PCR Detection and Assessment of Viability Methods for the Detection of *Legionella* species in Potable Water Supplies

Project No: WQI-699528-95

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Executive Summary

Legionella are Gram-negative bacteria that are ubiquitous in the aquatic environment. These bacteria are responsible for causing legionellosis, which can present in one of two clinical manifestations, Legionnaires' Disease in which there is a primary pneumonia and a high mortality rate or Pontiac Fever, a milder self-limiting form of the disease. This organism has been referred to as the "miserable invisible", because the disease is difficult to detect in patients and the bacteria presents detection challenges in environmental samples as well. Although, culture has been the "gold standard" for detection, there have been documented outbreaks were environmental culture results were uniformly negative during testing that followed the outbreak. In these cases, it was only through the use of direct detection methods testing employing the Polymerase Chain Reaction (PCR) or DFA on environmental samples and examination of patient data that the causative agent was identified as *Legionella*. It has been postulated that those *Legionella* cells may have entered a viable but non-culturable (VBNC) state due to unfavorable environmental conditions, such as pH, temperature, presence of disinfectants or a combination of pressures.

Direct pathogen detection methods such as PCR and immunofluorescent staining are rapid and sensitive, however these methods are unable to determine the viability of the cells detected. Currently the primary method to determine culturability of *Legionella* cells isolated from environmental samples is through culturing on artificial media, a laborious process that often requires weeks for confirmation.

It has been demonstrated that *Legionella* cells can enter a viable but non-culturable state and remain infectious. For the detection of VBNC cells, direct detection methods must be used in conjunction with vital stains which target cellular physiology. A primary aim of the research conducted in this study was to develop a method that combined immunofluorescent staining with simultaneous vital staining, in conjunction with culture and molecular testing. Once such a method was developed, it would be possible to apply it to environmental samples to determine the frequency of viable *Legionella* cells throughout two potable water distribution systems that employed different disinfectants, chlorination, chloramination and ozonation.

In this study, twenty San Francisco Bay Area municipal water system sampling sites were tested for the presence of *Legionella* species employing the PCR, direct fluorescent antibody staining (DFA) with simultaneous viability staining and culture on buffered charcoal yeast extract agar amended with α-ketoglutarate, glysine and antibiotics, vancomycin, polymyxin B and cycloheximide (BCYE-αGVPC). Samples were analyzed by PCR using the Perkin-Elmer EnviroAmp® Legionella Kit. DFA staining was performed on concentrated samples employing a polyclonal antibody specific for *L. pneumophila* serogroups 1-7 and 6 additional *Legionella*

species. Of the water samples studied 83% were positive for *Legionella* species by the PCR method and 83% were DFA positive, however in six samples, PCR and DFA results did not correlate. Approximately 13% of the *Legionella* positive samples were identified as *L. pneumophila* by PCR. PCR results correlated to DFA staining results whereas none of the *Legionella* species detected in the water samples were culturable on either selective or non-selective BCYE-α media.

Initially several different viability stains were evaluated for their performance in conjunction with DFA staining for *Legionella*, these included AODC and the tetrazolium salts; INT, CTC, XTT, and MTT. Viability staining methods were evaluated on several *Legionella* strains, including two environmental *L. pneumophila* strains seeded into tap water microcosms for a three month period. Cell numbers decreased one order of magnitude within the first week and stabilized thereafter. INT and CTC viability staining was performed in conjunction with plate counts to determine the decrease in viability/culturability over time.

Viability of all heterotrophic plate count (HPC) bacteria and *Legionella* was monitored in municipal waters by simultaneous AODC-CTC or DFA-CTC (5-cyano-2,3-ditolyl tetrazolium chloride), staining respectively. CTC staining yielded superior results over the other tetrazolium salts and was the easiest to visualize microscopically over a variety of sample matrices. Interestingly 23% of the *Legionella* samples positive by DFA-CTC staining were viable, 13% at a concentration of greater than 100 cells/ml and 7% at concentrations less than 100 cells/ml. This data supports the potential for sporadic cases of legionellosis which are observed globally. The observation that none of the 86 samples yielded culturable *Legionella* and that only 23% of the samples detected by direct methods were indeed viable, helps to explain the sporadic nature of legionellosis outbreaks.

Introduction

By far the most famous outbreak of Legionnaires' Disease occurred at the American Legion convention in Philadelphia, Pennsylvania during the summer of 1976. Of the 2,500 participants, 221 became infected and 34 died (Fraser et al. 1977). The source of the outbreak was believed to be the air conditioning system. A major delay in uncovering the agent of this outbreak was the lack of microbiological media to grow this newly recognized pathogen. Initial identification was made almost a year later, using immunological staining, followed by screening many different media combinations until one was identified that could support the growth of *Legionella*. Today there are currently more than 34 species of *Legionella* and over 50 serogroups. To date *L. pneumophila* reportedly accounts for close to 85% of the legionellosis cases reported to the Centers for Disease Control and Prevention (CDC). The natural habitat for *Legionella* spp. is the aquatic environment. *Legionella* spp. have been isolated from lakes, streams, ponds, and rivers, although outbreaks are rarely traced to these sources. Man-made aquatic habitats often provide a means of amplification and dissemination of the bacteria, enabling human infection to occur. Man-made reservoirs include humidifiers, hot tubs, cooling towers, showerheads, dental turbines, vegetable misting machines, and any other device which can hold water for prolonged periods of time and can generate an aerosol.

Goals of this Research

- Develop a rapid staining method that can supplement culture and be combined with direct fluorescent antibody staining and PCR.
- Determine the viability status of *Legionella* cells detected by molecular methods and immunofluorescent staining methods.
- Apply the viability staining method to environmental samples collected from several environmental sites throughout the distribution systems of local utilities.

For years, methodologies and technologies that have gained widespread acceptance in the field of clinical diagnostics have managed to find their way into environmental applications, molecular methods are no exception. The benefits of rapid, specific and sensitive detection of pathogens in clinical specimens by molecular methods have not gone unnoticed and have been modified to examine environmental samples. Conventional microbiological detection methods have relied heavily on culture employing a diverse range of microbiological media. Over the years it has become more and more apparent that many microorganisms are fastidious in their nutritional and growth requirements. This is particularly true for the *Legionella* species, although they are frequently detected by direct fluorescent antibody staining and PCR, culture results do not correlate well with the direct detection data, either PCR or DFA (Hussong et al. 1987, Paszko-Kolva et al. 1991, Palmer et al. 1993, Yamamoto et al. 1993). In addition to the poor culture recovery rate of *Legionella*, the typical time required to observe colonies on selective media is

approximately 3-7 days. Because legionellae are human pathogens, it is important that when an outbreak occurs or high concentrations are detected that remediation measures be initiated immediately. Waiting a week or longer for culture results is not acceptable under these circumstances. Molecular methods such as PCR can offer results within one day and have been used routinely by the CDC in suspected *Legionella* outbreaks.

There have been several instances during which culture techniques have failed to uncover *Legionella* species as the source of an outbreak (Miller et al., 1993 et al., CDC Public Hearing, 1994). In those instances, it was only through the application PCR and/or DFA that *Legionella* was identified as the causative agent of the outbreak. PCR avoids extensive incubation periods associated with culture, is specific and very sensitive. Although both PCR and DFA techniques are well suited for the rapid detection of *Legionella* species in environmental samples, neither method can determine culturability or viability. To measure culturability, concentrated samples are plated on artificial media, typically Buffered Charcoal Yeast Extract Agar (BCYEα). To measure bacterial viability a number of vital dyes have been utilized simultaneously with immunofluorescent antibody stains.

There are many published reports demonstrating that certain Gram-negative bacteria isolated from environmental and clinical samples are able to enter a viable but non-culturable (VBNC) state. These bacteria may undergo physiological and morphological changes prior to entering a dormant state. Dormancy is defined as a rest period of reversible interruption in the phenotypic development of a bacterium. These bacteria may remain viable under adverse conditions. *Legionella pneumophila* has been reported to enter the VBNC state in tap water and in drinking water, accounting for the difficulty and frequent failure in culturing the bacterium from these sources. Published data indicated that VBNC legionellae are lethal to chicken embryos (Hussong et al., 1987). It is important therefore to determine the viability of legionellae from those positive water samples which do not produce visible colonies on laboratory culture media. There are a number of methods for detecting and enumerating these bacteria including radiolabeled substrate uptake, detection of respiratory activity and differential staining. The enumeration of VBNC by some tetrazolium salts like INT, CTC is one method for the evaluation of legionellae cell viability determinations in water samples. The INT and CTC experiments were therefore used to enumerate the viability of the bacteria in the environmental samples with simultaneous DFA staining for *Legionella*.

Description of Water Sources Examined in this Study

Alameda County Water District (ACWD)

The Alameda County Water District serves the cities of Union City, Newark, and Fremont in the southwest corner of Alameda County. The service population in June 1994 consisted of 281,700 persons being served through a total of 70,230 service connections, with an average daily consumption of 41 mgd (million gallons

per day). The demands are divided into 70% residential, 13% industrial, 11% commercial, and 6% miscellaneous.

The District utilizes a variety of water sources to meet demands. Two well fields are maintained to produce about 40% of the District's water. The balance of production to meet demands is provided by the other surface water sources and treatment plants. About 20% of the demand is met by water purchased from the San Francisco Water Department (SFWD) with the remaining 40% coming from the California State Water Project.

State Water Project. ACWD receives its allotment from the State Water Project's California Aqueduct through the South Bay Aqueduct which takes water from the California Aqueduct after the Delta pumping plant. Both Water Treatment Plant 2 (WTP2) and Mission San Jose Water Treatment Plant (MSJWTP) use full conventional treatment technology to treat this water supply.

Groundwater. The Alameda County Water District draws groundwater from the Niles Cone aquifer which resides directly below the District's service area. The District maintains two well fields equipped with a total of seventeen wells for this source of supply. The District also operates a groundwater recharge facility that captures surface runoff from Alameda Creek by diverting the runoff to recharge ponds that allow percolation to the Niles Cone aquifer. Although located in nearby proximity to the District's well fields, the recharge operation does not influence the groundwater supply, i.e., the groundwater system is not known to be a groundwater under the direct influence of a surface water (Acevedo 1995). Since the groundwater system is not under the direct influence of a surface water source, no disinfection is required.

San Francisco Water Department. The District also purchases water from the San Francisco Water Department (SFWD). The primary source of this water supply is snowmelt from the Sierra Nevada mountain range that feeds the Tuolomne River watershed. The water is then collected in Hetch Hetchy Valley behind SFWD's O'Shaughnessy Dam in Yosemite National Park. SFWD disinfects, but does not filter this source, before distributing it to the retailers and consumers.

Water Treatment. As is typical of many groundwaters, water from the District's groundwater supply is very hard. In order to decrease the hardness of the groundwater supply, it is mixed or blended with the soft water purchased from the SFWD. Although SFWD water is disinfected with chlorine before blending, the groundwater is not. As the groundwater is not disinfected, the final disinfectant residual of the blended product is increased before being delivered to the consumers.

Water from the South Bay Aqueduct receives full-conventional treatment at one of two treatment plants. The Mission San Jose Water Treatment Plant (MSJWTP) is the older of the two facilities and consists of, prechlorination, flash-mix for coagulant addition, uplflow clarification, dual-media filtration, postfilter

disinfection with chlorine, pH adjustment with caustic, fluoridation, and finished water ammoniation for distribution system chloramination. Under the Surface Water Treatment Rule (SWTR), the plant must achieve a 3-log *Giardia* and 4-log virus removal/inactivation (Acevedo 1995). The state has granted the filtration process in the plant with a 2.5-log removal for *Giardia* and a 2.0-log removal for viruses. The balance of the removal/inactivation required under SWTR must be achieved by disinfection practices.

The second plant, known as Water Treatment Plant 2 (WTP2), must meet identical SWTR water quality requirements (Acevedo 1995) to those of MSJWTP. About two years old, this plant relies on predisinfection with ozone, flash-mix coagulation, flocculation, horizontal sedimentation, dual-media filtration, postfilter disinfection with hypochlorite, pH adjustment with caustic soda, fluoridation, and ammonia addition for finished water chloramination to meet water quality goals.

East Bay Municipal Utility District

The East Bay Municipal Utility District (EBMUD) serves the western Contra Costa and Alameda Counties stretching from the city of Crockett in the north to Hayward and San Lorenzo in the south. Their service area also extends east from the shores of San Francisco Bay to Walnut Creek and San Ramon. The service population in 1995 consisted of 1.2 million persons being served through a total of 330,836 general and residential service connections. There are 19,845 commercial and 2,163 industrial service connections. The average daily water production is 192 million gallons per day.

EBMUD depends entirely upon surface water supplies to meet demands. Through a system of reservoirs and aqueducts surface water is supplied to six water treatment plants prior to distribution. The primary source of water for this utility is the Moukelumne river watershed which covers an area that extends from an area just south of Lake Tahoe in a southwesterly direction down the western side of the Sierra Nevada mountain range. Snow melt from the watershed is the primary source of water for the Moukelumne river. Water is stored behind Pardee Reservoir located in the foothills of the Sierra Nevada mountain range. The water from this reservoir is chlorinated before it flows through a closed aqueduct to the service area where the water is treated before distribution. A portion of the water brought into the service area is also stored in local reservoirs that also receive and store local runoff before treatment.

EBMUD Water Treatment.

All six of the EBMUD plants must comply with the provisions of the SWTR and meet a 3-log *Giardia* and 4-log virus inactivation/removal. Water brought into the service area by the aqueduct is treated in the service area by three water treatment plants that provide direct filtration (no flocculation or settling) and disinfection. One of the direct filtration plants treats water from the Briones Reservoir before the water is distributed to consumers.

Two other service area reservoirs supply three conventional water treatment plants (coagulation, flocculation, sedimentation, filtration, and disinfection) two of which can provide ozonation between sedimentation and filtration. The third plant is an older conventional plant that has not been upgraded to include ozone.

The Surface Water Treatment Rule (SWTR) states that it is intended to regulate *Legionella* sp. The goal of the rule is to provide protection from *Legionella* (Malcolm Pirnie, Inc. and HDR Engineering 1991). "Inactivation levels have not been set for *Legionella* because the required inactivation of *Giardia* cysts will provide protection from *Legionella*." However, it clear from the SWTR guidance manual (Malcolm Pirnie, Inc. and HDR Engineering 1991) that such a goal is assumed, but is never confirmed as a footnote, in the guidance manual, explains that, "These CT's [for *Giardia*] are two to three times higher than that which is needed to achieve a 3-log inactivation of *Legionella*." As no disinfection or CT tables are setup for *Legionella* it is assumed that achieving a 3-log inactivation of *Giardia* is sufficient protection from *Legionella*. One has to wonder if such an assumption is correct.

A typical conventional water treatment plant with high rate dual-media filters is required to achieve an overall 3-log removal/inactivation of *Giardia* in the plant. Usually the filters are granted 2-log removal credit for *Giardia*, which means that only 1-log inactivation of *Giardia* is achieved by disinfection. Therefore, the margin of safety built into the USEPA assumption regarding *Legionella* control may not be valid. Indeed this shortcoming may be recognized by the statement contained in the SWTR guidance manual. "However, this level of disinfection cannot assure that all *Legionella* will be inactivated and that no recontamination or regrowth in recirculating hot water systems of buildings or cooling systems will occur."

To its credit the SWTR Guidance manual does recommend that hospitals and other institutions with potential for regrowth conduct routine monitoring of their hot water systems, using the analytical procedures for Legionellaceae in the 16th Edition of Standard Methods. However, the concern that *Legionella* organisms may be breaking through conventional water treatment barriers remains.

As conventional methods for detecting Legionellaceae do not assess infectivity, or virulence, this survey will serve to further our knowledge of how well our conventional water treatment barriers are functioning. Because of the concern to immunocompromised or immunosuppressed individuals this survey may also serve to highlight potential sources or incidents that might lead to "seeding" of the distribution systems.

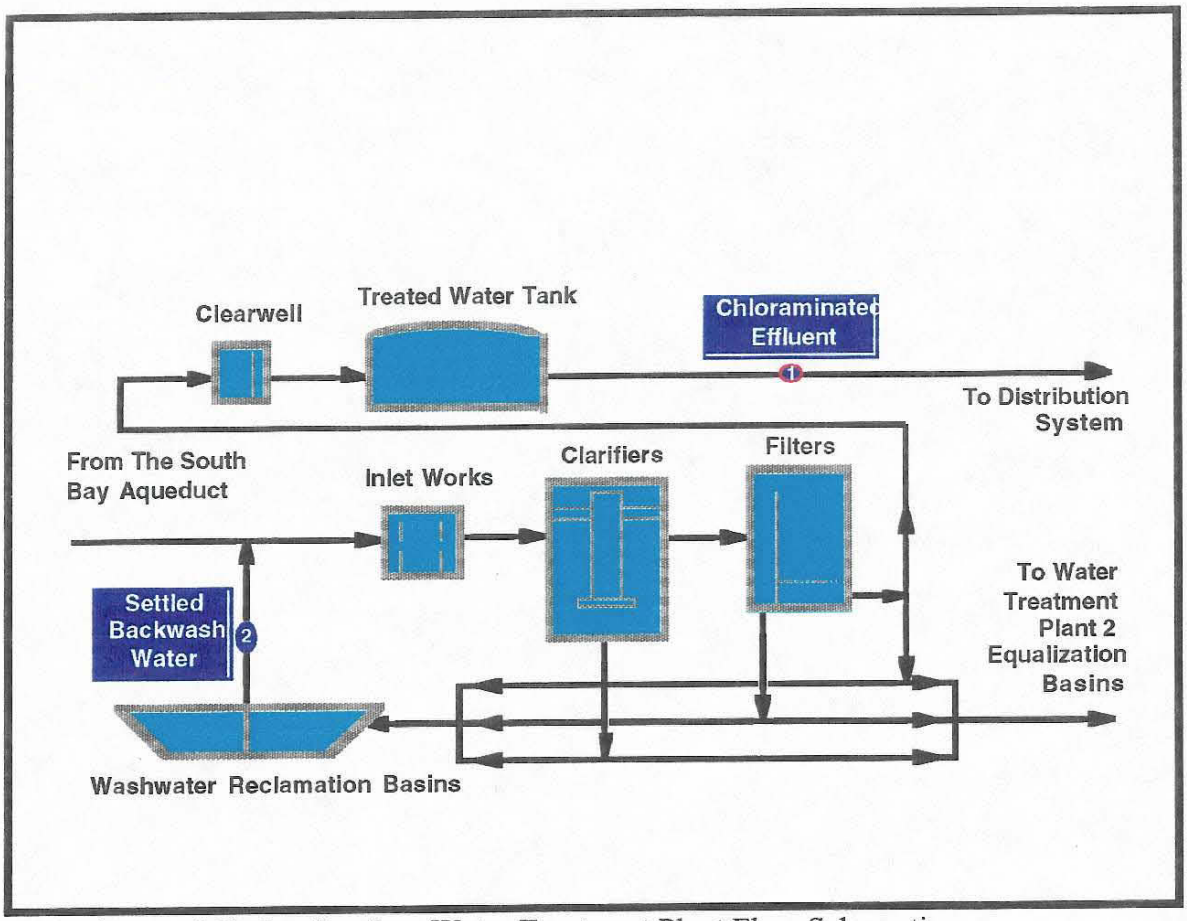

Figure 1. Mission San Jose Water Treatment Plant Flow Schematic

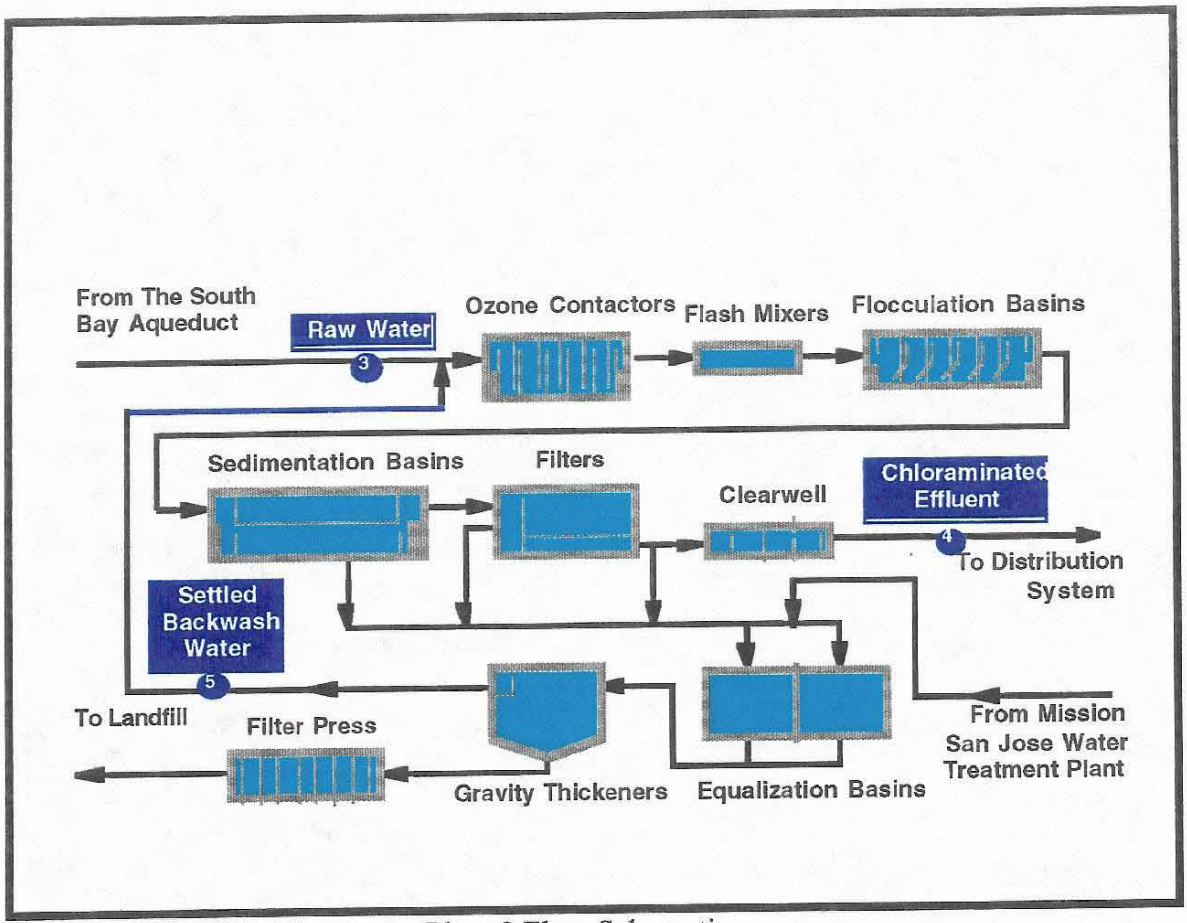

Figure 2. Water Treatment Plant 2 Flow Schematic

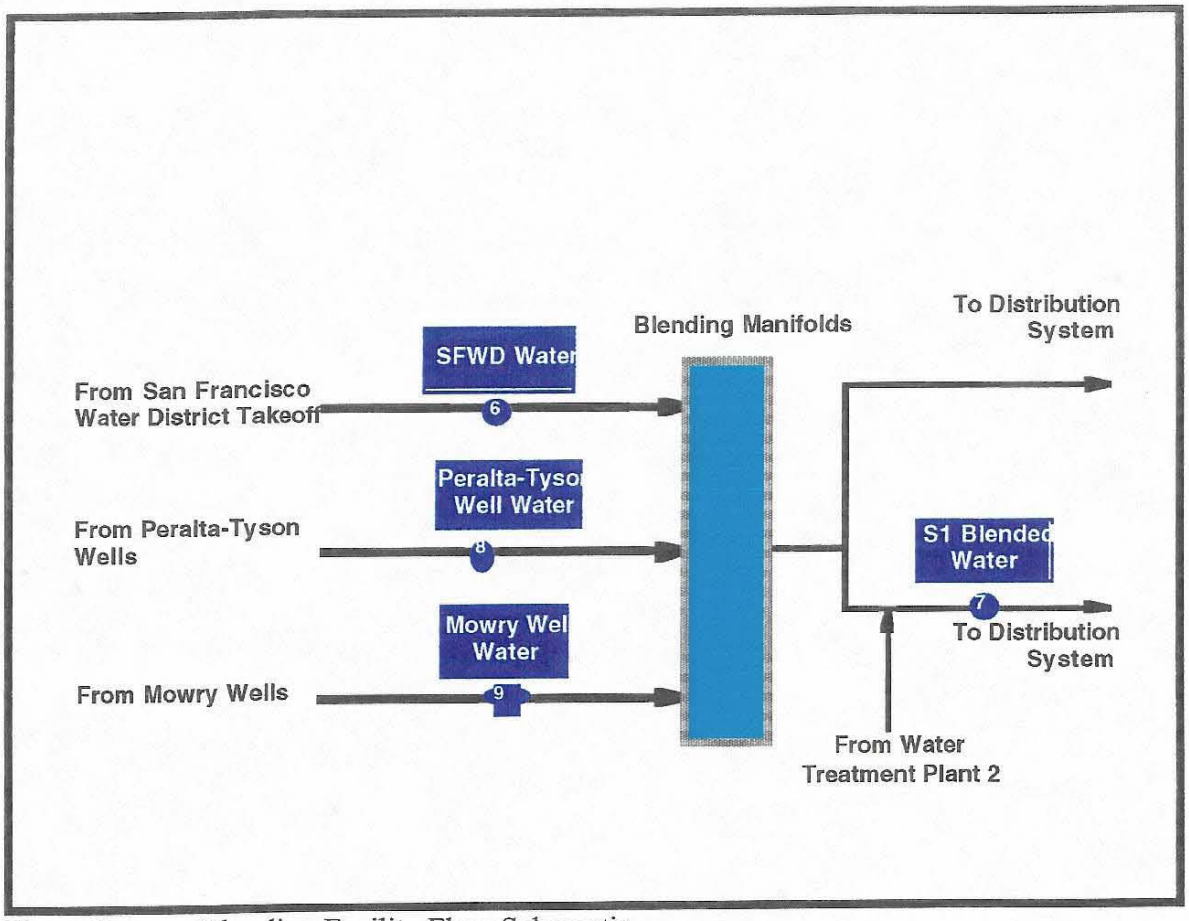

Figure 3. Blending Facility Flow Schematic

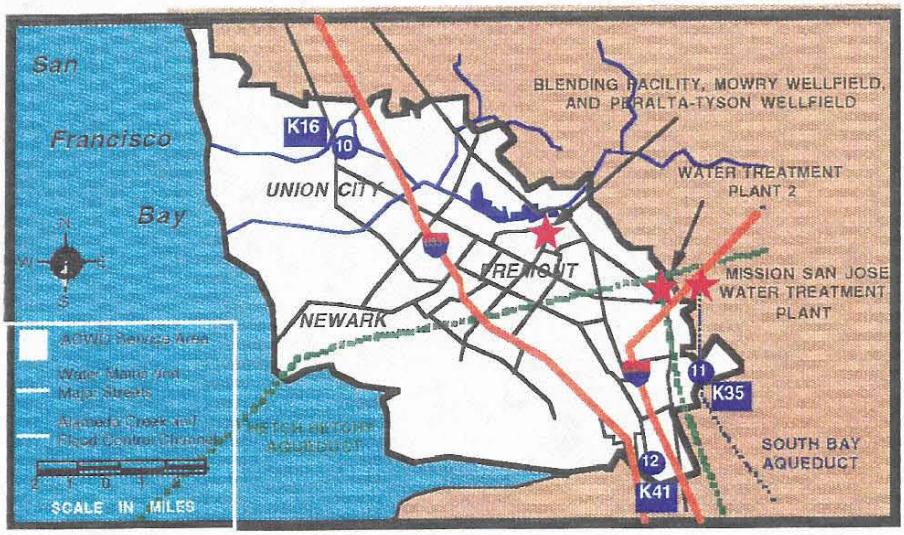

Figure 4. Alameda County Water District Service Area and Distribution System Sample Sites.

Materials and Methods

Sample Collection

All samples were collected in sterile 250 ml polypropyelene bottles and returned to the laboratory immediately for processing. Alameda county water sampling sites are listed in Table 1. Bottles collected for microbiological analyses contained a sufficient volume of 2% sodium thiosulfate to neutralize any chlorine in the samples. Excess sample was stored at 4° C in the event that further testing was required.

Table 1. Alameda County Water District Sample Locations.

Sample Site Number	Sample Site Descriptor
1	MSJWTP Chloraminated Finished Water
2	MSJWTP Settled Backwash Water
3	WTP2 Raw Water
4	WTP2 Chloraminated Finished Water
5	WTP2 Settled Backwash Water
6	SFWD Water Supply
7	S1 Blended Water (July and August)
8	Peralta-Tyson Well (groundwater)
9	Mowry Well (groundwater)
10	K16 Distribution System Sample
11	K35 Distribution System Sample
12	K41 Distribution System Sample

Chemical

The physical and chemical water quality analyses for pH, turbidity, temperature, and chlorine residual were performed by a state certified water quality laboratory using the standard methods identified in the 18th edition (1992). Table 2 is a summary of methods used for pH, turbidity, temperature, and chlorine residual.

Table 2. Physical and Chemical Water Quality Analyses

Water Quality Test	Standard Method
pH	4500-H^+ B
Turbidity	2130B
Temperature	2550B
Chlorine Residual	4500-Cl D

Bacteriological - Heterotrophic Plate Counts and Coliform Enumeration

Microbiological testing for total coliforms, fecal coliforms, and *E. coli* was done by standard method membrane filtration (9222B). Total and fecal coliforms were also

done by the standard method presence/absence multiple tube fermentation technique (9223B) because total coliforms from the membrane filtration technique were not enumerated above 20 colonies (drinking water procedures).

Microcosm Construction

Microcosms were constructed as previously described (Paszko-Kolva et al., 1993a) Briefly, five hundred milliliters of sterile tap water were added to one liter acid washed bottles, prior to seeding the microcosms with *Legionella pneumophila*. A three day culture of *L. pneumophila* serogroup 1 and a three day culture of *L. pneumophila* serogroup 5 were harvested and washed three times in sterile tap water. After the final wash, cells were suspended in filter sterilized tap water and inoculated into the microcosms in duplicate at a concentration of 10^7 cells/ml. Cells were enumerated by acridine orange direct count, DFA staining, PCR and culture. Cell viability was examined employing various vital dyes including CTC and INT, while culturability was followed by plating on BCYEα media.

Microscopic Evaluation of Vital Stains

A number of tetrazolium salt dyes were evaluated as indicators of viability in respiring bacteria when used in conjunction with direct fluorescent antibody staining for *Legionella* species. Based on reports in the literature the following dyes were evaluated for their ability to detect viability in bacterial cells; MTT = (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, XTT = 3'{1-[phenylamino)-carbonyl]-3,4-tetrazolium}-bis (4-methoxy-6-nitro)benzene-sulfonic acid hydrate, (CTC) = 5-cyano-2,3-ditolyl tetrazolium chloride, INT = 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride and (AODC) acridine orange (Hobbie et al., 1977; Bowden, 1977; Kogure, et al. 1979; Gerlier and Thomasset, 1986; Rodriguez et al., 1992; Thom et al., 1993; Rodlev and King, 1993). Based on preliminary performance studies, only CTC and INT were further investigated for use with the DFA stain.

Tetrazolium dyes have been evaluated by others (Gerlien et al., 1986; Vessey et al, 1990; and Rodriguez et al., 1992) to directly observe respirometrically active bacteria in environmental samples. When tetrazolium salts are utilized as vital stains they compete directly with molecular oxygen as artificial electron acceptors. Due to the reducing power of the electron transport system, CTC is converted into insoluble fluorescent formazan crystals which accumulate in metabolically active bacterial cells. The CTC-formazan is microscopically visualized under longwave UV light (>350 nm). Because of the fluorescence associated with crystal formation using CTC, microscopic visualization is much easier when compared to visualization of accumulated crystals employing INT staining. When using INT, bright field optics are employed to visualize a dull-red intracellular crystal deposit. This accumulation is indicative of metabolically active bacteria.

CTC and INT were both evaluated on pure cultures of 4 strains of *Legionella;* (*L. pneumophila, L. pneumophila* L-100, *L. jordanis,* and *L. gormanii*) and also when mixed with heterotrophic bacteria. (For mixed suspensions, each strain of

Legionella was mixed with four other non-*Legionella* bacteria in suspensions of different concentrations) for evaluation of the simultaneous DFA-CTC or DFA-INT staining procedure. The uptake of INT and CTC was nearly identical, as seen employing Acridine Orange and DAPI, respectively. However, INT accumulation could not always be viewed clearly when used in conjunction with DFA especially in cases where samples contained considerable debris. CTC on the other hand, could easily be viewed, even in the presence of debris. For this reason CTC was chosen to monitor the respiratiory activity of the *Legionella* cells detected in the water samples.

For the analysis of the environmental samples, ten ml of each water sample was concentrated by centrifugation. One ml of each concentrate was transferred into a sterile plastic 24 well tissue culture plate (Corning, NY). One-hundred microliters of a 50mM stock of CTC was added to each well for a final concentration of 5 mM. The plates were incubated at 37° C in a CO_2 incubator (5% CO_2) with 100 rpm shaking for 24 hours. Following incubation, the contents of each of the wells were fixed with 1% formalin for 1 hour after which the DFA staining was performed. For DFA staining, the contents of each well were filtered through a 0.2 µm black polycarbonate filter (Poretics, NJ). The filter was placed on a clean glass slide, 40 µl of FITC labeled *Legionella* antibody conjugate Poly II (SciMedix, NJ) was placed in the center of the filter. A coverslip was placed over the antisera and the sample was placed in a humid chamber and incubated for 37°C for 40 minutes. Following incubation, the coverslip was removed and the filter was rinsed with 1ml of PBS (pH 7.6) via filtration, then soaked in PBS for 10 minutes, and then rinsed with 10 ml of filter-sterilized deionized water. The filter was placed on a clean slide and fluorescent antibody mounting fluid was then added with a clean coverslip. The slides were then visualized under an epifluorescent microscope and fluorescent cells were enumerated under oil immersion at a magnification of 100X (Zeiss, NY).

Culture and Immunological Methods for the Environmental Detection of *Legionella* species

Suitable samples for environmental analysis include water, swab, filter, and in rare cases air samples, all collected in sterile containers or on media and immediately transported to a laboratory for analyses. The type of sample collected will vary depending on the source. In some cases it is impossible to collect water so a swab sample is substituted. Swab samples are also used to collect *Legionella* present in biofilms. Likewise, the volume of water (250-1,000 ml) collected will vary according to the source. Typically larger volumes of water are collected from natural sources such as lakes, since lower numbers of legionellae are present. Smaller water (10-100 ml) volumes are collected from home showerheads and other man-made devices that frequently harbor higher concentrations of cells since they provide favorable growth conditions for legionellae.

For the purpose of this study, 250 ml water samples were collected in sterile polyropylene containers (Nalgene, Rochester, NY) and transported immediately to

the laboratory for analysis. An appropriate volume of a 2% sodium thiosulfate solution was added to samples containing chlorine in an attempt to neutralize the disinfectant prior to analysis.

Following sample collection, microorganisms are usually concentrated by centrifugation or filtration and plated on Buffered Charcoal Yeast Extract agar amended with a-ketoglutarate and glycine (BCYEα). *Legionella* exhibit increased resistance to many antibiotics, heat and low pH. Therefore, heat and acid treatments of concentrates and the addition of antibiotics (such as cyclohexamide, vancomycin, and polymyxin B) to the media (BCYEαGVPC) aid in inhibiting the growth of competing non-legionellae bacteria. These strategies are necessary since *Legionella* is a slow grower, typically requiring 3-7 days and up to 21 to form visible colonies on artificial media. Despite these efforts, *Legionella* colonies are often overgrown by background flora. The sequential culturing method was employed with those samples which were positive by PCR and DFA according to the method of Shahamat et al. 1991.

Polymerase Chain Reaction

Environmental researchers have used PCR to detect a variety of microorganisms, including *Legionella* in potable water supplies (Bej et al. 1991). The first commercially available PCR kit for *Legionella* detection was introduced by Perkin-Elmer called the EnviroAmp® Legionella Kit. PCR is a process of enzymatic synthesis by which a specific target DNA sequence is amplified and then detected by hybridization on a solid support or by gel electrophoresis.

The EnviroAmp kit contains highly specific primers and probes which are complementary to the conserved regions in the 5S ribosomal RNA (rRNA) gene and the macrophage infectivity potentiator (*mip*) gene. *Legionella* species are identified by the presence of the targeted sequence of the 5S rRNA gene while *L. pneumophila* is identified by the presence of the *mip* gene (MacDonell, 1987; Engleberg et al. 1989). The *mip* gene is highly conserved and specific to *L. pneumophila* although *mip*-like genes have been detected in other *Legionella* species. Detection of *Legionella* spp. and *L. pneumophila* is accomplished by hybridization of resultant PCR products to their respective complementary probes. The EnviroAmp kit is able to detect 25 species and 15 serotypes of *Legionella.* Only *L. pneumophila* , the most clinically significant group, can be speciated.

The kit employs a reverse dot blot methodology. PCR products are labeled with biotin and hybridize to probes immobilized to nylon membranes (Figure 5).

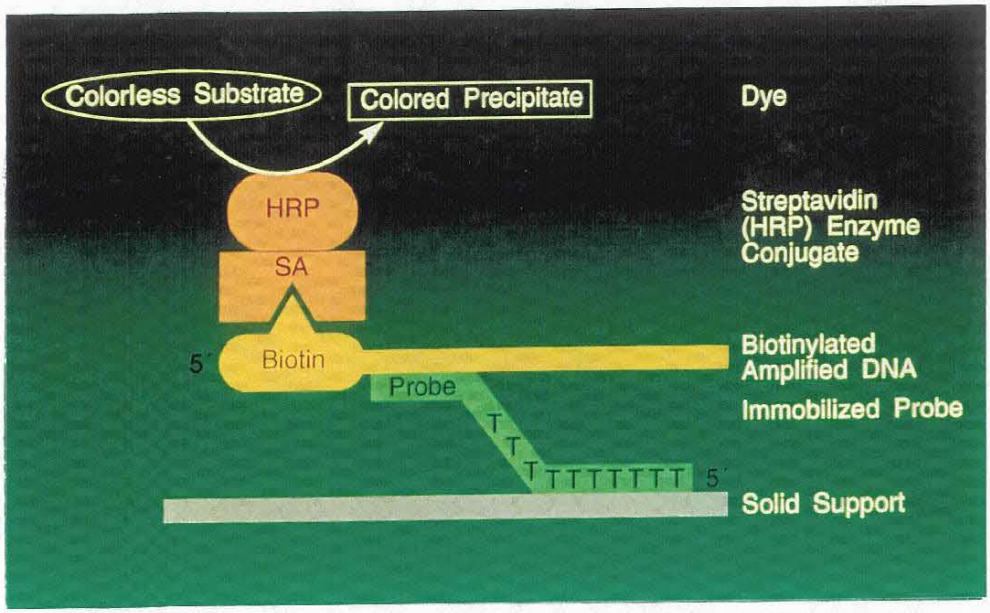

Figure 5. The EnviroAmp utilizes a reverse dot blot methodology to indicate the presence or absence of specific amplified sequences.

Following hybridization and stringent washing of the nylon membranes, bound PCR products are detected by incubating with an enzyme conjugate (horseradish peroxidase-streptavidin), followed by adding tetramethylbenzidine. A negative control is included in the assay to insure that hybridization conditions are optimized. An internal positive control is also included to indicate the presence of substances that may interfere with the PCR process. The detection limit of the EnviroAmp Legionella kit is as low as 10 *Legionella* cells/mL. Certain substances such as humic acids and iron, if present in water samples, may inhibit PCR and subsequently affect the level of sensitivity. However, a number of strategies have been employed to effectively remove or reduce these inhibitors. Bovine serum albumin has significantly helped in overcoming inhibition in the PCR, it is believed to bind inhibitors and also stabilize the ampliTaq. The EnviroAmp kit has been evaluated on a variety of samples, including potable water systems, hot tubs, cooling towers, sewage treatment effluent and even ocean waters.

Results interpretation is rather simple. If the positive control is indeed positive, as indicated by a blue dot next to the "+" symbol and the "L" and "p" spots are white, then the sample was negative for *Legionella* species (Figure 6). If the "L" dot is positive as indicated by a blue dot and the "p" dot is negative (white) then *Legionella* species were present. The nylon membranes of the EnviroAmp kit also provides semi-quantitative results. The concentration of the positive control is equal to approximately 10^3 *Legionella* cells, if the intensity of the sample is less than that of the control dot, then less than 10^3 *Legionella* cells were present in that sample. Conversely, if the intensity of the dot is much darker than the control then greater than 10^3 *Legioenlla* cells were present in the sample.

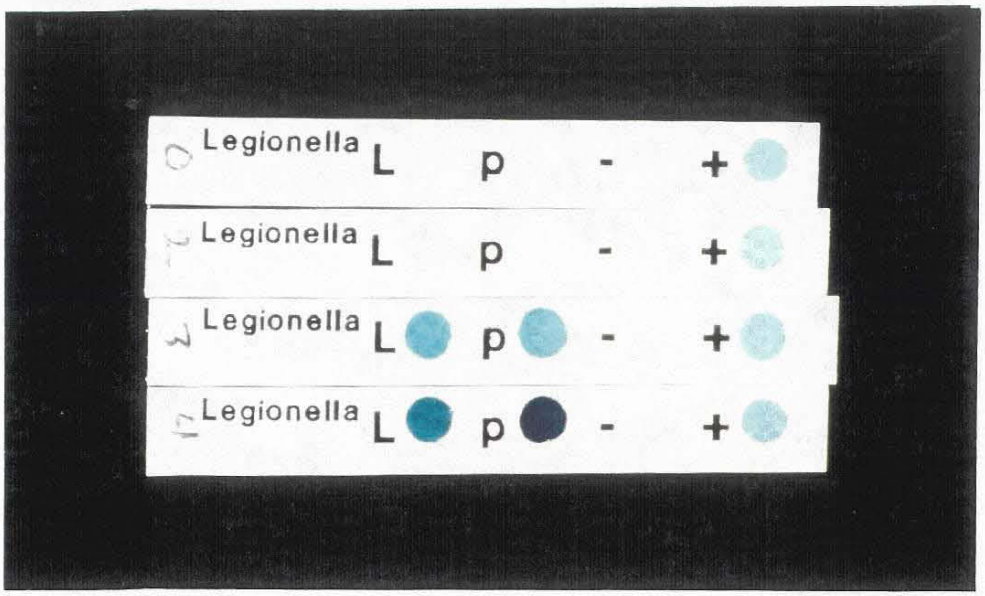

Figure 6. Comparison of *Legionella pneumophila* control DNA. The first strip is negative, the second indicates the presence of less than 10^3 *Legionella* cells/ml, the next strip has equal to 10^3 cells/ml and the last strip has greater than 10^3 cells/ml.

Results

Table 3 (in Appendix A) lists all the results for the abiotic parameters and microbiological testing for all 86 municipal water samples that were collected from July to November. The range of pH values was from 6.7 - 9.4, temperatures ranged from 13.2°C- 25°C, turbidity from 0.01 - 23.50 NTU, free chlorine levels ranged from 0.01 - 1.5mg/L. As expected, heterotrophic plate counts did not correlate well with acridine orange direct counts. Values for total and fecal coliforms were typically very low.

Of the samples analyzed for *Legionella* by direct methods, 83% were positive by DFA, 83% were positive by PCR for *Legionella* species while 13% were positive for *Legionella pneumophila* by PCR. However, 4 samples were inhibited in the PCR, all four samples were positive by DFA. Five environmental samples were positive by PCR, yet negative by DFA and one sample was negative by PCR yet positive by DFA. Vital staining was used in conjunction with DFA staining as shown in figure 7. Examination of DFA-CTC results indicate that (16/71) 23% of the *Legionella* cells which were detected were viable, while (2/16) 13% of those viable cells were identified as *L. pneumophila*, the species reportedly responsible for the majority of the outbreaks. If we compare raw surface water, chlorinated surface water, groundwater, finished water and recycled backwash water samples, the percent positive for *Legionella* by PCR is 93%, 90%, 67% 71% and 95%, respectively. Culture results on (BCYEαGVPC) were uniformly negative. There was no correlation observed between the abiotic or HPC and total coliform counts with those of *Legionella*. Table 4 provides detailed information on positive samples according to water type. It is interesting to note that groundwater and finished water had the fewest percent positives for *Legionella*. Also, not surprising, recycled backwash water had the highest percent of *Legionella* positives.

The term "Raw Surface Water Source" (Table 4) is meant to indicate raw oruntreated surface waters in order to differentiate these samples from those samples collected from surface waters that are chlorinated before being conveyed to a treatment facility. The surface waters that are chlorinated before treatment were categorized as "Chlorinated Surface Water." All "Groundwater Sources" were collected prior to the addition of chlorine and any additional treatment. "Plant Finished Water" refers to water leaving a treatment plant after treatment that at a minimum includes filtration and disinfection. "Distribution System Samples" were collected from dedicated sampling stations within the distribution system. "Recycled Backwash Samples" refers to samples taken from streams that have been used to backwash filters and is being returned to the front of the treatment plant in order to recover the water.
Both participating utilities are meeting or exceeding the disinfection and filtration requirements of the SWTR. This means that the utilities are providing inactivation/removal sufficient to achieve a 3-log inactivation/removal of *Giardia* and a 4-log inactivation/removal of viruses. While the finished water from the water treatment plants tested positive for *Legionella* sp. by PCR in 71% of the samples (15), only two samples also tested positive for *L. pneumophila* by PCR. The most important observation from this group of samples is that only one of the plant finished water samples (out of 25) was also DFA/CTC positive. Based on this survey, the frequency with which viable *Legionella* sp. can be detected in the finished water is a rare event.

Table 4. Sample Breakdown by Sample Location

Water Source	Total Number of Samples	PCR Positives for *Legionella* sp.	% PCR Positive for *Legionella* sp.	PCR Positive for *L. pneumo.*	% Positive for *L. pneumo.*	DFA/ CTC Positive	%DFA/ CTC Positive
Raw Surface Water Source	15	14	93	3	20	3	20
Chlorinated Surface Water	10	9	90	2	20	2	20
Groundwater Sources	6	4	67	1	17	1	17
Plant Finished Water	21	15	71	2	10	1	5
Distribution Systems	14	10	71	2	14	3	21
Recycled Backwash	20	19	95	1	5	7	35

Comparison of the DFA analyses to the PCR results (Table 5) shows the frequency of positives to between the assays to be similar. When the PCR positive samples are examined, it is clear that there are far fewer positives for *L. pneumophila* than for *Legionella* sp. The corresponding DFA/CTC results also indicate the frequency with which viable *Legionella* sp. are found is low.

Table 5. Comparison of PCR Results to DFA

	Total Number of Samples	PCR Positives for *Legionella* sp.	DFA Positives
Raw Surface Water Source	15	14	14
Chlorinated Surface Water	10	9	10
Groundwater Sources	6	4	4
Plant Finished Water	21	15	15
Distribution Systems	14	10	10
Recycled Backwash	20	19	19

The highest frequency of DFA/CTC positives occurred in the "Recycled Backwash." Such a result is not unexpected as conventional treatment plants that include filtration will concentrate particulate matter and microbiological pathogens in the media only to have these "particulates" resuspended when the filter is cleaned or backwashed. The resuspended particulates, if not properly removed by settling or inactivated by disinfection can be introduced back to the head of the water treatment plant.

Comparison of the positive results from the "Chlorinated Surface Water" samples to the "Raw Surface Water Source" shows the frequency of positive samples to be equal for both the PCR and DFA tests. Identical frequencies for the viability staining also occurred. Because of *Legionella* sp. documented resistance to chlorine, these results are not all that surprising. However, in examining the results from the "Chlorinated Surface Water" sample set, the data is more interesting.

All of the "Chlorinated Surface Water" samples were positive by PCR for *Legionella* sp. except for one sample which was inhibited. However, the two PCR positive samples for *L. pneumophila* occurred in the sample set from the unfiltered surface water source with only one of these samples also showing evidence of viability by the DFA/CTC analysis. The unfiltered chlorinated surface water source had one additional DFA/CTC positive sample which was also PCR positive for *Legionella* sp. Since two of the five samples collected from the unfiltered chlorinated surface water source were both the DFA/CTC positive, this supply can provide a source of inoculum for the distribution system. There were three DFA/CTC positives in the set of distribution system samples, one of which corresponded to a *L. pneumophila* positive by PCR. The most interesting observation is that the free and total chlorine residuals on all three of the positive DFA/CTC samples were equal. This is a strong indication that the water samples were primarily from the unfiltered water source entering the distribution system.

A second set of "Chlorinated Surface Water" samples was collected from a source that was filtered and disinfected before distribution. Although 2 of the 5

finished water samples from this plant did show signs of *Legionella* sp. (by PCR) in the finished water, none of the positive samples were viable as measured by the DFA/CTC analysis.

While both DFA and PCR indicate the presence of *Legionella* sp. in 71 percent of the "Finished Water" samples, the DFA/CTC analysis indicates that very few of these organisms are viable. Only 1 of the 21 "Finished Water" samples was DFA/CTC positive. This is a good indication that conventional water treatment plants operating to cover a range of source water qualities are providing adequate barriers to prevent the passage of pathogens into the drinking water supply.

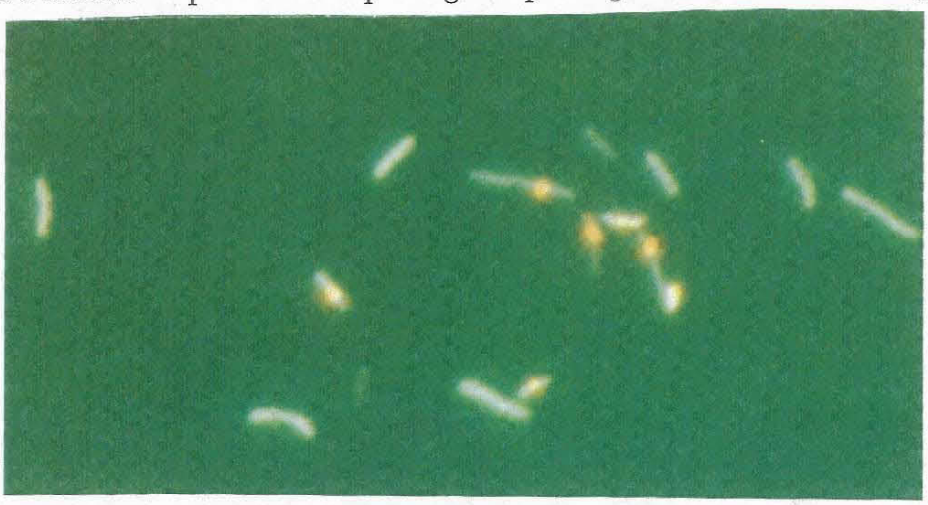

Figure 7. Fluorescent photomicrograph (original magnification 1000X) of *Legionella pneumophila* serogroup 1 stained with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC). Note that respiring cells stained with DFA-CTC accumulate an insoluble red fluorescent CTC-formazan, while non-viable cells remain green.

Microcosms were constructed primarily to evaluate vital stains and to follow the viability and culturability of *Legionella* cells over time. Two sets of microcosms were constructed that initially contained 1.4×10^7 cells/ml. Over a three month period the *L. pneumophila* cells inoculated into the microcosm declined in culturability by one log after the first month and then remained stable at 1.3×10^6 cfu/ml. However, viable counts determined by CTC staining, decreased considerably less than the plate counts (mean value for three month period; 5.7×10^6 cells/ml). Acridine orange direct counts and DFA staining remained constant over the three month period (1.0×10^7 cells/ml).

Discussion

Because *Legionella* are fastidious organisms with a complex ecology, they are inherently difficult to culture. It has also been demonstrated that certain species of heterotrophic plate count bacteria secrete bactericins which are inhibitory to *Legionella* species. *Legionella* are also able to survive in biofilms, responding to nutrient conditions and often shielding themselves from harsh environmental conditions such as disinfectants (Cargill, et al., 1992; Yu and McFeters, 1994).

Legionella are also frequently isolated in association with protozoa, which can sequester the *Legionella* cells from disinfectants (Paszko-Kolva et al., 1991). Additionally, it has also been observed that *Legionella* which are stressed or injured due to environmental pressures or disinfection treatments may be viable but rendered non-culturable (Hussong et al, 1987). In this state, the *Legionella* cannot multiply to form colonies on the standard culture media, yet they remain viable and potentially infectious. Therefore, culture methods do not provide the total solution to *Legionella* detection and direct methods, such as DFA and PCR must be utilized. In addition, the results obtained utilizing these different methods do not often correlate there are several explanations for these discrepancies. Appendix C gives a breakdown of the various scenarios, comparing DFA, PCR and culture results.

The DFA test is based on the principle that antibodies bind to both species and serogroup specific antigens to which they are directed against. Using anti-*Legionella* antisera conjugated to a fluorescent label, typically fluorescein isothiocyanate (FITC), *Legionella* cells present in a sample will bind with the antibody conjugate. When the excitation wavelength of the ultraviolet light hits the FITC, a brilliant apple green color is emitted. Limitations of DFA staining include potential cross reactions with other indigenous flora and the requirement for a highly trained analyst, benefits include speed and low reagent costs.

PCR targets the genetic information of microorganisms which offers a much higher level of discrimination and improved accuracy. Despite the use of bovine serum albumin, an agent which is believed to bind inhibitors and stabilize the Taq polymerase, a few samples were still inhibited and unable to yield a result. However, the benefits of PCR, primarily the ability to detect over 25 species of *Legionella*, (commercially available DFA reagent targets 8 species), objective results, speed and accuracy, outweigh the potential problems associated with alternative detection methods. (Paszko-Kolva et al., 1995).

Upon reviewing the data, we found that one utility had more inhibition of their samples when analyzed by PCR than the other utility. We believe that the higher incidence of inhibition is due to high iron levels. One utility utilized ferric chloride in their conventional treatment plants while the other utility used alum. All inhibited samples were re-run in the presence of bovine serum albumin employing an enhanced "clean-up" protocol. This was sufficient in the majority of the samples to overcome the inhibition, however despite this treatment less than 5% of the samples remained inhibited.

Of the samples analyzed for *Legionella* by direct methods, 83% were positive by DFA, 83% were positive by PCR for *Legionella* species while 13% were positive for *Legionella pneumophila* by PCR. However, 4 samples were inhibited in the PCR, all four of these samples were positive by DFA. Five environmental samples were positive by PCR, yet negative by DFA and one sample was negative by PCR yet positive by DFA. These discrepant results can easily be explained, the PCR assay is directed against 25 *Legionella* species, while the DFA reagent employed in this study

only detects eight different *Legionella* species. There a several explanations for the one sample that was positive by DFA and not PCR, first the antisera may have cross-reacted with a non-*Legionella* bacteria or the cells detected were antigenically intact but devoid of nucleic acid.

In addition to PCR, direct fluorescent antibody (DFA) staining is another method for the direct detection of *Legionella* in environmental samples (Cherry et al. 1978). The commercially available antisera used in this study was directed against eight different species of *Legionella*, while the PCR could detect greater than 25 species. However, when more samples are positive by PCR than DFA, as in this study, one possible explanation is that the PCR assay is able to detect over 25 species of *Legionella* while the DFA method employed in this study could only detect eight *Legionella* species.

Because of cultural and immunofluorescent *Legionella* detection limitations, molecular detection methods are evolving to become the methods of choice for rapid, direct screening, in conjunction with culture methods to obtain culturability information. Indepth interpretation of results for PCR, culture, and DFA are discussed in (Appendix C). Prior to viability staining, the primary method to obtain both viability and culturablility information was through plating on artificial media. The use of viability stains in conjunction with immunofluorescent stains is not a new idea (Vesey, et al., 1990), however, this is the first time that DFA-CTC staining has been combined with PCR, which adds to the power of discrimination.

Results of this study compare well with other studies that examined environmental waters for the presence of *Legionella* species (States et. al., 1987; Paszko-Kolva et al., 1991; Fram and Oberst, 1994; Paszko-Kolva et al., 1993c; Palmer et al, 1995). The addition of vital stains to our study in conjunction with PCR/DFA and culture allowed a more accurate picture of the ecology of this microorganism. It is apparent from the results of our study that although the *Legionella* cells are quite ubiquitous throughout the distribution system, only 23% detected by PCR and DFA were actually viable.

Samples collected in the distribution were collected from dedicated sampling stations that tap the transmission lines below grade. The sampling stations themselves were covered and locked to protect the faucet or sample tap from vandalism. Because the stations were allowed to discharge for a period of time before the sample was collected, the sample was actually more representative of free-swimming organisms. While these free-swimming organisms may have been released from biofilms coating the walls of the distribution system, they were not representative of the biofilm population. Therefore, the information generated by this study does not answer questions, such as the frequency with biofilms release organisms into the water supply. Only information regarding the frequency of occurrence and the impact of water source may be derived from these test results.

However, it should be kept in mind that this 23% can seed the endpoints of the distribution systems, where optimal conditions may exist allowing the cells to multiply to high numbers and potentially cause disease. Because *Legionella* are oligotrophic they can survive for long periods of time (>3 years under low nutrient conditions (Paszko-Kolva et al., 1992). Since *Legionella* is so ubiquitous in the aquatic environment, most individuals are constantly exposed and illicit an antibody response, the majority of the cases of legionellosis are believed to be self-limiting in healthy hosts (Paszko-Kolva et al., 1993b).

This study represents a complete analysis of a potable water distribution system employing multiple detection methods for the presence of *Legionella* species. And the results demonstrate that surveys for pathogens (either bacterial, viral or protozoal) in the environment should never rely on a single test. Employing multiple detection methods allows researchers to better understand the ecology of such pathogens and can provide valuable insight into remediation strategies.

In order to provide a more complete picture of what these viability results might mean to the general service population, the morbidity reports for legionellosis in Alameda County were obtained and reviewed. The intent of retrieving this information was to determine if there had been any significant change in the incidence of legionellosis during the period of study compared to the previous year. Although disease reporting is believed to underestimate the true incidence of disease (Kramer et. al. 1996), the current disease surveillance programs are the only means of measuring the incidence rates of disease in the local population without launching a more concerted effort.

The Alameda County Morbidity Report for the cities served by the Alameda County Water District show the incidence of legionellosis to be zero for the years 1994 and 1995. During this time, there were no reported cases of legionellosis reported in the tri-city area served by the Alameda County Water District.

EBMUD serves Alameda and Contra Costa counties and the morbidity statistics for the Alameda county cities served by EBMUD is summarized in Table 6. While there were reported cases in the EBMUD service area, the number of cases in Alameda County increased by 1, which does not constitute a significant increase in legionellosis between the two years. The incidence rate is low (compared to the national incidence rate of 0.6/100,000). The morbidity statistics do not indicate the source of the disease, i.e., how the person contracted the disease. However, the low incidence rate does not indicate a major outbreak and is probably more of a reflection of the endemic rates of legionellosis in the population. It should also be noted that person to person transmission has not been reported to date, explaining why large outbreaks are rare, given the ubiquity of this bacteria.

Occurrence of the disease is sporadic. Again this information used in concert with the viability staining results provide an indication that plants meeting the requirements of the SWTR are providing what may be construed as adequate protection against *Legionella* sp. However, present information is not sufficient to determine if treatment requirements are overly conservative or merely adequate to control the pathogenic agent of legionellosis.

Table 6. Annual Legionellosis Morbidity Summary for Cities in Alameda County served by the East Bay Municipal Utility District (Oliver 1996).

	1994	1995
Albany	0	0
Alameda	0	0
Castro Valley	0	0
Emeryville	0	0
Hayward	1	0
Oakland	3	2
Piedmont	0	0
San Leandro	0	1
Total:	4	3

In conclusion, this report serves as a first step to gaining a better understanding of the occurrence and survial of *Legionella* species in the natural aquatic environment. This study has also allowed us to develop and test a simultaneous staining method for the direct detection and viability determination of *Legionella* species, together with molecular confirmation employing PCR. Since a only a limited number of samples were collected in this study, it is impossible to make any general statements concerning water treatment. We can however state that, as expected, *Legionella* was detected in the waters of Northern California. This is not suprising since numerous reports have documented the detection of *Legionella* in the fresh waters of the East coast, both Northern and Southern areas as well as the waters of Southern California. Future studies should utilize the techniques used in this research to survey high-risk sites suspected of being positive for *Legionella* species, such as hospitals and nursing homes.

Conclusions

1. The results of this study confirm the ubiquity of *Legionella* in municipal water supplies in the Northern California area.

2. There was good agreement between the two direct detection methods for *Legionella,* DFA and PCR, while, as expected there was no correlation to culture.

3. Of the 71/86 (83%) positive samples detected by DFA, only 23% of the cells were viable as determined by simultaneous DFA-CTC staining, of these, (2/16) or 13% were identified as *L. pneumophila.* PCR results indicated that 71/86 or (83%) were positive while 4/86 or (4.6%) of the samples were inhibited.

4. *Legionella* cells are able to pass infrequently through conventional water distribution systems and remain viable, although at low concentrations.

5. From the viability tests and the morbidity report, it appears as though conventional water treatment plants are performing well enough to prevent *Legionella* sp. from being a major public health threat in the drinking water supply.

6. The supply with the greatest potential for seeding the distribution system with *Legionella* sp. is the chlorinated unfiltered surface water source because the frequency with which viable *Legionella* sp. were found in these samples was greater than for the chlorinated filtered water source.

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East Bay Municipal Water Utility
Perkin-Elmer Applied Biosystems

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APPENDIX A

Table 3

NWRI Legionella Project

Cumulative Data Sheet

Chronologically Sorted

Sample Description		pH	Turbidity	Temp	Chlorine		HPC	Coliform*			LegionelND Analyses									
Source	Date		NTU	°C	Free	Total	cfu/mL	Total	Fecal	*E. Coli*	PCR**				Concentration	DFA	LegionelND*	LegionelND	HPC	HPC	AODC
					mg/L	mg/L					L	p	-	+	>10³ or <10³***	score	DFA/CTC	cells/ml	CTC	cells/ml	cells/mL
MSJWTP TW	7/25/95	7.8	0.20	23.7	0.11	1.91	<1	<1	NA		•			•	<10³	3+	neg.	neg.	pos.	<100	1.80E+05
MSJWTP SBW	7/25/95	7.8	3.00	21.4	0.05	0.14	9	<1	NA		•			•	<10³	3+	pos.	<100	pos.	>1000	2.04E+05
WTP2 RW	7/25/95	8.0	19.50	21.9	0.02	0.02	825	20	NA	20	•			•	>10³	(-)	neg.	neg.	pos.	>1000	1.36E+06
WTP2 TW	7/25/95	9.3	0.17	22.2	1.50	1.77	6	<1	NA		•			•	>10³	(-)	neg.	neg.	pos.	>1000	3.12E+05
WTP2 SBW	7/25/95	6.7	4.40	22.6	0.01	0.01	>5500	36	NA	36	•			•	>10³	3+	neg.	neg.	pos.	<1000	1.36E+06
SFWD	7/25/95	9.2	0.92	12.4	0.84	0.92	5	1	NA	<1	•			•	<10³	3+	neg.	neg.	pos.	>1000	4.53E+05
S1	7/25/95	7.7	0.85	16.3	0.63	0.85	<1	<1	NA		•			•	<10³	(-)	neg.	neg.	pos.	>1000	1.77E+05
K16	7/25/95	7.4	0.12	22.0	0.47	0.50	<1	<1	NA					•	negative	(-)	neg.	neg.	neg.	neg.	2.91E+05
K41	7/25/95	8.4	0.13	21.6	0.07	0.10	<1	<1	NA		•			•	<10³	3+	neg.	neg.	pos.	<100	3.71E+05
FP WC Inf	8/2/95	8.5	0.71	19.3	0.24	NA	0	<2	<2	<2	•			•	<10³	3+	neg.	neg.	pos.	>1000	2.91E+05
FP WC Eff	8/2/95	8.6	0.12	20.3	0.89	NA	0	0	0	0				•	negative	(-)	neg.	neg.	pos.	>1000	4.53E+05
FP WC RS	8/2/95	7.9	3.2	20	0.02	NA	280	14	<2	<2	•			•	<10³	3+	pos.	<10	pos.	>1000	1.94E+05
FP SP Inf	8/2/95	7.3	2.1	20	NA	0	1100	13	<2	<2	•			•	>10³	(-)	neg.	neg.	pos.	>1000	1.02E+06
FP SP Eff	8/2/95	8.6	0.06	18	NA	1.53	39	0	0	0	•			•	>10³	3+	pos.	<10	pos.	>1000	2.04E+06
FP SP RS	8/2/95	7.6	2.5	16	NA	0.03	580	4	<2	<2	•			•	>10³	3+	pos.	<100 (clumps)	pos.	>1000	4.08E+05
FP S Eff	8/2/95	8.6	0.01	21	NA	0.91	0	0	0	0	•			•	>10³	3+	neg.	neg.	neg.	neg.	4.08E+05
FP S RS	8/2/95	7.7	1.9	21	NA	0.06	2900	<2	<2	<2	•			•	>10³	3+	neg.	neg.	pos.	>1000	2.04E+05
FP USL Inf	8/2/95	7.8	0.83	21	0.03	NA	CG	6	<2	<2	•	•		•	>10³	3+	neg.	neg.	pos.	>1000	1.36E+06
RB TW	8/2/95	8.4	0.22	22	NA	0.07	12	7	<2	<2	•			•	<10³	3+	pos.	<100	pos.	>1000	1.33E+05
MSJWTP TW	8/15/95	6.9	0.08	24.2	1.52	1.78	<1	<1	NA		•			•	<10³	3+	neg.	neg.	pos.	>1000	1.85E+05
MSJWTP SBW	8/15/95	7.3	1.75	25.0	0.01	0.08	725	<1	NA		•			•	<10³	3+	pos.	<100 (clumps)	pos.	>1000	1.85E+05
WTP2 RW	8/15/95	8.0	17.00	23.7	0.07	0.10	3180	20	NA	20	•			•	>10³	3+	neg.	neg.	pos.	>1000	2.04E+05
WTP2 TW	8/15/95	8.8	0.11	24.8	1.25	1.42	<1	<1	NA		inhibited				inhibited	3+	neg.	neg.	pos.	>1000	2.91E+05
WTP2 SBW	8/15/95	7.4	4.56	24.9	0.01	0.03	30250	10	NA	10	•			•	>10³	3+	pos.	<100	pos.	>1000	4.08E+06
SFWD	8/15/95	9.4	0.67	13.5	0.87	0.87	<1	<1	NA		inhibited				inhibited	3+	neg.	neg.	pos.	>1000	2.55E+05
S1	8/15/95	7.7	0.27	16.6	0.89	0.90	<1	<1	NA					•	negative	(-)	neg.	neg.	pos.	<100	3.14E+05
K16	8/15/95	7.6	0.13	22.8	0.50	0.55	<1	<1	NA		•			•	>10³	3+	pos.	<10	pos.	>1000	1.02E+05
K41	8/15/95	9.4	0.16	24.3	1.00	1.17	<1	<1	NA		•			•	<10³	3+	neg.	neg.	pos.	>1000	2.14E+05
MSJWTP TW	9/12/95	7.7	0.67	21.2	1.32	1.68	<1	<1	NA		•	•		•	>10³	3+	neg.	neg.	pos.	>1000	4.53E+06
MSJWTP SBW	9/12/95	7.6	23.50	19.7	0.05	0.33	4185	10	NA	<1	•			•	<10³	3+	neg.	neg.	pos.	>1000	4.49E+06
WTP2 RW	9/12/95	8.0	1.90	21.3	0.02	0.02	1800	20	NA	20	•			•	>10³	3+	neg.	neg.	pos.	>1000	5.16E+06
WTP2 TW	9/12/95	7.7	0.06	20.6	1.23	1.29	<1	<1	NA		•			•	>10³	3+	neg.	neg.	pos.	>1000	5.28E+06
WTP2 SBW	9/12/95	7.6	2.28	18.7	0.02	0.02	>55000	40	NA	10	•			•	>10³	3+	neg.	neg.	pos.	<1000	5.22E+06
SFWD	9/12/95	8.6	0.34	13.5	0.97	1.11	<1	<1	NA		•	•		•	>10³	3+	pos.	<100	pos.	>1000	4.08E+06
M	9/12/95	7.6	0.13	17.1	0.04	0.06	15	<1	NA		•	•		•	>10³	4+	neg.	neg.	pos.	>1000	4.08E+06
PT	9/12/95	7.5	0.14	18.6	0.00	0.02	47	<1	NA		•			•	<10³	4+	pos.	<10 (clumps)	pos.	>1000	4.08E+06
K16	9/12/95	7.2	0.11	21.0	0.31	0.44	<1	<1	NA		•			•	>10³	3+	neg.	neg.	pos.	>1000	5.81E+06
K41	9/12/95	8.3	0.11	21.3	0.04	0.16	<1	<1	NA		•	•		•	<10³	3+	neg.	neg.	pos.	>1000	4.77E+06
FP WC Inf	9/20/95	8.6	0.78	20.6	0.21	NA	3	<2	<2	<2	•			•	<10³	3+	neg.	neg.	pos.	<100	8.16E+05
FP WC Eff	9/20/95	8.9	0.01	21	0.94	NA	0	0	0	NA	•			•	<10³	3+	neg.	neg.	neg.	neg.	1.50E+06
FP WC RS	9/20/95	8.0	2.08	ND	ND	NA	660	9	<2	<2	•			•	<10³	3+	pos.	<100	pos.	>1000	2.19E+06
FP S Eff	9/20/95	8.5	0.02	21	1.36	NA	0	0	0	NA	•			•	>10³	3+	neg.	neg.	pos.	>1000	1.43E+06
FP S RS	9/20/95	7.7	1.72	21	<0.05	NA	850	140	<2	<2	•			•	>10³	3+	neg.	neg.	pos.	>1000	8.16E+05
FP USL Inf	9/20/95	8.0	0.32	ND	<0.05	NA	3400	7	<2	<2	•			•	>10³	4+	pos.	<100	pos.	>1000	2.40E+06
RB TW	9/20/95	7.2	0.27	14	NA	NA	310	<2	<2	<2	•	•		•	<10³	3+	neg.	neg.	pos.	>1000	1.43E+06
FP WC Inf	10/4/95	8.6	0.78	20.6	0.21	NA	3	<2	<2	<2	•			•	>10³	4+	neg.	neg.	pos.	>1000	5.83E+05

NWRI Legionella Project

Cumulative Data Sheet

Chronologically Sorted

Sample Description		pH	Turbidity	Temp	Chlorine		HPC	Coliform*			LegionelND Analyses									
Source	Date		NTU	°C	Free	Total	cfu/mL	Total	Fecal	*E. Coli*	PCR**				Concentration	DFA	LegionelND*	LegionelND	HPC	HPC	AODC
					mg/L	mg/L					L	p	-	+	$>10^3$ or $<10^3$***	score	DFA/CTC	cells/ml	CTC	cells/ml	cells/mL
FP WC Eff	10/4/95	8.9	<0.01	21	0.94	NA	0	0	0	0	•			•	$<10^3$	4+	neg.	neg.	pos.	>1000	2.04E+05
FP WC RS	10/4/95	8.0	2.08	ND	ND	NA	660	9	<2	<2	•			•	$>10^3$	3+	neg.	neg.	pos.	<100	2.04E+06
FP S Eff	10/4/95	8.5	0.02	21	1.36	NA	0	0	0	0	•			•	$<10^3$	4+	neg.	neg.	pos.	>1000	4.08E+05
FP S RS	10/4/95	7.7	1.72	21	<0.05	NA	850	140	<2	<2	•			•	$>10^3$	4+	neg.	neg.	pos.	>1000	8.16E+05
FP USL Inf	10/4/95	8.0	0.32	ND	NA	<0.10	3400	7	<2	<2	•	•		•	$>10^3$	3+	neg.	neg.	pos.	>1000	2.27E+05
RB TW	10/4/95	7.2	0.27	14	NA	NA	310	<2	<2	<2	•			•	$>10^3$	4+	pos.	<10	pos.	>1000	5.10E+05
MSJWTP TW	10/17/95	7.7	0.05	18.1	0.80	1.74	<1	<1	NA	+	•			•	$<10^3$	3+	neg.	neg.	pos.	>1000	1.02E+05
MSJWTP SBW	10/17/95	7.3	3.51	16.7	0.03	0.19	<10	<1	NA		•	•		•	$>10^3$	3+	neg.	neg.	pos.	>1000	1.02E+06
WTP2 RW	10/17/95	7.7	5.24	17.4	0.03	0.03	360	10	NA		•			•	$>10^3$	3+	neg.	neg.	pos.	>1000	4.08E+06
WTP2 TW	10/17/95	8.5	0.08	18.4	1.27	1.41	<1	<1	NA		•	•		•	$>10^3$	3+	neg.	neg.	pos.	>1000	1.02E+05
WTP2 SBW	10/17/95	7.3	2.08	17.0	0.01	0.03	28560	<1	NA		•			•	$>10^3$	4+	neg.	neg.	pos.	>1000	1.02E+06
SFWD	10/17/95	9.2	0.33	13.2	1.00	1.10	<1	<1	NA		•	•		•	$>10^3$	3+	neg.	neg.	pos.	>1000	4.53E+05
M	10/17/95	7.6	0.05	15.9	0.01	0.01	<1	<1	NA		•			•	$<10^3$	3+	neg.	neg.	pos.	>1000	4.08E+05
PT	10/17/95	7.5	0.09	16.7	0.05	0.07	<1	<1	NA		•			•	$<10^3$	3+	neg.	neg.	pos.	>1000	1.02E+05
K16	10/17/95	7.1	0.14	19.5	0.43	0.43	<1	<1	NA					•	negative	(-)	neg.	neg.	pos.	>1000	2.04E+05
K35	10/17/95	8.4	0.19	20.1	0.27	0.33	<1	<1	NA		•			•	$>10^3$	3+	neg.	neg.	pos.	>1000	3.40E+05
K41	10/17/95	8.2	0.15	19.3	1.06	1.06	<1	<1	NA		•	•		•	$>10^3$	3+	pos.	<100	pos.	>1000	1.96E+05
FP WC Inf	10/18/95	8.7	0.34	19	0.22	NA	1	<2	<2	<2	•			•	$>10^3$	3+	neg.	neg.	pos.	>1000	5.10E+05
FP WC Eff	10/18/95	8.9	0.04	19	0.79	NA	0	0	0	0				•	negative	(-)	neg.	neg.	pos.	>1000	3.40E+05
FP WC RS	10/18/95	7.9	3.7	19	NA	0.12	140	2	<2	<2	•			•	$>10^3$	3+	neg.	neg.	pos.	>1000	8.16E+05
FP S Eff	10/18/95	8.5	0.08	19	0.87	NA	0	0	0	0	•			•	$<10^3$	4+	neg.	neg.	pos.	>1000	2.04E+05
FP S RS	10/18/95	7.6	0.74	NA	NA	NA	14000	80	<2	<2	•			•	$>10^3$	4+	pos.	>100	pos.	>1000	8.16E+05
FP USL Inf	10/18/95	7.8	0.53	20	NA	0.1	240	4	2	2	•			•	$>10^3$	4+/3+	neg.	neg.	pos.	>10	4.08E+05
RB TW	10/18/95	8.3	0.22	19	NA	<0.1	38	11	<2	<2	inhibited				inhibited	4+	neg.	neg.	pos.	>1000	5.83E+05
MSJWTP TW	11/7/95	8.4	0.06	16.7	0.09	1.70	<1	<1	NA	-				•	negative	(-)	neg.	neg.	pos.	>1000	5.83E+05
MSJWTP SBW	11/7/95	7.7	0.85	15.2	0.03	0.13	<1	<1	NA	-	•			•	$<10^3$	4+	neg.	neg.	pos.	>1000	4.08E+05
WTP2 RW	11/7/95	7.6	5.20	16.1	0.02	0.03	230	20	NA	+	•			•	$>10^3$	4+	neg.	neg.	pos.	>1000	5.10E+05
WTP2 TW	11/7/95	8.6	0.06	16.6	1.48	1.58	<1	<1	NA	-	•			•	$<10^3$	4+	neg.	neg.	pos.	>100	4.08E+05
WTP2 SBW	11/7/95	7.1	2.70	15.2	0.01	0.02	>5500	>9	NA	+	•			•	$>10^3$	4+	neg.	neg.	pos.	>1000	2.04E+05
SFWD	11/7/95	8.9	0.32	13.4	1.03	1.11	1	<1	NA	-	•			•	$<10^3$	3+	pos.	<100	pos.	>1000	5.10E+05
M	11/7/95	7.6	0.05	16.5	0.02	0.03	<1	<1	NA	-				•	negative	(-)	neg.	neg.	neg.	neg.	4.80E+05
PT	11/7/95	7.6	0.06	17.1	0.02	0.02	5	<1	NA	-				•	negative	(-)	neg.	neg.	pos.	>1000	4.80E+05
K16	11/7/95	7.4	0.12	20.0	0.24	0.27	<1	<1	NA	-				•	negative	(-)	neg.	neg.	pos.	>1000	2.04E+05
K35	11/7/95	8.0	0.20	17.0	0.55	1.11	<1	<1	NA	-	•			•	$<10^3$	3+	neg.	<100	pos.	>1000	4.08E+05
K41	11/7/95	8.3	0.13	19.0	0.52	1.64	<1	<1	NA	-	•			•	$<10^3$	(-)	neg.	neg.	pos.	>1000	3.40E+05
FP WC Inf	11/8/95	8.6	0.34	17.1	0.2	NA	1^p	0^	0^	NA	•			•	$<10^3$	4+	neg.	neg.	neg.	neg.	4.08E+06
FP WC Eff	11/8/95	8.7	0.03	17.6	0.85	NA	0	0	0	0				•	negative	(-)	neg.	neg.	neg.	neg.	5.10E+05
FP WC RS	11/8/95	7.9	9.3	16.6	0.24	NA	ND	17	<2	<2	inhibited				inhibited	3+	neg.	neg.	pos.	>10	8.16E+05
FP S Eff	11/8/95	8.4	<0.01	NA	NA	NA	0	0	0	0				•	negative	3+	neg.	neg.	pos.	>100	8.16E+05
RB TW	11/8/95	8.3	0.25	16	0.09	NA	ND	8	ND	ND	•			•	$<10^3$	4+	neg.	neg.	pos.	>100	1.02E+06

APPENDIX B
Presentations:

Arvik, T., M. Shahamat[1], C. Thio, R. Sakaji, H. Lai and C. Paszko-Kolva. 1996. PCR detection and viability assessment studies for *Legionella* species in potable water supplies. ASM Annual Meeting, New Orleans, LA., 19-23 May.

Lye, D., M. Sparks, H. Cook, T. Arvik, and C. Paszko-Kolva. 1996. Efficacy of nucleic acid decontamination procedures by PCR. ASM Annual Meeting, New Orleans, LA., 19-23 May.

Sakaji, R. H., C. Paszko-Kolva, C. Thio, H. H. Lai, and T. Arvik. 1996. The detection of *Legionella* species in potable water supplies using ground and surface waters. AWWA, WQTC Conference, Boston, Mass. Nov. 18-21.

PCR Detection and Viability Assessment of *Legionella* Species in Potable Water Samples.

T. ARVIK[1], M. SHAHAMAT[2], C. THIO[1], R. SAKAJI[3], H. LAI[4] & C. PASZKO-KOLVA*[1].
[1]Perkin-Elmer, Applied Biosystems Division, Foster City, CA, [2]University of Maryland Biotechnology Institute, Baltimore, MD, [3]Alameda County Water District, Fremont, CA, and [4]East Bay Municipal Utility District, Alameda, CA.

Twenty San Francisco Bay Area municipal water system sampling sites were tested for *Legionella* species via polymerase chain reaction (PCR), direct fluorescent antibody staining (DFA) and culture on buffered charcoal yeast extract agar amended with α-ketoglutarate and antibiotics (BCYE-α). Of the water samples studied, greater than 80% were positive for *Legionella* species by the PCR method. Approximately 15% of the *Legionella* positive samples were identified as *L. pneumophila* by PCR. PCR results correlated closely to DFA staining results whereas none of the *Legionella* species detected in the water samples were culturable on selective and non-selective BCYE-α agar. Samples were analyzed by PCR using the Perkin-Elmer EnviroAmp® Legionella Kit. DFA staining was performed on concentrated samples (10-20X) using black polycarbonate 0.22 μm filter membranes and a polyclonal antibody specific for *L. pneumophila* serogroups 1-7 and 6 additional *Legionella* species. Viability of all HPC bacteria and *Legionella* was monitored in municipal waters by simultaneous INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride) and DFA staining. Viability of two environmental *L. pneumophila* strains in tap water was studied through weekly monitoring of duplicate, mono-culture microcosms, each inoculated with $1X10^7$ cells per mL. Cell numbers decreased one order of magnitude within the first week and stabilized thereafter. INT viability staining was performed in conjunction with plate counts to determine the decrease in viability/culturability over time.

Efficacy of Nucleic Acid Decontamination Procedures by PCR. DENNIS LYE[1,2]*, MELVIN SPARKS[2], HAROLD COOK[2], TOREY ARVIK[3], and CHRISTINEPASZKO-KOLVA[3]. [1]Northern Kentucky University, Highland Heights, KY., [2]National Exposure Research Laboratory, Environmental Protection Agency, Cincinnati, OH., [3]Perkin-ElmerApplied Biosystems Division, Foster City, CA 94404.
Because PCR-based methods detect signals from culturable, viable but non-culturable, and nonviable sources, decontamination of nucleic acid probe signal from sample preparation apparatus contaminated by *Legionella pneumophila* was monitored. *Legionella pneumophila* cells treated with ultraviolet light, chlorine, hydrogen peroxide, heat, alcohol, and iodine were monitored under laboratory conditions using the EnviroAmp[R] Legionella PCR Kit for detection of residual DNA. The complete absence of cellular DNA signal was achieved by exposure to a 2% sodium hypochlorite solution for one hour at 60 C. Three month-old microcosms containing *Legionella pneumophila* (10^7cells/ml) suspended in sterile tap water were also exposed to sodium hypochlorite, hydrogen peroxide, and autoclaving treatments. After *5*, *30*, *60*, and *120* minute exposure times, samples were collected and analyzed by culture on BCYE, PCR and gel electrophoresis. Nucleic acid probe signal (at 10^5 cells/ml) was completely removed by a combination of exposures to hydrogen peroxide and autoclaving.

Abstract for AWWA Water Quality Technology Conference 1996

The Detection of *Legionella* species in Potable Water Supplies using Ground and Surface Water Sources

Sakaji, R.H.; Paszko-Kolva, C.; Thio, C.; Lai, H.H.; Arvik, T.

Background: Early water system surveys for *Legionella* served to illustrate the ubiquitous nature of this organism. The public health concerns raised by this as well as other organisms has prompted Federal and State agencies to promulgate water quality regulations, i.e., the Surface Water Treatment Rule (SWTR), to control the occurrence of this organism in potable water supplies.

Objective: The means for evaluating the effectiveness of the SWTR with respect to *Legionella* is not addressed in the regulation. A five month survey of two water utilities in the San Francisco Bay Area was conducted in the fall of 1995 to demonstrate the applicability of PCR technology for surveying *Legionella* sp. and to provide further insight on the occurrence and ecology of *Legionella* in drinking water.

Findings/Results: The results from this survey study reemphasize the ubiquitous nature of *Legionella* sp. in source, finished, and recycled backwash water. The findings also compare the results gathered from the PCR to traditional, more time consuming immunofluorescent antibody staining and *Legionella* culturing methods. Traditional microbiological water quality parameters of coliform counts, heterotrophic plate counts, and acridine orange direct counts were also determined.

Although the surveys did indicate the presence of *Legionella* sp. and *Legionella pneumophila* in the finished water, public health statistics on legionellosis in one service area showed no reported cases during the period of study and no change in the number of cases from the previous year. Similar results were observed in the morbidity statistics for the second service area. There was no significant change in the number of legionellosis cases between 1994 and 1995 with an incidence rate of about half of the national average. (0.6 per 100,000).

Relevance to Industry: Properly used, PCR technology in conjunction with conventional Legionella cultural techniques and a disease surveillance program can provide utilities with an improved means of monitoring current levels of water treatment. Use of PCR may provide an analytical methodology for the semiquantitative enumeration of *Legionella* missing from the SWTR. Therefore, PCR may provide a means of evaluating the effectiveness of water treatment processes in removing this pathogen.

APPENDIX C

Explanation of Results by Culture, DFA and PCR

Summary of Hypothetical Results Possible by PCR, DFA, and Culture Methods

	L[1]	Conc.	p[2]	Conc.	-[3]	+[4]	Interpretation	DFA[5]	DFA[6]	Culture[7]	Species	Agreement
	5S	cells/ml	*mip*	cells/ml			of PCR Result	L.p. 1-6	poly	BCYEα	Recovered	
I	+	$=10^3$	+	$=10^3$	-	+	*L. pneumophila*	+	-	Positive	*L. pneumophila*	Excellent
	+	$=10^3$	-	NA	-	+	*Legionella* sp.	-	+	Positive	*Legionella* sp.	Excellent
	+	$>10^3$	+	$>10^3$	-	+	*L. p. + Legionella* sp.	+	+	Positive	*L. p. + Legionella* sp.	Excellent
	-	NA	-	NA	-	+	Negative	-	-	Negative	Negative	Excellent
II	+	$=10^3$	-	NA	-	+	*Legionella* sp.	-	+	Negative	NA	Good
	+	$=10^3$	+	$=10^3$	-	+	*L. pneumophila*	+	-	Negative	NA	Good
	+	$>10^3$	+	$<10^3$	-	+	*L. p. + Legionella* sp.	+	+	Negative	NA	Good
III	-	NA	-	NA	-	+	Negative	-	+	Positive	*Legionella* sp.	Fair
	+	$<10^3$	-	NA	-	+	*Legionella* sp.	-	-	Positive	*Legionella* sp.	Fair
	+	$=10^3$	-	NA	-	+	*Legionella* sp.	-	+	Positive	*L. pneumophila*	Fair
	+	$=10^3$	+	$=10^3$	-	+	*L. pneumophila*	+	-	Positive	*Legionella sp.*	Fair
IV	+	$<10^3$	+	$<10^3$	-	+	*L. pneumophila*	-	+	Negative	NA	Poor
	+	$>10^3$	-	NA	-	+	*Legionella* sp.	+	-	Negative	NA	Poor
	-	NA	-	NA	-	+	Negative	-	-	Positive	*Legionella sp.*	Poor
V	-	NA	-	NA	-	-	Inhibition	-	-	Variable	Variable	Unknown
	-	NA	-	NA	-	-	Inhibition	+	+	Variable	Variable	Unknown
	-	NA	-	NA	-	-	Inhibition	+	-	Variable	Variable	Unknown
	-	NA	-	NA	-	-	Inhibition	-	+	Variable	Variable	Unknown

[1]"L" represents the "L" dot on the nylon membrane of the EnviroAmp PCR kit which contains the 5S rRNA sequence of *Legionella*.
[2]"p" represents the "p" dot on the nylon membrane of the EnviroAmp PCR kit and represents the *mip* gene sequences.
[3]"-" represents the negative control that contains a one base mismatch to th esequence in the PCR product created from the amplification of the internal positive control.
[4]"+" represents an internal positive control which consists of a synthetic DNA sequence that is co-amplified with the primers used for the *mip* gene which is included in the reaction mix.
[5]DFA L.p. 1-6 represents direct fluorescent antibody (DFA) staining employing anti-*L. pneumophila* serogroups 1-6 antisera.
[6]DFA poly represents direct fluorescent antibody staining employing a polyvalent conjugate containing, *L. bozemannii, L. jordanis, L. micdadei, L. longbeachae, L. gormanii,* and *L. dumoffii.*
[7]Culture indicates plating on BCYEα (buffered charcoal yeast extract agar amended with α-ketoglutarate and antibiotics) with heat or acid treatment, incubated in 2.5% CO2 at 37 C for up to 3 weeeks.

POSSIBLE EXPLANATIONS FOR DISCREPANCIES

I Excellent Agreement

In these situations, there was excellent agreement between all three techniques, polymerase chain reaction (PCR), direct fluorescent antibody staining (DFA), and culture.

II Good Agreement

In these three hypothetical situations, both PCR and DFA results are in agreement, in one instance the PCR was positive for at least two species of *Legionella*, which was confirmed by DFA results. However, the culture results were uniformly negative. There are several explanations for these culture results, discounting laboratory error, the *Legionella* cells may be dead, injured, due to harsh environmental conditions (disinfection), or they may have entered a viable but non-culturable state (refs).

III Fair Agreement

In these four situations, two of the three techniques are in agreement. In the first situation PCR is negative, yet culture and DFA were positive for *Legionella*. One possible explanation is that the Legionella cells may have been present at concentrations below the detection limit of PCR (~10 cell/ml). The suggested sample volume for concentration and analysis for the PCR kit is 10 ml, and ca. 200 ml for DFA. In the second instance, PCR and culture results are in agreement yet DFA is negative. The negative DFA result may have been due to shielding of the *Legionella* by debris. In the third and fourth instances, PCR and DFA results are in agreement, however culture results yielded different species than detected by PCR and DFA. In these instances, the species (detected by PCR and DFA) may have been dead, injured, or non-culturable. The species detected by culture may have been sequestered within protozoa subsequently shielding cells from detection by PCR and DFA.

IV Poor Agreement

In the first two instances, PCR and DFA results are not in agreement and culture results are negative. There are several possible explanations, first, the antisera used in the DFA may have cross-reacted with the non-legionellae cells in the sample or the primers used in the PCR may have cross-reacted (refs). However, these instances are rare and typically cross-reacting species can be recovered by culture and re-tested and discrepancies defined.

V Agreement Unknown

In this hypothetical group, the PCR was inhibited due to the presence of iron, humic or other substances which inhibit the taq polymerase. An analogous situation exists in DFA staining with high turbidity samples, when excessive shielding by debris occurs. However, inhibition and shielding typically occurs in less than 5% of samples. In addition clean-up procedures have been developed to remove inhibitors of PCR and DFA dilution procedures exist.