Journal of Industrial Microbiology (1996) 16, 73–78

Survival of protozoa in cooling tower biocides

EE Sutherland[1] and SG Berk

Center for the Management, Utilization, and Protection of Water Resources, Tenessee Technological University, Cookeville, TN 38505, USA

Protozoa from cooling towers may serve as hosts for legionellae, but such protozoa have not been examined with respect to effects of cooling tower biocides. In this study, two ciliate species, *Tetrahymena* sp and *Colpoda* sp, and two amoebae species, *Vannella miroides* and *Acanthamoeba hatchetti*, were isolated from a cooling tower and tested for survival in the presence of four cooling tower biocides. The protozoa were exposed for 24 h to a thiocarbamate compound, an isothiazolone compound, quaternary ammonium compounds (QAC), and tributyltin neodecanoate with quarternary ammonium compounds (TBT/QAC). After exposure, cells were examined for viability. The highest concentration of each biocide in which cells could survive was compared to the manufacturers' recommended maintenance dosage (MRMD) of the biocides. *Tetrahymena* and *Colpoda* survived concentrations within the range of the MRMD of thiocarbamate and QAC. *Vannella* and *Acanthamoeba* survived concentrations within the MRMD of thiocarbamate, isothiazolone, and QAC. *Colpoda* cysts and *Acanthamoebae* cysts remained viable after exposure to concentrations much greater than the MRMD of thiocarbamate, isothiazolone, and QAC. None of the protozoa in any stage could survive the MRMD of TBT/QAC. These results show that protozoa indigenous to cooling towers may survive the recommended concentration of certain biocides, and this information may be important in devising procedures for eradicating hosts for legionellae.

Keywords: cooling tower; biocides; amoebae; ciliates; protozoa; legionellae

Introduction

Cooling towers of air-conditioning systems can provide suitable environments for microbial life including algae, bacteria and protozoa [6, 15, 22] because of the warm water, sunlight and nutrients available. Both protozoa and *Legionella pneumophila* have been found in cooling towers implicated as a source of legionellosis outbreaks [15]. Barbaree *et al* [3] examined cooling tower waters associated with an outbreak of legionellosis and isolated both amoebae and ciliates. Rowbotham [20] was the first to provide evidence that amoebae could serve as hosts for *Legionella*. Using amoebae of the genera *Acanthamoeba* and *Naegleria*, Rowbotham showed that when *L. pneumophila* was provided as a food source, the bacteria could replicate and survive intracellularly within the amoebae. The importance of protozoa as protective niches for legionellae and other bacteria was reviewed recently by Barker and Brown [5].

Since Rowbotham's observations, several other groups have also found intracellular survival and multiplication of *L. pneumophila* in amoebae. Tyndall and Dominigue [27] showed interactions between amoebae (*Naegleria* and *Acanthamoeba*) and *L. pneumophila*. They suggested that amoebae may support growth of legionellae in conditions unfavorable to the bacteria alone. Anand *et al* [2] noted the presence of *L. pneumophila* within early cysts of amoebae. The protection of legionellae inside cysts could allow *L. pneumophila* to survive in harsher conditions than those in which *L. pneumophila* alone could survive. Several others have also shown intracellular multiplication of *L. pneumophila* within amoebae [9,11,19,28]. Fields *et al* [10] found that *L. pneumophila* replicates within the ciliate *Tetrahymena pyriformis*, and survival of *L. pneumophila* was observed in *T. vorax* by Smith-Somerville *et al* [23]. Such observations have raised speculation that protozoa may serve as hosts for legionellae, thereby protecting legionellae from the effects of biocides [18] and contributing to the distribution of legionellae in the environment.

Although biocide efficacy has been examined for *L. pneumophila*, little work has been done with biocides and protozoa from cooling towers. Cursons *et al* [7] tested *Acanthamoeba* and *Naegleria* with four different disinfectants: chlorine, chlorine dioxide, ozone, and deciquam 222. Barker *et al* [4] exposed *Acanthamoeba polyphaga* to three different biocides: polyhexamethylene biguanide (PHMB), benzisothiasolone (BIT), and an isothiazolone compound. However, the effects of PHMB on *A. polyphaga* were obtained at only 10–20% of the concentrations used for sanitation of swimming pools. Concentrations of BIT used were much lower than those normally used in practice, and concentrations of isothiazolone were higher than those normally used in cooling tower water treatment. Srikanth and Berk [25] studied effects of low concentrations of biocides on cooling tower amoebae, and found that population growth rate was increased compared with controls which were not exposed to biocides. Kilvington [13] tested effects of biocides on *A. polyphaga* and *N. fowleri*, using both the trophozoite and cysts forms; however, *A. polyphaga* was isolated from a case of human keratitis, and *N. fowleri* was isolated from a thermal spring. Effects of chlorine were evaluated ealier [8] using cysts of *Naegleria* and *Acanth-*

Correspondence: SG Berk, Center for the Management, Utilization, and Protection of Water Resources, Tennessee Technological University, Box 5033, Cookeville, TN 38505, USA
[1]Present address: Genetics Associates, Inc, 121 21st Avenue N, Nashville, TN 37203, USA
Received 26 July 1995; accepted 8 November 1995

amoeba which were not isolated from cooling towers. Kilvington and Price [14] also tested chlorine against *A. polyphaga* cysts which contained *L. pneumophila*. They found that cysts could protect *L. pneumophila* from 50 mg L^{-1} free chlorine.

Since protozoa may harbor legionellae, protozoa from cooling tower waters should be examined for effects of cooling tower biocides. In the present study, four biocides commonly used in cooling tower systems were screened using protozoa isolated from a cooling tower.

The objectives of the present study were to determine: 1) the minimum lethal concentration of each biocide, and thereby the highest concentration of each of the biocides in which protozoa could survive; 2) whether the protozoa could survive the manufacturers' recommended maintenance dosage (MRMD) of each of the four biocides; 3) whether protozoa could survive in filter-sterilized cooling tower water collected one hour after addition of biocides to a cooling tower; and 4) whether protozoan species which initially could not survive the MRMD could survive after the addition of sterilized biofilm material.

Materials and methods

Protozoa isolation and culture

Protozoa were isolated from the pool of a cooling tower on the campus of Tennessee Technological University in Cookeville, TN, USA. Amoebae were isolated by placing a drop of water with biofilm material collected from the cooling tower pool onto a non-nutrient agar plate seeded with a lawn of *Escherichia coli*; the *E. coli* culture was obtained from Dr Susan Goss (Biology Department, Tennessee Technological University). After amoebae grew on the plate, a single cell was removed using a sterile microcapillary tube, and placed onto a fresh *E. coli*-seeded plate. The amoebae were identifed by Dr TK Sawyer of Rescon Associates, Inc, Royal Oak, MD. Ciliates were isolated by placing a drop of biofilm material onto an *E. coli*-seeded non-nutrient plate to culture them. They were then transferred manually through drops of Tris-buffered saline solution (TBSS): 2 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1 mM Tris, pH 6.8–7.2, until a single ciliate per drop was observed. The single cell was transferred to a liquid medium to achieve a clonal culture. The cilitates were identified morphologically by use of 'An Illustrated Guide to the Protozoa' [16].

Four species of protozoa were used in the study: one cyst-forming amoeba, *Acanthamoeba hatchetti*; one amoeba which does not form cysts, *Vannella miroides*; one cyst-forming ciliate, *Colpoda* sp; and one ciliate, a *Tetrahymena* sp which did not form cysts. The amoebae were maintained on *E. coli*-seeded non-nutrient agar plates at 24°C, and cells used in experiments came from 48-h-old cultures. *Tetrahymena* was maintained in TBSS amended with washed *E. coli* as a food source. *Colpoda* was maintained in a cereal leaves medium. One liter of water containing one gram of dehydrated cereal leaves (Sigma, St Louis, MO, USA) was boiled for 5 min, then filtered through a Whatman no. 4 (9.0 cm) Qualitative filter (Whatman International, Maidstone, UK) and sterilized by autoclaving it. *Colpoda* isolates described above were added to the medium along with an inoculum of *E. coli* as supplemental food. No subsequent transfers from this initial culture required addition of *E. coli* since sufficient numbers of bacteria were carried along with the *Colpoda* to serve as an inoculum in fresh cereal leaves medium. Ciliates were maintained at 24–26°C, and cells to be tested came from 24 to 48-h-old cultures. All culture transfers and experiments were carried out in a laminar-flow hood. Plates of amoebal cultures were rinsed with TBSS to harvest cells. Amoebae remained in the rinse (approximately 5 ml) for 0.5–1.0 h to allow cells to adapt to the osmolality of the TBSS prior to diluting them for enumeration. *Tetrahymena* cells were concentrated in the TBSS in which they were maintained. *Colpoda* cells from cereal leaves media were washed twice by repeated centrifugation ($104 \times g$ for 1–2 min) and resuspended in TBSS. The cells did not appear to be affected by centrifugation. The *Colpoda* cells were allowed to adjust to TBSS as described above for amoebae. *Tetrahymena* cells were adjusted to an average of 80 per 15 μl, and all other species were adjusted to a range of 125 to 135 cells per 15 μl.

Biocides

Four commonly used biocides obtained from Nash-Chem (Nashville, TN, USA) were used in the study. Biocides included a thiocarbamate compound, isothiazolones, quaternary ammonium compounds (QAC), and tributylin neodecanoate mixed with quaternary ammonium compounds (TBT/QAC). Table 1 lists the biocides and their active ingredients.

A series of 1:2 dilutions of biocides was made using TBSS. The pH of the minimum lethal concentration (MLC) of each biocide was compared with that of the TBSS alone.

Test procedure

Feeding stages: Protozoa in their vegetative (feeding) stage were exposed to a range of concentrations of each biocide to determine the MLC and, thereby, the highest concentration in which they could survive. Seventy-five microliters each of biocide solution and washed protozoan suspensions were placed into wells of microplates. TBSS in place of biocide solutions served as a control. The exposure

Table 1 Composition of biocides tested

Biocides	Active ingredents	% of Formulation
Thiocarbamate	Potassium dimethyldithiocarbamate	50.0
Isothiazolone	5-Chloro-2methyl-4-isothiazolin-3-one	1.15
	2-Methyl-4-isothiazolin-3-one	0.35
QAC	Poly [oxyethylene (dimethyliminio) ethylene- (dimethyliminio) ethylene dichloride]	15.0
TBT/QAC	Alkyl (C_{12}, 61%; C_{14}, 23%, C_{16}, 11%, C_8 & C_{10}, 2.5%, C_{18}, 2.5%) dimethyl benzyl ammonium chloride	9.0
	Tributyltin neodecanoate	5.0
	Alkyl (C_{14}, 58%; C_{16}, 28%; C_{12}, 14%) dimethyl benzyl ammonium chloride	4.5
	Alkyl (C_{14}, 90%; C_{16}, 5%; C_{12}, 5%) dimethyl ethyl ammonium bromide	1.5

period was 24 h at 26°C, and experiments were run in triplicate. After the exposure period, wells were examined for living cells, using an inverted microscope. Amoebae were considered living if they displayed normal morphology and contractile vacuole activity. Ciliates were considered viable based on motility and/or filter-feeding. When viability of remaining cells was difficult to determine, 100 μl of the suspension was drawn off and replaced with 200 μl of fresh medium containing *E. coli*. Viable cells retain their normal morphology and divide. The MLC was determined by finding the lowest concentration in which no viable cells were found in any of the three wells. The concentrations in which the protozoa could survive were then compared with the MRMD to determine whether protozoa could survive in any portion of the range of MRMD. *Acanthamoeba* grown on heat-killed *E. coli* was also exposed to the biocides, and results were compared with those using *Acanthamoeba* grown on living *E. coli* to determine whether live bacteria would have an effect on survival of the amoebae. All experiments were conducted at least three times.

Cyst stages: Cysts of the cyst-forming protozoa were also exposed to a range of concentrations of each biocide to determine the MLC. Sterile coverslips were placed in a broth of *E. coli* to allow a biofilm of bacteria to form on the coverslips. The coverslips were then transferred to an *E. coli*-seeded plate which was inoculated with *Acanthamoeba*. For tests with *Colpoda* cysts, sterile coverslips were placed in a petri dish containing cereal leaves media and *E. coli*. The medium was then inoculated with *Colpoda*. Natural starvation-induced cysts were allowed to form and adhere to the coverslips. Cysts on coverslips were then exposed to the biocides in sterile staining jars in duplicate for 24 h at 26°C. TBSS alone served as a control. After the exposure period, cysts were rinsed by soaking in sterile TBSS for 30 min, and then each coverslip was transferred to fresh media containing *E. coli* to test for viability. Viable cells excyst in the presence of fresh media and bacteria. Coverslips containing cysts were examined for excystment for up to 6 days. The MLC was determined by finding the lowest concentration in which excystation was not observed. The concentrations in which any cysts remained viable after biocide exposure were then compared with the MRMD to determine whether cysts were viable after exposure to concentrations in any portion of the range of the MRMD. All cyst experiments were conducted at least three times.

Cooling tower water: In order to determine whether protozoa could survive in cooling tower water that was being treated with biocides, water was collected from the pool of the cooling tower 1 h after addition of QAC or thiocarbamate (on separate days), and the cooling tower water was filter-sterilized to remove any protozoa. The actual concentration of biocides was not determined. Amoebae were collected from plate cultures by rinsing the plate with filtered cooling tower water. *Tetrahymena* was centrifuged and resuspended in the cooling tower water. *Colpoda* was washed by repeated centrifugation and resuspended in filtered cooling tower water. Protozoa were suspended in TBSS alone as a control. After protozoa were suspended in filtered cooling tower water or TBSS, they were transferred to triplicate wells of microplates for 24 h at 25°C. After the exposure period, cells were examined for viability as described above. If cyst-forming protozoa could not survive the cooling tower water in their feeding stage, cysts were then exposed to the cooling tower water for 24 h and examined for viability as described above.

Biofilm protection: Those species which did not survive the MRMD of any one of the biocides were then exposed to the effective biocides with the addition of treated biofilm material to determine whether the biofilm material would provide protection from the biocides. Biofilm material was collected from the pool of a cooling tower by scraping the sides of the pool. Thin layers of biofilm material were spread in glass petri dishes, placed under a UV light within a hood for 17 h, and allowed to air-dry. Dried biofilm material was then scraped from the glass dishes, plated onto an *E. coli*-seeded plate and observed for the growth of protozoa. After it was determined that there was no growth, 0.5 mg ± 0.01 mg of UV-treated biofilm material was added per ml of biocide solution. Biocide solutions were made as described above. Solutions containing biofilm material were allowed to stand 30 min to allow the biofilm to react with biocides, prior to adding the protozoa. Biofilm material mixed with TBSS alone served as a control to determine whether material that leached from the biofilm material would kill the cells. Protozoa were exposed to the biocide/biofilm solutions in microplates for 24 h at 26°C in triplicate. After the exposure period, cells were examined for viability as described above.

Results

Minimum lethal concentrations

Table 2 contains the MLC data. The MLC (the lowest concentration in which all cells were killed in every replicate of every experiment) of thiocarbamate was well above the MRMD for all species and cysts forms. The MLC against amoebae and cysts of both cyst-forming species fell within or above (depending on species) the MRMD range of isothiazolones. The MLCs of QAC against all species and the cysts fell within or above the MRMD range, but the MLC of TBT/QAC was below the MRMD for all feeding and cyst forms.

Table 2 Minimum lethal concentrations of biocides for protozoa

Biocide	MLC (ppm) for:						MRMD
	Tetrahymena	*Colpoda*		*Vannella*	*Acanthamoeba*		
		feeding stage	cyst stage		feeding stage	cyst stage	
Thiocarbamate	244	977	31 250	3906	3906	125 000	10–30
Isothiazolone	31	31	7813	122	244	31 250	35–219
QAC	122	61	488	61	61	62 500	8–80
TBT/QAC	15	15	31	61	31	122	156

Survival in thiocarbamate

The highest concentrations in which living cells were present after exposure in any of the wells of any replicated experiment is reported in Table 3 as the survival concentration. The next highest concentration of the dilution series is the concentration in which all protozoa were killed in every well of each replicated experiment, and this is reported in Table 2 as the MLC. After protozoa in the feeding stage were exposed to the thiocarbamate compound for 24 h, the highest concentration in which *Tetrahymena* survived was 122 ppm (Table 3). This occurred in two of nine samples in the three experiments. *Colpoda* survived 488 ppm thiocarbamate in two of nine samples, *Vannella* survived 1953 ppm in three of 15 samples, and *Acanthamoeba* survived 1953 ppm in one of nine samples (Table 3). All protozoa in the feeding stage survived the MRMD of the thiocarbamate, 10–30 ppm. The two amoebae were more tolerant to biocides than the two ciliates. Cysts of *Colpoda* and *Acanthamoeba* survived higher concentrations than did protozoa in the feeding stage. *Colpoda* cysts remained viable after exposure to 15 625 ppm in six of six samples, and *Acanthamoeba* cysts were still viable after exposure to 62 500 ppm in four of six samples (Table 3). Cysts survived concentrations much higher than the MRMD.

Survival in isothiazolone

When exposed to isothiazolones, *Tetrahymena* survived 15 ppm in three of nine samples, and *Colpoda* survived 15 ppm in only one of nine samples (Table 3). After 24 h, no *Colpoda* in the feeding stage were present, however there were several cysts which were not initially present. After removal of a portion of the suspensions and addition of fresh media containing *E. coli*, cysts from one well excysted. Survival at 15 ppm is reported because the biocide may have induced encystment which resulted in a viable cyst. Previous work [26] demonstrated that certain biocides can induce cyst formation. The MRMD of isothiazolones (35–219 ppm) was lethal to the ciliates in feeding stages. The amoebae, however, survived within the MRMD range. *Vannella* survived 61 ppm in nine of 12 samples, and *Acanthamoeba* survived 122 ppm in one of 12 samples (Table 3). Cysts of *Colpoda* and *Acanthamoeba* withstood much higher concentrations than the feeding forms. *Colpoda* cysts were viable after exposure to 3906 ppm in five of six samples, and *Acanthamoeba* cysts were viable after exposure to 15 625 ppm in two of six samples (Table 3).

Table 3 Highest biocide concentrations in which protozoan survival occurred

Biocide	Biocide concentration (ppm) against:						MRMD
	Tetrahymena	*Colpoda*		*Vannella*	*Acanthamoeba*		
		feeding stage	cyst stage		feeding stage	cyst stage	
Thiocarbamate	122	488	15 625	1953	1953	62 500	10–30
Isothiazolone	15	15	3906	61	122	15 625	35–219
QAC	61	31	244	31	31	31 250	8–80
TBT/QAC	8	8	15	31	15	61	156

Survival in QAC

After exposure to QAC, the highest concentration in which the protozoa survived were: 61 ppm for *Tetrahymena* (one of nine samples), 31 ppm for *Colpoda* (three of nine samples), and 31 ppm for both *Vannella* and *Acanthamoeba* (six of nine samples each) (Table 3). All species in the feeding stage survived within the range of the MRMD of 8–80 ppm. Cysts of *Colpoda* remained viable after exposure to 244 ppm in one of six samples, and cysts of *Acanthamoeba* were still viable after exposure to 31 250 ppm in two of six samples (Table 3). As with other biocides, cysts survived concentrations much greater than the MRMD.

Survival in TBT/QAC

After exposure to TBT/QAC, concentrations in which protozoa survived were: 8 ppm for *Tetrahymena* (one of nine samples), 8 ppm for *Colpoda* (nine of nine samples), 31 ppm for *Vannella* (four of 12 samples), and 15 ppm for *Acanthamoeba* (12 of 12 samples) (Table 3). None of the species survived the MRMD of 156 ppm; however, as with other biocides, the amoebae tolerated higher concentrations than ciliates. Cysts of *Colpoda* were viable after exposure to 15 ppm in seven of eight samples, and *Acanthamoeba* cysts were viable after exposure to 61 ppm in five of 10 samples (Table 3), but these were below the MRMD.

Order of toxicity, based on MLCs

The order of biocide toxicity to protozoa in the feeding stage was determined. For all four species, TBT/QAC was the most toxic, and thiocarbamate was the least toxic. The order of toxicity of the other two biocides varied, depending on species. QAC was the second most toxic biocide for the amoebae, and isothiazolones were the second most toxic for the ciliates. The order of toxicity was the same for cysts as for the feeding stages.

Survival in cooling tower water

Tetrahymena, Vannella, and *Acanthamoeba* survived in the filter-sterilized water collected after addition of thiocarbamate and QAC (used separately), as expected from MLC results. However, *Colpoda* survived only in the water collected after addition of QAC, but not after addition of thiocarbamate. *Colpoda* cysts, however, remained viable after 24-h exposure to the cooling tower water containing each biocide.

Experiments with biofilm

In experiments designed to determine whether UV-treated biofilm material would protect the protozoa, neither ciliate species survived the MRMD of isothiazolones or TBT/QAC with biofilm addition; however *Tetrahymena, Acanthamoeba*, and *Vannella* were provided some protection from the TBT/QAC. Addition of biofilm allowed *Tetrahymena* to survive 31 ppm, an increase of four-fold greater concentration of TBT/QAC than that of experiments in which no biofilm material was added. *Acanthamoeba* survived 31 ppm TBT/QAC, a two-fold greater concen-

tration. Although this was only an increase up to the next concentration tested, viability was not as difficult to determine as when tested with TBT/QAC alone. *Vannella* survived in only two of 12 wells without biofilm, whereas they survived in five of six wells with biofilm, although the concentrations in which they survived did not change. The addition of such treated biofilm does not represent a true living system, but does provide organic loading to the system, which may influence toxicity.

Heat-treated bacteria

Acanthamoeba grown on heat-killed *E. coli* showed the same response as *Acanthamoeba* grown on live *E. coli* after exposure to thiocarbamate, isothiazolones, or TBT/QAC. However, after exposure to QAC, *Acanthamoeba* grown on heat-killed *E. coli* survived 122 ppm, four-fold higher than when grown on live *E. coli*.

Discussion

Compounds used in this study have also been tested by others for their efficacy against *L. pneumophila*. Soracco *et al* [24] tested two compounds with *L. pneumophila*, a thiocarbamate similar to the thiocarbamate of the present study, and an isothiazolone compound which was the same as that used in the present study. The thiocarbamate compound, sodium dimethyldithio-carbamate, was effective in killing the bacterium at the manufacturers' recommended dosage. In the present study, protozoa survived in concentrations of thiocarbamate approximately 10- to 200-fold higher (depending on species) than the MRMD of 10 ppm. The isothiazolone compound used by Soracco *et al* [24] was also effective in controlling the growth of *L. pneumophila* at the pulse dose concentration, whereas in the present study, both amoebae survived concentrations of isothiazolones within the MRMD. From the present study it appears that *L. pneumophila* within protozoa may not be killed by the biocides. Kilvington and Price [14] concluded that *Acanthamoeba* containing *L. pneumophila* could protect the bacteria from 50 ppm free chlorine, and Kilvington [13] showed that *Acanthamoeba* cysts could protect *L. pneumophila* from isothiazolone. Furthermore, Barker *et al* [4] showed that *L. pneumophila* is more resistant to biocides after growth in amoebae.

A. hatchetti, the cooling tower isolate used in the present study, survived concentrations of 122 ppm isothiazolone, with cysts surviving 15 625 ppm. Kilvington [13] used the same compound under similar conditions and found it was amoebacidal to *A. polyphaga* at only 5 ppm and cysticidal at 150 ppm. This is a 25-fold difference for the vegetative form and over a 100-fold difference for cysts compared with results of the present study. The difference may be explained by species differences or by the fact that *A. polyphaga* was isolated from a case of human keratitis, whereas the *A. hatchetti* was from a cooling tower. It has been shown that *A. hatchetti* from a cooling tower is more resistant to cooling tower biocides than *A. hatchetti* which was not isolated from a cooling tower [25]. When testing the efficacy of cooling tower biocides against protozoa, cooling tower isolates should be used rather than protozoa from other sources, since cooling tower isolates may be more resistant.

From results of the laboratory tests, we predicted that the feeding forms of the protozoa should survive in the cooling tower when both thiocarbamate and QAC are used. This hypothesis was supported by the 'field' study in which cooling tower water was tested. The one exception was that feeding stages of *Colpoda* did not survive the 24-h exposure to the water after thiocarbamate was applied. On two occasions using *Colpoda*, the pH of the cooling tower water was slightly higher than when other species were tested, and the higher pH may have affected the cells. Also, when biocides are added to the tower on campus, they are not always accurately measured, and it is possible that concentrations greater than the MRMD were used on those occasions. Furthermore, 24-h exposures are longer than the protozoa would normally be exposed to in a cooling tower system.

For all of the tests without biofilm material added, the experimental conditions represented a 'worst case scenario' with respect to survival of the protozoa. Testing took place in a 'clean system' using 24-h exposures. Unlike in an actual cooling tower, there was no addition of makeup water, no loss by drift, no biofilm adsorption or heating. Such factors could result in decreased biocide concentrations. In a cooling tower that receives slug doses, the protozoa would not be exposed to a constant concentration for 24 h. Therefore, if protozoa survived the 24-h exposure in the laboratory, they would most likely survive in an actual cooling tower. The converse is not necessarily true, ie those that do not survive the experimental conditions may still survive in an actual cooling tower due to decreasing biocide concentrations and protective factors such as biofilms. Keevil and Mackerness [12] reported that bacteria in biofilms are more resistant to biocide treatments. Although none of the species could survive the MRMD of TBT/QAC in either the feeding or cyst stages, *Colpoda*, as well as another amoeba not used in the study, was isolated from the cooling tower during the time that TBT/QAC was being used in the tower. Most likely, the algal biofilm present in the pool of the cooling tower protected the protozoa by adsorbing the biocide or by physically sheltering the protozoa from exposure. Another possibility is that cysts of these organisms entered the pool when the biocide concentrations were very low. Although the TBT/QAC was the most effective against protozoa, this biocide has been abandoned by some cooling tower operators. It is no longer used on the Tennessee Technological University campus because of foaming problems.

The pH values of most of the MLCs for protozoa in the feeding states were similar to the pH of the TBSS, 6.8–7.2; therefore pH can probably be ruled out as a factor in the death of cells. The pH values of the concentrations that killed cysts, however, ranged from 5.05 to 11.74, and most were either lower or higher than that of the TBSS. In these cases, however, the concentrations required to kill cyst forms would not normally be encountered in a cooling tower.

Procedures of the American Society for Testing and Materials [1] for evaluating the efficacy of cooling tower biocides take into consideration only the effects on bacteria and fungi, as have other investigations [17,21]. Protozoa, however, should also be included when examining biocide

efficacy because of their association with *Legionella*, and because some amoeba species alone can be human pathogens. Suggestions for future research on biocides and amoebae include: 1) use of cooling tower isolates rather than commercially available cultures, 2) an exposure period of 3–6 h, which may be more appropriate than 24 h, and 3) adjustment of pH to 8.0–9.0 to more closely resemble that of a cooling tower.

The procedure and conditions of the present laboratory screening tests were designed to provide guidelines for determining biocide sensitivity of protozoa native to cooling towers. Maintaining lethal concentrations in cooling towers is probably not feasible; however, data obtained from this study should be useful for guiding cooling tower operators and biocide manufacturers in controlling protozoan populations. Such information may lead to more effective disinfection strategies.

Acknowledgements

This work was supported by a graduate research grant to EES from the American Society of Heating, Refrigerating and Air-Conditioning Engineers; and by the Center for the Management, Utilization and Protection of Water Resources of Tennessee Technological University. We thank Ken Meredith, heating and air-conditioning mechanic at Tenessee Technological University, for his assistance in this project.

References

1 American Society for Testing and Materials. 1985. E-645. Standard test methods for efficacy of microbiocides used in cooling systems. In: Annual Book of ASTM Standards, pp 188–191, American Society for Testing and Materials, Philadelphia, PA.

2 Anand CM, AR Skinner, A Malic and JB Kurtz. 1983. Interaction of *L. pneumophila* and a free living amoeba (*Acanthamoeba palestinensis*). J Hyg Camb 91: 167–178.

3 Barbaree JM, BS Fields, JC Feeley, SW Gorman and WT Martin. 1986. Isolation of protozoa from water associated with a legionellosis outbreak and demonstration of intracellular multiplication of *Legionella pneumophila*. Appl Environ Microbiol 51: 422–424.

4 Barker J, MRW Brown, PJ Collier, I Farrell and P Gilbert. 1992. Relationship between *Legionella pneumophila* and *Acanthamoeba polyphaga*: physiological status and susceptibility to chemical inactivation. Appl Environ Microbiol 58: 2420–2425.

5 Barker J and MRW Brown. 1994. Trojan Horses of the microbial world: protozoa and the survival of bacterial pathogens in the environment. Microbiology 140: 1253–1259.

6 Bear JC. 1988. Selecting chemical treatment equipment. ASHRAE 30: 39–41.

7 Cursons RTM, TJ Brown and EA Keys. 1980. Effect of disinfectants on pathogenic free-living amoebae: in axenic conditions. Appl Environ Microbiol 40: 62–66.

8 DeJonckheere J and H van de Voorde. 1976. Differences in destruction of cysts of pathogenic and nonpathogenic *Naegleria* and *Acanthamoeba* by chlorine. Appl Environ Microbiol 31: 294–297.

9 Fields BS, GN Sanden, JM Barbaree, WE Morrill, RM Wadowsky, EH White amd JC Feeley. 1989. Intracellular multiplication of *Legionella pneumophila* in amoebae isolated from hospital hot water tanks. Curr Microbiol 18: 131–137.

10 Fields BS, EB Shotts Jr and WT Martin. 1984. Proliferation of *Legionella pneumophila* as an intracellular parasite of the ciliated protozoan *Tetrahymena pyriformis*. Appl Environ Microbiol 47: 467–471.

11 Holden EP, HH Winkler, DO Wood and ED Leinbach. 1984. Intracellular growth of *Legionella pneumophila* within *Acanthamoeba castellanii* Neff. Infect Immun 45: 18–24.

12 Keevil CW and CW Mackerness. 1990. Biocide treatment of biofilms. Inter Biodeter 26: 169–179.

13 Kilvington S. 1990. Activity of water biocide chemicals and contact lens disinfectants on pathogenic free-living amoebae. Int Biodeterior 26: 127–138.

14 Kilvington S and J Price. 1990. Survival of *Legionella pneumophila* within cysts of *Acanthamoeba polyphaga*. J Appl Bacteriol 68: 519–525.

15 Kurtz JB, CLR Bartlett, UA Newton, RA White and NL Jones. 1982. *Legionella pneumophila* in cooling tower systems. J Hyg Camb 88: 369–381.

16 Lee JJ, SH Hutner and EC Bovee. 1985. An Illustrated Guide to the Protozoa, 629 pp. Society of Protozoologists, Lawerence, Kansas.

17 McCoy WF, JE Ridge and ES Lashen. 1986. Efficacy of biocides in a laboratory model cooling tower. Mat Perform 95: 9–14.

18 Meitz A. 1986. Clean cooling systems minimize Legionella exposure. Heat Piping Air Cond 58: 99–102.

19 Newsome AL, RL Baker, RD Miller and RR Arnold. 1985. Interactions between *Naegleria fowleri* and *Legionella pneumophila*. Infect Immun 50: 449–452.

20 Rowbotham T: 1980. Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. J Clin Pathol 33: 1179–1183.

21 Singer M. 1976. Laboratory procedures for assessing the potential of antimicrobial agents as industrial biocides. Process Biochem 11: 30–35.

22 Smith AL and RA Muia. 1973. Identify and control microbiological organisms in cooling water systems. Power 117: 17–19, 91–95.

23 Smith-Sommerville H, VB Huryn, C Walker and AL Winters. 1991. Survival of *Legionella pneumophila* in the cold-water ciliate *Tetrahymena vorax*. Appl Environ Microbiol 57: 2742–2749.

24 Soracco RJ, HG Gill, C Fliermans and DH Pope. 1983. Susceptibilities of algae and *Legionella pneumophila* to cooling tower biocides. Appl Environ Microbiol 45: 1254–1260.

25 Srikanth S and SG Berk. 1993. Stimulatory effects of cooling tower biocides on amoebae. Appl Environ Microbiol 59: 3245–3249.

26 Sutherland ES, S Srikanth and SG Berk. 1992. Biocide-induced encystment of amoebae and ciliates from a cooling tower, abstr Q-14, p 337. Abstr 92nd Annual Meet Am Soc Microbiol, New Orleans, LA.

27 Tyndall RL and EL Domingue. 1982. Cocultivation of *Legionella pneumophila* and free-living amoebae. Appl Environ Microbiol 44: 954–959.

28 Wadowsky RM, A Fleisher, NJ Kapp, M El-Moufte, JN Dowling, RM Agnostini and RB Yee. 1993. Multiplication of virulent and avirulent strains of *Legionella pneumophila* in cultures of *Hartmannella vermiformis* In: *Legionella*. Current Status and Emerging Perspectives (Barbaree JM, RF Breiman and AP Dufour, eds), pp 145–147, American Society for Microbiology, Washington, DC.