

DEVELOPMENT OF FLUORESCENT *IN SITU* HYBRIDIZATION
FOR DETECTION OF *L. PNEUMOPHILA* USING SOLID
PHASE LASER CYTOMETRY

by

Kien Seng Lim

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Dr. Barry H. Pyle

Approved for the Department of Microbiology

Dr. Mark Jutila

Approved for The Graduate School

Dr. Ronald W. Larsen

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ABSTRACT

Legionella pneumophila was first identified and documented after a pneumonia outbreak during an American Legion convention held in Philadelphia in 1976, with 25 casualties. *L. pneumophila* is a gram negative bacillus found mostly in fresh water environments. It is known as a facultative intracellular parasite of protozoa in its natural habitat, and can also infect macrophages in humans when contaminated aerosols are inhaled. The current detection procedure for *L. pneumophila* is a culture based method, which is slow and may not detect viable but non-culturable cells; presence of *L. pneumophila* usually takes 3 to 10 days to confirm by this method, due to its slow growth. New more reliable, rapid and sensitive methods are needed. In this research, a method coupling fluorescent *in situ* hybridization (FISH) and ScanRDI solid phase laser cytometry was developed to detect *L. pneumophila* relying on growth with extended incubation to obtain cultures. Published FISH methods were optimized by addition of HCl and Tween 20 incubation, dextran sulfate, helper probes and tyramide signal amplification to increase fluorescence intensity of probed *L. pneumophila*. ScanRDI settings were also adjusted to be able to detect TSA-FISH probed *L. pneumophila*. The resulting TSA-FISH method coupled with ScanRDI was able to detect almost 90% of *L. pneumophila* cell compared to plate counts. This optimized TSA-FISH method was also used on *Escherichia coli* and *Aeromonas hydrophila*; the ScanRDI detected 79.5% and 39.7% of these organisms respectively compared to plate counts. The results suggested that *L. pneumophila* can be detected and enumerated using the method developed in this research. It can be completed within 12 hours, which is a significant improvement compared to at least 3 days for current detection methods. The TSA-FISH method can be used for detection and enumeration of at least 2 microorganisms, *L. pneumophila* and *E. coli*. For reliable detection and enumeration of other organisms such as *A. hydrophila*, procedures need to be further optimized and ScanRDI settings adjusted for each organism. It is anticipated that several microbes could be detected on one membrane filter using appropriate probes, procedures and ScanRDI settings.

INTRODUCTION

Water and Contamination

Background

Water is an important component of living organisms; it is needed for sustaining life. We need clean water for our daily activities including drinking, cooking, bathing and cleaning (Gleick, 1998). On average, every person in the U.S.A. uses about 100 gallons daily which is twice as much as their European counterparts (United States Environmental Protection Agency [USEPA, n.d.]). It is very important to make sure that portable water is free of hazardous contaminants, be they microbial or chemical. In this study, novel methods of detecting microbial contaminants were explored and evaluated. The overall objective is to develop a method for simultaneous detection of several specific target microorganisms in water samples without the need for preliminary culturing, by coupling methods such as fluorescent *in situ* hybridization (FISH) and the ScanRDI (AES-Chemunex) solid phase laser cytometry (SPLC) system.

Using FISH to probe bacteria in a sample with species-specific probes, there is no need for overnight culturing of water samples to amplify numbers of microbial cells before detection (Bouvier & Giorgio, 2003). The techniques described in this study can be completed within a working day, which is rapid compared to classic microbial detection methods that usually require culturing before biochemical or molecular identification can be completed (Buchbinder, Trebesius & Heeseman, 2002).

The ScanRDI SPLC can be used to detect fluorescing cells that have been

captured on membrane filters and probed using FISH (AES Chemunex, 2011). Since this method, including sample processing, filtering, FISH staining and scanning, is anticipated to take about a day to confirm the presence and number of *Legionella pneumophila* and other specific microbial target cells in water samples it would be suitable for investigation of an outbreak or for routine monitoring of water sources, systems or supplies.

The microorganism of interest in this study is the Gram-negative bacterium *L. pneumophila*, the causative agent of Legionnaires' disease (McDade et al., 1977). *Legionellae* are intracellular bacteria that multiply in natural habitats by parasitism of protozoa (Fields, 1993) or in human macrophages during infection (Berrington & Hawn, 2013). *L. pneumophila* is a fastidious and slow growing bacterium that takes about 3 days to grow in laboratory cultures (Steinert, Em ödy, Amann & Hacker, 1997), requiring specialized media (Edelstein, 1981). Due to its slow growth, fastidious requirements and unreliable detection using culture methods, an optimized FISH procedure coupled with SPLC which avoids the need for culturing would provide reliable results within a shorter time.

Pathogens in Potable Water

Every year, millions of people, mostly from developing countries, die from diseases contracted from consuming unsafe drinking water; these include 1.6 million deaths from diarrheal diseases and tens of thousands from schistosomiasis (World Health Organization (WHO), 2013). Unsafe or contaminated water does not only harbor gastrointestinal disease-causing bacteria; respiratory disease causing bacteria are also

found to be common contaminants. *L. pneumophila* is a bacterium that causes pneumonia (McDade et al., 1977). According to the Centers for Disease Control and Prevention (CDC), between 2000 and 2009 the number of reported cases of legionellosis increased from 1,110 to 3,522 in the United States (CDC, 2011). Cases of *L. pneumophila* infections are frequently underreported but mortality of infections in humans is estimated to be as high as 40% for nosocomial cases and as high as 50% for patients with predisposing conditions (WHO, 2007).

Due to the high number of deaths associated with microbially contaminated water, the World Health Organization (WHO) and The United Nations Children's Fund (UNICEF) work jointly to help increase the accessibility of clean water and sanitization through joint monitoring programs for water supply and sanitation and promoting improved hygiene behavior (WHO, 2013). These and a few other programs carried out by WHO are intended to halve the number of people without sustainable access to safe drinking water and basic sanitation by the year 2015 (WHO, 2013).

With public health and safety in mind, the United States Environment Protection Agency (USEPA), through the Clean Water Act (USEPA, 2000), administers regulations to ensure that drinking water is free of contaminants that are a threat to consumers. Contaminants in water, such as microorganisms, are regulated based on a maximum contaminant level goal (MCLG) for each organism (USEPA, 2009) to ensure a safe level of contaminants in drinking water that will reduce the risk of harmful health effects. Waterborne pathogens include but are not limited to bacteria and viruses, and they can be divided into those that cause gastrointestinal effects and others that cause respiratory

disease (Table 1).

Bacterial pathogens in drinking water can cause self-limiting to severe diseases, including but not limited to gastroenteritis (*e.g.*, Enteropathogenic *Escherichia coli* such as *E. coli* O157:H7), gastric ulcers (*e.g.*, *H. pylori*), and respiratory infections (*e.g.*, *L. pneumophila* and *M. avium*) (USEPA, 2012). Bacteria such as *E. coli*, *A. hydrophila* and *H. pylori* can be found in water sources contaminated with fecal material from infected warm-blooded animals or wastewater sources (Sokolova et al., 2012). When these

Table 1. Microbial Contaminants listed on the Contaminant Candidate List 3 – CCL regulated by USEPA (adapted from USEPA, 2012).

^G Gastrointestinal disease causing

^R Respiratory disease causing

Microbial contaminant	Information
<i>Caliciviruses</i> ^G	Virus (includes Norovirus) causing mild self-limiting gastrointestinal illness
<i>Campylobacter jejuni</i> ^G	Bacterium causing mild self-limiting gastrointestinal illness
<i>Escherichia coli</i> (0157) ^G	Toxin-producing bacterium causing gastrointestinal illness and kidney failure
<i>Helicobacter pylori</i> ^G	Bacterium sometimes found in the environment capable of colonizing human gut that can cause ulcers and cancer
Hepatitis A virus ^G	Virus that causes a liver disease and jaundice
<i>Salmonella enteric</i> ^G	Bacterium causing mild self-limiting gastrointestinal illness
<i>Shigella sonnei</i> ^G	Bacterium causing mild self-limiting gastrointestinal illness and bloody diarrhea
<i>Adenovirus</i> ^R	Virus most commonly causing respiratory illness, and occasionally gastrointestinal illness
<i>Enterovirus</i> ^R	Group of viruses including polioviruses, coxsackie viruses and echoviruses that can cause mild respiratory illness
<i>Legionella pneumophila</i> ^R	Bacterium found in the environment including hot water systems causing lung diseases when inhaled
<i>Mycobacterium avium</i> ^R	Bacterium causing lung infection in those with underlying lung disease, and disseminated infection in the severely immune-compromised

bacteria are ingested from drinking contaminated water, they may colonize the gastrointestinal tract (Tarr, 1995; Vale & V for, 2010; Pablos et al., 2010) or the respiratory tract when aerosols contaminated with respiratory disease agents have been inhaled (Pierce & Sanford, 1973)

Because most microbial pathogens are typically present in water at extremely low numbers, even in outbreak situations, the organisms can be difficult to detect and identify using traditional culture methods. Therefore, indicator bacteria including coliforms, *E. coli*, enterococci and clostridia have been used for over a century as indicators of fecal pollution due to presence in higher numbers (Tallon, Magajna, Lofranco & Leung, 2005). In order to make sure that potable water is free from contaminants; *E. coli* and other coliform bacteria with a fecal origin, normally known as the fecal coliform bacteria are monitored to ensure their number are not over the MCLG (Bej et al., 1990). Total coliforms include both fecal species and others that represent the natural microbial inhabitants of the water. Fecal coliforms may be more reliable indicators of fecal pathogens because they include species that typically originate in feces of warm blooded animals. Total coliforms can also be used as indicators of presence of fecal contamination since they include fecal species in addition to some that proliferate in the environment (Elmund, Allen & Rice, 1999).

According to EPA standards, the maximum contaminant level for fecal and total coliform bacteria is <1 per 100 ml in drinking water and the detection of any fecal or total coliforms is indicative of a potential health hazard for consumers. Because *Legionellae* are not of fecal origin, it has been suggested that heterotrophic bacterial plate counts or

concentrations of trace elements such as manganese can be used to indicate the likely presence of *Legionellae* in water samples (Bareglini et al., 2011); manganese-oxidizing bacteria are identified as major biofilm contributors, allowing colonization by *L. pneumophila*.

Legionellae in Water

The overall goals of this study were to improve detection of waterborne pathogens including bacteria and protozoa in drinking water by developing more rapid, specific and sensitive techniques than those in current use. *L. pneumophila* bacteria are frequently found in water supplies, distribution systems and cooling equipment and they can cause serious pulmonary infections (WHO, 2007); the infectious dose is assumed to be as low as a single organism (WHO, n.d.). Thus, it is important to be able to detect very low numbers of these bacteria in water. *Legionellae* are also slow growing, fastidious bacteria so that enrichment on solid or in liquid media is required for current methods (Steinert, Emödy, Amann & Hacker, 1997). Bacterial pathogens such as *L. pneumophila* may become established in water contaminated with other pathogens, and also in otherwise relatively uncontaminated water (Stout, Yu & Best, 1985; Vaerewijck et al., 2005). A more direct, rapid and sensitive detection method would address limitations of current detection methods for *L. pneumophila*, which include culturing, direct cell staining and the polymerase chain reaction (PCR).

L. pneumophila was named after it was discovered to be the cause of a pneumonia outbreak during an American Legion convention in Philadelphia in 1976 (Fraser et al., 1977). The genus *Legionella* was named for the Legionnaires that were affected in that

first known outbreak, and the species *pneumophila* means ‘lung-loving’ (Abu Kwaik, et al., 1998). Pontiac fever and Legionnaires’ disease are caused by *L. pneumophila* when aerosolized water droplets containing the bacteria are inhaled during showering or washing, or exposure to other man made water based equipment such as cooling towers, evaporative condensers, fluid coolers and humidifiers (WHO, 2007). Pontiac fever causes flu-like symptoms and is the milder form of Legionnaires’ disease without pneumonia symptoms (Kaufmann et al., 1981) and the infection develops and resolves quickly while Legionnaires’ disease has a longer incubation period and requires more extensive care for it to resolve. For Legionnaires’ disease, death may follow in about 15% of those infected (United States Department of Labor, 1999).

Some species of *Legionella* are known as opportunistic pathogens as they mainly infect only certain susceptible individuals, many of whom may have compromised immune systems (Berry, Xi & Raskin, 2006; von Reyn et al., 1994). These pathogenic species include *L. pneumophila*, *L. micdadei*, *L. dumoffii* and *L. bozemanii* (Abu Kwaik et al., 1998; Muder & Victor, 2002). Immuno-compromised individuals may include the very young, the very old, and those whose immunity is impaired by other infections such as HIV/AIDS and during or after organ transplants or chemotherapy.

L. pneumophila is a unique resident of the water environment in that it is an intracellular parasite of amoeba (Abu Kwaik et al., 1998). It can survive in water as a free living organism, but amoebae including but not limited to the genus *Acanthamoeba* (Barker et al., 1992) and *Naegleria* (Huang & Hsu, 2010) are required for it to multiply in the environment. The ability of *Legionellae* to multiply within amoebae as an

intracellular parasite also enables *Legionellae* to survive phagocytosis by amoeboid human macrophages and gives them the ability to manipulate human macrophages the way they do with amoebae in water (Rowbotham, 1980). Besides the protection acquired from replicating within amoebae, viable *L. pneumophila* have been observed within vesicles expelled by *Acanthamoeba* spp. that were exposed to biocides inside cooling towers (Berk, Ting, Turner & Ashburn, 1998). *L. pneumophila* that replicated within amoebae were also found to be very resistant to a wide range of detrimental environmental conditions such as fluctuations in temperature, osmolarity, pH, and exposure to oxidizing agents (Abu Kwaik, Gao, Harb & Stone, 1997); they may also become resistant to disinfectants/biocides such as polyhexamethylene biguanide, benzisothiazolone, and 5-chloro-N-methylisothiazolone (Barker et al., 1992) which are typically used to control *Legionellae* in water systems. *L. pneumophila* has also been observed to be resistant to antibiotics such as rifampicin and erythromycin (Moffie & Mouton, 1988) that are typically used to control *Legionellae* infections.

L. pneumophila is able to evade the host's immune system phagocytic functions by producing exocellular substances including exopolysaccharide and specific enzymes (Cazalet et al., 2004). When these infectious bacteria are phagocytized by host cells, they may become intracellular parasites which enable them to hijack the cellular mechanisms of the host cells to reproduce or to avoid the host's immune system (Barker et al., 1992). *L. pneumophila* usually cause disease in the lower respiratory tract and may have minor effects on the gastrointestinal tract which may be less serious than infection in the lower respiratory tract (Berrington & Hawn, 2013; Horsburgh Jr, 1991).

Current *Legionellae* Detection

Culture methods may not be as reliable when fastidious and slow growing bacteria are the target of detection, compared to detection of less fastidious, faster growing bacteria such as *E. coli*. *L. pneumophila* is an example of fastidious, slow growing bacteria that require specific nutrients found in buffered charcoal yeast extract (BCYE) medium to grow. The cells normally take 48 to 72 hours of incubation for colonies to be visible on solid media (Steinert, Em ödy, Amann & Hacker, 1997). Besides being slow growing and fastidious bacteria, viable but non culturable bacterial cells may limit the reliable detection of waterborne pathogens such as *L. pneumophila*. (Rompr é et al., 2002). Current detection of *L. pneumophila* includes a culture based method (USEPA, 2000), which usually takes several days for confirmation of cellular presence in contaminated water. It is because of these disadvantages, detection methods other than those based on culture need to be used to obtain more reliable detection and enumeration, which allow for confirmation of false negative results, and assessment of false positives. These alternative methods are frequently based on molecular analysis of samples, including PCR or direct cell staining including FISH (Aurell et al., 2004). Following direct cell staining, samples may be analyzed using epifluorescent microscopy, flow cytometry or SPLC. Since the infectious dose of *L. pneumophila* is assumed to be a single organism (WHO, n.d.), this further justifies the need to develop a more sensitive detection method to be able to detect a single cell in a given sample.

PCR. The polymerase chain reaction is one of the many techniques developed to detect microorganisms without the need for culturing (Josephson et al., 1991) and is a

sensitive and qualitative method used to detect the presence of microorganisms. Species specific primers are widely available and at an affordable price. Once DNA is extracted from samples, detection of pathogens can be completed in a few hours using an automated thermo cycler. PCR can be done to detect a single microorganism (Malorny et al., 2003) or in a multiplex reaction to detect multiple target microorganisms (Edwards & Gibbs, 1994). The presence of microorganisms can be predicted based upon gel electrophoresis and visualization of PCR products. Advantages of PCR include its speed, high specificity and sensitivity (Bej et al., 1990). There is also no need for culturing before detection and this saves time and labor. The sensitivity of PCR can be problematic when used for detection of microorganisms from environmental samples, due to the presence of other contaminants that can be found in environmental samples (Picard et al., 1992). As a result, DNA extraction efficacy can be reduced drastically. The sensitivity of PCR also means that fragments of naked nucleic acids can give false positive results from environmental or laboratory samples. The need to extract nucleic acids is also another problem for PCR, because during the concentration of water samples loss of microorganisms and nucleic acids is known to occur (Long, Zhu, Zhang & Shi, 2008), making it difficult to extract sufficient DNA for detection of all microorganisms in the concentrated sample.

Cell Staining. Direct cell staining and microscopy is a common method for detection, enumeration and confirmation of microorganisms. Samples of microorganisms are normally fixed onto glass slides or captured on membrane filters for various staining methods. Depending on the type of staining, microorganisms might have to go through

fixation and permeabilization for dyes to be able to penetrate into the cell; different types of dyes have been developed to stain components of a cell (O'Brien & Bolton, 1995).

Nucleic Acid Dyes. Nucleic acid dyes like SYBR Green or DAPI are used to stain any biological cells in a non-specific fashion for rapid visualization by fluorescence microscopy (Noble & Fuhrman, 1998). Some dyes, including SYTOX Green nucleic acid stain or a LIVE/DEAD Bacterial Viability Kit (Invitrogen), can be used to differentiate between living and dead cells (Roth, Poot, Yue & Millard, 1997; Boulos et al., 1999). Once the cells are stained with the appropriate dye, the filters can be transferred to a suitable fluorescence system for observation.

Fluorescent Antibody (Fab) Labeling. Microorganisms may be labeled with species specific antibodies conjugated to fluorogenic dyes for differentiation (Radbruch, 2000). Depending on the antibody and type of staining being used for the study, some dyes have variable specificity to the cell structure that is being stained (Sternberger, Hardy, Cuculis & Meyer, 1970). For example, when using antibody conjugated dyes to stain antigens on cells, in depth knowledge of both the antibody conjugated dye and antigen on the cells has to be acquired before staining, due to the nature of antibody-antigen reactions, cross reactivity can cause binding to non-target antigens (Mighell, Hume & Robinson, 1998). For this reason, Fab labeling is not suitable for detecting *Legionellae* in environmental water samples because of false-positive and false-negative results.

Fluorescent in situ Hybridization (FISH). Besides labeling the structures of

bacteria with antibody conjugated dyes for visualization, nucleic acid staining is also available for labeling cells with nucleic acid probes designed based on published nucleic acid sequences (Amann, 1995). Fluorescent *in situ* hybridization (FISH) is a molecular method used to label cell with species specific oligonucleotide probes conjugated with a fluorochrome to visualize microorganisms under an epifluorescence microscope without the need of cell culturing (Amann, Fuchs & Behrens, 2001). Typical procedures for FISH include cell fixation, cell permeabilization, probe hybridization and lastly visualization (Amann & Fuchs 2008). An advanced FISH method has been developed to increase the fluorescence intensity of labeled cells includes an extra step called signal amplification that is introduced after the probe hybridization step. This variation of FISH is known as catalyzed reporter deposition fluorescent *in situ* hybridization (CARD-FISH) (Chao et al., 1996). The tyramide signal amplification (TSA) method used in this study was first applied in immuno-assays to detect specific cell proteins (Speel, Hopman & Komminoth, 1999).

In TSA, oligonucleotide probes are conjugated to horse radish peroxidase (HRP) instead of fluorochrome (Schönhuber, Fuchs, Juretschko & Amann, 1997). Once the HRP probes are hybridized to the target nucleic acid sites, fluorochrome labeled tyramide is introduced in the amplification step to make the cells fluoresce more brightly (Litt & Bobrow 2002). The difference between conventional FISH and CARD-FISH is that in conventional FISH, the intensity of labeled cell is limited by the amount of fluorochrome conjugated probe being able to attach the target nucleic site, in CARD-FISH, fluorescence intensity is increased because a high number of fluorochrome labeled

tyramide molecules can enter the cell to react with the HRP probe at the target nucleic acid site and be deposited in the cell to give increased fluorescence intensity (Speel, Hopman & Komminoth, 2006).

The FISH technique for detection of microorganisms is generally very reliable. By using a general probe EUB338 for detection of eubacteria, an average percentage of cells detected were reported to be 56% (Bouvier & Del Giorgio 2003). Although some procedures for FISH probing may be completed within a few hours (Manz et al., 1992; Manz et al., 1995) other FISH methods require a longer hybridization time of 15 hours for best results (Kirschner et al., 2012). In addition, the cost of oligonucleotides labeled with an enzyme such as HRP is significantly greater than those labeled with fluorochromes such as FITC or TRITC.

CARD-FISH, specifically TSA-FISH, due to its specificity and increased fluorescence (Sekar et al., 2003), and SPLC were used in combination to stain, detect, enumerate and visualize target microorganisms in water sample. The general procedures for this method involve filtering a water sample suspected to contain target microorganisms through a suitable membrane filter, specific labeling of microorganisms on the membrane filter using CARD-FISH followed by detection, enumeration and visual confirmation with a SPLC system.

Detection And Enumeration Methods

Epifluorescence Microscopy. Epifluorescence microscopy is a method that has been used to visualize and enumerate microorganisms that are stained with a dye that

between 450nm and 490nm for cells labeled with a FITC type of fluorochrome (Yeh, Godshalk, Olson & Kelly, 1987). Other fluorochromes such as DAPI require lower excitation wavelengths around 360nm. Cells that are stained for epifluorescence microscopy are more easily visible due to the fluorescence from the nucleic acid dye compared with the use of non-fluorescent colored stains. A limitation of epifluorescence microscopy is that around 10^5 cells per filter are required for reliable enumeration (Lisle, Hamilton, Willse & McFeters, 2004). On the other hand, SPLC can detect a single cell or particle on a similar filter.

Hence, the use of a more sensitive method of detection would be required unless very large volumes of a sample can be filtered to provide enough cells for detection by epifluorescence microscopy.

Flow Cytometry. Flow cytometry is an automated method based on the principles of fluorescent microscopy, developed to rapidly enumerate fluorescently labeled microorganisms. In flow cytometry, cells are not attached to glass slide or filter, as is done in many microscopic methods. Labeled cells are injected into a flow cytometer, where they are carried by a fluid through a flow cell. The fluid stream containing labeled cells passes through a laser detector, which will differentiate the wavelength from the fluorescence emitted by the labeled cells (Radcliff & Jaroszeski, 1998). A flow cytometer detects both forward and backscatter, providing a plot of individual cell fluorescence. In fluorescence activated cell sorting (FACS) the flow cytometer is coupled with a cell sorter allowing it to sort the cells into different containers. As the cells pass through the laser detector, scattered light from the cells also generates information about the cells.

Information such as size, shape and structure, cell mass and bacterial growth is collected by sensitive detectors called photomultiplier tubes; cells can also be sorted based on this information besides sorting based on fluorescence wavelength (Herzenberg et al., 2002). There are multiple varieties of flow cytometers for different applications; some are capable to analyze cells quickly with up to 100,000 cells per second with low sorting capability, while others are able to sort up to 30,000 cells per second (Nunez, 2001). Flow cytometry can be a very useful method for detection of water pathogens with direct staining followed by high speed cell analysis. Flow cytometry was developed for larger cell types such as eukaryotic cells. More recent versions allow for analysis of cells such as bacteria that are typically smaller than eukaryotic by three orders of magnitude. Bacterial cells are also variable; their size, shape and cell content can vary based on growth condition (Akerlund, Nordström & Bernander, 1995), and these differences may affect cell enumeration results.

Solid Phase Laser Cytometry. SPLC is a combination of rapid detection and microscopic verification (Broadaway, Barton, & Pyle, 2003; Pyle, Broadaway & McFeters, 1999). The ScanRDI (AES-Chemunex) is an SPLC system that can detect down to a single labeled cell or particle on a 25mm diameter membrane filter. In SPLC, a water sample is filtered through a membrane that will trap microorganisms; the microorganisms on the membrane filter can then be stained with a fluorogenic dye (Broadaway, Barton, & Pyle, 2003). The membrane filter is processed by the ScanRDI SPLC system which will scan, detect and count the number of fluorescently stained cells as fluorescence events over the entire surface of the membrane filter in around 3 minutes

(Van Poucke & Nelis, 2000). The positions of fluorescence events on the membrane filter are mapped and recorded on a computer once the scanning of the membrane is completed. The operator can then remove the membrane filter from the SPLC, and place it on an automated microscope stage attached to the computer. The position of each fluorescent event is mapped and used to visually locate each particle on the filter and verify if it represents a target cell. Advantages of SPLC include its sensitivity, speed and ability for visual verification by epifluorescence microscopy. The SPLC has a detection sensitivity of one cell or fluorescent particle per membrane filter, so that the lower detection limit depends on the volume of water sample that can be passed through the membrane filter. Being able to detect all the fluorescence events on the membrane filter reduces the error associated with traditional cell counting methods using membrane filters. SPLC is more rapid and sensitive than traditional culture methods (Mignon-Godefroy, Guillet & Butor, 1997). The ability of visual verification provides a way to minimize false positive results as the microscopist is able to locate and examine specific fluorescence events to determine their authenticity as target cells.

There are also limitations associated with SPLC. For example, the fluorogenic labels have to be specific for organisms to be clearly labeled in relation to background fluorescence from unspecific labeling of debris observed in some water samples (Lemarchand, Parthuisot, Catala & Lebaron, 2001). The *ScanRDI* system software is designed to discriminate/differentiate between target labeled cells and other particles or debris on the filters. Depending on the method of staining, the overall detection time can be increased significantly when cells do not fluoresce brightly enough. Since the

ScanRDI has a single laser that provides excitation at around 488nm, it can only be used to analyze samples stained with FITC or other labels that are activated at around 488 nm (Broadaway, Barton, & Pyle, 2003).

Experimental Approach

Based on the information reviewed above, the development of a more reliable, rapid and sensitive method would be based on coupling TSA-FISH with SPLC to label, detect and enumerate *L. pneumophila* on membrane filters. Following initial experiments, it was necessary to include several steps to improve the reliability of TSA-FISH labeling with *L. pneumophila*, such as a short term preincubation to increase rRNA content, HCl and Tween 20 treatments (Chin et al., 2003; Coonen, Dumoulin, Ramaekers & Hopman, 1994), dextran sulfate incubation (van Gijlswijk, Wiegant, Raap & Tanke, 1996; Wahl, Stern & Stark, 1979), use of helper probes (Baudart & Lebaron, 2010) and signal amplification using tyramide (TSA). For SPLC, it was necessary to adjust some ScanRDI settings to ensure detection of the majority of labeled *L. pneumophila* while limiting the number of false positive results, (e.g. non-legionella bacteria or debris in water). The experiments described in the chapters that follow were devised to optimize the results of CARD-FISH coupled with SPLC for detection of *L. pneumophila* in water. An environmental strain of *E. coli* was used in similar experiments to compare results with those for *L. pneumophila*.

Chapter 2 outlines the materials and methods used for these experiments. Chapter 3 includes the results of these experiments, while Chapter 4 is a discussion of the results, along with Chapter 5 for conclusions and possible future work that could be done.

References for all chapters are listed together, at the end of this dissertation.

Hypothesis

Legionella pneumophila can be detected using optimized Fluorescent in situ Hybridization coupled with ScanRDI solid phase laser cytometry.

CHAPTER 2

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The bacterial strains used in this study and their origins are listed in Table 2. *E. coli* and *A. hydrophila* were streaked onto R2A plates and incubated overnight at 37 °C inside a Ziploc bag. *L. pneumophila* was streaked onto BCYE plate and incubated at 37 °C for 65 $\pm$ 5 hours inside a Ziploc bag containing a piece of wet paper towel to prevent BCYE agar from drying up during the long incubation period. Stocks of bacterial strains were harvested from plates and stored in suspensions made up of water and glycerol (1:1) at -80 °C.

Table 2. Origin of bacterial strains used in this study.

Bacterial strain	Origin or strain number
<i>Escherichia coli</i>	Drinking water distribution system ^a
<i>Aeromonas hydrophila</i>	Drinking water distribution system ^a
<i>Legionella pneumophila</i>	ATCC 33153

^a Strain provided by D. Smith, South Central Connecticut Regional Water Authority, New Haven.

Media and Reagents

Lyophilized media were prepared according to manufacturers' instructions and autoclaved at 121°C for 15 min and tempered at 46°C for limited periods of time before pouring plates, unless specified otherwise. Reagent grade water, 18 meg-ohms (Millipore

Synergy), was used for all media and buffers.

Media.

R2A agar (Difco): 9.1g of agar mix was dissolved into 500ml of water. R2A broth (Teknova): 1.575g of R2A broth mix was dissolved in 500ml of water.

Buffered charcoal yeast extract (BCYE) #1099 broth was prepared according to the product information sheet provided with the freeze dried *Legionella pneumophila* ATCC 33153 vial. For 1 liter of BCYE #1099 broth; 10g of yeast extract, 10g of N-2-acetamido-2-amino ethane sulphonic acid (ACES) and 2g of activated charcoal were mixed into 1 liter of water and autoclaved. L-cysteine HCl.H₂O (0.4g) (Nutritional Biochem), and 0.25g of ferric pyrophosphate (Sigma) were dissolved in separate 10ml aliquots of water, both of which were filter sterilized (0.22um pore 37mm diameter mixed cellulose filters from). The autoclaved mixture was tempered to ca. 50°C, before adding the filter sterilized L-cysteine and ferric pyrophosphate. BCYE #1099 broth medium was used for initial rehydration of freeze dried *L. pneumophila* ATCC 33153.

BCYE Agar (Difco) was prepared by adding 18.5 g of BCYE base to 500ml of water and adjusting to pH 7.1-7.2 with 1N KOH before autoclaving. After autoclaving and tempering to ca. 55°C, 5ml of rehydrated Bacto Legionella agar enrichment was added aseptically to the BCYE agar mix in a 55 °C water bath. Filter sterilized 1N HCL or 1N KOH was used to aseptically adjust the final pH of the BCYE agar to pH 6.85-7.0 before pouring into Petri dishes.

Reagents.

Hybridization buffer was freshly made for every experiment before the hybridization step: 0.9M of NaCl from 5M stock solution), 20mM of Tris-HCl (Fisher) pH 7.2 from 1M stock solution, 0.01% sodium dodecyl sulfate (SDS; Thermo) from a 1% stock solution, 20% formamide (Sigma) and 1ng/ul of TSA-FISH and Helper probes for the respective experimental microorganism. Wash buffer: 180mM NaCl, 20mM Tris-HCl pH7.2, 5mM EDTA (Sigma) and 0.01% of SDS. To make 100ml of wash buffer, 1.04g of NaCl, 0.32g of Tris-HCl was added to 90ml of water and adjusted to pH 7.2 before adding 0.186g of EDTA and water to make 100ml total volume. The mixture was autoclaved and allowed to cool before adding 1ml of sterile 1% SDS.

TNT buffer: 0.1M Tris-HCl pH7.5, 0.15M NaCl and 0.05% Tween 20 (Sigma). To make 1L of TNT buffer, 15.79g of Tris-HCl was added to 900ml of water and adjusted to pH7.5, before adding 8.8g of NaCl and 0.5ml of Tween 20. After filling to a final volume of 1L with water, it was autoclaved.

TE buffer: 100mM Tris-HCl pH 8.2 and 50mM EDTA. Tris-HCl (15.8g) was added to 900ml of water and adjusted to pH 8.2 with 18.6g of EDTA added before adding water to a final volume of 1ml and autoclaved.

Ethanol series: Stock 95% ethanol was diluted with water to obtained bottles of 50%, 80% and 94% ethanol. After filter sterilizing, these solutions were stored at -20 °C.

Lysozyme was prepared by adding 250ug of FISHER lysozyme BP5251 (20,000Unit/mg) to 1ml of TE buffer, and aliquoted into 500ul amounts in microcentrifuge tubes and stored at -20 °C.

Paraformaldehyde: Stock 16% solution (Thermo) was diluted to 4% paraformaldehyde with filtered autoclaved ultrapure water (FAUP) for cell fixation procedure, and stored at 4 °C for up to 2 weeks.

HCl (0.02N) and Tween 20 (0.2%) were prepared by adding an appropriate amount of each reagent into FAUP and filter sterilized.

Dextran sulfate (Acros) was added into hybridization buffer and tyramide signal amplification mixture at ca. 0.1g/ml.

Oligonucleotide Probes

Probes used in these experiments are shown in Table 3. Those designated as -HRP were conjugated with Horse Radish Peroxidase that was required for Tyramide Signal Amplification. Specific probe sequences for non-eubacteria (NONEUB-HRP), eubacteria (EUB338-HRP), *E. coli* (Colinsitu-HRP), *A. hydrophila* (Aer66-HRP) and *L. pneumophila* (LEGPNE1-HRP) were obtained from Kjellerup et al., (2005), Amann et al., (1990), Regnault et al., (2000), Geisenberger et al., (1999) and Manz et al., (1995) respectively. Helper probe sequences for *E. coli* (HColin_L and HColin_R) were obtained from Regnault, B., S. Martin-Delautre, et al., (2000), while helper probe sequences for *A. hydrophila* (Aer66H21 and Aer66H57) and *L. pneumophila* (LEGPNE1H307 and LEGPNE1H343) were designed according to literature (Fuchs, Glöckner, Wulf & Amann, 2000). The helper probe sequences were designed to target sequences adjacent to the target sequence of the TSA-FISH probes. Sequences for helper probes of *A. hydrophila* and *L. pneumophila* were obtained by Basic Local Alignment

Search Tool (BLAST) searching TSA-FISH probe sequences to find sequences adjacent to the target site of TSA-FISH probes.

Preparation and Filtration of Cell Suspensions

Several colonies were picked and mixed into 1ml of FAUP in a 1.5ml microcentrifuge tube by vortexing for 3 intervals of 20 seconds to make a cell suspension with a Klett colorimeter (Klett-Summerson) reading of 10 units by adding the initial cell suspension to a 0 Klett reading test tube containing 7ml of FAUP. At a Klett reading of 10 units, *E. coli* and *A. hydrophila* had ca. 1×10^7 cells/ml while *L. pneumophila* had ca. 1×10^6 cells/ml. Cell suspensions were diluted to obtain 1×10^4 cells/ml and 1×10^3 cells/ml for optimization and Scan analysis respectively. For TSA-FISH Fluorescent microscopy studies, 1ml of 1×10^4 cells/ml cell suspension was filtered through a 25-mm-diameter membrane filter (black polyester ChemFilters CB04 from Chemunex) with 5ml of FAUP in a filtration system as shown in Figure 1. For TSA-FISH-SPLC studies, 100ul of 1×10^3 cells/ml cell suspension was spotted directly onto the center of the membrane filter and filtered by applying vacuum.

Table 3. List of probe names, sequence and specificity. HRP, Horse Radish Peroxidase; upstream helper probes HColin_L, Aer66H21 and LEGPNE1H307 and downstream helper probes HColin_R, Aer66H57 and LEGPNE1H343.

Probe	Sequence (5'-3')	Specificity
NONEUB-HRP	ACTCCTACGGGAGGCAGC	Negative Control
EUB338-HRP	GCTGCCTCCCGTAGGAGT	Most bacteria
Colinsitu-HRP	GAGACTCAAGATTGCCAGTATCA G	<i>E. coli</i>
HColin_L	ATGCAGTTCCCAGGTTGAG	Helper probe for Colinsitu-HRP
HColin_R	ACCTGGAATTCTACCCCCCTCTA C	Helper probe for Colinsitu-HRP
Aer66-HRP	CTACTTCCCGCTGCCGC	<i>A. hydrophila</i>
Aer66H21	GATTGTGTACGTTTCAGCT	Helper probe for Aer66- HRP
Aer66H57	AACGATGAAAACGGCCGC	Helper probe for Aer66- HRP
LEGPNE1-HRP	ATCTGACCGTCCCAGGTT	<i>L. pneumophila</i>
LEGPNE1H307	ACACTTTAAGGACCCGAA	Helper probe for LEGPNE1-HRP
LEGPNE1H343	TTATGACCAACTGAGCTC	Helper probe for LEGPNE1-HRP

Cell Filter Preparation for TSA-FISH

Membrane filters (AES-Chemunex CB04 M/100511/R/1-1) with captured target cells were placed on cellulose pads flooded with 650ul of R2A broth with 0.1mg/ml of chloramphenicol/nalidixic acid and incubated at 37 °C for 4 hours. Filters were transferred onto a cellulose pad flooded with 650ul of 4% paraformaldehyde for fixation at room temperature (RT) for 1 hour.

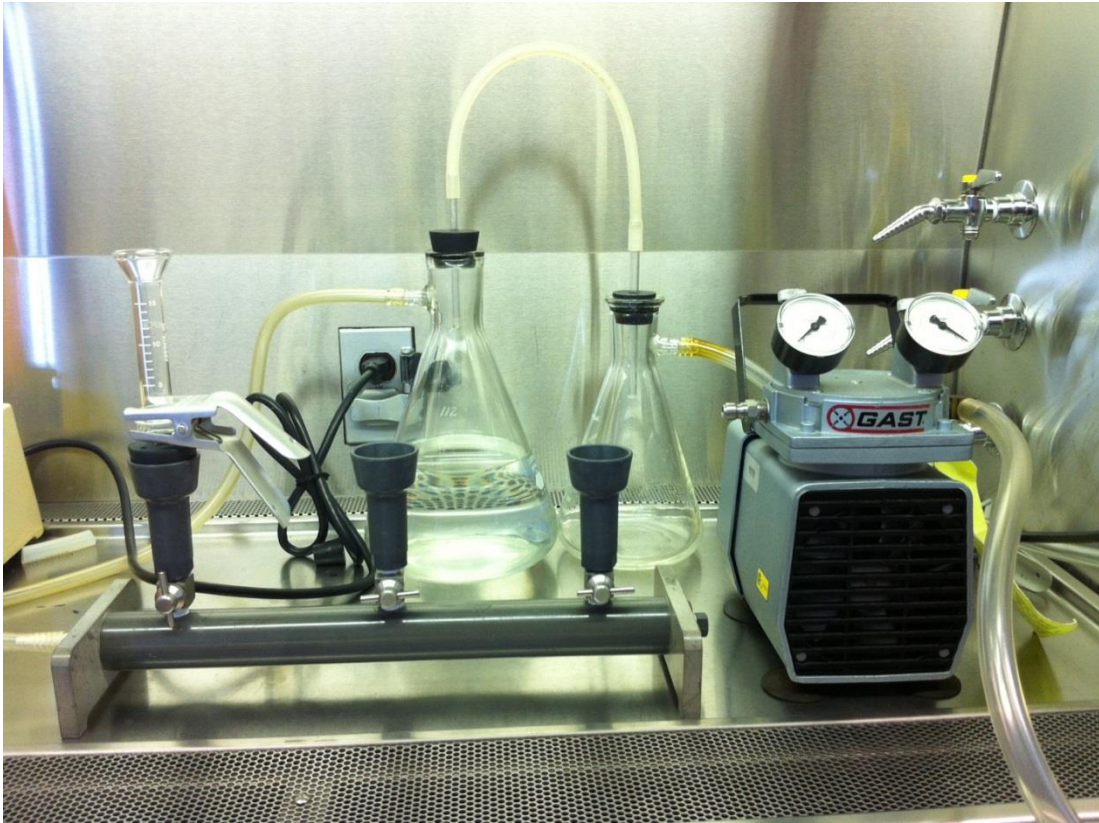


Figure 1. Filter system set up in a laminar flow hood.

After the fixation step, cells on each filter were dehydrated by placing the filter over a series of cellulose pads soaked with 650ul of 50%, 80% and 94% of ethanol for 4 min each. After dehydration, each filter was placed over a 76ul drop of TE buffer to rinse at RT for 5 min. Cells were permeabilized by placing each membrane filter on 100ul of lysozyme at RT for 20min. Lysozyme and other endogenous enzymes were inhibited by placing each membrane filter onto a 76ul drop of 0.02N HCL at RT for 20min. A second cell permeabilization step was done by transferring each membrane filter onto a 76ul drop of 0.2% Tween 20 PBS pH7.6 for 20min at 37 °C. Each membrane filter was finally rinsed on a 76ul drop of TE buffer at RT for 5min before hybridization.

Fluorescent *in situ* Hybridization

Prepared cells on membrane filters were placed onto 100ul of hybridization buffer containing 0.1g/ml of dissolved dextran sulfate and 1pmol of TSA-FISH and Helper probes, in a Falcon 35x10 mm easy grip Petri dish and placed into a sealed humidifying container (Fig. 2) inside a 48 °C water bath to hybridize for 2 hours. After 2 hours of hybridization, membranes were transferred onto a new 35x10 mm plate containing 100ul of wash buffer and returned to the humidifying container and incubated in the 48 °C water bath for 30min to wash off excess probe. Each membrane filter was rinsed by transferring onto 76ul of TNT buffer for 5min. Tyramide signal amplification was carried out by placing the membrane filter onto 76ul of FITC tyramide (1/50) for 10min at RT in dark to develop fluorescence. The membrane was washed again by placing it onto 76ul of TNT buffer for 5 min at RT in the dark, before examining with an epifluorescence microscope or analyzing with a *ScanRDI* solid phase laser cytometer (AES-Chemunex, BioMérieux) (Fig. 3). Figure 4 provides an overview of the TSA-FISH procedure used in this study, starting from cell suspension preparation, filtration, preparation for TSA-FISH and the TSA-FISH procedure.

Microscopy and Photography

Cells were observed using a Zeiss Axioskop 50 epifluorescence microscope system with an Axiocam 412 digital color camera using Axiovision software (Zeiss) and computer to view and save micrographs of observed cells at a total magnification of 1000x with the filter set appropriate for FITC (Ex/Em 460-500nm/515-550 Chroma

41001). The typical camera setting was 150mSec. For some samples, the camera exposure time was increased to detect faintly stained cells.



Figure 2. A humidifying container. It is made up of a plastic container with an air tight cover fitted with floral foam which was flooded with FAUP.

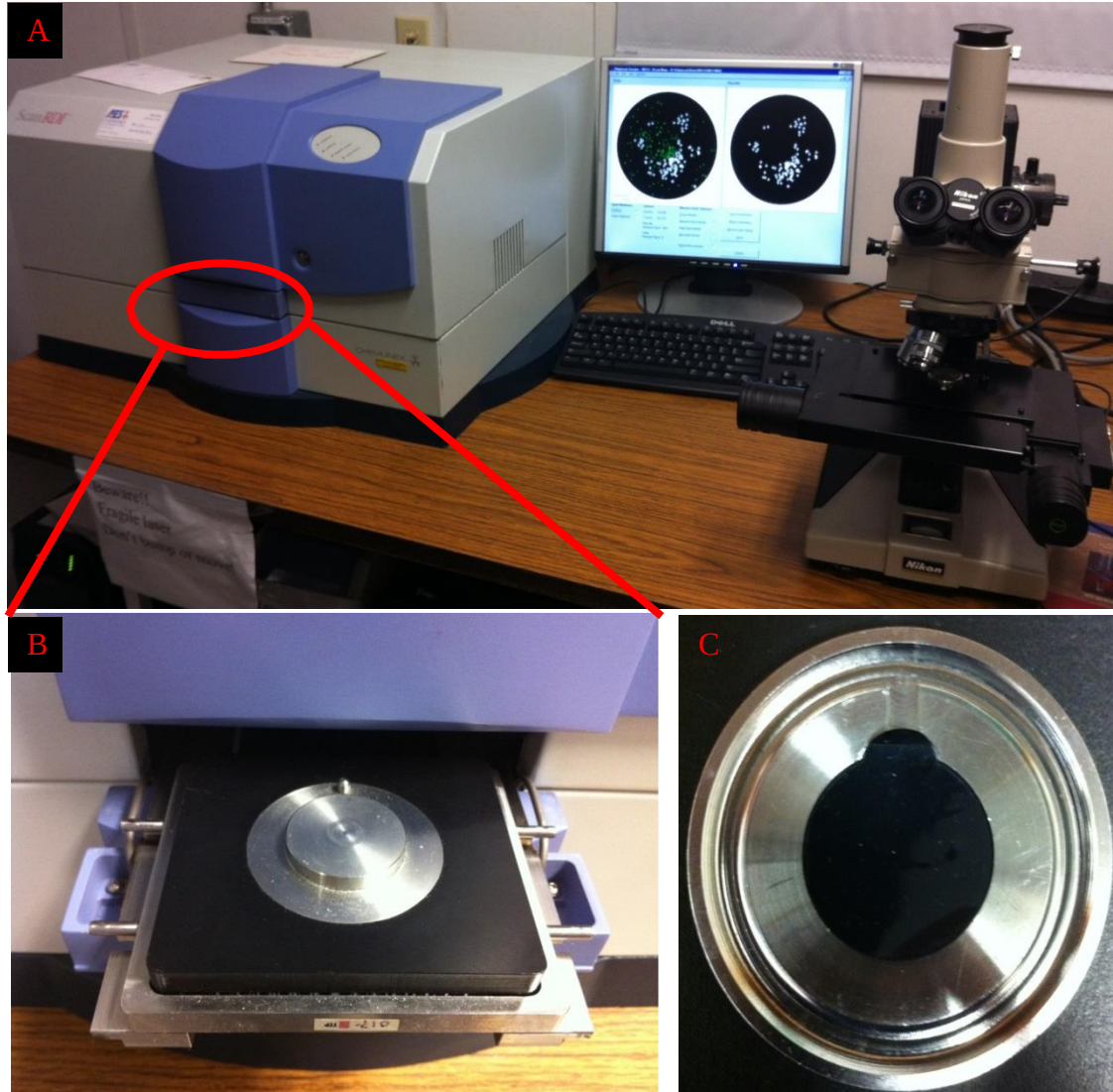


Figure 3. The solid phase laser cytometer (SPLC) system used in this study. The system includes the *ScanRDI*, a computer and an epifluorescence microscope (A). The carrier (C) which will move the filter holder (C) containing the membrane filter into the *ScanRDI* for scanning and recording of fluorescence events.

Data Analysis

All trials were replicated at least in triplicate unless otherwise stated. The mean and standard deviation were calculated to determine the mean cell count and maximum, average and minimum fluorescence intensity units in Microsoft Excel.

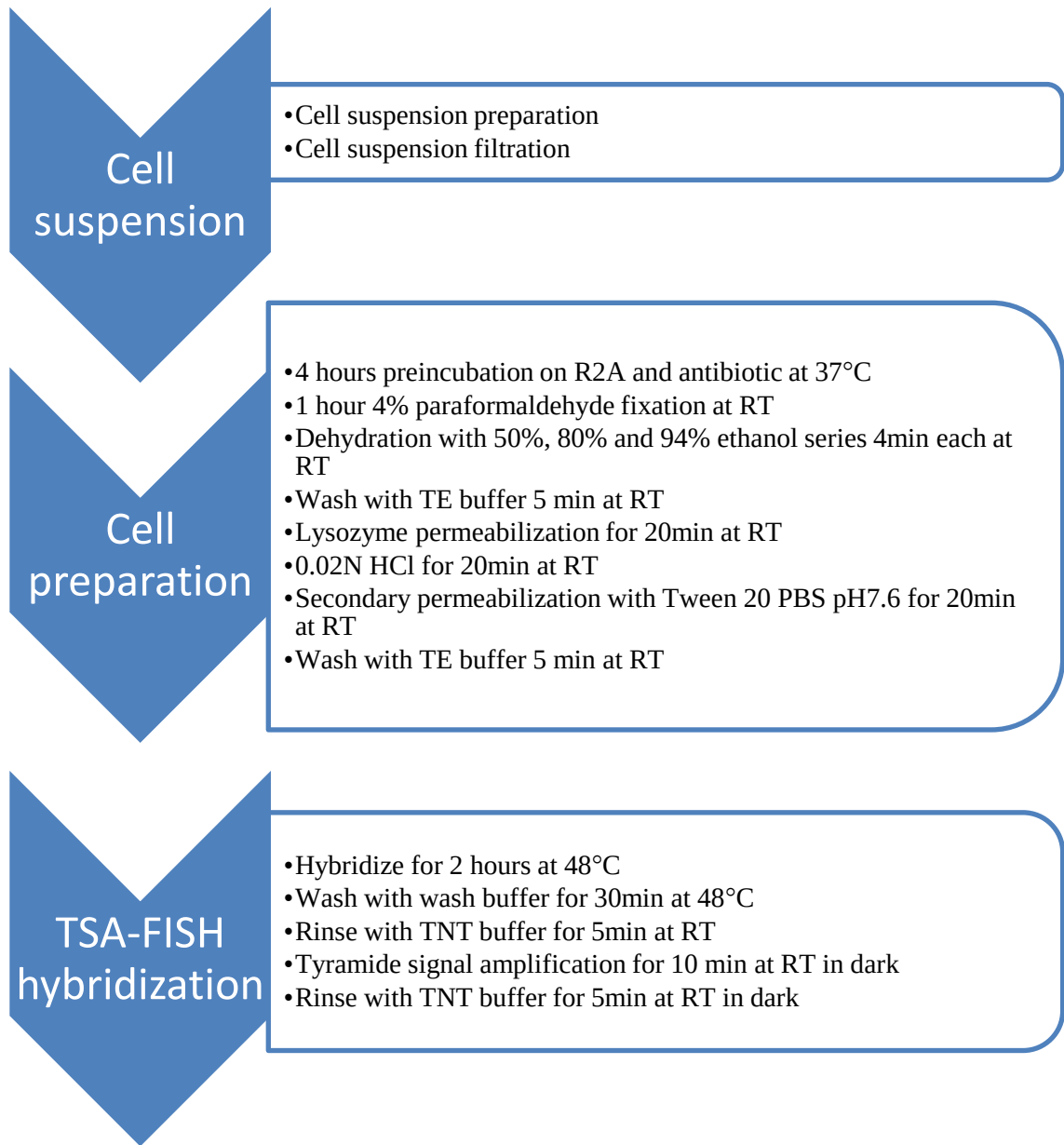


Figure 4. An overview of the TSA-FISH procedure being developed and used in this study.

Microscopic Quantification of Cell Counts

Cell counts were estimated by counting 20 random fields on the membrane filter with an ocular grid of $9.80\text{E}+3 \text{ um}^2$. The average cell count from the 20 random fields was multiplied by a microscope factor (2.32×10^4) that took into account the total area of 20 fields and the total filtration area on the membrane filter.

Fluorescence Intensity Determination

Micrographs were captured for each stained (SYBR Green/ TSA-FISH) membrane filter. Each micrograph was taken when 30 to 40 cells were observed within the ocular grid. Cell numbers in samples were adjusted to achieve these numbers. Three micrographs were taken for each stained membrane filter, and since each treatment was done in duplicate six micrographs were taken for fluorescence intensity determination using the MetaMorph microscopy automation & image analysis software. Fluorescence intensities determined by the software were tabulated using Microsoft Excel to calculate the mean and standard deviation of the maximum, average and minimum fluorescence intensity of the stained cells.

ScanRDI Application Development

Multiple membrane filters of TSA-FISH stained *L. pneumophila* were analyzed by the ScanRDI system and manually validated using the baseline application (Fig. 5A & 6) in the system to obtain data needed to set up a new application (Fig. 5B & 7) with higher threshold coefficients and lower peak intensities that was adapted to detect and

enumerate TSA-FISH stained *L. pneumophila*. A total of 3287 *L. pneumophila* TSA-FISH fluorescent events profile data were collected to create a new application for the ScanRDI system to specifically detect and enumerate TSA-FISH stained *L. pneumophila*. From these 3287 TSA-FISH fluorescent events, 1364 were positively validated as fluorescent events of TSA-FISH stained *L. pneumophila*; the remainder were negatively validated as fluorescent events of autofluorescent particles and false positive fluorescent events recorded by the ScanRDI system. Using these data, an application with a higher fluorescent threshold for detection was developed to detect TSA-FISH stained *L. pneumophila* that consistently exhibited lower fluorescence intensities in this study. Another application developed for a separate study (Broadaway, 2013) was also used for detection of *E. coli* and *A. hydrophila* (Fig. 5C & 8).

ScanRDI Analysis

Each labeled membrane filter was transferred on to a filter holder (Fig. 3C) with 100ul of glycerol to retain moisture and a support membrane. The filter holder was then placed into the ScanRDI system (Fig. 3) to detect and record fluorescence events. Once the scanning was completed and analyzed which typically took 3-5 min, the filter holder was transferred into an epifluorescent microscope Nikon Optiphot microscope with B2A epifluorescence filters and fluorescence events were verified visually using the filter map generated by the ScanRDI software to confirm each fluorescent event recorded by the system. By selecting particles shown in the primary/data map window (all particles detected), it was possible to determine true positive results from false positive (a detritus

particle) or false negative (no particle seen) results (Fig. 9). The results window contains fluorescence events that the ScanRDI system confirmed to be positively stained cells after sieving through the fluorescence profile of the fluorescence event using the threshold and discrimination of the application developed.

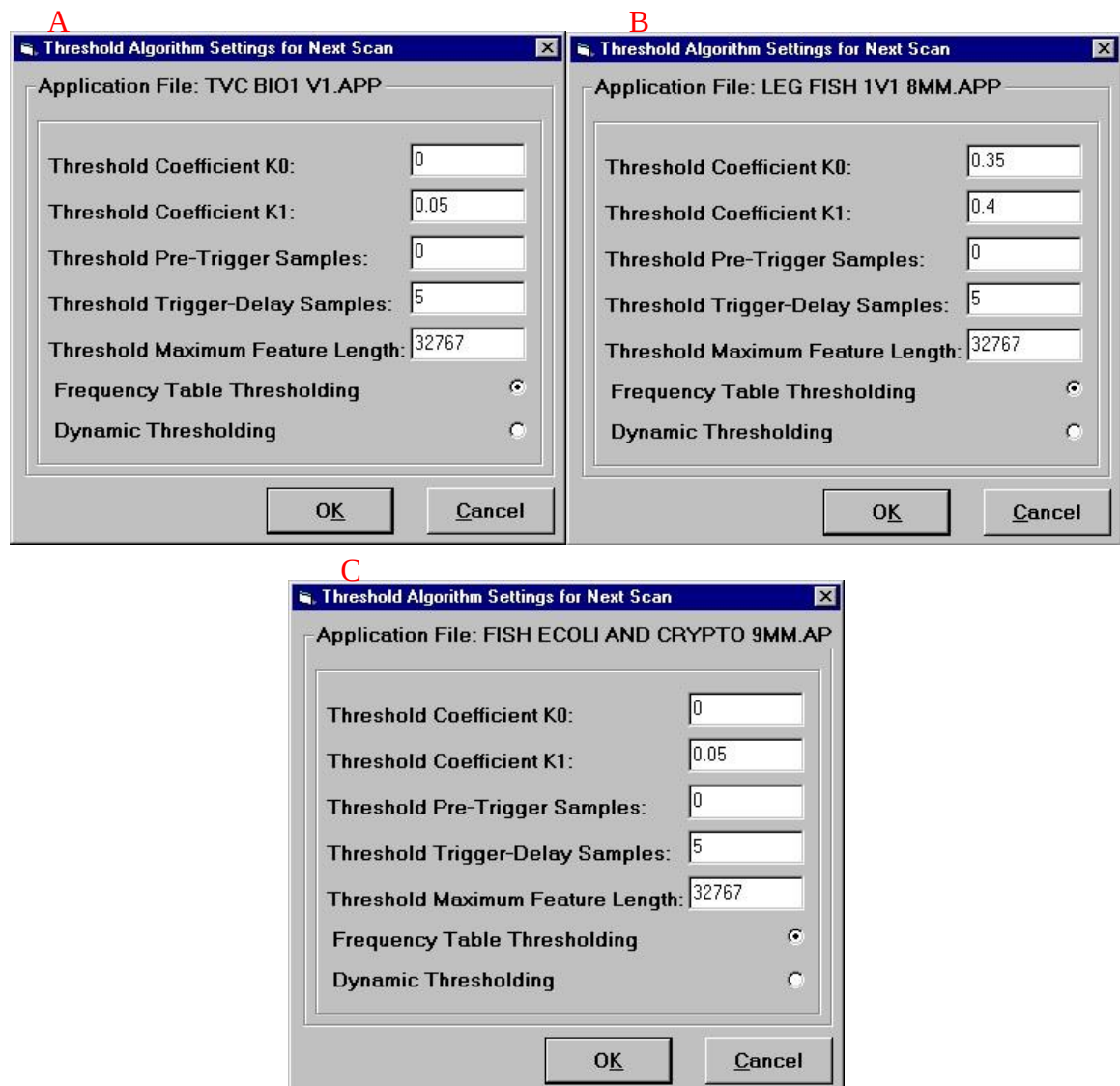


Figure 5. Threshold algorithm settings of ScanRDI applications specific to total viable count (TVC) (A), *L. pneumophila* (B) and *E. coli* and *Cryptosporidium* (C).

Discriminant Settings for Next Scan

Application File: TVC BI01 V1.APP

Size:

Reject Single Line Spots: ☒ of Length Less Than: 500 Samples

Reject Spots On Lines: ☒ Minimum: 1 Lines. Maximum: 50 Lines

Reject Spots On Samples: ☒ Minimum: 1 Samples. Maximum: 250 Samples

Intensity:

S/P Area Ratio: ☒ Minimum Value: 0 Maximum Value: 1.2

T/P Area Ratio: ☒ Minimum Value: 0.4 Maximum Value: 1.4

Use Peak Intensity: ☒ Minimum Value: 250 Maximum Value: 65535

Use Specific Intensity (AS): ☒ Minimum Value: 3

Use Specific Intensity (HW): ☒ Minimum Value: 10

Shape:

Use MultiPeaks ☒ Maximum Number of Peaks Per Line: 2

Use MultiWiggles ☒ Maximum Number of Inflections Per Line: 6

Use Half-Width: ☒ Maximum Number of Samples: 15

Use 2D Gaussian: ☒ Maximum Value: 1500

Apply 2D Gaussian to spots with a Peak Intensity less than: 4000

OK

Cancel

Figure 6. Discrimination settings for ScanRDI application specific to TVC.

Discriminant Settings for Next Scan

Application File: LEG FISH 1V1 8MM.APP

Size:

Reject Single Line Spots: ☒ of Length Less Than: 500 Samples

Reject Spots On Lines: ☒ Minimum: 2 Lines. Maximum: 23 Lines

Reject Spots On Samples: ☒ Minimum: 7 Samples. Maximum: 71 Samples

Intensity:

S/P Area Ratio: ☒ Minimum Value: 0 Maximum Value: 1.475

T/P Area Ratio: ☒ Minimum Value: 0 Maximum Value: 2.003

Use Peak Intensity: ☒ Minimum Value: 45 Maximum Value: 540

Use Specific Intensity (AS): ☒ Minimum Value: 0.09

Use Specific Intensity (HW): ☒ Minimum Value: 1.955

Shape:

Use MultiPeaks ☒ Maximum Number of Peaks Per Line: 4

Use MultiWiggles ☒ Maximum Number of Inflections Per Line: 11

Use Half-Width: ☒ Maximum Number of Samples: 15

Use 2D Gaussian: ☒ Maximum Value: 2516

Apply 2D Gaussian to spots with a Peak Intensity less than: 4000

OK

Cancel

Figure 7. Discrimination settings for ScanRDI application specific to *L. pneumophila*.

Discriminant Settings for Next Scan

Application File: FISH ECOLI AND CRYPTO 9MM.APP

Size:

Reject Single Line Spots: ☒ of Length Less Than: Samples

Reject Spots On Lines: ☒ Minimum: Lines. Maximum: Lines

Reject Spots On Samples: ☒ Minimum: Samples. Maximum: Samples

Intensity:

S/P Area Ratio: ☒ Minimum Value: Maximum Value:

T/P Area Ratio: ☒ Minimum Value: Maximum Value:

Use Peak Intensity: ☒ Minimum Value: Maximum Value:

Use Specific Intensity (AS): ☒ Minimum Value:

Use Specific Intensity (HW): ☒ Minimum Value:

Shape:

Use MultiPeaks ☒ Maximum Number of Peaks Per Line:

Use MultiWiggles ☒ Maximum Number of Inflections Per Line:

Use Half-Width: ☒ Maximum Number of Samples:

Use 2D Gaussian: ☒ Maximum Value:

Apply 2D Gaussian to spots with a Peak Intensity less than:

OK

Cancel

Figure 8. Discrimination settings for ScanRDI application specific to *E. coli* and *Cryptosporidium* (Broadaway, 2013).

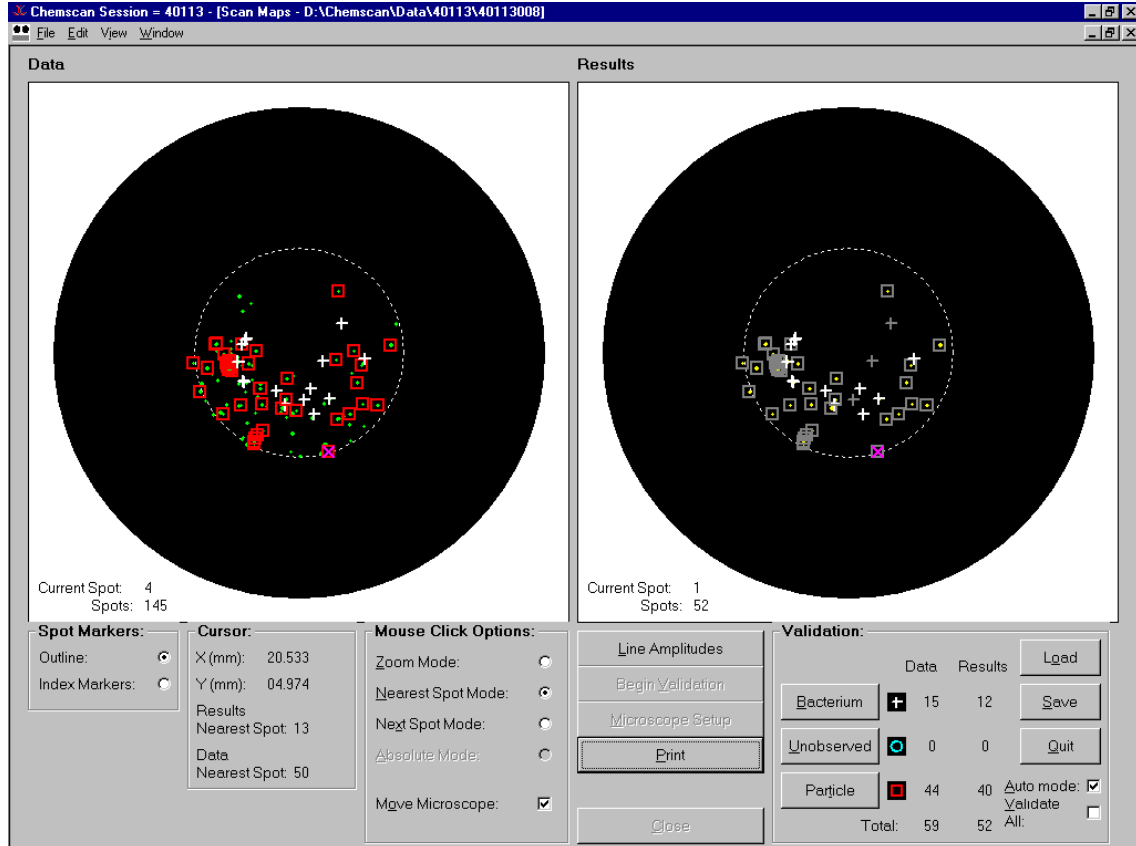


Figure 9. Maps of fluorescence events plotted by the ScanRDI system. The data map includes all fluorescence events detected by the system, while the results map contains fluorescence events of particles the system determined to be cells.

CHAPTER 3

RESULTS

TSA-FISH and ScanRDI

This study was carried out to develop a method to detect water borne pathogens without the need of cultivation. This was done by coupling two methods; specific cell labeling using TSA-FISH and the ScanRDI system for detection, analysis and enumeration. FISH is a species specific cell probing method used to detect microorganisms in environmental samples (Amann, 1995). The ScanRDI system is capable of detecting fluorescently labeled cells captured on membrane filters. The system is able to detect and discriminate fluorescent events on the filter membrane and discriminate fluorescent cells from non-cell particles when used with applications specifically developed for detection of particular microorganisms. Fluorescent events detected by the system can then be verified microscopically to determine if fluorescent events are positive for cells of interest (AES Chemunex, 2011). To achieve the goal of this study, established methods for FISH were explored and adapted to work on probing cells filtered onto membrane filter with a ScanRDI system.

The goal of this study was achieved by addition and optimization of steps that were shown to increase fluorescence intensity of TSA-FISH probed cells when using the method of Baudart et al. (Baudart & Lebaron, 2010). The additional steps included preincubation, HCl incubation, Tween 20 PBS pH6.7 incubation, addition of dextran sulfate and use of helper probes to obtain optimum fluorescence intensity.

E. coli TSA-FISH

Preliminary trials to establish TSA-FISH were carried out on *E. coli* according to the method reported by Baudart and Lebaron (2010) to assess its effectiveness for probing *E. coli* in this study. The *E. coli* cells were effectively stained with TSA-FISH (Fig. 10B) with CB04 black polyester 0.4µm pore size membrane filters as required for ScanRDI analysis (AES Chemunex, 2011). These polyester membrane filters with proprietary coatings were also compatible with the TSA-FISH reagents in that neither the base membrane nor coatings were damaged by the reagents such as ethanol. Fluorescent cells (Fig. 10B) were captured in micrographs under normal camera settings used for capturing SYBR Green stained *E. coli* cells (Fig. 10A). While the TSA-FISH-labeled cells were not as bright as those stained with SYBR Green, they were easily detectable visually through the microscope and in micrographs.

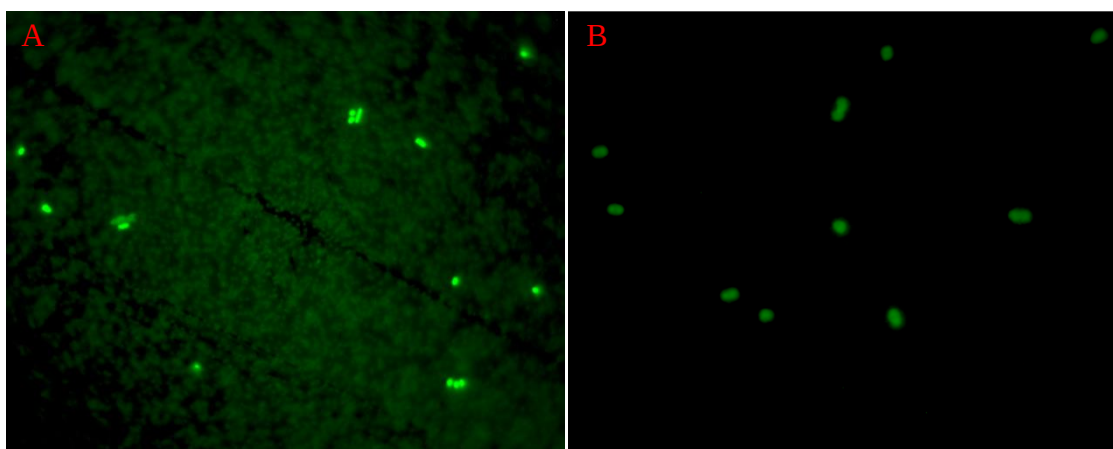


Figure 10. Micrograph of *E. coli* stained with SYBR Green (A) and the TSA-FISH method reported by Baudart *et. al.* (2010) (B).

Legionella pneumophila TSA-FISH

TSA-FISH was initially done on *L. pneumophila* according to methods reported by Baudart and Lebaron (2010) and Manz et al. (1995). Cells were barely visible through the epifluorescence microscope, and micrographs of *L. pneumophila* stained with these methods were only obtainable when the exposure time was raised substantially over the normal exposure time of 150mSec for fluorescence intensity determination. Besides the difficulties of capturing micrographs of *L. pneumophila*, very few cells were stained and observed with these methods when compared to cells being stained and observed on membrane filters stained with SYBR Green.

Legionella pneumophila TSA-FISH on Glass Slides

Since attempts to label *L. pneumophila* on membrane filters by TSA-FISH were thus far unsuccessful, alternate FISH methods were explored for *L. pneumophila*. Adapting the FISH method to be used with filter membranes to label *L. pneumophila* on glass slides was first explored, as it was found that *L. pneumophila* was being effectively TSA-FISH probed when cells were being fixed on glass slides for the procedure (Grimm et al., 1998). *L. pneumophila* could be stained by TSA-FISH with sufficient fluorescence for capturing micrographs (Fig. 11). The method reported by Baudart and Lebaron (2010) was adapted for these trials and was successful in TSA-FISH probing *L. pneumophila* that had been fixed onto glass slides.

Varying concentrations of LEGPNE1-HRP probe used for TSA-FISH staining *L. pneumophila* in wells on glass slides were used to determine optimum probe

concentration in terms of fluorescence. Samples were incubated with hybridization buffer containing different concentrations (1pMol/ul and 3.5pMol/ul) of LEGPNE1-HRP probe at 48 °C for 2 hours and fluorescence intensity of samples were determined. Resulting fluorescence intensity of samples with different probe concentrations showed little difference. Samples with 1pMol/ul (Fig. 11B & 12B) of probe had similar fluorescence intensity to that of samples with higher probe concentration (Fig. 11C & 12C).

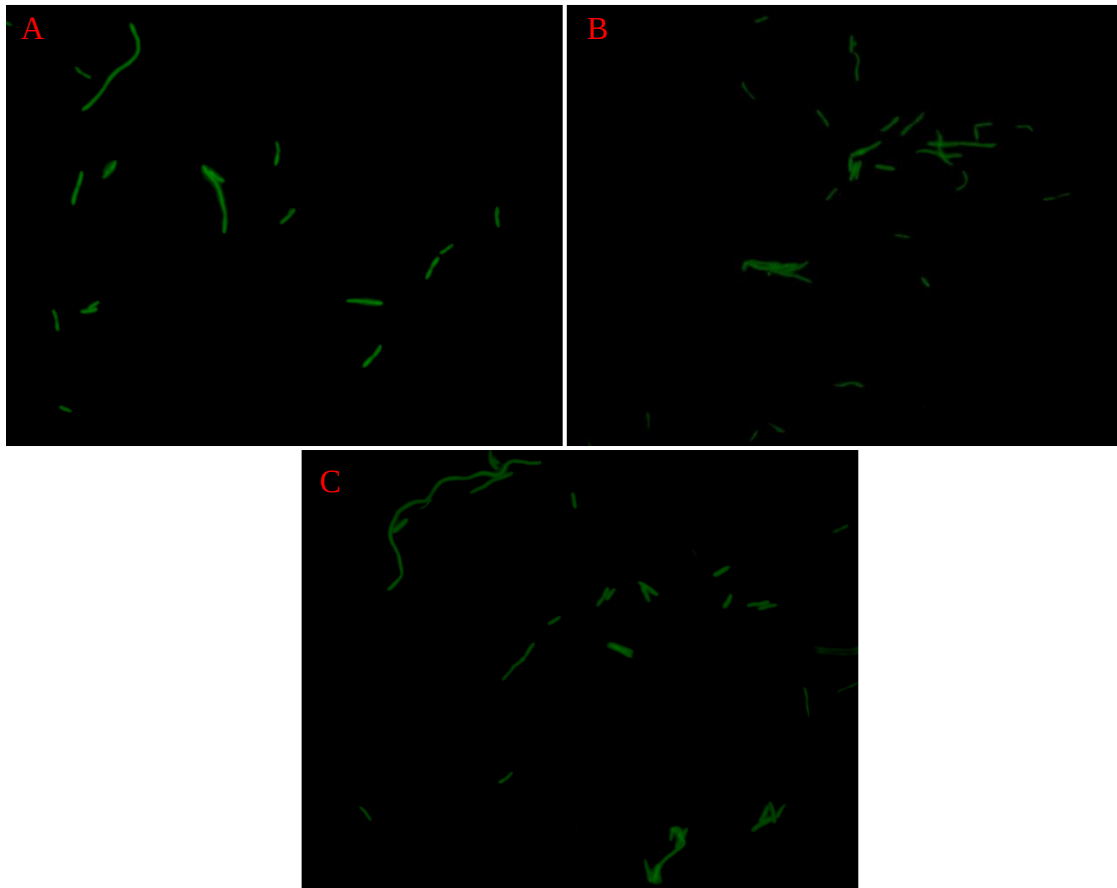


Figure 11. Micrograph of *L. pneumophila* TSA-FISH with different concentrations of LEGPNE1-HRP. SYBR Green stained *L. pneumophila* (A), *L. pneumophila* TSA-FISH with 1pmol/ul LEGPNE1-HRP (B) and *L. pneumophila* TSA-FISH with 3.5pmol/ul LEGPNE1-HRP (C).

Biotin and HRP Probes

Due to the higher cost of HRP conjugated probes and potential difficulties with uptake of the probe into *Legionella* cells because HRP is a very large molecule; the properties of a biotin conjugated probe were compared to an HRP conjugated probe. Experiments were set up to find out if it was efficacious to replace the HRP conjugated probe with a biotin conjugated probe. Results suggested that biotin conjugated probes nonspecifically bound to non *L. pneumophila* and non-cell particles (debris) found on the surface of glass slides. No micrographs were obtained due to the fact that the fluorescent particles observed were too faint to be recorded by photomicrography.

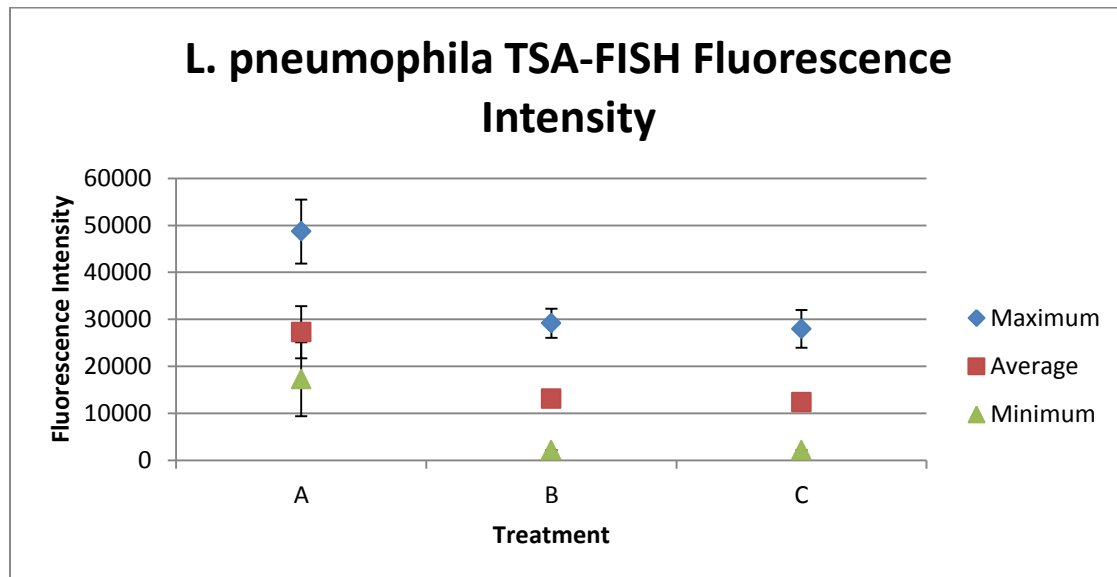


Figure 12. Fluorescence intensity of *L. pneumophila* samples probed with different concentrations of LEGPNE1-HRP probe: SYBR Green stained *L. pneumophila* (A), *L. pneumophila* TSA-FISH with 1pmol/ul LEGPNE1-HRP (B) and *L. pneumophila* TSA-FISH with 3.5pmol/ul LEGPNE1-HRP (C).

L. pneumophila TSA-FISH on Membrane
Filters with Chloramphenicol Preincubation

Since the method reported by Baudart and Lebaron (2010) was effective for *L. pneumophila* TSA-FISH on glass slides, this method was carried out on *L. pneumophila* filtered onto CB04 membrane filters, but this resulted in *L. pneumophila* cells that

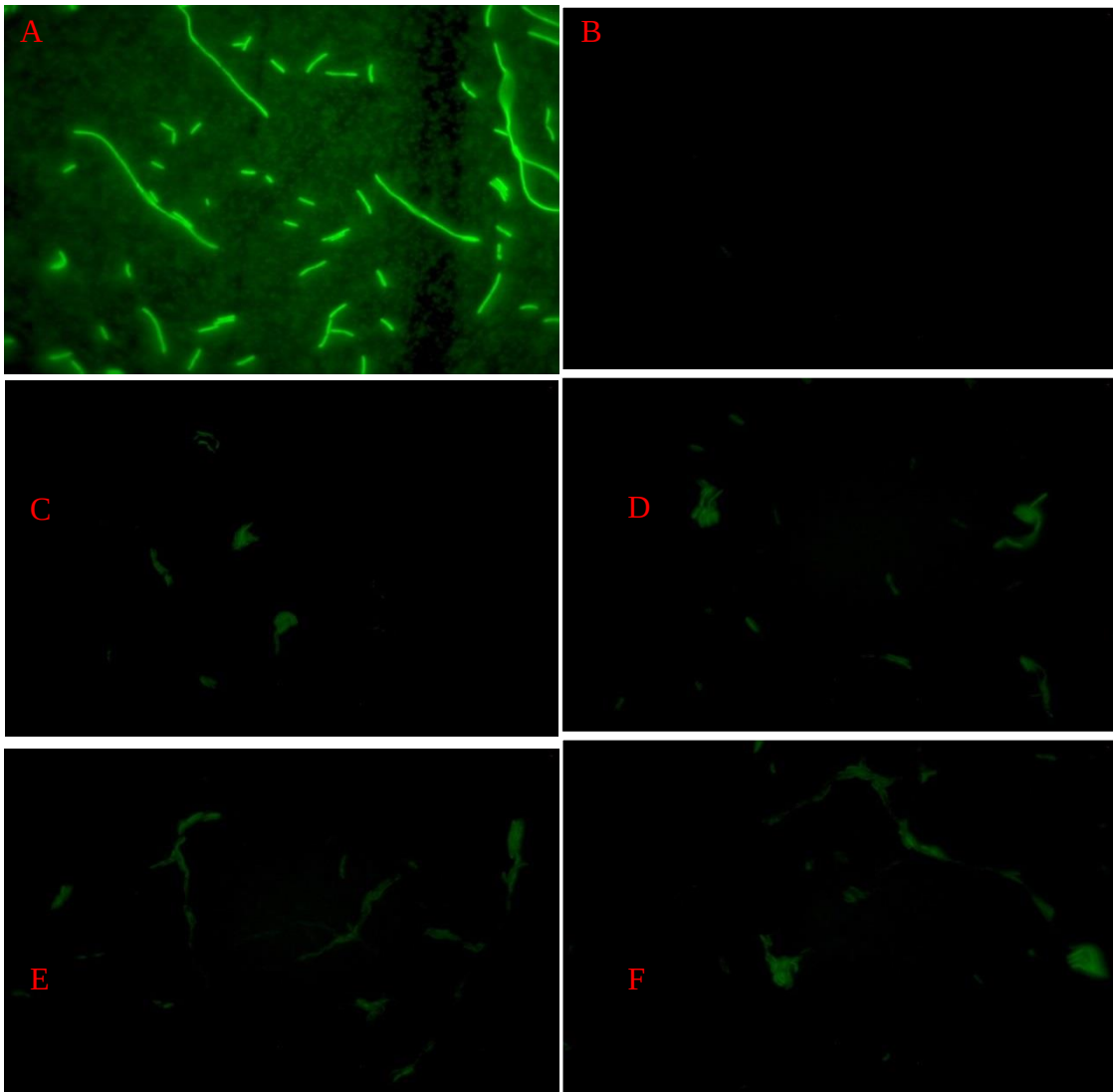


Figure 13. Micrograph of *L. pneumophila* stained with TSA-FISH preincubated on chloramphenicol for different durations. SYBR Green stained *L. pneumophila* without chloramphenicol preincubation (A), *L. pneumophila* TSA-FISH without chloramphenicol preincubation (B), *L. pneumophila* TSA-FISH with chloramphenicol preincubation (C, D, E, F), 0.5, 1, 2 and 3 hours respectively.

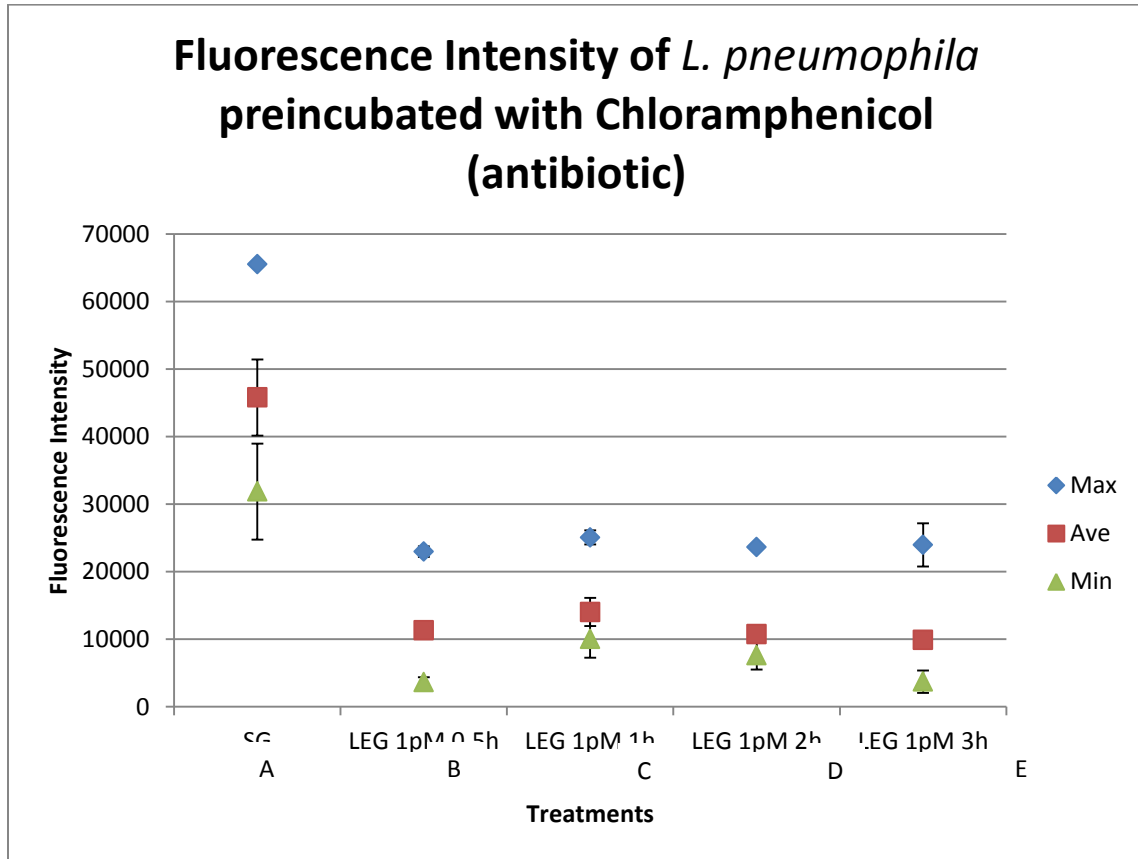


Figure 14. Fluorescence intensity of TSA-FISH stained *L. pneumophila* preincubated on chloramphenicol for different duration. SYBR Green stained *L. pneumophila* without chloramphenicol preincubation (A), *L. pneumophila* TSA-FISH with chloramphenicol preincubation (B, C, D, E) for 0.5, 1, 2 and 3 hours respectively.

fluoresced very faintly and not sufficiently bright for micrography. Since cells were labeled, although faintly, this suggested that the method does introduce the HRP probes into the cells for probing. Thus, ways to increase the number of probe molecules entering the cells and binding to target rRNA were explored.

Chloramphenicol inhibits protein synthesis and rRNA degradation which causes increased rRNA for better TSA-FISH probing (Ouverney & Fuhrman, 1997). Filtered *L. pneumophila* cells were preincubated on chloramphenicol for different durations (Fig. 13 & 14), TSA-FISH stained *L. pneumophila* were observed and fluorescence intensities

were at values that the camera was able to detect via micrographs (Fig. 14). It can be observed from the micrographs that with no preincubation (Fig. 13B), no cells were visible in the micrograph. Starting with 0.5 through 3 hours of preincubation with chloramphenicol (Fig. 13C-F), TSA-FISH probed *L. pneumophila* cells were detected.

While longer preincubation did not necessarily equate to higher fluorescence intensities, by examining the fluorescence intensity graph (Fig. 14), it was decided that 0.5 hour (Fig. 14B) of preincubation was enough to improve the fluorescence intensity of TSA-FISH probed *L. pneumophila* cells.

Besides using chloramphenicol, the effect of nalidixic acid's cell enlargement property on microorganisms by inhibiting DNA synthesis and preventing cell division (McKay, 1992), was examined. For *L. pneumophila*, no enlargement of *L. pneumophila* cells was observed, but when applied to *E. coli* and *A. hydrophila* there was an increase in cell size and presumably ribosome content; this made visualization of cells easier (results not shown). Due to the increase in cell size for *E. coli* and *A. hydrophila*, nalidixic acid was used for their preincubation treatment with R2A broth. Since nalidixic acid had no beneficial effect on *L. pneumophila* and it has been known to cause *Legionellae* cells to lyse on incubation (Hussong et al., 1987), it was decided to continue using chloramphenicol rather than nalidixic acid for *L. pneumophila* preincubation treatment.

0.02N HCl and 0.2% Tween 20 Incubation

HCl incubation is used in TSA-FISH procedures as an agent to denature proteins including added lysozyme and endogenous peroxidases and increase probe penetration (Chin et al., 2003). Tween 20 (Coonen, Dumoulin, Ramaekers & Hopman, 1994) incubation was also introduced as a second step for permeabilization after lysozyme treatment. These additional steps were introduced into the TSA-FISH method reported by Baudart and Lebaron (2010), and tested on *E. coli* (Fig. 15 & 16). Introduction of 0.2% HCl incubation to the TSA-FISH method decreased both cell count and fluorescence intensity of *E. coli* (Fig. 16C), but with the introduction of the Tween 20 incubation step, both cell counts and fluorescence intensities of *E. coli* were increased (Fig 16 D & E). Besides the fluorescence intensity plot on the graph, there was also a visible difference in brightness on the micrograph (Fig. 15D & E) showing the effects of HCl and Tween 20 on *E. coli* TSA-FISH.

The same treatments were carried out on *L. pneumophila*, but the same effect observed on *E. coli* was not observed with *L. pneumophila* TSA-FISH staining. *L. pneumophila* cells were still too faint for micrograph capture for fluorescence intensity analysis.

Effect of Dextran Sulfate

Dextran sulfate is known to improve localization of fluorescent tyramide (van Gijlswijk, Wiegant, Raap & Tanke, 1996) and accelerates hybridization (Wahl, Stern & Stark, 1979) when included into TSA-FISH procedures. Effects of dextran sulfate were examined by adding 1mg/ul of dextran sulfate (dextran sulfate, Acros) to the hybridization buffer and tyramide signal amplification solution (Kirschner et al., 2012). Effects of dextran sulfate were observed when 1mg/ul of dextran sulfate was added to the hybridization buffer (Fig. 17B). The maximum, average and minimum fluorescence intensities of TSA-FISH labeled *L. pneumophila* were increased after the addition of dextran sulfate (Fig. 18). Additional experimentation of addition of dextran sulfate to the tyramide signal amplification solution also exhibited an increase in maximum, average and minimum fluorescence intensities of TSA-FISH labeled *L. pneumophila* (Fig. 19).

With the addition of dextran sulfate to both the hybridization and tyramide signal amplification steps, *L. pneumophila* cells fluoresced bright enough for micrograph capture (Fig. 17C & 19). In addition to the increase in fluorescence intensity, cell counts of TSA-FISH stained *L. pneumophila* increased to become closer to the numbers in SYBR Green stained samples (Fig. 19).

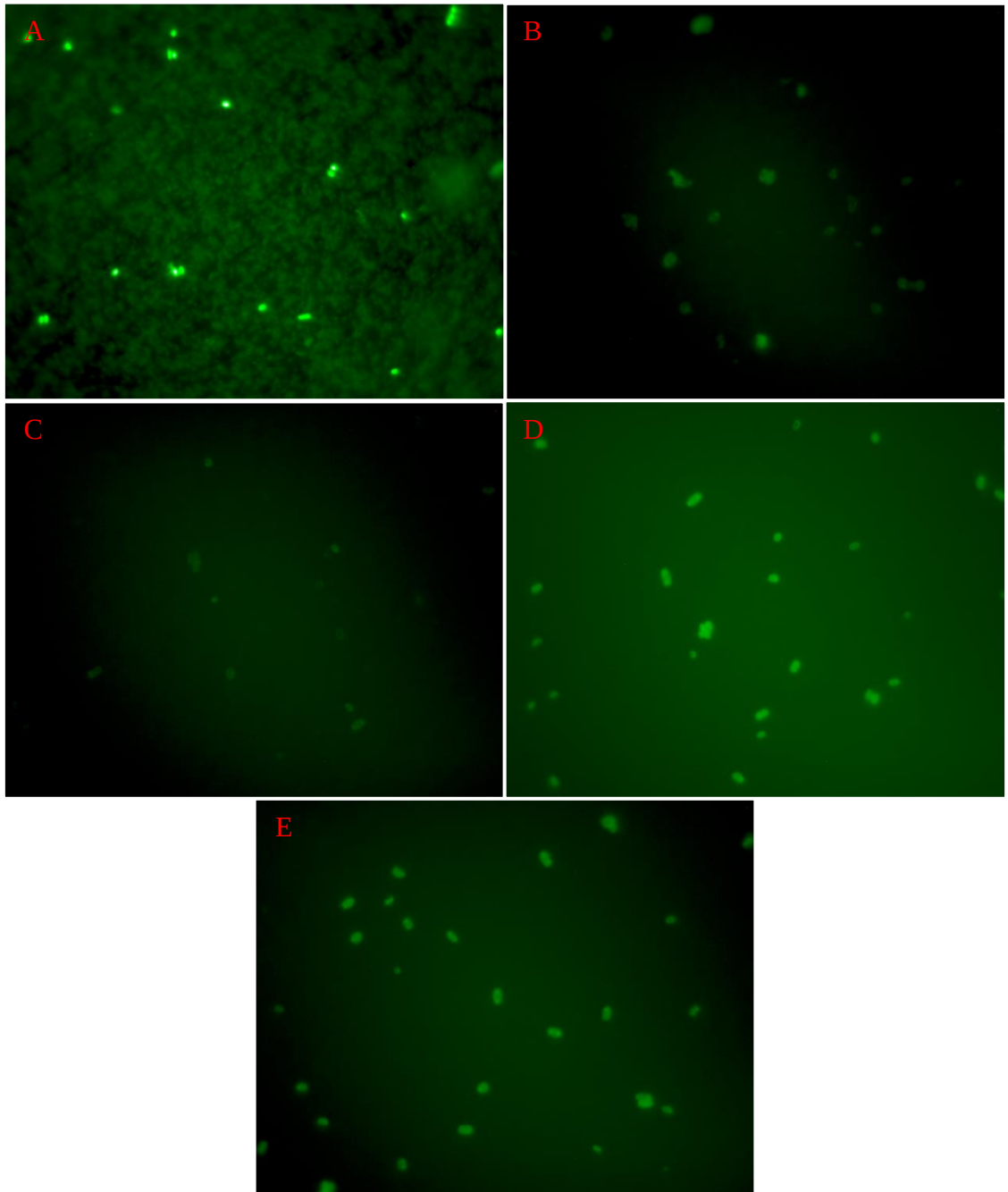


Figure 15. Micrographs of *E. coli*, SYBR Green stained *E. coli* (A), TSA-FISH stained *E. coli* with lysozyme permeabilization (B), TSA-FISH stained *E. coli* with lysozyme permeabilization followed by 0.02% HCl incubation (C), TSA-FISH stained *E. coli* with lysozyme permeabilization followed by 0.2% Tween 20 permeabilization (D) and TSA-FISH stained *E. coli* with lysozyme permeabilization followed by 0.02% HCl incubation and 0.2% Tween 20 permeabilization (E).

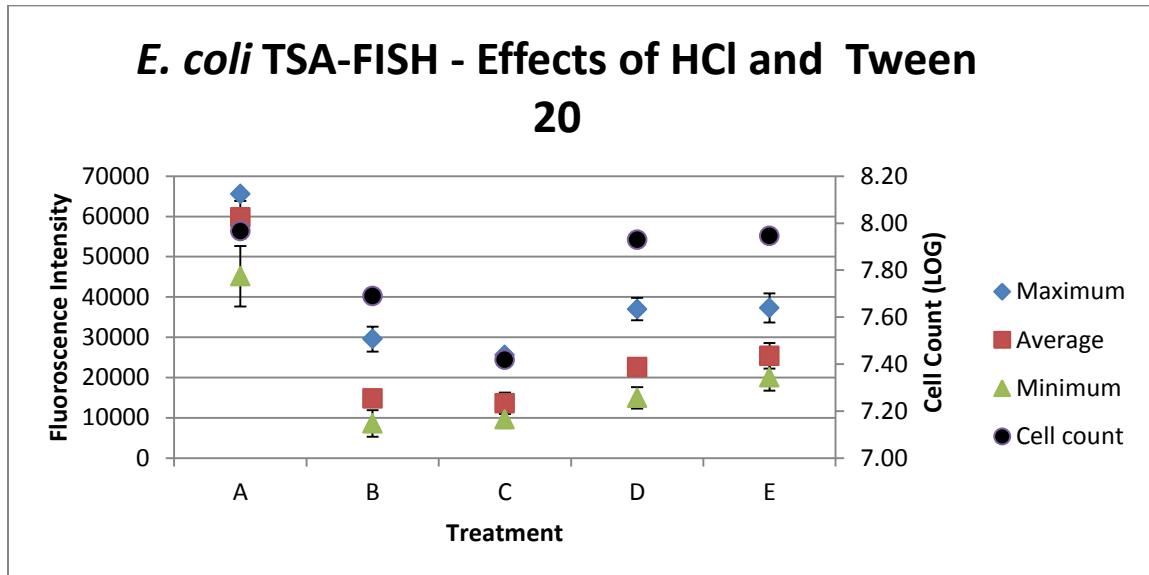


Figure 16. Fluorescence intensity of *E. coli*, SYBR Green stained *E. coli* (A), TSA-FISH stained *E. coli* with lysozyme permeabilization (B), TSA-FISH stained *E. coli* with lysozyme permeabilization followed by 0.02% HCl incubation (C), TSA-FISH stained *E. coli* with lysozyme permeabilization followed by 0.2% Tween 20 permeabilization (D) and TSA-FISH stained *E. coli* with lysozyme permeabilization followed by 0.02% HCl incubation and 0.2% Tween 20 permeabilization (E).

Helper Oligonucleotide Probes

Unlabeled helper oligonucleotide probes are known to increase accessibility of a TSA-FISH probe binding site and hybridization signal (Fuchs, Glöckner, Wulf & Amann, 2000). To test if it was possible to increase the fluorescence intensity of TSA-FISH probed *L. pneumophila* cells, helper oligonucleotide probes LEGPNE1H307 and LEGPNE1H343 were added to the hybridization buffer at a final concentration of 1pMol/ul each. The results (Fig. 20) were not as expected, since no significant difference in fluorescence intensity was observed between samples with helper oligonucleotide probes (Fig. 20B) and samples without helper oligonucleotide probes (Fig. 20C).

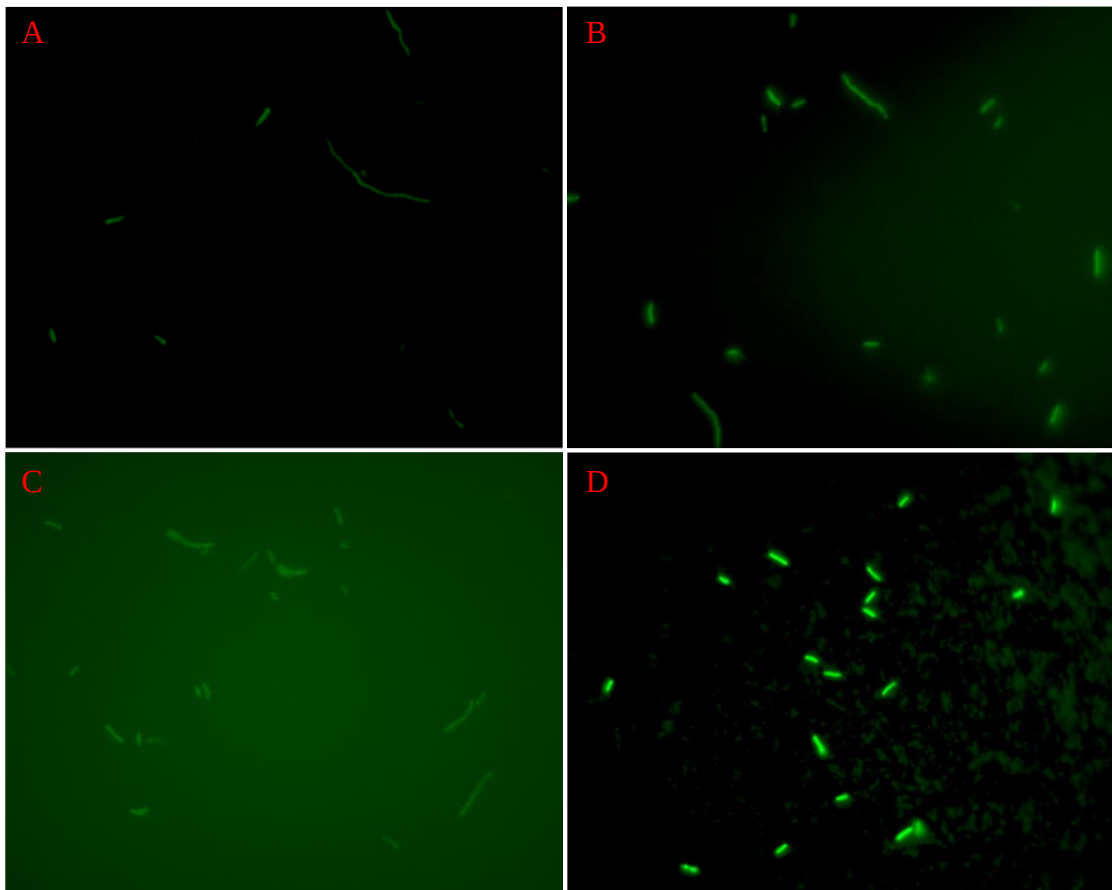


Figure 17. Effect of dextran sulfate on TSA-FISH of *L. pneumophila*. (A) *L. pneumophila* TSA-FISH control with no dextran sulfate. (B) *L. pneumophila* TSA-FISH with dextran sulfate in hybridization buffer. (C) *L. pneumophila* TSA-FISH with dextran sulfate in hybridization and tyramide signal amplification mixture. (D) SYBR Green stained *L. pneumophila*.

ScanRDI Results

TSA-FISH detection of *A. hydrophila*, *E. coli* and *L. pneumophila* was carried out on separate membrane filters and probed with their respective HRP conjugated probes using the final TSA-FISH method outlined in the materials and methods section (Fig. 4). TSA-FISH probed *A. hydrophila*, *E. coli* and *L. pneumophila* were scanned by the

ScanRDI system and fluorescent events were verified. Results of the ScanRDI verified detection numbers of *A. hydrophila*, *E. coli* and *L. pneumophila* were compared to spread plate counts, the resulting percentages were 39.7%, 79.5% and 89.1% of plate count numbers respectively.

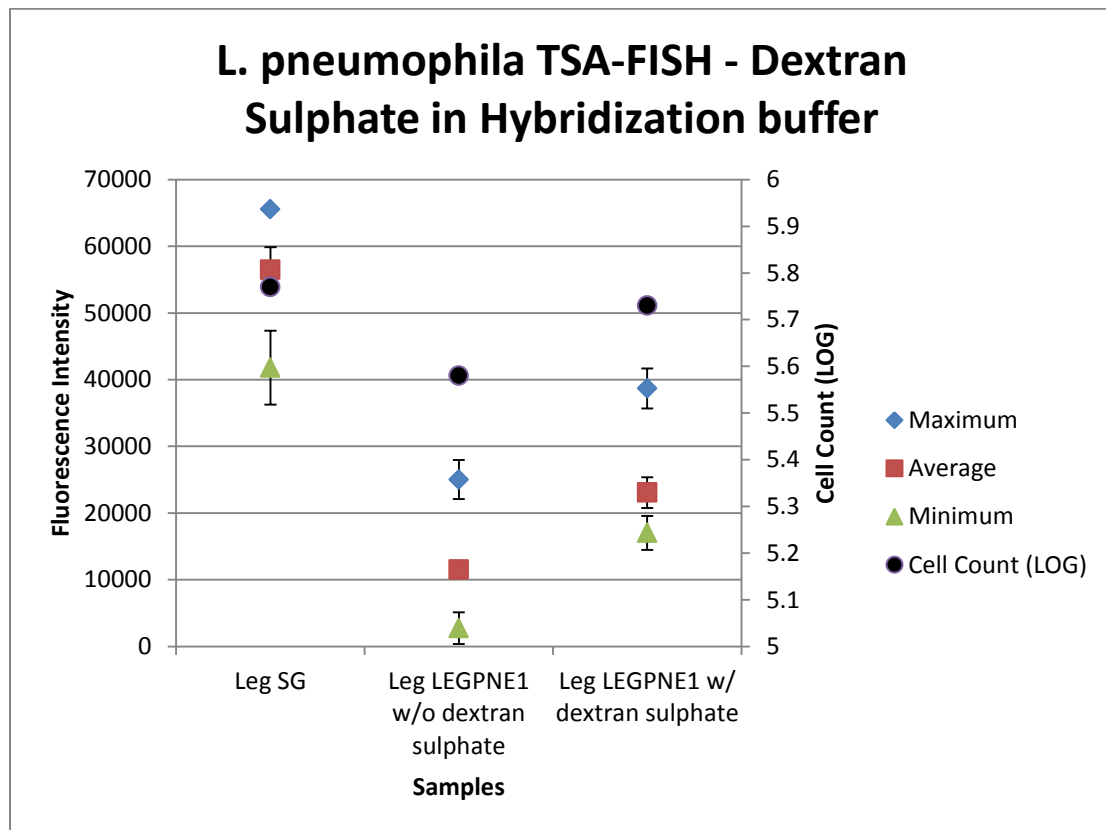


Figure 18. Fluorescence intensity and cell count of *L. pneumophila* stained with SYBR Green (Leg SG), TSA-FISH control without dextran sulfate in hybridization buffer (Leg LEGPNE1 w/o dextran sulfate) and TSA-FISH with dextran sulfate in hybridization buffer (Leg LEGPNE1 w/ dextran sulfate).

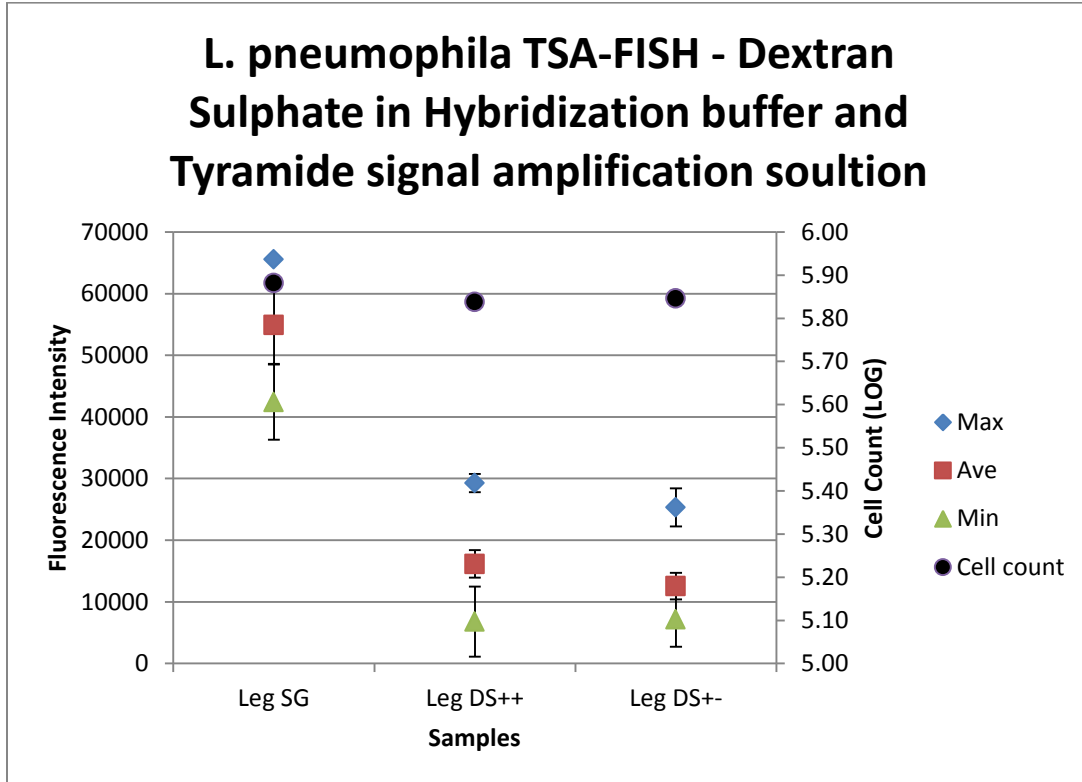


Figure 19. Fluorescence intensity and cell count of *L. pneumophila* stained with SYBR Green (Leg SG), TSA-FISH with dextran sulfate in hybridization buffer and tyramide signal amplification mixture (DS++) and TSA-FISH with dextran sulfate in hybridization buffer only (DS+-).

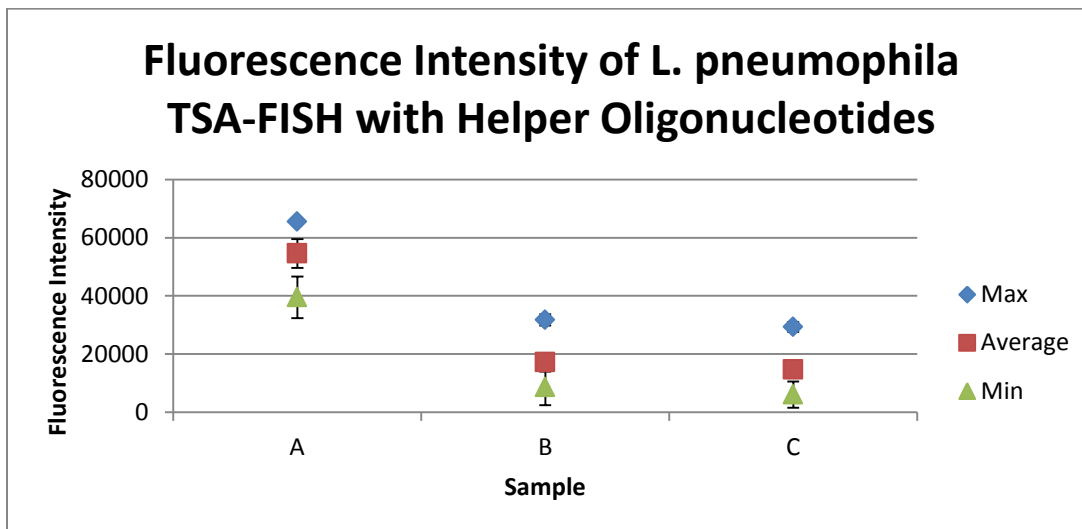


Figure 20. Fluorescence intensity of *L. pneumophila* stained with SYBR Green (A), TSA-FISH with helper probes (B) and TSA-FISH without helper probes (C).

CHAPTER 4

DISCUSSION

TSA-FISH Procedure Development

The TSA-FISH procedure was first carried out according to the method described by Baudart and Lebaron (2010) to find out if it was possible to replicate the results reported with the three bacterial species that were used in this study. The FISH method reported by Baudart and Lebaron (2010) was carried out on *E. coli*, and by following the method exactly, as expected, it was possible to probe the *E. coli* strain that was used in this study as reported in the results section (Fig. 10). The TSA-FISH probed *E. coli* (Fig. 10B) fluoresced bright enough for the camera to take micrographs at normal settings with an exposure time of 150mSec.

Since the *E. coli* strain used in this study was readily TSA-FISH probed with the method reported by Baudart and Lebaron (2010), it was thought that similar results could be obtained with *L. pneumophila*. The exact procedure reported by Baudart and Lebaron (2010) was used as a control with *L. pneumophila*. The results were not as productive as those observed for *E. coli*; very few *L. pneumophila* cells were observed and due to low fluorescence at very low intensities that were not detectable by the microscope camera at normal settings and exposure time which is why there are no micrographs for this trial.

TSA-FISH Procedures for *L. pneumophila*

Subsequently, it was observed that two significantly different methods reported by Manz et al. (1995) and Declerck et al. (2003) were used to specifically probe for *L. pneumophila* using FISH. It was reported in these papers that the resulting FISH probed *L. pneumophila* cells were easily detectable (Manz et al., 1995; Declerck et al., 2003) with low background fluorescence (Declerck et al., 2003), but when *L. pneumophila* TSA-FISH was carried out separately according to the two methods, the results did not correspond to previously reported results. The methods were repeated in three experiments, and the number of cells observed per field was significantly lower than that observed on SYBR Green stained samples and TSA-FISH fluorescence intensity was again too low for camera detection. Possible explanations for the difference in observed results and the expected results from those papers included the need of high fluorescence intensity in this study than that of Manz et al. (1995) and Declerck et al. (2003). In this study, the objective was to achieve high fluorescence intensity from TSA-FISH probed *L. pneumophila* so that the ScanRDI system could detect and enumerate the fluorescence events. In the studies of Manz et al. (1995) and Declerck et al. (2003), the objective was to observe the presence of *L. pneumophila* and was not limited by the fluorescence intensity of the FISH probed cells. Manz et al. (1995) used an exposure time of 2-8s for epifluorescence micrography (150mSec was used in this study); the exposure time used by Declerck et al. (2003) was not specified.

TSA-FISH Procedures on Glass Slides

Since the FISH methods using membrane filters reported by Manz et al. (1995) and Declerck et al. (2003) did not initially work well in this study, alternate methods were investigated to find out if it was possible to TSA-FISH probe *L. pneumophila* with fluorescence intensities that would be detectable by the epifluorescence microscope camera at settings used in this study. Methods suggesting the feasibility of TSA-FISH probing *L. pneumophila* with promising fluorescence intensity were collected and compared. The method reported by Grimm et al. (1998) was considered, and the main difference between Grimm's method and those of Manz et al. (1995) and Declerck et al. (2003) is that *L. pneumophila* were FISH probed on a glass slide instead of on a membrane filter. When *L. pneumophila* was TSA-FISH probed using Grimm's method (1998) on a glass slide, the results were encouraging. Cells were bright and were detectable by the epifluorescence microscope camera at normal settings and exposure time. Since Baudart's method (2010) was able to probe *E. coli* readily, it was decided to find out if using Baudart's method (2010) to FISH probe *L. pneumophila* on glass slide would produce the same results as that of Grimm's method (1998). The Baudart method (2010) was tested on glass slides and *L. pneumophila* cells fluoresced brightly and were detectable by the epifluorescence microscope camera at normal settings and exposure time (Fig. 11). Since this method successfully labeled cells that were bright enough for micrograph imaging, it was used as a model to examine factors that might affect the brightness of TSA-FISH probed cells.

Probe Concentrations

Varied probe concentrations were examined by comparing the resulting fluorescence intensity, captured in micrographs, produced by hybridizing cells with 1pMol/ul and 3.5pMol/ul of probe in the hybridization buffer. The results (Fig. 11& 12) showed that there was no appreciable difference found between the used of the two concentrations used for the hybridization of probe to cells, both samples probed with 1pMol/ul and 3.5pMol/ul produced fluorescence intensity close to 30,000 fluorescence units (Fig. 12B and 12C) which is above the intensity of about 20,000 units required for ScanRDI detection. From this result, the small difference in fluorescence obtained at between 1 and 3.5pmol/ul of probe concentration did not have an adverse effect on the fluorescence intensity of *L. pneumophila*. Although increasing probe concentrations would increase the cost of each experiment, it would be interesting to examine if higher concentrations of probe would affect the results found in this study. This could be tested in future experiments.

Biotin and HRP Probes

Another aspect of the type of probe used was also examined, because the HRP-conjugated probes were generally more expensive, and the HRP molecule is quite large which might limit penetration of the probes into cells, we were interested in substituting HRP-conjugated probes with less expensive and more permeable probes. Biotinylated probes were considered as a candidate substitute because biotinylated probes are less expensive than HRP-conjugated probes and the biotin molecule is smaller than HRP and

therefore likely to penetrate fixed cells more readily. Experiments were done using glass slides to examine effects of biotinylated probes when used for TSA-FISH probing *L. pneumophila* and compared to HRP conjugated probes. *L. pneumophila* cells probed with HRP conjugated probes produced results observed in Fig 11 & 12. When probed with biotinylated probes, cells were not bright enough for micrograph capturing, and bright fluorescent particles were observed, probably due to non-specific binding of the streptavidin to other biotin molecules besides the ones found conjugated to the probes.

Antibiotic Preincubation

With the Baudart FISH method (2010) probing effectively on glass slides, it was thought that methods used by others with *L. pneumophila* filtered on membranes would produce the same results. After repeating the experiment on membrane filters instead of glass slides, *L. pneumophila* cells again did not fluoresce bright enough for micrograph capture (Fig. 13B). Since rRNA sequences were being probed, ways of increasing rRNA content in cells were examined to find out if by increasing rRNA content will lead to increased fluorescence intensity due to increase binding of probes within the target cells.

Antibiotics such as chloramphenicol are known for their effects on the accumulation of stable rRNA in bacterial cells (Ouverney & Fuhrman, 1997), due to its ability of inhibiting protein synthesis by preventing peptidyl transferase activity of the bacterial ribosome. With that information, experiments were set up to pre-incubate filtered *L. pneumophila* on R2A broth containing 0.1mg/ml of chloramphenicol to find out if it would increase the fluorescence intensity of TSA-FISH probed *L. pneumophila*.

The pre-incubated TSA-FISH probed *L. pneumophila* cells fluoresced at intensities that were detectable by the epifluorescence microscope camera (Fig. 13C-F). Even though cells were pre-incubated on R2A broth with chloramphenicol for different durations, there were no major differences in the fluorescence intensity (Fig. 14). It could be observed that the fluorescence from the TSA-FISH probed cells were not as evenly distributed within the cells (Fig. 13C-F), especially longer cells, as observed in the SYBR Green stained cells (Fig. 13A). Although this pre-incubation step seemed to increase fluorescence intensity of TSA-FISH probed *L. pneumophila*, the uneven distribution of fluorescence became problematic when there was a need to determine cell counts, when a long cell can be miscounted as multiple cells due to the appearance of fluorescing sections of that single long cell.

Effects of HCl and Tween 20

With the introduction of chloramphenicol pre-incubation the fluorescence intensity of *L. pneumophila* was increased to a level where minimal fluorescence could be detected by the epifluorescence microscope camera (Fig. 13C-F). Additional treatments that were known to possibly increase fluorescence intensity in TSA-FISH were examined. Firstly, incubation with HCl was introduced to inactivate endogenous peroxidase that might react with the tyramide used in the tyramide signal amplification step. This step was included to make sure that the tyramide would only react with the horse radish peroxidase that was bound to the rRNA after the hybridization step, and not with endogenous peroxidases

Another additional step was the introduction of Tween 20 incubation as a secondary permeabilization step for *L. pneumophila*. This step was introduced because Tween 20 is a non-specific permeabilization agent that it was hoped would be able to permeabilize the cell membrane of *L. pneumophila* (Amano & Williams, 1983; Hindahl & Iglewski, 1984). The experiment was carried out on *E. coli* first, due to its faster growth than *L. pneumophila* and also because it is one of the bacteria that was to be included in detection and enumeration by the ScanRDI system for this project. The results (Fig. 15 & 16) were encouraging when *E. coli* cells were treated with Tween 20. Fluorescence intensity of samples not treated with Tween 20 (Fig. 15B & 15C) were less than 30,000 fluorescence units, while those treated with Tween 20 (Fig. 15D & 15E) were close to 40,000 fluorescence units. Besides having increased fluorescence intensity, cell counts were also increased for both samples treated with Tween 20, the resulting cell counts being close to those of SYBR Green stained samples (Fig. 15A). From this result, it seemed that Tween 20 had a significant effect on both fluorescence intensity and cell counts than HCl. When samples were treated with HCl only (Fig. 15C), there was decrease in both fluorescence intensity and cell count, but in combination with Tween (Fig. 15E) there was a slight increase in minimum and average fluorescence intensities. Therefore, it was decided to include both HCl and Tween 20 treatments in the TSA-FISH method for *L. pneumophila*.

Since the additional steps in the TSA-FISH method resulted in increased fluorescence intensity and cell counts of *E. coli*, the method was tested on *L. pneumophila*. The resulting fluorescence intensity was similar to that of cells treated with

chloramphenicol pre-incubation (Fig. 13B-E), although cells fluoresced with uneven distribution of fluorescence throughout the cells, which would make microscopic counting and possibly SPLC more problematic

Effects of Dextran Sulfate

Since several additional steps described above effectively increased the fluorescence intensity and cell counts of *E. coli* but not *L. pneumophila*, it was decided to continue applying those additional steps with *L. pneumophila*, because these treatments did not decrease fluorescence intensity or cell counts. Further treatments that might further increase the fluorescence intensity of TSA-FISH probed *L. pneumophila* cells were investigated. A recently published method used to quantify *L. pneumophila* employing a CARD-FISH procedure (Kirschner et al., 2012) was considered. The differences between Kirschner's method and the method used previously in this study were the reagents used in the permeabilization, hybridization and amplification steps. In addition, dextran sulfate was used in the hybridization and amplification steps because dextran sulfate is able to accelerate hybridization of probe to target site (Wahl, Stern & Stark, 1979) and improve localization of fluorescent tyramide in CARD-FISH (van Gijlswijk, Wiegant, Raap & Tanke, 1996). The effect of dextran sulfate was examined by adding 1mg/ul of dextran sulfate (Kirschner et al., 2012) to the hybridization buffer and tyramide signal amplification solution.

When the addition of dextran sulfate was first introduced to the hybridization step, the results (Fig. 17B and 18C), showed that dextran sulfate was able to increase both

fluorescence intensity and cell counts of TSA-FISH probed *L. pneumophila*. Since Kirschner's method (2012) also called for dextran sulfate in the amplification step, another experiment was set up to find out if there was any difference between samples that had dextran sulfate in the hybridization step and dextran sulfate in both hybridization and amplification steps. It was observed that samples with dextran sulfate in both hybridization and amplification steps (Fig. 19B) gave slightly higher fluorescence intensity than those that had dextran sulfate only in the hybridization step (Fig. 19C). After the introduction of dextran sulfate, it could be observed that the distribution of fluorescence found in the cells was more even (Fig. 17B-C), as uneven distribution of fluorescence was observed (Fig. 13C-F) before dextran sulfate was introduced into the TSA-FISH procedure.

Effects of Helper Probes

Another approach that might increase fluorescence intensity is using unlabeled helper oligonucleotide probes to increase accessibility of TSA-FISH probe binding site (Fuchs, Glöckner, Wulf & Amann, 2000). Fuchs reported that unlabeled oligonucleotides or helper probes that target sequences adjacent to the TSA-FISH probe target site were able to increase accessibility of TSA-FISH probe to the target sequence. Increase in accessibility to target sequence was made possible when helper probes hybridize to sequences adjacent to the target sequence, opening up a space for the TSA-FISH probe to gain easy access to target sequence for hybridization. Helper probes for *L. pneumophila* were designed according to the Fuchs, Glöckner, Wulf & Amann (2000) and incorporated

into the TSA-FISH method developed in this study thus far, according to the methods reported by Baudart Lebaron (2010). There was slight increase in fluorescence intensity in samples with helper probes (Fig. 20B) compared to that of samples without helper probes (Fig. 20C). Since there was a small increase in intensity, addition of helper probes was included as another modification to the method reported by Baudart (2002). Helper probes are relatively inexpensive and easily incorporated into the TSA-FISH reagents.

Generating a *L. pneumophila* Specific ScanRDI Application

With the modified Baudart method (2002) that included chloramphenicol pre-incubation, HCl incubation, Tween 20 incubation, addition of helper probes and addition of dextran sulfate to hybridization and amplification steps being able to produce TSA-FISH probed *L. pneumophila* cells that fluoresce with sufficient intensity, filtered *L. pneumophila* were probed with this optimized TSA-FISH method for detection and enumeration by the ScanRDI system. These samples were used for setting up an application, i.e. adjusting discriminants in the ScanRDI system to specifically detect and enumerate TSA-FISH probed *L. pneumophila* cells. In order to do that, the default total viable count (TVC) application (Riepl et al., 2011) on the system was used to detect and enumerate the number of cells found on the filters, but since the TSA-FISH probed *L. pneumophila* cells had lower fluorescence intensity than what was detectable by the default TVC application (Fig. 5A & 6), the threshold for fluorescence intensity detection was increased for the default application to be able to detect the *L. pneumophila* cells.

Membrane filters with TSA-FISH probed *L. pneumophila* were repeatedly scanned along with the increasing thresholds of the default application until the majority or all of the cells on the membrane filters were detected, enumerated and verified as positive *L. pneumophila* cells. When that was achieved, a large library of data listing the fluorescence intensity profiles of positive and negative fluorescence events were collected and analyzed to generate a new application (Fig. 5B & 7) specific for the detection and enumeration of TSA-FISH probed *L. pneumophila* cells. The minimum and maximum peak intensities of the default application (Fig. 6) and *L. pneumophila* specific application (Fig. 7) indicate that the overall fluorescence intensity of TSA-FISH probed *L. pneumophila* was much lower, which was reflective of what was seen in the fluorescence intensity results when comparing TSA-FISH probed samples to SYBR Green stained samples.

Detection and Enumeration by ScanRDI

With the development of the TSA-FISH probed *L. pneumophila* specific application (LEGAPP), the detection and enumeration capability of the ScanRDI was tested against spread plate counts. Using the LEGAPP to detect *L. pneumophila* cells on membrane filters, the average result of three experiments was 89.1% of spread plate counts. Besides testing the effectiveness of the TSA-FISH method on *L. pneumophila*, effectiveness of the method on *E. coli* and *A. hydrophila* was also examined. The results for *E. coli* and *A. hydrophila* were obtained using an application optimized in another study (Broadaway, 2013) for simultaneous detection and enumeration of *E. coli* and

Cryptosporidium. Using that application, detection of *E. coli* and *A. hydrophila* was 79.5% and 39.7% of spread plate counts respectively. The detection rate for *E. coli* and *L. pneumophila* were relatively high compared to that for *A. hydrophila*, which is probably due to the specificity of the procedures and *ScanRDI* applications, since no specific application had been optimized for detection and enumeration of *A. hydrophila*, and the TSA-FISH procedures had not been optimized for that organism. It is possible that if a procedure and application specific for *A. hydrophila* were optimized, the detection and enumeration rate could be increased to levels comparable with those for *E. coli* and *L. pneumophila*.

CHAPTER 5

CONCLUSIONS

L. pneumophila TSA-FISH and ScanRDI

In this study, it has been demonstrated that it is possible to detect and enumerate the causative agent of Legionnaires' disease, *L. pneumophila*, within an experimental environment using an automated detection and enumeration SPLC system (ScanRDI). The TSA-FISH procedure developed to probe *L. pneumophila* in this study was based upon previous method reported by Baudart and Lebaron (2010) with additional treatments such as antibiotic preincubation, HCl incubation, Tween 20 PBS pH 7.6 permeabilization, introduction of dextran sulfate to hybridization buffer and TSA reagents and introducing the use of helper oligonucleotides to increase the fluorescence intensity of probed *L. pneumophila* cells.

The detection and enumeration method described in this study was able to provide confirmative results for the presence of *L. pneumophila* spiked into clean water samples with about 90% of cells detectable and verified through the TSA-FISH method and ScanRDI system compared to plate counts. This compares favorably to an average percentage of a variety of bacterial cells reported by Bouvier & Del Giorgio 2003 were 56% .Using this method, results for the presence and enumeration of *L. pneumophila* in spiked clean water samples were obtainable within 10 hours, which is a significant improvement over the time needed for detection and confirmation of *L. pneumophila*

when compared to current culture procedures which take at least 3 days to obtain preliminary results with culture methods.

The TSA-FISH detection method could be applied to environmental samples for detection and enumeration of *L. pneumophila*, and this would be a significant improvement over existing methods as the need for environmental samples to be incubated overnight or longer in enrichment medium to increase cell numbers before detection and enumeration can be eliminated, which would ultimately reduce the detection time. With the shorter time needed to confirm presence of *L. pneumophila* during an outbreak, it may be possible to help control an outbreak within the shortest time possible to prevent health risks to the public.

TSA-FISH and ScanRDI

In this study, the method developed was effective for probing *L. pneumophila* and *E. coli*; this aspect of being able to use a single method to probe multiple organisms is one of the key targets of this research. Since the optimization of the method developed in this study was carried out in parallel for both *L. pneumophila* and *E. coli*, it was not a surprise that detection of *A. hydrophila* cells with ScanRDI was not as effective. This suggested that in order to better detect *A. hydrophila*, the TSA-FISH method may have to be optimized with the three different microorganisms in parallel optimization experiments. Besides the optimization of the developed method, the ScanRDI settings may have to be also adjusted to specifically detect *A. hydrophila* alone or all three species of microorganisms.

Use of ScanRDI settings adjusted to detect multiple species of microorganisms on a membrane filter was proved to be effective in another study (Broadaway, 2013).

Probing of both *E. coli* and *Cryptosporidium* on a membrane filter was possible when both microorganisms were used together for the TSA-FISH method optimization process and adjusting the ScanRDI settings.

With the pattern observed in this study and the other study (Broadaway, 2013), the results suggests that multiple species of microorganisms, including *L. pneumophila*, *E. coli*, *A. hydrophila*, *Cryptosporidium*, *Giardia* and *Helicobacter pylori*, could possibly be detected together on a single membrane filter if the TSA-FISH method is further optimized and evaluated to accommodate the different characteristics of each microorganisms involved. If it is not possible to detect all of them on a using a single optimized TSA-FISH method on a single membrane filter with a procedure to detect them all, then several optimized TSA-FISH methods may be needed in parallel, using more than one membrane filter for optimal detection of multiple species of microorganisms in a single sample. Whether or not it is possible for probing multiple species of microorganisms on a single membrane filter, routine monitoring or emergency detection could be done in a relatively short time and precautionary steps can be taken quickly to prevent further dissemination of several harmful microbial pathogens.

REFERENCES CITED

- Abu Kwaik, Y., Gao, L. Y., Harb, O.S., & Stone, B.J. (1997). Transcriptional regulation of the macrophage-induced gene (gspA) of *Legionella pneumophila* and phenotypic characterization of a null mutant. *Molecular Microbiology*, 24(3), 629-642.
- Abu Kwaik, Y., Gao, L.Y., Stone, B.J., Venkataraman, C., & Harb, O.S. (1998). Invasion of Protozoa by *Legionella pneumophila* and Its Role in Bacterial Ecology and Pathogenesis. *Applied and Environmental Microbiology*, 64(9), 3127-3133.
- AES Chemunex. (2011). ABRASP Meeting 2011: Ultra-rapid Microbiology using ChemScan® RDI & Regulatory Requirements for Validation of the RMM. Visited May 16 2013.
<http://www.abrasp.org.br/downloads/2011/APRESENTA%C3%87%C3%83O%20bIOMERIEUX%20.pdf>
- Akerlund, T., Nordström, K., & Bernander, R. (1995). Analysis of cell size and DNA content in exponentially growing and stationary-phase batch cultures of *Escherichia coli*. *Journal of Bacteriology*, 177(23), 6791-6797.
- Amann, R. I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., & Stahl, D.A. (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Applied and Environmental Microbiology*, 56(6), 1919-1925.
- Amann, R. (1995). *In situ* identification of micro-organisms by whole cell hybridization with rRNA-targeted nucleic acid probes. *Molecular Microbial Ecology Manual*. (A.L. Akkermans, J. Elsas, & F. Bruijn, Ed.). Netherlands: Springer
- Amann, R., Fuchs, B.M., & Behrens, S. (2001). The identification of microorganisms by fluorescence *in situ* hybridisation. *Current Opinion in Biotechnology*, 12(3), 231-236.
- Amann, R. & Fuchs, B.M. (2008). Single-cell identification in microbial communities by improved fluorescence *in situ* hybridization techniques. *Nature Reviews Microbiology*, 6(5), 339-348.
- Amano, K. & Williams, J.C. (1983). Peptidoglycan of *Legionella pneumophila*: apparent resistance to lysozyme hydrolysis correlates with a high degree of peptide cross-linking. *Journal of Bacteriology*, 153(1), 520-526.
- Aurell, H., Catala, P., Farge, P., Wallet, F., Brun, M.L., Helbig, J.H., . . . Lebaron, P. (2004). Rapid Detection and Enumeration of *Legionella pneumophila* in Hot Water

Systems by Solid-Phase Cytometry. *Applied and Environmental Microbiology*, 70(3), 1651-1657.

Bargellini, A., Marchesi, I., Righi, E., Ferrari, A., Cencetti, S., Borella, P., & Rovesti, S. (2011). Parameters predictive of *Legionella* contamination in hot water systems: association with trace elements and heterotrophic plate counts. *Water Research*, 45(6), 2315-2321.

Barker, J., Brown, M.R., Collier, P.J., Farrell, I., & Gilbert, P. (1992). Relationship between *Legionella pneumophila* and *Acanthamoeba polyphaga*: physiological status and susceptibility to chemical inactivation. *Applied and Environmental Microbiology*, 58(8), 2420-2425.

Baudart, J. & Lebaron, P. (2010). Rapid detection of *Escherichia coli* in waters using fluorescent *in situ* hybridization, direct viable counting and solid phase cytometry. *Journal of Applied Microbiology*, 109(4), 1253-1264.

Bej, A.K., Steffan, R.J., DiCesare, J., Haff, L., & Atlas, R.M. (1990). Detection of coliform bacteria in water by polymerase chain reaction and gene probes. *Applied and Environmental Microbiology*, 56(2), 307-314.

Berk, S.G., Ting, R.S., Turner, G.W., & Ashburn, R.J. (1998). Production of Respirable Vesicles Containing Live *Legionella pneumophila* Cells by Two *Acanthamoeba* spp. *Applied and Environmental Microbiology*, 64(1), 279-286.

Berrington, W.R., & Hawn, T.R. (2013). Human Susceptibility to Legionnaires' Disease. In *Legionella. Methods in Molecular Biology*, 954, 541-551.

Berry, D., Xi, C., & Raskin, L. (2006). Microbial ecology of drinking water distribution systems. *Current Opinion in Biotechnology*, 17(3), 297-302.

Boulos, L., Prévost, M., Barbeau, B., Coallier, J., & Desjardins, R. (1999). LIVE/DEAD® BacLight™: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. *Journal of Microbiological Methods*, 37(1), 77-86.

Bouvier, T. & Del Giorgio, P.A. (2003). Factors influencing the detection of bacterial cells using fluorescence *in situ* hybridization (FISH): A quantitative review of published reports. *FEMS Microbiology Ecology*, 44(1), 3-15.

Broadaway, S.C, 2013. Detection of *Cryptosporidium* and *E. coli* Using Fluorescent *In situ* Hybridization and Solid Phase Cytometry (Master's Thesis).

Broadaway, S.C., Barton, S.A., & Pyle, B.H. (2003). Rapid Staining and Enumeration of Small Numbers of Total Bacteria in Water by Solid-Phase Laser Cytometry. *Applied and Environmental Microbiology*, 69(7), 4272-4273.

Buchbinder, S., Trebesius, K., & Heesemann, J. (2002). Evaluation of detection of *Legionella* spp. in water samples by fluorescence *in situ* hybridization, PCR amplification and bacterial culture. *International Journal of Medical Microbiology*, 292(3-4), 241-245.

Cazalet, C., Rusniok, C., Brüggemann, H., Zidane, N., Magnier, A., Ma, L., . . . Buchrieser, C. (2004). Evidence in the *Legionella pneumophila* genome for exploitation of host cell functions and high genome plasticity. *Nature genetics*, 36(11), 1165-1173.

Centers for Disease Control and Prevention. (2011). Morbidity and Mortality Weekly Report (MMWR) – Legionellosis – United states, 2000 – 2009. Visited May 25 2013. <http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6032a3.htm>

Chao, J., DeBiasio, R., Zhu, Z., Giuliano, K.A., & Schmidt, B.F. (1996). Immunofluorescence signal amplification by the enzyme-catalyzed deposition of a fluorescent reporter substrate (CARD). *Cytometry*, 23(1), 48-53.

Chin, S. F., Daigo, Y., Huang, H.E., Iver, N.G., Callagy, G., Kranjac, T., . . . Caldas, C. (2003). A simple and reliable pretreatment protocol facilitates fluorescent *in situ* hybridisation on tissue microarrays of paraffin wax embedded tumour samples. *Molecular Pathology*, 56(5), 275-279.

Coonen, E., Dumoulin, J.C.M., Ramaekers, F.C., & Hopman, A.H. (1994). Optimal preparation of preimplantation embryo interphase nuclei for analysis by fluorescence *in situ* hybridization. *Human Reproduction*, 9(3), 533-537.

Declerck, P., Verelst, L., L. Duvivier, L., Van Damme, A., & Ollevier, F. (2003). A detection method for *Legionella* spp in (cooling) water: fluorescent *in situ* hybridisation (FISH) on whole bacteria. *Water Science and Technology*, 47(3), 143-146.

Edelstein, P.H. (1981). Improved semiselective medium for isolation of *Legionella pneumophila* from contaminated clinical and environmental specimens. *Journal of Clinical Microbiology*, 14(3), 298-303.

Edwards, M.C. & Gibbs, R.A. (1994). Multiplex PCR: advantages, development, and applications. *Genome Research*, 3(4), S65-S75.

Elmund, G.K., Allen, M.J., & Rice, E.W. (1999). Comparison of *Escherichia coli*, Total Coliform, and Fecal Coliform Populations as Indicators of Wastewater Treatment Efficiency. *Water Environment Research*, 71(3), 332-339.

Fields, B. (1993). *Legionella and protozoa: interaction of a pathogen and its natural host. Legionella: current status and emerging perspectives*. (J.M. Barbaree, R.F. Breiman, & A.P. Durfour, Ed.). Washington, DC: American Society for Microbiology.

Fraser, D.W., Tsai, T.R., Orenstein, W., Parkin, W.E., Beecham, H.J., Sharrar, R.G., . . . Brachman, P.S. (1977). Legionnaires' disease: description of an epidemic of pneumonia. *The New England Journal of Medicine*, 297(22), 1189-1197.

Fuchs, B.M., Glöckner, F.O., Wulf, J., & Amann, R. (2000). Unlabeled Helper Oligonucleotides Increase the *In Situ* Accessibility to 16S rRNA of Fluorescently Labeled Oligonucleotide Probes. *Applied and Environmental Microbiology*, 66(8), 3603-3607.

Gleick, P.H. (1998). Water In Crisis: Paths To Sustainable Water Use. *Ecological Applications*, 8(3), 571-579.

Geisenberger, O., Ammendola, A., Christensen, B.B., Molin, S., Schleifer, K.H., & Eberl, L. (1999). Monitoring the conjugal transfer of plasmid RP4 in activated sludge and *in situ* identification of the transconjugants. *FEMS Microbiology Letters*, 174(1), 9-17.

Grimm, D., Merkert, H., Ludwig, W., Schleifer, K.H., Hacker, J., & Brand, B.C. (1998). Specific Detection of *Legionella pneumophila*: Construction of a New 16S rRNA-Targeted Oligonucleotide Probe. *Applied and Environmental Microbiology*, 64(7), 2686-2690.

Herzenberg, L.A., Parks, D., Sahaf, D., Perez, O., Roederer, M., & Herzenberg, L.A. (2002). The History and Future of the Fluorescence Activated Cell Sorter and Flow Cytometry: A View from Stanford. *Clinical Chemistry*, 48(10), 1819-1827.

Hindahl, M.S. & Iglewski, B.H. (1984). Isolation and characterization of the *Legionella pneumophila* outer membrane. *Journal of Bacteriology*, 159(1), 107-113.

Horsburgh Jr, C.R. (1991). Mycobacterium avium complex infection in the acquired immunodeficiency syndrome. *New England Journal of Medicine*, 324(19), 1332-1338.

Huang, S.W. & Hsu, B.M. (2010). Survey of Naegleria and its resisting bacteria-Legionella in hot spring water of Taiwan using molecular method. *The journal Parasitology Research*, 106(6), 1395-1402.

Hussong, D., Colwell, R.R., O'brien, M., Weiss, E., Pearson, A.D., Weiner, R.M., & Burge, W.D. (1987). Viable *Legionella pneumophila* not detectable by culture on agar media. *Nature Biotechnology*, 5, 947 – 950

Josephson, K.L., Pillai, S.D., Way, J., Gerba, C.P. & Pepper, I.L. (1991). Fecal Coliforms in Soil Detected by Polymerase Chain Reaction and DNA-DNA Hybridizations. *Soil Science Society of America Journal*, 55(5), 1326-1332.

Kaufmann, A. F., McDade, J. E., Patton, C.M., Bennett, J.V., Skaliy, P., Feeley, J.C., . . . Brachman, P.S. (1981). Pontiac Fever: Isolation of the Etiologic Agent (*Legionella pneumophila*) And Demonstration Of Its Mode Of Transmission. *American Journal of Epidemiology*, 114(3), 337-347.

Kirschner, A.K., Rameder, A., Schrammel, B., Indra, A., Farnleitner, A.H., & Sommer, R. (2012). Development of a new CARD-FISH protocol for quantification of *Legionella pneumophila* and its application in two hospital cooling towers. *Journal of Applied Microbiology*, 112(6), 1244-1256.

Kjellerup, B.V., Veeh, R.H., Sumithraratne, P., Thomsen, T.R., Buckingham-Meyer, K., Frønd, B., & Sturman, P. (2005). Monitoring of microbial souring in chemically treated, produced-water biofilm systems using molecular techniques. *Journal of Industrial Microbiology and Biotechnology*, 32(4), 163-170.

Lemarchand, K., Parthuisot, N., Catala, P., & Lebaron, P. (2001). Comparative assessment of epifluorescence microscopy, flow cytometry and solid-phase cytometry used in the enumeration of specific bacteria in water. *Aquatic Microbial Ecology*, 25(3), 301-309.

Lisle, J.T., Hamilton, M.A., Willse, A.R., & McFeters, G.A. (2004). Comparison of Fluorescence Microscopy and Solid-Phase Cytometry Methods for Counting Bacteria in Water. *Applied and Environmental Microbiology*, 70(9), 5343-5348.

Litt, G. J., & Bobrow, M. N. (2002). Tyramide Signal Amplification. In *Rapid Detection of Infectious Agents* (pp. 159-173). US: Springer

Long, F., Zhu, X.N., Zhang, Z.M., & Shi, X.M. (2008). Development of a quantitative polymerase chain reaction method using a live bacterium as internal control for the detection of *Listeria monocytogenes*. *Diagnostic Microbiology and Infectious Disease*, 62(4), 374-381.

Malorny, B., Tassios, P.T., Rådström, P., Cook, N., Wagner, M., & Hoorfar, J. (2003). Standardization of diagnostic PCR for the detection of foodborne pathogens. *International Journal of Food Microbiology*, 83(1), 39-48.

Manz, W., Amann, R., Ludwig, W., Wagner, M., & Schleifer, K.H. (1992). Phylogenetic Oligodeoxynucleotide Probes for the Major Subclasses of Proteobacteria: Problems and Solutions. *Systematic and Applied Microbiology*, 15(4), 593-600.

- Manz, W., Amann, R., Szewzyk, R., Szewzyk, U., Stenström, T.A., Hutzler, P., & Schleifer, K.H. (1995). *In situ* identification of Legionellaceae using 16S rRNA-targeted oligonucleotide probes and confocal laser scanning microscopy. *Microbiology*, 141(1), 29-39.
- McDade, J.E., Shepard, C.C., Fraser, D.W., Tsai, T.R., Redus, M.A., & Dowdle, W.R. (1977). Legionnaires' disease: isolation of a bacterium and demonstration of its role in other respiratory disease. *The New England Journal of Medicine*, 297(22), 1197-1203.
- McKay, A.M. (1992). Viable but non-culturable forms of potentially pathogenic bacteria in water. *Letters in Applied Microbiology*, 14(4), 129-135.
- Mighell, A.J., Hume, W.J., & Robinson, P.A. (1998). An overview of the complexities and subtleties of immunohistochemistry. *Oral Diseases*, 4(3), 217-223.
- Mignon-Godefroy, K., Guillet, J.G., & Butor C. (1997). Solid phase cytometry for detection of rare events. *Cytometry*, 27(4), 336-344.
- Moffie, B.G. & Mouton, R.P. (1988). Sensitivity and resistance of Legionella pneumophila to some antibiotics and combinations of antibiotics. *Journal of Antimicrobial Chemotherapy*, 22(4), 457-462.
- Muder, R.R. & Victor L.Y. (2002). Infection Due to Legionella Species Other Than L. pneumophila. *Clinical Infectious Diseases*, 35(8), 990-998.
- Noble, R.T. & Fuhrman, J.A. (1998). Use of SYBR Green I for rapid epifluorescence counts of marine viruses and bacteria. *Aquatic Microbial Ecology*, 14(2), 113-118.
- Nunez, R. (2001). Flow cytometry: principles and instrumentation. *Current Issues in Molecular Biology*, 3, 39-45.
- O'Brien, M.C. & Bolton, W.E. (1995). Comparison of cell viability probes compatible with fixation and permeabilization for combined surface and intracellular staining in flow cytometry. *Cytometry*, 19(3), 243-255.
- Ouverney, C.C. & Fuhrman J.A. (1997). Increase in Fluorescence Intensity of 16S rRNA *In situ* Hybridization in Natural Samples Treated with Chloramphenicol. *Applied and Environmental Microbiology*, 63(7), 2735-2740.
- Pablos, M., Remacha, M.A., Rodríguez-Calleja, J.M., Santos, J.A., Otero, A., & García-López, M.L. (2010). Identity, virulence genes, and clonal relatedness of Aeromonas isolates from patients with diarrhea and drinking water. *European Journal of Clinical Microbiology & Infectious Diseases*, 29(9), 1163-1172.

Picard, C., Ponsonnet, C., Paget, E., Nesme, X., & Simonet, P. (1992). Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction. *Applied and Environmental Microbiology*, 58(9), 2717-2722.

Pierce, A.K., & Sanford, J.P. (1973). Bacterial contamination of aerosols. *Archives of Internal Medicine*, 131(1), 156-159.

Pyle, B.H., Broadaway, S.C., & McFeters, G.A. (1999). Sensitive Detection of *Escherichia coli* O157:H7 in Food and Water by Immunomagnetic Separation and Solid-Phase Laser Cytometry. *Applied and Environmental Microbiology*, 65(5), 1966-1972.

Radbruch, A. (2000). *Flow cytometry and cell sorting*. Springer Verlag.

Radcliff, G. & Jaroszeski, M. (1998). Basics of Flow Cytometry. *Methods in Molecular Biology*, 263, 1-24.

Regnault, B., Martin-Delautre, S., Lejay-Collin, M., Lefèvre, M., & Grimont, P.A. (2000). Oligonucleotide probe for the visualization of *Escherichia coli*/*Escherichia fergusonii* cells by *in situ* hybridization: specificity and potential applications. *Research in Microbiology*, 151(7), 521-533.

Riepl, M., Schauer, S., Knetsch, S., Holzhammer, E., Farnleitner, A.H., Sommer, R., & Kirschner, R.A. (2011). Applicability of solid-phase cytometry and epifluorescence microscopy for rapid assessment of the microbiological quality of dialysis water. *Nephrology Dialysis Transplantation*, 26(11), 3640-3645.

Roth, B.L., Poot, M., Yue, S.T., & Millard, P.J. (1997). Bacterial viability and antibiotic susceptibility testing with SYTOX green nucleic acid stain. *Applied and Environmental Microbiology*, 63(6), 2421-2431.

Rompré A., Servais, P., Baudart, J., de-Roubin, M.R., & Laurent, P. (2002). Detection and enumeration of coliforms in drinking water: current methods and emerging approaches. *Journal of Microbiological Methods*, 49(1), 31-54.

Rowbotham, T.J. (1980). Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. *Journal of Clinical Pathology*, 33, 1179-1183.

Schönhuber, W., Fuchs, B., Juretschko, S., & Amann, R. (1997). Improved sensitivity of whole-cell hybridization by the combination of horseradish peroxidase-labeled oligonucleotides and tyramide signal amplification. *Applied and Environmental Microbiology*, 63(8), 3268-3273.

- Sekar, R., Pernthaler, A., Pernthaler, J., Warnecke, F., Posch, T., & Amann, R. (2003). An Improved Protocol for Quantification of Freshwater Actinobacteria by Fluorescence *In Situ* Hybridization. *Applied and Environmental Microbiology*, 69(5), 2928-2935.
- Sokolova, E., Aström, J., Pettersson, T.J., Bergstedt, O., & Hermansson, M. (2012). Estimation of pathogen concentrations in a drinking water source using hydrodynamic modelling and microbial source tracking. *Journal of Water and Health*, 10(3), 358-370.
- Speel, E.J., Hopman, A.H., & Komminoth, P. (1999). Amplification Methods to Increase the Sensitivity of *In situ* Hybridization: Play CARD(S). *Journal of Histochemistry & Cytochemistry*, 47(3), 281-288.
- Speel, E.J.M., Hopman, A.H., & Komminoth, P. (2006). Tyramide Signal Amplification for DNA and mRNA *In situ* Hybridization. *Methods in Molecular Biology*, 326, 33-60.
- Steinert, M., Emödy, L., Amann, R., & Hacker, J. (1997). Resuscitation of viable but nonculturable *Legionella pneumophila* Philadelphia JR32 by *Acanthamoeba castellanii*. *Applied and Environmental Microbiology*, 63(5), 2047-2053.
- Sternberger, L.A., Hardy, P.H., Cuculis, J.J., & Meyer, H.G. (1970). The unlabeled antibody enzyme method of immunohistochemistry preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes. *Journal of Histochemistry & Cytochemistry*, 18(5), 315-333.
- Stout, J.E., Yu, V.L., & Best, M.G. (1985). Ecology of *Legionella pneumophila* within water distribution systems. *Applied and Environmental Microbiology*, 49(1), 221-228.
- Tallon, P., Magajna, B., Lofranco, C., & Leung, K.T. (2005). Microbial Indicators of Faecal Contamination in Water: A Current Perspective. *Water, Air, and Soil Pollution*, 166(1-4), 139-166.
- Tarr, P.I. (1995). *Escherichia coli* 0157: H7: clinical, diagnostic, and epidemiological aspects of human infection. *Clinical Infectious Diseases*. 20(1), 1-10.
- United States Department of Labor. (1999). OSHA Technical Manual (OTM) Section III: Chapter 7 Legionnaires' Disease. Visited May 17 2012
http://www.osha.gov/dts/osta/otm/otm_iii/otm_iii_7.html
- United States Environmental Protection Agency. (n.d.). Water Trivia Facts. Visited February 10 2013. http://water.epa.gov/learn/kids/drinkingwater/water_trivia_facts.cfm
- United States Environmental Protection Agency. 2000. *Legionella*: Drinking Water Fact Sheet. Visited February 10 2013.

http://water.epa.gov/action/advisories/drinking/upload/2009_02_03_criteria_humanhealth_microbial_legionellafs.pdf

United States Environmental Protection Agency. (2009). National Primary Drinking Water Regulations. Visited February 10 2013.
<http://water.epa.gov/drink/contaminants/upload/mcl-2.pdf>

United States Environmental Protection Agency. (2012). Contaminant Candidate List 3 – CCL: Microbial Contaminants. Visited February 10 2013.
<http://water.epa.gov/scitech/drinkingwater/dws/ccl/ccl3.cfm#microbial>

Vaerewijck, M.J., Huys, G., Palomino, J.C., Swings, J., & Portales, F. (2005). Mycobacteria in drinking water distribution systems: ecology and significance for human health. *FEMS Microbiology Reviews*, 29(5), 911-934.

Vale, F.F., & V for, J.M. (2010). Transmission pathway of *Helicobacter pylori*: Does food play a role in rural and urban areas?. *International journal of food microbiology*, 138(1-2), 1-12.

van Gijlswijk, R.P., Wiegant, J., Raap, A.K., & Tanke, H.J. (1996). Improved localization of fluorescent tyramides for fluorescence *in situ* hybridization using dextran sulfate and polyvinyl alcohol. *Journal of Histochemistry & Cytochemistry*, 44(4), 389-392.

Van Poucke, S.O. & Nelis, H. J. (2000). Rapid detection of fluorescent and chemiluminescent total coliforms and *Escherichia coli* on membrane filters. *Journal of Microbiological Methods*, 42(3), 233-244.

von Reyn, C.F., Marlow, J.N., Barber, T.W., Falkinham, J.O., & Arbeit, R.D. (1994). Persistent colonisation of potable water as a source of *Mycobacterium avium* infection in AIDS. *The Lancet*, 343(8906), 1137-1141.

Wahl, G.M., Stern, M., & Stark, G.R. (1979). Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. *Proceedings of the National Academy of Sciences*, 76(8), 3683-3687.

World Health Organization. (2007). *Legionella* and the Prevention of Legionellosis. Visited May 17 2013.
http://www.who.int/water_sanitation_health/emerging/legionella.pdf

World Health Organization. (2013). Water Sanitation and Health: Health through safe drinking water and basic sanitation. Visited May 17 2013.
http://www.who.int/water_sanitation_health/mdg1/en/

World Health Organization. (n.d.). *Legionella*. Visited June 17 2013.
http://www.who.int/water_sanitation_health/dwq/admicrob4.pdf

Yeh, T.Y., Godshalk, J.R., Olson, G.J., & Kelly, R.M. (1987). Use of epifluorescence microscopy for characterizing the activity of *Thiobacillus Ferrooxidans* on iron pyrite. *Biotechnology and Bioengineering* ,30(1), 138-146.