Effects of culture conditions on the susceptibility of Legionella pneumophila to iodine disinfection
by Kari Lisa Cargill

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Microbiology
Montana State University
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Abstract:
Control of Legionella pneumophila in drinking water systems is dependent on an accurate assessment of the efficacy of the chosen disinfectant at concentrations acceptable for use in potable water. Susceptibility of L. pneumophila to disinfecting agents is strongly affected by culture conditions under which it is grown. Thus agar-grown cultures may exhibit a different sensitivity than those grown in an environment more closely resembling that found in natural and manmade water systems. The goal of this study was to examine the effects of various growth conditions on the susceptibility of L. pneumophila to iodination.

L. pneumophila cultures were grown in well water, on rich agar media, in coculture with amoebae, and attached to stainless steel surfaces. Legionellae grown in water cultures in association with other microorganisms were less sensitive to disinfection by chlorine and iodine than were agar-passaged cultures. Differences in sensitivity to disinfection between water-cultured and agar-grown legionellae were determined by comparing CxT values and molar CxT values (mCxT). There was a greater difference in CxT values when the disinfecting agent was iodine (1500x) as compared with chlorine (68x). Iodine was 50x more effective than chlorine when used in agar-grown cultures but was only twice as effective when tested against water-grown Legionella cultures. CxTxS values, which take into consideration the percent surviving bacteria, were used to compare sensitivities in very resistant populations such as those found in a biofilm. These comparisons showed legionellae associated with stainless steel surfaces were 135x more resistant than were unattached legionellae and were 210,000x less sensitive than were agar-grown cultures. These results indicate that the conditions under which legionellae are grown can dramatically affect their susceptibility to some disinfectants and must be considered when evaluating the efficacy of a disinfecting agent.

EFFECTS OF CULTURE CONDITIONS ON THE SUSCEPTIBILITY OF

LEGIONELLA PNEUMOPHILA TO IODINE DISINFECTION

by

Kari Lisa Cargill

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APPROVAL

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Kari Lisa Cargill

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

Control of _Legionella pneumophila_ in drinking water systems is dependent on an accurate assessment of the efficacy of the chosen disinfectant at concentrations acceptable for use in potable water. Susceptibility of _L. pneumophila_ to disinfecting agents is strongly affected by culture conditions under which it is grown. Thus agar-grown cultures may exhibit a different sensitivity than those grown in an environment more closely resembling that found in natural and manmade water systems. The goal of this study was to examine the effects of various growth conditions on the susceptibility of _L. pneumophila_ to iodination.

L. pneumophila cultures were grown in well water, on rich agar media, in coculture with amoebae, and attached to stainless steel surfaces. Legionellae grown in water cultures in association with other microorganisms were less sensitive to disinfection by chlorine and iodine than were agar-passaged cultures. Differences in sensitivity to disinfection between water-cultured and agar-grown legionellae were determined by comparing CxT values and molar CxT values (mCxT). There was a greater difference in CxT values when the disinfecting agent was iodine (1500x) as compared with chlorine (68x). Iodine was 50x more effective than chlorine when used in agar-grown cultures but was only twice as effective when tested against water-grown _Legionella_ cultures. CxTxS values, which take into consideration the percent surviving bacteria, were used to compare sensitivities in very resistant populations such as those found in a biofilm. These comparisons showed legionellae associated with stainless steel surfaces were 135x more resistant than were unattached legionellae and were 210,000x less sensitive than were agar-grown cultures. These results indicate that the conditions under which legionellae are grown can dramatically affect their susceptibility to some disinfectants and must be considered when evaluating the efficacy of a disinfecting agent.

INTRODUCTION

There are several important factors to be considered when determining the efficacy of a disinfecting agent for use in a water system. These include the type of disinfectant, the microorganisms being tested, culture conditions under which the microorganisms are grown, the presence or absence of other microorganisms in the water system and biofilm formation.

This study concerns the use of iodine as a disinfectant in water systems. Iodine has been used in drinking water but has been primarily limited to a small scale or emergency treatment (Black et al., 1968; Gottardi, 1983). The National Aeronautics and Space Administration (NASA) has used anion exchange resins containing iodine on the Space Shuttle (Marchin et al., 1985; Taylor et al., 1970). The water system is "fill and draw" with a short duration time of about seven days on the shuttle. In addition, NASA is planning to use these resins on Space Station Freedom in conjunction with other methods to disinfect potable water and recycled wastewater (Colombo and Greenley, 1980). During the extended lifetime of this vehicle in space, water will be recycled and stored. This will require effective disinfection within the water system.

Bacteria belonging to the family Legionellaeceae deserve special attention since they are ubiquitous in water systems and grow under oligotrophic conditions (Wadowsky et al., 1982). _Legionella_ spp. are also the causative agent of Legionnaires disease and the less serious Pontiac fever. Since legionellosis appears to be spread by the airborne transmission of legionellae it could pose a special threat in the microgravity environment of operational spacecraft where aerosols from liquids may be a problem.

There are now 34 recognized species within the genus _Legionella_ as well as several serotypes (Winn, 1988). This organism was first isolated in 1976 following an outbreak of pneumonia in Philadelphia at an American Legion convention (Fraser et al., 1977).

Legionnaires disease, the pneumonic form, is characterized by a relatively long incubation period of 2-10 days, with a low attack rate (less than 1-4%), and a 15-20% fatality rate (Fraser and McDade, 1979). In contrast, Pontiac fever has a short incubation period averaging 36 hours, recovery within 48 hours, a high attack rate (up to 95% of persons exposed) and is a nonfatal flu-like illness (Winn, 1988). Pontiac fever may attack healthy adults but legionellosis usually only affects persons with predisposing factors. These may

include being over 50 years of age, heavy smoking or drinking, or being immunocompromised (Barbaree et al., 1987; Meyer, 1983). The disease is not spread person to person, rather its mode of transmission is through aerosols containing the bacterium. It is treatable with antibiotics such as erythromycin. Reasons for the existence of two different disease states caused by legionellae are unclear. Theories include differences in host susceptibility, a possible hypersensitivity reaction to protozoa associated with legionellae, or different species and strains of Legionella (Fields et al., Abstr. Annu. Meet. Am. Soc. Microbiol., 1990).

Legionellae are freshwater bacteria which are gram-negative rods of 2-6 um in length and are fastidious with unusual nutrient requirements. This accounts for the difficulty in isolating these organisms. Legionellae can obtain all carbon and energy requirements from just nine amino acids (Ristroph et al., 1980; Tesh and Miller, 1982; Warren and Miller, 1979). The media used to isolate Legionella spp. are supplemented with ferric pyrophosphate and L-cysteine. Growth is aerobic and occurs over a wide pH and temperature range. Legionella spp. have been isolated from waters ranging from 6-65 C with 35 C being optimal for growth and survival. The presence of unusual fatty acids seen in other thermophilic bacteria indicates

a possible adaptation to higher temperatures (Moss et al.,
1977).   Legionellae are resistant to pH as low as 2
although the optimal pH when grown on artificial media is
6.9 (Fliermans et al., 1981; Wadowsky et al., 1985).

Legionella has been detected in a wide range of water
including both natural and manmade systems. Lakes,
rivers, puddles, and even soil are environments that
support growth of legionellae (Barbaree et al., 1987).
Cooling towers have been implicated in a number of
outbreaks of legionellosis but other manmade systems are
also good reservoirs for the bacterium (England et al.,
1982). These may include faucets, showerheads, hot water
tanks, humidifiers, whirlpools and swimming pools (States
et al., 1987a; Stout et al., 1985; Wadowsky et al., 1982,
Witherell et al., 1988).

The species of Legionella most commonly associated
with disease is L. pneumophila although others have been
implicated in human disease. Several species of
Legionella have been isolated from environmental sites
that have never been implicated in a disease situation and
so may not be virulent for man. Conversely, there are
some clinical Legionella spp. that have not been isolated
from any water system. This may be due to the difficulty
in isolating legionellae from water where they may be
present in very low numbers. A recent study indicates

that viable but nonculturable legionellae might occur in some aquatic systems and may cause these bacteria to be underreported (Hussong et al., 1987).

Isolation procedures for retrieving _Legionella_ spp. from water systems have mainly focused on perfecting selective media. The base of most of these media is yeast extract with charcoal added (Feeley et al., 1979). In addition to solid media, a broth has been developed which does not require the addition of charcoal (Ristroph et al., 1980). All of these media contain L-cysteine and ferric pyrophosphate. Improvements have included the addition of a buffering agent (Pasculle et al., 1980), antibiotics such as vancomycin and polymyxin B (Edelstein and Finegold, 1979; Edelstein, 1981), and the dyes bromthymol blue and bromocresol purple (Vickers et al., 1981). These media are selective for _Legionella_ spp. and exclude many other bacteria which may be present in water samples.

In order to enhance recovery of _Legionella_ spp. and ensure the elimination of other bacteria other methods have been employed. These include treating the sample with heat or acid prior to plating. Both methods destroy other waterborne bacteria but legionellae are more resistant (Bopp et al., 1981; Dennis et al., 1984).

Membrane filtration has been used to concentrate

samples in which Legionella spp. are present in low numbers (Gorman et al., 1983; Orrison et al., 1981). A direct fluorescent antibody test is also available to aid in identification of Legionella cultures (Cherry et al., 1978) although there can be cross-reactions with several common water bacteria such as Flavobacterium spp., Pseudomonas spp. and Alcaligenes spp. which appear to share similar surface antigenic determinants (Tison and Seidler, 1983). Despite these advances there are still considerable problems in isolating legionellae from some water sources.

The control of Legionella spp. in water systems is difficult for many reasons. Since it is ubiquitous in fresh water, erradication is unrealistic and most experts agree that it is not necessary. Legionellae can survive in hot water systems as they tolerate higher temperatures than do most other water bacteria. Studies show that Legionella spp. also have a high tolerance for some disinfecting agents, including chlorine. This characteristic aids in their persistence after traditional treatments have eliminated other organisms (Kuchta et al., 1983; Skaliy et al., 1980).

Evidence that Legionella spp. interact with other microorganisms indicates another possible protective mechanism. Some of the microorganisms which have been

associated with enhanced growth and survival of legionellae include other bacteria, cyanobacteria, algae and protozoa (Hume and Hann, 1984; Tison et al., 1980). Studies show that _Legionella_ spp. can grow in satellite formation around nonlegionellae bacterial colonies, including _Flavobacterium_ spp., _Pseudomonas_ spp., _Alcaligenes_ spp. and _Acinetobacter_ spp., when grown on L-cysteine-deficient media (Stout et al., 1985; Wadowsky and Yee, 1983). It has been suggested that the other bacteria provide L-cysteine which the legionellae require for growth. This indicates that it may be possible for legionellae to obtain nutrients from other organisms in the environment but this has not been demonstrated in natural water systems. Other studies demonstrated stimulation of growth of legionellae when algal extracts were added to the culture (Bohach and Snyder, 1983; Pope et al., 1982). Again, this cross-feeding has not been shown to occur in the natural environment. Some bacteria, such as pseudomonads, rather than enhancing growth appear to be inhibitory to _Legionella_ spp. The association between free-living protozoa and _Legionella_ spp. may most closely approximate what actually occurs in water systems. Legionellae have been shown to multiply intracellularly within amoebae and ciliated protozoa (Barbaree et al., 1986; Fields et al., 1984; Fields et al., 1986; Rowbotham,

1984; Wadowsky et al., 1988). In addition, protozoa have been isolated from water implicated in outbreaks of legionellosis although it has not been demonstrated that any naturally-occurring protozoa harbored intracellular legionellae. Nonetheless, interactions such as these are likely to occur in natural water systems where legionellae are found and may confer additional resistance to disinfection, heat and low pH. These interactions may also help explain the pathogenesis of _Legionella_ spp. which cause an intracellular infection; growing within macrophages in the lungs and monocytes in the blood (Eisenstein, 1987).

Disinfection schemes which are most successful against _Legionella_ spp. include hyperchlorination and heat treatment. Chlorine levels must be higher than those normally used to disinfect potable water (up to 2 ppm) (Hsu et al., 1984; Winn, 1988) and the water temperature must be at least 70 C for several days (Wadowsky et al., 1982; Wadowsky et al., 1985) to achieve appreciable disinfection. Either treatment alone is not as effective as when used together and there is usually regrowth of _Legionella_ spp. shortly following treatment. Other treatments which have proven to be somewhat successful include the use of ozone (Domingue et al., 1988; Edelstein et al., 1982; Muraca et al., 1987) or ultraviolet light

(Dutka, 1984; Knudson, 1985; Muraca et al., 1987). Since legionellae are resistant to low pH but are fairly sensitive to higher pH levels, it has been recommended that large facilities such as cooling towers be maintained at an elevated pH (States et al., 1987b).

Legionellae are more resistant to several disinfecting agents than some other waterborne bacteria (Gerba et al., 1987; Kuchta et al., 1983). These bacteria include some coliforms which are used as indicator organisms to determine bacteriological water quality. Thus, elimination of the indicator bacteria may not guarantee the absence of legionellae from a water system.

Conditions under which waterborne bacteria are grown can be an important factor in determining disinfection susceptibility. Previous studies have shown that organisms grown under nutrient-limited conditions may be less sensitive to certain disinfectants than are the same bacteria cultivated in richer media (Brown and Williams, 1985; Carson et al., 1978). The species and strain of the organism being studied may determine whether this holds true. For instance, Pyle and McFeters (1989) found most strains of Pseudomonas exhibited an increased sensitivity to iodine disinfection when grown under nutrient limitation while one strain became less sensitive.

L. pneumophila has been shown to be much more

resistant to chlorination when grown in water culture than when cultivated in a rich medium (Kuchta et al., 1985). This may be due to a difference in nutrient conditions, growth rate, physiological changes or a reflection of interactions occuring between the legionellae and other microorganisms present in the water culture.

Another important consideration in water disinfection is the occurrence of biofilms. Bacteria, including legionellae, associated with surfaces may be significantly less susceptible to antimicrobial agents than planktonic cells (Costerton et al., 1987; LeChevallier et al., 1984; LeChevallier et al., 1988; Pyle and McFeters, 1990). Therefore, it is important to simulate the conditions under which a disinfectant will be used in order to accurately predict effectiveness in the field.

The goal of this study was to determine the effects of various culture conditions on the susceptibility of Legionella spp. to iodination. These conditions included the level of nutrients in the growth medium, presence or absence of other microorganisms, and attachment to stainless steel surfaces. This study focused on Legionella spp. since it is a potential pathogen which is commonly found in water systems and whose susceptibility to iodine disinfection was not known. Legionellae were grown in water cultures in an attempt to determine

sensitivity to iodine in conditions similar to those found
in actual water systems rather than in pure cultures under
highly artificial laboratory conditions.

MATERIALS AND METHODS

Media

Differential Glycine-vancomycin-polymyxin B Agar (DGVP)

The selective and differential medium DGVP (Wadowsky et al., 1981) was used to plate Legionella cultures. This medium allows growth of legionellae while inhibiting growth of most other water bacteria. L. pneumophila colonies grown on DGVP have a characteristic cut glass appearance and a bluish tint. These colonies were unable to grow on unsupplemented buffered yeast extract agar which confirms their identification as legionellae.

The formulation for this medium is in Table 1.

Buffered Charcoal Yeast Extract Agar (BCYE)

BCYE (Pasculle et al., 1980) was used to grow pure cultures of Legionella since it is not selective for legionellae in mixed culture. The formulation is equivalent to DGVP but without the dyes, antibiotics and glycine.

Unsupplemented Buffered Charcoal Yeast Extract Agar (UNBCYE)

UNBCYE (Feeley et al., 1979) was used to distinguish

between legionellae and nonlegionellae bacteria. It is similar to BCYE but lacks the L-cysteine and ferric pyrophosphate which are essential for the growth of *Legionella* spp. Thus, colonies which grown on UNBCYE are not legionellae. Growth on BCYE and inability to grow on UNBCYE are indicative of *Legionella* spp.

Table 1. Differential glycine-vancomycin-polymyxin B agar

Yeast extract	10.0 g
ACES buffer	10.0 g
Glycine	6.0 g
Activated charcoal	2.0 g
Distilled water to	1.0 l

Adjust pH to 6.9 with 1 N KOH.

Agar	17.0 g
Bromthymol blue	0.001 %
Bromcresol purple	0.001 %

Autoclave 121 C, 15 min.
Filter sterilize the following and add:

L-cysteine	0.4 g
Ferric pyrophosphate	0.25 g
Polymyxin B sulfate	100 U/ml
Vancomycin	5 ug/ml

Yeast Extract Broth (YEB)

YEB (Ristroph et al., 1980) was utilized for growth of legionellae in a liquid medium other than water. It consists of yeast extract, ACES buffer, L-cysteine and ferric pyrophosphate in the amounts used in the DGVP medium. These components are added to distilled water, pH adjusted and filter sterilized through a 0.22 um filter.

R2A

R2A (Reasoner and Geldreich, 1985) medium was prepared as directed by the manufacturer (Difco). This medium was used for growth of heterotrophic plate count (HPC) bacteria from water cultures.

Freshwater Amoeba Agar (FWA)

FWA is recommended by ATCC for growth of Hartmanella vermiformis. It contains: yeast extract (0.1 g), malt extract (0.1 g), agar (10.0 g) and distilled water (1 liter) and is adjusted to pH 7.

Pseudomonas Agar F

This medium was used to grow P. aeruginosa cultures isolated from water cultures. It consists of: tryptone (10.0 g), proteose peptone (10.0 g), dipotassium phosphate (1.5 g), magnesium sulfate (1.5 g), agar (15 g) and distilled water (1 liter) at pH 7.

Culture water

Tap water was suggested to maintain Legionella water cultures (Yee and Wadowsky, 1982). Water from the Bozeman water system was consistently unable to support viable Legionella cultures. Since copper sulfate in the water can pose a problem (States, 1985), water analysis was done by the Chemistry Station Laboratory, Montana State University. No copper sulfate was detected in our

samples. Tap water aged in sunlight for several weeks retained a slight chlorine residue and also failed to maintain the legionellae water cultures. Other attempts to condition the water included boiling for five minutes, pasteurizing at 60 C for 30 min, and autoclaving at 121 C for 15 min; none of these methods was successful. When well water from a home situated in the Gallatin Canyon was substituted, consistent growth of L. pneumophila was achieved. Gallatin Canyon well water was thus used for water cultures and as a diluent for disinfection experiments throughout the study. Creek water was also used and allowed growth of legionellae for a limited number of culture transfers.

Cultures

Water Cultures

Water cultures containing L. pneumophila serogroup 1 and other unidentified water microorganisms were obtained from the Department of Water, Pittsburgh, PA. The water cultures were maintained in well water which had been filtered through a 0.22 um membrane filter (Number GSWPO47SO, Millipore, Bedford, MA). Cultures were transferred every 21 days (when growth was in late-exponential to early-stationary phase) by inoculating 5 ml aliquots of the culture into 95 ml of the filtered well

water and incubating at 35 C in the dark.

Plate-grown Cultures

Agar-passaged cultures were obtained as colonies grown from water cultures on the selective DGVP medium. Legionellae were identified by their ability to grow on DGVP and BCYE, inability to grow on UNBCYE, by a direct fluorescent antibody test (SciMedX, Denville, NJ; Cherry et al., 1978), and by their characteristic colony morphology when grown on DGVP. Stock cultures of these organisms were suspended in a solution of 50% glycerol/50% yeast extract broth (vol/vol) and stored at -70 C.

Broth Cultures

L. pneumophila cultures were also maintained in YEB. Attempts to grow legionellae in dilute broth solutions (10% and 1%) were only partially successful.

Cocultures

Cocultures of legionellae were grown in association with hartmanellid amoebae as recommended by others (Navratil et al., Abstr. Annu. Meet. Am. Soc. Microbiol., 1990; Kellogg, Abstr. Annu. Meet. Am. Soc. Microbiol., 1990). L. pneumophila (ATCC number 33152) was grown on DGVP plates. The protozoan Hartmanella vermiformis (ATCC number 30966) was grown on FWA. Each culture was harvested from the plates using filtered well water,

centrifuged (5000 X g for 10 min), and the pellet was resuspended in well water. These suspensions were used to inoculate 100 ml filtered well water to obtain a final concentration of approximately 1500 amoebae/ml and 1500 legionellae/ml. The cocultures were kept at room temperature.

Pseudomonads

A heterotrophic bacterial strain was isolated from the water cultures obtained from Pittsburgh after a number of transfers with well water. This strain was able to grow on the selective DGVP medium and was inhibitory to the growth of L. pneumophila. The isolate was identified by an API/NFT test strip (API Analytab Products, Plainview, NY) as Pseudomonas aeruginosa. It was maintained on Pseudomonas F agar and was used in biofilm experiments.

Disinfecting Agents

Chlorine Solution

Chlorine solutions were prepared as described by LeChevallier et al. (1988). A 5% sodium hypochlorite solution was used to prepare stock solutions and concentrations were measured by amperometric titration (APHA, 1989).

Iodine Solution

Iodine solutions were prepared as described by others (Pyle and McFeters, 1989). Iodine concentrations were determined by amperometric titration (APHA, 1989). No chlorine or iodine demand was detected in the disinfection test systems.

Sodium Thiosulfate Solution

The sodium thiosulfate used to neutralize chlorine and iodine was prepared using distilled water to make a 10% solution and filter sterilized through a 0.22 um filter (Millipore).

Disinfection Protocol

Water cultures were disinfected two to three weeks following a culture transfer when legionellae were in exponential phase and numbers were between 10^3 and 10^5 cfu/ml. Chlorine or iodine was added directly to these water cultures.

Agar-grown legionellae were scraped from DGVP plates, suspended in filtered well water and centrifuged (5000 X g for 10 min). Cells were washed three times in this manner and resuspended in well water at a density of 10^5 to 10^6 cells/ml. Direct microscopic observation revealed monodispersed bacterial suspensions. Disinfectant was then added.

Cocultures were established using legionellae and hartmanellid amoebae in filtered well water which were allowed to grow for three days before disinfectant was added.

Disinfection experiments were done using 50 ml samples of cultures in a 100 ml Erlenmeyer flask held at room temperature (20-25 C), pH 6.8 to 7.2. Samples were taken at timed intervals over a one hour period and the disinfectant neutralized with sodium thiosulfate (0.01% final concentration) (APHA, 1989). Appropriate dilutions were made with sterile distilled water blanks and 0.1 ml samples were spread onto DGVP plates in three replicates. Plates were incubated at 35 C and counts were made when Legionella colonies were apparent (5-7 days).

Heterotrophic plate count (HPC) bacteria were monitored during disinfection experiments by plating samples on R2A medium and incubating at 35 C overnight.

Biofilm Formation and Disinfection

Water Cultures

Disinfection experiments were performed with biofilms of water-grown L. pneumophila on stainless steel (316) coupons. Coupons (12 X 76 mm) were placed in 400 ml water cultures in a biofilm apparatus (Pyle and McFeters, 1990) with a magnetic stirrer. All coupons were parallel to the

water flow. Biofilms were allowed to form for three days before iodination. Both coupons and water suspensions were sampled at various timed intervals following disinfection. Coupons were removed, placed in sterile sodium thiosulfate (0.01%) for five minutes and rinsed with sterile water to remove unattached cells. The biofilm was scraped from the coupon into 10 ml sterile water using a rubber scraper and shaken vigorously for about 10 seconds. Appropriate dilutions of both the scraped biofilm cells and the water culture suspension were prepared with sterile distilled water and spread onto DGVP plates in triplicate samples. Viable counts were made after incubating at 35 C for 5-7 days. Coupons that had been scraped were stained with acridine orange (Hobbie et al., 1977) and observed through an epifluorescence microscope to ensure that the majority of cells were removed by the scraping procedure.

Broth Cultures

Broth cultures were also used for biofilm disinfection experiments as a rich-grown sample. Legionellae were inoculated into tubes of YEB and allowed to grow at 35 C until the broth was turbid (5 days). Fifty ml of this suspension was added to 350 ml sterile distilled water to achieve approximately 5 X 10^4 cfu/ml. This was stirred for four hours in the biofilm apparatus

distilled water to achieve approximately 5 X 10^4 cfu/ml.
This was stirred for four hours in the biofilm apparatus
and cells were allowed to attach to the stainless steel
coupons. In order to avoid chlorine demand by the organic
material in the medium, the broth suspension was removed
and sterile distilled water was added to the biofilm jar.
Disinfectant was added and coupons were scraped at timed
intervals as described previously. Dilutions of the
scraped cells were made and plated onto DGVP medium for
enumeration.

Pseudomonas biofilm

.The P. aeruginosa culture isolated from water
cultures was grown on Pseudomonas Agar F. Cells were then
washed from the plates, suspended in well water,
centrifuged and sonicated. Some of this suspension was
added to 400 ml well water in a biofilm apparatus to a
final concentration of approximately 10^6 cfu/ml. Samples
of the suspension and scrapings from the coupons were made
over 72 hours to follow the development of the biofilm and
the survival of the pseudomonads in water culture.

Heat Treatment

Heat treatment was used to eliminate other
heterotrophic bacteria from water cultures and cocultures
prior to disinfection since growth of several of these

organisms was inhibitory to legionellae on DGVP plates. Prior to disinfection, water cultures were placed in a 50 C water bath for 30 minutes. This removed all of the contaminating bacteria while L. pneumophila survived, although at slightly lower numbers. Cultures were allowed to cool to room temperature before disinfectant was added. To determine the effect of this treatment on disinfection susceptibility of L. pneumophila, iodination was performed on unheated controls and on heat-treated water cultures. In order to discover whether heat had any lasting effects on sensitivity of legionellae to disinfection, some heat-treated cultures were allowed to recover for six days at 35 C (the temperature normally used to maintain these cultures).

Fluorescent Antibody Test

A commercially prepared direct fluorescent antibody (DFA) (SciMedX, Denville, NJ) was used to microscopically examine bacteria grown on plates, in water cultures and in biofilms. Better results were obtained with pure cultures of L. pneumophila since the DFA can cross-react with several organisms found in natural water samples. These may include pseudomonads, flavobacteria and Alcaligenes spp. (Tison and Seidler, 1983).

CxT Value Determination

Concentration (mg/L) multiplied by time (min) to achieve 99% reduction in cfu of disinfected samples (CxT_{99} value) was used as a method to compare various results. Since CxT_{99} values do not take into account the difference in molarity between similar concentrations (in mg/L) of chlorine and iodine, molar CxT_{99} values ($mCxT_{99}$) were used to compensate for these differences. Each CxT_{99} value was divided by the molecular weight of the disinfecting agent used to obtain the $mCxT_{99}$ value to be used in comparisons between these two disinfectants. At neutral pH the predominant forms of the halogens was hypochlorous acid ($HOCl^-$) and iodine (I_2) (Dychdala, 1983) so molecular weights used in calculations was 35 and 253 respectively.

In some instances 99% kill was not achieved so it was not possible to calculate the CxT_{99} values. In order to compare these results with findings in other experiments CxTxS values were calculated. This value is the product of the CxT value multiplied by the percent surviving bacteria.

RESULTS

Disinfection Experiments

Chlorine and iodine were used to disinfect cultures of L. pneumophila grown under various conditions to determine their susceptibility to these disinfectants. Legionellae grown on DGVP agar plates, in well water cultures, in broth, in cocultures and attached to stainless steel surfaces were exposed to the disinfectants.

Chlorine

Chlorination of L. pneumophila was used as a standard by which to compare subsequent experiments using iodine disinfection. These results (Fig. 1) show that when this organism was grown under oligotrophic conditions in well water it was markedly less sensitive to chlorination than following cultivation on a rich agar medium.

Iodine

Disinfection studies were performed using various levels of iodine. A concentration of 16 ppm (or mg/L) iodine was required before more than a 2 log decrease in

viability was observed in a one hour contact period with the water-grown bacteria (Fig. 2). Low concentrations (less than 4 ppm) of iodine caused an increase in L. pneumophila colony forming units as compared with an untreated control. At 8 ppm a biphasic curve was seen with relatively rapid initial disinfection followed by a plateau.
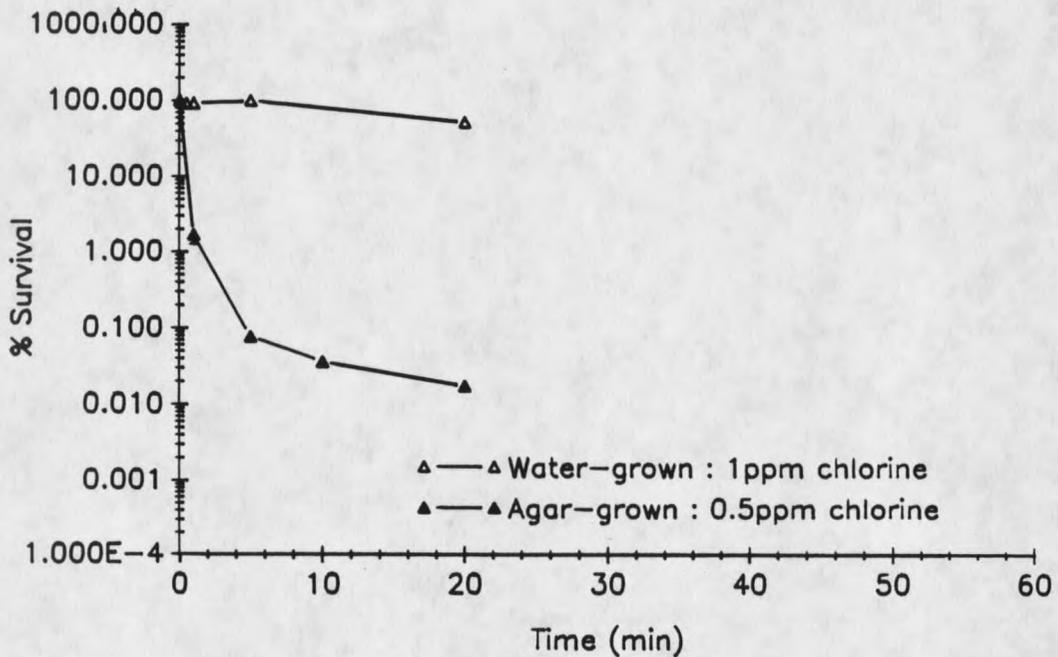

Figure 1. Chlorination of water-grown versus agar-grown L. pneumophila.
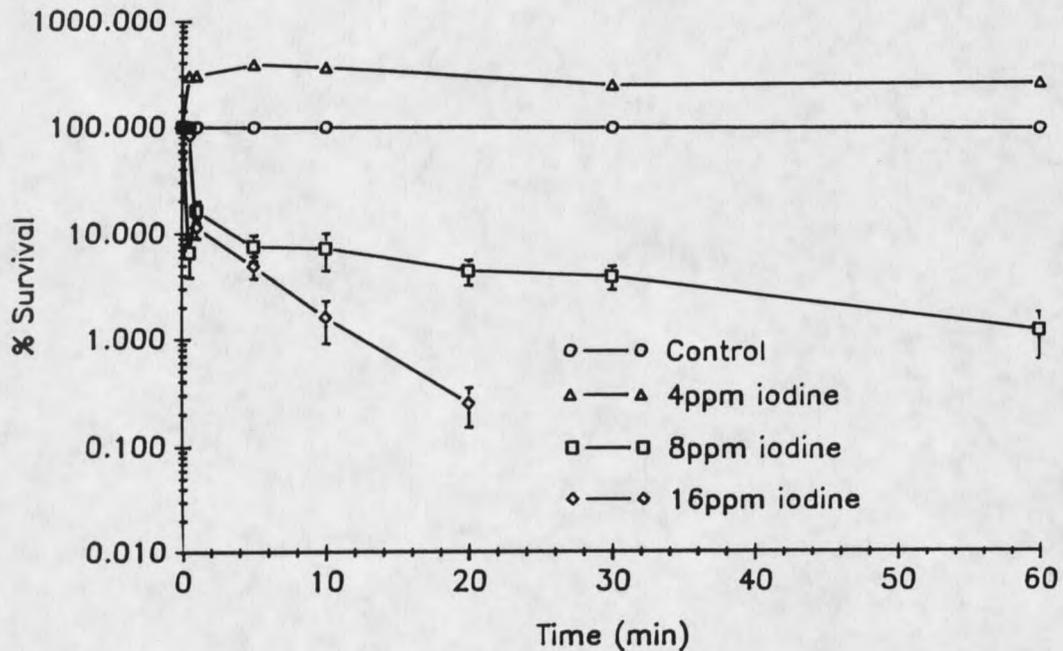
Figure 2. Iodination of water-grown L. pneumophila at
various concentrations.

Differences in sensitivity to iodine were determined
in both water-grown and agar-grown cultures. The results
(Fig. 3) indicate that there was a greater distinction
than was seen with chlorination (Fig. 1). Water-grown
cultures were much more resistant to a concentration of 16
ppm iodine than were agar-grown legionellae exposed to 0.5
ppm iodine. Water-grown cultures were not affected by a
concentration of only 0.5 ppm (data not shown).
Disinfection of water cultures over longer time periods
(up to 48 hours) at 8 ppm iodine also failed to eliminate
all legionellae (Fig. 4).