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# Detection and Eradication of a Non-*Legionella* Pathogen in a Cooling Water System

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## ABSTRACT

Measurements of bacteria found in cooling water systems are often used to indicate the bacterial level both in the cooling water and on the tower surfaces. Populations of planktonic bacteria are determined as an indication of the performance of the biocide programs used to maintain microbial control. In this case history of a cooling water system where an oxidizing biocide was used, the average bacterial count did not far exceed  $10^5$  CFU/mL, yet a number of workers became ill with symptoms much like those of legionellosis. The symptoms were flu-like and subsided in 24-48 hours. Results from a series of microbiological and biochemical tests indicated that the causal organism was not *Legionella* but a motile *Aeromonas* species. The causative agent was identified as an endotoxin produced by *Aeromonas hydrophila*. The original biocide program did not prevent proliferation of this microorganism. This article details the microbiological and biochemical analyses, which detected *Aeromonas* in biofilm from the cooling tower. Changes to a combination biocide program, which proved successful in eradicating the problem and maintaining microbial control in the tower, are described.

## INTRODUCTION

A variety of detrimental and sometimes injurious microorganisms can be found in industrial water systems such as cooling towers, airwashers, HVAC and paper mills. In these systems, it is not unusual to find large populations of aerobic bacteria such as *Pseudomonas*, *Klebsiella*, *Enterobacter*, *Acinetobacter*, and *Bacillus* species in the bulk water. Occasionally, *Legionella* is detected in the bulk water of cooling towers and airwashers. In some cases, plant personnel become ill with legionellosis. In other cases, the symptoms resemble those of legionellosis but are not due to *Legionella pneumophila*. Rather, the symptoms are induced by a different microorganism, exposure to which is not documented well in the literature.

To put this case history and laboratory study in perspective, one needs to review recent findings of problematic microorganisms pertinent to water.

Table 1: Pathogens Found in Drinking Water Systems	
TYPE OF MICROORGANISM	EXAMPLE
<b>A. New Pathogens</b>	
Bacteria	<i>Legionella pneumophila</i> , <i>Pseudomonas aeruginosa</i> <i>Aeromonas hydrophila</i> , <i>A. caviae</i> , <i>A. sobriae</i>
Environmental mycobacteria	<i>Mycobacterium intracellulare</i> , <i>M. chelonae</i> , <i>M. fortuitum</i> , <i>M. avium</i>

Cyanobacteria	Toxins from <i>Microcystis</i> , <i>Planktothrix</i> , <i>Anabaena</i> , <i>Aphanizomenon</i> , <i>Oscillatoria</i>
<b>B. Pathogens of Fecal Contamination</b>	
Enteric viruses	Hepatitis A, rotaviruses
Bacteria	<i>Campylobacter jejune</i> <i>Escherichia coli</i> O157:H7, <i>E. coli</i> O26 and O103 <i>Helicobacter</i> <i>pylori</i>
Protozoa	<i>Cryptosporidium</i> <i>parvum</i> , <i>Giardia</i> sp.

Several new pathogens have recently been found growing in water systems. Some of these have originated from fecal sources, while others arose from unknown sources of contamination (see Table 1) (Szewzyk et al, 2000).

For example, on the top of the list, *Legionella pneumophila* has been well recognized as a nosocomial pathogen for approximately 10 years, and cooling towers have been implicated as sources for outbreaks of community and nosocomial Legionnaires' disease (Muder *et al*, 1982; Call *et al*, 1999). However, other pathogens of serious concern (for example, *Aeromonas* sp.) have been detected in drinking water systems and have been known to cause illnesses often confused with legionellosis or influenza (Call *et al*, 1999). While these problematic microorganisms were found in drinking water, it is plausible that they would also be found in cooling water.

## Aeromonas

*Aeromonas* sp. are members of the family *Vibrionaceae*. They are Gram-negative, non-sporeforming, facultatively anaerobic, rod-shaped bacteria. *A. hydrophila* is a motile species and grows optimally at 30°C, and as most aeromonads, can also grow at 37°C.

Aeromonads cause diarrhea; however, the pathogenesis of this symptom is unclear. A variety of potential toxins include a cytotoxic beta-hemolysin, an extracellular cytotoxic enterotoxin, hemoagglutinins, an enterovasive factor, proteases, and elastases (Hunter, 1977). These result in clinical features such as flu-like symptoms, including mild diarrhea, and may involve development of a fever, abdominal pain, and bloody diarrhea. A small proportion of adults exposed to *A. hydrophila* had developed chronic colitis (Hunter, 1977).

## Biofilm Bacteria

The second important microbiological factor of concern in cooling systems is microbial deposition on surfaces. When bacteria attach to surfaces, some express genes to make slime exopolymer. This results in a phenotype, which one observes as mucoid colonies on a Petri dish or slime masses on cooling tower or airwasher surfaces. The growth of biofilms is determined by nutritional and environmental conditions, as well as other factors mentioned below. Of the biofilms we have observed in cooling systems, the slime exopolymer consists of approximately 70-80% carbohydrate and 20-30% protein (Wiatr, unpublished data).

Two genera of bacteria that can grow into a biofilm are *Aeromonas* and *Pseudomonas*. Both are opportunistic pathogens, which can, in the right circumstances, lead to human health problems. In terms of cooling water treatment, both can also indirectly lead to corrosion problems by metabolizing nitrogen-based corrosion inhibitors and releasing gaseous compounds.

*Pseudomonas* can reduce nitrite to  $N_2$ , and *Aeromonas* also consumes nitrite to produce  $NH_3$ . Either of the processes can result in loss of corrosion inhibitor. In biofilms in which the growth of these bacteria is favored and is positively regulated by compounds such as homoserine lactone and other cell-signaling compounds, the bacteria can grow up in “towers.” Fluid transport through these biofilm structures facilitates contact with microbial enzymes and promotes rapid chemical turnover and loss of corrosion protection. The enzymes they produce can be involved in rapid turnover of several chemical compounds. Thus, the presence of either of these bacteria growing in a biofilm community can lead to a loss of chemical protection and result in corrosion problems.

## Field Site

The field test site was a steel mill in the midwestern United States. The mill brought in railroad cars transporting iron parts, melted the iron down, and used the molten iron to manufacture steel products. The cooling towers, referred to as Cooling Tower A and Cooling Tower B for this study were mechanical-draft crossflow towers. Cooling Tower A cooled water for the blast furnace operation, while Cooling Tower B cooled the electroplating operations, heat exchangers and air compressors. The makeup water for both towers was mill water. The fill was polyvinylchloride, but some wood was in the towers. Characteristics of the cooling tower included exposure to sunlight and to warm, oxygenated waters. The plant did not chemically clean the cooling tower surfaces. Both cooling towers were analyzed for problematic bacteria. Cooling water was sampled from the cold well of the blast furnace recycled water system. Treatment consisted of bleach fed until a free residual halogen (typically 0.1 mg/L as free  $Cl_2$ ) was reached. Nonoxidizing biocide was reportedly slug dosed at 50 mg/L product (1.50% combination of isothiazolinones mentioned above) once per week.

Individuals who worked near these towers experienced flu-like symptoms. Initially two individuals became ill, followed by eight more, and then the total increased to 26. The symptoms were severe enough to cause the workers to be absent from work for a minimum of 24 to 48 hours. If they returned to work after only 24 hours, they had to return home to rest and missed additional workdays until their recovery was complete. All workers interviewed stated that they had to remain in bed and rest, and did not feel like eating. Therefore, this article presents evidence of a situation in which individuals who worked near and around a cooling tower suffered from symptoms similar to those involved in legionellosis, but were not exposed to legionellae. They were instead exposed to *Aeromonas hydrophila*. This article points out the results of an oxidizing and nonoxidizing biocide and their performance against the bacterial population growing in the cooling tower, including *A. hydrophila*.

## MATERIALS AND METHODS

**Microorganisms.** Standard pure cultures were made from a single colony isolates of the bacteria *Pseudomonas aeruginosa* ATCC 15442 and *Aeromonas hydrophila* ATCC 23211, as well as *Pseudomonas* sp. and *Aeromonas* sp. isolated from the cooling water.

**Media and Chemicals.** Bacteria were propagated in dilute tryptic soy broth (TSB) and were enumerated on tryptone glucose beef extract agar (TGE) supplied by Difco (Detroit, MI). Additional ingredients such as salts and anhydrous dextrose used to supplement TSB were from J.T. Baker (Phillipsburg, NJ). Antibiotics were from Sigma Chemical Co. (St. Louis, MO).

For field studies, 1 liter samples of cooling water were drawn and the microorganisms were cultured based on 0.5, 1.0 or 10 ml subsamples. 2.0 x 0.5 ml of the sample was inoculated onto Levine eosine methylene blue medium and MacConkey medium, which were used for enumeration of Gramnegative enteric bacteria. *Pseudomonas* isolation agar (PIA) supplemented

with carbenicillin (200 µg/ml) was used to enumerate pseudomonads. Additional antibiotics were used at the following concentrations (mg/ml as indicated in parentheses): for *Pseudomonas aeruginosa* carbenicillin (250), gentamycin (10), and/or HgCl<sub>2</sub> (10); for *Pseudomonas fluorescens* gentamycin (10). The media were incubated at 35±1°C, for 48 hours, except for PIA which was incubated at 30±1°C for 48 hours. Total coliform testing was conducted by filtering 25 ml of cooling water through a 0.45 µm membrane filter. The membrane filter was incubated on a pad containing MF coliform medium for 24 hours. Fecal coliforms were determined similarly on a membrane filter incubated, on a pad soaked with 1.8 ml M-FC medium, for 24 hours at 44.5°C. Blue colonies were counted as fecal coliforms. All other aerobic and facultative anaerobic bacteria were incubated at 48 hours at 35°C, then 24 hours at 24°C. Sulfate reducing bacteria were grown in modified Postgate's medium. *Clostridium*, yeast and mold were determined as previously described (Wiatr, 1991).

**Isolation and Biochemical Testing.** The dominant Gram negative bacteria isolated from MacConkey medium were transferred to trypticase soy agar (TSA). Five colonies were suspended in 0.85% sterile saline and identified by the API 20E method. This method involves the determination of the biochemical profiles based on an inoculation of miniature cupules containing dehydrated substrates in a plasticized strip. The inoculum (McFarland 0.5 ml) and incubation were standard, and color changes were read visually. Reagents were added to some of the cupules prior to reading. In addition, the organisms were restreaked on TSA, and were allowed to grow for 24-48 hours at 28°C for identification by gas chromatography.

**Table 2: Composition of Microbes Found in Cooling Tower A & B<sup>a</sup>**

	<b>TOWER A</b>	<b>TOWER B</b>
<b>Total Aerobic Bacterial Plate Count<sup>b</sup></b>	120,000,000	100,000,000
<i>Pseudomonas</i>	10,000	400
<i>Enterobacter</i>	600	300
<i>Escherichia coli</i>	<1	<1
Pigmented bacteria (yellow)	30,000,000	330
<b>Anaerobic Bacteria<sup>c</sup></b>		
Sulfate reducing bacteria <sup>c</sup>	3	1
<i>Clostridium</i>	1	<1
<b>Fungi<sup>d</sup></b>		
Yeast	<10	<10
Mold	<10	<10

a. Microbiological counts are expressed as CFU/ml of cooling water sampled.

b. Aerobic bacteria were counted on TGE. *Pseudomonas* was enumerated on PIA.

c. Anaerobic bacteria were determined by sulfate reducing medium of Postgate and thioglycollate medium (*Clostridium*).

d. Fungi were determined on tartrate-acidified potato dextrose agar (PDA) and unacidified PDA.

**Gas Chromatography.** *Aeromonas* and *Pseudomonas* species were identified by gas chromatography using Microbial Identification System (MIS) software. Approximately 40 mg of grown cells was harvested and the fatty acid methyl esters from the microbial cells were extracted by organic solvents. The final extract was transferred to a vial for GC analysis. The resulting chromatogram of each fatty acid methyl ester profile was compared to a reference library in the MIS, yielding a printout of the most probable identification of the organism. The software has a dendrogram function that further allowed comparison of the isolates in the database to each other.

**Limulus Amebocyte Lysate Analysis.** Endotoxin was determined by the *Limulus polyphemus* Amebocyte Lysate gelation technique performed on serial dilutions from  $10^{-1}$  to  $10^{-6}$ , and interdilutions of  $10^{-4}$  : 1:2, 1:4, 1:8.

**Biocides.** The oxidizing biocide was NaOCl, which was introduced from a 12.5% concentration. The nonoxidizing biocides consisted of 50% 1,5-pentanedial, 20% 2,2-dibromopropionamide, 1.15% 5-chloro-2-methyl-4-isothiazolin-3-one+0.35% 2-methyl-4-isothiazolin-3-one, 60% polyquat or poly[oxyethylene(dimethyleneiminio) ethyl-ene(dimethyliminio)ethylene dichloride), and a combination of the isothiazolinones above and polyquat (WSKT-10). The concentrations of actives used in the study are given in the tables in the “results” section below.

## RESULTS

The types of microbes which were found in Cooling Towers A and B are given in Table 2. The aerobic bacteria in the cooling water of both towers were approximately 10<sup>8</sup> per ml. The *Enterobacter* count was <1000 organisms/mL, and the *Escherichia coli* were <1/mL, which is the detection limit of the test. The *Pseudomonas* sp. were enumerated at 10<sup>3</sup> and 4x10<sup>2</sup> per ml for Towers A and B, respectively. Since *Pseudomonas* sp. and the pigmented bacteria in Tower A were approximately 2-4 log greater than those in Tower B, a small number of sulfate reducing bacteria (SRB) were also found. A single colony of SRB was grown. No clostridia were detected. Yeast and mold did not grow on either tartrate-acidified or unacidified potato dextrose agar (PDA.)

Under the phase-contrast microscope neither *Gallionella* nor *Sphaerotilus* were found. A few protozoa were found in Tower A but not in Tower B cooling water. A few nonfilamentous algae were also found in Tower A but not in Tower B.

Since the total aerobic plate counts of both cooling towers were 10<sup>8</sup> initially, we examined both towers. However, the aerobic bacterial plate counts, *Pseudomonas*, and yellow pigmented bacterial counts were found to be consistently higher in Cooling Tower A than Tower B. The number of personnel who grew ill worked around Tower A but not around Tower B. Consequently, this study gradually became focused on the cooling system of Tower A only.

Bacteria from samples of cooling water from Tower A were isolated on antibiotic-containing blood agar plates and MacConkey agar. However, cultures which yielded yellow colonies that were oxidase positive were considered presumptive for *Aeromonas* sp (Hunter, 1977). Samples were also re-plated on MacConkey agar in duplicate. The predominant colonies were subjected to API20E biochemical testing for identification (see Table 3). Five colonies were selected. All five were confirmed biochemically as *A. hydrophila*. These were also confirmed as *A. hydrophila* by re-plating on TSA and conducting MIS-gas chromatography. Additional work was performed to determine whether the causative agent was a toxin. Samples of the cold well recycled blast furnace water were submitted for amoebocyte lysate assay (LAL) and were found positive for lipolase endotoxin, as pointed out in Table 3.

Samples of the cold well-recycled blast furnace water were analyzed also for numerous



bacteria and fungi when the tower was treated chemically. The samples were collected after the Cooling Tower A bleach treatment (NaOCl) where the free residual was measured as 0.2 mg/L (as Cl<sub>2</sub>). As the data indicate in Table 4, the heterotrophic bacterial count or aerobic bacterial count was measured at 5,300,000 per ml sample. However, 24 hours later, when the isothiazolin chemistries were used at 70 mg/L product (1.05 mg/L active), a decrease of 1.8 log<sub>10</sub> in aerobic bacteria count was observed. However, the *Pseudo-monas* count data did not indicate a significant log<sub>10</sub> decrease (5.11 vs. 4.95). The *Enterobacter* also did not change significantly, but the *Escherichia coli* level dropped 1 log<sub>10</sub>. The yellow pigmented bacteria population was decreased >2 log<sub>10</sub> (from 3x10<sup>3</sup> to less than 10<sup>1</sup>). No anaerobic bacteria were detected after dosing with a combination of chlorine and isothiazolinones. Fungi were also not detected. The prominent Gram-negative bacterium was found to be *Aeromonas hydrophila* (Table 4). In addition, other Gram-negative bacteria were found, but were not listed in the table because they were not found as prominent colonies in the water samples. These were *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Pseudomonas* sp. The endotoxin level from *Aeromonas hydrophila* was found to be 250 ng/ml (nanogram per milliliter).

**Table 3: Microbiological and Biochemical Testing for the Identification of Enterobacteriaceae**

Characteristic	<i>Aeromonas hydrophila</i>
Oxidase	+b
Catalase	+
Motility	+
Dnase	+
Indole	+
H <sub>2</sub> S on triple sugar iron agar	-
Voges-Proskauer	+
Urea hydrolysis	-
Lysine decarboxylase	+
Ornithine decarboxylase	-
Arginine dihydrolase	+
Growth on KCN medium	+
Esculin hydrolysis	+
Beta-hydrolysis on sheep blood agar	+
Sensitivity to O/129	-
Growth on 6.5% NaCl	-

Gelatin hydrolysis at 22 °C	+
Gas from glucose	+
<b>Acid From:</b>	
Glucose	+
Sucrose	+
Lactose	+
Maltose	+
Cellobiose	-
L-arabinose	+
Raffinose	-
myo-inositol	-
D-sorbitol	+
Mannitol	+
a. Incubation is at 29 or 37 C for 18 h but checked for changes in 2 days. b. + Represents growth or a positive test/ - is absence of growth or negative result.	

The biocide treatment was increased. The isothiazolin concentration was increased to 200 mg/L product (3.00 mg/L total active isothiazolinones). Based on data of several nonoxidizing biocides, this dose was known to decrease the level of the laboratory grown *Aeromonas hydrophila* strain in 3 hours (See Figure 1a, Nonoxidizing Biocide C). However, this dose was ineffective in the cooling water of Tower A. A significant effect was not observed over time. The reason for failure of the nonoxidizing biocides to do well can be seen from the results in Figure 1a-1d.

The results in Figures 1a and 1b indicate that the nonoxidizing biocides B and E performed well against the laboratory strain *Aeromonas hydrophila* ATCC 23211. At 3 hours, biocides C and E did well at higher dosages. At 7 hours, biocides A and E killed well at all concentrations while biocides B, C, and D at their higher dosages killed well vs. the control. (Figure 1b). The results in Figure 1c and 1d, however, indicate that most biocides tested did not kill the wild type *Aeromonas hydrophila*. At 3 hours, only nonoxidizing biocide E at the highest dose provided a significant kill, which was slightly greater than a one log<sub>10</sub> kill. Figure 1d indicates that all dosages of nonoxidizing biocide E gave a kill at 7 hours. That is, the lowest dose gave a 1 log kill, the second and third dosages gave >2 log kill, while the highest dose provided a 4 log kill. None of the other nonoxidizing biocides affected the wild type aeromonad.

Figure 2 indicates the heterotrophic plate count (HPC) and the *Pseudomonas* sp. count for a field trial. These are data collected subsequent to the testing done for Table 4. The counts were determined on samples taken approximately 24 hours after the addition of the biocides. The results indicate the effects of the isothiazolinones and the combination of the polyquat plus isothiazolinones on the bacterial population of Cooling Tower A. At day 1 the HPC was  $2.9 \times 10^6$



CFU/ml and the *Pseudomonas* counts were  $3.1 \times 10^5$ . After the concentration of the active isothiazolinones were increased to 3 mg/L at day 1, the total bacterial population was still not reduced significantly ( $2.9 \times 10^6$  /day 1 to  $9.3 \times 10^5$ /day 2); likewise, the *Pseudomonas* sp. population remained almost the same ( $3.1 \times 10^5$  to  $2.8 \times 10^5$ ). Continued slug dosing of 3 mg/L isothiazolinones at days 5,7, and 9.5 did not have a significant effect. The bacterial counts essentially remained at the same log level. At day 10.5 the total bacterial level reached  $8.5 \times 10^7$  and the *Pseudomonas* count reached  $1.01 \times 10^6$ . While the 3 mg/L active isothiazolinones dose was effective against the laboratory strain *A. hydrophila* (Figure 1a and Figure 1b), this concentration did not have a strong effect against the field bacterial population in Cooling Tower A (Figure 2, Figure 1c and 1d). Clearly, the dose of isothiazolinones alone was inadequate against the wild type bacteria.

With the aerobic bacterial level at  $8.5 \times 10^7$  and the *Pseudomonas* count increasing 1 log<sub>10</sub> (Figure 2), it would be difficult to bring the cooling tower under control. The endotoxin produced by *A. hydrophila* also had to be reduced. Consequently, the combination of polyquat and isothiazolinones was introduced to the cooling water at days 14 and 15, and samples again were drawn for analyses approximately 24 hours later. The results in Figure 2 indicate that the combination caused an overall > 4 log reduction in both HPC and pseudomonads by day 16. A return to 70 mg/L isothiazolinones two days later allowed regrowth even though chlorine was present at 0.3 mg/L (as free residual Cl<sub>2</sub>) as well. Three days later the mill wanted to return to the combination of polyquat plus isothiazolinones. The combination resulted in 3 log decreases in HPC and *Pseudomonas* counts to <1000 and 10 CFU/ml, respectively.

In another set of tests, the total aerobic and pigmented bacteria were counted before and after treatment using the combination of the polyquat and the isothiazolinones. The bacterial counts determined during the standard mill biocide treatment program are illustrated in the bar graph at day 1 in Figure 3a. The effect of the combination of polyquat and isothiazolinones is given at days 16, and 30 (Figure 3a). The concentration of the endotoxin produced by *Aeromonas hydrophila* was measured by LAL. The results are indicated in Figure 3b.

In Figure 3a, the first set of bars indicates that with a 70 mg/L isothiazolinones biocide treatment, the HPC was  $2.9 \times 10^6$  and the pigmented bacteria were  $3.1 \times 10^5$ . The same cooling water sample was measured for endotoxin concentration and was found to contain 1,000 ng/ml (see Figure 3b.)

The second bar in Figure 3a indicates an overall decrease of 2 log approximately in both types of counts at 16 days, while Figure 3b indicates a drop in endotoxin to 250 ng/ml at the same time point. Finally, the third set of bars indicates a decrease to <1000 CFU aerobic bacteria/ml and to <10 *Pseudomonas* per ml sample at 30 days. The final count indicates when the combination of polyquat plus isothiazolinones were applied at the highest dose which had an effect against the wild type bacteria at 3 h in the laboratory study (Figure 3c) and the greatest effect at 7 hours (Figure 3d). For this combination of nonoxidizing biocides, the dose-response relationship is clear in both the HPC and *Pseudomonas* count (Figure 2 at 30 days; Figure 3a). A concomitant reduction of endotoxin data (Figure 3b) occurred as well. It should be noted that since the endotoxin concentration was reduced to <100 ng/ml, no workers were ill. After the Cooling Tower A was under control one month, no additional workers were reported as becoming ill.

**Table 4: Composition of Microbes Found in Cooling Water Tower A – After Teatmenta**

	NaOCI	Nonoxidizing Biocide
Total Aerobic Bacterial Plate Count <sup>b</sup>	5,300,000	830,000
Pseudomonas	130,000	90,000

Enterobacter	640	400
Escherichia coli	220	16
Pigmented bacteria	1000	10
Anaerobic Bacteria <sup>c</sup>		
Sulfate reducing bacteria	3	<1
Clostridium	1	<1
Fungi <sup>d</sup>		
Yeast	<10	<10
Mold	<10	<10
Dominant Gram negative bacterium <sup>e</sup>	Aeromonas	Aeromonas
<p>a. Microbiological counts are expressed as CFU/ml of cooling water sampled. Tower A was treated with 0.2 mg/L free residual chlorine alone. Subsequently, the tower was treated with a nonoxidizing biocide.</p> <p>b. Aerobic bacteria were determined by standard procedures on TGE.</p> <p>c. Anaerobic bacteria were determined on sulfate reducing medium of Postgate and thioglycollate medium (Clostridium).</p> <p>d. Fungi were determined at &lt;10 CFU/ml on both tartrate-acidified and unacidified PDA.</p> <p>e. The dominant Gram negative bacteria were isolated from MacConkey medium and were transferred to TSA. They were identified by the API 20 method, given in table 3, then speciated by MIS-GC analysis.</p>		

## DISCUSSION

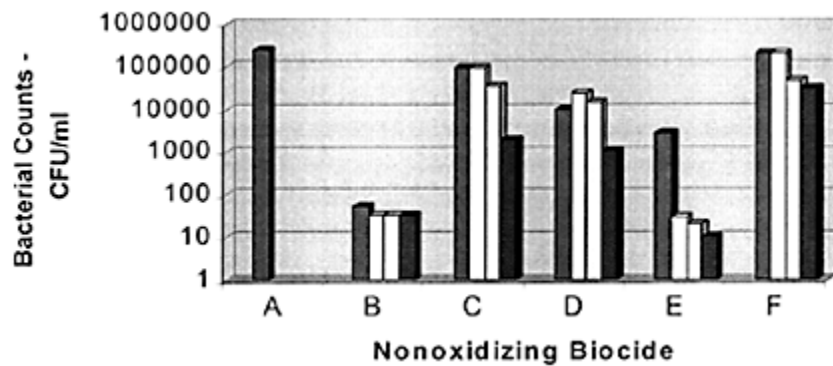
The data in Table 2 clearly indicate that the bacteria in Cooling Tower A were not under control using the steel mill's program of chlorine and isothiazolines (50 mg/L as product). Since the illnesses occurred with personnel who also worked near Tower A, this study focused on that cooling tower. Based on standard microbiological testing, the microorganisms suspected to be the cause of disease were *Aeromonas* sp. or *Pseudomonas* sp. *Aeromonas* was distinguished by biochemical tests, then determined conclusively by gas chromatography to be *Aeromonas hydrophila*. In addition, endotoxins produced by *A. hydrophila* were determined to be present by the LAL test.

In this case study, isothiazolinones and glutaraldehyde were effective against laboratory strains of *A. hydrophila* ATCC 23211, but were not effective against the wild type *A. hydrophila* strain found in Cooling Tower A. A similar conclusion was drawn from the lab testing of *P. aeruginosa* ATCC 15442 and the wild type *Pseudomonas* strain, which also was isolated from Cooling Tower A (data not shown).

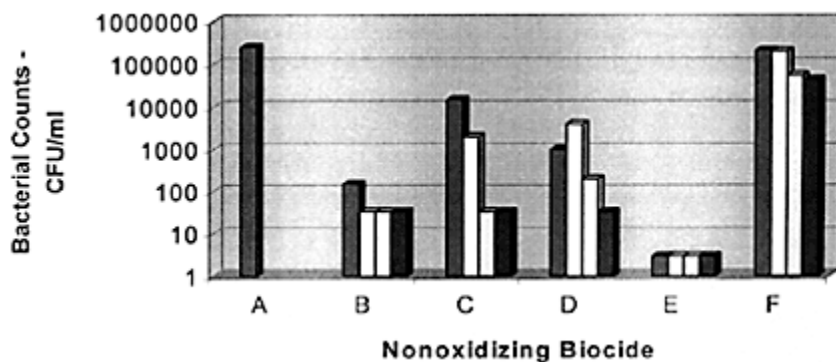
The results in Figures 3c and 3d clearly indicate that Biocide E is effective against both wild type strains. These are biocides isothiazolin and polyquat (polyionene). In a previous study, these biocides were found synergistic versus other bacteria (Lukanich, 1997). The biocide combination is called WSKT. Application with that biocide twice per week reduced the level of *A. hydrophila* 3 log<sub>10</sub>, with no changes in the chlorine program, suggesting that the combination of nonoxidizing biocides was necessary for the control of *A. hydrophila*. The combination nonoxidizing biocide also was successful in reducing the aeromonad endotoxin, as indicated by Figure 3b.

**Figures 1a-1b** were plotted from the bacterial counts of an *Aeromonas hydrophila* ATCC 23211 laboratory strain challenged by various nonoxidizing biocides at 3 and 7 hours and plated on TGE. Likewise, **Figure 1c-1d** consists of data of bacterial counts of an *Aeromonas hydrophila* wild type strain, which was isolated from the recycled water in the cold well of the cooling tower. In each graph, letter A represents an average of duplicate negative (untreated) controls for each set of experiments; B represents counts obtained following treatment with 50% glutaraldehyde at 25, 50, 75 and 100 mg/L active concentration; C with 1.50% isothiazolin combination used at 0.75, 1.13, 1.5, 3.0 mg/L active; D with the polyquat used at 0.6, 2.0, 6.0 and 12.0 mg/L active; E is the combination of isothiazolins plus the polyquat used at the respective concentrations of 0.07+0.6, 0.35+3.0, 0.7+6.0, and 1.4+12.0; F involved treatment with 20% DBNPA at 4, 8, 10, and 20 mg/L active.

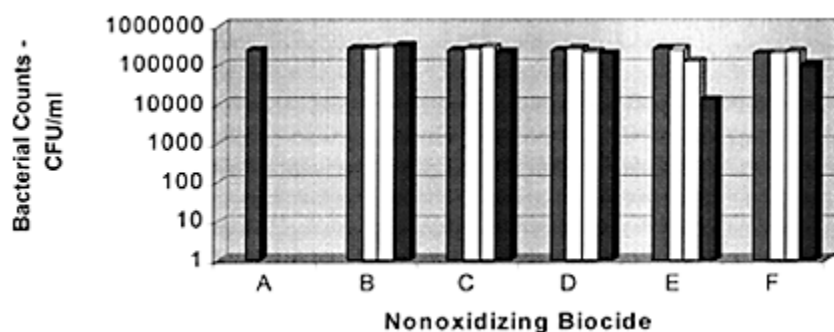
**Figure 1a: Nonoxidizing Biocides vs. *Aeromonas hydrophila* ATCC 23211 - 3 hours**



**Figure 1b: Nonoxidizing Biocides vs. *Aeromonas hydrophila* ATCC 23211 - 7 hours**



**Figure 1c: Nonoxidizing Biocides vs. *Aeromonas* WT - 3 hours**



The mill followed the same treatment regimen of Cooling Tower A they were used to; that is, they treated with chlorine and isothiazolinones (70 mg/L). However, the results in Figure 2 indicate that the cooling water still contained  $2.9 \times 10^6$  CFU/ml aerobic bacteria and was still positive for *A. hydrophila*. However, the addition of the synergistic combination of nonoxidizing biocides polyquat plus isothiazolines proved to be effective. This combination decreased the wild type bacteria and *Aeromonas* in the laboratory studies (Figures 1c and 1d) and in the field test (Figures 2 and 3a). This combination of biocides also reduced the concentration of the endotoxin (Figure 3b).

While the origin of the problem with *Aeromonas*, its endotoxin, and overall makeup of the bacterial population could not be known, it is worth discussing the treatment situation which led to it. To control the bacterial population, chlorine was relied on as the primary treatment. However, chlorine alone was inadequate.

It is not unusual that chlorine alone does not kill *Aeromonas* bacteria. *Aeromonas* is isolated from chlorinated waters (Burke *et al*, 1984). Makerness *et al* found that *A. hydrophila* survived a monochloramine concentration of 0.3 mg/l for 21 days.

Being careful to monitor cooling water for both chlorine and bacteria is important. Monitoring water for microorganisms in the water and in biofilms provide insight on how well they are controlled in the planktonic and the sessile (attached) state. For example, earlier *Aeromonas* was cited as a pathogen (Table 1) with other microorganisms found in drinking water, which is typically chlorinated (Szewzyk, *et al*, 2000). Nakano *et al* studied for one year the distribution of *Aeromonas* sp. in aquatic environments and identified 2,444 isolates. They found that *A. hydrophila* was a predominant species in clean river samples; *A. sobria* in stagnant waters. *A. caviae* strains are commonly isolated from marine waters as well. *Aeromonas* sp. were discovered on reverse osmosis membrane surfaces.<sup>10</sup> These findings suggest that it is important to treat drinking water and RO systems for the bacteria of interest.

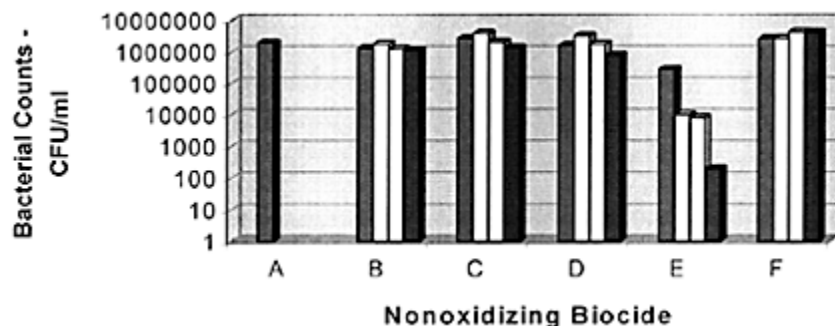
This article shows that *Aeromonas hydrophila* was found in cooling water systems. Up to this time, however, *Aeromonas* has not been discussed as a cause of flu-like symptoms in an industrial community, particularly where cooling towers were in use.

This article discusses the efficacy of several biocides against *Aeromonas* found in a cooling tower and provides evidence that the combination of the polyquat and isothiazolinones was clearly effective. The article also describes a case history in which *Aeromonas* induced flu-like symptoms in personnel working at or near a cooling water system and points out the alleviation of those symptoms after treatment with the same combination of biocides.

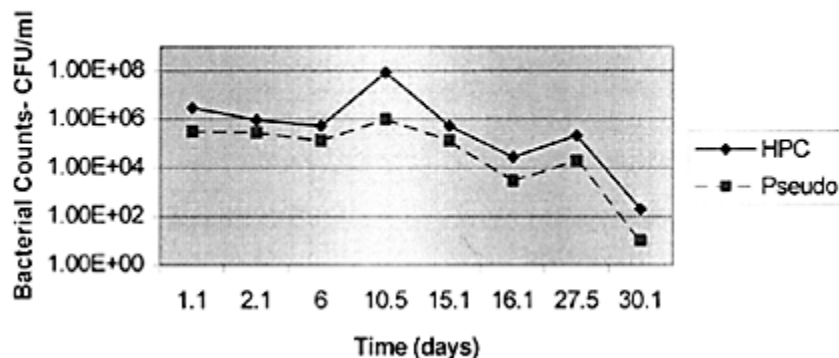
The symptoms due to *Aeromonas* were severe for 24-48 hours. This is not surprising, based on the membrane biochemistry and the molecular biology of *Aeromonas*. *Aeromonas hydrophila* has evolved well developed genetic and biochemical systems for causing pathogenesis. *A. hydrophila* produces a virulence factor called an endotoxin that is the lipopolysaccharide (LPS) component in the outer cell membrane, which is released during the growth and the breakdown of its cells (Bone, 1991). The toxicity of endotoxin resides in the lipid A fraction which can cause high fever, septicemia, shock, and respiratory tract problems (Bone, 1991). *Aeromonas* is

known to secrete proteins through a type II secretion system, much like *P. aeruginosa* secretes toxin A. *A. hydrophila* secretes amylase and protease enzymes in particular, which can digest starch and proteins, respectively (Haker, 2000). It is also well known that *Aeromonas* strains are resistant to penicillin, ampicillin, piperacillin, as well as second and third generation cephalosporins, and that these strains can develop resistance to these drugs (Balows *et al*, 1991). Because of these potentially toxic LPS and enzymes and resistance to several antibiotics, it is mandatory to take all precautions in testing waters from an industrial system and to treat them aggressively to kill off potentially harmful bacteria, such as *Aeromonas* sp.

**Figure 1d: Nonoxidizing Biocides vs. Aeromonas WT - 7 hours**



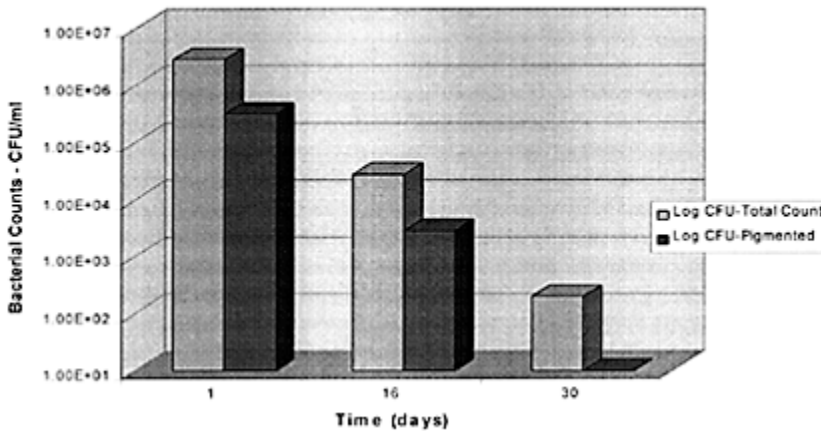
**Figure 2: Bacterial Kill vs. Time - Using Nonoxidizing Biocides**



**Figure 2** illustrates the bacterial kill of the heterotrophic plate count (HPC) and *Pseudomonas* counts over time. The treatments were given approximately one day prior to sampling of the cooling water for microbiological analysis. At day 1, the result is for the treatment using 1.05 mg/L active isothiazolines the day before; at days 2, 5, 7 and 9, 3.0 mg/L isothiazolinones; at days 14 and 15, the combination of polyquat and isothiazolins; at days 19, 23, and 26.5, the mill returned to treatment using 1.05 mg/L isothiazolines, and the result is given as the data point at 27.5 days. At day 29, the combination of

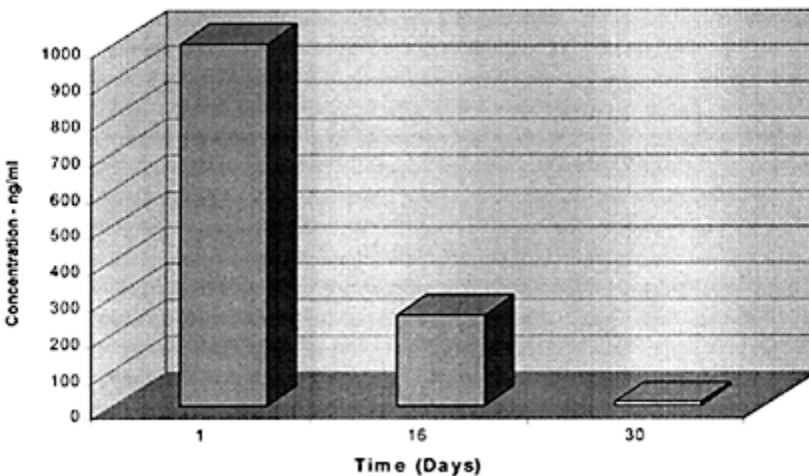
polyquat and isothiazolines was dosed. No nonoxidizing biocide treatment was dosed at day 30.

**Figure 3a: Total Heterotrophic and Pigmented Bacterial Count**



**Figure 3a** illustrates the total bacterial and pigmented bacterial counts as determined on TGE at 1, 16, and 30 days. **Figure 3b** indicates the gradual reduction of endotoxin concentration at the same time points.

**Figure 3b: Endotoxin Concentration**



## CONCLUSIONS

*Aeromonas hydrophila* was found to grow up in the cooling tower with *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Pseudomonas* sp.

*Aeromonas* can produce an endotoxin in a cooling water environment and the endotoxin can induce flu-like symptoms in individuals who work around that cooling tower.



If the bacterial population is treated with a proper biocide program, the endotoxin produced by *Aeromonas* was shown to decrease and the corresponding flu-like symptoms were observed to cease.

An appropriate biocide program was required to maintain in check bacteria, including *Aeromonas* and *Pseudomonas* species.

Chlorinating alone was inefficient and insufficient.

A nonoxidizing biocide, such as isothiazolin at sublethal dosage, even in the presence of free residual chlorine, was proven to be inadequate at controlling wild type bacteria in the cooling system over time.

A combination of nonoxidizing biocides proved to provide the best control of the wild type microorganisms, including *Aeromonas*, in the cooling tower vs. bleach alone or bleach plus one nonoxidizing biocide.

Reduction in planktonic bacteria does not necessarily indicate reduction in bacteria at surfaces in a cooling system.

The presence of pigmented bacteria, the appearance of atypical colors surfaces or slime masses on cooling tower surfaces, and atypical differences between past and present performance of a cooling system may indicate problems of microbial deposition. It is important to observe the appearance of the deposition during weekly inspections, since bacteria can grow up well in depositions in a short time. If found early and treated with an appropriate biocide program, the microbial deposits are more easily controlled and eliminated. When one has less control of the situation, one finds that the microbial populations will grow more uncontrolled, leading to major problems with the cooling systems and even the health of the workers. The frequency of *Aeromonas*, in particular, in causing these problems will be the subject of a different study.

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“The purpose of this quiz is to ensure the CWT (Certified Water Technologist) has read and understands the technical paper or article. The quiz answers are based strictly on the content and perspective of this article. The AWT and Certification Committee make no representation to the factual content of the article. Each article has been reviewed and the Certification Committee has made every attempt to avoid articles with misleading statements. Any questions concerning the scoring of any quiz will be referred back to the article for clarification”.

Analyst Article – “*Detection and Eradication of a Non-Legionella Pathogen in a Cooling Water System*”, By Christopher L. Wiatr, Buckman Laboratories.

1. The mirco-organism that caused the illness at the steel mill was:
  - a. *Legionella pneumophila*
  - b. *Staphylococcus*
  - c. *Aeromonas Hydrophila*
  - d. *Lactobacillus*
2. Which tower was determined to be the problem tower?
  - a. Tower A
  - b. Tower B
  - c. Towers A & B
  - d. Neither towers were a problem; another piece of equipment was the problem.
3. The biocide treatments used for these towers were:
  - a. Stabilized bromine and glutaraldehyde
  - b. Bleach and isothiazolins
  - c. Bleach and glutaraldehyde
  - d. Ozone
4. For Tower A, a combination of polyquat and isothiazolines resulted in the following:
  - a. A >4 log reduction in both HPC and *pseudomonads*.
  - b. Excessive foaming in the tower.
  - c. No change in aerobic bacterial levels.
  - d. A gradual 2 log increase in aerobic bacterial levels.
5. In the case study isothiazolines and glutaraldehyde were effective against:
  - a. Wild type *A. hydrophila*
  - b. Wild type *P. aeruginosa*
  - c. Laboratory strains of *A. hydrophila*
  - d. Wild type *staphylococcus*
6. *Aeromonas* can produce an endotoxin that can:
  - a. Induce vomiting
  - b. Induce flu-like symptoms
  - c. Induce headaches and blurred vision
  - d. Induce other sensitivities to harmful bacteria.

7. Chlorinating alone was:
  - a. The most effective at controlling *Aeromonas* and *Pseudomonas* species.
  - b. Able to provide a 4 log decrease in bacteria counts.
  - c. Inefficient and insufficient.
  - d. Effective and sufficient
8. In this paper, isothiazolin at a sublethal dosage was:
  - a. Able to control wild type bacteria.
  - b. Unable to control wild type bacteria.
  - c. Able to control wild type bacteria only with chlorine.
  - d. Able to control wild type bacteria only with chlorine dioxide.
9. Biocides used in this paper included all of the following except:
  - a. NaOCl
  - b. Polyquat
  - c. 1,5-pentanedial
  - d. Ozone
10. *Aeromonas* sp are members of the family:
  - a. *Enterobacteriaceae*
  - b. *Vibrionaceae*
  - c. *Pseudomonadaceae*
  - d. *Thiotrichaceae*