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# Stimulatory Effect of Cooling Tower Biocides on Amoebae

SUJATA SRIKANTH AND SHARON G. BERK*

*Center for the Management, Utilization and Protection of Water Resources, Tennessee Technological University, Cookeville, Tennessee 38505*

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**Two species of amoebae were isolated from the cooling tower of an air-conditioning system and examined for effects of exposure to four cooling tower biocides, a thiocarbamate compound, tributyltin neodecanoate mixed with quaternary ammonium compounds, another quaternary ammonium compound alone, and an isothiazolin derivative. The amoebae isolated were *Acanthamoeba hatchetti* and a *Cochliopodium* species. Two other amoeba cultures, an *A. hatchetti* culture and *Cochliopodium bilimbosum*, were obtained from the American Type Culture Collection (ATCC) and were also tested. The cooling tower isolates were more resistant to most of the biocides than the ATCC isolates were. The isothiazolin derivative was the least inhibitory to all four amoeba isolates, and tributyltin neodecanoate mixed with quaternary ammonium compounds was the most inhibitory to three of the four isolates. After exposure to lower concentrations of the biocides, including for one strain the manufacturer's recommended concentration of one biocide, the cooling tower amoeba populations increased significantly compared with unexposed controls, whereas the ATCC isolates were not stimulated at any of the concentrations tested. In some cases, concentrations which stimulated cooling tower amoebae inhibited the growth of the ATCC isolates. These results suggest that cooling tower amoebae may adapt to biocides, underscoring the need to use freshly isolated cooling tower organisms rather than organisms from culture collections for testing the efficacy of such biocides. The stimulatory effect of biocides on amoeba populations is an alarming observation, since these organisms may be reservoirs for legionellae. Biocides used to control microbial growth may actually enhance populations of host organisms for pathogenic bacteria.**

Cooling tower systems have been found to contain *Legionella* spp. (10, 16, 17, 25), and outbreaks of legionellosis have been traced to such systems (5). Amoebae have also been isolated from cooling tower samples which were positive for *Legionella pneumophila* (16). Since intracellular reproduction of *L. pneumophila* within amoebae has been demonstrated (1, 6, 11, 18, 21), amoebae have been suggested as potential reservoirs for legionellae, possibly protecting the legionellae from the effects of biocides and disinfectants (14, 15). If amoebae or other protozoa serve as hosts for intracellular bacteria such as *L. pneumophila*, it is important to examine factors that affect the survival and growth of such host populations in treated waters.

Early investigations of disinfection in water treatment focused primarily on concentrations of chlorine that were cysticidal for pathogenic amoebae, such as *Entamoeba histolytica*, in natural waters (12). Little is known today, however, about the effects of modern biocides on amoeba cysts and trophozoites in cooling systems. Most of the recent work has examined the effects of biocides on bacteria (3, 4, 7). In one recent study Kilvington (13) examined the activity of water biocides against cysts and trophozoites of *Acanthamoeba polyphaga* isolated from a human case of keratitis and *Naegleria fowleri* isolated from a thermal spring. Kilvington and Price (14) tested the survival of *A. polyphaga* cysts infected with *Legionella* sp. and exposed to chlorine. The antimicrobial properties of three biocides against *A. polyphaga* trophozoites have also been described recently (2). In this study, we examined the effects of four biocides on amoebae. The inhibitory and stimulatory effects of biocides on population growth were examined by using both cooling tower isolates and two species of amoebae obtained from the American Type Culture Collection (ATCC), Rockville, Md.

## MATERIALS AND METHODS

**Sources of protozoa.** Walls of the pool of an evaporative cooling tower were scraped with the rim of a sterile screw-cap test tube to collect biofilm material. One drop of biofilm material was aseptically transferred in a laminar flow hood to a nonnutrient agar (NNA) plate seeded with a lawn of 24- to 48-h old *Escherichia coli* (obtained from Susan Goss, Biology Department, Tennessee Technological University, Cookeville). The NNA plates were incubated at 24°C for 2 to 3 days. As amoebae grew and reproduced, they moved away from the inoculum drop and onto the side of the NNA plate devoid of bacteria. The amoebae dispersed sufficiently to allow transfer of a single cell to another seeded NNA plate; a sterile microcapillary tube (5 μl) was used to capture the amoeba. One amoebal isolate was identified by T. K. Sawyer (Rescon Associates, Royal Oak, Md.) as a member of a *Cochliopodium* species that most closely resembled *Cochliopodium minus*. Another amoebal isolate, *Acanthamoeba hatchetti*, was also obtained and identified in this manner.

Two other amoebae, an *A. hatchetti* strain and a *Cochliopodium bilimbosum* strain (*C. minus* is not available from the ATCC), were obtained from the ATCC (strains ATCC 30730 and ATCC 30937, respectively) and were not derived from cooling towers or treated water. *A. hatchetti* ATCC 30730 was isolated from sediments of Baltimore Harbor, and *C. bilimbosum* ATCC 30937 was isolated from a lake in Bethesda, Md. The cultures were maintained on *E. coli*-seeded NNA plates incubated at 24°C. In this study, the cooling tower was not examined for the presence of *Legionella* species.

**Biocides.** The following two biocides which were used in the cooling tower were tested: a thiocarbamate compound,

* Corresponding author.

MBC 350 (50% potassium dimethyldithiocarbamate [Nash-Chem, Nashville, Tenn.]), and MBC 120 (5% tributyltin neodecanoate mixed with quaternary ammonium compounds [TBT/QAC] [Nash-Chem]). The concentrations of the biocides in the tower at the time of isolation were not determined. Two additional biocides, an isothiazolin derivative, MBC 215 (5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one [Nash-Chem], and a quaternary ammonium compound (QAC), MBC 115 {poly[oxyethylene(dimethyliminio)ethylene (dimethyliminio)ethylene dichloride], [Nash-Chem]}, were also tested. The biocides were diluted with a Tris-buffered saline solution (TBSS) (2 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1 mM Tris, [pH 6.8 to 7.2]). The concentrations indicated below are the concentrations (by volume) of the whole product. In order to rule out the possibility that addition of chemicals to TBSS indirectly affected toxicity because of a change in pH or osmolality, the pH and osmolality values of the highest biocide concentrations in TBSS were measured and compared with the pH and osmolality of the TBSS alone, which was used as the control solution. Although other investigators use Page's amoeba saline (19) as the control solution, we found that addition of biocides shifted the pH too much for comparison with controls. In this study the pH and osmolality of the cooling tower water were not determined. However, in another study (23) the pH and osmolality of water in the same cooling tower were measured 1 h after application of QACs and were found to be 8.7 and 20 mosM, respectively; this water had no effect on amoebal survival compared with the TBSS which had a pH of 7 and an osmolality of 17 mosM. Range-finding tests were conducted to determine biocide concentrations that were lethal and stimulatory to the amoebae. These tests were conducted as described below for population growth.

**Determination of population growth.** Plates of amoeba cultures that were 24 to 48 h old were rinsed with TBSS to harvest cells. The amoebae were kept in the rinse (approximately 5 ml) for 30 min to allow the cells to adapt to the osmolality of the TBSS before they were diluted for enumeration. The amount of bacteria washed from the seeded plates was sufficient to allow population growth and prevent starvation-induced cyst formation. Aliquots (15 μl) were transferred to glass slides and examined microscopically to enumerate the trophozoites before the amoebae were dispensed into sets of triplicate wells in microplates to which equal volumes of various biocide dilutions (or TBSS for controls) were added. The mixtures of biocides and protozoa were incubated at 25 ± 1°C for 24 h. The temperature matched that of the cooling tower at the time that the amoebae were isolated. Since trophozoites of all isolates except *C. bilimbosum* did not adhere tenaciously to microplates, subsamples (15 μl) were removed after agitation to enumerate trophozoites in the biocides and controls. For *C. bilimbosum* three microscopic fields covering more than 75% of the bottom of the well, including edges and centers, were examined, and the cells in these areas were counted. A statistical test for homogeneity among fields was conducted to ensure that cells were distributed homogeneously. Also, initial counts of amoebae were made for all wells to ensure homogeneous distribution among replicates. All wells were sampled after 24 h to enumerate amoebae as described above. All amoebae enumerated were considered viable on the basis of movement and contractile vacuole activity. An analysis of variance followed by Dunnett's procedure of the Toxstat program of Gulley et al. (8) was used to determine differences between numbers of amoebae in controls and in different treatments after 24 h of exposure to the biocides. All experiments were conducted at least twice on separate days. Amoeba cultures for each test were started from cysts from an early transfer, and all repeated experiments were started with the same initial source of cysts of each isolate.

The MIC of each biocide was determined for each isolate. Various dilutions of biocides were tested, and those dilutions which resulted in statistically significant decreases in numbers of cells compared with controls were considered inhibitory concentrations. The lowest concentration tested which was inhibitory was defined as the MIC. The gaps between dilutions in the range-finding tests were never greater than a 1:2 dilution, but narrower gaps were used in the inhibitory ranges. The MICs reflected the levels of sensitivity of the organisms to the biocides. Also, concentrations at which stimulation of population growth occurred were determined in a similar manner. As used here (see Tables 2 and 3), the manufacturer's recommended concentration (or dose) was the concentration recommended for maintenance in a cooling system rather than the concentration recommended as an initial start-up dose for a cooling tower.

## RESULTS

**Biocides.** The osmolality and pH of the highest concentration of each biocide used were similar to the osmolality and pH of the TBSS. The osmolality values were 14 to 15 mosM for the control TBSS, 17 mosM for the highest thiocarbamate concentration, and 15 mosM for the highest concentrations of the other three biocides. The pH was 6.8 for TBSS, 7.1 for the thiocarbamate, 6.8 for TBT/QAC, 7.1 for QAC, and 7.1 for the isothiazoline derivative.

**Stimulation.** Initial counts revealed similar amoeba densities in all replicates (ca. 50 amoebae per 15 μl). Homogeneity tests revealed that there were no differences in numbers of *C. bilimbosum* between any two microscopic fields of the triplicate wells. The population growth of the cooling tower isolates was stimulated significantly in the presence of certain concentrations of all four biocides, as shown by the greater numbers of amoebae in biocide-exposed suspensions than in unexposed controls (Table 1). The ATCC isolates were not stimulated at any of the concentrations tested, including those which stimulated the cooling tower isolates. *Cochliopodium* sp. was significantly stimulated at the manufacturer's recommended maintenance concentration of thiocarbamate, at isothiazolin concentrations that were only 1.4- to 13-fold lower than the recommended concentration, at concentrations of QAC that were 3.2- to 160-fold lower than the recommended concentration, and at concentrations of TBT/QAC that were 45- to 75-fold lower than the concentration recommended by the manufacturer (Table 2). *A. hatchetti* was stimulated by thiocarbamate at concentrations that were 12- to 120-fold lower than the concentration recommended by the manufacturer, by isothiazolin at concentrations that were 7- to 200-fold lower than the recommended concentration, by QAC at concentrations that were 11- to 114-fold lower than the recommended concentration, and by TBT/QAC at 47- to 62-fold lower than the recommended concentration (Table 2). The increases in amoeba populations compared with control populations are shown in Table 1. In one case the population was double the control population after 24 h. All results were reproducible.

**Inhibition.** The lowest concentrations resulting in significant decreases in amoeba population densities after 24 h of exposure to the biocides are shown (as MICs) in Table 3. These data show that in all cases except two, the cooling

TABLE 1. Final population densities and levels of stimulation of cooling tower amoebae exposed to biocides

| Biocide | Concn (ppm) yielding maximum stimulation of: | | *A. hatchetti* | | | *Cochliopodium* sp. | | |
|---|---|---|---|---|---|---|---|---|
| | *A. hatchetti* | *Cochliopodium* sp. | No. of amoebae in control[a] | No. of amoebae in treated prepn[a] | % Stimulation | No. of amoebae in control[a] | No. of amoebae in treated prepn[a] | % Stimulation |
| Thiocarbamate | 1 | 8.3 | 200 ± 9 | 279 ± 8 | 40 | 410 ± 16 | 660 ± 32 | 64 |
| Isothiazolin | 5 | 16.6 | 241 ± 18 | 297 ± 5 | 23 | 169 ± 11 | 210 ± 5 | 25 |
| QAC | 0.6 | 0.6 | 198 ± 6 | 252 ± 38 | 27 | 169 ± 11 | 280 ± 7 | 66 |
| TBT/QAC | 2.5 | 2.5 | 230 ± 43 | 370 ± 40 | 60 | 162 ± 24 | 339 ± 15 | 100 |

[a] Average ± 1 standard error for the number of amoebae in 15-μl subsamples from three wells.

tower isolates were more resistant to the biocides than the ATCC isolates were. The exceptions were the experiments in which *C. bilimbosum* ATCC 30937 and the cooling tower *Cochliopodium* sp. exhibited equal sensitivities to the isothiazolin biocide and in which the *Acanthamoeba* isolates from the two sources were equally sensitive to the TBT/QAC biocide. The latter exception is discussed below.

The isothiazolin biocide was least toxic biocide for all isolates, and TBT/QAC was the most toxic biocide for all isolates except *A. hatchetti* ATCC 30730 (Table 3). The order of toxicity for the other two biocides varied depending on the isolate. Although the population growth rates decreased in the presence of certain concentrations of biocides, the organisms could still reproduce in the presence of inhibitory concentrations of some biocides. Although amoeba population growth was inhibited, final cell numbers in some tests were still 80% of control cell numbers after 24 h. The level of inhibition varied, depending on the species and the biocide. Only TBT/QAC was so toxic that no isolate survived at the recommended dose of 156 ppm. Table 3 shows that it may be possible for the cooling tower isolates to be unaffected at the manufacturer's recommended dose of thiocarbamate and isothiazolin and that both ATCC isolates could be inhibited by all of the biocides at the manufacturer's recommended doses. Tables 1 and 3 show that in certain cases concentrations that were inhibitory to the ATCC isolates were stimulatory to the cooling tower isolates.

## DISCUSSION

Our data suggest that amoebae from cooling towers may adapt to the biocides used to control microbial growth. The amoebae not only were not affected by concentrations of biocides that inhibited other isolates which were not isolated from cooling towers, but also reproduced faster in the presence of low concentrations of biocides, including (for one species) concentrations recommended by the manufacturer. These observations underscore the significance of using cooling tower isolates rather than culture collection strains for studies of cooling tower biocide efficacy.

TABLE 2. Stimulatory concentrations of biocides for cooling tower amoebae[a]

| Biocide | Stimulatory concn (ppm) for[a]: | | Manufacturer's recommended dose (ppm) |
|---|---|---|---|
| | *A. hatchetti* | *Cochliopodium* sp. | |
| Thiocarbamate | 0.25–0.8 | 5.5–10 | 10–30 |
| Isothiazolin | 1–5 | 17–25 | 35–219 |
| QAC | 0.7 | 0.5–2.5 | 8–80 |
| TBT/QAC | 2.5–3.3 | 2–3.3 | 156 |

[a] Ranges are the ranges of concentrations at which stimulation occurred as determined by repeated experiments. Single values indicate that repeated experiments yielded the same stimulatory concentration.

In this study the cooling tower isolates survived the manufacturer's recommended doses of all of the biocides except TBT/QAC. Many amoebae and ciliates form resistant cysts which may allow them to survive recommended biocide concentrations in cooling towers. This may explain our isolation of amoebae even though trophozoites could not survive the manufacturer's recommended concentration of TBT/QAC in vitro. Kilvington and Price (14) reported that *A. polyphaga* cysts could survive exposure to 50 mg of residual free chlorine per liter and that *L. pneumophila* could be recovered from the treated cysts. The effect of cooling tower biocides on amoeba cysts is currently under investigation. Barker et al. (2) showed that *A. polyphaga* could recover from a 20-h exposure to two biocides, Vantocil and benzisothiazolone; however, benzisothiazolone was used at much lower concentrations than the concentration used in practice. A third biocide, 5-chloro-*N*-methyl isothiazolone, which is very similar to the isothiazolin which we used, was toxic to the amoebae although it was used at a concentration at which our cooling tower isolates survived (32 ppm). Furthermore, our *Cochliopodium* isolate was stimulated by isothiazolin at concentrations of 17 to 25 ppm.

Although the cooling tower isolates presumably had never been exposed to the QAC which we used (MBC 115) or isothiazolin, with one exception they were still more resistant to these compounds, as determined by measuring MICs, than the ATCC isolates; the exception was *C. bilimbosum* exposed to isothiazolin. Perhaps other chemical aspects of the cooling tower environment can cause cooling tower amoebae to become more resistant to a variety of chemicals. Another exception was the fact that the ATCC strain of *A. hatchetti* had the same tolerance to TBT/QAC as the cooling tower isolate of *A. hatchetti*, although the relative toxicities of the four biocides were different for the two strains. *A. hatchetti* ATCC 30730 appeared to be relatively tolerant to TBT/QAC. This may have been due to its source, which was the sediment of Baltimore Harbor. Hallas and Cooney (9) reported 239.6 ppm of tin in sediments of Baltimore Harbor, and they isolated tin-resistant bacteria from such environments. This observation reveals the importance of knowing the source of cultures acquired for toxicity testing. At the time of this study, there was only one strain of *A. hatchetti* available from the ATCC.

In this study we could not distinguish between the possibility that a biocide had an inhibitory effect on the overall growth rate of a population and the possibility that a certain percentage of the population died while the survivors reproduced. However, the former possibility is more likely, since

TABLE 3. MICs of cooling tower biocides

| Biocide | MIC (ppm) for[a]: | | | | Manufacturer's recommended dose (ppm) |
|---|---|---|---|---|---|
| | *A. hatchetti* cooling tower isolate | *A. hatchetti* ATCC 30730 | *Cochliopodium* sp. cooling tower isolate | *C. bilimbosum* ATCC 30937 | |
| Thiocarbamate | 17 | 0.5 | 62 | 5 | 10–30 |
| Isothiazolin | 50 | 16.5 | 100–166 | 166 | 35–219 |
| QAC | 12–25 | 0.7 | 12.5–25 | 6 | 8–80 |
| TBT/QAC | 5.5 | 5.5 | 12.5 | 2 | 156 |

[a] Single values indicate that repeated experiments yielded the same MIC. Ranges are given when repeated experiments gave different MICs.

we did not observe cells that looked stressed or were lysing at the MICs of the biocides used after 24 h. All trophozoites were morphologically similar to controls. Although growth rates were suppressed at certain biocide concentrations, the surviving cells could theoretically repopulate the cooling tower environment when the biocide concentrations decreased to noninhibitory or stimulatory levels.

For various reasons cooling tower biocide concentrations decrease rapidly as the biocides circulate through the cooling system (24). Also, thick biofilms may prevent initial concentrations from reaching amoebae or other protozoa embedded within the biofilms. It is reasonable to assume that stimulatory concentrations of biocides exist where viable trophozoites are found. In this study, stimulation of amoeba population growth occurred at the manufacturer's recommended concentration of one biocide, as well as at 6- to 200-fold lower than the concentrations recommended for other cooling tower biocides.

The mechanism(s) by which the biocides inhibit or stimulate the cooling tower amoebae is not known. Low concentrations of the pesticide carbaryl(naphthylmethylcarbamate) have been reported to stimulate freshwater algae (22). In that study Stadnyk and Campbell suggested that degradation of carbaryl may have led to availability of nitrogen for the algae. Perhaps protozoa can utilize degradation products of the thiocarbamate biocide.

Cooling tower biocides are used to decrease the degree of biofouling, particularly by algae, and are not meant to disinfect the system. However, recent reports of pathogens in cooling towers has led to investigations of effects of commonly used biocides on amoebae and bacteria. The stimulatory effect of cooling tower biocides on amoeba populations may be important in the epidemiology of legionellosis, since amoebae in treated waters have been implicated as host organisms for *Legionella* spp. *Acanthamoeba* spp. have been shown to become infected with legionellae, and a *Cochliopodium* species has been isolated along with legionellae (20), although its ability to support intracellular growth of the bacteria has not been studied. Members of the genus *Cochliopodium*, however, cannot be ruled out as possible hosts for legionellae in treated water. Ironically, biocides used to control microbial growth may actually increase populations of host organisms for *L. pneumophila* if concentrations fall to stimulatory levels. Furthermore, amoebae alone may be pathogenic (the ATCC *A. hatchetti* strain was categorized as a class III pathogen), and such biocides may increase the numbers of pathogenic amoebae.

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