Effects of culture conditions and biofilm formation on the iodine susceptibility of *Legionella pneumophila*

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The susceptibility of *Legionella pneumophila* to iodination was studied with cultures grown in well water, on rich agar media, and attached to stainless-steel surfaces. *Legionella pneumophila* 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 $C \times T$ values (concentration in milligrams per litre multiplied by time in minutes to achieve 99% decrease in viability) and $C_M \times T$ values (concentration in molarity). Iodine (1500 x) gave a greater difference in $C_M \times T$ values than did chlorine (68 x). Iodine was 50 times more effective than chlorine when used with agar-grown cultures but was only twice as effective when tested against water-grown *Legionella* cultures. $C \times T \times S$ values ($C \times T$ multiplied by percent survivors), which take into consideration the percent surviving bacteria, were used to compare sensitivities in very resistant populations, such as those in biofilms. Water cultures of legionellae associated with stainless-steel surfaces were 135 times more resistant to iodination than were unattached legionellae, and they were 210 000 times more resistant 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.

Key words: *Legionella pneumophila*, iodine, disinfection, growth conditions, biofilms, water.


La sensibilité à l'iode de *Legionella pneumophila* a été étudiée à l’aide de cultures placées dans de l’eau de puits, sur gélose riche et attachées à des surfaces d’acier inoxydable. Les *L. pneumophila* ont été moins sensibles à la désinfection par le chlore et l’iode pour les cultures dans l’eau de puits, en association avec d’autres microorganismes, que pour les cultures repiquées sur gélose. Les différences de sensibilité à la désinfection entre les légionelles cultivées dans l’eau et les légionelles cultivées sur gélose ont été déterminées en comparant les valeurs de $C \times T$ (concentration en milligrammes par litre multipliée par le temps en minutes pour atteindre une réduction de la viabilité de 99%) et les valeurs $C_M \times T$ (concentration en molarité). L’iode (1500 x) a donné une plus grande différence que le chlore (68 x) en valeurs $C_M \times T$. L’iode a été 50 fois plus efficace que le chlore lorsqu’il a été utilisé contre des cultures placées sur gélose, mais seulement 2 fois aussi efficace lorsqu’il a été vérifié contre les *Legionella* cultivées dans de l’eau. Les valeurs de $C \times T \times S$ ($C \times T$ multipliée par le pourcentage de survivantes) qui prennent en considération le pourcentage de bactéries survivantes, ont été utilisées pour comparer les sensibilités de populations très résistantes comme celles qui se retrouvent dans les biofilms. Les cultures en milieu aqueux de légionelles associées à des surfaces en acier inoxydable ont été 135 fois plus résistantes à l’iode que les légionelles libres, et 210 000 fois plus résistantes que les cultures sur gélose. Ces résultats indiquent que les conditions sous lesquelles sont cultivées les légionelles peuvent affecter dramatiquement leur sensibilité à certains désinfectants. Ces conditions doivent être considérées au moment d’évaluer l’efficacité d’un désinfectant.

*Mots clés*: *Legionella pneumophila*, iodine, désinfection, conditions de croissance, biofilms, eau.

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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.

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Bacteria belonging to the family Legionellaceae deserve special attention, since they are ubiquitous in water systems and grow under oligotrophic conditions (Colbourne and Dennis 1989; Lee and West 1991; Wadowsky et al. 1982). Legionella spp. are also the causative agents of Legionnaires' disease and the less serious Pontiac fever (Fraser et al. 1977). Since legionellosis appears to be spread by the airborne transmission of legionellae, it could pose a special threat in the closed microgravity environment of operational spacecraft, where aerosols from liquids may be a problem.

Legionellae are more resistant to several disinfecting agents than some other waterborne bacteria (Landeen et al. 1989; Kuchta et al. 1983). These other bacteria include some coliforms that are used as indicator organisms to determine bacteriological water quality. Thus, elimination of indicator bacteria may not guarantee the absence of legionellae from a water system.

Conditions under which waterborne bacteria are grown can determine disinfection susceptibility. 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. 1972, 1978). The species and strain of the organism being studied may determine whether this holds true. For instance, Pyle and McFeters (1989) found that one strain of Pseudomonas exhibited increased sensitivity to iodine disinfection when grown under nutrient limitation, whereas another strain became less sensitive.

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

**Legionella pneumophila** can utilize nutrients produced by other microorganisms such as bacteria (Stout et al. 1985; Wadowsky et al. 1982), algae (Hume and Hann 1984; Pope et al. 1982; Tison et al. 1980). They grow intracellularly within amoebae and ciliated protozoa (Barbace et al. 1986; Fields et al. 1984; Rowbotham 1984; Tyndall and Domingue 1982). These interactions are likely to occur in natural water systems and may confer additional resistance to disinfection.

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, 1988; Pyle and McFeters 1990). Therefore, it is important to simulate the conditions under which a disinfectant will be used 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 **L. pneumophila** to iodination. These conditions included the level of nutrients in the growth medium and attachment to stainless-steel surfaces. We attempted to determine whether legionellae grown in water cultures were more resistant to
iodination than those grown on plates of an enriched medium and whether legionellae attached to stainless-steel coupons were more resistant to disinfection with iodine.

**Materials and methods**

**Cultures**

Water cultures containing *L. pneumophila* serotype 1 and other unidentified water microorganisms were obtained from the Department of Water, Pittsburgh, Pa. The water cultures were maintained in well water that had been filter-sterilized through a 0.22-µm membrane filter (catalogue No. GSWP047SO, Millipore Corp., Bedford, Mass.). Cultures were incubated at 35°C in the dark and transferred every 21 days, when in log phase growth, by inoculating 5-mL portions into 95 mL of filtered well water.

Agar-passaged cultures were obtained as colonies grown from water cultures on differential glycine–vancomycin–polymyxin B agar (DGVP) medium (Wadowsky and Yee 1981). Legionellae were identified by their ability to grow in unsupplemented buffered charcoal–yeast extract agar (UNBCYE) (Wadowsky et al. 1982), and by a direct fluorescent antibody test (Cherry et al. 1978; SciMedX, Denville, N.J.).

**Chlorine and iodine solutions**

Chlorine and iodine solutions were prepared as reported by others (LeChevallier et al. 1988; Pyle and McFeters 1989). Halogen concentrations were determined by amperometric titration (American Public Health Association 1985). No chlorine or iodine demand was observed in the disinfection test systems.

**Disinfection protocol**

Water cultures were disinfected 2–3 weeks following a culture transfer, when legionellae were actively growing, based on plate counts (data not shown), and numbers were between 10^2 and 10^5 cfu/mL. Chlorine in the form of sodium hypochlorite (1 ppm final concentration) or iodine (16 ppm final concentration) was added directly to these water cultures. The selective DGVP medium allowed isolation of *Legionella* spp. and excluded the growth of other bacteria present, with the exception of a strain of *Pseudomonas aeruginosa*. Growth of this bacterium on DGVP was inhibitory to *L. pneumophila*. Heat treatment of the water cultures (50°C for 30 min) eliminated the pseudomonads, while the legionellae persisted, although at lower numbers.

Agar-grown legionellae were scraped from DGVP plates, suspended in filter-sterilized well water, and centrifuged (5000 × g for 10 min). Cells were washed three times in this manner and resuspended in well water at a density of 10^7–10^8 cfu/mL. Direct microscopic observations revealed monodispersed bacterial suspensions. Disinfectant was then added.

Disinfection experiments were done using 50-mL samples in 100-mL Erlenmeyer flasks stirred magnetically at room temperature (20–25°C), pH 6.8–7.2. Samples were taken at timed intervals over a 1-h period, and the action of the disinfectant was stopped with sodium thiosulfate (0.01% final concentration; American Public Health Association 1985). Appropriate dilutions were made with sterile distilled-water blanks, and 0.1-mL samples were plated on DGVP medium in three replicates. Cultures were incubated at 35°C and *Legionella* colonies were counted when visible (5–7 days). Variability was determined by calculating the standard deviation of the mean.

**Biofilm apparatus**

The vessel used was a wide-mouth pint Mason jar (Kerr). Instead of the regular sealing lid, a disk was cut out of 1 mm thick stainless steel, and a circular arrangement of six holes (16 mm in diameter) was drilled in it (Fig. 1a) to accommodate No. 1 rubber stoppers. A cut across the base of each stopper allowed a metal coupon to be suspended inside the jar. The stoppers were inserted to bring each coupon parallel to the wall of the jar (Fig. 1b) and thus parallel to the circulation of culture medium. The jar, containing the culture and a magnetic stir bar, was placed on a magnetic stirrer. The culture was stirred at ca. 300 rpm while incubating at room temperature for up to 3 days.

Biofilms were formed on flat coupons of mechanically polished 316 stainless steel (12 × 76 mm). Coupons, prepared as described previously (Pyle and McFeters 1990), were removed from the apparatus as required, and the rubber stopper was replaced to allow continued incubation under aseptic conditions.

**Biofilm disinfection**

After incubation in the biofilm apparatus, coupons and water suspensions were removed for disinfection. Coupons were placed in sterile sodium thiosulfate (0.01%) and rinsed with sterile water to remove unattached cells. The biofilm was scrapped, using a rubber scraper, from each coupon into 10 mL sterile distilled water and plated on DGVP medium for enumeration as above. Coupons that had been scraped were stained with acridine orange (Hobbie et al. 1977) and observed through an epifluorescence microscope to ensure that most cells were removed.

**Results**

Chlorine and iodine were used to disinfect cultures of *L. pneumophila* grown under various conditions to deter-
mine their susceptibility to these conditions. Legionellae grown on DGVP plates, in well-water cultures, or attached to stainless-steel surfaces were exposed to the disinfectants.

Chlorination of *L. pneumophila* was used as a standard against which iodine disinfection was compared in subsequent experiments. When grown under oligotrophic conditions in well water, this organism was markedly less sensitive against which iodine disinfection was compared in subsequent experiments. When grown under oligotrophic conditions in well water, this organism was markedly less sensitive to chlorination than following cultivation on a rich agar medium (Fig. 1).

Disinfection studies were performed using various levels of iodine. A concentration of 16 mg iodine per litre was required with the water-grown bacteria before more than a 2 log decrease in viability was observed in a 1-h contact period (Fig. 3). Low concentrations (less than 4 mg/L) of iodine caused an increase in *L. pneumophila* colony-forming units as compared with an untreated control. Differences in sensitivity to iodine were determined in both water-grown and agar-grown cultures. Comparison of Figs. 2 and 4 indicated a greater distinction than was seen with chlorination. Water-grown cultures were much more resistant to 16 mg iodine/L than were agar-grown legionellae exposed to 0.5 mg iodine/L. Disinfection of water cultures over longer times (up to 48 h) at 8 mg iodine/L also failed to eliminate all legionellae (data not shown).

In disinfection experiments using the biofilm system (Fig. 5), attached organisms were less susceptible to 16 mg iodine/L than were unattached legionellae. In addition, *L. pneumophila* in suspension within the biofilm system were less sensitive to 16 mg iodine/L than those in water cultures where no biofilm system was present (see Fig. 4).

*C × T* values were calculated for the time (minutes) it took to achieve 99% reduction in viability at a particular concentration of disinfectant. As shown in Table 1, the *C × T* value for water-grown legionellae was 68 times that of agar-grown legionellae when chlorine was the disinfectant, whereas there was a 1500-fold difference when iodine was used.

Molar *C × T* values (*Cₘ × T*) were used to obtain comparable data on the effects of chlorine and iodine. *C × T* values did not take into account the difference in molarity between similar concentrations (mg/L) of chlorine and iodine. To compensate for these differences we divided each *C × T* value by the molecular weight of the halogen used. For agar-grown legionellae, iodine appeared to be 50 times more effective than chlorine (Table 1). The difference with water-grown cultures was less dramatic, with iodine being about twice as effective as chlorine in eliminating *L. pneumophila*.

Iodination of biofilms did not achieve 99% kill (see Fig. 5); therefore, *C × T* values could not be calculated for attached or suspended cells. To compare these results with findings in other experiments we calculated *C × T × S* values (Table 2), which are the product of the *C × T* value multiplied by the percent of cells surviving at that time. Attached legionellae had *C × T × S* values 2.8 times greater than those in suspension within the biofilm apparatus. Suspended cells were 48 times more resistant to iodine than those in water cultures where no stainless steel-associated biofilm was present. *C × T × S* calculations also revealed that attached cells were 135 times more resistant than water-grown legionellae and approximately 210 000 times more resistant to iodine than agar-grown *L. pneumophila*.

**Discussion**

The conditions under which *L. pneumophila* was grown affected its susceptibility to iodine disinfection. We also examined the susceptibility of the cultures to chlorine, since this halogen is a more common water disinfectant and provided a comparison with subsequent iodination experiments. The observation that legionellae grown in water cultures containing other microorganisms without added nutrients were more resistant to chlorine than those grown on a rich nutrient medium is similar to those reported by Kuchta et al. (1985). In addition, our results demonstrated a parallel effect of culture conditions with iodine as the disinfecting agent. Cultures grown on enriched nutrient media were significantly more sensitive to iodine than water cultures.

Low concentrations of iodine initially caused an increase in *L. pneumophila* colony-forming units as compared with a control water culture. Possible explanations for this phenomenon may be the dispersal of legionellae from aggregates, release from intracellular growth within protozoa, or the activation of some protective physiological mechanism (Matin et al. 1989). However, microscopic examination
using direct fluorescent antibodies failed to show any evidence of bacterial aggregation.

There have been many studies showing intracellular growth of legionellae within various protozoa (Barbaree et al. 1986; Fields et al. 1984; Tyndall and Domingue 1982). Protozoa have also been isolated from water in which legionellae have been detected (Barbaree et al. 1986). They may be natural hosts for Legionella spp. in water systems, and the thicker cell envelopes and cyst stages found with protozoa may contribute to their reduced susceptibility to many disinfecting agents. The increase in L. pneumophila numbers at low concentrations of iodine may have been due to the release of intracellular legionellae from damaged protozoa. Higher concentrations of disinfectant may have killed extracellular legionellae before they could be detected. Although studies reporting the susceptibility of common water protozoa generally have shown them to be resistant to levels of disinfectants used in water treatment (King et al. 1988), they may be more sensitive to iodination.

The resistant fraction of bacteria detected over the course of the experiments may cause a biphasic survivor curve for L. pneumophila and Escherichia coli treated with chlorine dioxide (Berg et al. 1988). Therefore, the plateau portion of the disinfection curves may be due to a resistant subpopulation of legionellae in these experiments.

Another aspect of this study was to determine whether L. pneumophila formed biofilms on stainless steel and whether this would affect their susceptibility to iodine disinfection because of the importance of attached bacteria in the disinfection of potable water systems (LeChevallier et al. 1988). Most viable cells in chlorinated water systems are attached to surfaces (Ridgway and Olson 1982). Since water cultures containing other water microorganisms in addition to the legionellae were used in these experiments, the resulting biofilm was also a mixed culture.

Our results do not establish whether L. pneumophila would attach to stainless-steel surfaces in the absence of these other organisms. However, pure cultures of agar-grown cells were able to form biofilms (data not shown). Attached water-grown legionellae were significantly more resistant to iodination than legionellae in water cultures where no stainless-steel coupons were present. This agrees with other studies that have shown organisms associated with biofilms to be more resistant to disinfection (Costerton et al. 1987; LeChevallier et al. 1984, 1988; Pyle and McFeters 1990). Possible explanations for this enhanced resistance may include protection due to attachment and aggregation on surfaces, protective extracellular material, or differences in the physiology of attached organisms. Also, legionellae in suspension within the biofilm apparatus were more resistant to iodination than were those where no biofilm was apparent. This effect has been reported previously (Pyle and McFeters 1990) and may be attributed to cells that have been sloughed from the surface but retain some of the protective mechanisms suggested for attached bacteria. This may explain the observation that Legionella spp. often recolonize water systems following treatment by hyperchlorination or superheating (Muraca et al. 1987).

Direct comparisons of disinfecting agents were difficult when only \( C \times T \) values were used because it was not always possible to achieve 99% lethality with the disinfectant concentrations used. When \( C \times T \) data cannot be obtained for resistant cultures such as biofilms, \( C \times T \times S \) values, which take into account the percent surviving bacteria, may be useful. These calculations show that attached water-grown legionellae were 135 times more resistant to iodination than those where no biofilm was present on stainless-steel coupons, and about 210,000 times more resistant than agar-grown cultures. Since water-grown and attached legionellae may be particularly insensitive to halogenation, alternative disinfection strategies such as electrochemically produced Cu and Ag ions may be appropriate in combination with iodination or chlorination (Landeen et al. 1989; Pyle et al. 1991).

An approach used to quantify differences observed was to substitute molar concentration for weight/volume in \( C \times T \) calculations to obtain an alternative assessment of the relative efficacy of iodine versus chlorine in treatment of Legionella cultures. Iodine was found to be 50 times more effective than chlorine in treating agar-grown legionellae and twice as effective against water cultures. Davis (1962) also noted that iodine may be more effective than chlorine, even though the latter is more reactive than iodine. Apart from a simple oxidizing effect, it is possible that differences in molecular form, persistence, or binding to cell structures may be involved in the relative efficacies of these halogens (Davis 1962).

Culture conditions affected the susceptibility of L. pneumophila to iodination. Agar-grown cultures were much more sensitive than water-grown cultures, which were in turn, markedly more sensitive than legionellae associated with biofilms. These findings suggest that L. pneumophila was more susceptible to iodination as compared with chlorination, especially in agar-grown cultures. The results indicate that disinfection experiments using Legionella spp. grown on rich media may not accurately predict the effectiveness of iodine and other disinfectants against legionellae grown
in water systems or attached to surfaces. Therefore, data for these culture conditions should be included in the evaluation of disinfectant efficacy since they are representative of the ambient microbiological environment within potable water systems.

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