COOLING TOWER LEGIONELLA PNEUMOPHILA STUDY CDC JOINT RESEARCH PROJECT MARCH 28-AUG. 15, 1194

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Abstract

Since the late 1970's, there have been many small and large outbreaks of Legionnaires' disease. Most of these outbreaks have been traced to either cooling tower water systems or potable water systems. Although the bacteria most often associated with Legionnair's disease, *Legionella pneumophila*, is present in many waater systems, outbreaks are usually a result of large concentrations of *Legionella pneumpohila.*

This paper is a report of the work conducted in association with the Centers for Disease Control at their facility in Atlanta, GA. A model cooling tower was constructed that recirculated water and rejected heat through evaporation. This model system was inoculated with know concentrations of bacteria and amoebae. It was allowed to reach equilibrium and then biocides, such as ozone chlorine, chlorine dioxide, and monochloramine, were applied to determine their efficacy in controlling the total bacteria and the legionella in particular.

The results of this study are summarized in this paper.

Introduction

The focus of this research is to determine the relative efficacy of different biocides in controlling panktonic *Legionella pneumophila* in a simulated cooling tower environment. These same biocides will also be used to establish their efficacy in eliminating fully developed biofilms, like those found in potable and cooling water plumbing systems.

Background

Since its initial isolation in 1977, *Legionella pnuemophila* has been found to be associated with sporadic and large scale outbreaks of Legionnaires' Disease. It is estimated that there are between 10,000 and 20,000 cases of Legionnaires Disease annually in the United States.

The genus of bacteria which cause the disease have proven to be unusual pathogens in many respects. The legionellae were originally considered transitory contaminants of the environment, but have since been recognized as natural components of freshwater ecosystems. These bacteria are known to survive as intracellular pathogens of freshwater protozoa and multiply in these cells in a manner analogous to their infection of human alveolar macrophages. Studies have shown that multiplication within these freshwater protozoa can protect the bacteria from the effects of biocides. In addition these protozoa exist as part of a complex microbialbiofilm in these aquatic environments, that serve as physical barriers to biocides.

Because reservoirs of legionellae have been implicated in outbreaks of Legionnaires Disease in cooling towers, evaporative condensers, and plumbing systems, the effectiveness of various biocides, both common and novel, with respect to the prevention of legionnaires and related respiratory diseases needs to be evaluated..

There are many biocides available. Some of these contain heavy metals, organic molecules, or amine structures. Historically, the most common biocide used in comfort cooling towers and similar equipment have been halogenated compounds that contain chlorine and/or Bromine. Recent research has also shown that ozone is an acceptable biocide when used in these systems.

Several studies of the effectiveness of biocides for the control of *Legionella p*. have been published in the past. All of the studies were performed in the laboratory and used pure cultures of the bacteria in sterile water. Although these simplified systems of testing biocides were easy to perform, they have little relevance to applications in cooling towers or plumbing systems (England, A.C.,et al. Failure of Legionella pneumophila sensitivities to predict results from disinfectant treated air conditioning cooling towers. 1982. Appl. Envion. Microbiol. 43:240-244). In this project we will develop a model cooling tower and cooling tower basin in which legionellae grow in conjuction with other naturally occurring aquatic organisms.

Cooling towers have been used for reducing the temperature of liquids for the past century. By evaporating a percentage of the water that recirculates through the system, the remainder of the water is cooled. These systems are used alone or with compressive chillers and/or heat exchangers to cool a wide variety of gas or liquid systems.

Comfort cooling, especially in drier climates, has made extensive use of cooling towers. Commercial, industrial, and medial office buildings use cooling towers to reduce their air conditioning bill.

To operate efficiently cooling tower need a variety of water treatment. Corrosion and scal inhibitors are often used to control the natural tendencies of

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the water to corrode or precipitate. Over the years there has been intensive research into these areas. Many chemical treatments have been discontinued for environmental reasons. Some chemical treatments have been replaced by newer or superior chemicals or groups of chemicals.

Cooling towers, because they are west and warm, are ideal locations for biological growth. Most treatment systems include a biocide. Lately there have been several epidemics which have been closely associated with bacteria, known to be in cooling towers. This has caused the acceptable level of bacteria in cooling towers to be reconsidered.

In the recent past a cooling tower was considered to be in acceptable biological control with bacterial populations of under 100,000 colony forming units per milliliter (cfu/mL). With planktonic bacteria populations in this region, the drift leaving a cooling tower could contain billions of potentially harmful bacteria.

Often this was the best that could been done without using dangerous chemicals or inducing excessive corrosion. The danger associated with immune suppressed people at a hospital and cooling tower drift became quite clear.

The majority of cooling towers are currently treated with chlorine in various forms. Chlorine is very inexpensive and easy to use. Unfortunately many organism become immune to high levels of chlorine and bacteria levels can build. Shock treatments are then used, either with extremely high concentrations of chlorine or another biocide.

Among the new generation of biocides ozone has demonstrated the ability to be a candidate to replace most biocides in cooling tower applications.

Ozone is a gas which has to be generated on site. This prevents problems with storage and handling. Ozone is also be thousands of time more effective than chlorine, chlorine dioxide, and hypochlorous acid for control of spores, bacteria, and virus.

As a cooling tower treatment ozone has only recently become a viable option. The combination of ozone generators with computers has made a system which is highly reliable. The ozone residual in the water is controlled on a feed back loop. This maximizes biological control yet minimizes corrosion. Well controlled ozone systems have bacteria populations well under 1,000 cfu's and often less than the makeup water to the cooling tower.

The research in this paper is a comparison of well maintained and controlled chlorine and ozone systems. Concentrations necessary for effective bacterial and especially Legionella p. control are considered.

Experimental

Bacterial cultures and amoeba, which are known to reside in cooling tower and potable water systems with Legionella pneumophila, were grown to simulate a typical cooling tower environment. These included:

1. *Legiionella pneumophila serogroup 1, Rl-243*
2. *Acinetobacter*
3. *P.Stutzeri*
4. *Comamonas testosteroni*
5. *P.paucimobillis*
8. *A hydophila*
9. *Alcaligenes*
10. *P.aeroginosa*
11. *P.climinuta*
12. *P. vesicularis*
13. *P.methylobacterium extorquens*
14. *Hartmannella veriformis*

These bacteria, initially frozen, were thawed. The Rl-243 was plated on Buffered Charcoal Yeast Extract (BCYE) agar and the other bacteria were plated on blood agar. Cultures were incubated for 24 hours at 35°C. To 10 mL of steriale buffered saline solution, bacteria samples, excluding RI-243, were added to produce a suspension with an absorbance of 0.955 using a Beckman spectrophotometer at 540 nanometers with 1 cm path length. The Rl 243 was suspended in sterile endotoxin free water. This absorbance indicated a bacterial concentration of 1×10^9 cfu/mL.

1.8×10^7 Hartmannella veriformis amoebas were suspended in 10 milliliters of buffered saline solution to achieve a concentration of 1.8×10^6.

To produce an environment conducive to the production of a consistent, large quality of bacteia and amoeba, a biological growth reactor or chemostat was used. The suspensions of amoeba and bacteria were added to the chemostat.

The Chemostat

The chemostat used in these experiments was a 12 liter carboy. It was equipped with an air sparger, nutrient feed, stirrer, and located in a temperature controlled room. The chemostat was connected to a pump and the liquid from the chemostat was pumped through a biological sampling devise made by Calgon Corp. The biological sampling devise contains stainless steel substrate which develop a biofilm. The biofilm is thought to be an integral part in the sustained, continuous development of the biological population in the chemostat.

Liqid in the chemostat was withdrawn under vacuum and added to the cooling tower before each experiment. This controlled, defined bacterial population make the quantification of individual biocides more evenly attained.

R2A nutrient is pumped into the chemostat at a rate of 10 gallons per 14 days. The R2A concentration is 2.2 grams per 12 liters of sterile water.

The Cooling Tower

The model cooling tower was filled with water and allowed to operated overnight. The tap water used had a conductivity of 75 microsiemens per centimeter.

After one day of operation a 1.3 liters sample from the chemostat was used to provide a level of biological activity in the model cooling tower. The tower continued to run for 24 hours before the introduction of the biocide. This time helped the microbes acclimatize to the cooling tower.

The fully functional model cooling tower is pictured in Figure 1. It is a forced draft style system with PVC nozzles and fill. It is equipped with a recirculating pump in the basin and a heat source to facilitate evaporation. A separate recirculation system has locations for a sampling port, and ORP probe. The system has a total volume of 12 liters. A supply of potable water was used to replenish water lost by evaporation. A water analysis of the supply water is in Figure 2. The cooling tower was located in a biosafety cabinet.

Sodium Hypochlorite

Commercially available 5.25% sodium hypochlorite wa diluted to keep a residual of 1.0 free chlorine during the experiment. Before the experiment started a biological sample was taken. Before and after each sample was taken, the sample port was swabbed with alcohol and dried, a 10 mL sample was taken at each time interval for bacterial examination.

Another sample was taken in one hour, after which the chlorine concentration was again adjusted to 1.0 parts per million (ppm). The final sample was taken at 2 hours, after which the experiment was stopped. Free chlorine was determined with a Hach test kit.

After the experiment was concluded the water feed to the basin was turned off and the basin water was emptied into a glass carboy and autociaved for disinfection. The model cooling tower was allowed to run empty with the fans on to dry the system.

Ozone

The Oxidation Reduction Potential (ORP) of the tap water was initially at 200 mv. After being stripped of residual chlorine for a day the ORP was reduced to 175 mv. After the sample from the fermentor was added to the model cooling tower, the ORP was reduced to 150 mv. Ozone was added at a rate of 100 on the Aalborg rotameter, or 288 mL/min, at 1.5% weight ozone from an ozone generator[1]. The ozone concentration reached 0.1 ppm within 10 minutes. The ORP was stable st 250 mv.

Again samples were taken at 0, 1, and 2 hours. Ozone concentration was determined using a spectrophotometer[2] and Potasium Indigo Trisulfonate dye. After the experiment the water to the cooling tower was turned off, the cooling tower fans, pump, and heater turned off, the ORP probes removed and stored for future use and the cooling tower drained and dried. The water from the cooling tower was autoclaved and discarded.

Sample Preparation

One milliliter of sample was diluted with 9 mL of sterile endotoxin free water and shaken to ensure homogeneity. From that solution one milliliter was removed and added to another 9 mL of sterile endotoxin free water. In this way the sample was serially dilted four more times to produce six samples, diluted one through six logs.

At time zero, one hour, and two hours a sample was taken to determine the quanity of *Legionellap.* and total bacteria in the cooling tower. These microbes were serially dilated and plated on BCYE agar and BCYE with antibodies to reduce bacteria otheer than Legionella. The samples that were to be plated on BCYE with antibodies were acidified for 15 minutes with a KCI solution. These samples were then neutralized with KOH solution and serially diluted. This technique also reduces the number of total bacteria and makes the *Legionellap.* colonies more detectable.

100 microliters of sample were pipetted from each dilution onto the different culture medium. The solutions were plated using the spread-plate technique to ensure an even distribution and incubated for 48 hours. To accurately read the bacterial colonies on the plates, a total of 3 to 300 colonies are necessary. When the plates are within this number, they can be read, multiplied by the dilution factor and recorded.

Results

Samples were tested for the presence of amoeba. Amoeba were found in the samples taken from the chemostat. No amoeba were found in samples taken from the cooling tower. This indicated all amoeba were attached to the cooling tower surfaces.

No biofilm was detected inside the biofilm sampling devise. This prevented testing the biocides against the fully developed biofilm. There was a noticeable biofilm in the tygon tubing which connected the chemostat to the biological sampling devise.

The data that were recorded fro the two experiments follow.

Conclusions

The chemostat was run over three months sustaining a population of bacteria and amoeba.

Both biocides reduced the amount of *Legionellap.* substantially. The continuously controlled Ozone removed the *Legionellap.* completely and was three orders of magnitude more effective in killing the total bacteria than chlorine.

The amount of ozone used was one tenth the amount of chlorine used. In work done by Nalco, this concentration of ozone would be no more corrosive than oxygenated water itself.

Endnote

[1]TriOx model 400 Ozone generator
[2]Hach Dr2000

Table 1

Time	Disinfectant	Total Bacteria	Legionella P.
0	Chlorine	4,650,000	58,000
1 hour	Chlorine	3,880,000	30,000
2 hour	Chlorine	3,110,000	2,600
0	Ozone	14,000,000	29,000
1 hour	Ozone	19,000	0
2 hour	Ozone	6,000	0