	13.16
	b	12.42
	mean	12.79
Amplicon length		386
Number of copies		3.07×10^{10}

(http://cels.uri.edu/gsc/resources/cndna.html)

DNA was quantified as seen in Table 4.4, and a qPCR was run with SsoAdvanced qPCR mix using JP primers and the adapted Wellinghausen protocol as described in Section 3.2.3.

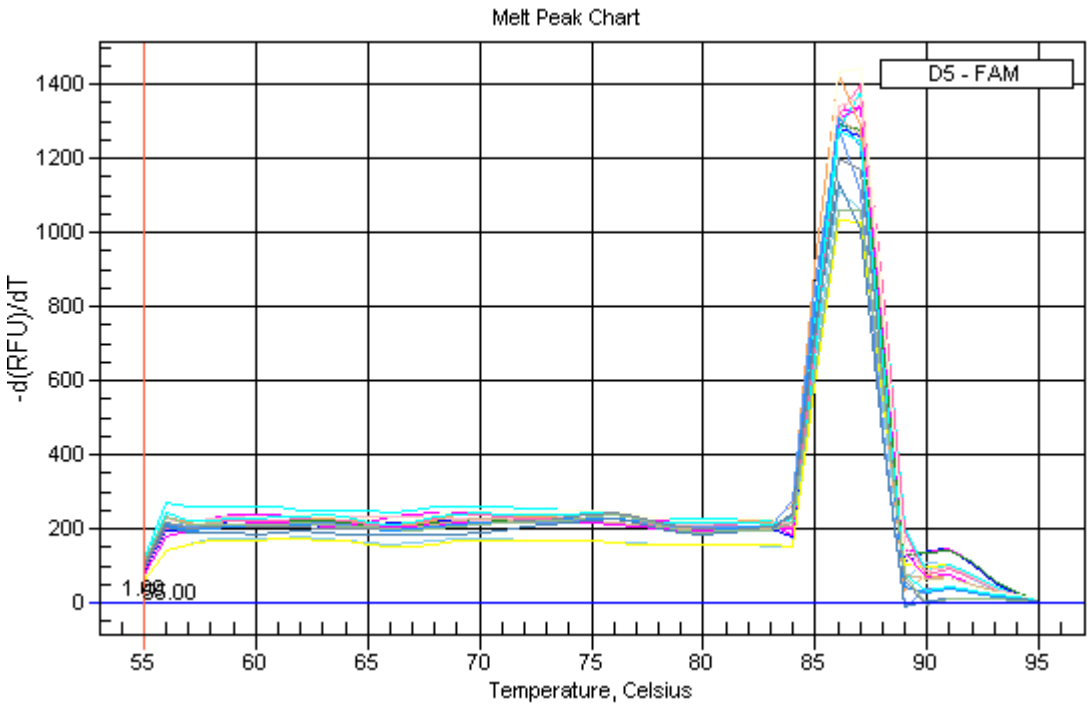


Figure 4.4 Melt Peak Graph for *L. pneumophila* Sg 1 OLDA

The melt peak derived from the *L. pneumophila* Sg1 OLDA template DNA (Figure 4.4) showed that primer-dimers did not occur. The melt curve reflects the length of the PCR product and its GC content, so a sharp, centralised peak represents amplicons of a similar size and composition. The standard curve created from quantified *L. pneumophila* Sg 1 OLDA DNA (Figure 4.6) shows that the standard created accurately represents a starting concentration of 10^3 DNA template copies and can be extrapolated to represent lower quantities. The results from the standard curve confirm the suitability of these primers for use with the adapted Wellinghausen protocol for qPCR of samples containing *Legionella* spp. The standard curve slope is used to determine the amplification efficiency of the PCR assay and should fall within an optimal range of 90-110%. For the qPCR assay based on the *L. pneumophila* Sg1 OLDA standard curve, the efficiency was 97.08%, confirming these primers' suitability for use.

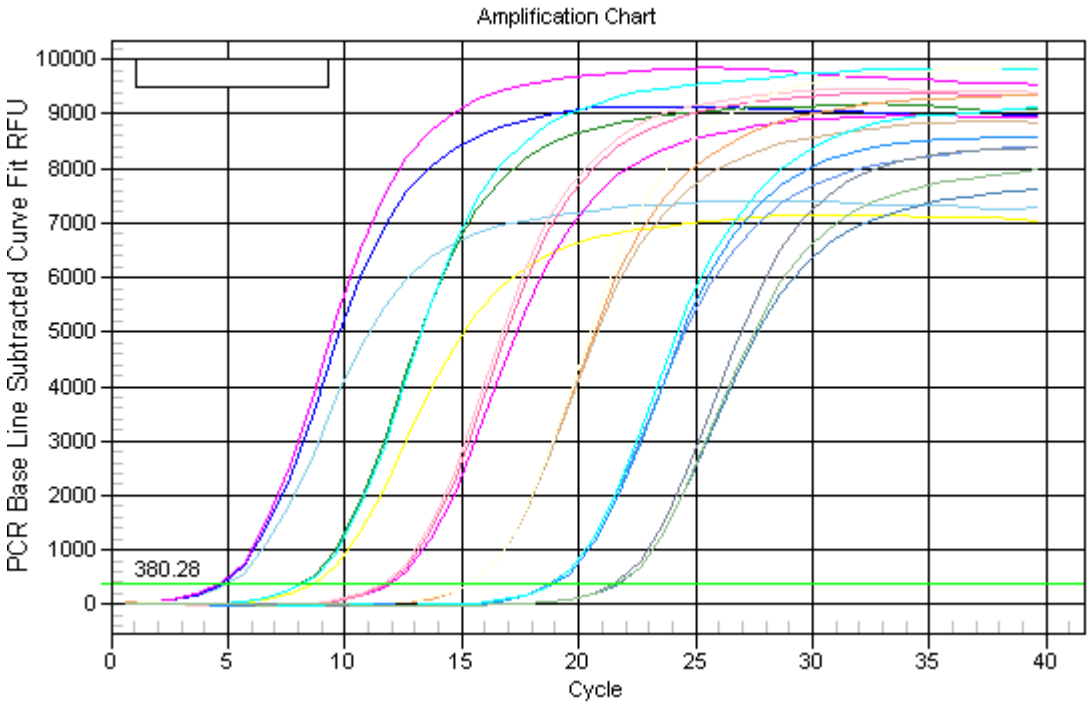


Figure 4.5 Amplification Graph for *L. pneumophila* Sg 1 OLDA

The amplification chart shows parallel lines in triplicate approximately three cycles apart, representing a dilution factor of 10. The lines are a typical shape of a PCR curve, including the exponential phase when the reaction is uninhibited and the plateau phase, which occurs when more

or more of the components of the reaction, such as polymerase or nucleotides, are used up and become a limiting factor to the reaction. In the curved section where the exponential phase begins, the cycle threshold (C_T) value plots against starting DNA concentration to create the standard curve. The standard curve can then be used to determine the starting concentration of DNA in samples where only the C_T value is known (Figure 4.6).

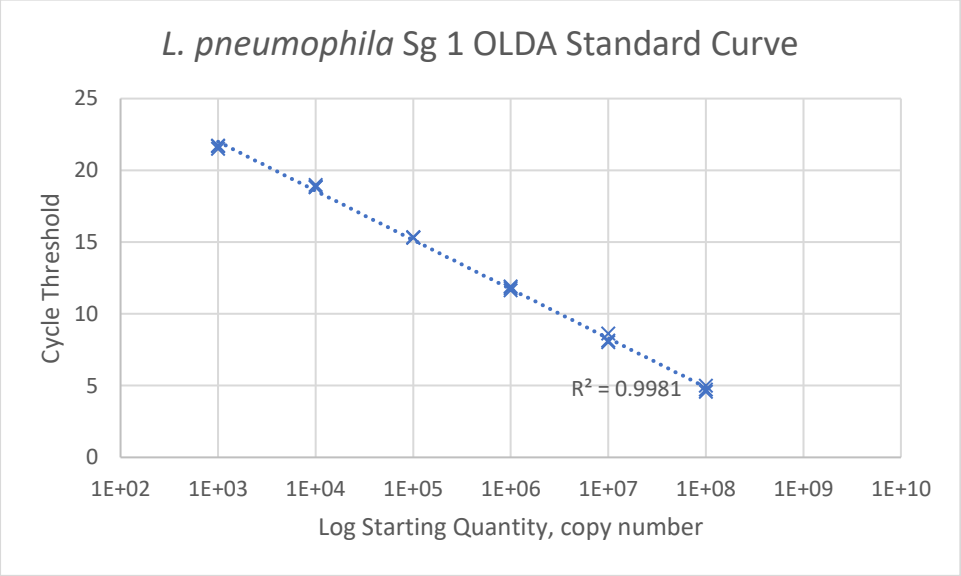


Figure 4.6 Standard Curve for *L. pneumophila* Sg 1 OLDA. Slope = -3.394. Efficiency = 97.08%.

4.3.3 Development of DNA extraction protocol

The results of DNA extraction after qPCR varied (Figure 4.7). For methods 1, 2, 4, 5, 6 and 7, the amplicon copy number for the neat sample was lower than or similar to the amplicon copy number for the 10^{-1} sample, indicating that these samples suffered inhibition during PCR. Despite this inhibition, Sample 4 showed the highest copies at 10^{-1} and 10^{-2} dilutions. The two dilutions appeared to be different by a single magnitude, indicating that this sample had the highest level of *Legionella* spp. in the initial sample. As the sample compost type had been used for all samples, and all samples spiked in the same way with *Legionella* spp. Sample 4 was chosen as the best DNA extraction method to use for future work. Tween and TTSP were added to samples and processed in a stomacher blender (as described in Section 4.2.3), before the filtrate was placed into a centrifuge

tube and centrifuged at 1000g for 5 mins. The supernatant was then placed into a clean centrifuge tube and centrifuged for 10mins at 10000g, before the pellet was used in the MoBio kit.

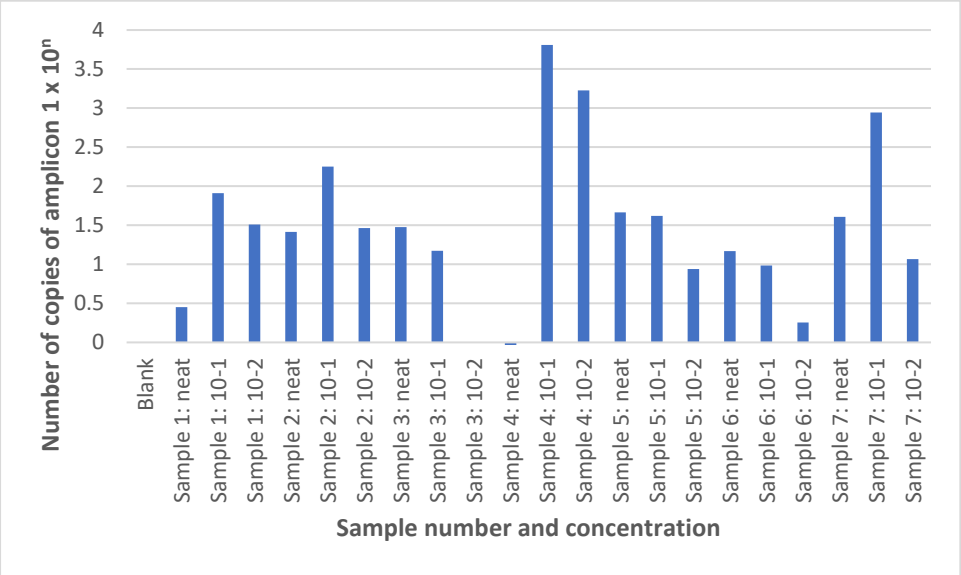


Figure 4.7: qPCR of 7 DNA extraction test samples neat, at 10^{-1} and 10^{-2}

In the inhibition test, the number of amplicon copies expected in the UV-irradiated samples was 10^{-5}, while a 1-magnitude difference in spacing was expected in the unirradiated samples (Figure 4.8) and show that inhibition occurred in neat samples, but that contaminants, specifically humic acids from compost, have not affected other dilutions. Therefore, DNA samples extracted from compost should be diluted at 10^{-1} in future work.

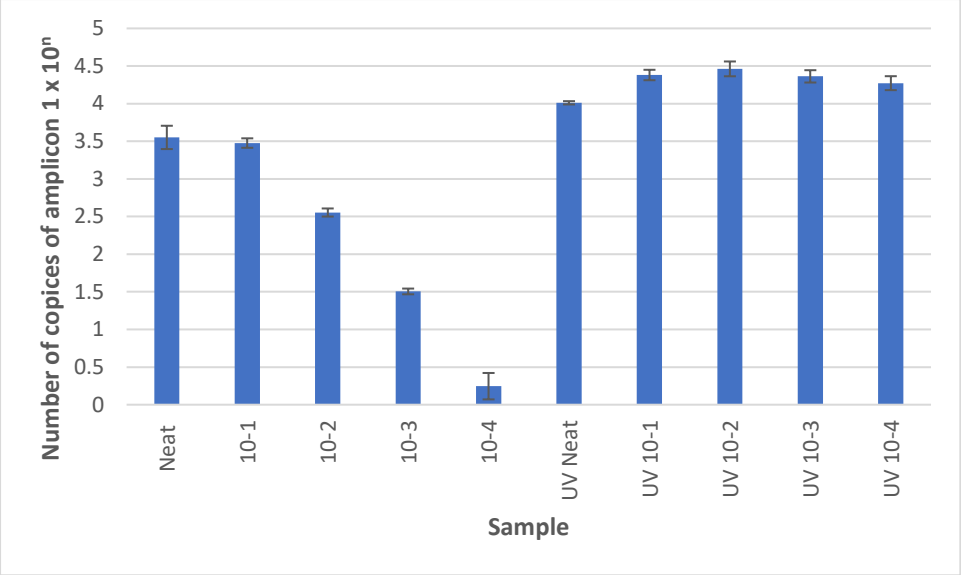


Figure 4.8: Results from UV Inhibition test

4.4. Discussion

This chapter investigated the use of JP primers to identify *Legionella* spp. when used with DNA extracted directly from compost samples. In Chapter 3, the samples tested by PCR showed 100% positivity when tested for *Legionella* spp., using the JP primers. The investigations outlined above were performed to confirm that this was a valid result. These primers have been used in numerous other studies, successfully identifying *Legionella* spp. in domestic and environmental water samples, clinical specimens and biofilm samples (Dobrowsky *et al.* 2014, Edagawa *et al.* 2008, Jonas *et al.* 1995, Sheehan *et al.* 2005), including qPCR of water samples (Wellinghausen *et al.* 2001). Since completing this work, a study in Canada used these primers to identify *Legionella* spp. directly in soil samples and found 100% (n=16) positive by PCR (Marchand *et al.* 2018).

The work in this chapter was performed with the intent to confirm primer suitability for use with complex soil and compost samples and enable the quantification of *Legionella* spp. Thus, developing the ability to examine conditions in which these organisms proliferate—conditions that may enhance public health risk to these organisms.

To confirm that the primer set was specific for *Legionella* spp. in compost samples, first, samples were run on a 1% agarose gel to examine amplicon size. Second, primers were examined *in-silico* against a database of nucleotide sequences to identify potential targets for cross-reaction. Subsequently, a denaturing gradient gel electrophoresis was performed, and bands were later sequenced to ensure that only *Legionella* spp. were positively identified in these samples. In the 1% agarose gel run post-PCR, all bands, including controls, showed the same size bands, an initial indication that the amplicons are similar and coming from the same organism (**Error! Reference source not found.**2). Furthermore, *in-silico* analysis showed a 100% match for *Legionella* spp. in the ProbeMatch tool when both primers entered the search (Table 4.2). These initial results indicated that these primers are suitable for use in mixed-species samples.

DGGE results were not as expected as each lane, including the controls, contained more than one band. Different band sizes indicate amplicons of the same size but with different sequences. Therefore, in theory, different vertical bands in a DGGE gel may represent a different bacterial species (Muyzer *et al.* 1993). The prominent bands representing *L. pneumophila* Sg 1 OLDA and *L. longbeachae* Sg1 controls were located at different distances from the loading well, indicating, as expected, that the sequence for these species is different (Figure 4.2). Therefore, different bands in the gel may represent different *Legionella* spp. within the same sample. A number of the compost samples in Chapter 3 were positive for more than one species of *Legionella* by culture. However, positive controls *L. pneumophila* Sg 1 OLDA and *L. longbeachae* Sg1 also show multiple bands, which is not expected as these were pure cultures taken from individual colonies. Another possible explanation is that base mismatches may have been introduced during PCR amplification, resulting in amplicons of the same size with differing sequences (Speksnijder *et al.* 2001, Zijnge *et al.* 2006).

The clearest DGGE bands, darkest in colour, were in Lanes 2, 8 and 11, representing the two positive controls and compost sample 23, respectively. Higher intensity bands are likely representative of a higher abundance of DNA in the sample (Muyzer *et al.* 1993). Unusually, bands seen in Lane 11 and

the control lanes were comparable in intensity when *Legionella* spp. was not cultured from Sample 23. This is especially true when examining the bands from Sample 15 (Lane 13) – the sample with the highest number of organisms isolated by culture, which does have a clear band, but this is not as intense as those seen for Lanes 2, 8 and 11. Thus, it is possible that a high number of dead *Legionella* organisms were present in Sample 23, killed during the compost manufacturing process, or viable but not culturable (VBNC) organisms present, possibly due to unfavourable storage conditions.

With the exception of blank and negative controls, all excised DGGE gel bands were positive for *Legionella* spp. after PCR with JP primers. The DNA extract from these bands was sent to GATC Biotech for sequencing, and all sequences had a band length between 266-348 bases long, except for Band p, which was 85 bases long. This amplicon had travelled farther to the bottom of the gel; it may represent an anomalous amplicon fragment, or a primer-dimer. All sequences showed a good match to *Legionella* spp. When entered into a nucleotideBLAST and SeqMatch search (Table 4.2), only DNA from *Legionella* spp. was represented in the PCR amplicons. There is limited evidence in the literature for the use of DGGE for the investigation of *Legionella* spp. However, in a study published after this chapter's work, Huang *et al.* (2017) used this method to investigate *Legionella* spp. in river water. They observed similar results to those found above, notably multiple bands observed from positive control and experimental samples, all identified as *Legionella* spp. following sequencing.

It should be noted that some Qiagen DNA extraction kits have been identified as causing contamination in PCR assays looking for *Legionella* spp. (van der Zee *et al*. 2002). Here, a Qiagen DNA clean-up kit was used, and it is not clear if this kit is affected by the same manufacturing contamination. However, the main aim for performing this method was to determine if the Jonas primers amplified any non-Legionella organisms, and this was satisfied as all sequences showed a high percentage match to *Legionella* spp.

In combination with other results discussed in this section, these results indicate the suitability of these primers for the identification of *Legionella* spp. from mixed DNA samples extracted from

compost material. The results also confirm that all of the samples in Chapter 3 were positive for *Legionella* spp. and may have been undetectable by culture due to low numbers, which would be expected due to the fastidious nature of *Legionella* spp. Cell death, likely due to the high temperatures in the composting process, may also explain the lack of culture. As noted above, positive PCR results in culture-negative soil samples were also observed in a study by Marchand *et al.* (2018) after the work for this chapter had been carried out.

After the suitability of the primers had been confirmed, it was necessary to develop a reliable DNA extraction protocol for use with compost samples larger than 0.25g, an amount considered relatively small to be representative. The qPCR results of seven protocols were directly compared, and Method 4 was chosen, as it had the highest starting concentration of DNA after a qPCR at dilutions of both 10^{-1} and 10^{-2} (Figure 4.7). Interestingly, the direct kit method produced the second-highest starting concentration of DNA (Method 7), which is surprising due to the considerably smaller sample of compost used but may be due to the likelihood of increased inhibitors in larger samples. Method 1 resulted in the third-highest concentration of DNA being extracted. This method used the same centrifuge protocol as Method 4, but Method 4 included TTSP and Tween 80 in the initial stages. The addition of TTSP enabled the removal of more cells from compost material, increasing available cells for DNA extraction. The worst performing methods were 3 and 6, which used filter paper instead of centrifugation to collect the cells before use in the kit. The presence of the filter paper likely impaired extraction, and although an additional shredding/bead-beating was used, this may not have been enough or may have damaged cells too much leading to shearing of DNA in samples. The chosen method described above involves removing cells from compost material using a laboratory blender, using methods based on those by Ernebjerg and Kishony (2012), before DNA extraction using the MoBio PowerLyser PowerSoil DNA extraction kit. Numerous DNA extraction protocols from soil have been described (Miller *et al.* 1999, Zhou *et al.* 1996); however, the kit was chosen for its speed, replicability and success at extracting DNA in Chapter 3.

While PCR can provide strong evidence of DNA present in a sample, it does not definitively prove the presence of colony-forming bacteria units. A positive result may occur due to the presence of live cells but may also be a result of VBNC, dead cells, or potentially exogenous DNA fragments or remnant DNA in the sample. It should also be noted that more than one copy of 16SrRNA may be present in an organism e.g. 7 in *E.coli* and 6 in *Salmonella typhimurium* (Yamamoto et al, 1993) and the exact copy number of the 16S rRNA gene in *Legionella* is not known (Wellinghausen et al, 2001). As observed in Chapter 3, it is likely that a variety of *Legionella* spp are present in the composts being investigated and the 16SrRNA copy number may vary species to species. The likely variation in the copy number is a further reason why this method should only be considered as semi-quantitative. Furthermore, while the purpose of this chapter is to confirm the specificity of these primers for use with DNA extracted from compost, there is potential for cross-reaction in any PCR reaction, especially in complex environmental samples. This should also be taken into account. Regardless of this fact, the methods described in this chapter have been robustly tested and are suitable for future use in semi-quantitative PCR experiments using DNA extracted from compost samples, both in Chapter 5 and additional work.

5. THE EFFECT OF STORAGE CONDITIONS ON *LEGIONELLA* SPP. IN COMPOST

5.1. Introduction

Cases of Legionnaires' disease where *L. longbeachae* is the aetiological agent have been widely linked to gardening activities (Lindsay *et al.* 2012, den Boer *et al.* 2002, Cramp *et al.* 2010). Cases reported range in severity from an outbreak of Pontiac Fever (Cramp *et al*. 2010) to Legionnaires' disease requiring ICU treatment (Potts *et al.* 2013). The link between gardening and *L. longbeachae* was first made by Steele *et al*. (1990a), who isolated *L. longbeachae* from potting soils after a statewide outbreak in South Australia between 1988-1989. Since then, *L. longbeachae* has been isolated from compost and potting mixes in Japan, Switzerland, Greece, Scotland, and the USA (Koide *et al*. 2001, Casati *et al.* 2009, Velonakis *et al.* 2010, Lindsay *et al.* 2012, Duchin *et al.* 2000), but a description of isolation from water in the literature is limited (Lau *et al*. 2013, Thornley *et al*. 2017).

Work by Potts *et al*. (2013) examining a cluster of six *L. longbeachae* infections in Scotland did not identify a common product or manufacturer; however, they did isolate the organism in growing media from 5/6 of the cases. In addition, it was noted that all of the growing media had been stored inside the house, greenhouse, car, polytunnel, shed or garage of the infected individual. This, combined with the higher-than-average temperatures seen in Scotland when this cluster occurred, led the authors to suggest that climatic conditions and storage of the growing media may have enabled high levels of growth, leading to an increased risk of human infection.

A case of *L. longbeachae* infection has been reported in a greenhouse repairman; however, the organism was not isolated from the patient or environmental samples (Eitrem *et al*., 1987). Several other studies have examined the greenhouse environment for *Legionella* spp. Using a mixed latex agglutination test consisting of *L. longbeachae* Sg 1 and 2, *L. bozemanii* Sg 1 and 2, *L. dumoffii, L.*

gormanii, L. jordanis, L. micdadei and *L. anisa,* Stojek and Dutkiewicz (2002) did not find these organisms in 18 tap water samples, 14 soil samples, 14 growing media samples or six samples of air collected in modern greenhouses at gardening farms. The authors found the organisms in 3/36 samples from outdoor taps used for watering plants in outdoor gardens and 2/20 water samples from indoor taps used in greenhouses or foil tunnels. Zietz *et al*. (2006) isolated *L. pneumophila* Sg 6 from 10% of greenhouse water misting systems tested (2/20 systems) and experimental and commercial slow sand filters, such as those used in horticultural irrigation systems, were found to support *Legionella* spp. (Calvo-Bado *et al*. 2003). The majority of research linking *Legionella* spp. and greenhouses relates to the water supply. Work examining the role of greenhouse storage on the survival or proliferation of *Legionella* spp. in compost is limited (Lindsay *et al.* 2012, Schwake *et al.* 2014).

In Chapter 3, the potential for greenhouse storage to increase levels of legionellae in compost was noted, based on observations of amoebal enrichment and a preliminary study by Lindsay *et al*. (2012). The optimal growth temperature for Legionellae is 35°C (Fields *et al*. 2002 in Zietz 2006) and standard agar culture conditions utilise a humid chamber. A limited increase of legionellae was seen in some, but not all, amoebal enrichment studies in both Chapter 3 and work completed by Koide *et al*. (2001), which may suggest that these organisms may proliferate more readily in warm, humid conditions provided in a greenhouse. Therefore, the work in this Chapter aims to investigate the effect of greenhouse storage on levels of *Legionella longbeachae* in compost, using the semi-quantitative PCR method developed in Chapter 4.

5.2. Methods

Three distinct experiments were carried out to determine the effect of greenhouse storage on *Legionella* spp. in compost; these were spiked greenhouse (SGH), unspiked greenhouse (NSGH) and incubator (INC), and each was used to represent different possible greenhouse conditions. These experiments are summarised in Table 5.1 and described in full below. The work in this chapter was

performed sequentially, with observations and results from the previous experiment providing information which was then used to adapt the methodology for the next experiment. To compare the different experimental setups used in SGH, NSGH and INC, graphs displaying the total bacterial population of samples, *Legionella* spp. specific population in samples, *Legionella* spp. as a percentage of the bacterial population, and percentage change between Day 0 and Day 14 samples have been created. All results are examined collectively in the discussion.

Table 5.1: A summary of Greenhouse style experiments performed

Experiment Name	**Experiment Setup**	**Experiment duration**
Spiked Greenhouse (SGH)	Compost batches (same brand) were placed in individual bags, spiked with *L. pneumophila* and/or *L. longbeachae* and/or *Acanthamoeba castellanii,* and stored at both 15°C and under propagator greenhouse conditions. In addition, 10g samples were removed from each bag at Day 0, 3, 7, 14, 21, 28, 42 and stored at -80°C before testing.	42 days
Unspiked Greenhouse (NSGH)	Batches of four different compost brands were stored in pots at both 15°C and under humid propagator greenhouse conditions without the addition of *Legionella* spp. or *A. castellanii.* Batches were watered to represent gardening activities. 5g samples were removed at Day 0 and Day 14 and stored at -80°C before testing.	14 days
Incubator (INC)	Compost batches (same brand) were spiked with *Legionella* spp. and/or *A. castellanii,* as per SGH, and placed in perforated bags in sealed, humid glass jars and stored in an incubator at 15°C, 30°C and 37°C. 10g Samples removed at Day 0 and Day 14 and stored at -80°C before testing.	14 days

The standard compost used in all experiments was Compost 15 from the compost isolation experiments in Chapter 3 and was used to develop the DNA extraction methods (Section 4.2.3.). It also contained the most *Legionella* spp. of those growing media tested in Chapter 3; it is therefore known to support the survival of these organisms. Three additional composts were used in the NSGH trial to compare the standard compost type. *Legionella* strains used to spike compost samples were *L. pneumophila* Sg 1 OLDA and *L. longbeachae* Sg1, both isolated from compost in Chapter 3. In addition, *Acanthamoeba castellanii* (CCAP 1501/1A) were maintained in axenic culture at room temperature using proteose peptone glucose (PPG) as described in Section 3.2.2. Compost samples were spiked using the method outlined in Section 4.2.3.

It was not possible to set up experiments in a traditional greenhouse due to safety constraints in spiking samples with *Legionella* spp., which are biohazard Category II pathogens (ACDP 2021). Therefore, a heated propagator and a standard laboratory incubator were used to represent a greenhouse environment in the experiments described in this chapter. The Royal Horticultural Society recommends ventilation in greenhouses over 25-27°C (https://www.rhs.org.uk/advice/profile?pid=732). When the temperature in a domestic greenhouse was measured throughout June – August 2012, the highest temperature observed with full ventilation was 27°C, while the highest temperature measured with limited ventilation was 37°C (personal communication, Mrs Elizabeth Currie). Commonly available fully enclosed electric propagators only reached a maximum of 23°C, too low a temperature to represent a greenhouse during summertime. The chosen propagator consisted of a small flexible plastic polytunnel containing a heat mat controlled by an adjustable thermostat, regulated by a probe placed into a compost control sample. An example of the setup is shown in Figure 5.1. This setup ensured that the heat mat responded to a drop in the internal compost temperature, not just the ambient temperature of the propagator. As the propagator was not fully enclosed, a polystyrene mat was also added below the heat mat as an insulator to reduce heat loss into the laboratory environment. A temperature and humidity monitor was also placed into the mock greenhouse.

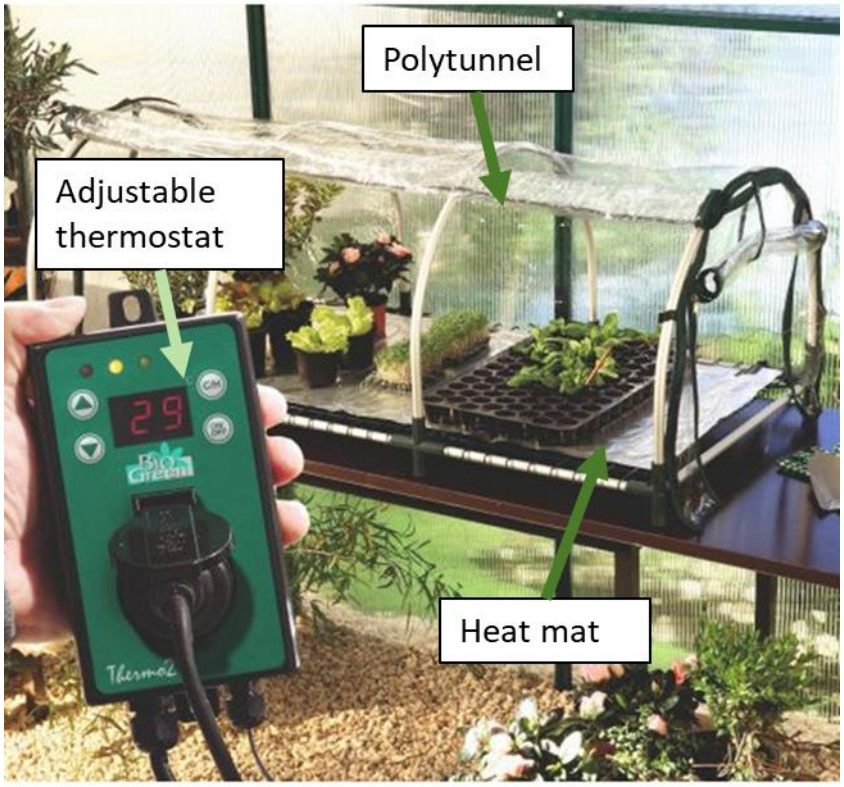


Figure 5.1: Example of the greenhouse set up. Image adapted from https://www.thepolytunnelcompany.ie/product/seed-propagator-with-heating-mat-thermostat

Both the propagator and the incubator were set up using an electric timer to create temperature cycling to represent natural heating and cooling of a greenhouse for 24 hours in high summer in Scotland (http://www.scotlandinfo.eu/daylight-hours-sunrise-and-sunset-times/). This was 18h on/6h off for SGH, representing daylight hours at midsummer; however, the cycling times were reassessed due to evaporation during SGH. The timer was set to 16h on/8h off for NSGH and INC based on average sunlight hours in August which is when a cluster of *L. longbeachae* infections related to gardening occurred in Scotland in 2013 (Potts *et al.* 2013).

5.2.1 DNA extraction and PCR

When all samples had been obtained, they were processed using the DNA extraction method described in Section 4.2.3. All NSGH samples were 5g rather than 10g, and so the method was

adjusted proportionally to use 20ml of Tween and TTSP for these samples. This is the only adjustment made from the DNA extraction method developed in the previous chapter.

The primers used in this Chapter were:

a) JP primers which were used in Chapter 3 and 4, and are used to amplify a *Legionella* specific region of the 16SrRNA gene, thus identifying *Legionella* spp present in the samples.
b) Muyzer primers which are used to amplify a variable V3 region of the 16SrRNA gene, and used in this study as a marker of the general bacterial population present in the samples. These primers were used for the first time in this chapter and additional information on their use is outlined below.

A summary of these primers can also be seen in Table 3.1.

The DNA extract was diluted to 10^{-1}, and 3µl was used in a PCR reaction with JP primers as described in Chapter 4 and Muyzer primers, as described below.

Semi-quantitative PCR was also performed on samples using primers that amplify a variable region of the 16S rRNA gene (Muyzer et al. 1993) to estimate the whole bacterial population of each compost sample. As described in Chapter 4, it is necessary to develop a standard curve as a reference point against which the quantity of target DNA in the unknown sample can be compared. Therefore, DNA was prepared, purified and quantified from cultures of *Legionella longbeachae* Sg1 and *Legionella pneumophila* Sg1 OLDA, again, as described in Chapter 4, using Muyzer primers (Muyzer *et al.* 1993) in place of JP primers. An amplicon length of 193bp as described by Muyzer *et al.* (1993) was used for quantification, and PCR protocol was adapted from the same study for use with Sso Advanced SYBR Green Mastermix: 1 x 98°C for 3 mins, 40 x 94°C for 15s, 60°C for 15s and 55°C for 15s. Fluorescence was measured at the end of each cycle, and a melt curve was carried out between 55-95°C, with temperature increasing by 1°C after each six-second cycle.

Each PCR reaction for SGH and INC was performed in duplicate. Each PCR reaction was performed in triplicate for NSGH, with the mean and standard deviation for each data set represented graphically in the results for the following experiments. In addition, the amount of *Legionella* as a proportion of the total bacterial population was estimated using the results from the JP (*Legionella*) and the Muyzer (16S) assays. These results are also displayed graphically below. Unfortunately, sample sizes were too small to undertake meaningful statistical analyses.

5.2.2 Spiked greenhouse (SGH)

A six-week experiment was set up using unsterilised compost spiked with *Legionella* spp. to represent long-term compost storage in a greenhouse setting. The main aim of this experiment was to determine if the storage of compost in greenhouse conditions results in increased *Legionella* spp. numbers. Seven batches of compost (250g) from the same bag were spiked with sterile dH_2O, *L. pneumophila* or *L. longbeachae* and/or *A. castellanii* in the same manner and concentrations as described in Section 4.2.3. A summary of each batch can be seen in Table 5.2. Solutions containing *L. pneumophila* or *L. longbeachae* and/or *A. castellanii* were made up to the same volume (2.5ml) with sterile dH_2O before compost batches were spiked to prevent the introduction of variation in moisture content through the addition of different volumes of liquid.

The seven batches were stored at 15°C, and seven batches were prepared in the same way for greenhouse storage. Due to the safety aspect of incubating compost spiked with *Legionella* spp. at increased temperatures, this work was carried out in a CAS BioMAT 2 Class II microbiological safety cabinet. Batches in the biosafety cabinet were stored in a clear sealed plastic bag, surrounded by a black plastic bag to reduce UV impact. The bags were placed directly on the heat mat of the propagator greenhouse and turned every 2-3 days to allow even heat distribution throughout the sample. On days 0, 3, 7, 14, 21, 28, and 42, each batch was mixed by manipulating the bag for 2 minutes. Samples weighing 15g±0.5g were removed from each compost batch and frozen at -80°C until needed. Two control batches were set up as described in Table 5.2: On each sampling day,

samples were taken from Batch 1 and moisture content measured using British Standard BS EN 13040:2007, while Batch 2 had samples removed for molecular analysis (further described in Section 5.2.1).

Table 5.2: Summary of batches included in Spiked Greenhouse Experiment. All added organisms were made up in solution, with a total volume of 2.5ml added to each batch.

Compost Batch	Added organisms
1	Control. Sterile dH_2O added. For moisture content analysis.
2	Control. Sterile dH_2O added. For molecular analysis.
3	*L. pneumophila* ($5x10^3$ cells/g)
4	*L. longbeachae* ($5x10^3$ cells/g)
5	*A. castellanii* ($5x10^2$ cells/g)
6	*L. pneumophila* ($5x10^3$ cells/g) and *A. castellanii* ($5x10^2$ cells/g)
7	*L. longbeachae* ($5x10^3$ cells/g) and *A. castellanii* ($5x10^2$ cells/g)

5.2.3 Unspiked greenhouse (NSGH)

This experiment was used to determine if naturally occurring *Legionella* spp. and protozoa in four composts could be amplified during the common greenhouse activities of potting plants and growing seedlings, including watering. Batches of compost were not spiked with *Legionella* spp. during this study, and as a result, it was not deemed necessary to keep these composts contained within a Biosafety cabinet, therefore allowing a mock greenhouse environment more realistic to life than the set up in the SGH experiment above. This was a 14-day long experiment using four different commercially available multipurpose composts, this time was chosen to represent situations where consumers may store compost bags after purchase, but to minimise drying seen in the spiked greenhouse experiment. Composts 1, 2 and 3 contained peat, while compost 4 was peat-free. Compost 1 was the same compost type used for SGH and INC, as described above (Section 5.2). It

was known that this compost brand supported naturally occurring, culturable *Legionella* spp. Thus, it was likely that the compost used already contained these organisms based on Chapters 3 and 4. After purchase, compost was refrigerated until use (within two days of purchase).

Two batches of each compost brand, comprising 15g of compost per batch, were placed into a seed tray in individual pots. Each pot within the seed tray had its own draining system (Figure 5.2) to prevent potential cross-contamination via water flow. Next, each compost brand was watered with 20ml sterile distilled water, while the remaining batch was an unwatered control. Following this initial setup, the seed tray was incubated at 15°C, and 10ml sterile distilled water was added to the watered batches every 2-3 days.

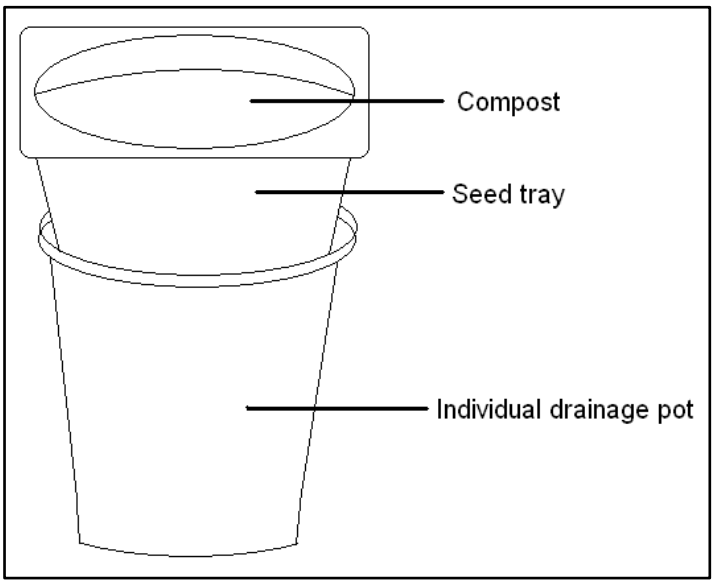


Figure 5.2 : Individual drainage system for compost batches used in NSGH to prevent cross-contamination through water flow.

A second seed tray was created to the same specifications and incubated in the mock greenhouse for a 16hr/8hr cycle. As described above, watered batches had 10ml sterile distilled water added every 2-3 days. A temperature and humidity monitor was added to the mock greenhouse set up, with readings taken throughout the experiment; soil temperature was also measured using a probe, as described in Section 5.2. The greenhouse batches were held on a shelf within the mock greenhouse, added to allow air movement while keeping compost away from the heat mat's

potentially drying direct heating element, which was identified as a potential problem in the previous experiment. To create higher humidity in the mock greenhouse than in the previous experiment, the seed trays were left open, and a tray with 750ml of sterile distilled was placed directly onto the heat mat under the shelves. The tray was refilled every 2-3 days with 750ml sterile distilled water to compensate for evaporation and moisture escaping from the mock greenhouse environment. A BCYE-α agar settle plate was left open in the mock greenhouse. Every three days, the plate was removed and incubated at 37°C for 3-7 days in a humid chamber as described in Chapter 3 to check for the potential presence of *Legionella* spp. in water aerosols released during watering.

The initial water content of each compost type was determined using British Standard BS EN 13040:2007 to establish starting differences between the batches. At the end of the experiment, the moisture content was not tested due to the health and safety risk posed by placing samples with a potentially increased bacterial load of *Legionella* spp. into a communal oven. After 14 days in the greenhouse, the batches were frozen at -80°C prior to DNA extraction and analysis. In addition to testing using semi-quantitative PCR for 16S rRNA and JP genes as described in Section 3.2.3, samples from the NSGH were tested for *Acanthamoeba* spp. using JDP primers with specifications in Table 3.1 to determine whether any *Acanthamoeba* spp. were present in the different compost batches.

5.2.4 Incubator (INC)

The NSGH experiment successfully increased the humidity of the greenhouse compared to the spiked greenhouse experiment; however, the difference in the microbial composition of each compost brand used in the unspiked greenhouse experiment did not allow for direct comparison between samples in different conditions. The aim of this experiment was similar to the spiked greenhouse experiment: to compare the growth of *Legionella* spp. in batches of compost stored at 15°C, with the growth of *Legionella* spp. in batches of compost stored in a greenhouse setting. These experiments were completed with compost batches spiked with *Legionella* spp. and *A. castellanii*

and used the same compost used in SGH and as brand 1 in NSGH. Each experiment also contained an unspiked control. As these composts were spiked, it was not possible to use the setup as used in the NSGH experiment due to safety reasons: the composts were not contained, which may have created a risk to laboratory users via potential inhalation of the test organisms during manipulation. As low humidity levels were seen when the mock greenhouse was placed into the Biosafety cabinet, a new mock greenhouse setup was devised to prevent moisture loss into the environment.

Five batches of compost (200g) were spiked with *L. longbeachae, L. pneumophila, Acanthamoeba* and *L. longbeachae, Acanthamoeba and L. pneumophila* as described in Section 5.2.2. These batches and an additional unspiked control batch (200g) were placed in bags within separate sealed glass jars in a laboratory incubator set at 30°C (mock greenhouse). Each jar contained a 15ml sterile distilled water in an open glass universal vial to provide humidity by evaporation. In order to fully represent the bag used to contain commercially available compost, the bags used were pierced on each side multiple times using a sterile needle to allow airflow throughout the batches. The neck of the bags was left open, and the jars were sealed using a screw-top lid. This setup was repeated, with jars stored in an incubator set at 15°C (stable control).

Day 0 samples weighing 15g±0.5g were taken from each batch, and the remaining composts were placed in the incubators for two weeks, after which an endpoint Day 14 samples were taken. The 30°C experiment was run on a 16hr-on/8hr-off heating cycle, the stable control composts were maintained at 15°C throughout. Humidity levels were not measured in this experiment as it was a closed system: the jars were not touched until the 14-day experimental period was finished to maintain steady moisture content and temperature level. As with the NSGH experiment, the 14 day time period was chosen to represent situations where consumers may store compost bags after purchase, but to minimise drying seen in the spiked greenhouse experiment. All samples were stored at -80°C before DNA extraction and semi-quantitative PCR were carried out as described in Section 3.2.3.

5.3. Results

5.3.1 Spiked Greenhouse

Compost batches spiked with *Legionella* spp. and/or *Acanthamoeba* spp. were stored in greenhouse conditions, and samples were taken on Day 0, 3, 7, 14, 21, 28, 42. Day 0, Day 14 and Day 42 samples were tested for *Legionella* spp. using the JP semi-quantitative PCR assay. The mean temperature of compost over the experiment was 29.8°C (range 25-34°C), the mean air temperature in the greenhouse was 30.4°C (range 25.8-36°C), and the mean humidity in the mock greenhouse was 23.4% (range 10-53%). The complete data set can be seen in Appendix 1. The working nature of the biosafety cabinet means that air is removed from the setup via a top filter. This resulted in low ambient humidity values: A full table of temperature and humidity values can be seen in Appendix 1. Figure 5.3 shows the difference between the moisture content of samples taken at days 0, 3, 7, 14, 21, 28, 42. The moisture content of the samples taken from Batch 1 ranged between 55.88% - 60.66% for batches stored at 15°C and between 57.4% - 61.69% for batches stored in the mock greenhouse setup. During the experiment, condensation could be seen on the surface of the inner plastic bag, indicating that this moisture may have been lost in an open system due to evaporation. While bags were turned every 2-3 days, direct contact with the heat mat likely influenced water movement in the samples. This is a factor that was taken into consideration when designing further experiments. The regular turning and manipulation of each bag before samples were taken would have enabled the reintroduction of evaporated water on the surface of the bags back into the compost. This is likely why the moisture content remained consistent throughout the experiment (Figure 5.3). The complete data set can be seen in Appendix 1. The dip in moisture content observed for both experimental conditions at Day 28 potentially indicates that batches were not mixed as thoroughly on this day as on previous days.

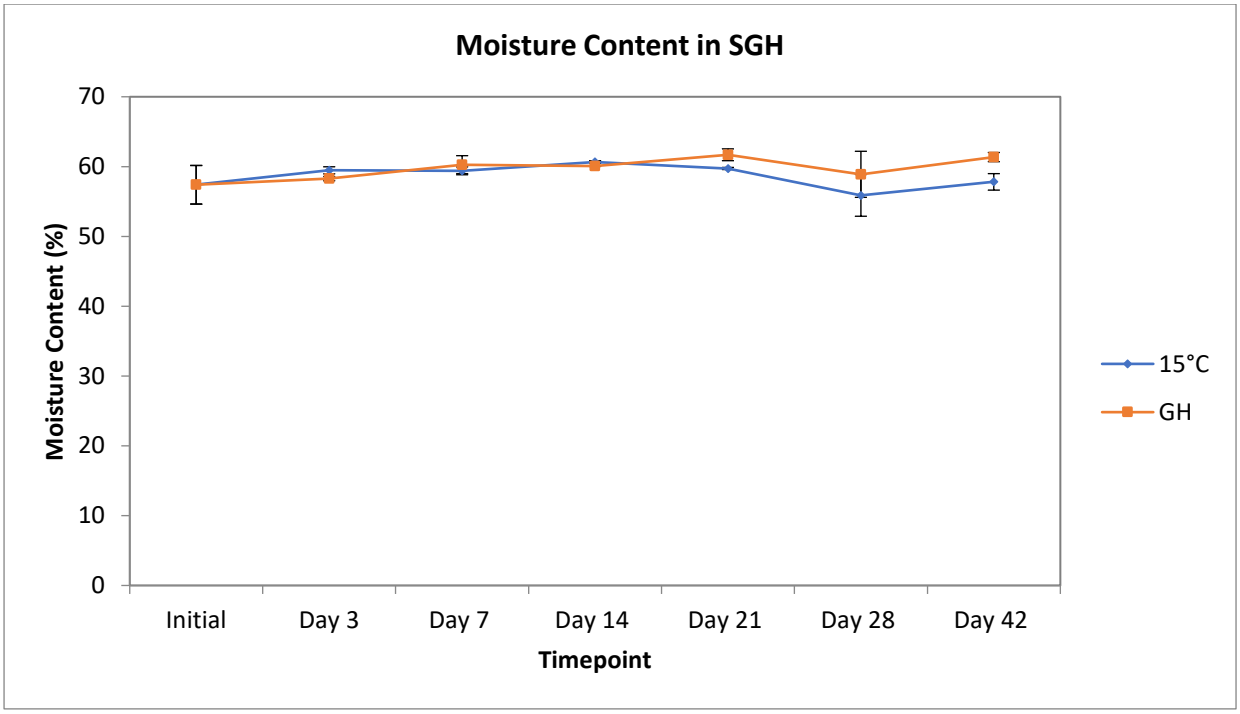


Figure 5.3: Moisture content of control compost (Batch 1) measured at different time points during the spiked greenhouse experiment

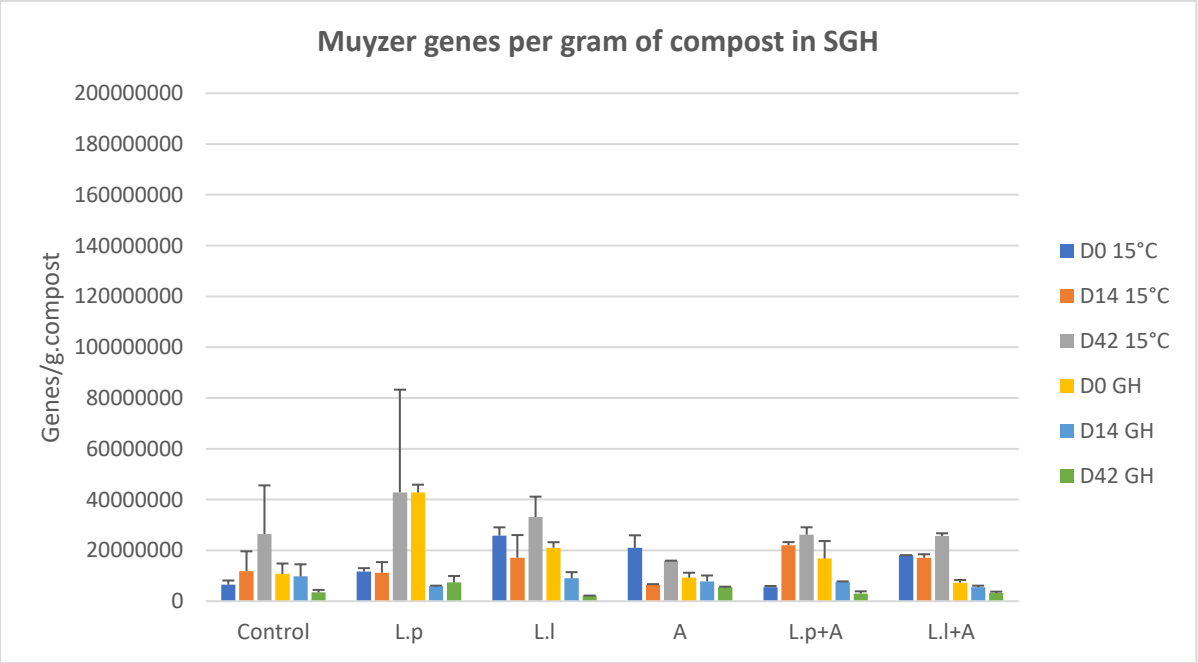


Figure 5.4: Muyzer genes/g.compost in SGH. "Control" represents an unspiked batch; "L.p" represents a batch spiked with *Legionella pneumophila,* "L.l" represents *Legionella longbeachae;* "A" represents batch spiked with *Acanthamoeba castellanii;* "L.p and A" represents *Legionella pneumophila* and *Acanthamoeba castellanii*, and "L.l and A" represents *Legionella longbeachae* and *Acanthamoeba castellanii.*

The general bacterial population present in the SGH compost batches was estimated at Day 0 (D0), Day 14 (D14) and Day 42 (D42) using the Muyzer primers, with results shown in Figure 5.4. With the exception of the batch with *Legionella pneumophila* added to it (Batch 3), there were stepwise reductions in the number of gene copies per gram in compost over time for batches stored in the greenhouse, with highest numbers being seen at D0 and the lowest at D42. This is not true for batches stored at 15°C, where a reduction in the number of copies from D0 to D14 occurs in 4 of 6 batches, but at D42, the number of genes per gram increase above D0 levels, except the batch with *A. castellanii* added.

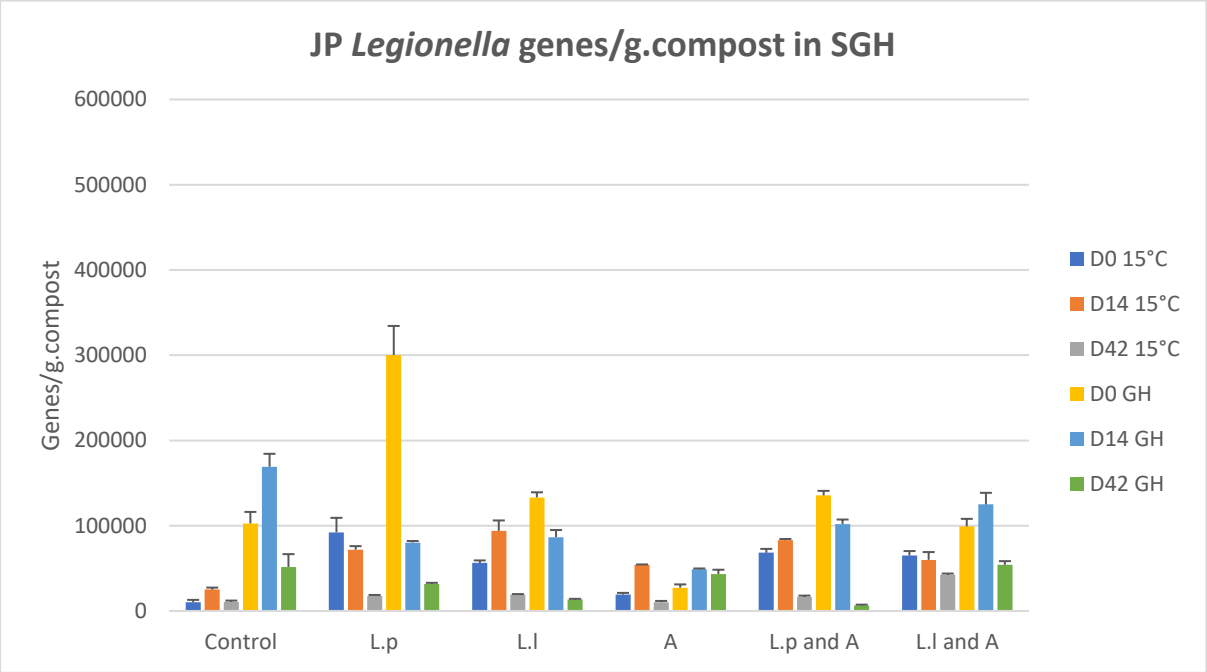


Figure 5.5: JP *Legionella*-specific 16S genes/g.compost in SGH. "Control" represents an unspiked batch; "L.p" represents a batch spiked with *Legionella pneumophila,* "L.l" represents *Legionella longbeachae; "*A" represents batch spiked with *Acanthamoeba castellanii; "*L.p and A" represents *Legionella pneumophila* and *Acanthamoeba castellanii*, and "L.l and A" represents *Legionella longbeachae* and *Acanthamoeba castellanii.*

When examining the results from the JP primers in Figure 5.5, there is not such a clear pattern in the number of copies of *Legionella*-specific 16S genes between batches. However, where *Legionella* spp. had been added to the compost, results from batches stored in the greenhouse appear to match those of the bacterial population (Figure 5.4) by reducing between Day 0 and Day 42, except for the

batch with *A. castellanii* added, where the number of *Legionella*-specific 16S genes slightly increases between D0 and D42 for those samples stored in the greenhouse setting.

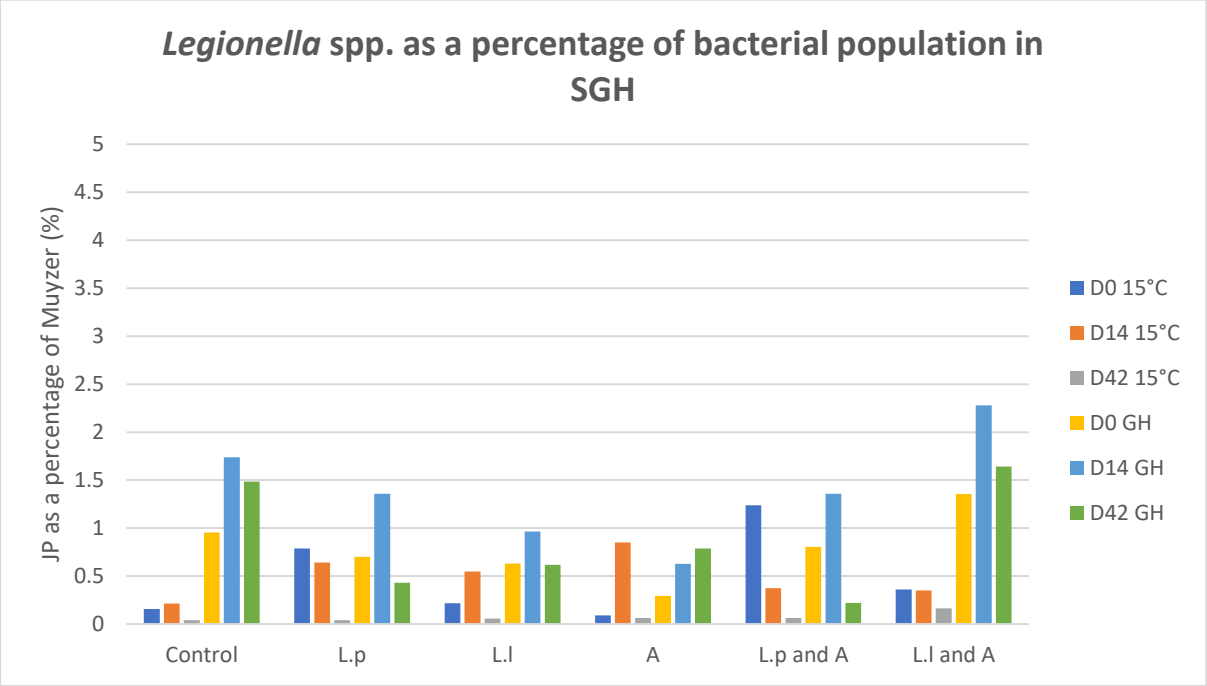


Figure 5.6: *Legionella* spp. as a percentage of bacterial population in SGH. "Control" represents an unspiked batch; "L.p" represents a batch spiked with *Legionella pneumophila,* "L.l" represents *Legionella longbeachae;* "A" represents batch spiked with *Acanthamoeba castellanii;* "L.p and A" represents *Legionella pneumophila* and *Acanthamoeba castellanii*, and "L.l and A" represents *Legionella longbeachae* and *Acanthamoeba castellanii.*

When examining *Legionella* spp. as a percentage of the bacterial population, the percentage of *Legionella* spp. genes in all batches were lowest at 15°C on Day 42, having decreased in size from Day 14, as shown in Figure 5.6. Except for the batch with only *A. castellanii* added, the percentage of *Legionella* spp. genes were highest in each batch at Day 14 after greenhouse storage. Generally, *Legionella* spp. represent a higher percentage of the bacterial population in batches stored in the greenhouse than at 15°C. However, the graphs above are difficult to compare due to possible differences in starting inoculum in the compost.

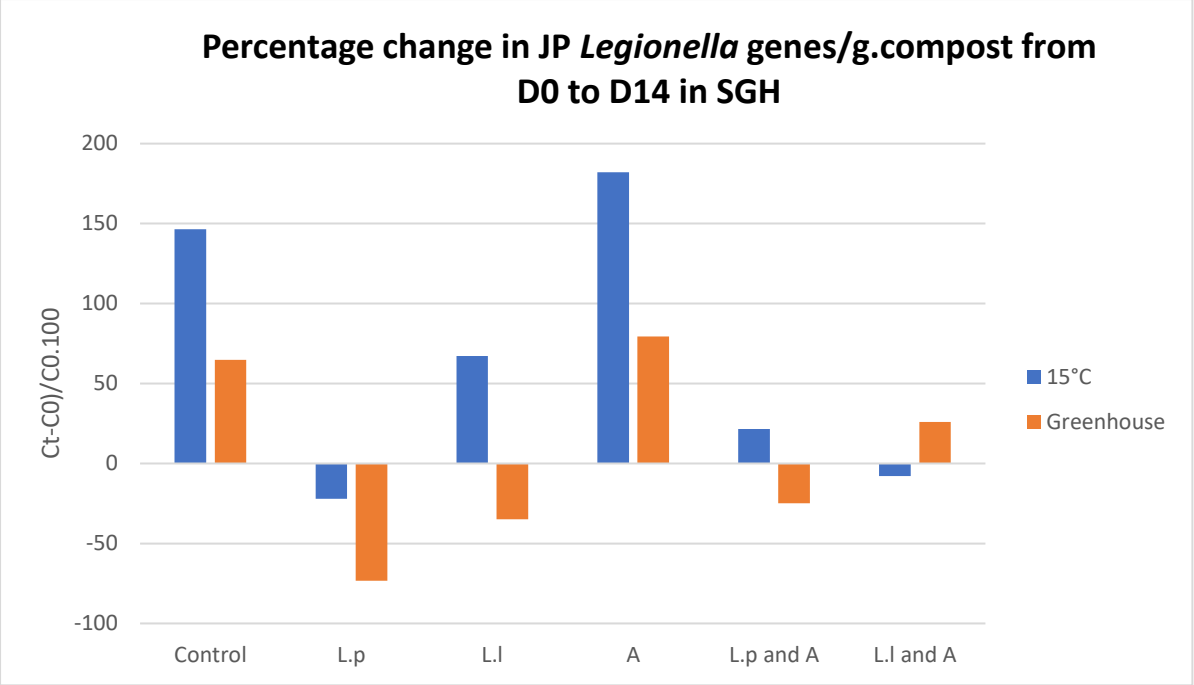


Figure 5.7: Percentage change in JP Legionella genes/g.compost from D0 to D14 in SGH. "Control" represents an unspiked batch; "L.p" represents a batch spiked with *Legionella pneumophila,* "L.l" represents *Legionella longbeachae;* "A" represents batch spiked with *Acanthamoeba castellanii;* "L.p and A" represents *Legionella pneumophila* and *Acanthamoeba castellanii*, and "L.l and A" represents *Legionella longbeachae* and *Acanthamoeba castellanii.*

To account for differences in starting concentration of *Legionella* spp. due to the variable nature of compost, the percentage change in *Legionella* genes from Day 0 to Day 14 was determined for each experimental setup using the equation $\left(\frac{C_T - C_0}{C_0} \times 100\right)$ where C_T represents Day 14 and C_0 represents Day 0. From Day 0 to Day 14, the percentage difference in *Legionella* genes in batches stored at 15°C increased in the control sample, and in the batches spiked with *L. longbeachae, A. castellanii* and *L. pneumophila & A. castellanii,* as seen in Figure 5.7. For batches stored under greenhouse conditions, the percentage difference in *Legionella* genes increased in the control sample and the batch spiked with *A. castellanii.* However, the change was not as large as for samples stored at the lower temperature. The only condition where the percentage change from D0 to D14 was greater in the greenhouse batch compared to the 15°C batch was in those spiked with *L. longbeachae* & *A. castellanii.*

Overall, there was a lack of clear and consistent trends in the data obtained from the spiked greenhouse experiment, meaning this study was inconclusive. However, the low humidity and high level of evaporation was potentially a factor in the behaviour of compost organisms within these composts, and future experiments were amended to consider this.

5.3.2 Unspiked greenhouse

Batches of four different compost brands were stored at 15°C and in greenhouse conditions. Samples were taken on Day 0 and 14 and tested for *Legionella* spp. using the JP semi-quantitative PCR assay. The mean temperatures of soil and air in the greenhouse over the run were 29°C (range 28 - 30°C) and 30.9°C (range 29.2-32.1°C) respectively, while the mean humidity in the greenhouse during the run was 75.2% (range 40-90%). A complete table of results can be seen in Appendix 1. The average soil and air temperature in this experiment were similar to those seen in SGH; however, average humidity was much higher when compared with SGH, up from 23.4%, which is a difference of 51.8%. No growth was observed on the agar settle plate.

The unwatered compost batches stored in the mock greenhouse contained lower levels of 16S genes per gram of compost than the watered compost batches stored in the mock greenhouse at Day 14 for all four compost brands. In addition, when compared at Day 14, the unwatered batches stored in the mock greenhouse also contained lower levels of 16S genes per gram of compost than the unwatered batches stored at 15°C.

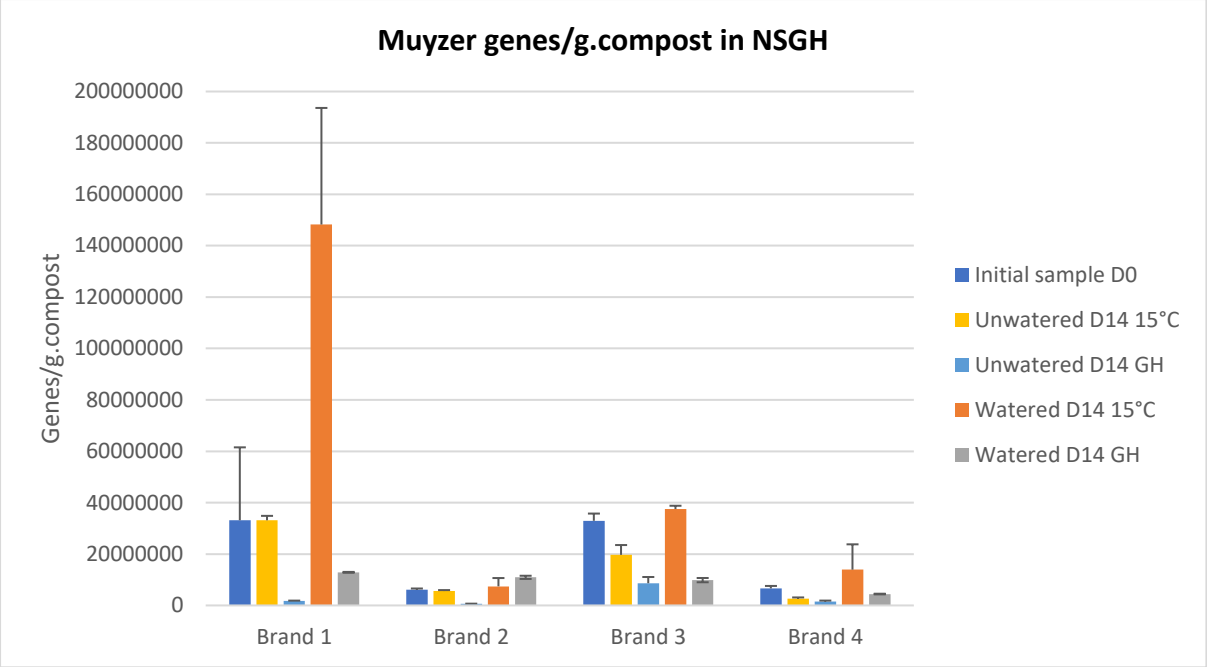


Figure 5.8: Muyzer genes/g.compost in NSGH. Sample 1 represents batches from the standard compost used across all experiments, Sample 2, 3 and 4 represent batches from other compost brands. GH represents samples stored in the mock greenhouse, and 15°C represents control samples

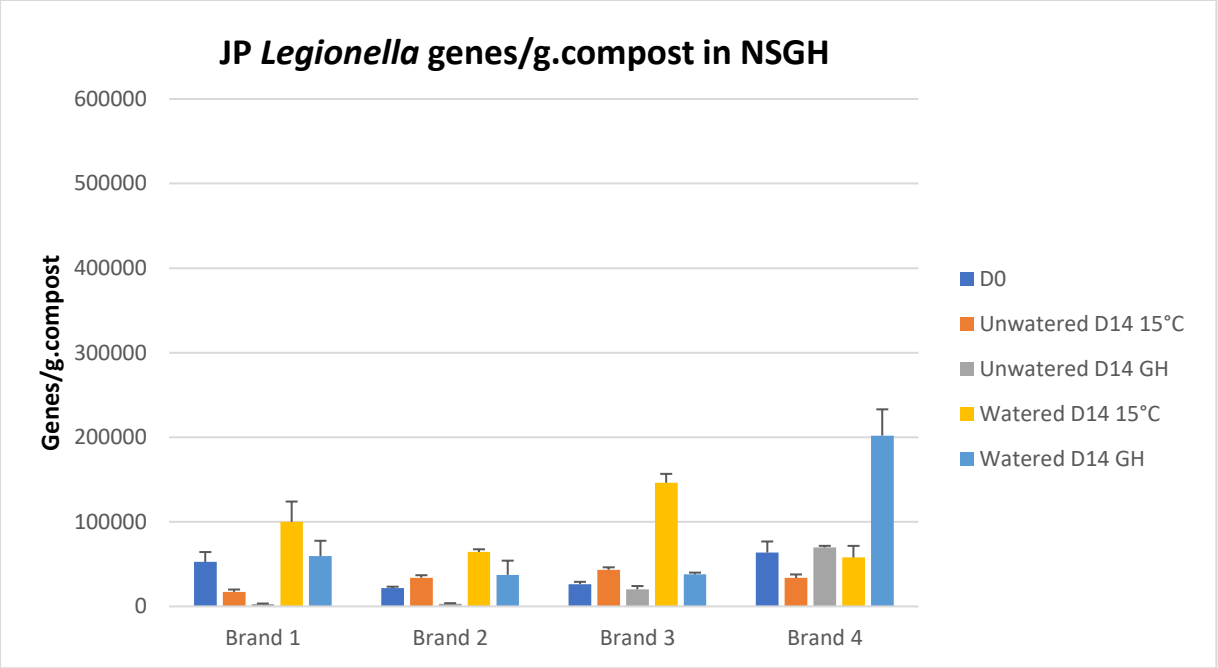


Figure 5.9: JP *Legionella* genes/g.compost in NSGH. Sample 1 represents the standard compost used across all experiments, Sample 2, 3 and 4 represent three different commercially available compost types

When the greenhouse batches were watered, all batches showed more *Legionella* spp. genes per gram of compost than the initial sample after 14 days. The opposite is true for the number of *Legionella* spp. genes in the unwatered greenhouse batches at day 14, which were consistently lower than the number of genes seen in the initial batches, except compost Brand 4. However, these differences are insufficient to conclude when the error is considered. Compost Brand 4 is also against the trend when comparing Day 0 with Day 14 watered control (15°C) batches: the number of *Legionella* genes per gram of compost increases after 14 days in compost brands 1, 2, and 3, whereas the number of genes decreases slightly in compost Brand 4. This trend continues when comparing Day 0 with Day 14 greenhouse samples: while there is an increase in the number of *Legionella* genes per gram of compost after 14 days in compost Brands 1, 2, and 3, this is not sufficient to draw conclusions; however, the number of genes increases in compost Brand 4. These differences can be seen even when error bars are taken into consideration.

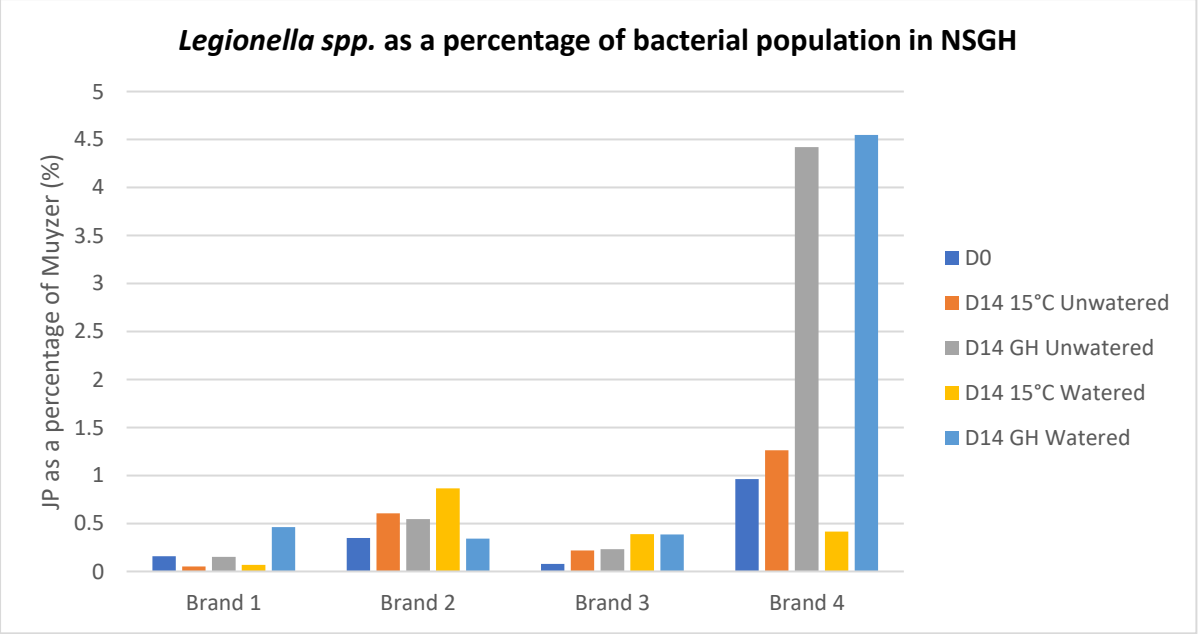


Figure 5.10: *Legionella* spp. as a percentage of the bacterial population in NSGH

When compost Brand 4 was stored in the greenhouse, JP genes represented a much larger percentage of the bacterial population in both watered and unwatered batches after 14 days than any other batches. In this experiment, the proportion of *Legionella* spp. in the bacterial population increased from Day 0 to Day 14 for nearly all batches stored in all conditions. This is with the exception of Compost brand 1, which only saw *Legionella* spp. increase its percentage of the bacterial population in batches stored in the greenhouse and watered.

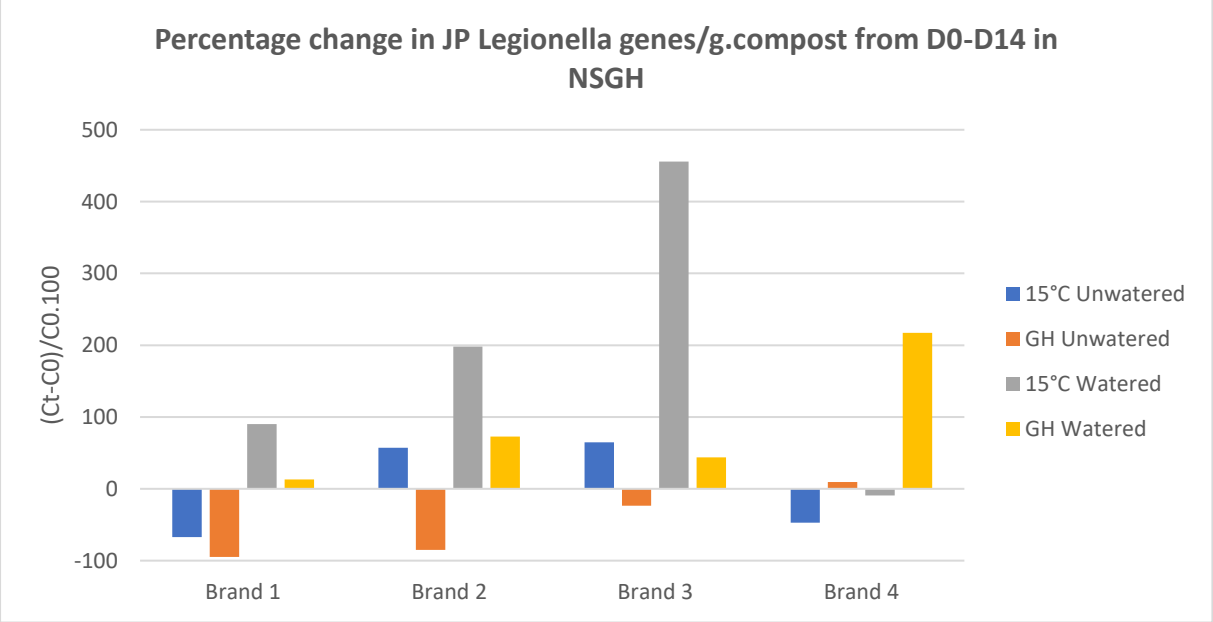


Figure 5.11: Percentage change in JP *Legionella* genes/g.compost from D0 to D14 in NSGH

As with SGH, the data was normalised using the equation $\left(\frac{C_T - C_0}{C_0} \times 100\right)$ to allow direct comparison despite differences in starting inoculum. Compost Brand 4 again shows a different pattern when comparing Day 0 and Day 14 in the unspiked greenhouse. While the percentage change from Day 0 to Day 14 decreases in the unwatered greenhouse batches and increases in the watered greenhouse batches in compost brands 1, 2 and 3, the opposite is true for compost Brand 4. For the 16 samples shown, the percentage change in JP amplicons increased in 10/16 samples. Of the six samples where

a decrease was observed, 5/6 were unwatered samples. All of the samples in the watered greenhouse set up increased from Day 0 to Day 14 (n=4).

Table 5.3: The presence or absence of *Acanthamoeba* (JDP) genes in NSGH. 'ND' represents none-detected, and '+ve' represents a positive result

	Brand 1	**Brand 2**	**Brand 3**	**Brand 4**
Day 0	ND	ND	ND	+ve
Day 14 15°C Unwatered	+ve	ND	+ve	+ve
Day 14 GH Unwatered	ND	+ve	+ve	+ve
Day 14 15°C Watered	ND	ND	ND	+ve
Day 14 GH Watered	ND	+ve	ND	+ve

Table 5.3 shows the results from PCR of each sample with JDP primers. *Acanthamoeba* spp. were present consistently in compost Brand 4. In contrast, results from other brands indicate that these organisms may have been present in low numbers initially, with different conditions resulting in the growth of amoebae. Most growth in Brands 1 – 3 appears to occur when batches were unwatered, with 4/6 batches positive for JDP after 14 days compared with 1/6 watered batches.

The results from the unspiked greenhouse experiment showed overall that the greenhouse setting enabled the growth of *Legionella* spp. when the batches were watered but not when the batches were unwatered. While the watered samples also showed an increase in *Legionella* genes from D0-D4 at 15°C, the impact on unwatered samples is less clear. Compost Brand 4 was the peat-free brand, and organisms in this compost appeared to behave differently to the peat-containing brands 1-3. This is true both in the greenhouse setting and at 15°C. Variations in this experiment were likely due to diversity between bacterial communities present in each sample, possibly due to differences between components used to formulate each compost type.

5.3.3 Incubator

From Day 0 to Day 14, the number of Muyzer amplicons in all samples declined at 15°C except the two batches with *Legionella* spp. and *A. castellanii* added to them which increase after 14 days. For

batches stored at 30°C, there is also a decrease in the number of Muyzer amplicons in each sample, except for the batch with *L. pneumophila* and *A. castellanii* added, and the batch spiked with *L. longbeachae*.

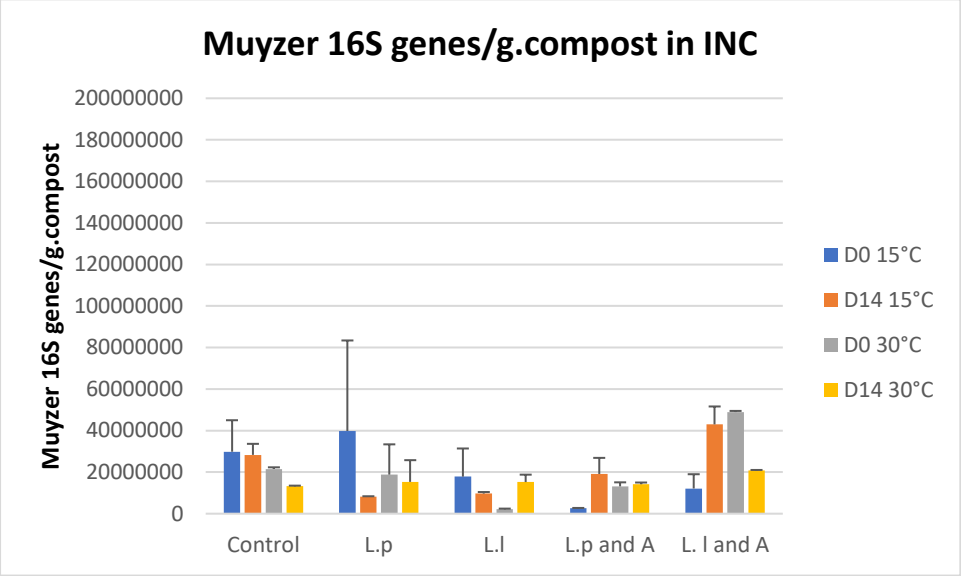


Figure 5.12: Muyzer 16S genes/g.compost in INC. "Control" represents an unspiked batch; "L.p" represents a batch spiked with *Legionella pneumophila,* "L.l" represents *Legionella longbeachae;* "A" represents batch spiked with *Acanthamoeba castellanii;* "L.p and A" represents *Legionella pneumophila* and *Acanthamoeba castellanii*, and "L.l and A" represents *Legionella longbeachae* and *Acanthamoeba castellanii.*

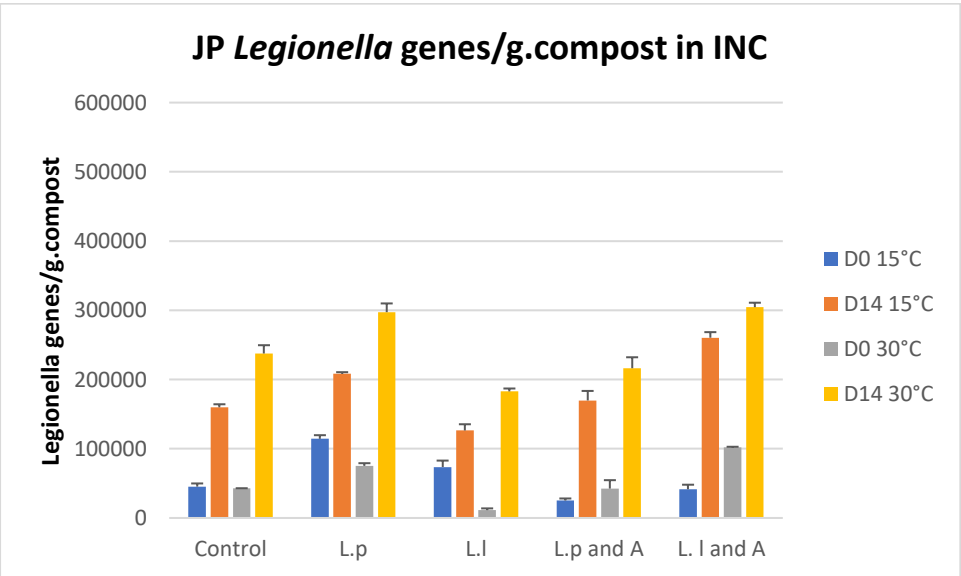


Figure 5.13: JP *Legionella* genes/g.compost in INC. "Control" represents an unspiked batch; "L.p" represents a batch spiked with *Legionella pneumophila,* "L.l" represents *Legionella longbeachae;* "A" represents batch spiked with *Acanthamoeba castellanii;* "L.p and A" represents *Legionella pneumophila* and *Acanthamoeba castellanii*, and "L.l and A" represents *Legionella longbeachae* and *Acanthamoeba castellanii.*

At both 15°C and 30°C, the number of *Legionella* spp. genes in samples taken from each compost batch increased after 14 days of storage, indicating that the INC setup was favourable for the growth of *Legionella* spp.

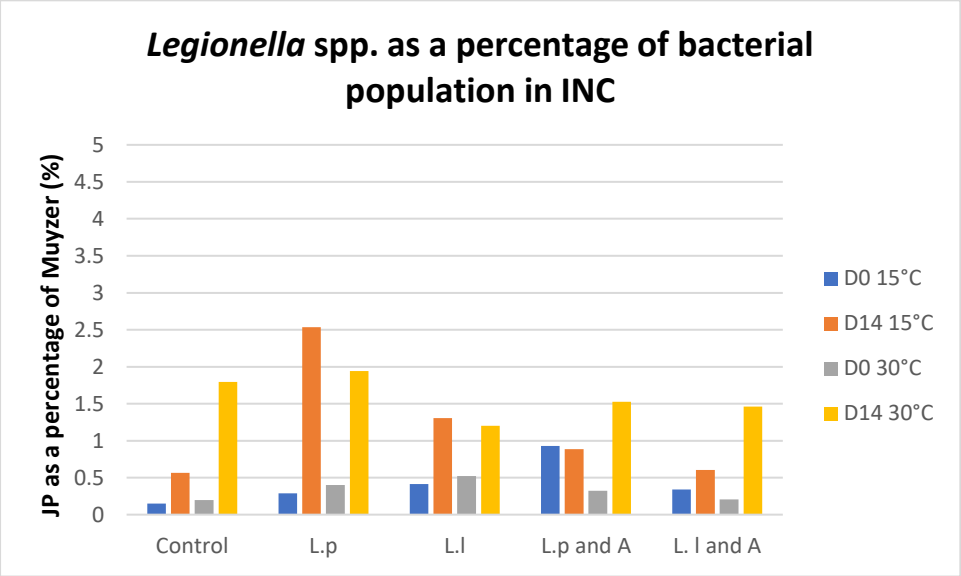


Figure 5.14: *Legionella* spp. as a percentage of bacterial population in INC. "Control" represents an unspiked batch; "L.p" represents a batch spiked with *Legionella pneumophila,* "L.l" represents *Legionella longbeachae;* "A" represents batch spiked with *Acanthamoeba castellanii;* "L.p and A" represents *Legionella pneumophila* and *Acanthamoeba castellanii*, and "L.l and A" represents *Legionella longbeachae* and *Acanthamoeba castellanii.*

When looking at JP *Legionella* spp. in comparison with Muyzer 16S genes, the proportion of JP Legionella genes increased in all samples after 14 days in the INC set up except samples spiked with *L. pneumophila* and *A. castellanii* at 15°C. This indicates that for most samples, the INC mock greenhouse conditions were favourable to the growth of *Legionella* spp. both at 15°C and 30°C. For composts spiked with only *Legionella* spp., there is more of an increase between Day 0 and Day 14 in composts stored at 15°C than in composts stored at 30°C. In contrast, the opposite is true for the control compost and composts with *Legionella* spp. and *A. castellanii* added*,* where *Legionella* spp. JP genes make up a larger percentage of the population in composts after 14 days at 30°C.

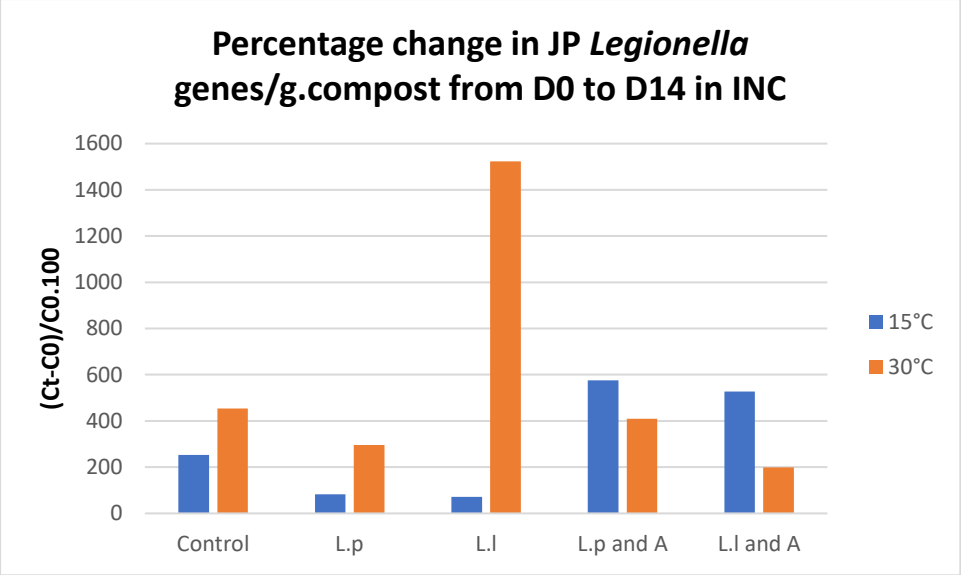


Figure 5.15: Percentage change in JP Legionella genes/g.compost from D0 to D14 in INC. "Control" represents an unspiked batch; "L.p" represents a batch spiked with *Legionella pneumophila,* "L.l" represents *Legionella longbeachae;* "A" represents batch spiked with *Acanthamoeba castellanii;* "L.p and A" represents *Legionella pneumophila* and *Acanthamoeba castellanii*, and "L.l and A" represents *Legionella longbeachae* and *Acanthamoeba castellanii.*

Once again, the data has been normalised to allow direct comparison between experimental setups despite differences in starting inoculum. The number of *Legionella* spp. genes in all composts stored in the INC mock greenhouse set up increased from Day 0 to Day 14, whether stored at 15°C or 30°C. The control batches and batches spiked with only *L. pneumophila* or *L. longbeachae* saw a higher increase in *Legionella* genes when stored at 30°C compared with 15°C. In contrast, compost spiked with both *Legionella* spp and *A. castellanii* saw more of an increase in numbers after 14 days at 15°C.

Overall in the incubator experiment, the number of *Legionella* spp. JP genes in most samples increased from Day 0 to Day 14 regardless of the storage temperature. Due to the nature of compost, it is still likely that the bacteria profile within each sample was not identical and therefore had the potential to act differently under different conditions. Differences were observed when amoebae were added to the compost mixture, indicating that the naturally occurring protozoa and the bacterial species present impact the ecology of *Legionella* spp. in the compost environment.

5.4. Discussion

The experiments in this chapter were designed to determine the effect of greenhouse storage on the survival and proliferation of *Legionella* spp. in compost. A mock greenhouse setup was devised, and a series of experiments performed sequentially using composts either unspiked or spiked with *Legionella* spp. and/or *Acanthamoeba* spp. Adaptations to the experimental setup were made with each subsequent experiment to improve upon the previous setup. Samples were analysed using the DNA extraction and semi-quantitative PCR methods developed in Chapter 4.

Culture of samples was considered; however, it would not be possible to accurately count the number of *Legionella* spp. present on a culture plate due to compost's high microbial diversity, causing plate overcrowding. In addition, while the use of sterilised compost would have made culture possible, biofilms with or without the influence of amoebal hosts are likely crucial in the maintenance of a *Legionella* spp. population in the compost environment, as discussed in the literature review (Section 2.1.6.3), and sterilised compost would have prevented interactions between *Legionella* spp. and other bacteria or protozoan hosts; therefore, the compost environment would not be accurately represented.

Several factors are considered when using PCR to examine organisms in a complex community such as compost. First, the irregular distribution of organisms within the compost matrix means that two different batches, and the samples taken from them, are highly unlikely to contain the same proportions of microflora, even when taken from the same initial source of compost. This can be seen when comparing D0 data in Figure 5.4 and Figure 5.12. The samples were prepared in the same way, with care taken to ensure thorough mixing; however, there is a lack of consistency across the samples. This highlights the problematic nature of working with compost and may be due to due to hotspots of *Legionella* spp. within the compost matrix, which remained despite mixing. The percentage change from D0 to D14 has been shown to account for this, in Figure 5.7, Figure 5.11, Figure 5.15. Using these protocols in future work would be beneficial to simplify the experiment and

test one batch multiple times to allow for robust statistical analysis to be performed and determine the measurement of uncertainty.

Similarly, the actual quantity of *Legionella* spp. present in each sample cannot be accurately determined, as PCR assays cannot distinguish between live and dead cells. Identifying increased numbers of amplicons from Day 0 to Day 14 in most samples in the INC experiment, Figure 5.15, may suggest that the growth of *Legionella* spp. has occurred over time, as dead cells would not increase in number. However, this should be interpreted with caution due to the semi-quantitative nature of the assay used. Decreases in the number of amplicons were also observed between Day 0 and Day 14 (see Figure 5.7: (SGH) and Figure 5.11 (NSGH)). In SGH, batches of the same brand of compost were spiked with different combinations of *Legionella* spp. and/or *Acanthamoeba castellanii* and examined after storage at 15°C and in greenhouse conditions.

The low humidity and high level of evaporation was likely a factor in the behaviour of compost organisms within these composts, and this study was inconclusive. The following experiment, NSGH, involved batches of four different compost brands stored in pots at both 15°C and greenhouse conditions without the addition of *Legionella* spp. or *A. castellanii*. Batches were watered to represent gardening activities, and overall results suggest that watered samples were more conducive to the proliferation of *Legionella* spp. than unwatered samples. A reduction in DNA possibly occurred, for example, if DNA was from lysed cells and desiccated within the compost environment. Cell rupture due to drying and rewetting soil is widely documented (Salemac *et al.* 1982, Fierer *et al.* 2003, Turner *et al.* 2003).

Katz and Hammel (1987) found that *L. pneumophila* was sensitive to drying, with viable organisms undetected in some, but not all, samples after 90mins of drying. Steele *et al*. (1990a) also found drying to be detrimental to the survival of *Legionellae* in soil samples. Two soil samples known to be positive for *L. longbeachae* were dried at 36°C for seven days; the organisms were isolated from undried samples held at room temperature and at 36°C after seven days, but not from dried

samples. In 11 of the 12 SGH-experimental batches, JP-amplicon amounts (Figure 5.5:) were lower at Day 42 than at Day 0; and of these, 6/11 increased from Day 0 to Day 14 but decreased from Day 14 to Day 42. This is likely due to increasing temperatures being optimal for growth and some water remaining in the samples; however, there was a decrease in JP amplicons by Day 42. The samples had undergone many more drying and rewetting cycles at this point.

Lysis of cells due to drying or death would release DNA into the compost. When extracellular DNA was spiked into soil samples by Frostegård *et al* (1999), less than 6% was recovered after DNA extraction, and the same would likely be true for DNA released after cell lysis. The DNA extraction protocol developed in Chapter 4 was designed to recover whole cells from the compost samples and would be unlikely to recover extracellular DNA accurately. In the SGH and NSGH experiments described above, composts becoming dried out were always rewetted when mixed, and condensation was reintroduced to compost when batches were watered or when samples were added to the Tween 80 solution during DNA extraction. In support of this, and in contrast to SGH and NSGH, an increase in gene number was observed for all samples at both temperatures from Day 0 to Day 14 in the final experiment (INC). All samples in this experiment were stored in humid glass jars in an incubator, which would have reduced drying, evaporation, and cell rupture.

Compost samples in the SGH experiment became dried out due to evaporation, as a result of increased temperatures and low humidity seen within the experimental setup, but this was not reflected in the results from moisture content analysis of each batch (Figure 5.3) because the compost was stored in plastic bags. Moisture evaporating out of the batches condensed on the inner surface of the bags. This moisture was reintroduced when the compost was mixed prior to each time point sample being taken, meaning that the measured moisture content remained constant. At the same time, bacteria were subjected to the drying and rewetting pattern shown above, potentially causing cell lysis and the release of DNA. Figure 5.4 shows an apparent decrease in overall Muyzer

amplicons per gram of compost in all greenhouse samples as time progresses from Day 0 to Day 42, but not in the 15°C sample, likely due to increased evaporation at the higher temperature.

Evaporation is lower in higher humidity settings such as the INC set up. Humidity was identified as one of the most critical factors in isolating *Legionella* spp. from compost samples by Conza *et al* (2013), who cited Fisman *et al* (2005), who found that increasing humidity and increasing average monthly temperature correlated with increased incidence of *Legionella* spp. In addition, Karagiannis *et al.* (2009) documented that warm wet weather (+17.5°C) was associated with sporadic legionellosis in the Netherlands. Conza *et al* (2014) also identified a higher level of compost heap contamination with *Legionella* spp. in southern Switzerland, which has warmer summers and a higher level of rainfall than the north of the country. The effect of climate on the prevalence of *Legionella* spp. was also examined by Graham *et al* (2012) who state that during spring in New Zealand, a seasonal spike in cases of legionellosis is consistently seen. They also mention that more outbreaks occur in this season where compost is the source than outbreaks where cooling towers are the source. Average temperatures in Spring in New Zealand range from 16-19°C, compared with an average of 20-25°C in the summer (https://www.newzealand.com/uk/feature/new-zealand-climate-and-weather/). The increase may be due to an increase in gardening activity during this period, but when examined with regards to the INC experiment, showing there are increases in JP genes at 15°C as well as at 30°C, this may indicate that humidity has more of an influence on *Legionella* spp. proliferation than increased temperatures. Potentially, in optimal conditions, there is an increase in *Legionella* spp. numbers at low temperatures, but an even higher increase when the temperatures also rise, which is valid for INC samples that were not spiked with amoebae (Figure 5.15). In work published after the experiments in this chapter were completed, Schwake *et al.* (2014) cultured naturally occurring *Legionella* spp. from compost stored at 25°C, 32°C and 37°C. They observed growth in samples stored at all three temperatures, with a higher level of growth observed with increasing temperature. At all three temperatures, the growth rate was higher from Day 0 to Day 4 compared to the rate of growth from Day 4 to Day 8. They were only able to monitor growth

for eight days due to the overgrowth of culture plates and suggested that competition from other organisms may have been responsible for these results due to inhibition caused. When discussing the microbiome, the authors also observed the potential for *Legionella* spp. to use fungi as a source of nutrition, but they did not examine the samples for the presence of other bacterial species or free-living amoebae.

Amoebal hosts may have the potential to change the behaviour of bacteria within this environment; for example, Murga *et al.* (2001) found that *L. pneumophila* was unable to replicate in a biofilm without the presence of a host species. Bigot *et al.* (2013) suggested that multiplication within amoebae may enhance the ability of *Legionella pneumophila* to colonise biofilms. In NSGH, Batch 4 was the only compost consistently positive for *Acanthamoeba* spp. under all conditions (Table 5.3), indicating a consistent population of these host species present in this material. Batches 1, 2 and 3 showed a decrease from Day 0 to Day 14 in the number of JP amplicons present in unwatered samples held in the greenhouse setting, which would have experienced drying due to the increased temperature and evaporation. Batch 4 showed opposite results, with a slight increase in *Legionella* genes from Day 0 to Day 14 greenhouse unwatered samples. All samples showed an increase in the number of JP amplicons present from Day 0 to Day 14 in the watered greenhouse set up, while only 1-3 showed an increase when watered at 15°C. It is possible that the *Acanthamoeba* spp. present in Batch 4 influenced the survival of *Legionella* spp. either negatively through predation or positively as a protective host dependent on the storage temperature.

Acanthamoeba polyphaga was shown to protect *Pseudomonas paucimobilis* in soil from drying (Bryant *et al* 1982). Similarly, Berk *et al* (2003) found that *L. pneumophila* contained in vesicles expelled from protozoans were protected from drying for ten days, whereas *L. pneumophila* alone did not survive. In INC, where the humidity was less variable, the number of JP amplicons in all batches increased from Day 0 to Day 14 regardless of the storage temperature. However, in batches where *A. castellanii* were added to the compost mixture, the increase was smaller for samples

stored at 30°C than samples stored at 15°C. This is the opposite of samples where *A. castellanii* were not added and, as above, suggests that the impact of *A. castellanii* on the survival of *Legionella* spp. in compost may be temperature and humidity dependent.

An additional explanation for the observed differences in the pattern of JP amplicons present in Batch 4 NSGH may be differences in bacterial community structure, as this compost was peat-free. In contrast, compost batches 1, 2 and 3 contained peat. While *Legionella* spp. were isolated from both peat-containing and peat-free composts in Chapter 3, it may be that the presence of peat is detrimental to the growth of *Legionella* spp. The use of peat is further discussed in Chater 3 (Section 3.4). The use of different components as feedstock may affect a number of factors including pH, chemical make up and microbiome of the final compost. Compost batch 4 in NSGH also had the largest increase in JP amplicons from Day 0 to Day 14 when compared with the other three brands. Compost is a complex media, and there are a number of factors which is not possible to control for which may impact the growth and survival of different organisms. For example, Schwake *et al.* (2014) supplemented one batch of compost with calcium silicate to increase the pH and found a twofold decrease in *Legionella* spp. isolated compared to the compost, which had not been supplemented. Whiley and Taylor (2014) outlined how the availability of trace elements has been shown to improve *Legionella* spp. growth in culture, and suggested this could potentially affect the ability of *Legionella* spp. to proliferate in compost. The quantity and balance of chemical and biological elements will vary depending on compost components and where they have been sourced. The impacts of these variables were mitigated in part in the SGH and INC experiments by using the same brand of compost throughout but may go some way to explain differences observed in NSGH.

As noted in the literature review (Section 2.2.2), changing the compost microbiome may lead to differences between organisms, predator-prey interactions, and biofilm formation. More specifically, differences in numbers of JP genes present in different samples at different temperatures may be

due to differences in the species of *Legionella* present: potentially, some species of *Legionella* have an optimal multiplication temperature lower than the perceived optimal 36°C (Table 2.1). The jars used in the INC experiment eliminated the influence of humidity fluctuations on the samples by preventing evaporation and found JP genes in samples spiked with *Legionella longbeachae* increased more than any other sample after 14 days at 30°C. This also suggests that differences between samples may be due to differences between *Legionella* species. While the number of JP genes in the control sample and the sample spiked with *L. pneumophila* both increased after 14 days, this was not to the same extent as the increase seen for the *L. longbeachae* sample. It may be the case that different species of *Legionella* proliferate at different temperatures and are dependent on the microbiome. However, to the best of my knowledge, research investigating this in detail has not been performed.

In the studies described in this Chapter, the Jonas primers were chosen to indicate how *Legionella* spp. behave under different conditions, and the assays above cannot distinguish between species present. Work published after the work in this chapter was completed differentially identified *Legionella* spp, *Legionella pneumophila, Legionella pneumophila* Sg 1 and *Legionella longbeachae* in soil samples by direct qPCR using individual assays (Marchand *et al* 2018). After completing experimental work for this thesis, some multiplex assays have also been developed, one of which identified *Legionella* spp, *Legionella pneumophila* and *Legionella pneumophila* Sg1 in environmental water samples (Collins *et al* 2015), and a second, which was developed using isolates of non-*L. pneumophila* species from clinical and environmental samples (Benitez and Winchell 2016). Neither of these studies describes using a multiplex assay to examine compost materials. In order to build upon the work described in this chapter, future work distinguishing which species of Legionella are present in each compost sample, at what proportion, and how these different species react to humidity and temperature changes may help to elucidate how these organisms can replicate to infective levels. This might be achieved through development of a suitable multiplex qPCR for detection of key species in compost, or through comparative study of phenotypic bench and

molecular analysis. Work examining the impact of various compost component material on the bacterial community and amoeba host species present may also be of benefit to further understanding of *Legionella* spp in the compost environment.

6. BRIEF ANALYSIS OF WINDROW COMPOST PRODUCTION

6.1. Introduction

Work completed in Chapter 5 describes the effect of greenhouse storage on *Legionella* in consumer compost post-purchase; however, in Chapter 3, *Legionella* spp. were isolated from compost immediately after purchase, before storage in the home environment. Although it is possible that storage prior to purchase, for example, indoors in shops and garden centres, may provide increased temperatures and humidity, leading to a proliferation of *Legionella* spp, it is likely that compost is already contaminated with *Legionella* spp during the production process and before packaging and transport. In Australia, Hughes and Steele isolated *Legionella* spp. in all compost samples taken from six large scale composting facilities (Hughes & Steele, 1994). Casati and colleagues isolated *Legionella* spp. from six out of eight green waste collection centres in Switzerland, three of which were composting facilities; fresh green waste was negative for *Legionella* spp by culture at 7 out of 8 facilities, whereas compost was always positive (Casati *et al.,* 2010). These results mirrored those of Steele *et al*., who, with the exception of pine sawdust, did not isolate *Legionella* spp. from fresh component materials but did isolate these organisms from composted components (Steele *et al.* 1990b). It may be that *Legionella* spp. are present in green waste in low numbers, which increase during structured composting or long-term storage as communities establish themselves (Steele *et al.* 1990b). Alternatively, as suggested by Casati *et al*., wind and rain contamination may introduce *Legionella* spp. into the green waste/compost piles at any point throughout the storage and composting process (Casati *et al.,* 2010).

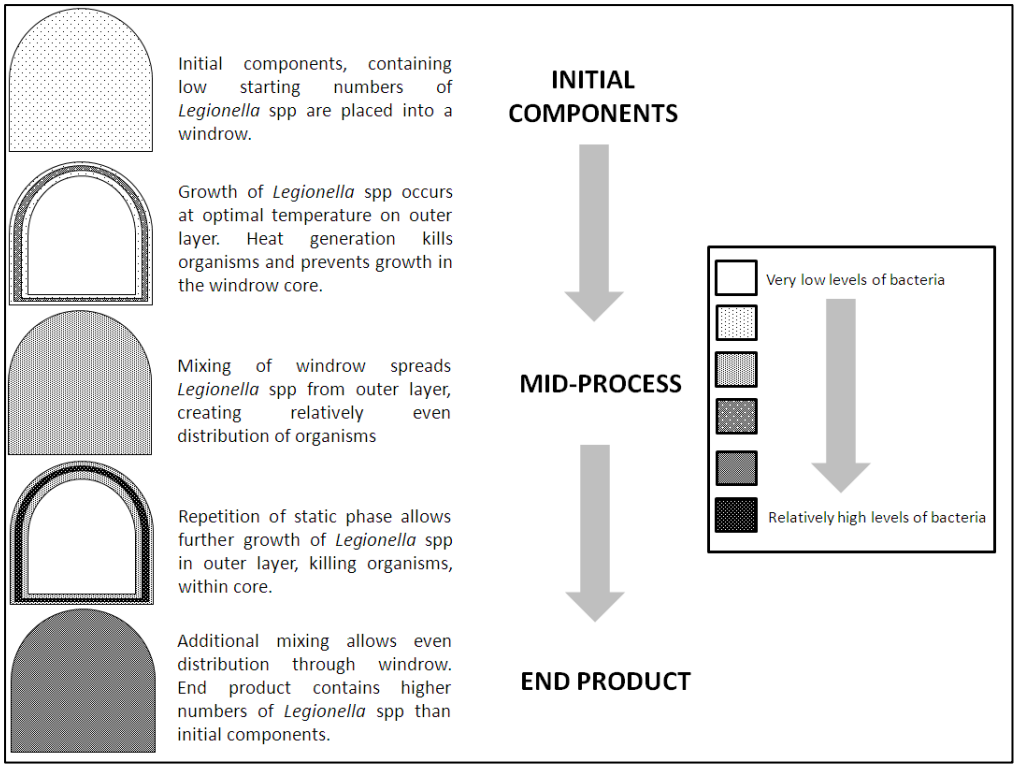


Figure 6.1: Theoretical diagram of windrow cross-sections representing the survival and proliferation of *Legionella* spp in windrows during the composting process

Bacterial diversity in compost is known to decrease at temperatures above 60°C (Strom, 1985); however, it is unclear whether windrows used in compost production reach this temperature throughout. Shepherd *et al.* (2007) found that if compost piles were not turned, *Escherichia coli* O157:H7 survived for months at the outer surface, and this may also be the case for *Legionella* spp. Conza *et al*. (2013) took temperatures and samples near to the surface (30cm depth) of compost heaps from four facilities. They found that compost temperatures positive for *Legionella* spp. were significantly lower (mean temperature 38.69°C) than compost heaps unfavourable for *Legionella* spp. (mean temperature 53.24°C). There are several scenarios where the outer layer of the windrow is likely to be cooler than the core, including lower ambient temperatures, rainfall and wind. These lower temperatures on the outer surface of the windrow could enable *Legionella* spp. to survive and proliferate under certain conditions during the composting process. A diagram outlining this

theoretical mechanism for *Legionella* spp. proliferation can be found (Figure 6.1) and an example of windrow composting can be seen in Figure 6.2.

This chapter identifies the presence or absence of *Legionella* spp. at different stages of the composting process in order to give an overview of potential contamination points or stages during the process.

6.2. Methods

To determine whether *Legionella* spp. could be isolated from different stages of the composting process, access to a processing facility was necessary. Contact was made with the Organics Recycling Group, which led to a connection with the Group Manager for Scotland, Jenny Grant, who facilitated contact with composting sites. Three sites initially showed interest; however, due to the sensitive nature of this topic, only one processing facility in Scotland agreed to be tested on a single occasion only and under the condition of anonymity.

The outdoor site used domestic mixed green waste from local authority collections and distillery waste as feedstock without peat. These components were transported to the site and placed on a mixed-component pile before being shredded and added to windrows (dimensions L40mxW6mxH3m). These dimensions can be seen in context in Figure 6.2 which shows an example of windrow composting. Each batch of feedstock then underwent a 12-week composting process, with five turns during this period. The resulting compost was screened, with large fragments sent to the biomass industry and small fragments kept for domestic and agricultural composts. The finest particles were retained to create the highest quality products. Screening is followed by four weeks of maturation before the final product is ready; this was stored before and after packaging before transport to retailers or direct sale to consumers. The site manager described that the facility adheres to the BSI PAS 100 guidelines (British Standards Institute, 2018), and windrow temperatures were measured weekly at a depth of 1.5m and ranged 65-80°C. At the site, moisture

was added to the windrows when required to prevent excess airborne particles; however, this was rarely needed due to rainfall which kept the windrows damp. Triplicate samples were taken from: a) the outside layer of the initial feedstock pile (mixed shredded components), b) the six-week windrow core, c) the six-week outer windrow, and d) the outside layer of the mature, end-product pile (16 weeks).

Windrows at different stages of the composting process: microorganisms on the external surface are more exposed to UV and unfavourable weather conditions than those in the core.

Figure 6.2: Example of windrow composting. Image adapted from https://blog.soil3.com/3-ways-to-celebrate-international-compost-awareness-week

The samples were all processed using the same method: 20 g was suspended in 200 ml sterile H2O, mixed and left to settle for 15 mins. The samples were then added to a filter Stomacher bag as used in Section 4.2.3 and blended on low for 30 s, followed by a break for 1 min and another 30 s in the Stomacher laboratory blender on low. The filtrate was added to a 50 ml sterile centrifuge tube, and an aliquot of 500 µl was diluted 10^{-1} in sterile distilled water. From the diluted solution, 200 µl was acid-treated 1:1 with 200 µl HCl-KCl pH 2.2. After 15 mins of acid treatment, 50 µl was plated onto BCYE-α and grown at 37°C in humid conditions for 3-10 days (limit of detection 4.0 x 10^3 CFU/g. compost). Legionella-suspect colonies were sub-cultured on GVPC and cysteine negative agar plates, as described in Chapter 3. This method was repeated for overgrown plates, with the stomacher filtrate diluted 10^{-2} in sterile distilled water instead of 10^{-1}.

In addition to culture, DNA was extracted from each sample using the method described in Section 4.2.3. The DNA extract was diluted to 10^{-1} and 3 µl used in a PCR reaction with JP primers, as also described in Chapter 4.

6.3. Results

Samples from a green waste composting facility were tested by culture and PCR for *Legionella* spp. Cultures were negative for *Legionella* spp. for all samples tested, i.e. from the mixed components, windrows and end product. At six weeks, higher bacterial numbers from outer windrow were seen in comparison with core samples (Figure 6.3), however as noted, none of the colonies were identified as potential *Legionella* spp.

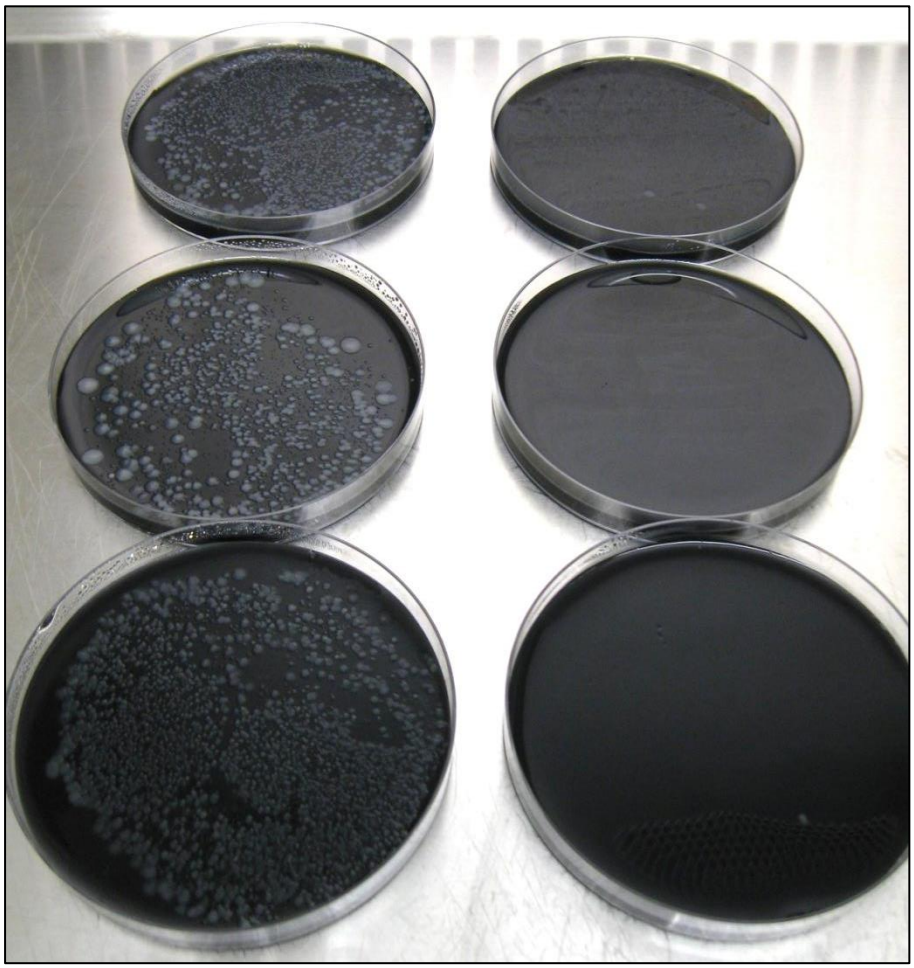
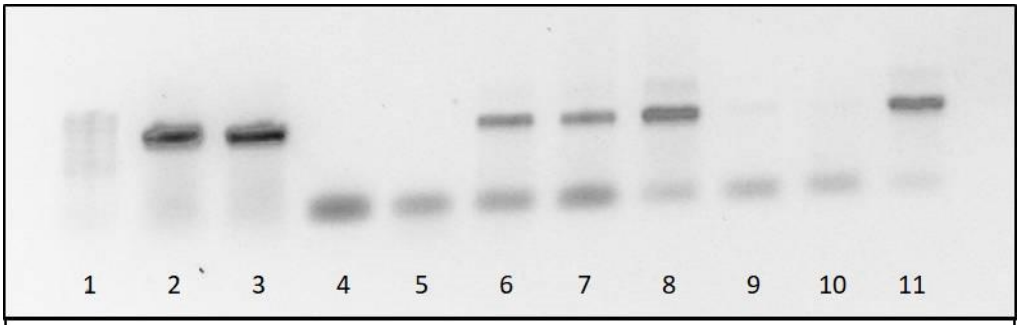


Figure 6.3: Triplicate samples on BCYE-α agar after eight days incubation in humid conditions at 37°C. Left-hand side plates represent samples from week six taken from the outer layer of the windrow. Right-hand side plates represent samples from week six taken from the core of the windrow.

A gel containing PCR products for samples taken from the green waste composting facility can be seen in Figure 6.4. Positive controls (Lanes 2&3) and blanks (Lanes 4&5) gave results as expected. All experimental samples were positive, with the component sample (Lanes 6, 7, 8) and the 16week end product sample (Lane 11) showing stronger bands than both the inner and outer 6-week windrow samples (Lanes 9&10), which were only faintly positive for the JP amplicon, indicating a much lower concentration of starting DNA in these samples.



Key:

1. 50-500bp ladder
2. **+VE CONTROL** *L. pneumophila* Sg 1
3. **+VE CONTROL** *L. longbeachae* Sg 1
4. **BLANK** Primers and nuclease free water
5. **BLANK** Primers and sterile distilled water
6. Component Sample 1
7. Component Sample 2
8. Component Sample 3
9. 6 week core windrow sample
10. 6 week outer windrow sample
11. End product

Figure 6.4: An electrophoresis agarose gel image of JP PCR products from the green waste composting site

6.4. Discussion

This study examined samples from a green waste processing facility, including green waste and distillery waste as mixed components, mature end-product and samples taken from windrow core and outer surface halfway through processing. *Legionella* spp. were not isolated by culture from any of the samples investigated in this study; however, molecular tests were positive. This result is similar to that observed in Chapter 3, where *Legionella* spp were not isolated using culture from four of the samples (numbers 4, 6, 13 and 17) but were identified by the *Legionella* specific PCR, indicating the presence of *Legionella* spp in these samples. Similar results have been published since

the experimental work in this thesis was completed: Marchand *et al.* (2018) investigated sixteen soil samples for *Legionella* spp. using culture- and PCR-based methods and found that all the samples were positive by PCR. In contrast, only one of the samples was culture-positive. As discussed in Section 3.4, there are several possible explanations for differences between PCR and culture results. These include bacterial numbers below detection limits, dead or damaged bacteria with viable DNA (Clark & Hirsch, 2008), or the presence of viable but non-culturable (VBNC) organisms. All scenarios require *Legionella* spp. presence at some point in the samples, regardless of whether they remain viable or not.

The introduction of the EU Landfill Directive 1999/31/EC and commitment by the British Government in the 2011 White Paper *The natural choice: securing the value of nature* (DEFRA, 2011), to the reduction in peat usage in compost is likely to lead to an increase in composted green waste as a component of commercial composts. However, the components used to create a compost can be highly variable, and the combination can affect the population of microorganisms present during processing. For example, a study by Neher *et al.* (2013) examined three different compost 'recipes' including the components manure, silage, hay and hardwood, using high-throughput sequencing to determine bacterial and fungal species present. They found that the microbial community structure of the compost end-product was significantly different dependent on the components used. In a separate experiment, the authors examined the bacterial and fungal communities in three different composting methods where the same recipe was used. They found that the communities present in composts created by the windrow, aerated static pile and vermicompost methods differed by the method used. However, the communities in the windrow and aerated static pile were more similar than the community in the vermicompost. *Legionella*'s phylogenetic class, gamma-proteobacteria, was consistently among the top three bacterial classes with the highest proportion in the compost community, regardless of the method used. Nevertheless, it would not be appropriate to conclude the meaning of this result specifically for *Legionella* spp. in compost without further research due to the diverse nature of this bacterial class.

In addition to the effect of components used and the preparation method on the population of organisms present in compost, the organisms present can, in turn, affect the growth of pathogens in compost. For example, a study by Pietronave *et al.* (2004) looked at the role of indigenous microflora on the growth of gastrointestinal pathogens in compost and found that the most probable number per gram of *Salmonella arizonae* spiked into compost samples increased in sterilised composts at room temperature and at 37°C, and decreased in non-sterilised composts held at the same temperatures over 30 days.

It was not possible to test individual green waste components in this study as they had already been mixed on a pile at the site; however, previous work by Steele *et al.* examined potting soil components and only isolated *Legionella* spp from 17.6% (3/17) fresh components (Steele *et al.,* 1990b). In contrast, *Legionella* spp were present in 72.7% (8/11) composted components tested. These results were mirrored in a study by Casati *et al.* (2010) who found *Legionella* spp in all compost end product tested (3/3 sites), but only isolated these organisms from fresh green waste samples in 1/8 sites tested. The samples in both studies used culture methods similar to those described in Section 3.2.2 to isolate *Legionella* spp. However, samples were not further investigated using more sensitive methods such as amoebal co-culture or PCR, which could indicate that limit of detection is the reason for the observed difference.

In work described in this chapter, PCR results suggest the presence of *Legionella* spp, whether alive or dead, in the mixed green waste components. This supports the theory proposed by Steele *et al.* (1990b), who suggested that small numbers of *Legionella* spp may be present in components. It is, therefore, possible that low numbers of *Legionella* spp are present in fresh green waste components and may multiply to detectable levels during the composting process. In this scenario, *Legionella* spp are likely to be present throughout the composting process, re-establishing numbers following a temperature peak, after maintaining a limited population on the cooler outer surface of the windrow. Patel *et al.* (2015) found that physically covering windrows with a layer of finished

compost increased the length of time the windrows remained at 55°C or above, leading to decreased survival rates of *E. coli* O157:H7 and *Salmonella* spp in the windrows. This may be a viable method for increasing effectiveness of heat inactivation treatments, however may be counterintuitive if the layer of finished compost is contaminated with *Legionella* spp, thus reintroducing these organisms to the windrow.

The mixed green waste component samples tested in this study were negative by culture but positive by PCR for *Legionella* spp. Samples taken from the six-week windrow and mature end product were also negative by culture. While the mature end product showed a solid, positive band for *Legionella* spp PCR, samples taken from the 6-week windrow were only weakly positive (Lanes 9 & 10, Figure 6.3). The inner core of the windrows reaches between 65-80°C during the composting procedure, which is enough to kill *Legionella* spp. When samples cultured from the core and the outer layer of the week-six windrow were compared, a higher level of microbial growth was observed on the samples taken from the outer layer (Figure 6.2). It is, therefore, possible that the outer layer holds the key to the proliferation of these organisms. Taylor *et al*. (2013) suggested that legionellae may lie dormant, replicating within a biofilm when conditions are favourable, while work by Temmerman *et al.* (2006) suggested that *Legionella* spp. can recolonise the environment after heat treatment by feeding on dead cells created during the heating process. Although these studies apply to biofilms in a water environment, it is possible that they can be applied to biofilms that occur in a compost environment. Storage time would allow communities to re-establish themselves, including the production of biofilms likely to support growth of *Legionella* spp. This is supported by the work of Conza *et al.* (2013) who suggested that outdoor storage of compost may increase the number of *Legionella* spp present, possibly due to the increasing likelihood of contamination by *Legionella* spp with increasing storage time. The storage of mature compost may therefore provide an opportunity for *Legionella* spp to re-establish and increase its population size.

It is likely that additional factors also contribute to the survival or reduction of *Legionella* spp in compost. For example, work carried out in Chapter 5 showed that a lack of moisture impacted the proliferation of *Legionella* spp in a compost environment. Pietronaye et al (2004) showed that bacterial populations were more stable in compost samples with higher moisture concentrations (40% and 80% compared with 10%). In samples seeded with *Salmonella arizoniae*, the pathogen concentration declined faster at 10% moisture than at 80% moisture. Kim and Jiang (2010) also showed the role of moisture in the growth of pathogens in dairy compost and suggested compost should be kept as dry as possible to reduce growth. Samples investigated in this chapter were taken in mid-autumn after a period of dry weather, and water had not been added to the windrows during this time. It is possible that the outer layer of the windrow had a lower moisture content than the inner layer due to evaporation, and this may have impacted the survival of *Legionella* spp. on the outer layer of the windrow.

Drying or UV desiccation may cause lysis of *Legionella* spp. on the windrow surface; however, all windrows were stored outdoor within proximity to one another, under the same climatic conditions. Therefore, samples taken from the outer layer of all windrows would be expected to show similar results. This was not the case in this study, as samples from the component pile and the final product windrow were taken from the outer surface, and both showed strong positive bands, while the sample taken from the outer surface of the Week 6 windrow showed a very pale band (Figure 6.3). The pale bands observed from samples taken from the outer and inner parts of the six-week windrow may indicate that organisms present in the components reduce in number due to high temperatures in the composting process, before increasing in number when conditions become more favourable.

While heat may reduce numbers or eliminate *Legionella* spp fully from compost windrows during the composting period, existing low numbers, or bacteria reintroduced through environmental contamination may increase in numbers when left at lower temperatures conducive to *Legionella*

spp growth. Casati *et al.* suggested that windrows were contaminated by exposure to wind or rain, presumably carrying *Legionella* spp in bioaerosols (Casati *et al.* 2010). Numerous studies have associated sporadic Legionellosis with rainfall (Fisman *et al.* 2005, Garcia-Vidal *et al.* 2013*, Hicks et al.* 2007) and work performed in the UK identified *Legionella* spp. in collected rainwater, including *L. pneumophila*, which was identified in aerosols created by a fine spray hosepipe setting, but not in other common garden aerosols (Steege and Moore 2018). *Legionella* spp. present in rain would have the potential to contaminate windrows directly, while bioaerosols may be a mode for wind transmission from one compost windrow to another.

Several studies have identified *Legionella* spp. in compost bioaerosols. Authors in Canada sampled the air at three different compost facilities (domestic, manure and carcass) before, during and after workers undertook compost manipulation activity (Bonifait *et al.* 2017). They examined aerosols using culture and RT-PCR, and while they did not identify *Legionella* spp using culture, or in domestic compost bioaerosols, they did identify *Legionella* spp using qPCR at the manure and carcass compost sites. They also noted that organisms were present in more samples taken after manipulation than before manipulation. Other pathogens, including *Aspergillus fumigatus,* were identified in control samples taken when manipulation of compost had not occurred, indicating that even static windrows are capable of emitting bioaerosols. Similar work performed in the UK identified *L. pneumophila* and *Legionella* spp. by qPCR, but not by culture, from samples of green-waste compost aerosolised in a controlled experimental set up room 2.25m by 4m (Nasir *et al*. 2018).

Windrows observed in the present study were in close proximity to one another (<5m) with weekly turning and it is not unreasonable to consider that bioaerosols released from these windrows may include *Legionella* spp in low numbers which could contaminate neighbouring windrows. Cross-contamination of windrows and, therefore, re-introduction of *Legionella* spp. to heat-treated windrows may also occur due to contamination of the surrounding area, including machinery and facilities. For example, the screening apparatus used at the site described in this study to separate

compost into varying particle sizes was shared between multiple composting sites and posed the potential for cross-contamination of composts after the heated stage. Similar cross-contamination may also occur on worker clothing or hands when moving between windrows. Contamination of later-stage windrows and mature end product storage piles due to contaminated machinery, workers or by subsequent wind or rainfall may reintroduce these organisms to the compost material.

In work outlined in this chapter, *Legionella* spp. were identified by PCR but not by culture, which aligns with results in several other studies which examined compost and compost aerosols, as described above (Bonifait *et al*. 2017, Nasir *et al.* 2018). This is likely due to the high sensitivity of PCR compared to culture, as work in Chapter 3 and work by Marchand *et al.* (2018) both identified *Legionella* spp in compost samples using both culture and PCR, but found that more samples were positive using PCR. *Legionella* spp. have also been identified from bioaerosols using amoebae co-culture to increase sensitivity: Conza *et al.* (2013) isolated *Legionella* spp from bioaerosol samples taken 5m from compost heaps. However, further work to differentiate between live/dead cells should be considered. As described in Chapter 2, propidium monoazide has been suggested as a method to differentiate live cells from dead cells using PCR methods. However, due to the toxicity of this method, it has been suggested that this would not be suitable for use with environmental samples (Taylor, et al., 2014).

Fainter bands identified in PCR products from the 6-week windrow samples compared with samples taken at other stages of the process may suggest that the heat generated during the composting process works to reduce the concentration of *Legionella* spp. However, the bold bands observed in PCR products from samples taken in the compost end product at 16 weeks suggests an increase in *Legionella* spp in the second half of processing. This may be due to the regrowth of a small number of pathogens that survived the composting process or were reintroduced through cross-contamination. There are some potential routes for cross-contamination as described above,

including wind, rain, bioaerosols, workers hands and clothing, machinery, or a combination of these. Further work to identify if contamination occurs and by what method using qPCR would be helpful to investigate this further. Examination of windrows at different times of year and under different climatic conditions may aid understanding in this area. This study was relatively small for significant conclusions to be drawn; however, results indicate that the composting process is likely to reduce the number of *Legionella* spp. present. It follows that steps could be taken to optimise this process, and to minimise contamination in order to reduce the potential risk posed by these organisms.

7. CONCLUSIONS AND FUTURE WORK

7.1. Conclusions

This chapter outlines the conclusions for the work described in this thesis and proposes areas for future work in this field. The overarching aim of the work outlined in this thesis was to investigate the ecology of *Legionella* spp. in compost. The literature review highlighted key gaps in the literature, specifically around the lack of studies completed using species other than *L. pneumophila* Sg1 and the lack of studies examining environmental *Legionella* spp. in areas other than the water habitat. In order to build on scientific understanding in these areas, three key questions were identified. These were, 1) To what extent are *Legionella* spp. present in UK composts? 2) Do storage conditions impact the survival and proliferation of *Legionella* spp? and, 3) Are *Legionella* spp. detectable during the manufacture of compost products?

In order to answer the first question, in Chapter 3, samples were obtained from 24 different compost formulations and examined for the presence of *Legionella* spp. using culture from initial samples and after an 8-week enrichment period. Fifteen of the twenty-four (62.5%) samples tested contained *Legionella* spp. Ten of twenty-four (41.7%) were positive after initial testing, and of the 22 samples which were re-tested after enrichment at 30°C, 13 (59.1%) tested positive after the eight-week incubation period. Twelve species were identified in the compost samples using *mip* gene speciation, with the most commonly isolated *Legionella sp*ecies was *L. sainthelensi*, which were present in 5 of the 24 (20.8%) samples, but only after the 8-week enrichment period. All samples (24/24, 100%) were positive for *Legionella* spp. by PCR. The work documented in Chapter 3 demonstrated that *Legionella* spp. are commonly present in compost products available in the UK.

Given that compost is widely used in the UK, yet the reported number of *Legionella* spp. infections remains relatively small, it is important to understand how storage conditions impact the survival and proliferation of *Legionella* spp. The work in Chapters 4 and 5 aimed to answer this question. In

Chapter 4, methods for DNA extraction and semi-quantitative PCR analysis were developed and assessed for suitability, respectively. *In silico* analysis and development of a standard curve for the Wellinghausen primers identified these as suitable for use in further experiments. Next, DGGE was investigated to determine if different *Legionella* spp. could be easily distinguished following molecular identification in a compost sample. Multiple bands were observed from positive control and experimental samples. All were identified as *Legionella* spp following sequencing; however, this method was most successful in control samples. Much more work would be needed before this method could be used robustly. Finally, DNA extraction protocols were compared, and the best performing protocol was chosen for use in future work. This involved adding Tween and TTSP to samples, before processing in a stomacher blender. The filtrate was placed into a centrifuge tube and centrifuged at 1000g for 5 mins. The supernatant was then placed into a clean centrifuge tube and centrifuged for 10mins at 10000g, before the pellet was used in the MoBio kit

Following on from the method development in Chapter 4, the chosen methods were used in Chapter 5 to investigate the behaviour of *Legionella* spp. in compost under three different mock greenhouse settings; spiked greenhouse (SGH), unspiked greenhouse (NSGH) and incubator (INC). Batches of compost were both spiked and unspiked with *Legionella* spp. and *Acanthamoeba* spp. They were examined to determine situations in which *Legionella* spp. may proliferate and, therefore, pose a risk to public health. A limitation of this work was the lack of repeat sampling performed, and overall, results were inconclusive due to the variable nature of the compost matrix. However, the experiments showed that humidity is likely a critical factor in the maintenance and proliferation of these organisms in compost. There was also evidence that *Legionella* spp. may behave differently in peat-containing composts than peat-free composts in the greenhouse setting. The greenhouse setup and DNA extraction process developed in this chapter are suitable for further studies which may include further investigation of the role of compost components e.g. peat, and exploration into the role of other species e.g. *Acanthamoeba* spp. in the survival and maintenance of *Legionella* spp. in compost.

In addition to investigating how Legionella spp. behave in a greenhouse setting, work was also performed in Chapter 6 to determine if *Legionella* spp. are detectable during the manufacture of compost products. Identifying *Legionella* spp. in compost is a sensitive issue, and there was difficulty engaging with compost manufacturers in Scotland. As a result, only one green waste processing site agreed to be tested on a single occasion. The site consisted of windrows which were turned weekly for 12 weeks before being left to mature into the end product. Green waste feedstock, mature product, and samples from the inner core and outer layer of a 6-week windrow were tested using culture and PCR. *Legionella* spp. were not identified using culture but were identified using PCR at all stages of the process; however, samples taken at the 6-week stage were lower in numbers than samples taken at other stages. Due to a lack of engagement from compost manufacturers, it was impossible to perform sufficient sampling in this experiment to draw firm conclusions. However, this study highlighted that *Legionella* spp. are present in components used to manufacture green waste composts, are likely inhibited at high temperatures inside the windrow and that numbers can recover in mature composts. Given that these organisms were identified through PCR but not culture indicates that they are only present in small numbers or in a VBNC state. This also suggests that the higher temperatures required for the PAS 100 process do work to remove these organisms to an extent but may not be sufficient for complete removal. Cross-contamination, for example, from aerosols, workers or machinery may re-introduce *Legionella* spp. to composts at a later stage.

This thesis provides an overview of *Legionella* spp. in the compost environment. A large proportion of the composts available to purchase in Scotland tested positive for *Legionella* spp, but this is not proportional to the case numbers observed in Scotland. Several possibilities may explain this; for example, it may be that the majority of species present are adapted to be non-pathogenic or cause mild disease; that diagnostic ascertainment is poor; that the organisms are present in insufficient quantities to cause infection; or a combination of these factors. Although it was impossible to draw firm conclusions from a number of the experiments performed, the work outlined here provides novel information that contributes to the knowledge of *Legionella* spp. behaviour in compost

materials.The methods outlined in this thesis could be used for environmental testing in an outbreak scenario or as a monitoring tool during the compost production process. If used routinely as part of the composting manufacturing process, over time, this could further clarify the ecology of *Legionella* spp in compost by identifying baseline levels, and changes in different processing scenarios such as warm weather. Peat is being phased out as a component of compost in the UK, and climate change is likely to have an impact on manufacturing and storage conditions. The methods could also be used to provide information on the impact of changing components and climate on the presence of *Legionella* spp in the compost environment over time.

When approaching this from a public health perspective, these organisms should still be considered during diagnostic investigation in patients presenting with an appropriate history. An epidemiological link between an environmental and clinical isolate of *Legionella longbeachae* has not been confirmed using genomics. The methods outlined in this thesis may also be used for further study to investigate potential for links between clinical infection and presence of these organisms in the compost environment. Rather than limit the use of compost, it is perhaps sensible to focus on how the proliferation of these organisms to infectious levels can be prevented. This may include advice to take common-sense precautions when opening bags and manipulating compost material. In Australia, these are general hygiene warning labels on compost bags to educate users of potential risks, and it would be beneficial to consider a similar scheme in the UK.

7.2. Research impact and future work

Work from Chapter 3 was published in *Clinical Microbiology and Infection* (Currie *et al*., 2013) and has had 25 citations (Scopus 31/07/2022). This work was picked up by a number of local, national and international news outlets including the BBC (Legionella 'common' in compost brands, Strathclyde University study finds - BBC News) and the Guardian newspaper (Does compost really pose a threat to our health? | Compost | The Guardian). In addition, one of the isolates from work in Chapter 3 was used by another group for further study in this field (Bacigalupe *et al*., 2017). Further

work summarising issues around compost and *Legionella longbeachae* infection was published in Perspectives in Public Health (Currie *et al*. 2015) and, to date, has had 20 citations (Scopus 31/07/2022). This paper highlighted that collaboration between growing media manufacturers and researchers should be used to improve knowledge in this field. However, more research is needed before definitive conclusions can be drawn. There are several ways in which the work outlined in this thesis could be expanded upon in the future, including:

Further engagement with environmental bodies and industry to explore routes by which the PCR method developed could be used as a monitoring tool during the compost production process.

Understanding the structure of the microbiome may provide additional insight into the behaviour of *Legionella* spp. and its interaction with other species, including free-living amoebae in compost under different conditions, for example, different components, moisture content and greenhouse storage.

Full characterisation of free-living amoebae species present in compost and studies to explore their interaction with different species of *Legionella* spp. at different temperatures and nutrient densities. This could also be applied to the characterisation of fungi in composts.

Work using methods developed in Chapter 4 to build on the experiments performed in Chapter 5 should use multiple repeats to ensure statistical analysis is possible so that firmer conclusions can be drawn.

Further engagement with industry to enable analysis of different composting sites at different times and under different conditions to fully explore the role of climate and feedstocks on the survival of these organisms during production and the use of optimised molecular methods to examine these samples. This would also enable the comparison of different types of composting, such as in-vessel and windrow. Long-term analysis could be used to monitor the impact of climate change and changing component feedstocks over time.

The work outlined in this thesis has added to the available knowledge of how *Legionella* spp. survive and proliferate in the compost environment. However, further work in these areas should be completed with the aim to minimise any risks posed to workers and members of the public exposed to these organisms in this environment.

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9. APPENDIX 1: TEMPERATURE AND HUMIDITY READINGS FROM GREENHOUSE EXPERIMENTS

Table 9.1: Temperature and humidity readings from SGH setup

Date	Soil temp (°C)	Air temp (°C)	Air humidity (%)
1/10/13	26	27.4	28
3/10/13	25	26.7	53
4/10/13	26	25.8	43
8/10/13	27	26.3	42
11/10/13	31	36	10
15/10/13	32	34.9	10
18/10/13	33	32.3	22
28/10/13	34	29.5	33
29/10/13	30	No reading	10
21/11/13	31	32.2	10
25/11/13	31	31.5	10
1/12/13	31	31.7	10
Mean	**29.75**	**30.39**	**23.42**

Table 9.2: Moisture content analysis of SGH temperature-control samples

Sample	Dry Tray Weight (M_T)	Wet soil and tray (M_W)	Dry soil and tray (M_D)	Moisture content: $\frac{M_W - M_D}{M_W - M_T} \times 100$	Mean (%)
Initial	1.29	8.81	4.3	59.97	57.40
	1.3	10.05	5.51	51.89	
	1.3	8.84	4.29	60.34	
Day 3 GH	1.29	8.51	4.32	58.03	58.27
	1.29	8.56	4.28	58.87	
	1.3	8.95	4.52	57.91	
Day 3 15°C	1.3	9.47	4.58	59.85	59.46
	1.29	8.88	4.32	60.08	
	1.3	8.16	4.15	58.45	
Day 7 GH	1.3	8.26	4.24	57.76	60.28
	1.31	8.07	3.88	61.98	
	1.29	9	4.29	61.09	
Day 7 15°C	1.3	7.69	3.88	59.62	59.39
	1.3	8.45	4.28	58.32	
	1.31	7.92	3.94	60.21	
Day 14 GH	1.31	7	3.58	60.11	60.08
	1.29	7.51	3.78	59.97	
	1.29	7.24	3.66	60.17	
Day 14 15°C	1.3	8.15	4.02	60.29	60.66
	1.31	8.22	4.01	60.93	
	1.3	8.51	4.13	60.75	
Day 21 GH	1.3	8.56	3.99	62.95	61.69
	1.3	8.57	4.06	62.04	
	1.3	10.67	5.04	60.09	
Day 21 15°C	1.32	9.66	4.69	59.59	59.73
	1.3	7.43	3.75	60.03	
	1.31	8.63	4.27	59.56	
Day 28 GH	1.3	10.13	5.33	54.36	58.89
	1.3	7.86	4.12	57.01	
	1.3	8.19	3.69	65.31	
Day 28 15°C	1.3	9	4.44	59.22	55.88
	1.3	7.95	4.06	58.50	
	1.3	7.29	4.3	49.92	
Day 42 GH	1.27	4.82	2.67	60.56	61.36
	1.25	4.25	2.37	62.67	
	1.32	4.59	2.6	60.86	
Day 42 15°C	1.28	4.75	2.67	59.94	57.81
	1.27	4.22	2.52	57.63	
	1.29	4.44	2.68	55.87	

Table 9.3: Temperature and humidity readings from NSGH set up n.b "*" represents reading taken after water tray was refilled

Date	Soil temp (°C)	Air temp (°C)	Air humidity GH (%)
7/12/13	29	30.4	79
9/12/13	29	31.3	*80
10/12/13	29	29.9	90
11/12/13	30	31.6	*80
12/12/13	30	31.6	75
13/12/13	30	32.1	*76
16/12/13	28	30.3	*70
17/12/13	29	30.6	84
18/12/13	29	32.1	40
19/12/13	28	29.2	78
Mean	**29.1**	**30.9**	**75.2**