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A COMPARATIVE STUDY USING POLYMERASE CHAIN REACTION, CULTIVATION AND IMMUNO-MAGNETIC SEPARATION FOR DETECTION, ISOLATION, AND IDENTIFICATION OF *LEGIONELLA* SPP. IN WATER AND BIOFILM SAMPLES FROM GROUNDWATERS

Teresa Jane Brooks

Thesis submitted to the Department of Biochemistry, Microbiology and Immunology in partial fulfillment of the requirements for the degree of Master of Science

University of Ottawa
Ottawa, Ontario, Canada

ABSTRACT

This study was carried out to determine the frequency and levels of occurrence of legionellae in groundwaters from a variety of sources in U.S. and Canada. A limited number of water samples from cooling towers were also tested because municipally treated waters are considered to be the main sources of *Legionella* in artificial habitats such as cooling towers, air conditioners, whirlpools, hot tubs and plumbing systems (Krammer and Ford, 1994).

Conventional procedures, including cultivation and polymerase chain reaction (PCR), were used to determine the presence of *Legionella* spp. in the water and biofilm samples during this study. A novel approach, using immuno-magnetic separation (IMS) in combination with cultivation or PCR, was also explored as an improved and rapid detection method for *Legionella* over conventional procedures.

In this study, the number of positive samples by conventional cultivation (29.1%) and the number of positive samples by semi-nested PCR (28.2%) were approximately equal. Analysis of the conventional cultivation samples revealed that more *Legionella* spp. were isolated using the selective media and treatments along with an incubation temperature of 30°C over the conventional 35°C. PCR inhibitors in the water and biofilm samples were a problem during this study.

The IMS-cultivation procedure used during this study on pure suspensions of Legionella gave average recovery rates of 10-20%. Both combined and uncombined IMS were used. When using *Legionella* suspended in a sterilized environmental sample, the recovery rates of the two techniques were comparable.

Because the IMS technique was continually being improved over the course of this study, all the samples received did not undergo the same procedure. Using only the IMS results from the samples undergoing the finalized IMS procedure and comparing this technique with conventional cultivation, IMS-cultivation recovered *Legionella* spp. from fewer samples and had a higher number of overgrown plates. On the other hand, the number of positive samples using IMS-PCR was similar to the results using PCR without prior IMS.

Overall, this study determined that *Legionella* were present in the environmental samples, including water and biofilm samples from underground sources, and can be isolated successfully using conventional cultivation and PCR techniques. Additionally, although the IMS procedure used in this study did not improve the cultivation and PCR results, it is still a promising method for improving *Legionella* recovery from environmental samples.

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1.0 BACKGROUND AND INTRODUCTION

In North America and elsewhere, waters from underground sources are regularly extracted for industrial, agricultural, community and personal uses (de Villiers, 1999). Depending on the intended use of such waters, guidelines are available for allowable levels of many chemical and biological contaminants (US EPA, 2001). One class of biological agents not yet included in such guidelines are members of the bacterial genus *Legionella.*

While the human health impact of legionellae as opportunistic pathogens is increasing with the mounting numbers of those immunocompromised due to increased life expectancy and acquired or induced immunosuppression, very limited information is available on the presence of these bacteria in groundwaters. As a result, this study was carried out to determine the frequency and levels of occurrence of legionellae in groundwaters from a variety of sources in U.S. and Canada. A limited number of water samples from cooling towers were also tested because municipally treated waters are considered to be the main sources of *Legionella* in artificial habitats such as cooling towers, air conditioners, whirlpools, hot tubs and plumbing systems (Krammer and Ford, 1994).

Conventional procedures, including cultivation and amplification by polymerase chain reaction (PCR), were used to determine the presence of *Legionella* spp. in the water and biofilm samples during this study. A novel approach, using immuno-magnetic separation (IMS) in combination with cultivation or PCR, was also explored as an improved and rapid detection method for *Legionella* spp. over conventional procedures.

1.1 Historical Perspective

The 1976 outbreak of pneumonia at an American Legion convention in Philadelphia, PA, later termed Legionnaires' Disease (LD) (Hacker *et al.*, 1993), was found to be caused by a Gram-negative, rod-like, facultative, intracellular bacterium (Hacker, Ott, Wintermeyer, Ludwig, and Fischer, 1993). This newly discovered pathogen, named *Legionella pneumophila,* was subsequently incriminated also as the causative agent of Pontiac fever, a milder infection of the respiratory tract (Hacker, Ott, Wintermeyer, Ludwig, and Fischer, 1993).

Since then, many more members of the genus *Legionella* have been isolated and implicated in the etiology of both community-acquired and nosocomial infections. *Legionella* is considered an opportunistic pathogen, causing disease normally only in individuals who are immunocompromised or have underlying lung conditions. It is estimated that 8,000 to 18,000 cases of LD occur in the United States each year (Kool, Carpenter, and Fields, 1999) with *L. pneumophila* accounting for 70-80% of the cases (Edelstein and Meyer R.D., 1994). *L. micdadei, L. longbeachae, L. dumoffii,* and *L. bozemanii* are among the more commonly incriminated species after *L. pneumophila* (Edelstein and Meyer R.D., 1994). It is believed that members of the genus *Legionella* cause 3 to 8% of the cases of community-acquired pneumonia that requires hospitalization (Benson and Fields, 1998).

1.2 The Family *Legionellaceae*

So far, a total of 41 *Legionella* species containing 62 serogroups have been characterized, with 21 of these being reported as pathogenic in humans (Miyamoto *et al.*, 1997);(Szewzyk *et al.*, 2000);(Winn, 1999) (Table 1-1). Bacteria known as *Legionella-*

like amoebal pathogens (LLAPs) have also been described. In addition to being labeled LLAPs, they have been identified as *L. lytica* and previously identified at *Sarcobium lyticum* (Winn, 1999). LLAPs are capable of multiplying in the cytoplasm of amoebae, but they are difficult to cultivate on media designed to support the growth of other *Legionella* species (Newsome *et al.*, 1998). Additional studies have shown that they could occasionally be cultured on buffered charcoal yeast extract (BCYE) agar (Giles *et al.*, 1995). Recently, the LLAPs have been renamed as *Legionella* species (Adeleke *et al.*, 2001).

In 1980 and 1981, it was proposed to reclassify certain members of the genus *Legionella* into two new genera: *Tatlockia* representing one species, *T. micdadei,* and *Fluoribacter* containing three species, *F. bozemanae, F. dumcjfii,* and *F. gormanii* (Bangsborg, 1997). Later, it was generally agreed to maintain the usage of a single genus (Bangsborg, 1997).

Table 1-1: Known *Legionella* sp. isolated from humans and the environment (Winn, 1999;Miyamoto, *et al.*, 1997).

Legionella sp.	Serogroup	Isolated from:		Auto-fluorescence
		Humans	Environment	
L. pneumophila	15	Yes	Yes	-
L. micdadei	1	Yes	Yes	-
L. bozemanii	2	Yes	Yes	BW
L. dumoffii	1	Yes	Yes	BW
L. feeleii	2	Yes	Yes	-
L. gormanii	1	Yes	Yes	BW
L. hackeliae	2	Yes	No	-
L. israelensis	1	Yes	Yes	-
L. jordanis	1	Yes	Yes	-
L. sainthelensi	2	Yes	Yes	-
L. longbeachae	2	Yes	Yes	-
L. maceachernii	1	Yes	Yes	-
L. oakridgensis	1	Yes	Yes	-
L. wadsworthii	1	Yes	No	YG
L. birminghamensis	1	Yes	No	YG
L. cincinnatiensis	1	Yes	No	-
L. anisa	1	Yes	Yes	BW
L. tucsonensis	1	Yes	No	BW
L. lansingensis	1	Yes	No	-
L. cherrii	1	Yes	Yes	BW
L. erythra	2	No	Yes	R
L. jamestownensis	1	No	Yes	-
L. parisiensis	1	Yes	Yes	BW
L. shakespearei	1	No	Yes	-
L. santicrucis	1	No	Yes	-
L. steigerwaltii	1	No	Yes	BW
L. adelaidensis	1	No	Yes	-
L. fairfieldensis	1	No	Yes	-
L. brunensis	1	No	Yes	-
L. moravica	1	No	Yes	-
L. quinlivanii	2	No	Yes	-
L. gratiana	1	No	Yes	-
L. quateirensis	1	No	Yes	-
L. nautarum	1	No	Yes	-
L. worsleiensis	1	No	Yes	-
L. londiniensis	1	No	Yes	-
L. geestiana	1	No	Yes	-
L. rubrilucens	1	No	Yes	R
L. spiritensis	2	No	Yes	-
L. waltersii	1	No	Yes	-
"*L. lytica*" (amoebal pathogens)	1	No	Yes	NA

BW - Blue-White, R – Red, YG – Yellow-Green, NA – Not available

1.3 Characteristics of *Legionellacae*

Legionella spp. are nutritionally fastidious, nonspore-forming, aerobic, Gram-negative bacteria (Rogers and Pascule, 1991). When taken from an autopsied lung or from environmental samples (like those grown in amoeba), they appear as coccobacilli or short rods, with nonparallel sides tapering to rounded ends (Rodgers, 1985; Edelstein and Meyer, 1994). The dimensions of freshly isolated cells are approximately 0.5 X 1 to 2 μm (Rodgers, 1985), but after passage on culture media, cells measured 0.3-0.7 μm in width X 2-3 μm in length. However, it is not uncommon to find forms up to 20 μm in length (Rodgers, 1985; Edelstein and Meyer, 1994). With the exception of *L. oakridgensis, L. nautarum,* and *L. londiniensis*, all *Legionella* are motile by means of polar or subpolar flagella, and these flagella appear to share a common antigen (Rodgers and Laverick, 1984).

1.4 *Legionella* Reservoirs

Legionella species are natural inhabitants of fresh water and soil. They have been isolated from lakes, rivers, streams, natural thermal lagoons, mud samples, and groundwater. These habitats have included temperatures ranging from 63°C to frozen rivers, lakes with an oxygen content below 0.2 ppm to greater than 15.0 ppm, pH ranging from 5.0 to 8.5 and with conductivities between 10 and 120 μS/cm (Fliermans, 1984). Although ubiquitous in surface waters, there is very limited information about their occurrence in groundwaters, many of which may not undergo full conventional treatment before reaching the consumer. Natural water sources are rarely connected to *Legionella*-induced disease because the conditions in these waters do not allow *Legionella* organisms to grow to high numbers. The only natural waters considered sources of outbreaks are

natural warm water spas with temperatures ranging from 30°C to 40°C, occasionally up to 60°C (Szewzyk *et al.*, 2000). The elevated water temperature may give *Legionella* a selective advantage over other microorganisms since it is able to grow at higher temperatures.

There is substantial evidence that *Legionella* spp. exist in nature by growing in free-living amoebae including *Acanthamoeba castellanii neff* (Holden *et al.*, 1984), *Acanthamoeba royreba* (Tyndall and Domingue, 1982), *Hartmanella vermiformis* (King *et al.*, 1991), *Naegleria fowleri* (Newsome *et al.*, 1985), *Naegleria lovaniensis* (Tyndall and Domingue, 1982), and *Tetrahymena pyriformis* (Fields *et al.*, 1986; Fields *et al.*, 1984). In the environment, the presence of naturally-occurring protozoa, blue-green algae, and other microorganisms may enhance the growth of legionellae (Wadowsky and Yee, 1985). Wadowsky and Yee (1985) also showed that in experimental settings, the presence of protozoa and blue-green algae helped stabilize *Legionella* in aerosols. Legionnaires' disease is contracted through breathing aerosolized particles of the bacterium. If aerosol stability is also increased outside the laboratory setting, the risk of contracting the disease could be increased if protozoa are also present. *Legionella* have been shown to utilize the extracellular products of blue-green algae for extracellular growth (Tison *et al.*, 1980). Tison *et al.* (1980) demonstrated that *L. pneumophila* could derive all of its nutritional requirements for growth through its association with *Fisherella* sp., a blue-green alga. Some bacteria that have been shown to stimulate the growth of *Legionella* are various species of genera including *Flavobacterium*, *Pseudomonas*, *Alkaligenes*, and *Acinetobacter* (Krammer and Ford, 1994).

In addition to natural reservoirs, *Legionella* spp. are frequently isolated from man-made water storage and distribution systems. These systems include showerheads, hot water storage tanks, and boiler outlets (Harley *et al.*, 1997). The presence of biofilm on the surfaces of these systems can harbor *Legionella* from the residual disinfectant and from increased temperatures that may be present. Most cases of infection from *Legionella* are associated with conditions that amplify and spread the bacilli such as heat-exchange devices and water distribution systems in large buildings (Edelstein and Meyer, 1994). Also implicated in disease have been whirlpool spas (Centers for Disease Control and Prevention, 1997), a decorative fountain (Hlady *et al.*, 1993), as well as respiratory therapy equipment (Arnow *et al.*, 1982).

1.5 Clinical Considerations

There are two diseases associated with *Legionella* species, i.e. Legionnaires' disease and Pontiac Fever. Both diseases can be caused by the same *Legionella* spp. (for example, *L. pneumophila*). The conditions under which an individual develops either Legionnaires' disease or Pontiac Fever are not fully understood but may depend on the health status of the individual, the degree of exposure to the organism, or the strain-specific virulence (Rogers and Pascule, 1991).

Pontiac Fever is a self-limiting, febrile illness usually affecting healthy individuals (Glick *et al.*, 1978). It was been shown to be the result of exposure to high levels of nonviable bacterial cells or bacteria unable to multiply in lung phagocytes (Fields *et al.*, 2001). Because it is self-limiting, this form of *Legionella* infection is not as much of a concern. On the other hand, the symptoms of Legionnaires' disease range from mild cough and low-grade fever to rapidly progressive pneumonia with multi-organ

failure and fevers in excess of 40°C (Lin *et al.*, 2001; Rogers and Pascule, 1991). The effects of the disease can be severe. During an outbreak it is fatal in 20 to 40% of cases (Kool *et al.*, 2000). Because of the seriousness of this disease, the presence of the causative bacterium in water supplies is a concern.

1.5.1 Mode of Transmission

Legionnaires' disease can be acquired by inhalation of aerosols or microaspiration of water containing legionellae (Edelstein, 1993). The critical value for infection was found to be approximately 10 CFU/mL (Krammer and Ford, 1994; US EPA, 1992). Legionellae-containing aerosols of 5 to 15 μm in diameter, can readily enter the respiratory tract and colonize the alveolar air spaces of the lung, infect the alveolar macrophages, and induce disease (Rogers and Pascule, 1991). Human-to-human transmission has not been observed (Hacker *et al.*, 1993). There is also no evidence to support drinking of contaminated water as a means of transmission (Edelstein and Meyer, 1994). Infections with one species, *Legionella longbeachae*, has been associated with gardening and the use of potting soil in Australia and Japan, (Koide *et al.*, 1999; Steele *et al.*, 1990) and more recently in the United States (Centers for Disease Control and Prevention, 2000). The mode of acquisition in these cases is thought to be by inhalation of particles containing the bacterium.

1.5.2 Intracellular Replication

Legionellae are phagocytized by host cells and reside, or even replicate, intracellularly (Horwitz, 1983a; Horwitz, 1983b; Horwitz and Maxfield, 1984). Entry into the host cell is generally through coiling phagocytosis (Horwitz, 1987). This type of phagocytosis is seen with other pathogens such as *Leishmania donovani, Chlamydia*

psittaci, Trypanosoma brucei, and *Borrelia burgdorferi* (Segal and Shuman, 1999), and can occur even with heat-killed *L. pneumophila* (Segal and Shuman, 1999). Nearly 50% of the internalized cells are killed during this process (Horwitz and Silverstein, 1981). The invasion is mediated by complement components, antibody Fc receptors, or parasite-directed endocytosis (Fernandez *et al.*, 1996).

Intracellular replication of *Legionella* spp. can occur in protozoan hosts as well as in human macrophages (Steinert *et al.*, 1997). Replication is affected by many factors including temperature, the nutritional condition of the host, and the availability of ferrous and ferric ions (Pope *et al.*, 1996; Steinert *et al.*, 1994). Tests using acanthamoebae have shown that, at 20°C, the phagocytized *Legionellae* are quickly digested, but at 35°C, extensive intracellular multiplication can occur (Tyndall and Domingue, 1982). To survive inside the host, *L. pneumophila* inhibits phagosome-lysosome fusion and phagosome acidification (Horwitz, 1983b; Horwitz and Maxfield, 1984). To be virulent, legionellae must subvert host phagosome processes at a very early stage in the internalization process (Fernandez *et al.*, 1996). To my knowledge, these initial processes have not been completely elucidated.

One of the differences between macrophages and protozoa as hosts for legionellae is the protozoa's ability to expel membrane-bound compartments that contain large numbers of living bacterial cells (Segal and Shuman, 1999). They are also capable of encapsulating the bacteria in a protective coating when in low moisture or poor nutrient environments (Segal and Shuman, 1999).

1.5.3 Host Immunity and Conventional Treatments

Immunity is provided by the cellular immune system and therefore any disease or medical treatment that reduces the systemic or local cellular immunity increases the risk of contracting Legionnaires' disease (Edelstein and Meyer, 1994).

To be effective in the treatment of legionellosis, antibiotics must be able to reach and kill legionellae inside the host cells (Rogers and Pascule, 1991). Such effective antibiotics include erythromycin, rifampicin, and the 5-fluoroquinolones (Fitzgeorge *et al.*, 1986) as well as the new generation of macrolides. *L. pneumophila* isolated from European water systems were susceptible to clarithromycin (MICs 0.03-0.5 mg/L), erythromycin (MICs 0.125-2.0 mg/L), ciprofloxacin (MICs 0.06-0.25 mg/L), pefloxacin (MICs 0.06-0.5 mg/L) and rifampicin (MICs 0.007-0.015 mg/L) with MIC (mean inhibitory concentration) being the lowest drug concentration at which no growth occurred (Koide *et al.*, 1999).

Early diagnosis of the disease usually leads to successful treatment, whereas inappropriate identification and therefore improper treatment often leads to a high number of fatalities (Rogers and Pascule, 1991). This high mortality rate is reflective of the immunocompromised states of the individuals who normally contract this disease (Rogers and Pascule, 1991).

1.6 Known Virulence factors

There are well-defined virulence factors in *L. pneumophila* (Winstanley and Morgan, 1997) although information on other *Legionella* spp. is not as complete. For *L. pneumophila*, virulence factors include the region of the genome containing the *icmwxyz* genes for intracellular replication (Brand *et al.*, 1994), the *dotA* gene that is involved in

intracellular replication and organelle recruitment (Berger and Isberg, 1993);(Berger *et al.*, 1994), and the Mip protein that is involved in initiation of intracellular infection of human macrophages as well as exhibiting peptidyl-prolyl cis/trans isomerase activity (Cianciotto *et al.*, 1989). These factors are all required for intracellular infection although the exact role each plays is not known (Segal and Shuman, 1997). In addition, the major outer membrane protein (MOMP), encoded by the *ompS* gene, binds complement components resulting in internalization of bacteria into macrophages (Bellinger-Kawahara and Horwitz, 1990). Other virulence factors are still being identified (Segal and Shuman, 1997). Of these virulence factors, only the Mip protein will be dealt with in greater detail since it is pertinent to this study.

1.6.1 Macrophage Infectivity Potentiator (Mip)

A bacterial protein, called macrophage infectivity potentiator or Mip, is important for optimal bacterial entry into cells (Edelstein and Meyer, 1994; Cianciotto *et al.*, 1989). Mip has been described as a highly basic (pI 9.8) membrane-associated protein of 24 kDa (Hacker *et al.*, 1993) expressed on the surface of the bacteria (Cianciotto *et al.*, 1989). Mip's role in infection is supported when the characteristics of infection are compared with the characteristics of the protein (Cianciotto *et al.*, 1989). Cationic polypeptides are known to enhance membrane interactions, and cationic lysosomotropic agents raise intralysosomal pH and inhibit phagosome-lysosome fusion (Cianciotto *et al.*, 1989). These effects are seen in *Legionella* infections (Cianciotto *et al.*, 1989).

In *Legionella* spp. the Mip protein is made from a monocistronic mRNA (Cianciotto *et al.*, 1989). The amino acid sequence of the Mip protein is highly conserved with conservation being highest in regions associated with PPIase (peptidyl

prolyl *cis trans* isomerase) activity (Ratcliff *et al.*, 1997). The complete sequence ranges from 232-251 amino acids with sequence conservation ranging from 82% to 99% (69-97% at the nucleotide level) (Ratcliff *et al.*, 1997). "Kyte and Doolittle hydophobicity plots and Chou and Fasman estimates of the secondary structure confirm significant conservation of the secondary structure (Ratcliff *et al.*, 1997)." Mip genes have been shown to have approximately 50% homology to genes found in other pathogenic bacteria including *Chlamydia trachomatis, Neisseria meningitidis,* and *Pseudomonas aeruginosa* (Hacker *et al.*, 1993; Ott, 1994).

The Mip protein has been associated with virulence although the Mip sequences are highly conserved in virulent and non-virulent strains of *L. pneumophila* (Hacker *et al.*, 1993). Mip proteins have been found in most other *Legionella* spp. irrespective of their association with disease (Ratcliff *et al.*, 1997). Since this protein is found on the surface of the bacteria and it is almost universal among *Legionella*, it was used as the target for the IMS method employed in this study (explained in further detail below).

1.7 Factors Influencing The Presence Of *Legionella* In The Environment

1.7.1 Temperature, and pH

One selective advantage that *Legionella* spp. have over other organisms is their ability to grow at temperatures between 45-55°C, although this is not their optimal growth range (Krammer and Ford, 1994). In experimental situations, 50°C for 30 minutes was found to have no adverse effect on the viability of legionellae, but longer heat treatments did reduce their numbers (Harley *et al.*, 1997). Legionellae are also acid tolerant (Harley *et al.*, 1997). A pH of 2.2 for 5 minutes has no adverse affect on their

viability (Harley *et al.*, 1997). These properties have been exploited to isolate *Legionella* from the samples received during this study (see below).

1.7.2 Salinity/Sodium Chloride

Experiments dealing with the effect of salinity on the growth of *Legionella* spp. give conflicting results. In one experiment, NaCl concentrations of 0.65% were found to inhibit the growth of virulent strains of *Legionella* (Marra, 1992). In a separate experiment, it was found that salt concentrations (ranging from 0.1% to 3% w/v NaCl) had no negative effect on survival of *L. pneumophila* at temperatures below 20°C. There was evidence of a possible protective effect of NaCl at 20 °C. However, when samples were incubated at 30°C and 35°C, higher salt concentrations (above 1.2%) caused a rapid decline in cell numbers. At these temperatures there appeared to be a protective effect only at concentrations between 0.1% and 0.5% (Heller *et al.*, 1998).

The specific role of sodium and chloride ions in the metabolism of *L. pneumophila* has not been examined (Heller *et al.*, 1998). Historically, sodium ions play a role in metabolic carrier systems as well as being important cofactors for enzymes (Heller *et al.*, 1998). There has been a mild association described for chloride ions and growth of *L. pneumophila* (States *et al.*, 1985).

1.7.3 Sediments

It was found that there is a direct correlation between the concentration of sediment, and the growth or survival of *L. pneumophila* in hot water tanks (100-120°F) (Stout *et al.*, 1985). In this experiment, the sediment contained 128 mg/L total organic content, along with copper, iron, and other inorganic compounds. The presence of other microflora (representing a number of heterotrophic species) also had a positive effect on

the growth or survival of the *Legionella* (Stout *et al.*, 1985). It was found that the combined presence of sediment and microflora had an even greater positive effect on the growth and survival of *L. pneumophila* than either one alone (Stout *et al.*, 1985).

1.8 Disinfection Methods for *Legionella* in Man-Made Systems

Many methods have been investigated for their effectiveness in eradicating legionellae (specifically *L. pneumophila*), including ozonation, UV radiation, elevated temperatures, and periodic hyperchlorinations. In addition to eradication methods, system design and material composition are taken into consideration to try and reduce the possibility of colonization in the systems by these bacteria.

1.8.1 Light and UV radiation

Ultraviolet radiation, generated by mercury lamps, has been demonstrated as an effective method for prevention of *Legionella* colonization of water fixtures under the following conditions. The UV radiation had to be continuous, near the point of use and combined with filtration (Lin *et al.*, 1992). Filtration was necessary to minimize the accumulation of scale on the quartz glass tubes. The major advantages of UV radiation include fewer disinfection by-products, it does not harm the plumbing and the system is easy to install. UV light does not provide any residual protection and does not penetrate well into biofilm. It also is only effective in water over short distances so it cannot be used as the sole method for disinfection of a water system (Lin *et al.*, 1992).

1.8.2 Silver-Copper Ionization

In a hospital in Germany, silver-copper ionization was used as a method to control L*egionella* at distal outlets. The investigation in this case revealed that there was no correlation between copper concentration and *Legionella* counts but there was a noted

effect of the silver ions and a decrease in the level of *Legionella* contamination. The susceptibility of *Legionella* spp. to the silver ions did appear to be short-lived. During this 4-year study, the silver concentration used (<10 μg/L) was not sufficient to keep the levels of *Legionella* low (Rohr *et al.*, 1999).

1.8.3 Hyperchlorination

It has been found that *Legionella* spp. have relatively high chlorine resistance (Krammer and Ford, 1994). It has been shown that *L. pneumophila* (both agar and non-agar passaged) were more resistant to chlorine than coliforms which are typically used to indicate sanitary quality of potable water (States *et al.*, 1987). The mechanism for chlorine resistance is not known, but might be due to changes in the cellular envelope or may be provided from the other microbiota in the natural environment (Krammer and Ford, 1994). It has been found that successive and temporary operations of hyperchlorination and temperature elevation result in only partial or temporary decontamination of hot water systems (Bornstein *et al.*, 1986; Kool *et al.*, 1999). This could be the result of *Legionella* being present in biofilms and within protozoa where these operations are unable to reach as well as an increased tolerance to heat and chlorination.

In one example, during hyperchlorination, *L. pneumophila* within amoebae were protected from high levels of chlorine making eradication difficult (Edelstein and Meyer, 1994). Effective decontamination of the system was obtained only when free chlorine concentration reached 5 ppm at tap outlets although such decontamination was not permanent (Bornstein *et al.*, 1986).

1.8.3.1 Chloramination vs. Chlorination

Monochloramine has been used as an alternative to free chlorine for water disinfection. It has been shown to penetrate better into biofilm than free chlorine (Kool *et al.*, 1999). In an investigation in which the U.S. Centres for Disease Control and Prevention (CDC) participated, it was seen that water samples from three municipalities that used free chlorine for disinfection tested positive for the presence of *Legionella* whereas four municipalities using monochloramine disinfection were negative (Kool *et al.*, 1999). The reasons for these results are not known but suggest that monochloramine is more effective in eliminating *Legionella* from the water system.

1.8.4 Thermal

Maintenance of high operating temperatures within a hot water system is generally successful in the elimination of *L. pneumophila*, although some bacteria may remain in the system despite the elevated temperature (Best *et al.*, 1983). In a hospital associated with sporadic infection with *L. pneumophila*, the hot water was maintained at 5 to 6° above the thermal threshold for suppression of *Legionella* multiplication (Struelens *et al.*, 1992) and superheating was performed but both measures failed to eradicate the microorganism (Visca *et al.*, 1999). In this same hospital, no legionellae were isolated from the cold water system or the cooling towers of the hospital air-conditioning system (Visca *et al.*, 1999).

In one experiment, it was found that the legionellae colonies were in highest numbers at 40°C making up about 50% of the total biofilm at this temperature, with the numbers falling off at temperatures on either side (Rogers *et al.*, 1994). This is important

in systems where the water may not be maintained at a high temperature and therefore increases the potential for infection (Rogers *et al.*, 1994).

1.8.6 Current Recommendations For Legionella Eradication

The reduction in the number of *Legionella* organisms in a contaminated system to acceptable levels is only possible, in most cases, with a combination of different measures, among them are changes in construction and maintenance, as well as, thermal or chemical disinfection, and UV irradiation (Szewzyk *et al.*, 2000).

The most commonly used methods for prevention and eradication of legionellae from distribution systems combine hyperchlorination with an increase in water temperature to above 50°C. Variations on the level of chlorination, the contact time for the high chlorine levels, water temperatures, and cleaning schedules have been explored and tested. Recommendations include adding 20 to 50 mg chlorine/L to water tanks and allow circulation until tap water reaches 2 mg of free chlorine /L and maintaining this concentration for 2 hours. It is also recommended that water be heated to 70°C in the tank to obtain 60°C at the water taps (Moreno *et al.*, 1997). These procedures should be performed at least once a year, before starting the system or after an actual or suspected outbreak of legionellosis (Moreno *et al.*, 1997).

1.9 Nutritional Requirements

In general, legionellae do not utilize simple carbohydrate sources (Rogers and Pascule, 1991). This was shown by the lack of oxidation or fermentation of available sugars (Rogers and Pascule, 1991). However, the Entner-Doudoroff and pentose phosphate pathways are involved in the catabolism of glucose, while the gluconeogenic anabolic enzymes of the Embden-Meyerhof pathway are responsible for sugar synthesis

and the Krebs cycle for carbon assimilation (Rogers and Pascule, 1991). It was found that amino acids such as arginine, threonine, methionine, serine, proline, isoleucine, valine, and cystine form the major sources of energy for the organism, with proline, serine and threonine being the preference for *L. pneumophila* (Tesh and Miller, 1982; Tesh *et al.*, 1983).

Legionellae have been shown to be able to survive under low nutrient and starvation conditions. *L. pneumophila* could maintain a vegetative state for at least 600 days in a low-nutrient environment (Szewzyk *et al.*, 2000). This ability has been attributed, in part, to their intracellular reserves of poly-3-hydroxybutyrate (PHB). PHB can be used as an endogenous source of carbon and energy in periods of unbalanced growth. This is consistent with findings of James *et al.* (1999) that PHB accumulation was enhanced during iron-limited growth.

With the exception of passaged strains of *L. oakridgensis,* all legionellae have an absolute nutritional requirement for L-cysteine (Rogers and Pascule, 1991). Additionally, while most bacteria require iron for growth and virulence, *L. pneumophila* (and probably other *Legionella* species) appear to require comparatively higher amounts to thrive (Viswanathan and Cianciotto, 2001).

1.9.1 The Particular Role of Iron for Legionella

Legionellae have a high metabolic requirement for iron (Horwitz, 2000). When inside a host macrophage, the iron is obtained from the host's labile iron pool (Byrd and Horwitz, 1989). If this pool has been reduced or eliminated by agents such as iron chelators, changes in pH preventing pH-dependent release of iron from iron-transferrin or iron-lactoferrin, or by gamma interferon which down-regulates transferrin expression, the

result is inhibition of the growth of the bacterium (Horwitz, 2000). Some mechanisms for iron acquisition from the host cell have been identified although others are probably also present. These include the expression of the ferric uptake regulation (Fur) protein, as well as production of compounds known as siderophores (Viswanathan and Cianciotto, 2001). Siderophores are compounds that bind iron with high affinity and are then transported back into the bacterium (Viswanathan and Cianciotto, 2001).

1.9.2 Laboratory Media Required for Legionella Growth

Legionella spp. will not grow on routine bacteriological media. Instead, a buffered charcoal yeast (BCYEα) medium or a semi-selective BCYEα medium is used (Edelstein and Meyer, 1994). The addition of charcoal prevents photochemical oxidation of media components. It also non-specifically absorbs toxic free radicals and fatty acids (Hoffman *et al.*, 1983). Exposure of this medium to light, particularly when hot, may result in accumulation of peroxides that are inhibitory to the bacteria (Hoffman *et al.*, 1983). α-Ketoglutarate, in conjunction with the charcoal, helps to scavenge toxic oxygen radicals and remove oleic acid that can be released from the yeast extract into the medium during autoclaving (Rogers and Pascule, 1991).

Although the *Legionella* colonies themselves are round and entire, the play of light on the surface produces an appearance of irregularity or complex internal structure. As colonial growth continues, the iridescence can be appreciated with the naked eye particularly where growth is confluent. In short, the observation of opaque bacterial colonies that have a "ground-glass" appearance and auto-fluorescence should be considered as presumptive identification of *Legionella* spp. or *Legionella*-like species.

Laboratory media must be supplemented with L-cysteine and with iron in order for legionellae to grow (Rogers and Pascule, 1991). *L. pneumophila* requires 400 mg/L of L-cysteine and 250mg/L of iron salts in the bacteriological media (Feeley *et al.*, 1979) in order to be cultivable. The presence of soluble iron in the media has been shown to stimulate growth (Rogers and Pascule, 1991). The balance between the amount of L-cysteine and iron added to the media is more important than these constituents being in high concentrations (Segal and Shuman, 1999). The addition of trace metals such as calcium, cobalt, copper, magnesium, manganese, nickel, vanadium, and zinc have been shown to enhance growth (Rogers and Pascule, 1991). Identification of specific important compounds is difficult since no single environmental factor has yet been described which accurately predicts environmental densities of legionellae.

1.10 Cultural Characteristics

When grown on solid media, *Legionella* colonies are round with an entire edge, glistening, and low convex to convex and measure 1 to 4 mm in diameter (Rogers and Pascule, 1991). The edge of the colonies, particularly on the first 2 days, appear pink or blue-green, while the center of the colony is grayish with a characteristic speckled opalescence resembling cut glass (Rogers and Pascule, 1991). The doubling time of *L. pneumophila* in a rich medium is approximately 2 hours (Marra, 1992).

As a means of presumptive identification of legionellae on laboratory media, species that will grow on BCYE with L-cysteine but not on BCYE plates without L-cysteine or on blood agar plates are considered as a member of the genus *Legionella* (Rogers and Pascule, 1991). Additional tests are required for confirmation (Rogers and Pascule, 1991).

1.11 Detection Methods

Several methods are available for isolation or detection of bacteria from water and biofilm samples. These include (but are not limited to) cultivation, polymerase chain reaction (PCR), direct fluorescent antibody staining, and immuno-magnetic separation.

1.11.1 Cultivation

Currently, culture is regarded as the "gold standard" for detection of legionellae. However, due to the fastidious nature of the organisms and problems inherent in specimen collection and transport, culture may have a sensitivity as low as 50 to 60% (Steele *et al.*, 1990; Gao *et al.*, 1999). The sensitivity of the method can be increased by membrane filtration or centrifugation, although these additional steps may reduce the numbers of detectable legionellae (Brindle *et al.*, 1987; Boulanger and Edelstein, 1995).

The media for culturing these bacteria have been described above (Edelstein and Meyer, 1994). Other conditions that are critical for *L. pneumophila* to replicate in bacteriological media are a pH near neutrality (between 6.85 and 6.95) (Hoffman *et al.*, 1983), absence of Na^+, and absence of oxidants (Segal and Shuman, 1999). All known species grow well in humidified air at 35°C, with some species growing better in 2.5 to 3.0% CO_2. The incubation time until the first growth is seen is approximately 3 days, but may range from 1 to 10 days (Edelstein and Meyer, 1994).

One problem of using cultivation to detect *Legionella* is the phenomenon of viable but non-culturable (VBNC) cells. This condition is the rule with legionellae rather than an exception (Wireman *et al.*, 2000). Also, the problem of overgrowth of the media by other microorganisms is a concern when using cultivation due to its low specificity (Ng *et al.*, 1997; Leoni and Legnani, 2001). During this study, the tolerance of

Legionella spp. to heat and acidic conditions (as stated earlier) were exploited to improve the recovery of *Legionella* by eliminating some of the microflora.

1.11.2 Direct Fluorescent Antibody Detection

Direct staining with a fluorescent antibody (DFA) is a rapid diagnostic test. As a detection method for *Legionella* spp., it lacks sensitivity and specificity (Helbig *et al.*, 1999). Its sensitivity is less than that of culture because it requires large numbers of organisms before they can be readily visualized (Stout and Yu, 1997). Also, false-positive results due to cross reactions with non-*Legionella* bacteria can occur depending on the specificity and quality of the antibody being used (Stout and Yu, 1997). Currently, DFA cannot be used to detect all types of *Legionella* because no genus-specific antibody has been developed commercially for this purpose. It is generally used to detect *L. pneumophila*, although reagents recognizing *L. micdadei* and *L. dumoffii* have been developed and successfully tested (Bangsborg, 1997).

1.11.3 Immunomagnetic Separation

Immunomagnetic separation (IMS) is a useful technique for concentrating and purifying samples using specific antibodies, paramagnetic particles, and magnetic concentration (Rochelle *et al.*, 1999). Integrated systems for IMS and PCR have recently been used to facilitate DNA diagnosis of some bacteria, viruses, and parasites (Biswas *et al.*, 1994; Muir *et al.*, 1993).

The immunomagnetic separation technique involves capturing cells using specific antibodies and paramagnetic beads. The beads allow the targeted organism to be held stationary with a magnet while the surrounding supernatant matrix is removed by repeated washing (Rochelle *et al.*, 1999). This technique helps to concentrate the target

cells and also to remove inhibitory substances (Rochelle *et al.*, 1999). Using a scanning electron microscope and by staining with acridine orange, attachment of the target bacteria to the beads can be verified (Benkirane *et al.*, 1995). It was found that the bacteria tended to bind several particles simultaneously, thus forming aggregates (Benkirane *et al.*, 1995; Roberts and Hirst, 1997). The bacteria that are captured on the magnetic beads usually remain viable and can continue to multiply if suitable nutrients are provided (Torensma *et al.*, 1993).

When carrying out IMS, there are three options for the addition of the antibody and paramagnetic beads (Roberts and Hirst, 1997). The direct method adds the paramagnetic beads directly to the target-specific antibody being used. The combined method involves binding the antibody and immunomagnetic particles together prior to introduction to the sample. The third option is the uncombined method where the organism is first incubated with antibody and added to the beads secondarily.

1.11.4 Polymerase Chain Reaction (PCR)

Detecting legionellae at the species or genus level has been carried out using PCR systems that amplify conserved regions of the 5S rRNA, the 16S rRNA, and the macrophage infectivity potentiator (Mip) genes (Jaulhac *et al.*, 1992; Helbig *et al.*, 1999; Lisby and Dessau, 1994). Use of the 16S rRNA gene as a target (LEG primers) was shown to be satisfactorily specific and sensitive for amplification for 29 *Legionella* species (Miyamoto *et al.*, 1997). This gene is a good choice since ribosomal RNA genes contain highly conserved regions that are good targets for primers. Between these conserved regions lies a variable region that can be amplified, sequenced and used to determine phylogenetic relationships between bacteria (Wilson *et al.*, 1997).

One major issue that needs to be addressed when using PCR as a method of detection is the presence of PCR inhibitors (Miyamoto *et al.*, 1997). For example, rust was found to be able to completely inhibit PCR in some instances (Maiwald *et al.*, 1994). Although dilution of a sample is a simple and useful method to reduce inhibitors, when trying to detect *Legionella*, this method could lead to under-estimations of the contamination. One possible solution for reducing the amount of inhibitors is the use of semi-nested PCR programs. Since only a small amount of product from the first step of the semi-nested procedure is amplified during the second step, the quantity of inhibitors is lower. As well, the additional amplification cycles increase the sensitivity of the semi-nested PCR assay (Miyamoto *et al.*, 1997).

There are possible biases associated with this technique. For example, in the context of quantitative analysis, little is known about the differential amplification rates of PCR primers due to base composition and the dynamics of mixed DNA amplification techniques (Wilson *et al.*, 1997). Also, this technique amplifies nucleic acids from both live cells, whether they are in a culturable or non-culturable state, and dead cells, making it impossible to know what condition the cell was in at the time of DNA extraction.

1.12 Objective of this study

This study was carried out to determine if *Legionella* are present in water and biofilm samples from groundwater sources. A limited number of distribution system and cooling tower samples, both water and biofilm, were also tested. Due to the serious nature of the illnesses caused by this bacterium, determining their presence is one step towards being able to predict and more effectively treat water to remove these bacteria. Conventional cultivation and semi-nested PCR was used for analysis of the samples.

Because both of these methods have their drawbacks for detection of *Legionella*, IMS was tested as a possible improvement for *Legionella* detection when combined with conventional cultivation and PCR

2.0 MATERIALS AND METHODS

2.1 *Legionella* Reference Strains

L. pneumophila (Bellingham-1, ATCC 43111), *L. micdadei* (Tatlock, ATCC 33218), *L. rubrilucens* (WA-270-C2, ATCC 35304) and *L. steigerwaltii* (strain SC-18-C9, ATCC 35302) were used as reference strains for conventional processing and immunomagnetic separation. *P. aeruginosa* (ATCC 49266) was used as specificity control for the primary monoclonal antibody (Mab) 22/1. *Legionella* strains used in this study were provided by the Central Public Health Laboratory (Toronto, Ontario, Canada).

2.2 Environmental Water and Biofilm Samples

2.2.1 Sample Collection

Water and biofilm samples were collected from water utilities in various states in the U.S. as well as from two provinces in Canada. Both warm and cold groundwater samples were collected. Three or four liters of water (depending on the water utility) were collected in one-liter Nalgene bottles. Polyvinyl chloride (PVC) pipe sections, water meters, coupons, or end-cap scrapings were used for the collection of biofilm samples. When using PVC pipe, the sections were attached to a sampling tap and water was allowed to run through them continuously for 7-60 days. Independent of the biofilm type, all biofilm samples were shipped in water from the same source to keep the biofilms from drying out in transit. Water and biofilm samples were shipped to the laboratory, on ice, by overnight priority courier service so that they could be processed within 24 hours of collection.

2.2.2 Water and Biofilm Sample Processing

Upon receipt of water samples, each liter of water was filtered through a sterile 47-mm diameter filter funnel assembly containing 0.45-μm porosity polycarbonate membrane filter (Millipore, Bedford, MA, USA). The filter was then aseptically removed and placed in a 50-mL centrifuge tube or similar-size vessel containing 10-mL sterile water. When more than one filter was necessary per liter, all the filters for that liter were added to the same tube and, for each additional filter, a 10-mL volume of sterile water was added. All filters were then vortexed (3 x 30s) to re-suspend the collected particles. One 10 mL filtered sample was kept at 35°C for 1 week (to enrich the *Legionella* for PCR processing) and another 10 mL filtered sample was sent for protozoa analysis. The other sample(s) were subsequently sonicated (sonicating water bath (Branson 1200, Panbury, CT, USA), 1 min). Glass tubes were used in the sonicating process because plastic does not conduct sonic pulses as well. One of the 10 mL sonicated and vortexed samples was used for cultivation on the day of its arrival.

Biofilm containers were scraped and resuspended in the water in which they arrived. If this volume was too small, sterile water was used for resuspension to a minimum volume of 20 mL. Any clumps of organisms and/or their aggregates as biofilms were then dispersed by vortexing the suspensions at full speed (3 x 30 seconds). Ten milliliters of the sample was stored at 35°C for 1 week to enrich the *Legionella* for PCR processing. An additional 10 mL was sent for protozoa analysis. The rest of the volume was sonicated (sonicating water bath, 1 min). Sonication was used to release intra-amoebal *L. pneumophila*, and possibly other species, thus increasing culture

sensitivity. The sonicated and vortexed sample was used for cultivation on the day of its arrival.

2.3 Cultivation

2.3.1 Water and Biofilm Samples

The cultivation of *Legionella* (summarized in Figure 1) was performed using three different types of semi-solid media, all based on buffered charcoal yeast extract (referred to at BCYEα). BCYEα supplemented with glycine, vancomycin, and polymyxin B was referred to as GVP. CCVC was BCYEα supplemented with cephalotin, colistin, vancomycin and cycloheximide. The semi-selective CCVC and GVP media were used to try and reduce the contaminating bacterial load during cultivation. Further details on the composition of these media are given in Appendix A.

In addition to the use of semi-selective media, the water and biofilm samples underwent heat and acid treatments. For heat treatment, 2 mL of the sample was placed in a 50°C water bath for 30 minutes. For acid treatment, 1 mL of the sample and 1 mL of the acid treatment reagent were combined (giving a pH of 2.2) and allowed to stand for 15 minutes. The heated samples were allowed to return to room temperature and the pH of the acidified ones were raised to pH 6.9 before cultivation.

One hundred μL of each sample (direct plating, acid treatment, and heat treatment) was placed onto each of the three different media in duplicate and incubated at 35°C and 30°C in a humidified (>50% RH) 2.5 to 5% CO_2 atmosphere for up to 14 days.

Each plate was viewed through a dissecting microscope every 72 hours to detect the presence of *Legionella* colonies that had a "mottled" surface, an iridescent red-blue-green shade, or a faceted "cut-glass" appearance.

Plates with *Legionella*-like colonies were placed in a biological safety cabinet and each suspect colony was aseptically picked and inoculated onto BCYEα agar, a BCYEα agar plate without L-cysteine and a blood agar plate and incubated for 48 hours.

Plates without growth on either BCYEα without L-cysteine or blood agar were re-incubated for an additional 24 hours. All colonies were checked for auto-fluorescence under long-wavelength UV light (UVS 11, Fisher Scientific, CA, USA).

Plates demonstrating growth on only BCYEα agar were presumed to be *Legionella*. Care was taken when the L-cysteine requirement for growth was used as a differential criterion for the presence or absence of *Legionella* in a sample because some strains are able to grow on BCYEα without L-cysteine even on their first isolation from environmental samples. Also, some *Legionella* species can grow on sheep blood agar and exhibit the typical "cut-glass" appearance. DNA was isolated from colonies suspected to be *Legionella* and PCR amplification was carried out. Nucleotide sequencing was used to identify individual *Legionella* species.

2.4 Polymerase Chain Reaction

2.4.1 Water and Biofilm Samples

A semi-nested PCR was performed on the water and biofilm samples that were not sonicated and had been left to incubate for a week at 35°C. For the first step of the semi-nested procedure, 45 μL of a master mix (Appendix A) was added to each tube along with 5 μL of sample that contained DNA to amplify. The second step of the semi-nested PCR used 45 μL of a master mix and 5 μL of the DNA product amplified in the first step.

The oligonucleotide primers were designed from the partial sequences of 16S rRNA genes of *Legionella* species that were obtained from the Ribosomal Database Project (Miyamoto *et al.*, 1997). For the first-step PCR, two oligonucleotides (LEG 225 and LEG 858) were used as amplimers enclosing a 654-bp fragment of the 16S rRNA gene. LEG 225 (5'-AAGATTAGCCTGCGTCCGAT-3') was located at positions 225 to 244 and LEG 858 (5'-GTCAACTTATCGCGTTTGCT-3') was complementary to positions 880 to 859. LEG 225 was first selected from a large database of the 16S rRNA gene.

For the second-step PCR, LEG 448 (5'-GAGGGTTGATAGGTTAAGAGC-3') and LEG 858 were used as another set of amplimers enclosing a 430-bp fragment of the 16S rRNA gene.

The thermocycler program is detailed in Appendix A.

Samples that were negative by the semi-nested procedure were tested for the presence of PCR inhibitors. DNA was added (2 μL of extracted DNA from *L. pneumophila*) to 3 μL of the sample. In this case only the first step of the semi-nested PCR was carried out.

2.5 DNA Isolation

DNA was isolated using the DNeasy Extraction Kit (Qiagen, Mississauga, ON, Canada) following the procedure for extraction from Gram-negative bacteria in cell culture, according to the manufacturer's instructions.

2.6 Agarose Gel Electrophoresis

To resolve the band of interest from PCR, agarose gel electrophoresis was performed. Agarose was used at 1% (w/v) in Tris-Borate-EDTA solution

(TBE)(Appendix A). The tracking dye was run ¾ of the way down the gel. The gel was then stained for 15 minutes in a 10 μg/mL solution of ethidium bromide, destained in 1X TBE for 15 minutes and placed on a UV transilluminator to resolve any bands. A picture was taken of the gel and data reported.

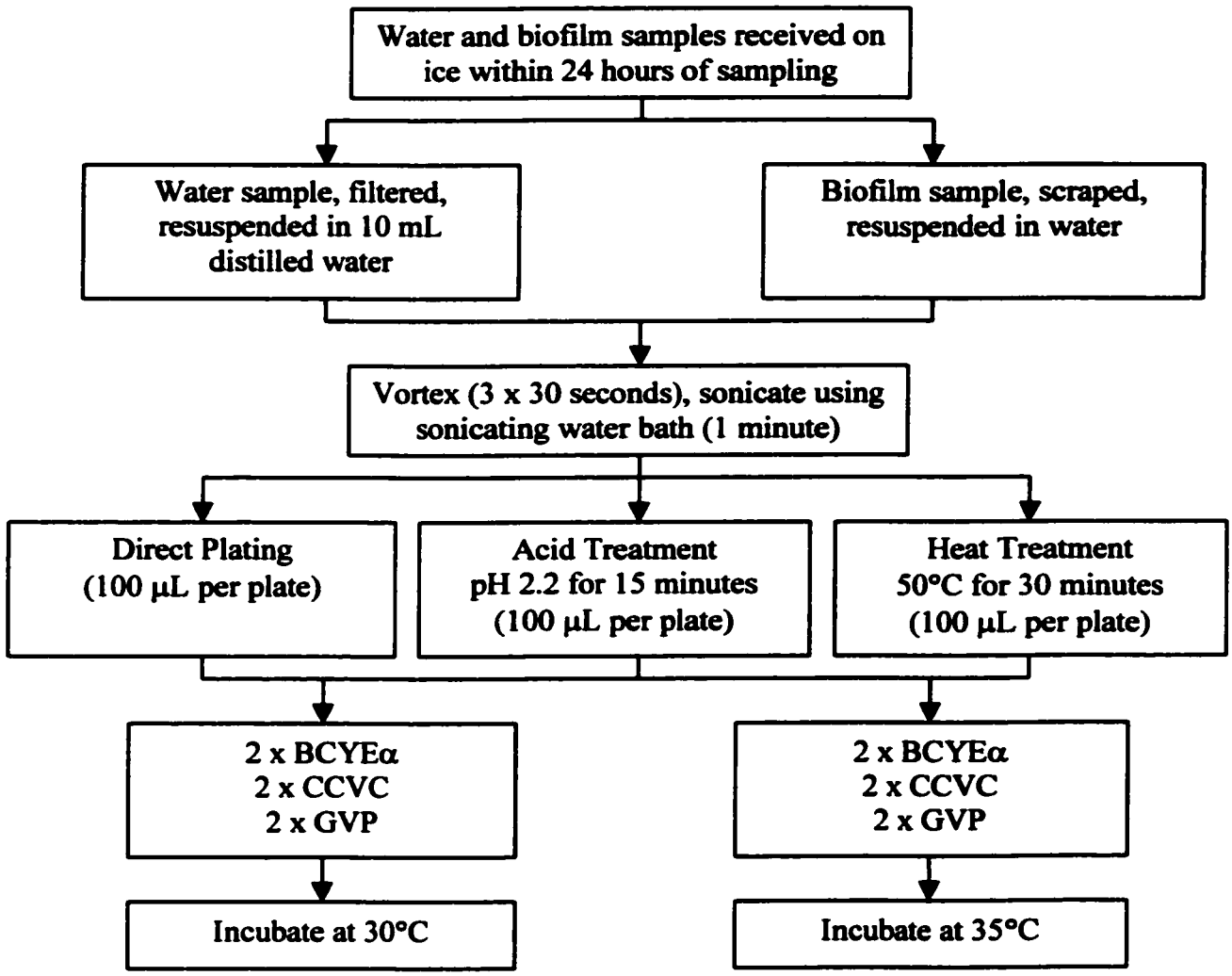

Figure 1: Outline of cultivation process for water utility samples

2.7 Nucleotide Sequencing

In the event of a positive PCR product from a single colony recovered from a water sample, DNA sequencing was commenced on the amplified 16S rDNA fragment. The positive band from PCR was run on an agarose gel as described above. The band was then excised from the gel and the DNA was isolated using a gel purification kit (Qiagen, Mississauga, ON, Canada). Once the DNA was isolated, sequencing was carried out at the University of Ottawa Biotechnology Research Institute (UOBRI). An automated sequencing system (Applied Biosystems - ABI 373 Stretch) using the BigDye™ Terminator cycle sequencing kit (#4303152) was used following the manufacturers instructions.

The sequences obtained were compared with available sequences in the DDBJ, EMBL and GenBank databases with the Gapped BLAST program through the Internet (http://www.ncbi.nlm.nih.gov).

2.8 Immuno-Magnetic Separation (IMS)

2.8.1 Antibodies

A mouse Mab (22/1; IgG1 subclass) able to recognize a *Legionella*-specific epitope of the Mip protein, was received as cell culture supernatant (Dr. Jergen Helbig, Insitiut für Medizinische Mikrobiologie und Hygiene, Universitätsklinikum der TU Dresden, Germany) and was purified using a protein-G column following manufacturer's instructions (Amersham Pharmacia Biotech, Inc., NJ, USA). Sheep anti-mouse IgG1-coated Dynabeads® M-450 (Dynal Inc., NY, USA) were used as the secondary antibody to detect *Legionella*-Mab 22/1 complexes following manufacturer's instruction. Non-

specific binding assays employed a commercially available affinity purified mouse IgG Fc antibody (Chemicon, Temecula, CA, USA).

2.8.2 Specificity Testing

Although Mab22/1 is known to be genus-specific for *Legionella*, its specificity for *Legionella* in IMS was confirmed earlier by Dr. Serge Riffard of this laboratory by flow cytometry against a strain of *P. aeruginosa*, representative of bacteria frequently encountered in environmental samples and known to cross-react with *Legionella* when using direct fluorescent antibody typing. One fraction of the purified Mab 22/1 was FITC-labeled using a FluoReporter® FITC Protein Labeling Kit following manufacturer's instructions (Molecular Probes Inc., Eugene, OR, USA). An Epics XL™ (Coulter Corp., Miami, FL, USA) cytometer equipped with a 15-mW, air-cooled, 488-nm-wavelength argon laser light allowing a 4-colour fluorescence analysis was used to measure forward angle light scatter (FSC), and fluorescence of the microbial cells. Set up of the flow cytometric assay was performed using pure cultures of *L. steigerwaltii* (strain SC-18-C9, ATCC 35302). Photo-multiplier tube voltage and spectral compensation had been initially set using cells single-stained with propidium iodide (PI) and FITC-labeled 22/1 antibody.

2.8.3 Isolation Procedure for Bacterial Cells Using Uncombined IMS

The Mab 22/1 (20 μL of a 1 μg/mL suspension) was added to pure bacterial suspensions or artificially contaminated natural samples. Mixtures were left at room temperature for 30 minutes with agitation. Sheep anti-mouse coated Dynabeads® was then added (15 μL of a 30 μg/μL suspension) and agitated at room temperature for 30 minutes. IMS was performed (target cells were attracted by applying a magnet at the

bottom of test tubes for 2 min). One washing step (with 1-mL sterile water) with the cells being attracted on the magnetic support was carried out. The concentrated cells were resuspended in 200 μL or 1 mL (depending on the experiment). The same bacterial suspensions without prior IMS were plated onto BCYEα to determine the recovery rates of the IMS procedure (sensitivity).

2.8.4 Isolation of Bacterial Cells Using Combined IMS

The Mab (10 μL of a 10 μg/mL solution) was added to 1 mL of the sheep anti-mouse Dynabeads and incubated with gentle agitation for 24 hours at 4°C. After the 24-hour incubation, the bead-antibody complexes were drawn to the side of the tube using a magnetic support and washed with 1 mL of PBS. The bead-antibody complexes were resuspended in the PBS wash solution and agitated for an additional 15 minutes at room temperature. This washing procedure was repeated with the final resuspension of the beads in 1 mL of PBS. The combined antibodies were stored at 4°C until use. Varying amounts of the combined antibody were added to the sample and allowed to incubate for 1 hour at room temperature with gentle agitation. IMS was then performed (target cells were attracted by applying a magnet at the bottom of test tubes for 2 min). One washing step (with 1-mL sterile water) with the cells being attracted on the magnetic support was done. The concentrated cells were resuspended in 1 mL of sterile distilled water. Controls were set up in parallel to determine the recovery rates when pure bacterial suspensions were used (sensitivity).

2.8.5 Non-Specific Binding Experiments

A variation of uncombined IMS was used to test non-specific binding. Varying concentrations of mouse IgG antibody (20 μL) were added to the secondary antibody (15

μL) and allowed to incubate for 30 minutes at room temperature. During the 30 minute incubation, the primary antibody (20 μL of a 1μg/mL solution) was added to the sample and incubated for 30 minutes. The combined secondary and mouse IgG antibody complex (35μL) was added to the sample already incubated with primary antibody and IMS was carried out as described for uncombined IMS above.

2.8.6 Preparation of Sterilized Environmental Samples Spiked with *Legionella*

An environmental biofilm sample received during this study was sterilized by autoclaving (20 minutes at 121°C, 15 psi). Using this sterilized sample instead of distilled water, 10-fold serial dilutions of a *Legionella* reference strain (strain used depended on experiment) were made and used for combined and uncombined IMS as outlined above.

2.8.7 Preparation of Spiked Environmental Samples for Decontamination Experiments

The environmental samples used were those remaining after conventional cultivation and PCR. In order to estimate the sensitivity of the IMS-cultivation assay under experimental conditions, serial 10-fold dilutions of *L. steigerwaltii* were prepared in sterile water and 100 μL of each dilution was added to 900 μL of environmental sample in order to obtain a range of concentrations. The spiked environmental samples (100 μL) were inoculated on BCYEα-based media (BCYEα and CCVC) as controls and were incubated for 72h at 35°C under 2.5% CO_2 and humidified air. IMS-cultivation was carried out using the uncombined IMS procedure, with the exception of the chlorine treatments (described below), using 1 mL of spiked environmental sample, and adding 15 μL of monoclonal primary antibody (1μg/mL) and 15μL of the secondary antibody.

After IMS, the tubes were resuspended in 1 mL of distilled water and one decontamination procedure was used on each IMS tube.

2.8.8 Decontamination Procedures on Spiked Environmental Samples

Low ionic strength water was made by adding 5 g of Chelex 100 resin (Biorad, CA, USA) to 100 mL of distilled water. The solution was allowed to sit overnight and the supernatant was removed and filter sterilized. The sterilized low ionic strength water was used in place of distilled water during the IMS procedures.

Heat and acid decontamination procedures were carried out as described for conventional cultivation.

Lysozyme (Pharmacia, USA) was diluted in distilled water from solid form (20100 U/mg) to give a range of concentrations. One mL of lysozyme solution was added to the 1 mL IMS resuspension and allowed to incubate for 1 hour at 35°C before being plated onto BCYEα, CCVC and GVP media and incubated at both 30°C and 35°C for 72 hours under the same conditions used for conventional cultivation.

The free chlorine present in test solutions was determined colorimetrically with the Hach (DR/820) colorimeter following manufacturer's instructions (Hach Company, Loveland, CO, USA). The free chlorine solution of interest was then used as a substitute for the distilled water wash during the uncombined IMS procedure. The chlorine solution was allowed to remain in contact with the cells for the desired time, it was then removed and the bead-cell complexes were resuspended in distilled water as normally done.

2.8.9 Preparation of Water and Biofilm Samples

The IMS procedure used during this study was continually improved upon as more sampling was carried out. For this reason, all the samples were not subjected to the same experimental procedure.

2.8.9.1 Samples Received August to November, 2000

During processing of the water and biofilm samples (refer to section 2.2.2), 10 mL of each sample was stored at 35°C for one week for PCR processing. Each sample was then vortexd (3 x 30s), sonicated (in a sonicating water bath for 1 minute) and centrifuged (20 minutes at 2500 rpm) to concentrate the sample to 2 mL. One mL was used for IMS processing following the procedure for uncombined IMS (refer to section 2.8.3) except 15 μL of 1 mg/mL primary antibody and 15 μL of secondary antibody were used and the bead-cell complexes were resuspended in a final volume of 200 μL. The remaining 1 mL sample was used for PCR processing (as described in section 2.4.1). After IMS, 20 μL of each sample was plated onto BCYEα, CCVC, and GVP in duplicate and incubated at 35°C with 2 to 5% CO_2 and humidified air. Plates were looked at every 3 days for 14 days. The remaining IMS sample (80μL) was used for IMS-PCR (section 2.8.10).

2.8.9.2 Samples Received January and February, 2001

The water and biofilm samples remaining after conventional cultivation were stored at 4°C overnight before IMS processing. The 5.8 mL of water sample and 5.8 mL of biofilm sample remaining after conventional cultivation were centrifuged for 20 minutes (2,500 rpm) and all of the supernatant removed except for 2 mL. One mL was used for IMS-PCR and the remaining 1 mL was used for IMS-cultivation. Uncombined

IMS was performed using 15 μL of 1 mg/mL primary antibody and 15 μL of secondary antibody. The bead-cell complexes were resuspended in a final volume of 1.6 mL for IMS-cultivation and 1 mL for IMS-PCR. The IMS-PCR samples were kept at -20°C until processed. From the IMS-cultivation samples, 100 μL was plated in duplicate onto BCYEα, CCVC, and GVP. The remaining 1 mL was used for acid treatment (pH 2.2 for 15 minutes) and plated in duplicate onto BCYEα, CCVC, and GVP. All plates were incubated at 35°C (with 2-5% CO_2 and humidified air) and looked at every 3 days for 14 days for *Legionella* colonies.

2.8.9.3 Samples Received March and April, 2001

The water and biofilm samples (after sample processing) were stored at 4°C overnight before IMS processing. 10 mL of biofilm sample and 1 liter of water (filter concentrated to 10 mL) were centrifuged for 20 minutes (2,500 rpm) and the all of the supernatant removed except for 1 mL. IMS was carried out as described for uncombined IMS with the final resuspension in 1 mL. From the 1 mL sample, 500 μL was stored for DNA extraction and IMS-PCR, 250 μL had 750 μL of sterile distilled water added for direct plating, 250 μL had 750 μL of sterile distilled water added for acid treatment (1 mL of acid treatment added to give a total of 2 mL), and neutralized with 0.1 M KOH to give a final pH of 6.9. One hundred μL of each sample was used for direct plating onto BCYEα, GVP, and CCVC in duplicate, one of each duplicate was stored at each 30 and 35°C and 100 μL of each sample was used for acid treatment plating onto BCYEα, GVP, and CCVC in duplicate, one of each duplicate was stored at each 30 and 35°C.

2.8.9.4 Samples received in May 2001

The water and biofilm samples (after sample processing) were stored at 4°C overnight before IMS processing. Ten mL of biofilm sample and 1 liter of water (filter concentrated to 10mL) were centrifuged for 20 minutes (2,500 rpm) and all of the supernatant removed except for 1 mL. IMS was carried out as described for uncombined IMS with the final resuspension in 1 mL. Additionally, the remaining 5.8mL for conventional cultivation for the water and biofilm samples was concentrated to 1 mL using the same method and combined IMS (20 μL of combined antibody) was performed as described earlier. After IMS the samples were inoculated onto media as outlined for samples received for March and April 2001.

2.8.9.5 Samples received in June and July 2001

Processing of the water and biofilm samples followed the same procedure used for samples received March and April, except combined IMS (20μL of combined antibody) was used instead of uncombined IMS.

2.8.10 IMS-PCR

The sample was subjected to DNA extraction using a commercially available kit (Dneasy Extraction kit, Qiagen, ON, Canada). Extracted DNA was concentrated to a 100 μL volume and 5 μL of this DNA extract was subjected to the semi-nested PCR (described previously) targeting a 16S rDNA fragment of *Legionella*. PCR products were then resolved by conventional agarose gel electrophoresis (1% gels), stained using an ethidium bromide solution, and viewed under UV light.

2.8.10.1 Sensitivity testing of IMS-PCR

Serial 10-fold dilutions of *L. steigerwaltii*, *L. micdadei*, *L. rubrilucens*, and *L. pneumophila* were prepared in sterile water with concentrations ranging from 10^5 to 10 CFU/mL. The dilutions were inoculated on BCYEα media to determine exact concentrations and incubated for 72 h at 35°C under 2.5% CO_2 and humidified air. IMS-PCR was performed as explained previously.

3.0 RESULTS AND DISCUSSION

3.1 Water and Biofilm Samples

Water utilities from the U. S. as well as Ontario and New Brunswick participated in this study to determine the presence of *Legionella* spp. in the water systems. Descriptions of the samples received from each utility are provided in Appendix D. In view of the nature of the agreement with the participating utilities, in this report, each utility identified only by the state or province of their location. With the exception of Water Utility A, each utility sent samples for processing only once during the study. Water Utility A sent samples on a monthly basis for the duration of the study. Some water utilities (including Water Utility A) used PVC pipe sections for the development of biofilms. PVC has been shown to enhance the growth of biofilms through release of organic substances that can be used as nutrients by the bacteria (Schofield and Locci, 1985). Both water and biofilm samples were collected since the presence of *Legionella* in biofilm is as important as its presence in water. Biofilm can become dislodged and resuspended in the water phase moving through the water systems.

3.2 Cultivation

3.2.1 Detection Limits for Cultivation

For cultivation, the lowest number of the target bacteria that can be detected is 1 colony forming unit (CFU) per plate. For the conventional cultivation technique used to process the water and biofilm samples received during this study (as described in Section 2), this limit varied according to the type of sample as well as with the selective treatment used during processing. In general, the detection limit for water samples was 100 CFU/L for direct plating and heat treatment. This limit increased to 206 CFU/L when using acid

treatment and conventional cultivation. Direct plating and heat treatment detection limits were 10 CFU/L for IMS-cultivation and acid treatment detection limits were 20.6 CFU/L. Detection limits for biofilm samples were highly variable depending on the type of sample received.

3.2.2 Conventional Cultivation

Conventional cultivation as a technique for isolating *Legionella* is not very specific. Direct inoculum of a water sample onto growth media containing L-cysteine, with or without the addition of selective elements, gives low sensitivity and low specificity (Leoni and Legnani, 2001). To increase the sensitivity of the method, filtration of the water samples was performed before inoculation onto the growth media. This increases the sensitivity of the method, but not the specificity. It has been shown that a reduction in detectable *Legionella* of 16-91% was found using filtration and 20-63% using centrifugation (Brindle *et al.*, 1987; Boulanger and Edelstein, 1995).

Even when using selective media, heat treatment, and acid treatment, the levels of heterotrophic bacterial contamination were still quite high. Figure 2 shows a typical cultivation plate. In this case, the *Legionella* colony was that of a blue-white auto-fluorescent species (Figure 3) making it easy to detect using long wave UV light.

The conventional cultivation results for all the utilities are found in Table 3-1 and Table 3-2. Overall, 29.1% of these samples were found positive for the presence of *Legionella* spp. The identification of each species should be taken with care because partial 16S rDNA segment sequencing is not the ultimate tool to definitively assign a species name to a strain.

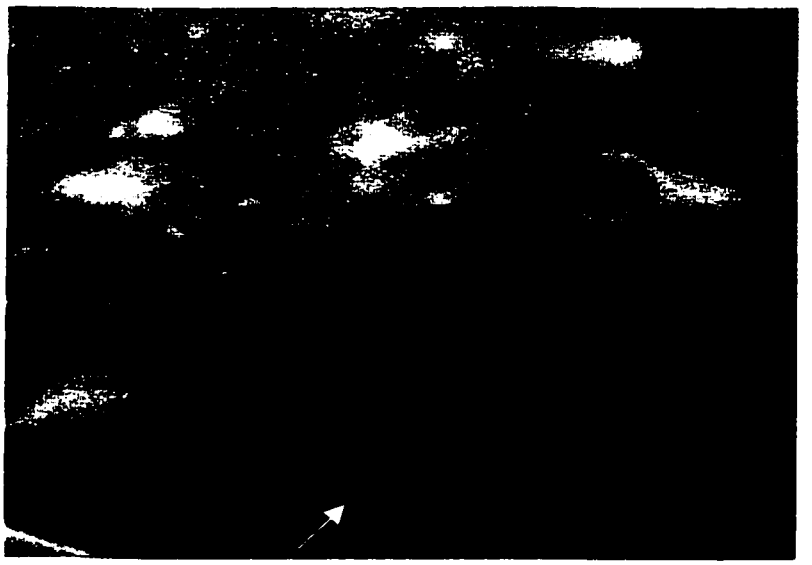

Figure 2 Appearance of a typical cultivation plate from environmental water and biofilm samples showing a *Legionella* colony (bottom just off-centre)

Figure 3 Typical *Legionella* plate (shown in Figure 2) under long wave UV light showing a blue-white auto-fluorescent *Legionella* colony.

Table 3-1 Cultivation results for Water Utility A (Florida)

Well ID	Date Received	Water (CFU/L)	Strain Identification	Biofilm	Strain Identification
103-1	04/04/2000	280	*L. geestiana*	None	N/A
136-1		None	N/A	None	N/A
103-2	12/05/2000	None	N/A	1 CFU/cm^2	No sequence available
136-2		None	N/A	None	N/A
103-3	20/06/2000	None	N/A	None	N/A
136-3		None	N/A	None	N/A
103-4	01/08/2000	None	N/A	None	N/A
138-4		431	No sequence available	None	N/A
103-5	19/09/2000	None	N/A	None	N/A
138-5		413	No sequence available	None	N/A
103-6	28/11/2000	None	N/A	None	N/A
138-6		None	N/A	None	N/A
103-7	09/01/2001	None	N/A	None	N/A
138-7		4600	*L. parisiensis*	None	N/A
103-8	09/02/2001	None	N/A	None	N/A
138-8		206	*L. parisiensis*	None	N/A
103-9	27/03/2001	150	*L.erythra*	None	N/A
		100	*L. gresilensis*		
138-9		206	*L. santicrucis*	None	N/A
		206	*L. gresilensis*		
		100	*L. sainthelensi*		
		206	LLAP		
103-10	02/05/2001	100	*L. dumoffii*	None	N/A
138-10		413	LLAP-2	None	N/A
103-11	01/06/2001	Not received	N/A	6.4 CFU/cm^2	*L. dumoffii*
				15.9 CFU/cm^2	*L. erythra*
		None		13.1 CFU/cm^2	*L. quateirensis*
138-11			N/A	None	N/A
103-12	06/07/2001	Not received	N/A	14.4 CFU/cm^2	*L. quateirensis*
		619		29.6 CFU/cm^2	*L. erythra*
138-12			*L.quateirensis*	None	N/A
KWII RW	21/11/2000	Not received	N/A	2.1x10^4 CFU scraped into 100mL of sterile water	*L. dumoffii*
KWII IFS	21/11/2000	Not received	N/A	None	N/A
05B	18/04/2001	Not received	N/A	4.0x10^3 CFU scraped into 80mL of sterile water	LLAP 10
15A	18/04/2001	Not received	N/A	None	N/A
21A	18/04/2001	Not received	N/A	None	N/A

Cont'd

Table 3-1 Continued

Well ID	Date Received	Water (CFU/L)	Strain Identification	Biofilm	Strain Identification
32G	18/04/2001	Not received	N/A	$2.1x10^3$ CFU $2.0x10^3$ CFU (each scraped into 100mL of sterile water)	*L. anisa* LLAP 10
72F	18/04/2001	Not received	N/A	None	N/A
94A	18/04/2001	Not received	N/A	$1.0x10^3$ CFU scraped into 100 mL of sterile water	LLAP 10
CT1	11/05/2001	Not received	N/A	None	N/A
CT2	11/05/2001	Not received	N/A	None	N/A
CT3	11/05/2001	Not received	N/A	None	N/A
CT4	11/05/2001	Not received	N/A	$1.5x10^4$ CFU scraped into 80mL of sterile water	*L. pneumophila*
CT5	11/05/2001	Not received	N/A	None	N/A
EMS	01/08/2001	None	N/A	None	N/A
JA	01/08/2001	$6.6x10^3$ $4.9x10^3$	*L. shakespearei* *L. pneumophila*	Not received	N/A
CRIM	01/08/2001	None	N/A	None	N/A
CH1	01/08/2001	$4.0x10^5$	*L. pneumophila*	None	N/A
CLW	01/08/2001	None	N/A	None	N/A

Table 3-2 Cultivation results for water and biofilm samples for Water Utilities C to Q

Water Utility	Well ID	Date	Water CFU/L	Strain Identification	Biofilm Type	Biofilm	Strain Identification
C	AZ (A)	17/05/	100	*L. rubrilucens*	None	N/A	N/A
Arizona	AZ (B)	2000	100	*L. pneumophila*	Coupons	3 CFU/cm^2	*L. anisa*
	AZ(BM)		N/A	N/A	Water meter	20 CFU/cm^2	*L. anisa*
D	EP	19/05/	None	N/A	Water meter	None	N/A
Texas		2000					
E	ST	14/06/	None	N/A	Water meter	None	N/A
Illinois		2000					
F	CS2	26/07/	100	*L. pneumophila*	Pipe section	10 CFU/cm^2	*L. pneumophila*
Texas	CS5	2000	183	*L. pneumophila*		5 CFU/cm^2	*L. pneumophila*
G	PEO	15/08/	None	N/A	Water meter	None	N/A
Illinois		2000					
H	PK	22/09/	None	N/A	Water meter	None	N/A
Illinois		2000					
I	MEX	11/10/	None	N/A	Water mater	None	N/A
Missouri		2000					
J	SF	15/02/	100	*L. donaldsonii*	Pipe sections	None	N/A
Michigan	MN	2001	None	None		None	N/A
K	NJ	11/04/	None	N/A	Water meter	118CFU/cm^2	ATCC 700511
New Jersey		2001					
L	OTT	25/04/	412	*L. quateirensis*	Scraped in water	6.2x10^4 CFU scraped in 100mL sterile water	*L. quateirensis*
Ontario		2001					
M	CH	24/05/	None	N/A	Water meter	None	N/A
Illinois		2001					
N	NB3	6/06/	None	N/A	Pipe sections	None	N/A
New	NB7	2001	None	N/A		None	N/A
Brunswick							
O	MO18	7/06/	100	*L. quateirensis*	Scraped in	None	N/A
Missouri	MO34	2001	None	N/A	water	None	N/A
P	NE54	10/07/	None	N/A	Pipe sections	None	N/A
Nebraska	NE66	2001	100	*L. quateirensis*		13.6 CFU/cm^2	*L. quateirensis*
			412	*L. dumoffii*			
Q	FK8	25/07/	None	N/A	Not received	Not received	N/A
Florida	FK13	2001	800	*L. pneumophila*		Not received	N/A

Results from Water Utility A (Table 3-1) show that not only are *Legionella* present in their groundwater systems, but also that the numbers of *Legionella* in the system seems to fluctuate from month to month. Prior to the beginning of this study, it was found that well #103 was positive and well #136 was negative for the presence of *Legionella*. During this study, *Legionella* were isolated from the first 2 samples received from well #103. These samples were collected during April and May 2000. The next 6 samples received from this well showed that *Legionella* concentrations had dropped to below the detection limits of the method. The last 4 sampling events for well #103, during March, April, May, and June 2001, once again showed the presence of *Legionella* in the groundwaters.

These results indicate a possible seasonal distribution of *Legionella* in the groundwater system. However, this assumption is not supported when examining the results from the second well from Water Utility A. For the first three months of the study (April to June 2000), well #136 was tested, but due to technical problems, samples from it were no longer available. Well #138 was selected as the replacement well. When looking at the results, this well almost consistently tested positive for *Legionella* although the types of *Legionella* did vary from month to month. Since *Legionella* was recovered at similar levels over the 10-month period, this does not support the theory of a seasonal variation.

One surprising finding was the isolation of LLAP strains using cultivation without additional supplements since LLAPs are considered as not-yet cultivable species under standard conditions. The LLAPs that were isolated were from plates incubated at 30°C. This lower temperature may be more favorable for their growth than the conventional

incubation at 35°C. The possibility that the isolate was misidentified is improbable since the sequence was 98% identical to LLAP-2, LLAP-9, LLAP-6, *L. lytica* and 97% identical to LLAP-7. In an experiment by Newsome *et al.*, (1998), it was found that a LLAP (identified as LLAP 14) was cultivable on BCYE differential and selective agars at room temperature and 30°C, but not at 37°C. This finding adds support to our previous conclusion of 30°C being a more favorable temperature for LLAPs.

It should be noted that the length of time allowed for biofilm growth on the PVC pipe sections received from Water Utility A was increased from 7 days to 15 days, and eventually to 30 days. It was not known whether *Legionella* were initial colonizing bacteria of biofilms so it was then decided to join two pipe sections in series to allow biofilm to develop for 60 days but to still be able to sample the wells each month. The PVC pipe sections received from all other water utilities allowed 30 days of biofilm growth on the pipe sections except for Water Utility F where only 15 days of growth was allowed.

In addition to groundwater well samples, Water Utility A was kind enough to provide additional types of samples for *Legionella* testing. KII RW and KII IFS samples were also taken from the raw water before any additional treatment but not directly from the groundwater well. Samples 05B, 15A, 21A, 32G, 72F and 94A were water meters taken from various locations in the distribution system that are fed by the same groundwater well field.

The remaining samples listed are those from cooling towers also within the same water distribution area as Utility A. Cooling towers are often implicated as the source of community acquired Legionnaires' disease. These additional samples give an indication

as to the wide spread presence of *Legionella* in the distribution system. The concentrations of the bacterium in the distribution system are, in general, 10- to 1000-fold higher than those found in the water and biofilm samples from groundwaters. This was expected since constructed systems act as natural amplifiers for *Legionella* and are often implicated in outbreaks of Legionnaires' disease (Edelstein and Meyer, 1994). These results seem to indicate that fewer types of *Legionella* are present in the distribution system although because of the lower number of samples received this difference could be artificial.

The *Legionella* concentration (specifically *L. pneumophila*) considered necessary to cause one or more sporadic cases of Legionellosis per year is 10^2 to 10^4 CFU per liter (Ezzeddine *et al.*, 1989). Other studies indicate that *Legionella* only appeared to present a health risk to humans when a threshold value estimated at 10^4 to 10^5 CFU/L is reached (Leoni and Legnani, 2001). It should be noted that although *L. pneumophila* was isolated from a small percentage of the groundwater well samples, it does appear to be the predominant species present in the cooling tower samples. The concentrations in the cooling tower and water distribution samples were in the range considered to be a potential health risk. Even some of the groundwater samples contained concentrations between the suggested 10^2 to 10^4 CFU/L necessary for sporadic cases of Legionellosis.

Assessment of the risk of Legionnaires' disease outbreaks based solely on counts of *Legionella* may be misleading. In one study, results taken from the same cooling tower were statistically unrelated when samples taken a week apart were compared (Bentham, 2000). Previous work has already demonstrated that *Legionella*

concentrations in a system may vary as much as 3 orders of magnitude within 10 minutes (Bentham and Broadbent, 1993)

All other water utilities that participated in this study submitted a single water and biofilm sample from each well that was included by the utility. Depending on the Water Utility, either one or two wells were sampled. The conventional cultivation results are shown in Table 3-2. Both warm and cold groundwater wells were included in the study. The field parameters measured for each water utility are listed in Table 3-4, Table 3-5, Table 3-6, and Table 3-7.

The results show that *Legionella* were present irrespective of the water temperature since they were isolated from the cold water samples received from Ontario, New Jersey, Nebraska and Michigan as well as in the warm water samples received from Texas, Florida, and Arizona. For this study, cold groundwater was defined as water at temperatures below 15°C.

The groundwater samples analysed during this study were designated as groundwater wells not under the influence of surface water. Therefore, the discovery of *Legionella* spp. in these wells was significant since only surface waters where historically considered to contain the bacteria. There are several possible explanations for the presence of *Legionella* spp. in these groundwater wells. The first, and most likely explanation, is even though these wells are not suppose to be under the influence of surface water, the bacteria, overtime, is still able to migrate to the water table through the overlying earth formations. Also, all groundwaters were at one time surface water that, sometime in the past, became no longer exposed to the direct influences of the earth's surface. Either of these explanations could account for the presence of *Legionella*.

Although not investigated in this study, due to the low nutrient content found in groundwater, the *Legionella* bacteria are probably not growing in the groundwater but are instead in a stationary phase allowing them to survive for extended periods of time.

It was observed during cultivation of the water and biofilm samples from Texas (designated CS2 and CS5) that the samples had few other types of contaminating bacteria. The groundwater temperatures for these sample was >40°C. Temperatures between 45°C and 55°C are not optimal for the growth of *Legionellae* but give them a selective advantage over other bacteria commonly found in drinking water (Krammer and Ford, 1994).

Table 3-4 Field parameters for well #103 from Water Utility A

Sampling Date	Pipe section Biofilm growth (days)	Water (°C)	Air (°C)	Dissolved Oxygen (mg/L)	Turbidity (NTU)	pH	Conductivity (mS/m)
March-April 2000	7	24.9	27.2	0.40	0.14	7.46	48.0
April- May 2000	14	25.3	29.4	0.45	0.16	7.45	4.17
May- June 2000	19	25.0	30.0	N/D	N/D	N/D	N/D
June- July 2000	18	25.2	31.7	0.55	0.26	7.65	43.9
August-September 2000	18	24.9	ND	0.51	0.29	7.65	47.2
November-December 2000	26	ND	ND	ND	ND	ND	ND
December-January 2001	33	24.3	23.0	0.50	0.22	7.53	45.8
January-February 2001	58	24.1	25.0	0.30	0.28	7.58	49.0
February-March 2001	31	24.4	23.0	0.55	0.16	7.49	43.0
April-May 2001	71	24.4	23.0	0.55	0.16	7.49	43.0
May-June 2001	29	ND	ND	ND	ND	ND	ND
June-July 2001	66	ND	ND	ND	ND	ND	ND

Table 3-5 Field parameters for well #136 from Water Utility A

Sampling Date	Pipe section Biofilm Growth (days)	Water (°C)	Air (°C)	Dissolved Oxygen (mg/L)	Turbidity (NTU)	PH	Conductivity (mS/m)
March-April 2000	7	25.0	25.6	0.45	0.21	7.52	45.1
April- May 2000	14	25.3	28.9	0.48	0.15	7.47	42.1
May- June 2000	19	N/D	N/D	N/D	N/D	N/D	N/D

Table 3-6 Field parameters measured for well #138 from Water Utility A

Sampling Date	Pipe section Biofilm Growth (days)	Water (°C)	Air (°C)	Dissolved Oxygen (mg/L)	Turbidity (NTU)	PH	Conductivity (mS/m)
June- July 2000	18	25.1	31.1	0.61	0.19	7.78	43.2
August-September 2000	18	25.1	ND	0.48	0.25	7.97	45.7
November-December 2000	26	23.7	25.1	0.50	ND	7.46	ND
December-January 2001	33	24.2	22.5	ND	0.36	7.58	43.2
January-February 2001	58	24.3	25.0	0.45	0.24	7.55	47.8
February-March 2001	31	23.6	23.0	0.48	0.22	7.38	41.1
April-May 2001	71	23.6	22.5	0.48	0.22	7.38	41.1
May-June 2001	29	25.3	30	0.61	ND	7.50	ND
June-July 2001	66	25.1	31.1	0.55	0.46	7.48	38.8

Table 3-7 Field parameters for water utilities involved in the study (other than Water Utility A well #103, well #136, and well #138)

Water Utility	Well ID	Water (°C)	Air (°C)	Dissolved Oxygen (mg/L)	Turbidity (NTU)	PH	Conductivity (mS/m)
A	KII RW	23.2	16	ND	ND	7.5	ND
	KII IFS	25.1	18	ND	ND	7.73	ND
	05B	29.4	ND	3.69	0.26	7.68	51.0
	15A	26.3	ND	3.92	0.29	7.71	50.8
	21A	26.3	ND	2.03	0.33	7.65	51.0
	32G	26.6	ND	5.82	0.33	7.78	51.9
	72F	26.3	ND	3.99	0.24	7.67	52.5
	94A	26.3	ND	6.43	0.30	7.84	50.4
	CT1	24.7	29	ND	2.30	9.9	4590
	CT2	20	27	ND	1.43	8.97	94.7
	CT3	25.0	31	ND	1.10	9.52	412.0
	CT4	25.7.	33	ND	0.27	8.63	51.1
	CT5	26.9	35	ND	5.58	8.88	80.3
	JA	30.1	33	ND	0.93	9.44	409.0
	CRIM	27.6	35	ND	11.5	9.40	390.0
	CH1	28.4	35	ND	3.2	9.42	382.0
	EMS	27.6	29.5	ND	18	8.89	81.6
	CLW	29.8	33	ND	1.9	9.68	1746.0
C	AZ	33	28	ND	ND	7.7	ND

Cont'd

Table 3-7 Continued

Water Utility	Well ID	Water (°C)	Air (°C)	Dissolved Oxygen (mg/L)	Turbidity (NTU)	PH	Conductivity (mS/m)
D	EP	25	ND	ND	0.19	8.2	94.6
E	ST	20	22.2	5.3	ND	7.2	ND
F	CS2	44.6	ND	0.86	0.09	8.12	ND
	CS5	40.5	ND	0.55	0.08	7.82	ND
G	PEO	ND	ND	ND	ND	ND	ND
H	PK	13.3	ND	ND	ND	7.2	ND
I	MEX	14.4	ND	ND	0.39	7.88	0.688
J	MN	11.8	-6.7	1.5	4.0	7.4	80.4
	SF	10.9	-6.7	5.8	2.3	7.3	99.4
K	NJ	12.3	ND	ND	ND	7.70	ND
L	OTT	9.0	ND	ND	0.15	7.6	ND
M	NB3	ND	ND	ND	ND	ND	ND
	NB7	ND	ND	ND	ND	ND	ND
N	MO18	14.5	23.4	4.1	21.9	7.47	64.3
	MO34	15.7	23.4	1.9	35.2	7.10	67.9
O	NE54	14.4	31.1	ND	0.14	7.2	45.2
	NE66	15.0	31.1	ND	0.28	7.3	59.0
P	FK 8	24.7	28	3.59	0.28	7.4	59.7
	FK 13	24.0	28	4.79	0.31	7.4	63.0

It has been found that *Legionellae* are only ubiquitous in fresh water with conductivity between 10 and 120 $\mu S\ cm^{-1}$ (Heller *et al.*, 1998). This is equivalent to 1 to 12 $mS\ m^{-1}$. The results indicate that although the conductivity is out of the range for ubiquity according to previous research, *Legionella* species have been isolated from these samples. Total organic carbon and turbidity have previously been positively associated with growth of *Legionella* spp. When looking at the samples from Water Utility A, no conclusions can be satisfactorily drawn since the turbidity did not fluctuate with the absence or presence of positive colonies. There has also been a positive correlation of Zn with the ability to support *Legionella* spp. growth (States *et al.*, 1987) although in this study Zn concentrations were not measured. Findings by another group indicated that there was no apparent correlation between the physical parameters of the water samples and the presence of *Legionella* (Lye *et al.*, 1997). Although statistical analysis correlating field parameters with the presence of *Legionella* was not done, visual inspection of the data does not indicate any obvious connections between measured field parameters and the presence of *Legionella*.

In order to make the conventional cultivation technique as specific as possible, selective media and selective treatments were used during cultivation (as outlined in Section 2). For each water or biofilm sample received, a total of 36 plates were inoculated for each subsample of interest. Using this method, 15.9% of the plates inoculated were overgrown with other microorganisms. Inoculated plates were considered overgrown when a large percentage of the plate was covered in a lawn of microorganisms making it impossible to visual detect *Legionella*. When comparing selective methods and media by looking at the types of plates that were overgrown,

15.4% were CCVC plates, 66.1% were BCYEα plates, and 18.2% were GVP plates. These numbers are expected since BCYEα does not contain any selective ingredients. If the types of treatments are compared instead of the types of media, 37.5% of the overgrown plates were the result of direct plating, 31.3% were from acid treatment, and 30.5% were from heat treatment. These results seem to indicate that the selective treatments were not any more successful at eliminating contaminating bacteria than plating the sample directly onto the media. When comparing the different combinations of media and treatments possible, only the combinations involving BCYE appeared to be significantly greater. The inoculated plates were also held at two different temperatures in an attempt to isolate a variety of species of *Legionella*. With regards to overgrown plates, 47.9% were from plates held at 30°C and 52.1% were plates at 35°C. Neither incubation temperature appeared to have a significant affect on reducing the problem of overgrowth.

The conventional cultivation results were also analyzed to determine which media and treatments were most effective for isolating *Legionella* from the environmental samples. Of all the plates inoculated with samples, only 4.4% were found positive for *Legionella*. The percentage was calculated using all of the samples received during this study. This number is not necessarily reflective of the effectiveness of the media or treatments used since some of the samples included in the calculation may not have had *Legionella* present or the *Legionella* was at concentrations below the detection limit of cultivation. This result does give an indication as to the degree of difficulty and the time consuming nature of isolating *Legionella* using conventional cultivation.

The effectiveness of the selective media and treatments was evaluated by comparing the treatments and media that were found positive for *Legionella* growth. When looking at only the 4.4% of positive plates, 41.3% of colonies were isolated from CCVC, 22.8% from BCYEα, and 35.3% were isolated from GVP. It was expected that the two selective media (CCVC and GVP) would be more favorable for *Legionella* growth since the numbers of contaminating bacteria present is lowered. Fewer numbers of contaminating bacteria not only make the *Legionella* colonies easier to identify since the chances of overgrowth is lowered, but also inhibitory effects on *Legionella* growth by the presence of other bacteria would also be reduced. When comparing the selective treatments for *Legionella* isolation, 29.3% of *Legionella* isolates were from direct plating, 38.3% were from acid treatment, and 32.3% were the result of heat treatment. As with the numbers of overgrown plates, there does not appear to be a significant difference in the treatments used for isolation from environmental samples. When comparing the different combinations of media and treatments possible, no single combination appeared to be better at isolating *Legionella*. In another, it was found that the highest frequency of positive samples was obtained by inoculating the sample after filter concentration and heat decontamination (Leoni and Legnani, 2001). They did not find any significant differences in the isolation frequencies between their direct inoculum technique and the technique involving concentration and acid decontamination although their acid decontamination differed in that it was only applied for 5 minutes whereas in this study 15 minutes at pH 2.2 was employed (Leoni and Legnani, 2001). This suggests that the longer acid treatment time was more effective in isolating *Legionella*, probably due to the

reduction in the numbers of contaminating bacteria. The heat treatments used in both studies were identical.

One finding that was surprising was that 59.3% of the *Legionella* were isolated from plates incubated at 30°C and the remaining 40.7% were isolated at 35°C. Most laboratories attempting to culture *Legionella* use only one temperature, and it is 35°C. A 35°C incubation temperature is used since it is known that *Legionella* are human pathogens and can multiply at this temperature. Adding a 30°C incubation temperature for the water and biofilm samples was done to more closely mimic the temperatures in the groundwater samples. When comparing the *Legionella* spp. present at 30°C and 35°C, pathogenic and non-pathogenic strains were found at both temperatures. These findings indicate that more species and perhaps different species could be isolated using the lower temperature.

A previous survey on 58 groundwater samples from areas of the United States reported only 7% of the 58 wells to be positive for *Legionella* using cultivation although species identification was not done (Lye *et al.*, 1997).

3.3 Semi-nested PCR Assay

3.3.1 Detection Limit for the Semi-Nested PCR Assay

Reference strains available in the laboratory were used to determine the detection limit for the semi-nested PCR procedure for conventional PCR as well as for IMS-PCR following the PCR experimental protocol described in Section 2.

It was found that the detection limit for the first step of the semi-nested PCR (without prior IMS) using *L. steigerwaltii* was between 10^2 CFU/mL and 10^3 CFU/mL (Figure 4). By performing the second step of this assay, the detection limit was lowered

to between 10^1 CFU/mL and 10^2 CFU/mL (Figure 5). Similar results were found using *L. micdadei, L. rubrilucens,* and *L. pneumophila.*

When uncombined IMS was performed prior to the DNA extraction and subsequent PCR, the detection limit for the first step of the semi-nested PCR was, on average, between 10^1 CFU/mL and 10^2 CFU/mL of the IMS isolated bacterial suspension. The detection limit did vary between *Legionella* species with the detection limit for *L. rubrilucens* being between 10^2 and 10^3 CFU/mL (Figure 6) and the detection limit for *L. micdadei* (Figure 9) being between 10^0 to 10^1 CFU/mL. *L. pneumophila* (Figure 7) and *L. steigerwaltii* (Figure 8) both had detection limits between 10^1 and 10^2 CFU/mL.

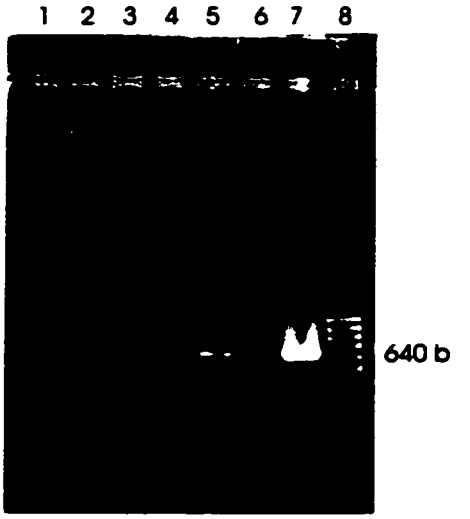

Figure 4: Detection limit for first step of semi-nested PCR procedure determined to be between $10^2$and 10^3 CFU/mL using *L. steigerwaltii* (lane 1:10^0 CFU/mL, lane 2: 10^1 CFU/mL, lane 3: 10^2 CFU/mL, lane 4: 10^3 CFU/mL, lane 5: 10^4 CFU/mL, lane 6: negative control, lane 7: positive control, lane 8: DNA ladder)

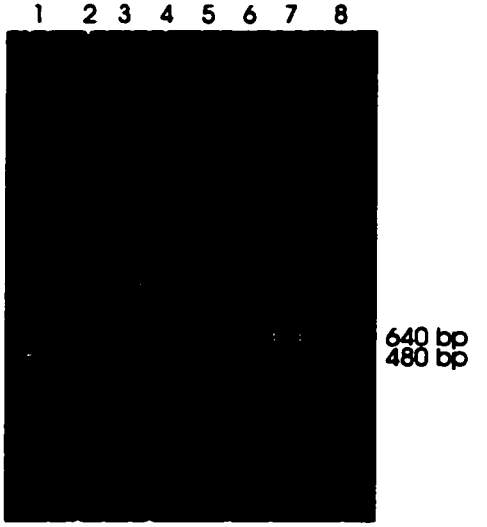

Figure 5: Detection limit for the second step of the semi-nested PCR procedure determined to be between $10^1$and 10^2 CFU/mL using *L. steigerwaltii* (lane 1:positive control, lane 2: negative control, lane 3: 10^1 CFU/mL, lane 4: 10^2 CFU/mL, lane 5: 10^3 CFU/mL, lane 6: 10^4 CFU/mL, lane 7: positive control from step 1, lane 8: DNA ladder)

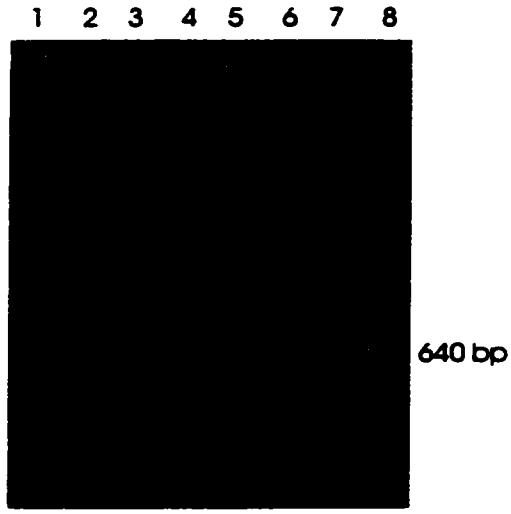

Figure 6: Detection limit for the first step of the semi-nested PCR using prior IMS selection for *L. rubrilucens* determined to be between $10^2$and 10^3 CFU/mL (lane 1:10^3 CFU/mL, lane 2: 10^2 CFU/mL, lane 3: 10^1 CFU/mL, lane 4: 10^0 CFU/mL (extrapolated), lane 5: 10^{-1} CFU/mL (extrapolated), lane 6: negative control, lane 7: positive control, lane 8: DNA ladder)

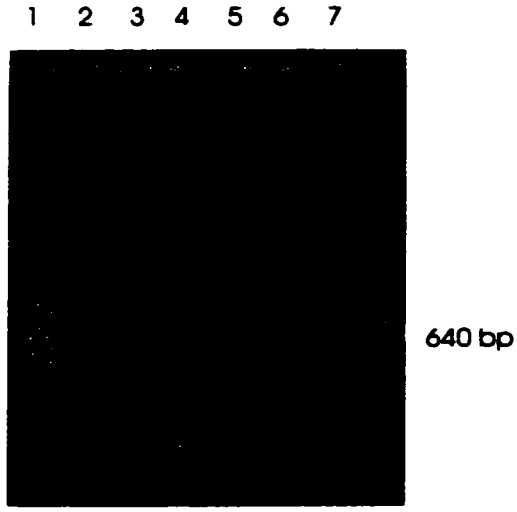

Figure 7: Detection limit for the first step of the semi-nested PCR using prior IMS selection for *L. pneumophila* determined to be between $10^1$and 10^2 CFU/mL (lane 1:DNA ladder, lane 2: 10^3 CFU/mL, lane 3: 10^2 CFU/mL, lane 4: 10^1 CFU/mL, lane 5: 10^0 CFU/mL (extrapolated), lane 6: 10^{-1} CFU/mL (extrapolated), lane 7: negative control, lane 8: positive)

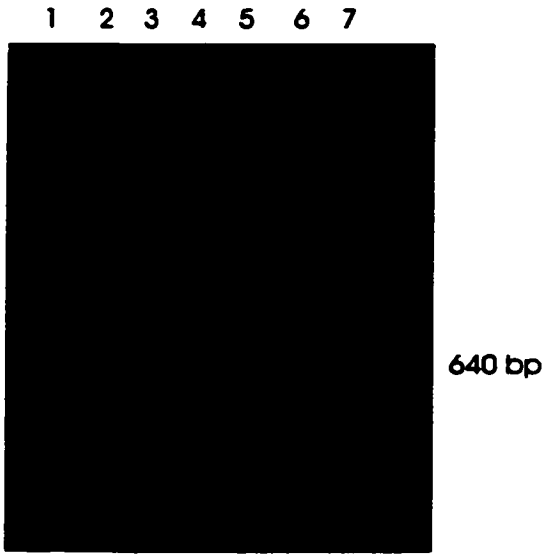

Figure 8: Detection limit for the first step of the semi-nested PCR using prior IMS selection for *L. steigerwaltii* determined to be between $10^1$and 10^2 CFU/mL (lane 1:positive control, lane 2: negative control, lane 3: 10^{-1} CFU/mL (extrapolated), lane 4: 10^0 CFU/mL (extrapolated), lane 5: 10^1 CFU/mL, lane 6: 10^2 CFU/mL, lane 7: 10^3 CFU/mL, lane 8: DNA ladder)

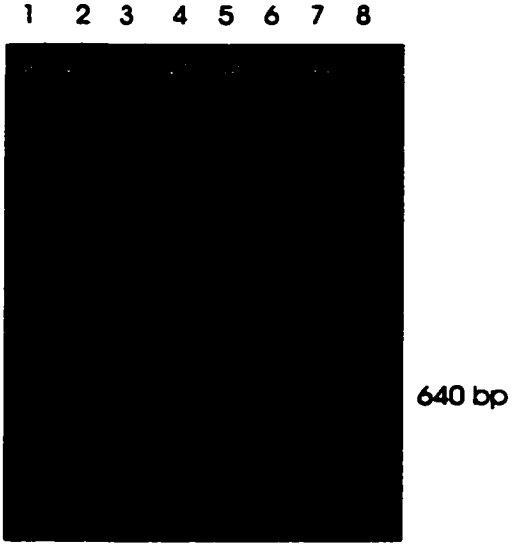

Figure 9 Detection limit for the first step of the semi-nested PCR using prior IMS selection for *L. micdadei* determined to be between $10^0$and 10^1 CFU/mL (lane 1:10^3 CFU/mL, lane 2: 10^2 CFU/mL, lane 3: 10^1 CFU/mL, lane 4: 10^0 CFU/mL (extrapolated), lane 5: 10^{-1} CFU/mL (extrapolated), lane 6: negative control, lane 7: positive control, lane 8: DNA ladder)

A possible explanation for the lower detection limit of the initial step of the semi-nested procedure using IMS is that more *Legionella* are actually being pulled down by the antibodies than is suggested by the plate counts. The aggregation of the bacteria with the antibody could lead to a lower CFU count and therefore a false lower detection limit. Since the recovery of *Legionella* from any given sample has been shown to be variable (sections 3.4.3.1 and 3.4.3.2), and if bacterial clumping with the antibodies has led to lower plate counts and therefore lower recoveries, it is then impossible to define an accurate lower detection limit for *Legionella* from an environmental sample using IMS-PCR. It should be noted that the detection limits are described in terms of CFU/mL because they refer to the detection limit of the process including the DNA extraction and the agarose gel visualization. The detection limits were expressed in this format for direct comparison to *Legionella* concentrations in typical groundwater samples.

3.3.2 Semi-Nested PCR on Environmental Water and Biofilm Samples

The results from the water and biofilm samples received are listed in Table 3-8 and Table 3-9. With the exception of the first samples received from Water Utility A (April 2000), all samples were incubated at 35°C for one week before PCR. The first samples from Water Utility A (April 2000) had PCR performed the day after the samples were received.

Table 3-8 Semi-nested PCR results for Water Utility A (Florida)

Well Identification	Date Received	Water	Biofilm
103-1	04/04/2000	-	-
136-1		-	-
103-2	12/05/2000	Inhibitors	Inhibitors
136-2		Inhibitors	Inhibitors
103-3	20/06/2000	-	-
136-3		Not received	-
103-4	01/08/2000	-	-
138-4		Inhibitors	-
103-5	19/09/2000	-	-
138-5		-	-
103-6	28/11/2000	-	-
138-6		-	-
103-7	09/01/2001	+	+ *(B and ES)
138-7		+	+ *(B and ES)
103-8	09/02/2001	-	Inhibitors *(B and ES)
138-8		-	Inhibitors *(B only)
103-9	27/03/2001	-	Inhibitors
138-9		-	Inhibitors
103-10	02/05/2001	-	Inhibitors
138-10		-	Inhibitors
103-11	01/06/2001	Not received	-
138-11		-	-
103-12	06/07/2001	Not received	+
138-12		-	-
KWII RW	21/11/2000	Not received	+
KWII IFS	21/11/2000	Not received	-
05B	18/04/2001	Not received	-
15A	18/04/2001	Not received	-
21A	18/04/2001	Not received	Inhibitors
32G	18/04/2001	Not received	Inhibitors
72F	18/04/2001	Not received	Inhibitors
94A	18/04/2001	Not received	Inhibitors

*Two biofilm samples were received for samples 7 and 8 for well 103 and 138, B was a PVC pipe section, ES was an end cap scraping resuspended in sterile water

Table 3-9 Semi-nested PCR results for water and biofilm samples from Water Utilities C to Q

Water Utility	Well Identification	Date Received	Water	Biofillm
C (Arizona)	AZ(A)	17/05/2000	+	Not applicable
	AZ(B)		+	+
	AZ(BM)		Not applicable	+
D (Texas)	EP	19/05/2000	+	Inhibitors
E (Illinois)	ST	14/06/2000	+	-
F (Texas)	CS2	26/07/2000	+	+
	CS5		+	+
G (Illinois)	PEO	15/08/2000	-	-
H (Illinois)	PK	22/09/2000	+	+
I (Missouri)	MEX	11/10/2000	-	-
J (Michigan)	SF	15/02/2001	-	-
	MN		-	-
K (New Jersey)	NJ	11/04/2001	-	-
L (Ontario)	OTT	25/04/2001	-	-
M (Illinois)	CH	24/05/2001	-	Inhibitors
N (New Brunswick)	NB3	6/06/2001	+	+
	NB7		+	+
O (Missouri)	MO18	7/06/2001	Inhibitors	Inhibitors
	MO34		-	Inhibitors
P (Nebraska)	NE54	10/07/2001	-	-
	NE66		Inhibitors	-
Q (Florida)	FK8	25/07/2001	-	Not received
	FK13		-	Not received

Using the semi-nested procedure, it was found that 28.2% of the samples tested were positive. In addition, 23.3% of the samples gave inconclusive results since they contained PCR inhibitors. Comparing the PCR results with the cultivation results (Table C-11 and Table C-12) it was found that several samples were PCR positive but were culture negative.

Positive PCR results on a sample that proved culture negative have several explanations. The PCR assay used in this study was shown to be *Legionella*-specific but the possibility of non-specific PCR reactions cannot be ruled out completely. Another possible explanation is the phenomenon of viable but non-culturable cells, giving a positive PCR signal but a negative cultivation result. Because of the non-specificity of the cultivation technique, other microorganisms are also grown at the same time as the *Legionella*. The presence of other microorganisms on the plate could mask the *Legionella* colonies and make them invisible to the observer (Ng *et al.*, 1997). They could also inhibit the growth of the *Legionella* either through substances they release into the surroundings or possibly by using up available nutrients in the medium, thus preventing the growth of the *Legionella* colonies.

Certain samples negative by PCR proved to be culture positive in this study. There are several likely reasons for this finding. It is possible that substances present in the water could have influenced the amplification yield (Maiwald *et al.*, 1994). Even though testing for inhibitors was carried out on the samples, a low amplification yield could give false- negative results. Amplification could still be taking place, but the lower amplification may not allow the *Legionella* in the environmental samples to be visualized.

Additionally, the detection limit for the semi-nested PCR was determined to be between 10^1 and 10^2 CFU/mL (or 10^4 to 10^5 CFU/L). All of the environmental samples tested during this study by cultivation were found to have *Legionella* concentrations lower than the detection limit of PCR. Although some of these samples tested positive for *Legionella* using the semi-nested PCR procedure, it is not surprising that many of the samples did not. Because viable-but-not-culturable cells are thought to be the rule with *Legionella* instead of the exception, the concentrations of *Legionella* found using cultivation may not accurately reflect the actual concentration of *Legionella* in the sample. Because of this inability to accurately predict the concentration of the bacteria in the samples, negative PCR results on culture positive samples could occur.

3.4 Immunomagnetic Separation (IMS)

To develop an immunocapture technique suitable for isolating bacteria from a sample of interest, in this case from water and biofilm samples, there are three main requirements. First, an antibody to a suitable cell surface component must be available. Second, non-specific attachment of the cells to the beads should be minimal and third, the bead-cell complexes must be sufficiently stable to be attracted towards a magnet (Morgan *et al.*, 1991).

To meet these requirements, a monoclonal antibody directed towards the macrophage infectivity potentiator (Mip) protein was selected. The Mip protein is a surface protein that was shown to be present in 29 of the 30 *Legionella* strains tested (Cianciotto *et al.*, 1990) and the Mab 22/1 monoclonal antibody selected was shown to recognize the Mip epitope common to all 34 *Legionella* species tested (Helbig *et al.*, 1995). Although not explored in this study, due to the difficulty of obtaining monoclonal

genus-specific antibodies, other surface proteins (ie. antibodies to a lippopolysaccaride, major outer membrane protein, or heat shock proteins) could also be used as targets for the IMS procedure. One advantage to using IMS is its ability to select cells of interest from a mixed population and pull them out of the solution. This helps in not only removing contaminating bacteria for use in combination with cultivation, but IMS also could remove growth inhibitory- or PCR inhibitory- substances present in the sample. One benefit to IMS is the fact that bacteria bound to magnetic beads usually remain viable and can be plated directly without having to be separated from the beads (Olsvik *et al.*, 1994).

3.4.1 Antibody Titration

In setting up an IMS procedure for isolating legionellae, titration of the primary and secondary antibody was performed to determine the optimum amounts for maximum recovery of *Legionella* species. Varying amounts of the primary antibody (at a concentration of 1 mg/mL) was used keeping the amount of secondary antibody added constant at 15 μL. It was found that the optimal amount of primary monoclonal antibody for 15 μL of secondary anti-mouse antibody was 15 μL (Table C-1).

Using the primary antibody at a concentration of 1 mg/mL quickly depleted the available stock of primary antibody. A new supply was received and purified. Titration of the primary antibody was redone using a range of concentrations from 1 mg/mL to 0.1 μg/mL. The optimal concentration was found to be 1 μg/mL (Figure 10). The same concentration was also found to be optimal when using *L. steigerwaltii* suspended in a sterilized biofilm sample (Table C-2) instead of distilled water (Table C-1). Varying the amount of primary antibody added at a concentration of 1 μg/mL with a constant amount

of secondary antibody (15 μL) showed the highest recovery of legionellae when 20 μL of the 1-μg/mL solution of primary antibody was added (Table C-3)

3.4.2 Specificity Testing

One problem that was encountered when IMS was used for the isolation of Legionella from environmental samples was other contaminating bacteria being co-enriched.

To determine if the primary antibody was binding to other bacteria, its specificity was tested against a strain of *P. aeruginosa* as representative of strains frequently encountered in environmental samples and known to cross-react with *Legionella* when using direct fluorescent antibody typing. This testing was done using flow cytometry and showed that the Mab 22/1 did not cross react with the strain of *P. aeroginosa* (data not shown).

Experiments were then carried out to determine if the secondary antibody was binding non-specifically to the surface of the *Legionella* as well as to other bacteria in the environmental samples. As an initial experiment, IMS was carried out on a biofilm sample but only the secondary antibody was added. After 2 days incubation, the plates were all overgrown with other contaminating bacteria. This observation led to two possible conclusions. Either the secondary antibody non-specifically bound to bacteria in the samples, or all of the bacteria were not being washed away during the washing step. The latter explanation probably accounts for the majority of the bacterial contamination.

To determine the extent of non-specific binding that occurred during the IMS procedure, pure suspensions of *Legionella* were used. Since the secondary antibody binds to the Fc portion of the mouse monoclonal primary antibody, serial dilutions of a

mouse IgG (initial concentration 1 mg/mL) were combined with the secondary antibody prior to its addition to the legionella-specific primary antibody.

As shown in Figure 11, the amount of non-specific binding that occurs during the IMS procedure, using a pure suspension, was approximately 15 % of the total number of bacteria found. As the amount of mouse IgG was decreased, the recovery of *Legionella* increased, proving that there is specific binding of the antibodies and that only a small percentage of the bacteria being isolated resulted from non-specific binding.

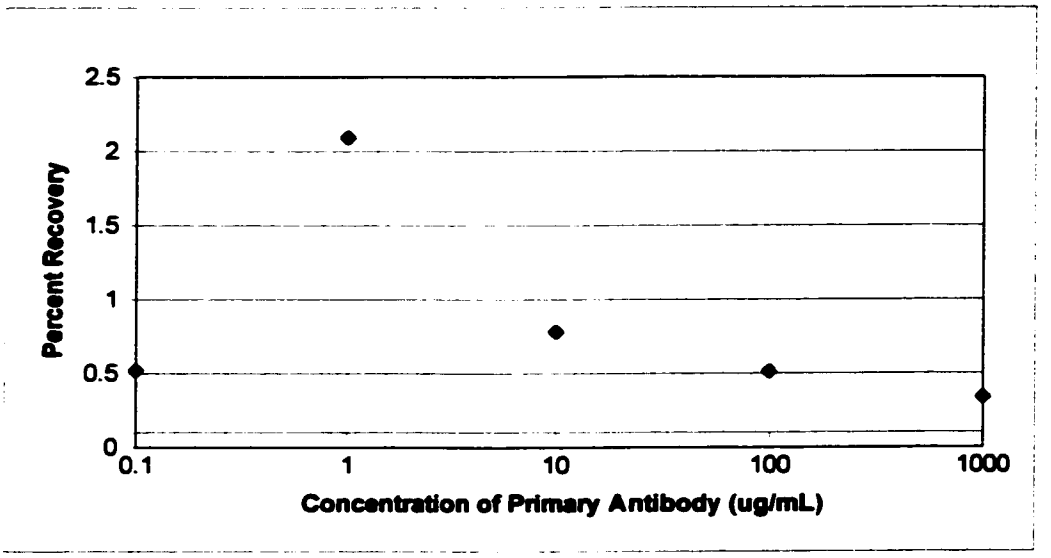

Figure 10 Titration of the primary monoclonal antibody (Mab 22/1) against *L.steigerwaltii* (3 day old culture) at a concentration of 10^3 CFU/mL to find the optimum concentration for *Legionella* recovery.

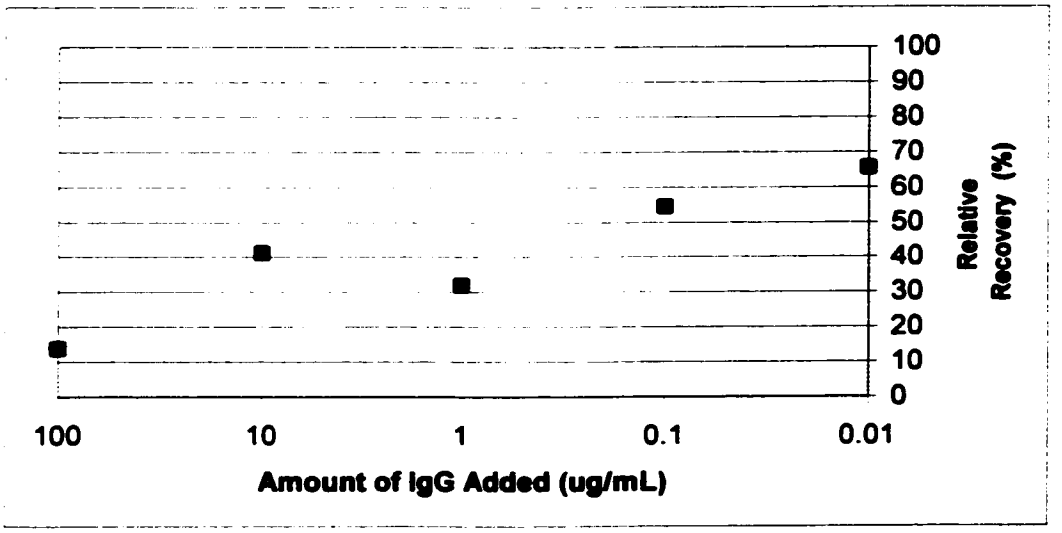

Figure 11 Relative recovery of *L. steigerwaltii* (3 day old culture) to test the degree of non-specific binding during IMS using a mouse IgG antibody (1 mg/mL) at various concentrations to block the binding site on the secondary antibody.

3.4.3 IMS Using Pure Suspensions of *Legionella*

It is generally accepted that IMS can be directed either at maximizing the capture of target cells and accepting a high contamination level or at obtaining a smaller population of pure target cells. It was decided that the procedure should be directed towards maximizing cell recovery and accepting a higher contamination level. The amount of contamination could be reduced using additional washing steps. In an experiment using *Pseudomonas putida,* it was shown that at least four washes were required to remove unattached cells from bead samples (Morgan *et al.*, 1991) but, during additional washing steps, target cells are also lost. The strength of the cell-bead interaction would influence the level of target bacteria loss during washing steps. Since the strength of the cell-bead interaction is unknown, only a single washing step was used. During the washing step, the cell-bead complexes were attracted towards the magnet and held at the side of the tube. The option of resuspending the beads in the wash and reattracting the cell-bead complexes to the magnet was explored (data not shown) but because the goal was maximum cell recovery, it was decided that the less disruption to the cell-bead complexes, the greater the cell recovery. Also, it was found that even though the magnetic beads are super-paramagnetic, with additional washing steps, the beads appeared to attach to each other (creating a film on the eppendorf tube) making it difficult to put them back into suspension.

Two types of IMS were explored to determine which method was the better choice for recovery of *Legionella* although both methods employed the single washing step with the cell-bead complexes attracted to the magnetic support.

3.4.3.1 Uncombined IMS

The first method, as outlined in Section 2, used the uncombined primary monoclonal (Mab 22/1) antibody and the secondary antibody with an incubation time between the additions of each. The recovery of *Legionella* using this method varied between species as well as between experiments. As shown in Figure 12, for 5 and 7 day old cultures, the recovery of *L. steigerwaltii* from distilled water using uncombined IMS was around 10% at concentrations between 10^2 and 10^3 CFU/mL. The 13-day-old culture had a peak recovery of around 3% for the same concentration range. In all cases the recovery decreased as the concentration increased. Similar recovery patterns were found using *L. rubrilucens* (Figure 13) with a recovery of approximately 6% in the range of 10^2 to 10^3 CFU/mL. The remaining two reference strains, *L. micdadei* and *L. pneumophila*, were also tested and it was found that the former had a recovery of approximately 6% in the range of 10^2 to 10^3 CFU/mL and the latter a 5% recovery in the same range (data not shown). These tests were carried out on cultures that were greater than 7 days old.

It has been reported in experiments with *Cryptosporidium parvum* that the recoveries for seeded one liter water were as high as 63-82% for flow-through magnetic columns but as low as 10% when a static separation system was used (Rochelle *et al.*, 1999). The IMS procedures used in this study are part of the latter group. If these results are typical, a flow-through system could increase the recoveries dramatically.

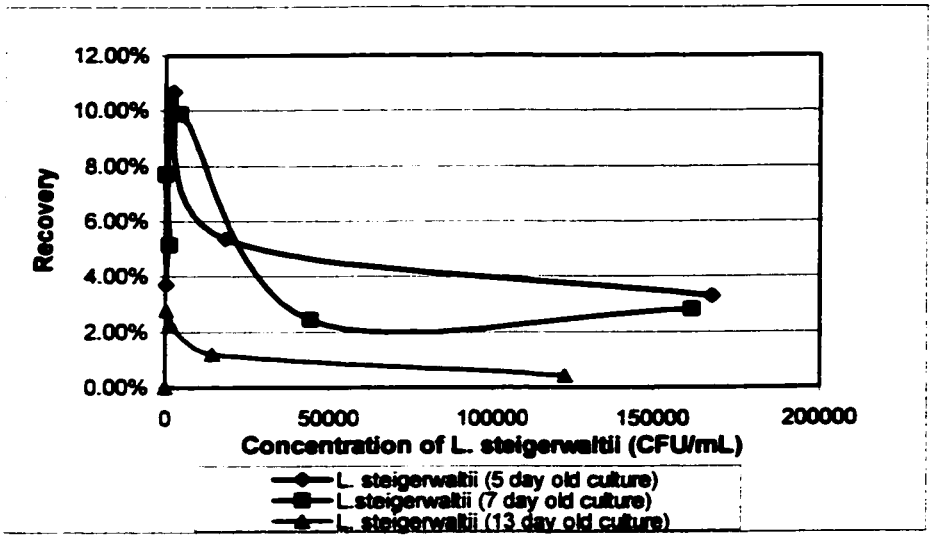

Figure 12 Recovery of *L. steigerwaltii* using IMS with uncombined antibodies (15μL of 1mg/mL primary monoclonal (Mab 22/1) antibody, 15μL of secondary antibody).

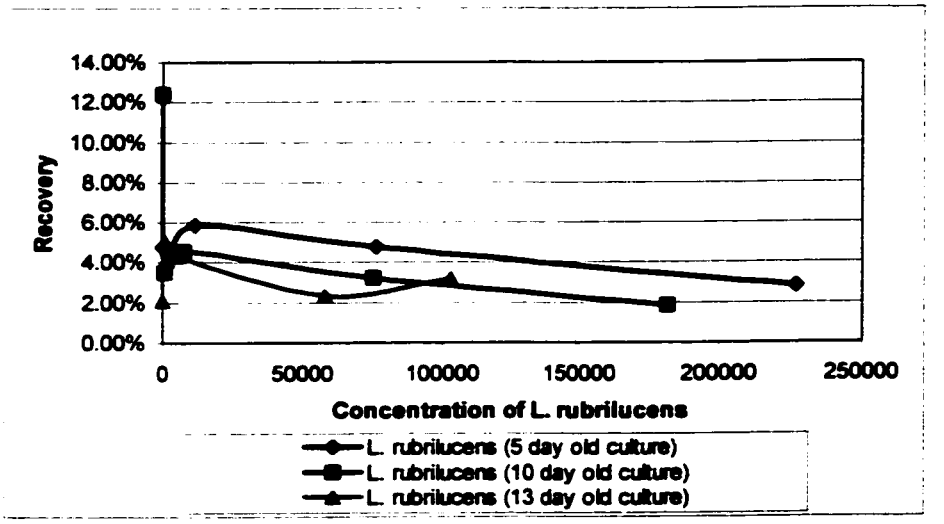

Figure 13 Recovery of *L. rubrilucens* using IMS with uncombined antibodies (15 μL of 1 mg/mL primary monoclonal (Mab 22/1) antibody, 15 μL of secondary antibody).

The recoveries of the *Legionella* reference strains indicate that the age of the organism used could affect the recovery efficiencies. This could be the result of a change in the number of surface antigens present or the condition of the antigen. Both would affect the binding by the antibody or the binding specificity (Rochelle *et al.*, 1999). The initial experiments with the reference strains showed a decrease in recovery when using older cultures of *Legionella*. After the second batch of primary antibody was purified and an optimum concentration (20 μL of a 1 μg/mL solution) was determined, the reference strains were re-tested and it was found that there still appeared to be higher recovery at the lower concentrations, although the age of the strain did not appear to have as much of an effect on the recovery as previously noted (Figure 14, and Figure 15). On average, the recoveries were still greatest between 10^2 to 10^4 CFU/mL for all reference strains.

3.4.3.2 Combined IMS

The second IMS method (outlined in Section 2) combined the antibodies prior to addition to the sample. The recoveries for the two methods are compared at two concentrations. The recovery was first tested in sterilized distilled water. These results are shown below.

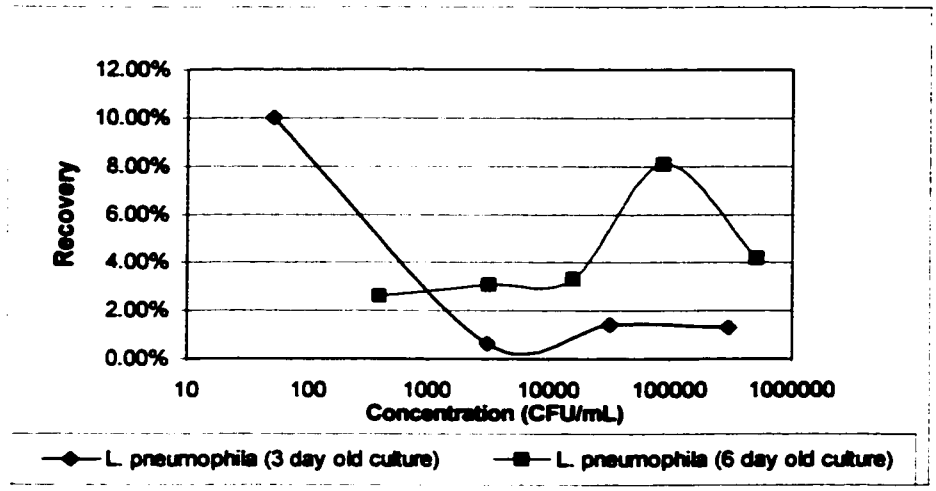

Figure 14 Recovery of *L. pneumophila* using IMS with uncombined antibodies (20µL of 1µg/mL primary monoclonal (Mab 22/1) antibody, 20 µL of secondary antibody).

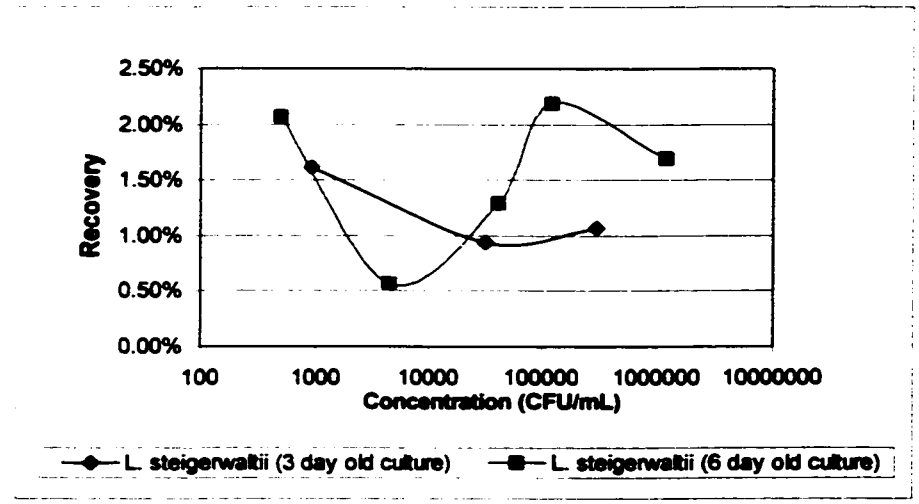

Figure 15 Recovery of *L.steigerwaltii* using IMS with uncombined antibodies (20µL of 1µg/mL primary monoclonal (Mab 22/1) antibody, 20µL of secondary antibody).

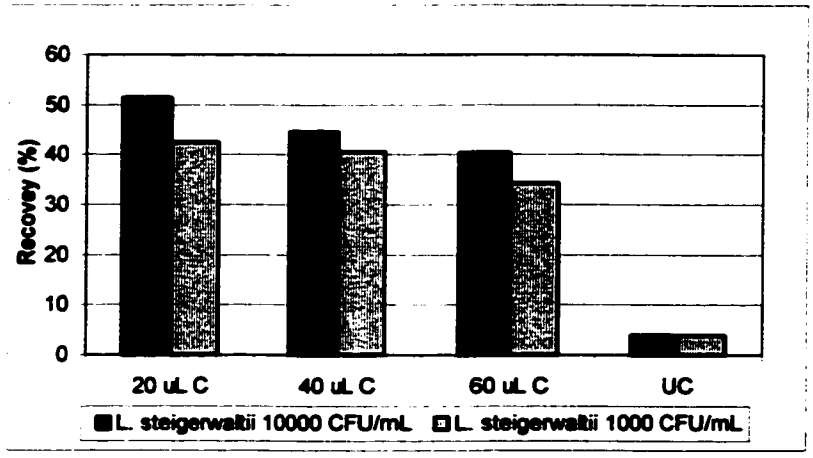

Figure 16 Average recovery of *L. steigerwaltii* (3 day old culture) using combined (20, 40 or 60 µL) and uncombined (20µL of 1µg/mL primary antibody, 20µL of secondary antibody) IMS in sterilized distilled water.

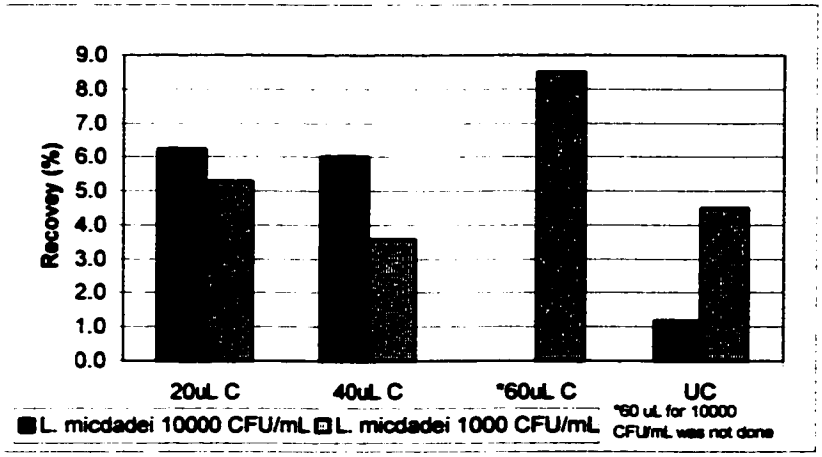

Figure 17 Recovery of *L. micdadei* (3 day old culture) using combined (20, 40 or 60 µL) and uncombined (20µL of 1µg/mL primary antibody, 20µL of secondary antibody) IMS in sterilized distilled water.

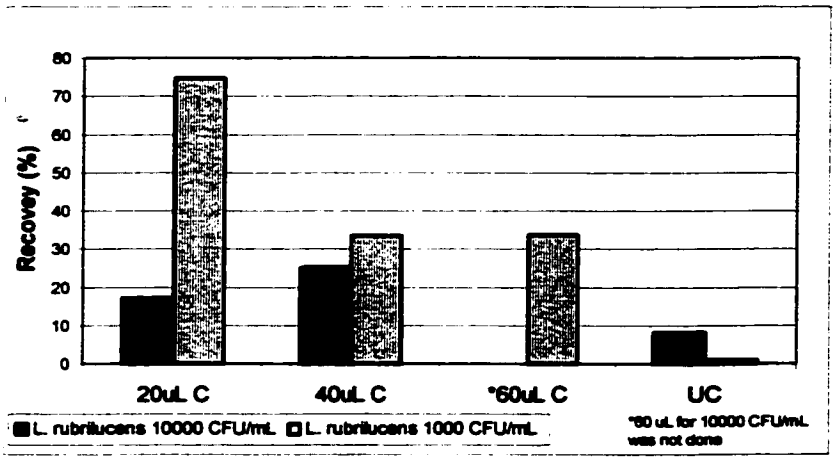

Figure 18 Recovery of *L. rubrilucens* (3 day old culture) using combined (20, 40 or 60 μL) and uncombined (20μL of 1μg/mL primary antibody, 20μL of secondary antibody) IMS in sterilized distilled water.

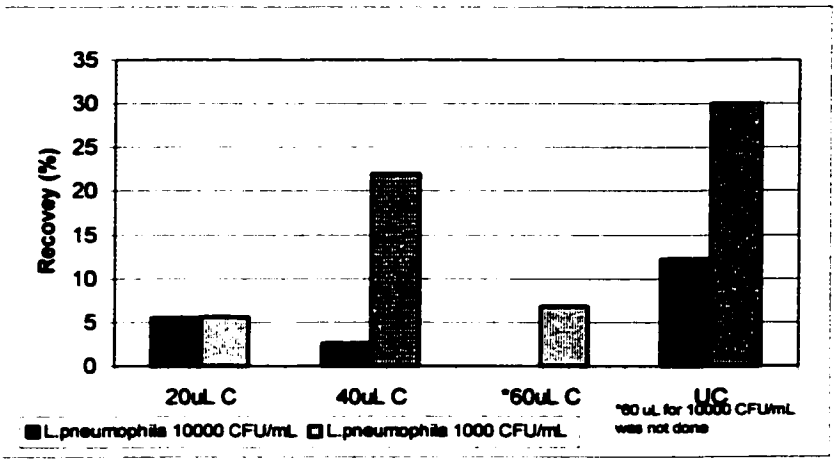

Figure 19 Recovery of *L. pneumophila* (3 day old culture) using combined (20, 40 or 60 μL) and uncombined (20μL of 1μg/mL primary antibody, 20μL of secondary antibody) IMS in sterilized distilled water.

The results show that for 3 of the 4 reference strains, the recovery was higher using the combined IMS method than when using the uncombined method although the amount of beads necessary to capture the largest target population also varied. A comparison of the recoveries proved difficult since they were highly variable between species as well as between treatments and concentrations. Although recoveries as high as 50-70% were obtained using *L. steigerwaltii* and *L. rubrilucens*, the average recovery when combining all reference strains and all combined antibody quantities was 28.3% $\pm$ 28.1 (n=27). Although it was found that the recoveries for each *Legionella* strain were variable, since each strain of *Legionella* was shown previously to bind to the Mab22/1 (Helbig *et al.*, 1995), it was assumed that the variability was due to the technique and not the *Legionella* strain and so the data (only from the same age of strains) for each of the *Legionella* reference strains tested were pooled. All comparisons were made using the data obtained with 3-day-old cultures. Average recoveries using 20 µL of combined antibody was 30.2% $\pm$ 29.8 (n=10), using 40 µL was 26.3% $\pm$ 26.1 (n=10), and using 60 µL was 28.4% $\pm$ 20.2 (n=10). There was no significant difference between the quantities of antibodies used (t = 0.05). The average recovery using uncombined IMS was only 7.3% $\pm$ 8.6 (n=10). From these results, combined IMS was gave significantly higher recoveries (95% confidence interval) for isolating *Legionella* from water samples.

Because the goal of using IMS was to employ this method to successfully isolate *Legionella* from environmental samples, a previously received environmental sample was sterilized and used to test IMS recovery in the presence of other organic material. These results are shown in Figure 20, Figure 21, Figure 22, and Figure 23.

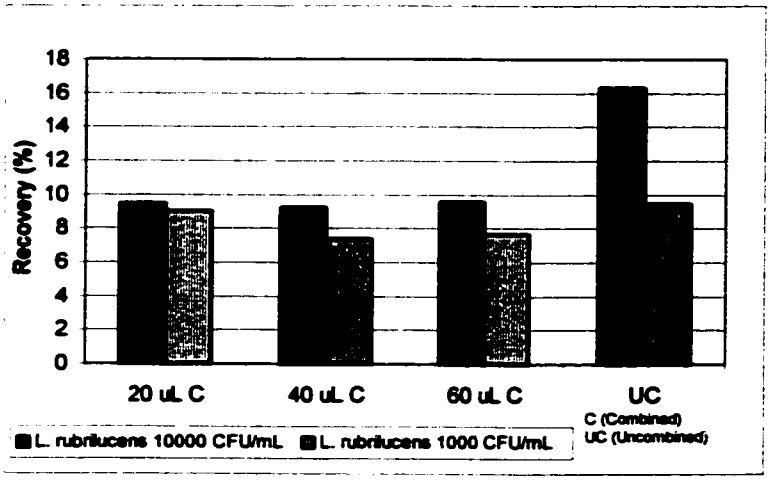

Figure 20 Recovery of *L. rubrilucens* (3 day old culture) using combined (20, 40 or 60 μL) and uncombined (20 μL of 1 μg/mL primary antibody, 20 μL of secondary antibody) IMS in a sterilized environmental water sample.

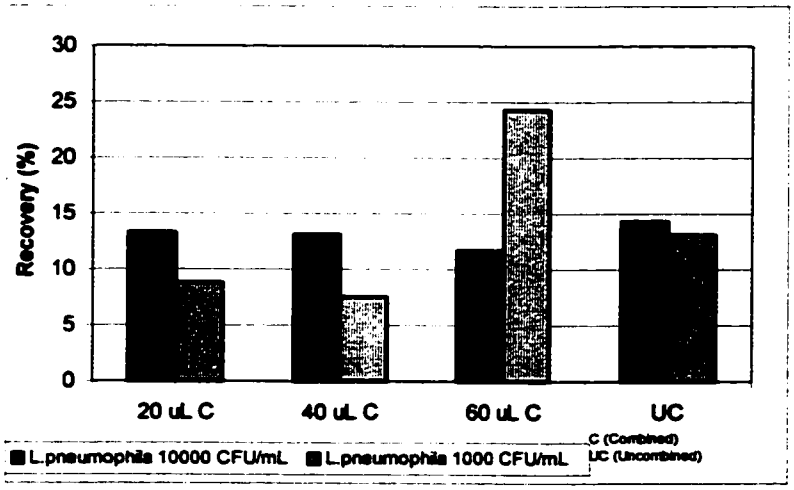

Figure 21 Recovery of *L. pneumophila* (3 day old culture) using combined (20, 40 or 60 μL) and uncombined (20μL of 1μg/mL primary antibody, 20μL of secondary antibody) IMS in a sterilized environmental water sample.

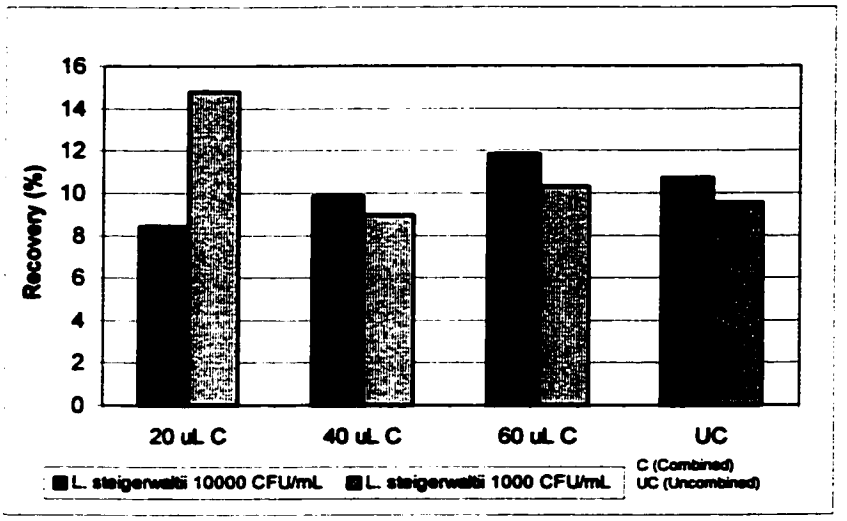

Figure 22 Recovery of *L. steigerwaltii* (3 day old culture) using combined (20, 40 or 60 μL) and uncombined (20 μL of 1 μg/mL primary antibody, 20 μL of secondary antibody) IMS in a sterilized environmental water sample.

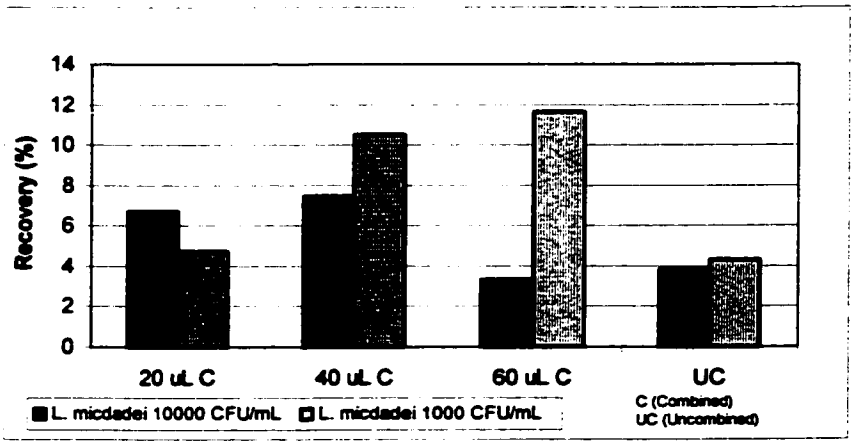

Figure 23 Recovery of *L. micdadei* (3 day old culture) using combined (20, 40 or 60 μL) and uncombined (20 μL of 1 μg/mL primary antibody, 20 μL of secondary antibody) IMS in a sterilized environmental water sample.

Comparisons of the recoveries using the sterilized environmental samples showed the following results. The average recovery when combining all reference strains and all combined antibody quantities was 11.6% ± 6.3 (n=30) compared to 14.5% ± 10.8 (n=10) for uncombined IMS. As done with the previous IMS results, it was assumed that the variability in the recovery was due to the technique and not the strain of *Legionella*, so the data (only from the same age of strains) for the different *Legionella* reference strains were pooled. All comparisons were made using the data obtained with 3-day-old cultures. When comparing the amount of antibody added for combined IMS, 20μL of antibody added had an average recovery of 9.2% ± 3.8, 40 μL added was 12.7% ± 8.1, and 60 μL of antibody added was 12.8% ± 6.3 (n=10). Using a 95% confidence level, there was no significant difference between these recoveries. When comparing these combined IMS recoveries with the uncombined IMS recoveries (14.5% ± 10.8), there was no significant difference between them (for the same 3 day old strains).

When comparing recoveries based on the age of the culture, 3-day-old cultures had recoveries of 9.2% ± 3.8 and 14.5% ± 10.8 (n=10), 8-day-old cultures had recoveries of 8.0% ± 6.1 and 13.7% ± 12.6 (n=12), and 14-day-old cultures had recoveries of 4.4% ± 3.7 and 13.4% ± 11.4 (n=4). These recoveries were from combined IMS and uncombined IMS, respectively. There was no significant difference between the recoveries using combined and uncombined IMS for the different ages of cultures (t=0.05). From the above results it can be concluded that the sterilized environmental water sample had similar recoveries for the combined and uncombined IMS procedures. This is contrary to the results found in distilled water but is a better indication of the

results expected when using IMS to extract environmental *Legionella* from a water or biofilm sample.

In addition to testing recoveries of reference strains, an environmental isolate (randomly chosen from the *Legionella* isolated using conventional cultivation) used at a single concentration, underwent uncombined IMS (using 20 µL of 1 µg/mL primary antibody and 20 µL of secondary antibody, suspended in distilled water) to assess whether the recovery was comparable to the recoveries obtain using the reference strains. Using an initial concentration of 3.0×10^4 CFU/mL, the recovery of the environmental strain was approximately 4.5%. This recovery is within the range found using the laboratory reference strains.

When looking at recoveries, it should be noted that each colony found was not necessarily the product of a single cell. Several cells might be attached to a cluster of beads to initiate a single colony (Olsvik *et al.*, 1994). This clustering of cells with beads acts to artificially lower the recoveries obtained in all the samples. For this reason, it is probable that although the recoveries obtained appear low, they could, in reality, be much higher. These aggregates could also partially explain the variability observed with the IMS technique since the amount and size of clusters is going to be inconsistent from sample to sample.

3.4.5 Susceptibility of Legionella Species to Various Stresses

Contaminating bacteria in the IMS-cultivation procedure was one of the major problems that needed to be addressed if this method were going to be applicable to environmental samples. To try and reduce the levels of heterotrophic bacteria cultivated with the *Legionella*, decontamination procedures were tested on reference strains to

determine their level of susceptibility. The goal was to find a decontamination procedure that had little or no affect on the recovery of the target bacteria. These decontamination procedures were used after IMS in order to circumvent the problem of possible conformational changes in the target epitope. Any conformational changes in the binding site could affect the ability of the monoclonal antibody to recognize, and bind to the surface of the bacteria. It has been shown that after heat treatment the cell shape becomes distorted, and the cell wall appears thickened, probably due to cytoplasm shrinkage (Harley *et al.*, 1997). Also, during acid treatment, there is severe shrinkage of cytoplasmic contents, and increased membrane permeability due to the low pH (Harley *et al.*, 1997). If these treatments, and others that affect the cells surface, were applied before IMS, there could be a decrease in target cell recoveries.

Treatments with heat, acid, chlorine, and lysozyme were tested. Also, the washing step was modified so that instead of a distilled water wash, a low ionic strength water wash (known to increase the recovery rate of *E. coli* O157 cells vs. non-O157 *E. coli* cells (Tomoyasu, 1999)) or a PBS-Tween-20 wash (PBS without sodium azide with 0.05% Tween-20) were employed.

3.4.5.1 Susceptibility to PBS-Tween 20 and Low-Ionic Strength Water Washes

The low-ionic strength wash and the PBS-Tween 20 wash both decreased the recovery of legionella (Table 3-10, Table 3-11, and Table 3-12) although the PBS-Tween 20 reduced the recovery rates more than the low-ionic strength wash. The results using *L. rubrilucens* and *L. micdadei* showed similar results (data not shown). Previous work using Tween 20 has shown that agglutination of the beads and non-specific attachment of the cell were both reduced when a PBS-Tween-20 wash were used (Morgan *et al.*, 1991).

These reductions would be helpful in improving the IMS procedure but because of the decrease in cell survival, neither method proved suitable for use with environmental samples.

Table 3-10 Results of uncombined IMS-cultivation recovery for *L. steigerwaltii* (3-day-old culture) using a low-ionic strength solution

Introduced inoculum (CFU/mL) [Controls]	Inoculum recovered after IMS (CFU/mL) [IMS]		Percentage of recovery for chelex-100 testing	
	In water	In Chelex-100	Compared to water	Compared to controls
$1.93\ 10^5$	$5.57\ 10^3$	$3.17\ 10^3$	56.9	1.64
$2.04\ 10^4$	$1.07\ 10^3$	$8.00\ 10^2$	74.8	3.92
$3.13\ 10^3$	$1.33\ 10^2$	$1.20\ 10^2$	90.2	3.83
$3.07\ 10^2$	-	-		
33	-	-		

Table 3-11 Results of uncombined IMS-cultivation recovery for *L. pneumophila* (3-day-old culture) using a low-ionic strength solution

Introduced inoculum (CFU/mL) [Controls]	Inoculum recovered after IMS (CFU/mL) [IMS]		Percentage of recovery for chelex-100 testing	
	In water	In Chelex-100	Compared to water	Compared to controls
TNTC	$9.10\ 10^4$	$1.12\ 10^5$	123	-
$2.25\ 10^5$	$3.53\ 10^4$	$2.81\ 10^4$	79.6	12.5
$4.60\ 10^4$	$4.52\ 10^3$	$3.80\ 10^3$	84.1	8.26
$1.20\ 10^4$	$8.60\ 10^2$	$6.40\ 10^2$	74.4	5.33
$1.15\ 10^3$	$1.80\ 10^2$	$1.00\ 10^3$	55.6	87.0

Table 3-12 Results of uncombined IMS-cultivation recovery for *L. pneumophila* (3-day-old culture) using a PBS Tween-20 solution

Introduced inoculum (CFU/mL) [Controls]	Inoculum recovered after IMS (CFU/mL) [IMS]		Percentage of recovery (%)	
	In water	In Chelex-100	Compared to water	Compared to controls
TNTC	$9.10\ 10^4$	$2.20\ 10^4$	24.2	-
$2.25\ 10^5$	$3.53\ 10^4$	$2.00\ 10^3$	5.67	0.89
$4.60\ 10^4$	$4.52\ 10^3$	$9.20\ 10^3$	200	20.0
$1.20\ 10^4$	$8.60\ 10^2$	$1.30\ 10^2$	15.1	1.08
$1.15\ 10^3$	$1.80\ 10^2$	20	11.1	1.74

3.4.5.2 Susceptibility to Chlorine

Initial susceptibility testing of the reference strain, *L. steigerwaltii*, to free chlorine levels was done using concentrations ranging from 0.5 ppm to 8 ppm with a contact time of 10 minutes. This initial experiment was a qualitative one to determine the chorine level required to have no growth observed when cultivated. This experiment showed that even at concentrations as high as 8 ppm free chlorine some *L. steigerwaltii* were still cultivatable (data not shown). To further test the susceptibility of the *L. steigerwaltii* reference strain, the same free chlorine concentration range was used with contact times of 1, 3, 5, and 10 minutes. IMS was carried out as described in Section 2 for uncombined IMS except the distilled water wash was replaced with a chlorine wash (desired concentration and contact time) and the beads were resuspended in the solution during the wash instead of remaining attached to the magnetic support. The results showed that when using free chlorine after the IMS procedure, the recovery of the reference strain used varied with concentration and contact time (Figure 24).

From this graph, with the exception of the extended contact times at 8 ppm, only low concentrations of free chlorine did not decrease the recovery of the *Legionella* reference strain tested. Experiments in the laboratory suggest that *Legionella* spp. have reduced resistance to disinfections such as chlorine, after prolonged cultivation on artificial media (Krammer and Ford, 1994). The increase in recovery with extended contact time at high concentrations of free chlorine was not expected and was not a consistent finding. In subsequent experiments (not shown), the high concentrations of free chlorine reduced the recovery of the *Legionella* reference strain to below 20%.

Other researchers have shown that *L. pneumophila* (ATCC33152) was effectively killed using an aqueous solution of chlorine at 2 ppm within 3 minutes (Miyamota *et al.*, 2000).

3.5.4.3 Susceptibility to Lysozyme, Heat and Acid Treatments

The second decontamination procedure tested was the lysozyme treatment. The results are shown in Table 3-13. With this treatment, there was less than a 1-$\log_{10}$ reduction and so further tests with this treatment were performed (Table C-14).

Heat (50°C for 30 minutes) and acid (pH 2.2 for 15 minutes) susceptibility testing of the *L. steigerwaltii* reference strain gave approximately an 80% reduction for each treatment. In addition, the heat and acid treatments were done consecutively on the same sample. The combination of both treatments reduced the recovery of the reference strain to approximately 50% of untreated sample (Table C-9).

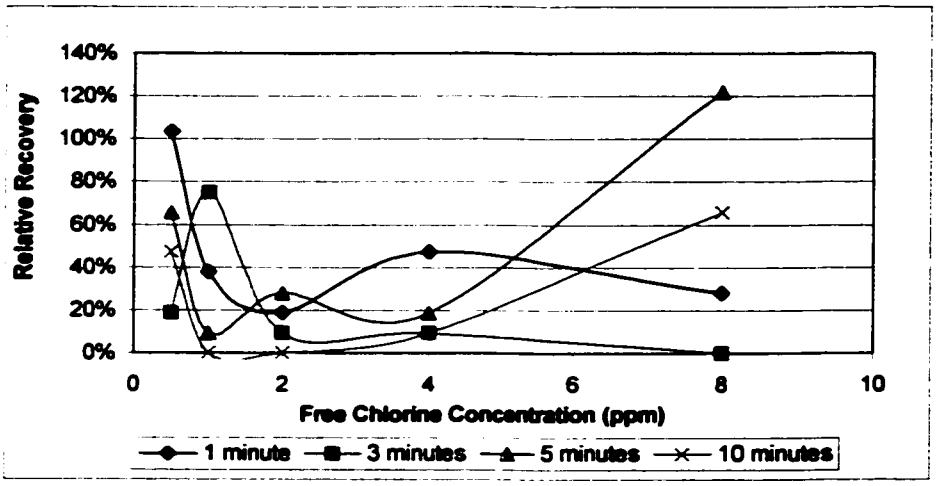

Figure 24 Relative recovery of *L. steigerwaltii* (4 day old culture) after treatment with free chlorine (0.5ppm, 1ppm, 2ppm, 4ppm, or 8ppm) for a set contact time (1, 3, 5, or 10 minutes).

Table 3-13 Susceptibility of *L. steigerwaltii* to lysozyme treatment (1 hour incubation at 35°C, diluted to 20 mL before plating 100 uL)

Sample	Plate Counts	CFU/mL	Log	Log Reduction
No lysozyme (control)	109, 56	825	2.92	
10 U	17, 7	120	2.08	0.84
50 U	96, 23	595	2.77	0.14
100 U	32, 84	580	2.76	0.15
200 U	24, 39	315	2.50	0.42
400 U	45, 98	715	2.85	0.06

3.4.6 Decontamination Procedures on Spiked Environmental Samples

Using the results obtained from the susceptibility testing of the *L. steigerwaltii* reference strain, environmental samples were artificially contaminated with known numbers of *L. steigerwaltii* (as outlined in Section 2 for preparation of spiked environmental samples for decontamination experiments) and the decontamination treatments were applied to try to reduce the contamination by other bacteria during IMS-cultivation.

Since it was shown that *Legionella* spp. (reference strain) could survive chlorine treatment up to concentrations of 8 ppm for 10 minutes (Figure 24), an environmental biofilm sample (stored at room temperature for 2 months) was spiked with a range of concentrations of *L. steigerwaltii* and treated with chlorine. It was found that only a very small amount of the *Legionella* added to the sample survived the treatment although most other bacteria were also killed in the process (data not shown). With this knowledge, it was decided that *Legionella's* resistance to chlorine was not clearly proven and so it was decided against using this method as a decontamination procedure for environmental samples.

Using the same two-month-old biofilm employed for chlorine testing, in addition to a one-week-old biofilm sample stored at room temperature, and a biofilm sample received the day of the experiment, lysozyme, heat, and acid treatments were tested for their abilities to reduce the contaminating bacteria. The biofilm sample used was previously found to be negative for *Legionella* spp. For each sample, the same amount of the reference strain was added to distilled water as a control and IMS was carried out on all samples. None of the treatments was capable of decreasing the numbers of

contaminating bacteria to levels that allowed *Legionella* to be detected when using a two-month-old environmental sample (plates were incubated at 35°C)(Table C-10). Using the same treatments on the biofilm sample stored for one week at room temperature and the biofilm sample received the same day, it was concluded that the disinfection treatments were only effective on recently sampled biofilms (Table 3-14 and Table 3-15).

Table 3-14 Decontamination procedures on an environmental biofilm sample (stored at room temperature for one week) spiked with *L. steigerwaltii* (Ls)(4 day old culture) to try and reduce heterotrophic bacteria

Treatment/Temp	Amount Plated (µL)	BCYE		GVP		CCVC	
		Ls CFU/ mL	Other CFU/ ML	Ls CFU/ mL	Other CFU/ ML	Ls CFU/ mL	Other CFU/mL
Heat/	40						
30		0	OG	0	OG	0	OG
35		0	OG	0	OG	0	OG
Acid/	40						
30		0	10^6	0	10^3	0	10^5
35		50	10^5	50	10^2	0	10^6
Chlorine/	20						
30		0	OG	0	OG	0	OG
35		0	OG	0	OG	0	OG
Lysozyme/	20						
30		0	OG	0	10^6	0	OG
35		0	OG	0	OG	0	OG
No treatment/	20						
30							
35		0	OG	0	OG	0	OG
		0	OG	0	OG	0	OG

OG - overgrown

Table 3-15 Decontamination procedures on an environmental biofilm sample (used the day it was received) spiked with *L. steigerwaltii* (Ls)(4 day old culture) to try and reduce heterotrophic bacteria

Treatment/Temp	Amount Plated (µL)	BCYE		GVP		CCVC	
		Ls CFU/ mL	Other CFU/ ML	Ls CFU/ mL	Other CFU/ ML	Ls CFU/ mL	Other CFU/ mL
Heat/	40						
30		0	10^4	25	0	0	10^4
35		750	10^4	750	10^4	25	10^4
Acid/	40						
30		3850	325	0	0	975	125
35		2725	150	375	25	3675	250
Chlorine/	20						
30		0	10^4	0	0	0	10^4
35		150	10^4	50	2125	0	10^4
Lysozyme/	20						
30		0	10^5	0	150	0	10^5
35		400	10^5	700	5000	100	10^5
Distilled water/	20						
30		0	10^4	0	100	0	10^4
35		1200	10^4	950	4000	1450	10^4

Heat and acid treatments had the highest recoveries for the majority of the samples although the numbers of contaminating bacteria in the heat treatment samples was about the same as for lysozyme and chlorine treatments and the distilled water control. In all cases, the acid treatment was the most effective at decontaminating the sample. Using this information, the heat and acid treatments were chosen as the decontamination methods for IMS-cultivation of environmental water and biofilm samples.

3.4.7 IMS-Cultivation on Environmental Water and Biofilm Samples

The preparation of the environmental water and biofilm samples for IMS and the IMS procedure changed during the course of this study (outlined in Section 2). IMS carried out on environmental samples during August, September, October, and November (2000) yielded no positive colonies. All the plates were overgrown with contaminating bacteria making it impossible to visually identify *Legionella* colonies, if present. The IMS procedure was altered for samples received in January and February 2001 (see Section 2). Storing the samples at 4°C overnight instead of using the enrichment combined with heat and acid treatments did reduce the extent of overgrowth on the plates. Even so, no positive colonies were isolated although positive colonies were found from some samples using conventional cultivation. The final significant change to the procedure involved the reduction in the concentration of the primary monoclonal antibody to 1 μg/mL. With these final alterations, positive colonies were isolated from both water and biofilm samples (Table 3-16 and Table 3-17). Uncombined IMS was employed for samples received up until May 2001. Since tests using reference strains of *Legionella* showed that combined IMS was as effective as uncombined IMS with regards

to recovery of samples, environmental samples received in May underwent both types of IMS. Similar results were found using both methods. Samples received in June and July were only subjected to the combined IMS procedure.

Problems using IMS-cultivation for recovery of environmental isolates appear to be a common. In an unrelated experiment using *Cryptosporidium parvum*, IMS with spiked environmental samples gave efficient recovery of microorganisms although naturally occurring organisms were not recovered from unspiked samples in all investigations (Rochelle *et al.*, 1999). Other organisms have been successfully isolated from samples using IMS-cultivation (Uyttendaele *et al.*, 2000). To our knowledge, IMS-cultivation using *Legionella* as the target organism has not been attempted. One problem with using IMS for environemental samples is the condition of the *Legionella* in the water or biofilm sample is unknown. The surface proteins could be altered preventing the antibody from binding and drawing the bacteria out of the solution. Additionally, the surface proteins could be present and in good condition, but physically unavailable for binding due to other contaminants in the sample. Even with these inherent difficulties, using the final alterations to the IMS procedure, it was possible to use IMS to isolate *Legionella* spp. from the water and biofilm samples. In this study, 25.9% of the samples were found to be positive.

The design of the IMS procedure lowers the detection limit by 10 fold over the conventional cultivation technique used in this experiment. This increase in sensitivity could allow for detection of bacteria that are on the borderline of detection using conventional cultivation. Experiments using *Listeria monocytogenes* showed that although the detection limit was lowered, the ratio of *L. monocytogenes* to non-*Listeria*

flora was not increased (Uyttendaele *et al.*, 2000). These findings are similar to ours using *Legionella* since the proportion of overgrown plates using the IMS procedure was 22.2% compared to 15.9% found using conventional cultivation.

The IMS-cultivation technique used during this study could be possibly be improved by making several changes to the IMS procedure. First, it is possible that a different monoclonal antibody, either to the Mip protein or to a different surface protein, would be more efficient at recovering the *Legionella* bacteria from the sample. Also, additional selective treatments or additional washing steps may help to reduce the number of contaminating microorganisms on the inoculated plates making it easier to visually detect the *Legionella* colonies. As mentioned previously, flow through columns, where the antibody is attached to the column and the water sample is passed through, may increase the recovery of *Legionella*. The latter recommendation would only be effective for water samples since biofilm samples would probably clog the column. These changes to the IMS procedure could increase the quantity of *Legionella* pulled out of the sample and make it easier to detect, not only by cultivation, but also by PCR or possibly other detection techniques.

Table 3-16 IMS-cultivation results for Water Utility A

Well ID	Date	Water (CFU/L)	Strain Identification	Biofilm	Strain Identification
103-9	27/03/	None	N/A	None	N/A
138-9	2001	40	*L. dumoffii*	None	N/A
		40	*LLAP* 2		
		40	*L. santicrucis*		
		40	*L. quateirensis*		
103-10	02/05/	None	N/A	None	N/A
138-10	2001	None	N/A	None	N/A
103-11	01/06/	Not received	N/A	5.2 CFU/cm^2 (C, UC)	*L. quateirensis*
	2001			1.3 CFU/cm^2 (C only)	*L. erythra*
				5.2 CFU/cm^2 (UC only)	*L. dumoffii*
138-11		None	N/A	None	N/A
103-12	06/07/	Not received	N/A	5.8 CFU/cm^2	*L. quateirensis*
138-12	2001	2.5×10^2	*L. quateirensis*	None	N/A
05B	18/04/	Not received	N/A	1.3×10^3 CFU scraped in	LLAP 10
	2001			80 mL water	
15A	18/04/	Not received	N/A	None	N/A
	2001				
21A	18/04/	Not received	N/A	None	N/A
	2001				
32G	18/04/	Not received	N/A	None	N/A
	2001				
72F	18/04/	Not received	N/A	None	N/A
	2001				
94A	18/04/	Not received	N/A	None	N/A
	2001				
CT1	11/05/	Not received	N/A	None	N/A
	2001				
CT2	11/05/	Not received	N/A	5.0×10^2 CFU scraped in	*L. erythra*
	2001			60 mL water	
CT3	11/05/	Not received	N/A	None	N/A
	2001				
CT4	11/05/	Not received	N/A	5.9×10^3 CFU scraped in	*L. pneumophila*
	2001			80 mL water	
CT5	11/05/	Not received	N/A	None	N/A
	2001				
EMS	01/08/	None	N/A	None	N/A
	2001				
JA	01/08/	8.9×10^3	*L.shakespearei*	Not received	N/A
	2001	4.9×10^2	*L. pneumophila*		
CRIM	01/08/	None	N/A	None	N/A
	2001				
CH1	01/08/	1.1×10^4	*L. pneumophila*	8.2×10^3 CFU scraped in	*L. pneumophila*
	2001			50mL water	
CLW	01/08/	3.3×10^2	*L. londiniensis*	None	N/A
	2001	1.3×10^3	*L. israelensis*		

C – Combined IMS, UC – Uncombined IMS

Table 3-17 IMS-cultivation results for water and biofilm samples from Water Utilities K to Q

Water Utility	Well ID	Date	Water CFU/L	Strain Identification	Biofilm Type	Biofilm	Strain Identification
K New Jersey	NJ	11/04/ 2001	None	N/A	Water meter	5.7 CFU/cm^2	*L. sp.* ATCC700511
L Ontario	OTT	25/04/ 2001	None	N/A	Pipe scraped	None	N/A
M Illinois	CH	24/05/ 2001	None	N/A	Water meter	None	N/A
N New Brunswick	NB3	6/06/ 2001	None	N/A	Pipe sections	None	N/A
	NB7		None			None	
O Missouri	MO18	7/06/ 2001	None	N/A	Scraped from pipe	$4.1x10^3$ CFU $2.0x10^3$ CFU scraped in 250mL water	LLAP sp. *L. quateirensis*
	MO34		N/A	N/A		None	N/A
P Nebraska	NE54	10/07/ 2001	None	N/A	Pipe section	None	N/A
	NE66		None	N/A		None	N/A
Q Florida	FK8	25/07/ 2001	None	N/A	Not done	N/A	N/A
	FK13		25	*L. pneumophila*		N/A	N/A

3.4.8 IMS-PCR on Environmental Water and Biofilm Samples

IMS-PCR was performed on the same samples as IMS-cultivation. The results of this testing are shown in Table 3-18 and Table 3-19. Using IMS-PCR, 18.9% of the samples were positive for the presence of *Legionella* and 28.3% of the samples contained inhibitors. It is possible that PCR inhibitors could be reduced using IMS-PCR if, during the IMS procedure, additional washing steps were employed. This was not done in this study because the IMS procedure was designed to maximize the cell recovery and not the purity of the target cells.

Table 3-18 IMS-PCR results for samples received from Water Utility A

Well Identification	Date Received	Water	Biofilm
103-9	27/03/2001	-	Inhibitors
138-9		-	Inhibitors
103-10	02/05/2001	+	Inhibitors
138-10		+	+
103-11	01/06/2001	Not received	-
138-11		-	-(UC), inh(C)
103-12	06/07/2001	Not received	-
138-12		-	Inhibitors
05B	18/04/2001	Not received	-
15A	18/04/2001	Not received	-
21A	18/04/2001	Not received	-
32G	18/04/2001	Not received	Inhibitors
72F	18/04/2001	Not received	Inhibitors
94A	18/04/2001	Not received	-
CT1	11/05/2001	Not received	-
CT2	11/05/2001	Not received	-
CT3	11/05/2001	Not received	Inhibitors
CT4	11/05/2001	Not received	Inhibitors
CT5	11/05/2001	Not received	Inhibitors
EMS	01/08/2001	Inhibitors	+
JA	01/08/2001	-	Not received
CRIM	01/08/2001	Inhibitors	Inhibitors
CH1	01/08/2001	-	Inhibitors
CLW	01/08/2001	-	-

Table 3-19 IMS-PCR results for Water Utilities K to Q

Water Utility	Well ID	Date Received	Water	Biofilm
K (New Jersey)	NJ	11/04/2001	-	Inhibitors
L (Ontario)	OTT	25/04/2001	+	-
M (Illinois)	CH	24/05/2001	-(C), +(UC)	-
N	NB3	6/06/2001	+	+
(New Brunswick)	NB7		+	+
O	MO18	7/06/2001	Inhibitors	Inhibitors
(Missouri)	MO34		-	-
P	NE54	10/07/2001	-	-
(Nebraska)	NE66		-	-
Q	FK8	25/07/2001	-	Not received
(Florida)	FK13		Inhibitors	Not received

3.5 Comparison of IMS and Conventional Cultivation and PCR Techniques

The purpose of using IMS during this study was to determine if using IMS isolation of bacterial cells prior to cultivation or PCR could enhance the detection of *Legionella* in water and biofilm samples. As seen in the conventional cultivation and semi-nested PCR results, neither of these methods separately detected *Legionella* in all the samples, but by combining both techniques *Legionella* could be detected in a greater number of samples. To try and increase the proportion of samples testing positive for *Legionella*, IMS was tested as a pre-selection technique before the conventional methods were applied.

3.5.1 IMS-Cultivation Versus Conventional Cultivation

When comparing the IMS-cultivation to the conventional cultivation technique (Table C-13 and Table C-14) several observations were made. It should be noted that comparisons were only made between the IMS-cultivation results for the samples that were subjected to the finalized IMS procedure and not with all the samples received in this study. Using IMS-cultivation, *Legionella* were isolated from 25.9% of the samples whereas using conventional cultivation 34.5% of the same samples were *Legionella*-positive. One of the major problems with using conventional cultivation is overgrowth by heterotrophic bacteria. In theory, IMS should reduce the levels of contaminating bacteria and therefore the proportion of overgrown plates. The IMS-cultivation results in this study do not support this theory. It was found that 22.2% of IMS-cultivation plates were overgrown compared to 15.9% for conventional cultivation. It should be noted that there were differences in the numbers and types of plates inoculated for each technique. Conventional cultivation used direct plating, heat treatment and acid treatment on the

three media totalling 36 plates for each sample. IMS-cultivation only used direct plating and acid treatment for a total of 12 plates for each sample. Because fewer plates were used for IMS-cultivation and the concentrations of *Legionella* in the samples was quite low, some strains of *Legionella* may not have been recovered. It is also possible that the monoclonal antibody was unable to bind to certain types of environmental *Legionella* or the epitopes on the surface of the bacteria were not in a condition favorable for binding. Improvements in the IMS technique could alleviate some of these problems (outlined in IMS-cultivation results).

3.5.2 IMS-PCR Versus Conventional Semi-Nested PCR

As stated earlier, 18.9% of the samples tested by IMS-PCR were positive while an additional 28.3% of the samples contained PCR inhibitors. If these results are compared to the same samples using semi-nested PCR with enrichment but no prior IMS (Table C-15 and Table C-16), (11.3% positive, and 24.5 % inhibitors) IMS-PCR did increase the proportion of positive samples detected but it did not act to decrease the PCR inhibitors present. As when comparing PCR results to cultivation results, IMS-PCR results compared to PCR results gave positive results by one method with negative results by the other and vice versa. Because IMS is an antibody- based technique, it requires binding of the antibody to the target epitope before the cell of interest can be pulled out of the mixed population of cells. Without proper binding, the target could be removed with the supernatant in the washing step. If this occurs, IMS-PCR cannot detect *Legionella* DNA even though it may have been present in the original sample and was therefore detected by the semi-nested PCR procedure. On the other hand, if the antibody binds well to the target epitope, the *Legionella* will be selectively enriched in the sample and IMS-PCR

will be positive. A positive result for the semi-nested procedure is also possible or, depending on the *Legionella* concentration, the bacteria could be below the detection limit giving a negative result.

As discussed earlier, the number of samples having PCR inhibitors was similar for the two PCR procedures. Improvements are necessary for both procedures to try and eliminate these from the samples. For IMS, an increased number of washing steps could reduce inhibitors. Post IMS treatments to reduce inhibitors could be applied to both samples. These may include changes to the DNA extraction procedure or additional clean-up steps.

4.0 CONCLUSIONS

At the start of this study, there were two main objectives. The first was to determine the presence of *Legionella* species in both water and biofilm samples from underground sources. Conventional cultivation and semi-nested PCR (of the 16S rDNA) were used for this purpose. The second objective was to determine the usefulness of applying an additional separation method, in this case IMS, as a means to improve the cultivation and PCR methods currently used. The following was concluded.

- The presence of Legionella in both water and biofilm from both warm and cold groundwaters was established in this study.
- *Legionella* spp. recognized as human pathogens were isolated from the water and biofilm samples in this groundwater study
- In this study, the number of positive samples by conventional cultivation and the number of positive samples by semi-nested PCR were approximately equal.
- The selective treatments used for cultivation were effective in reducing the amount of heterotrophic bacteria (fewer overgrown plates) and increasing the number of plates containing *Legionella* compared to using no selection procedures.
- Using reference strains, the recovery of *Legionella* using IMS was highly variable (making accurate quantification of the levels of *Legionella* difficult), although on average, the recovery was 10-20%.
- Combined and uncombined IMS gave similar results when used to isolate reference strains of *Legionella* suspended in a sterilized environmental sample received during this study

- **The IMS procedure used in this study, either combined with cultivation or PCR, did not increase the proportion of positive samples or decrease the levels of contaminants compared to the conventional cultivation and PCR methods.**

5.0 FUTURE RESEARCH

From the results of this study, several recommendations can be made for future experiments in this area.

- PCR should be used as a standard method to detect *Legionella* in environmental samples if an absence or presence result is all that is required since it is a fast and easy method for the detection of *Legionella*. Additional clean-up techniques to reduce the presence of PCR inhibitors should be explored.

- Cultivation should be used if more information about the quantity and strains of *Legionella* present are required. Since fewer *Legionella* colonies and more overgrown plates were found with the direct plating technique, its elimination from the procedure should be considered. Additionally, the higher number of *Legionella* isolated from plates incubated at 30°C suggests that this may be a more appropriate temperature for isolating *Legionella* from groundwater samples than using the conventional 35°C.

- During this study, the IMS method used did not improve the cultivation or PCR techniques over the conventional methods although further research using IMS is needed. An alteration of the procedure from isolating the maximum number of target bacteria to isolating the purest sample of target bacteria could reduce both the number of overgrown plates when combined with cultivation and the number of samples containing inhibitors when combined with PCR. Also, a change to a flow through system from the static system used in this study could increase the recovery of the bacteria.

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APPENDIX A FORMULATIONS

A.1 Media

Buffered Charcoal Yeast Extract α-ketoglutarate (BCYEα)

Norit SG charcoal	2 g	(Merck 102186)
Yeast extract	10 g	(Difco 0127-17-9)
ACES buffer	10 g	(Sigma A-9758)
Ferric pyrophosphate	0.25 g	(Pfaltz and Bauer, Inc. F00930)
L-cysteine HCl.H_2O	0.4 g	(Merck 102839)
Agar	17 g	(Difco 0145-17-0)
Potassium α-ketoglutarate	1 g	(Sigma K-2000)
Water	1 L	

Dissolve yeast extract, agar, charcoal, and alpha ketoglutarate in 440 mL water; boil. Dissolve 10 g ACES (N-(2-Acetamido)-2-amino-ethanesulfonic acid) Buffer in 500 mL warm water and add to the 440 mL above. pH is adjusted to 6.9 with 40 mL 1 N KOH. Autoclave 15 min 121°C. Cool at 50°C. Dissolve 0.4 g L-cysteine and 0.25 g ferric pyrophosphate in 10 mL of water each and filter sterilize separately (0.22 μm). After base has cooled, add cysteine, ferric pyrophosphate and dyes (such as bromothymol blue and bromocresol purple if necessary) in that order. Adjust to pH 6.9 with sterile 1N KOH if necessary and dispense.

GVP medium

(BCYEα + glycine + vancomycin + polymyxin B) - less inhibitory to some *Legionella* species

Glycine	0.3 %	
Polymyxin B	100 U/mL	selective agent (Gram -)
Vancomycin	5 μg/mL	selective agent (Gram +)(Sigma V-2002)

To cooled BCYEα-base with glycine (added at the end of boiling; Prolab 24403) add filter-sterilized antibiotics and mix. Adjust pH to 6.9 with sterile 1 N KOH and dispense.

CCVC medium

(BCYEα + cephalotin + colistin + vancomycin + cycloheximide)

Cephalotin	4 μg/mL	selective agent (gram +)
Colistin	16 μg/mL	selective agent (gram -)(Sigma C-4520)
Vancomycin	0.5 μg/mL	selective agent (gram +)(Sigma V-2002)
Cycloheximide	80 μg/mL	selective agent (fungal)

To cooled BCYEα-base add filter-sterilized antibiotics and mix. Adjust to pH 6.9 with sterile 1 N KOH and dispense.

A.2 Acid treatment reagents

Solution A: 0.2 M KCl (14.9 g/L in distilled water)
Solution B: 0.2 M HCl (16.7 mL/L 10 N HCl in distilled water)

Mix 18 parts of Solution A + 2 parts of Solution B, check pH against a pH 2.0 standard buffer. Dispense into screw-cap tubes in 1-mL volumes and sterilize by autoclaving.

A.3 Alkaline neutralizer reagent (0.1 N KOH)

Stock solution 0.1 N KOH (6.46 g/L in deionized water).

Dispense into screw-cap tubes in convenient volumes and sterilize by autoclaving.

A.4 PCR master mix

Composition for a 10 μl sample of DNA (total volume 100 μl):

- 10 μl 10x PCR Buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 0.1 mg/mL of gelatin)
- 6 μl (1.5 mM) of Magnesium Chloride.
- 6 μl (1 mM) of forward primer.
- 6 μl (1 mM) of reverse primer.
- 16 μl dNTP (0.2mM dATP, dGTP, dCTP, dTTP).
- 45 μl ultra-pure water
- 1 μl *Taq* polymerase

Semi-nested PCR components

	First Step PCR	**Second Step PCR**
10X PCR buffer	5.0 µL	5.0 µL
Forward Primer	3.0 µL (LEG 225)	3.0 µL (LEG 448)
Reverse Primer	3.0 µL (LEG 858)	3.0 µL (LEG 858)
DNTPs	8.0 µL	8.0 µL
Sterile water	21.50 µL	22.50 µL
$MgCl_2$ solution	3.0 µL	3.0 µL
BSA	1.0 µL	NOT ADDED
***Taq* polymerase**	0.50 µL	0.50 µL
Extracted DNA	5.0 µL	5.0 µL **(Amplified product from First Step PCR)**
Total volume	50 µL	50 µL

dNTPs solution (mix ready to use)

Mix 12.5 µL of each stock solution of dATP, dTTP, dCTP, dGTP (100 mM each from Pharmacia) with 950 µL of sterile water (dNTP mix 1.25 mM). Use 8 µL of this mix per 50 µL reaction volume (200 µM each).

Primers

Should be made in ready-to-use solutions of 10 pmoles/µL so that 3 µL the final concentration in the PCR assay is 30 pmol (0.3 µM) for each primer.

Thermocycler programs

Non-linking program #65 = first step semi-nested
1 min 30 s jump start at 95°C then press start again
30 cycles: 95°C 10 s
64°C 1 min
74°C 1 min
Post-dwell: 5 min 74°C

Non-linking program #64 = second step semi-nested
1 min 30 s jump start at 95°C then press start again
20 cycles: 95°C 30 s
66°C 1 min
74°C 1 min
Post-dwell: 5 min 74°C

A.5 Buffer

10 X TBE Buffer (TRIS-BORATE-EDTA concentrated 10 times)

Trizma Base	108 g	(Sigma T- 8524)
Boric acid	9,3 g	(Merck - 0165)
EDTA	5,84 g	(Sigma E- 3610)
H_2O QSP	1 Liter	
Autoclave 20 min at 115°C		

A.6 Agarose Gel Electrophoresis

Prepare 2.50 g agarose and add 250 mL of 1.0 X TBE buffer. Heat until agarose melts and cast the gel when cooled enough to touch. Once the gel is solidified in the casting tray, the PCR products and DNA ladder (GeneRuler 100bp DNA Ladder) can be combined with DNA loading buffer comprising of 0.09 % Bromophenol Blue and 0.09% Xylene Cyanol and placed in the wells of the gel. A sample lane breakdown follows:

Lane 1: DNA Ladder (1 kb ladder)
Lane 2: Positive Control (DNA extracted from a known *Legionella* species)
Lane 3: Negative Control (Product of a PCR run with no DNA)
Lane 4: Sample

Other lanes may be used for samples as well but a minimum of 4 lanes is required for running any given sample. The gel is then placed in a field of 5V/cm until the loading buffer and run approximately ¾ of the way down the gel.

APPENDIX B LIST OF ABBREVIATIONS

BCYE α– Buffered charcoal yeast extract
bp – base pair
C – Combined
CDC – U.S. Centre for Disease Control and Prevention
CCVC - BCYEα with colistin, cephalothin, cycloheximide, and vancomycin
CFU – Colony forming units
DFA – Direct fluorescent antibody technique
IMS – Immuno-magnetic separation
LD – Legionnaires' disease
LLAP – *Legionella*-like amoebal pathogens
Mab – Monoclonal antibody
MIC – Mean inhibitory concentration
min – Minutes
Mip – Macrophage infectivity potentiator
N/A – Not available
ND – Not done
NTU – Nephelometric turbidity units
PBS – Phosphate-buffered saline
PCR – Polymerase chain reaction
ppm – Parts per million
PVC – Polyvinyl chloride
sp. – Specie
spp. – Species
TBE – Tris-borate-EDTA solution
UC – Uncombined
UV – Ultraviolet
Zn - Zinc

APPENDIX C DATA

Table C-1 Determination of optimal amount of primary antibody (1mg/mL) using 15 µL of secondary antibody (*L. steigerwaltii* reference strain, 3 day old culture, suspended in sterile distilled water.)

Primary Antibody (µL)	CFU	CFU/mL	% Recovery
5	5, 61, 7	194.7	0.67
10	42, 42, 29	301.3	1.03
15	52, 48, 79	477.3	1.63
20	63, 25, 12	266.7	0.91
25	36, 33, 5	197.3	0.68
30	42, 34, 47	328	1.12
Control	265, 354, 256	29200	

Table C-2 Titration of primary antibody to determine optimal concentration when using 15 uL of secondary antibody (*L. steigerwaltii* reference strain, 3 day old culture, suspended in sterile environmental biofilm sample)

Sample	CFU	CFU/mL	% Recovery
Reference	326	32600	
1 ug/mL	593, 550	5715	**17.53**
0.5 ug/mL	230, 352	2910	8.93
0.1 ug/mL	155, 143	1490	4.57

Table C-3 Titration of 1 ug/mL solution of primary antibody to determine optimal amount when using 15 uL of secondary antibody (*L. steigerwaltii* reference strain, 3 day old culture, suspended in sterile distilled water)

Sample	CFU	CFU/mL	% Recovery
Reference	~300	~30000	
5 uL	18	180	0.60
10 uL	31, 27	290	0.97
20 uL	93,99	960	**3.20**
40 uL	78, 67	725	**2.42**
80 uL	24, 50	370	1.23

Table C-4 IMS experiment using *L. steigerwaltii* (3 day old culture) to determine the extent of non-specific binding by adding 15 µL of a series of serial dilutions of a mouse IgG to 20 µL of secondary antibody and 20 µL of primary antibody

IgG Added (ug/mL)	CFU	CFU/mL	% Recovery	CFU	CFU/mL	% Recovery
Reference	403, 336	36950		42, 38	4000	
100	109, 35	720	1.95	2, 1	15	0.38
10	18, 23	205	0.80	3, 6	45	1.13
1	26, 23	245	1.01	5, 2	35	0.88
0.1	48, 105	765	2.07	4, 8	120	1.50
0.01	103, 118	1105	2.99	11, 3	70	1.80
No IgG	106, 123	1145	3.10	8, 14	110	2.75

Table C-5 Recovery of *L. steigerwaltii* (concentration 7.2 x10^3 CFU/mL, 4 day old strain) after treatment with free chlorine for 1 minute

Chlorine (ppm)	CFU	CFU/mL	Relative Recovery (%)
0.5	5, 6	22	103.13
1	2, 2	8	37.50
2	0, 2	4	18.75
4	3, 2	10	46.88
8	2, 1	6	28.13

*Control (IMS +water wash) had a concentration of 21.3 CFU/mL used to calculate recovery

Table C-6 Recovery of *L. steigerwaltii* (concentration 7.2 x10^3 CFU/mL, 4 day old strain) after treatment with free chlorine for 3 minutes

Chlorine (ppm)	CFU	CFU/mL	Relative Recovery (%)
0.5	1, 1	4	18.75
1	4, 4	16	75.00
2	1, 0	2	9.38
4	1, 0	2	9.38
8	0, 0	0	0

*Control (IMS +water wash) had a concentration of 21.3 CFU/mL used to calculate recovery

Table C-7 Recovery of *L. steigerwaltii* (concentration 7.2 x 10^3 CFU/mL, 4 day old strain) after treatment with free chlorine for 5 minutes

Chlorine (ppm)	CFU	CFU/mL	Relative Recovery (%)
0.5	2, 5	14	65.63
1	0, 1	2	9.38
2	1, 2	6	28.13
4	1, 1	4	18.75
8	12, 1	26	121.88

*Control (IMS +water wash) had a concentration of 21.3 CFU/mL used to calculate recovery

Table C-8 Recovery of *L. steigerwaltii* (concentration 7.2 x10^3 CFU/mL, 4 day old strain) after treatment with free chlorine for 10 minutes

Chlorine (ppm)	CFU	CFU/mL	Relative Recovery (%)
0.5	0, 5	10	46.88
1	Cont, 0	0	0
2	0, 0	0	0
4	1, 0	2	9.38
8	5, 2	14	65.63

*Control (IMS +water wash) had a concentration of 21.3 CFU/mL used to calculate recovery

Table C-9 Susceptibility of *L. steigerwaltii* to heat (30 minutes at 50°) and acid treatments (pH 2.2 for 15 minutes)

Treatment	Average Plate Counts	Recovery
No treatment	*325	100%
Heat	*258	80%
Acid	*126	80%
Heat + Acid	326	50%
Acid + Heat	309	50%

*counted ¼ of the plate and used this count to determine the recovery obtained, therefore is only an approximate value

Table C-10 Decontamination procedures (heat, acid and lysozyme (200 U, 400 U, 800 U) treatments) used on an environmental biofilm sample (stored 2 months at room temperature) to reduce heterotrophic bacteria

Sample Treatment/	Distilled Water Sample			Biofilm Sample
Amount plated (μL)	CFU	CFU/mL	% Recovery	
No treatment / 100	7, 10	85	2.72	Overgrown
Heat / 100	14, 4	90	2.88	Overgrown
Acid / 100	1, 1	10	0.32	Overgrown
200 U / 200	3, 0	15	0.48	Overgrown
400 U / 200	0, 0	0	0	Overgrown
800 U / 200	0, 22	110	3.51	Overgrown

* Initial concentration of *L. steigerwaltii* (before IMS, 5 day old culture) was 3.13×10^3 CFU/mL

Table C-11 Comparison of semi-nested PCR and cultivation results for Water Utility A

Well Identification	Date Received	Water		Biofilm	
		PCR	Cultivation	PCR	Cultivation
103-1	04/04/2000	-	280 CFU/L	-	-
136-1		-	-	-	-
103-2	12/05/2000	Inhibitors	-	Inhibitors	1 CFU/cm^2
136-2		Inhibitors	-	Inhibitors	-
103-3	20/06/2000	-	-	-	-
136-3		Not received	-	-	-
103-4	01/08/2000	-	-	-	-
138-4		Inhibitors	431 CFU/L	-	-
103-5	19/09/2000	-	-	-	-
138-5		-	431 CFU/L	-	-
103-6	28/11/2000	-	-	-	-
138-6		-	-	-	-
103-7	09/01/2001	+	-	+ (B and ES)	-
138-7		+	4600 CFU/L	+ (B and ES)	-
103-8	09/02/2001	-	-	Inhibitors (B and ES)	-
138-8		-	206 CFU/L	Inhibitors (B only)	-
103-9	27/03/2001	-	150	Inhibitors	-
138-9		-	206	Inhibitors	-
103-10	02/05/2001	-	100	Inhibitors	-
138-10		-	413	Inhibitors	-
103-11	01/06/2001	Not received	Not received	-	15.9 CFU/cm^2
138-11		-	-	-	-
103-12	06/07/2001	Not received	Not received	+	29.6 CFU/cm^2
138-12		-	619	-	-
KWII RW	21/11/2000	Not received	Not received	+	2.1x10^4 scraped in 100 mL
KWII IFS	21/11/2000	Not received	Not received	-	-
05B	18/04/2001	Not received	Not received	-	4.0x10^3 scraped in 80 mL
15A	18/04/2001	Not received	Not received	-	-
21A	18/04/2001	Not received	Not received	Inhibitors	-
32G	18/04/2001	Not received	Not received	Inhibitors	2.1x10^3 scraped in 100mL
72F	18/04/2001	Not received	Not received	Inhibitors	-
94A	18/04/2001	Not received	Not received	Inhibitors	1.5x10^4 scraped in 80mL

B - pipe section biofilm, ES - Endcap scraping

Table C-12 Comparison of semi-nested PCR and cultivation results for Water Utilities C to Q

Water Utility	Well ID	Water		Biofilm	
		PCR	Cultivation	PCR	Cultivation
C (Arizona)	AZ (A)	+	100 CFU/L	Not received	N/A
	AZ (B)	+	100 CFU/L	+	3 CFU/cm^2
	AZ (BM)	Not received	N/A	+	20 CFU/cm^2
D (Texas)	EP	+	-	Inhibitors	-
E (Illinois)	ST	+	-	-	-
F (Texas)	CS2	+	100 CFU/L	+	10 CFU/cm^2
	CS5	+	183 CFU/L	+	5 CFU/cm^2
G (Illinois)	PEO	-	-	-	-
H (Illinois)	PK	+	-	+	-
I (Missouri)	MEX	-	-	-	-
J (Michigan)	SF	-	100 CFU/L	-	-
	MN	-	-	-	-
K (New Jersey)	NJ	-	-	-	118 CFU/cm^2
L (Ontario)	OTT	-	412 CFU/L	-	6.2x10^4 scraped in 100 mL water
M (Illinois)	CH	-	-	Inhibitors	-
N (New	NB3	+	-	+	-
Brunswick)	NB7	+	-	+	-
O (Missouri)	MO18	Inhibitors	100 CFU/L	Inhibitors	-
	MO34	-	-	Inhibitors	-
P (Nebraska)	NE54	-	-	-	-
	NE66	Inhibitors	412 CFU/L	-	13.6 CFU/cm^2
Q (Florida)	FK8	-	-	Not received	Not received
	FK13	-	800 CFU/L	Not received	Not received

Table C-13 Comparison of IMS-cultivation to conventional cultivation for Water Utility A

Well ID	Date Received	Water		Biofilm	
		IMS-Cultivaion	Cultivation	IMS-Cultivation	Cultivation
103-9	27/03/2001	-	150	-	-
138-9		40 CFU/L	206	-	-
103-10	02/05/2001	-	100	-	-
138-10		-	413	-	-
103-11	01/06/2001	-	Not received	5.2 CFU/cm^2	15.9 CFU/cm^2
138-11		-	-	-	-
103-12	06/07/2001	-	Not received	5.8 CFU/cm^2	29.6 CFU/cm^2
138-12		2.5x10^2 CFU/L	619	-	-
05B	18/04/2001	Not received	Not received	1.3x10^3 CFU scraped in 80 mL water	4.0x10^3 scraped in 80 mL water
15A	18/04/2001	Not received	Not received	-	-
21A	18/04/2001	Not received	Not received	-	-
32G	18/04/2001	Not received	Not received	-	2.1x10^3 CFU scraped in 100mL water
72F	18/04/2001	Not received	Not received	-	-
94A	18/04/2001	Not received	Not received	-	1.5x10^4 CFU scraped in 80mL water
CT1	11/05/2001	Not received	Not received	-	-
CT2	11/05/2001	Not received	Not received	5.0 x10^2 CFU scraped in 60 mL water	-
CT3	11/05/2001	Not received	Not received	-	-
CT4	11/05/2001	Not received	Not received	5.9x10^3 CFU scraped in 80 mL water	1.5 x 10^4 CFU scraped in 80 mL water
CT5	11/05/2001	Not received	Not received	-	-
EMS	01/08/2001	-	-	-	-
JA	01/08/2001	8.9 x10^3 CFU/L	6.6x10^3 CFU/L	Not received	Not received
CRIM1	01/08/2001	-	-	-	-
CH1	01/08/2001	1.1x10^4 CFU/L	4.0 x10^5 CFU/L	8.2x10^3 CFU scraped in 50 mL water	-
CLW	01/08/2001	1.3x10^3 CFU/L	-	-	-

Table C-14 Comparison of IMS-cultivation and conventional cultivation for Water Utilities K to Q

Water Utility	Well ID	Water		Biofillm	
		IMS-Cultivaion	Cultivation	IMS-Cultivation	Cultivation
K (New Jersey)	NJ	-	-	5.7 CFU/cm^2	118 CFU/cm^2
L (Ontario)	OTT	-	412 CFU/L	-	6.2x10^4 scraped in 100 mL water
M (Illinois)	CH	-	-	-	-
N (New	NB3	-	-	-	-
Brunswick)	NB7	-	-	-	-
O (Missouri)	MO18	-	100 CFU/L	4.1 x10^3 CFU scraped in 250mL	-
	MO34	-	-	-	-
P (Nebraska)	NE54	-	-	-	-
	NE66	-	412 CFU/L	-	13.6 CFU/cm^2
Q (Florida)	FK8	-	-	Not received	Not received
	FK13	25 CFU/L	800 CFU/L	Not received	Not received

Table C-15 Comparison of semi-nested PCR and IMS-PCR results for Water Utility A

Well Identification	Date Received	Water		Biofilm	
		PCR	IMS-PCR	PCR	IMS-PCR
103-9	27/03/2001	-	-	Inhibitors	Inhibitors
138-9		-	-	Inhibitors	Inhibitors
103-10	02/05/2001	-	+	Inhibitors	Inhibitors
138-10		-	+	Inhibitors	+
103-11	01/06/2001	Not received	Not received	-	-
138-11		-	-	-	- (UC) Inhibitors (C)
103-12	06/07/2001	Not received	Not received	+	-
138-12		-	-	-	Inhibitors
05B	18/04/2001	Not received	Not received	-	-
15A	18/04/2001	Not received	Not received	-	-
21A	18/04/2001	Not received	Not received	Inhibitors	-
32G	18/04/2001	Not received	Not received	Inhibitors	Inhibitors
72F	18/04/2001	Not received	Not received	Inhibitors	Inhibitors
94A	18/04/2001	Not received	Not received	Inhibitors	-
CT1	11/05/2001	Not received	Not received	Not done	-
CT2	11/05/2001	Not received	Not received	Not done	-
CT3	11/05/2001	Not received	Not received	Not done	Inhibitors
CT4	11/05/2001	Not received	Not received	Not done	Inhibitors
CT5	11/05/2001	Not received	Not received	Not done	Inhibitors
EMS	01/08/2001	Not done	Inhibitors	Not done	+
JA	01/08/2001	Not done	-	Not received	Not received
CRIM1	01/08/2001	Not done	Inhibitors	Not done	Inhibitors
CH1	01/08/2001	Not done	-	Not done	Inhibitors
CLW	01/08/2001	Not done	-	Not done	-

C – Combined IMS, UC – Uncombined IMS

Table C-16 Comparison of semi-nested PCR and IMS-PCR for Water Utilities K to Q

Water Utility	Well ID	Date Received	Water		Biofilm	
			PCR	IMS-PCR	PCR	IMS-PCR
K (New Jersey)	NJ	11/04/2001	-	-	-	Inhibitors
L (Ontario)	OTT	25/04/2001	-	+	-	-
M (Illinois)	CH	24/05/2001	-	- (C) + (UC)	Inhibitors	-
N (New Brunswick)	NB3	6/06/2001	+	+	+	+
	NB7		+	+	+	+
O (Missouri)	MO18	7/06/2001	Inhibitors	Inhibitors	Inhibitors	Inhibitors
	MO34		-	-	Inhibitors	-
P (Nebraska)	NE54	10/07/2001	-	-	-	-
	NE66		Inhibitors	-	-	-
Q (Florida)	FK8	25/07/2001	-	-	Not received	Not received
	FK13		-	Inhibitors	Not received	Not received

C – Combined IMS, UC – Uncombined IMS

APPENDIX D SAMPLE INFORMATION

Table D-1 Water and biofilm sample information for well #103 from Water Utility A

Sampling Date	Sample Type		Biofilm Resuspension (mL)	Water Resuspension (mL)
	Biofilm	Water		
March-April	PVC pipe 42 cm length, 3.7 cm diameter	3 l liter bottles of water	52	10 mL of water per liter filtered
April- May	PVC pipe 36 cm length, 4.8 cm diameter	3 l liter bottles of water	50	10 mL of water per liter filtered
May- June	PVC pipe 36 cm length, 4.8 cm diameter	3 l liter bottles of water	100	10 mL of water per liter filtered
June- July	PVC pipe 41 cm length, 5.1 cm diameter	3 l liter bottles of water	50	10 mL of water per liter filtered
August-September	PVC pipe 35 cm length, 5.0 cm diameter	3 l liter bottles of water	85	10 mL of water per liter filtered
November-December	PVC pipe 36 cm length, 5.0 cm diameter	3 l liter bottles of water	150	10 mL of water per liter filtered
December-January	PVC pipe 38 cm length, 5.0 cm diameter End-cap scraping	3 l liter bottles of water	100 100	10 mL of water per liter filtered
January-February	PVC pipe 38 cm length, 4.8 cm diameter End-cap scraping	3 l liter bottles of water	100 100	10 mL of water per liter filtered
February-March	PVC pipe 33 cm length, 5.0 cm diameter	3 l liter bottles of water	100	10 mL of water per liter filtered
April- May	PVC pipe 33 cm length, 5.0 cm diameter	3 l liter bottles of water	120	10 mL of water per liter filtered
May-June	PVC pipe 36.0 cm length 5.0 cm diameter	None	180	N/A
June-July	PVC pipe 31.0 cm length 5.0 cm diameter	None	700	N/A

Table D-2 Water and biofilm sample information for well #136 from Water Utility A

Sampling Date	Sample Type		Biofilm Resuspension (mL)	Water Resuspenstion (mL)
	Biofilm	Water		
March-April	PVC pipe 38 cm length, 3.7 cm diameter	3 1 liter bottles of water	105	10 mL of water per liter of water filtered
April- May	PVC pipe 36 cm length, 4.8 cm diameter	3 1 liter bottles of water	90	10 mL of water per liter of water filtered
May- June	PVC pipe 36 cm length, 4.8 cm diameter	None received	75	None received

Table D-3 Water and Biofilm sample information for well #138 from Water Utility A

Sampling Date	Sample Type		Water Resuspenstion (mL)	Biofilm Resuspension (mL)
	Biofilm	Water		
June- July	PVC pipe 36 cm length, 4.8 cm diameter	3 1 liter bottles of water	10 mL per liter of water filtered	50
August-September	PVC pipe 35 cm length, 5.0 cm diameter	3 1 liter bottles of water	10 mL per liter of water filtered	150
November-December	PVC pipe 36 cm length, 5.0 cm diameter	3 1 liter bottles of water	10 mL per liter of water filtered	150
December-January	PVC pipe 38 cm length, 5.0 cm diameter	3 1 liter bottles of water	10 mL per liter of water filtered	150
	End-cap scraping	None	N/A	100
January-February	PVC pipe 37 cm length, 4.8 cm diameter	3 1 liter bottles of water	10 mL per liter of water filtered	100
	End-cap scraping	None	N/A	100
February-March	PVC pipe 35 cm length, 5.0 cm diameter	3 1 liter bottles of water	10 mL per liter of water filtered	100
April-May	PVC pipe 35.0 cm length 5.0 cm diameter	3 1 liter bottles of water	10 mL per liter of water filtered	150
May-June	PVC pipe 36.0 cm length 5.0 cm diameter	3 1 liter bottles of water	10 mL per liter of water filtered	180
June-July	PVC pipe 31.0 cm length 5.0 cm diameter	3 1 liter bottles of water	10 mL per liter of water filtered	700

Table D-4 Water and biofilm information of water utilities C to Q

Water Utility/ Sample ID	Sample Type		Water Resuspension (mL)	Biofilm Resuspension (mL)
	Biofilm	Water		
C/				
AZ(A)	None	3 liters of water	10 mL per liter	None
AZ(B)	Water meter	3 liters of water	10 mL per liter	115
AZ(B/M)	PVC coupons	None	None	125
D/ EP	Water meter	4 liters of water	10 mL per liter	150
E/ ST	Water meter	4 liters of water	10 mL per liter	150
F/				
CS2	PVC pipe section (diameter 2.0 cm, length 35.5)	4 liters of water	10 mL per liter	115
CS5	PVC pipe section (diameter 2.0 cm, length 34.0 cm)	4 liters of water	10 mL per liter	115
G/ PEO	Water meter	4 liters of water	10 mL per liter	200
H/ PK	Water meter	4 liters of water	10 mL per liter	200
I/ MEX	Water meter	4 liters of water	10 mL per liter	200
J/				
SF	PVC pipe section (diameter 4.8cm, length 43 cm)	4 liters of water	10 mL per liter	150
MN	PVC pipe section (diameter 4.8 cm, length 43 cm)	4 liters of water	10 mL per liter	150
K/ NJ	Water meter	4 liters of water	10 mL per liter	100
L/ OTT	Scraping	4 liters of water	10 mL per liter	100
M/ CH	Water meter	4 liters of water	10 mL per liter	100
N				
NB3	PVC pipe (diameter 2.0 cm, length 35cm)	4 liters of water	10 mL per liter	115
NB7	PVC pipe (diameter 2.0 cm, length 36 cm)	4 liters of water	10 mL per liter	115
O/				
MO18	Scraping	4 liters of water	10 mL per liter	250
MO34	Scraping	4 liters of water	10 mL per liter	250
P/				
NE54	PVC pipe (diameter 2.3 cm, length 30.5cm	4 liters of water	10 mL per liter	150
NE66	PVC pipe (diameter 2.3, length 30.5 cm)	4 liters of water	10 mL per liter	150
Q/				
FK8	None	4 liters of water	10 mL per liter	None
FK13	None	4 liters of water	10 mL per liter	None

CURRICULUM VITAE

Teresa Jane Brooks
396 McArthur St. Apt. 1
Ottawa, ON K1K1G8
Home:(613)742-9357

EDUCATION

1995-1999 **University of Guelph**, Guelph, Ontario.

- Graduated with Bachelor of Science (Honours), Major – Biochemistry
- Co-operative education program

- Dean's Honour List
- Recipient of University of Guelph Entrance Scholarship (1995)
- Recipient of University of Guelph Deans Scholarship and Analytical Biochemistry Award (1999)

2000-2001 **University of Ottawa**, Ottawa, Ontario.

- Enrolled in the Department of Biochemistry, Microbiology and Immunology Masters' Program, maintaining an A average
- Recipient of University of Ottawa Tuition Scholarship (2000)
- Recipient of Ontario Graduate Students in Science and Technology Scholarship (2000)

PRESENTATIONS AND PUBLICATIONS

- 16th Eastern Canadian Symposium on Water Pollution Research (Occurrence of *Legionella* in groundwater: An ecological study - Oral presentation, 2000)
- Ontario Water Works Assocation (Isolation and Identification of *Legionella* in groundwaters – Oral presentation, 2001)
- Occurrence of *Legionella* in groundwater: an ecological study (Publication – ***Water Science and Technology***, Vol 43, No 12, pp 99-102)

WORK EXPERIENCE

April 2000-Sept 2000 **Laboratory Technician,**
Centre for Research on Environmental Microbiology, University of Ottawa, Ottawa, Ontario.

- Analyzed water and biofilm samples for the presence of specific micro-organisms using cultivation methods, polymerase chain reaction procedures and immunomagnetic separation
- Prepare periodic reports for the funding agency for the project

May 1999-Sept 1999 (co-op) **Laboratory Technician and Research Assistant,**
University of Guelph, Laboratory Services Division, Trace Contaminants Lab, Guelph, Ontario.

- carried out trace pesticide analysis of various matrices using liquid-liquid extractions and column chromatography
- involved in developing a method for analysis of trace contaminants in water samples using solid phase micro-extraction (SPME)and gas chromatography

Sept 1998-Dec 1998 (co-op) **Research Assistant,**
Uniroyal Chemical Ltd., Research Laboratories, Guelph, Ontario.

- synthesized and purified organic compounds using various organic techniques including precipitation, liquid-liquid extractions, recrystalization, and column and thin layer chromatography
- interpreted NMR, IR and Mass Spectra information for determination and confirmation of compound structures

Jan 1998-May 1998 (co-op) **Fertilizer Research Assistant,**
Fertilizer Section, Plant Products Division, Canadian Food Inspection Agency, Nepean, Ontario

- actively participated in the production of the Canadian Fertilizer Quality Assurance Report, including data entry in the computer system, verification of results with various laboratories and manufacturers in the industry
- performed a literature review for the resident toxicologist to be used to set safety standards for a certain contaminant in the environment

Jan 1997-May 1997 **Research Assistant,**
Atmospheric Environmental Services, Environment Canada, **(co-op)**
Downsview, Ontario

- Under guidance from the resident chemist, documented, in a report to be used by future co-op students, the process of updating the Envirodat Code Dictionary
- Proposed and designed inpart a graphical interface for the Envirodat system to replace the current text-based menus

SKILLS

- Working knowledge of computer packages including Groupwise, Windows, Microsoft Word, Microsoft Excel, Microsoft Powerpoint, Wordperfect, and Netscape
- Taken graduate level courses in virology and selected areas of microbiology including environmental health microbiology
- Prepared poster presentation for University of Ottawa Graduate Poster Day
- Gained proficiency in analytical, biochemistry, microbiology, and organic laboratory techniques
- Well developed planning and organizational skills
- Proficient at retrieving and analyzing papers and reports from various media sources including internet sites such as PubMed.

INTERESTS AND ACTIVITIES

- Enjoy reading, rollerblading, soccer and other outdoor activities.
- Volunteer at the Children's Hospital of Eastern Ontario