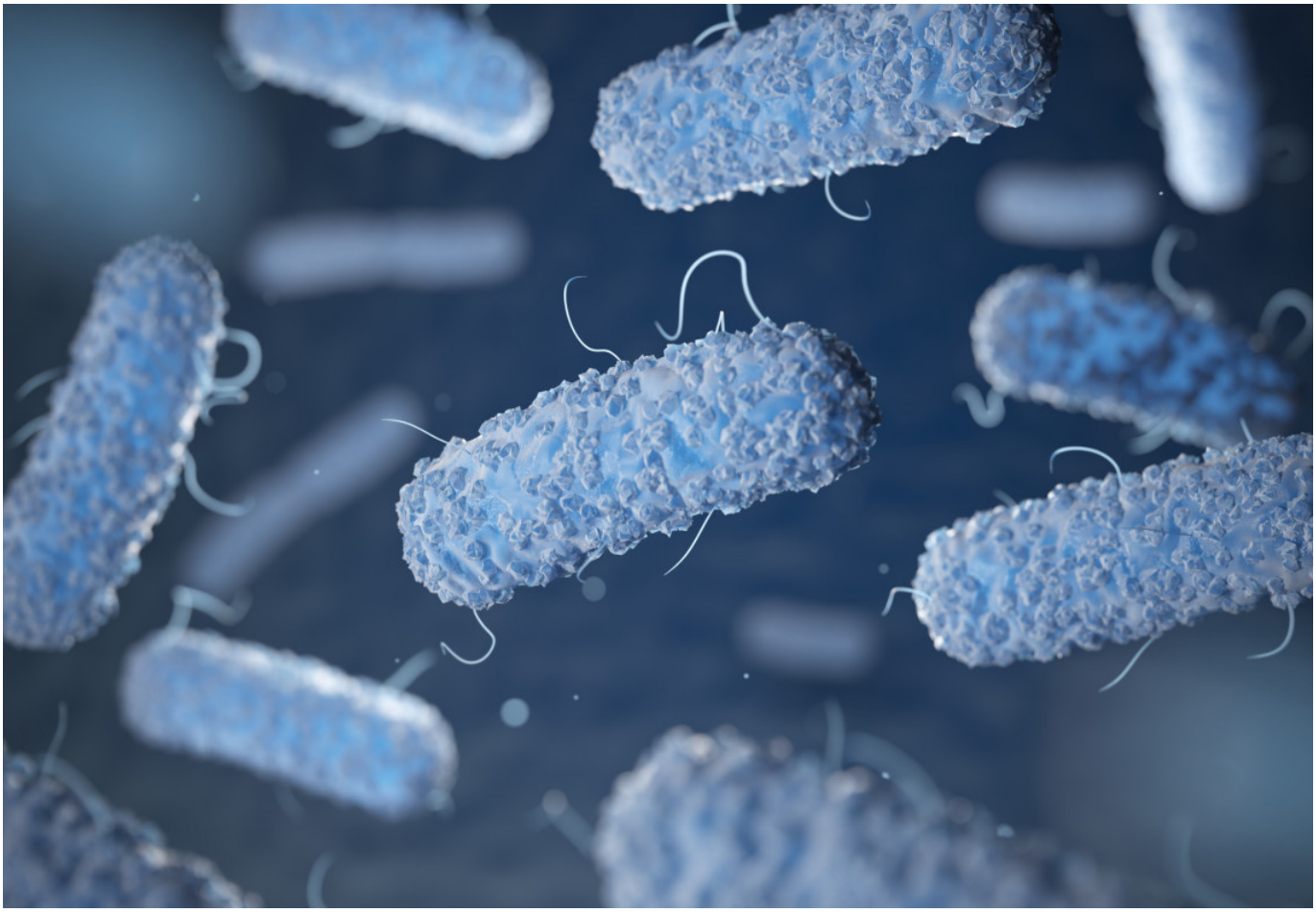
JC 9465

Legionella – A New Solution for the 21st Century



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JC 9465 CAN BE USED TO INACTIVATE LEGIONELLA IN LESS THAN 10 SECONDS. IT IS A SUPERIOR TECHNOLOGY- VERY EFFECTIVE AT ELIMINATING A MAJORITY OF WATERBORNE PATHOGENS QUICKLY.

1 BACKGROUND

1.1 LEGIONELLA BACTERIA

Legionella are natural inhabitants of water and can be detected in rivers, lakes, and streams. They are characterized as gram-negative, aerobic, unencapsulated bacilli. Currently, fifty Legionella bacteria species have been identified and 20 of them are known to be associated with human infection.

The major source of Legionella is water distribution systems of large buildings and cooling towers. Other sources include misting machines, humidifiers, whirlpool spas, fountains and hot springs. It can be transmitted by aspiration of contaminated water, use of respiratory-therapy equipment and inhalation of aerosol, when the bacteria are present.

Legionella was first discovered following a pneumonia outbreak at the 1976 Convention of the American Legion in Philadelphia. This onset of antibiotic resistant pneumonia was named Legionnaire's disease. There are two forms of Legionellosis, Pontiac fever, characterized by flu-like symptoms and the more severe, often lethal form of pneumonia, Legionnaires' Disease. The majority of human infections are caused by the species Legionella pneumophila.

1.2 CURRENT METHODS TO CONTROL LEGIONELLA

Legionella are relatively resistant to standard water disinfection procedures. They tend to grow in biofilms or slime, are difficult to penetrate, and are also protected by their symbiotic relations with other microorganisms. They are not eradicated by the conventional chlorination used to purify domestic water systems. Low and even non-detectable levels of the organism can colonize a water source and grow to high concentrations under the right conditions (See Additional Information-Legionella vs Chlorine).

The chemical treatments recommended for Legionella control include chlorine, chlorine dioxide, mono-chloramine, ozone and copper/silver ionization. Other, non-chemical treatments are filtration, heat and Ultra Violet radiation (UV).

The above alternatives can cause unwanted issues for the potable water system such as, corrosion, pitting of piping, disinfection byproducts that pose a health risk, gas leaks, taste/odor, and/or fall short of providing residual protection against recolonization.

Chlorine based chemicals, except for Chlorine Dioxide, are not effective at removing biofilm and cannot eliminate biofilm-associated pathogens. These chemicals cannot control recolonization. Chlorine Dioxide though, is slow reacting and needs plenty of contact time to attack biofilms.

Ozone has a short life and cannot protect the distribution system more than a few minutes. UV does not provide a residual disinfectant that can be measured. Both treatments need an application of chlorine residual.

Copper/silver ionization is effective at deactivating Legionella and biofilm. However, it renders insufficient reactivity as the ions travel further away from the electrodes that release them into the water flow. This treatment is not reliable in fast running water. The presence of chlorine can neutralize silver ions. Some microorganisms can become resistant. Metal precipitation needs to be filtered and little is known about health effects of long-term exposure to this methodology.

2 INTRODUCTION

JC 9465 IS AN ADVANCED OXIDATION FORMULA OF OXYGEN CHELATED MINERALS STABILIZED IN A WATER BASED SOLUTION.

Oxidation is the chemical reaction where an electron is taken. It transforms the physio-chemical properties of the oxidized matter.

When oxidation reaction takes place, the chemistry of the substance or microorganism that loses the electron is disrupted and cannot exist in its previous form.

Oxidants attract electrons and initiate oxidation.

At the same time there is a gain of electrons by oxidation, there is also a loss of electrons by reduction. This is known as oxidation-reduction reaction, or REDOX for short. Reductants are prompt to donate electrons to the oxidants.

Advanced oxidation was discovered in 1894 and first proposed for water treatment in the 1980s.

In a general sense, Advanced Oxidation Processes (AOPs), are chemical treatment procedures that catalyze oxidants and intensify the results.

In advanced oxidation processes, catalysts are a source of energy that helps the reagent act faster, stronger, and amplify the chain reaction. Most common catalysts are Ultra Violet Radiation (UV), light, heat, electricity and mineral salts. Oxidant reagents in advanced oxidation are ozone (O_3), Oxygen (O_2) and Hydrogen Peroxide (H_2O_2).

The selection of the reagent and the catalyst are first, a consideration of the unwanted materials and microbiological life that need to be removed from the water, and second, the feasibility and cost of the system. Because of the high reactivity of

advanced oxidation processes, the reagents and catalysts are combined and applied in situs at the time of use.

3 TECHNOLOGY

JC 9465 IS A SECOND-GENERATION ADVANCED OXIDATION PROCESS THAT DELIVERS CLEAN, PURIFYING ENERGY IN A SUSTAINABLE AND ECOLOGICAL MANNER.

JC 9465 comes as a transformation to already existing advanced oxidation processes. Its novel technology was able to stabilize the oxidation reagent together with the catalyst. This had not been accomplished before. Scientists had not been able to balance so much reactivity in such an effective way that it can maintain a steady state for a very long time without loss of potency.

Compared to all oxygen-based reagents JC 9465 ranks the highest at applying active energy to initiate oxidation, otherwise known as electrochemical potential.

REAGENT NAME	FORMULA	ELECTROCHEMICAL POTENTIAL (Volts)
Fluorine	F^2	3.06
JC 9465	**MxOyClz**	**2.8-2.9**
Hydroxyl Radical	OH^-	2.8
Oxygen Ion	O^-	2.42
Ozone	**O_3**	**2.07**
Hydrogen Peroxide	H_2O_2	1.78
Chlorine Dioxide	ClO_2	1.57
Hypochlorous Acid	HOCl	1.49
Chlorine Gas	Cl_2	1.36
Oxygen (Molecule)	O_2	1.23
Hypochlorite Ion	OCl^-	0.94
Sodium Hypochlorite	NaOCl	0.94
Hydroperoxide Anion	HO_2^-	-0.88
Perhydoxyl Radical	HO_2^-	-1.7
Superoxide Radical Anion	O_2^-	-2.4

In the efforts to efficiently control Legionella in water systems, this novel technology resolves a cluster of challenges that other technologies have not been able to conquer in a practical way.

Up until now, there has not been a satisfactory, all in one affordable solution, that could promise 100 % positive reassurance it will completely remove Legionella from a water distribution system and prevent regrowth.

The following table summarizes the features of JC 9465 relative to unavoidable considerations when using other treatments.

FEATURE	JC 9465	OTHERS
Corrosion	Non-corrosive	May create corrosion and/or pitting problems
pH	Is not sensitive to it and does not affect pH of the water	Chlorination makes the water more acidic
Biofilm	Destroys bio-films; even mature ones	ClO_2 and ionization need continuous application
Amoeba and protozoa	Will perish with treatment	Could be resistant
Crypto, Giardia	Will be eliminated. They cannot build resistance.	Cannot be killed with a Legionella protocol
Residual protection	Longest available	None or not effective enough. Often misleading.
Kill time	On contact	May require special system design
Temperature	Not affected	Affects chlorine and ozone
Potential hazards	None	Yes
Sustainable	Yes	Only ozone and ionization
Disinfection byproducts	None	Yes
Emerging contaminants	Will be removed	Ozone is effective
Capital investment	Minimal. A dosing pump and an ORP controller.	Significant. Sometimes cost prohibited.

4 TREATMENT

WITHOUT THE AVAILABILITY OF TREATMENTS THAT SUPPORTED COMPLETE ERADICATION OF LEGIONELLA FROM A WATER SYSTEM, CONTROLLING THE BACTERIA HAD BEEN ONLY ABOUT EFFORTS TO REDUCE THE CHANCES OF AN ONSET AND NOT ABOUT A PERMANENT SOLUTION THAT ELIMINATES THE POSSIBILITY OF ITS EXISTENCE.

Currently, we can only enforce water management plans to mitigate the risk of Legionella problems. These are programs designed to help maintain and protect the water systems.

The main goal of the plans is to reduce the risk of Legionella bacteria growing within the system, and most importantly, reduce the risk of Legionnaire's disease outbreak. The plans identify areas of risk for bacteria growth and potential areas for testing.

Is "reducing" the risk of Legionella growing in your system enough?

JC 9465 can completely eliminate the risk of having Legionella in your system.

Are risk "mitigation" protocols enough to protect lives?

With JC 9465 you can have zero risk treatment protocols.

JC 9465 is a reliable and affordable technology that can very effectively rid of Legionella bacteria from any system and at any scale. It provides a permanent solution for a worldwide problem that is just getting bigger and already running out of resources.

5 FUNDAMENTALS

VITALS	IMPERATIVES
Legionella Bacteria thrives where there is poor water flow and stagnant water.	JC 9465 will provide a residual protection that will continue to work and prevent bacterial growth in stagnant water
Reproduce in temperatures between 68° – 122°F. (Optimal growth range 95° – 115°F)	JC 9465 is effective in this temperature range. Is not a gas and stays soluble in solution.
Find essential nutrients for growth in micro-organisms including algae, flavobacteria and Pseudomonas.	JC 9465 will very quickly exterminate all waterborne micro-organisms and thus extinguishing essential food source.
Possess habits that increases survival when it senses the presence of biocides.	JC 9465 acts in nano-seconds and Legionella does not have time to detect a chemical attack and escape destruction.
Find shelter against chemical invasion in acanthamoeba and protozoa.	Amoeba and protozoa will be wiped out.
Proliferate in biofilms and slime which makes them very difficult to reach.	JC 9465 will destroy the bio-film from all surfaces in contact with water.

6 RELIABILITY

THE BACTERIAL ACTIVITY IN THE WATER CAN BE MONITORED WITH A SIMPLE DEVICE IN A CONSISTENT AND RELIABLE WAY.

The technology of JC 9465 found the perfect match with a monitoring tool known as ORP meter, or Oxidation-Reduction Potential. ORP is a term used frequently in the water treatment industry and is a measure of the cleanliness of the water and its ability to remove contaminants.

ORP is a convenient method to assess JC 9465's ability to perform this task. The ORP meter will indicate, in a quick and precise way, when JC 9465 has created an oxidative environment capable of causing a level of damage not compatible with the possibility of Legionella bacteria survival.

The higher the ORP level the more residual activity is available to protect the water and the quicker the bacteria are removed.

The International Ozone Institute published the following table that has become the industry standard. An ORP of 600 mV is considered disinfected water. The World Health Organization stated in the 1971 Guidelines for Drinking Water Quality that the oxidation reduction potential was the most accurate indicator of water quality and stablished 650 mV the recommended ORP level for potable water.

ORP Level	Coliform Count in 100 ml of Water
+200 mV	300
+300 mV	36
+400 mV	3
+600 mV	3

ORP level can also be viewed as the level of bacterial activity of the water because it has been demonstrated by several scientific studies that there is a direct link between ORP levels and bacteria count (CFU-colony forming units) in water.

A nationally recognized laboratory performed a study on the effectivity of JC 9465 and demonstrated that at an ORP of +650 mV Legionella bacteria was eliminated instantly (See Legionella Study Final Report).

The following chart is a good example. It lists a summary of results from various lab simulation and survey studies.

	Survival in seconds (s) or hours (h) at ORP (mV)		
Pathogen/Indicator	< 485	550 < × < 620	> 665
E. coli O157:H7	> 300 s	< 60 s	< 10 s
Salmonella spp.	> 300 s	> 300 s	< 20 s
Listeria monocytogenes	> 300 s	> 300 s	< 30 s
thermotolerant coliform	> 48 h	> 48 h	< 30 s

7 OTHER USES

- When used for cooling tower applications it controls scaling, corrosion and micro-bio-fouling.
- Removes sulfuric acid in wet scrubbers .
- In municipal water treatment, it is effective at removing all pathogens while being a green product.
- Does not create toxic by-products.
- It neutralizes pharmaceuticals and emerging contaminants.
- It will help improve and maximize the disinfection system.
- Very effective in reclaimed water treatment.
- Longest residual protection in the market.
- At an ORP above +700 mV JC 9465 actively disinfects everything it touches.
- In hospital settings, it can be used to wash and disinfect hands and wounds.
- Removes pathogens and pollutants on the surface of fruits and vegetables, meat, frozen food, seafood.
- Manufacture of anti-microbial ice.
- Surface cleaning, food preparation. Eliminates sources of food borne illness.
- Reduces major issues like spoilage, cross contamination and pesticides.

8 ACCREDITATIONS

- JC 9465 is NSF/ANSI 60 certified as a drinking water treatment chemical.
- JC 9465 is EPA FIFRA Registered -EPA Est. No. 92945-1
- JC 9465 is USDA Organic Certified
- All components are recognized GRAS (Generally Regarded as Safe) and considered safe for direct food contact by the Food and Drug Administration.
- The properties of JC 9465 satisfy the twelve principles of the Protection Agency (EPA) Green Chemistry Program.

9 LOGISTICS

- As of the Fall of 2013, there are three manufacturing plants nationwide and six international plants in Australia, Norway, Dominican Republic, Chile, Mexico, and Canada.
- Jenfitch, Inc. is the exclusive distributor of JC 9465. Call
- JC9465 has a conservative shelf life of 6 months. It can be stored on site and minimize the frequency of the shipments.

10 ADDITIONAL INFORMATION

Follow the links if active, or copy and paste on your browser:

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Mineral Oxychloride Solution

Evaluation of Biocide Efficacy Against Legionella pneumophila Serogroup 1 by Time-Kill Assay

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Introduction

According to the manufacturer, JC 9450/ JC 9465 liquid ozone is a non-toxic advanced oxidation process with a mineral oxychloride formula stabilized in a water-based solution. The chemistry of JC 9450/ JC 9465 is based on the principles of Fenton's reagent. It kills pathogens by cell lysis.

Special Pathogens Laboratory (SPL) performed an evaluation to determine its biocide efficacy against *Legionella pneumophilia* serogroup 1 (*Legionella*) using an in vitro time-kill assay. The efficacy was evaluated based on its oxidative reactivity in solution, as measured bythe oxidation-reduction potential (ORP), rather than its quantity in milligrams per liter (mg/L or ppm). The objective was to demonstrate the use of ORP measurement as a method to monitor the effective killing level of oxidant in the water. The ranges of ORP tested were 500-550 mV and 650-700 mV. Three exposure time points tested were: immediately after inoculation (~10 sec), 30 sec and 120 sec.

Methods

Test Solution Preparation

The JC 9450/ JC 9465 liquid ozone (oxidant), prepared 10/5/18, was provided by Jenfitch Inc. and stored in a cabinet in the dark at ambient temperature until use. A Milwaukee (MW 500) ORP meter was provided to perform the evaluation. Preparations were made in 1 L glass bottles filled with locally purchased distilled water (DW) that was filter sterilized before use. Prior to each solution preparation, the ORP probe was cleaned by lightly rubbing it with fine grit sand paper, followed by soaking the tip in white vinegar and rinsing with DW. Finally, it was submersed in a calibration standard at ORP of ~225 mV for about 5 min, followed by a second DW rinse.

The test solutions were prepared and tested, one at a time, using a magnetic stir bar and stir plate. The pH and ORP of the filter sterilized DW was recorded before adding oxidant. An intermediate solution of the oxidant at 100 ppm was made in filter sterilized DW and used to more accurately prepare the test solutions. The intermediate solution was added in varying volumes, incrementally, to the liter of DW in the glass bottle to reach the desired ORP range within 10-60 seconds. The quantity of oxidant in mg/L added was determined. Once the desired ORP range was reached, the solution was allowed to stabilize for 3 minutes to ensure the ORP stayed within the range. The ORP values after 3 min are given in Table 1.

Table 1. Concentrations of test solutions by ORP measurement and by mg/L (ppm) of oxidant added

Desired ORP Ranges	Test Solutions	
	ORP (mV) at 3 min	mg/L (ppm)
500-550 mV	505	0.38
650-700 mV	680	4.4

Inoculum Preparation

*Legionella (*ATCC strain 33152) was grown overnight on buffered charcoal yeast extract agar (BCYE) at 36±2°C with humidity. A dense cell suspension was made in sterile deionized (DI) water that approximately equaled a 4 McFarland turbidity standard with an expected *Legionella* concentration of ~$1x10^9$ colony forming units per milliliter (CFU/mL). Once 1 mL of inoculum was added to the test solutions, the expected final concentration of *Legionella* was ~$1x10^6$ CFU/mL.

Staggered Inoculation and Sampling

After the ORP stabilized, 1 mL of the *Legionella* inoculum was added to the 1 L test solution. At the immediate (~10 sec), 30 sec and 120 sec time points,1 mL aliquots were removed and transferred to sterile culture tubes containing 9 mL of 0.1% sodium thiosulfate in DI to neutralize the oxidant. For the viability control, 1 L of filtered sterile DW without oxidant was inoculated with the *Legionella* inoculum and sampled as the test solutions were.

Viable Bacteria Count and Analysis

Sodium thiosulfate tubes containing 1 mL of inoculated control or test solutions were held until all sampling was complete (approximately 15-30 minutes). The samples were then diluted in tubes of 9 mL sterile DI water and plated in duplicate to BCYE plates. The inoculated plates were incubated at 36±2°C with humidity for 6 days. Following incubation, colonies per duplicate plate were counted and averaged. The CFU/mL of viable *Legionella* were calculated for each time point and test solution. The log CFU/mL of viable *Legionella* recovered from tests were compared to the log CFU/mL of *Legionella* recovered from the viability controls to calculate log reduction.

Results

Preparation and testing was performed on 11/21/18.

The pH of the filter sterilized DW water, taken before solution preparations was 7.49. The ORP of the filter sterilized DW water averaged 419 mV at 3 minutes with readings between 200-300 mV during initial measurement. The pH of the viability control was 7.3.

The average concentration of *Legionella* in the viability controls was $5.98x10^6$ CFU/mL. Little to no reduction in *Legionella* was observed for the test solution at the ORP range of 500-550 mV. However, with increase in ORP to a range of 650-700 mV, the *Legionella* were reduced 100% compared to the viability control by 30 seconds. Compared to the viability control, the log reduction in the *Legionella* concentration in the 650-700 mV ORP test solution was 6.78, which demonstrates biocide efficacy. (Table 2)

Proper ORP measurement did depend on adequately cleaning and calibrating the probe; and minimizing the concentration adjustments of the solution in coordination with the timing of the reading.

Table 2. *Legionella* viability of control and test solutions with percent and log reductions.

Time Points (sec)	ORP (mV)	mg/L Added Oxidant	Viability (CFU/mL)		% Reduction	Log Reduction
			Test	Control		
Immediate	505	0.38	$4.30x10^6$	$6.45x10^6$	0	0
30			$5.45x10^6$	$5.35x10^6$	0	0
120			$4.40x10^6$	$6.15x10^6$	0	0
Immediate	680	4.4	$3.30x10^3$	$6.45x10^6$	99.95	3.30
30			0	$5.35x10^6$	100	6.78
120			0	$6.15x10^6$	100	

Conclusion

This evaluation demonstrated:

1. the oxidant, JC 9450/ JC 9465 liquid ozone, in a distilled water solution at an ORP range of approximately 650-700 mV killed 100% of *Legionella* present at a concentration of ~$6x10^6$CFU/mL by 30 seconds.
2. killing of *Legionella* was not observed at the lower ORP range of 500-550 mV.
3. that an ORP reading, if measured properly, can be a method to monitor the oxidation-reduction reactivity of JC 9450/ JC 9465 liquid ozone in a solution at a level that will kill *Legionella*.

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Susceptibility of *Legionella pneumophila* to Chlorine in Tap Water

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A study was conducted to compare the susceptibility of legionellae and coliforms to disinfection by chlorine. The chlorine residuals used were similar to concentrations that might be found in the distribution systems of large public potable water supplies. The effects of various chlorine concentrations, temperatures, and pH levels were considered. A number of different *Legionella* strains, both environmental and clinical, were tested. The results indicate that legionellae are much more resistant to chlorine than are coliform bacteria. At 21°C, pH 7.6, and 0.1 mg of free chlorine residual per liter, a 99% kill of *L. pneumophila* was achieved within 40 min, compared with less than 1 min for *Escherichia coli*. The observed resistance is enhanced as conditions for disinfection become less optimal. The required contact time for the removal of *L. pneumophilia* was twice as long at 4°C than it was at 21°C. These data suggest that legionellae can survive low levels of chlorine for relatively long periods of time.

During the past several years, *Legionella pneumophila* has been isolated from shower heads, taps, mixing valves, and hot water tanks of hospitals, hotels, and homes (7, 8, 25–27, 29). In a number of cases, the occurrence of legionellae in the plumbing systems was associated with disease; in other cases, it was not.

These bacteria have been found primarily in hot water systems. In particular, large numbers of legionellae have been detected in the sediment that accumulates at the bottom of institutional hot water tanks. Typically, the temperature at the bottom of the tanks, especially in hospital tanks intentionally maintained at relatively low temperatures (e.g., 43 to 55°C), falls within the optimal range for the growth of these organisms (19, 29). It has been shown experimentally that *L. pneumophila* grows in unsterilized tap water within the range of the temperatures found at the bottom of institutional tanks (31). This observation led to the hypothesis that hot water tanks act as breeding sites for the contamination of plumbing systems (29).

A question arises concerning the initial introduction of *L. pneumophila* into the hot water tanks. It has been suggested that plumbing systems may be seeded by small numbers of legionellae from public water supply reservoirs (25, 29). However, attempts to actually isolate these bacteria from the mains of water supplies have not been successful (12). Such evidence would be difficult to obtain since the legionellae may occur sporadically and in low numbers.

Legionellae in a public water supply would be exposed to chlorine concentrations that had been adjusted to control the presence of the indicator coliform bacteria. A number of studies have been conducted to determine the bactericidal effectiveness of a variety of disinfectants against *L. pneumophila* (11, 13, 15). Most of this work has been directed toward problem areas such as cooling towers and evaporative condensers of air conditioning systems. Skaliy et al. (24) found that free chlorine at concentrations of 3.3 and 6.6 mg/liter rapidly inactivated *L. pneumophila*. These relatively high chlorine concentrations were typical of those utilized in cooling towers. Wang et al. (30) examined the effectiveness of disinfectants at concentrations normally used in hospitals for the decontamination of tissues and surfaces. The investigation included the effect of relatively high concentrations of hypochlorite on both *L. pneumophila* and *Escherichia coli*. Their data suggested that legionellae might be somewhat more resistant to these high chlorine concentrations than are the coliform bacteria. They also raised the suspicion that the amount of residual chlorine recommended for standard water purification might not be sufficient for killing *L. pneumophila* when the bacteria are present in high numbers.

Our study pursued the question of *Legionella*

susceptibility to chlorine by examining the bactericidal effectiveness of chlorine at levels which might be found in public water distribution systems. A number of *Legionella* strains from several sources, both environmental and clinical, were examined for susceptibility to chlorine. A comparison was made with *E. coli, Klebsiella pneumoniae*, and *Enterobacter aerogenes* since these bacteria are members of the coliform group which is the commonly accepted microbial indicator for disinfection. Consideration was also given to measuring changes in susceptibility of *L. pneumophilia* to variations in chlorine concentration, temperature, and pH level that might be found in different water systems.

MATERIALS AND METHODS

Bacteria. A number of bacterial strains from various sources were used in this study (Table 1). Several environmental strains of *L. pneumophila* were isolated from samples collected from the Allegheny River in Pittsburgh, Pa. This river is the source of water for the municipal water supply system. To obtain these isolates, 20 liters of river water were concentrated to 10 ml by centrifugation on a Sorvall model RC-2B centrifuge that was equipped with a continuous-flow attachment. Due to the biological complexity of the river water, acid and heat enrichment procedures were used to exclude competing microorganisms. The concentrate was heated for 30 min at 50°C and then treated with 2 parts of a 0.2 M HCl–0.2 M KCl buffer solution (pH 2.2) (3, 31). The sample was then plated on a selective medium, differential glycine-vancomycin-polymyxin B agar (28). *L. pneumophilia* was identified on the basis of colonial morphology, the inability to grow on unsupplemented buffered charcoal-yeast extract agar, and the direct immunofluorescence test (6, 28). The environmental isolates used in this study had been subcultured three times on artificial medium before this experiment.

Environmental strains of *L. pneumophila* serogroups 1 and 6 and *Legionella micdadei* were also isolated from water and sediment which had been collected from the bottom of hospital hot water tanks by direct plating of the samples on differential glycine-vancomycin-polymyxin B agar.

The Centers for Disease Control-derived strain of *L. pneumophila* (Philadelphia 1), the clinical isolate of *L. micdadei* (EK), and the American Type Culture Collection-derived strains of *E. coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were kindly supplied by A. W. Pasculle of the Presbyterian-University Hospital, Pittsburgh, Pa.

Experimental procedure. The bactericidal effectiveness of chlorine was examined by inoculating tap water with known quantities of legionellae and treating these aquatic test systems with chlorine. The action of chlorine was stopped by the addition of 0.1 ml of a 10% (wt/vol) solution of sodium thiosulfate to a 10-ml sample. Viable counts of legionellae were obtained by plating both 0.1 and 0.5 ml of a test system on buffered charcoal-yeast extract agar (21). Colony counts were performed after incubation of the plates at 37°C for 7 days. Appropriate chlorine, bacteria, and thiosulfate controls were included in each experiment. The inhibition of *L. pneumophila* and the other bacteria by sodium thiosulfate was tested by the addition of the thiosulfate solution to test systems in the presence and absence of chlorine. The exposure of the bacteria in these test systems to the thiosulfate for up to 2 h did not affect their viability compared with control samples which did not contain sodium thiosulfate.

TABLE 1. Bacteria tested for chlorine resistance

Bacteria	Origin (strain)[a]
L. pneumophila	
Serogroup 1	Allegheny River
Serogroup 1	CDC (Philadelphia 1)
Serogroup 1	Hospital hot water tank
Serogroup 6	Hospital hot water tank
L. micdadei	Hospital hot water tank
	Clinical specimen (EK)
E. coli	ATCC (ATCC 25922)
	Allegheny River
S. aureus	ATCC (ATCC 25923)
K. pneumoniae	Allegheny River
E. aerogenes	Clinical specimen

[a] CDC, Centers for Disease Control; ATCC, American Type Culture Collection.

The basic experiments involved a comparison of an environmental isolate of *L. pneumophila* serogroup 1 from the Allegheny River with an American Type Culture Collection-derived strain of *E. coli*. Both bacteria were exposed to identical chlorine concentrations under the same environmental conditions. A free chlorine residual of 0.1 mg/liter was used as the "standard" chlorine concentration. The standard environmental conditions for the basic experiments consisted of pH 7.6 at 21°C. After the addition of chlorine, the sample was rapidly stirred for 30 s at 200 rpm with a Teflon-coated magnetic stirring bar and then slowly stirred (60 rpm) for the remainder of the experiment. The above chlorine concentration and environmental conditions were chosen to simulate conditions that might be found in the distribution of a large public water supply.

In addition to performing experiments under standard conditions, the comparison between *L. pneumophila* and *E. coli* was extended to other chlorine residuals and environmental conditions. In studying the effects of different chlorine concentrations, the same experiment was repeated under standard conditions of pH and temperature but at free chlorine residuals of 0.2 and 0.5 mg/liter. Temperature variations, 4 and 32°C, were tested with a standard chlorine residual of 0.1 mg/liter and pH 7.6. Similarly, the effect of pH 6.0, 7.0, and 7.6 was determined under standard conditions of 0.1 mg of total chlorine per liter at 21°C.

Test system and chlorine determination. The aquatic test system consisted of sterile 1-liter Erlenmeyer flasks containing 600 ml of tap water. The water was obtained from a tap in the municipal water distribution system. Tap water was used because the purpose of

TABLE 2. Comparison of chlorine demand of boiled tap water with demand of deionized, distilled water[a]

Boiled tap water		Deionized distilled water	
Total chlorine[b]	Free chlorine[b]	Total chlorine[b]	Free chlorine[b]
0.05	0.05	0.05	0.05
0.10	0.10	0.10	0.10
0.25	0.25	0.25	0.20
0.35	0.35	0.35	0.30
0.50	0.50	0.50	0.45
0.60	0.60	0.60	0.55

[a] Essentially chlorine demand-free.
[b] Milligrams per liter as determined by the amperometric method.

this study was to investigate the survival of legionellae in a municipal water system. Dechlorination of the tap water was accomplished by boiling it before use. The water was then buffered with a phosphate buffer. KH_2PO_4 (0.5 M) and K_2HPO_4 (0.5 M) were mixed and diluted to a final pH of 6.0, 7.0, or 7.6 (standard) and a final concentration of 10 mM. A 100-mg/liter stock chlorine solution was prepared by dissolving calcium hypochlorite in sterile, distilled, deionized water. A Milli-Q system (Millipore Corp., Bedford, Mass.) was used to deionize the water. Chlorination of the test system was achieved by adding precalculated volumes of this stock to the buffered tap water. Free and total chlorine concentrations were measured at the beginning and end of each experiment by the amperometric method (2) to ensure that no unexpected chlorine demand had appeared in the test system water. Free and total chlorine measurements were also performed at the end of each experiment to determine the degree of chlorine depletion. Chlorine loss never exceeded 10% during any of the experiments.

Initially, the chlorine demand of boiled tap water was compared with that of essentially demand-free, distilled, deionized water. Various amounts of hypochlorite were added to portions of each type of water, and the total and free chlorine concentrations were measured. Boiled tap water was found to be essentially demand-free (Table 2).

To prepare inocula for the test system, *Legionella* and non-*Legionella* bacteria were cultured on buffered charcoal-yeast extract agar at 37°C. Legionellae were incubated for 76 h, and the non-*Legionella* bacteria were incubated for 24 h. The bacteria were scraped from the plate, washed twice with 30 ml of distilled water, and then suspended in 5 ml of distilled water. This inoculum was added to the aquatic test system to achieve a bacterial density of ca. 3,000 CFU/ml. This density of *L. pneumophila* is within the range reported in contaminated hot water tanks (29).

RESULTS

The effect of chlorine on *L. pneumophila* at various concentrations of chlorine, contact times, pH levels, and temperatures is summarized in Fig. 1 to 3. The results are expressed in terms of percent survival at progressively longer

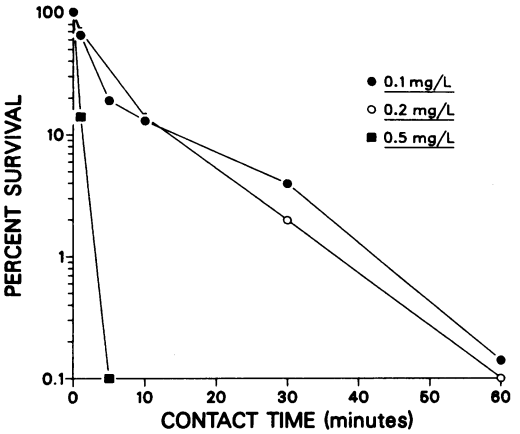

FIG. 1. Bactericidal effect of different concentrations of chlorine on *L. pneumophila* in tap water at pH 7.6 and 21°C.

times of exposure under each of the sets of conditions. *E. coli* was not detected in the samples within min 1 of treatment with chlorine. Identical results were obtained with *S. aureus* as well as with a strain of *K. pneumoniae* that had been isolated from a sample of river water. A river water sample containing a natural population of coliforms was also tested. These coliform bacteria were likewise killed within min 1 of treatment. Because the earliest sampling period after the addition of chlorine was 1 min, bacteria other than *L. pneumophilia* are not represented in the figures.

Under the standard conditions of pH 7.6, a temperature of 21°C, and a free chlorine residual of 0.1 mg/liter, a 99% kill of the legionellae did not occur until a contact time of between 30 and

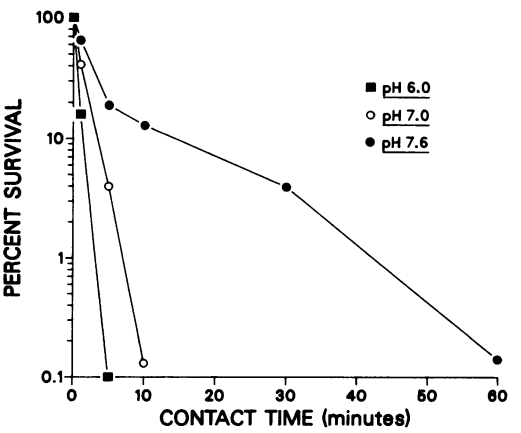

FIG. 2. Effect of pH on bactericidal activity of 0.1 mg of chlorine per liter on *L. pneumophila* in tap water at 21°C.

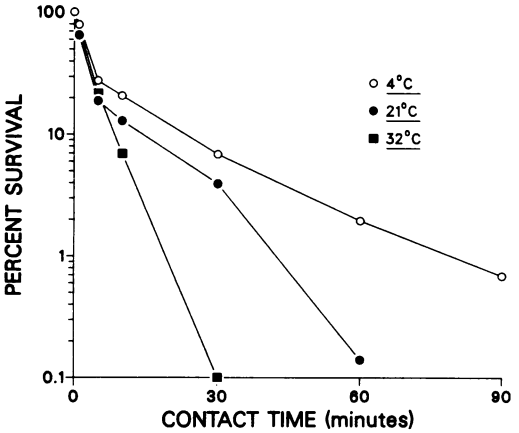

FIG. 3. Effect of temperature on bactericidal activity of 0.1 mg of chlorine per liter on *L. pneumophila* in tap water at pH 7.6.

60 min had elapsed. In addition to the standard bacterial concentration of 3,000 CFU/ml, a 10-fold increase and a 10-fold decrease in the number of bacteria were also tested. The kill rate was not affected by these changes. This latter finding is consistent with the observations of Butterfield et al. on other bacterial species (5). Increasing the total chlorine concentration (Fig. 1) predictably enhanced the bactericidal effect, resulting in a 99% kill within the first 5 min at a concentration of 0.5 mg/liter.

Decreasing the pH exerted an effect similar to that of increasing the chlorine concentration (Fig. 2). A contact time of ca. 40 min was required to eliminate 99% of the *Legionella* population at pH 7.6. In contrast, less than 10 min was required at pH 7.0 and less than 5 min was required at pH 6.0.

Temperature also exerted a dramatic influence on the chlorine disinfection of *L. pneumophilia* (Fig. 3). The time required for a 99% kill at 0.1 mg of chlorine per liter decreased from 40 min at room temperature to less than 30 min at the higher temperature of 32°C. At 4°C, between 60 and 90 min was required for a 99% kill.

In addition to examining the bactericidal effectiveness of chlorine on a strain of *L. pneumophila* that had been isolated from a river water sample, a number of other environmental and clinical isolates of legionellae were tested (Table 3). All of these isolates were studied under the standard conditions of 0.1 mg chlorine per ml, pH 7.6, and a temperature of 21°C. The contact times necessary to eliminate 99% of these populations were as long or longer than those required for the river isolate of *L. pneumophila* that had been used as the primary test organism. Long contact times were required for the clinical and environmental isolates of *L. pneumophila*, regardless of serogroup or origin, as well as for *L. micdadei*. These results indicate that legionellae can survive for relatively long periods of time at low concentrations of chlorine under a variety of temperatures and levels of pH.

DISCUSSION

Hypochlorites have been employed for the disinfection of water for potable use since 1894 (22). The basis for the establishment of effective levels of chlorine is the susceptibility of *E. coli* and other coliform bacteria. These bacteria have served as indicators of the bacteriological quality of water supplies since the publication of the first edition of *Standard Methods of Water Analysis* in 1905 (1). Some waterborne pathogens have been shown to be more resistant than the coliform bacteria to chlorine (4, 10, 16, 18, 20, 23). These reports and the incidence of diseases, such as hepatitis, giardiasis, and gastroenteritis, have periodically prompted reconsideration of the coliform bacteria as microbial indicators of water sanitary quality (17).

Levels of *L. pneumophila* ranging from 9×10^3 to 3.3×10^7 organisms per ml have been detected by direct immunofluorescence in sur-

TABLE 3. Survival of environmental and clinical *Legionella* isolates under standard conditions[a]

Bacteria	Source	% Legionellae surviving after following min of chlorine treatment:							
		1	5	10	30	60	90	120	150
L. pneumophila									
Serogroup 1	Allegheny River	65[b]	19	13	4	<1	<1	<1	<1
Serogroup 1	Hot water tank	56	19	20	17	6	<1	<1	<1
Serogroup 1	CDC[c] (Philadelphia 1)	85	20	11	10	5	3	<1	<1
Serogroup 6	Hot water tank	47	15	6	6	4	4	2	<1
L. micdadei	Hot water tank	31	9	6	4	3	2	<1	<1
	Clinical specimen	55	20	9	6	3	2	<1	<1

[a] Free residual chlorine, 0.1 mg/liter; temperature, 21°C; pH, 7.6.
[b] Compared with the concentration of legionellae before the addition of chlorine.
[c] CDC, Centers for Disease Control.

face waters (14). The recent detection of *L. pneumophila* in the plumbing systems of institutions has raised the suspicion that municipal drinking water systems serve as pathways for this contamination (9, 25). Our study directly involved a measurement of the effectiveness of chlorine in killing *L. pneumophila* and indirectly involved an assessment of the coliform bacteria as indicators of this process. Our results with *E. coli* are consistent with those of earlier workers: a 99% kill of these bacteria is achieved within a very short period of time. In contrast to these results, *L. pneumophila* may survive for periods of longer than 1 h under the same conditions. The bactericidal action of the chlorine is enhanced at higher temperatures and at lower pH levels. These findings are consistent with studies which were done with other bacteria (4, 5). Thus, the survival of *L. pneumophilia* in chlorinated waters may vary with the season and geographic area.

As stated previously, the criterion for a sanitary quality of water supplies is elimination of coliform bacteria. Our observation that legionellae are more resistant than coliform bacteria suggests the possibility that small numbers of legionellae may occasionally survive in waters that have been judged to be microbiologically acceptable. This difference in susceptibility to chlorine tends to increase as conditions become less optimal, e.g., higher pH, lower temperature, and lower chlorine concentration. These findings support the hypothesis that small numbers of legionellae may pass through public water supplies and subsequently contaminate internal plumbing systems. It should be noted that, to date, *L. pneumophilia* has not been isolated from water in reservoirs or in the water supply mains. Currently available methodology does not appear to be sufficiently sensitive to detect very low numbers of legionellae. Even if these bacteria are present in potable water, the extent of the hazard posed is not entirely clear. Plumbing systems and potable water have been shown to contain legionellae in some institutions in which outbreaks of Legionnaires disease were occurring (12, 25, 27). However, these bacteria have also been found in natural waters in the absence of any association with disease and in the plumbing systems of institutions in which no or only infrequent sporadic disease had been detected (14, 26, 29).

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