APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1995, p. 407–412 Vol. 61, No. 2
0099-2240/95/$04.00+0

Detection of Legionella Species in Reclaimed Water and Air with the EnviroAmp Legionella PCR Kit and Direct Fluorescent Antibody Staining

CAROL J. PALMER,[1]* G. FRED BONILLA,[1] BRUCE ROLL,[2] CHRISTINE PASZKO-KOLVA,[3] LOUIS R. SANGERMANO,[1] AND ROGER S. FUJIOKA[2]

Environmental Sciences Laboratory, County Sanitation Districts of Orange County, Fountain Valley, California 92728-8127[1]; Department of Microbiology, University of Hawaii, Honolulu, Hawaii 96822[2]; and ATL, Inc., Alta Loma, California 91701[3]

Received 25 April 1994/Accepted 16 November 1994

Reclaimed water is an important resource for areas with inadequate water supplies. However, there have been few studies on the variety of microorganisms found in this type of water, since typically reclaimed water is examined only for the presence of coliform bacteria. Many microorganisms, including the legionellae, are known to be more resistant to chlorine than are coliform bacteria. Previously, we detected $>10^3$ *Legionella* cells per ml in primary and secondary sewage effluents and observed no significant reduction in population numbers throughout the treatment process. In this study, we detected *Legionella* spp. in chlorinated effluent by using an EnviroAmp Legionella PCR kit and direct fluorescent antibody (DFA) staining. However, we were not able to isolate Legionella spp. from either natural or seeded reclaimed water samples. This suggests that the *Legionella* spp. detected by the PCR and DFA methods may be injured or viable but nonculturable after exposure to the high residual chlorine levels typically found in this type of water source. The numbers of coliform bacteria were low (<2 cells per 100 ml) in most reclaimed water samples and were not correlated with the presence or absence of *Legionella* spp. We also collected air samples from above a secondary aeration basin and analyzed them by using the PCR, DFA, and plate culture methods. *Legionella* spp. were detected in the air obtained from above the secondary basin with all three methods. We concluded that the PCR was superior to the culture and DFA methods for detecting *Legionella* spp. in environmental water samples.

Water reclamation or reuse of sewage effluent has become more popular during the past decade. Reuse of highly treated sewage effluent for irrigation, for aquifer recharging, and for watering greenbelts is an attractive alternative in many drought-prone areas with insufficient water supplies. However, except for standard coliform testing, few studies have been performed to evaluate the microbial contents of reused water. Since it is known that many microorganisms are more resistant to chlorine than coliform bacteria are or can be shielded from chlorination by protozoans (7, 8, 19), we decided to determine to what degree chlorine-resistant microorganisms are present in samples of reused water.

One group of organisms that are highly resistant to chlorine is the legionellae (8, 9). Legionellae are the etiological agents of both Legionnaires' disease and Pontiac fever (4, 23), and human infection occurs through inhalation of contaminated aerosols. Outbreaks of legionellosis have been traced to a number of potable water sources, including *Legionella*-contaminated water in cooling towers and air conditioners (3, 23), hot tubs (11), showerheads (20), public fountains (5), and even a supermarket vegetable-misting machine (10).

Legionella spp. are ubiquitous in environmental water and are capable of surviving extreme ranges of environmental conditions (6, 16–18). Paszko-Kolva et al. (16) found that both clinical and environmental isolates of *Legionella pneumophila* inoculated into drinking water and creek water survived for 2.5 years under low-nutrient conditions. In another study Paszko-Kolva et al. showed that temperature and natural predation have considerable influence on *L. pneumophila* persistence and distribution in natural aquatic environments since *L. pneumophila* survived longer in water kept at low temperatures, in which the metabolic activity of predators was found to be significantly lower (17).

Although *Legionella* spp. are considered ubiquitous, isolating *Legionella* spp. from environmental water samples can be difficult in some instances. For example, in previous studies we found that the plate culture method was the least sensitive method for detecting *Legionella* spp. in both sewage and ocean water (13, 14). A kit in which genetic amplification and detection methods are used, the EnviroAmp kit (Perkin Elmer, Foster City, Calif.), has recently been introduced to improve detection of *Legionella* spp. in environmental water samples. The advantages of this kit compared with the plate culture method for detecting environmental *Legionella* spp. were recently demonstrated during an outbreak of Pontiac fever associated with the use of a hot tub at a resort. The etiological agent, *L. pneumophila*, was not found when the plate culture method was used, and investigators were able to determine the cause of the outbreak only by using the PCR and direct fluorescent antibody (DFA) methods (12).

We previously used the EnviroAmp PCR kit, DFA staining, and culture methods to demonstrate that *Legionella* spp. survive both primary and secondary sewage treatment processes with only small decreases in population. In this study we compared these three methods for *Legionella* detection by using tertiary treated sewage effluents that are used as reclaimed

* Corresponding author. Mailing address: Environmental Sciences Laboratory, County Sanitation Districts of Orange County, P.O. Box 8127, 10844 Ellis Ave., Fountain Valley, CA 92728-8127. Phone: (714) 962-2411. Fax: (714) 962-2591.

water and aerosols obtained from above a secondary sewage treatment basin.

MATERIALS AND METHODS

Sample collection and processing. Reclaimed water was collected in sterile 250-ml bottles over a 6-month period. Samples were processed within 4 h after they were collected. Free and total residual chlorine contents were determined at the time of collection with a colorimeter (Hach, Loveland, Colo.). Total coliforms and *Escherichia coli* were enumerated from water samples with Colilert (Idexx, Portland, Maine) in most-probable-number format.

Sample sites. Reclaimed water samples were obtained from three areas in Southern California and one area in Hawaii. The samples obtained from Southern California sites included reclaimed water samples collected at three areas in Orange County. The samples obtained from sites GA1 through GA5 were chlorinated tertiary effluent samples that were collected from a landscape sprinkling system at a wastewater treatment plant (site GA). The sampling sites were located approximately 0.5 mi (ca. 0.8 km) from the point of chlorination. This reclaimed water system can also be supplied by nonpotable city water (through an air gap connection) if tertiary effluent is not available. After secondary treatment (an activated sludge treatment), the water underwent additional flocculation with 5 mg of alum per liter and 2 mg of anionic polymers per liter. The water was chlorinated so that the residual total chlorine content was at least 5 mg/liter after a contact time of at least 2 h.

The site MS water samples were collected from a public park sprinkler system. The water in this system includes reclaimed water from site GA; the site MS water received the same treatment as the site GA water described above. The park that receives the reclaimed water is located approximately 2 mi (ca. 3.2 km) from the treatment plant. Site MS1 water was collected from a test cock on a backflow device on the line feeding the irrigation system. Site MS2 water was collected at a nearby lake (which was filled with groundwater that had not been disinfected); this lake is the alternative water supply for the sprinkler system during the winter rainy season. Site MS3 and MS4 reclaimed water samples were collected directly from sprinkler heads within the park.

The site IR samples were reclaimed water samples collected from a water reclamation plant that produced chlorinated tertiary effluent for greenbelt watering. This water underwent activated sludge and dual-medium filtration (anthracite coal and sand filtration) before it was treated with enough chlorine so that the residual total chlorine content was 5 to 10 mg/liter with a minimum contact time of 2 h. Site IR1 samples were final effluent samples that were collected from a postchlorination point at the discharge port of the chlorine contact chamber. Site IR2 samples were water samples obtained from one of the agency's open reservoirs that receives reclaimed water. Sites IR3, IR4, IR5, IR6, and IR7 are dedicated sampling stations located throughout the greenbelt distribution system. These sites are within 7 mi (ca. 11.3 km) of each other and are between 1 and 7 mi (between ca. 1.6 and 11.3 km) from the initial site of chlorination.

The samples of reclaimed water obtained in Hawaii were collected after sewage underwent processing that included settling tanks, aeration basins (activated sludge treatment), and enough chlorination so that the residual total chlorine content was 1.0 to 1.5 mg/liter with a minimum chlorine contact time of 2 h. The treated effluent was discharged into a nearby lake. The lake water was subsequently used to irrigate sugarcane. Ten samples were collected over a 6-month period at a postchlorination point at the discharge port of the chlorine contact chamber. Lake water samples were obtained twice at each of five sites. Site FP was located approximately 400 yards (ca. 366 m) from the point of discharge. Sites E-1 and E-2 were closest to the point of discharge. Sites E-3 and E-4 were located at opposite ends of the lake.

Air sampling. Air samples were obtained from above secondary treatment (activated sludge) aeration tanks with a laboratory-designed vacuum apparatus. This apparatus consisted of a large portable vacuum pump (Nelson, Berkeley, Calif.) connected to the side port of a 1,000-ml Erlenmeyer vacuum flask. The flask contained an aquarium bubbling stone and was connected to a glass funnel which was used as an air collector. The apparatus was autoclaved and filled with 800 ml of sterile buffer water (1.25 ml of 0.3 M KH_2PO_4 and 5 ml of 0.4 M $MgCl_2 \cdot 6\ H_2O$ in 1 liter of distilled H_2O). The flask was placed in an ice cooler and kept at 4°C during the sampling period. A 6-ft (ca. 183-cm) piece of sterile latex tubing attached to a glass funnel was hung next to the secondary aeration tank approximately 4 ft (ca. 122 cm) above the water surface. The air sampler was run for 4 h. An air flow meter (Dwyer model 4″ VF Visi-Float) was used to monitor and maintain the air flow at a rate of 283 liters/h. The total volume of air sampled was approximately 1,139 liters. The air samples were transported at 4°C and were analyzed within 4 h after they were collected.

An Occupational Safety and Health Administration (OSHA)-approved impinger air-sampling device (model 224-44xr; SKC, Fullerton, Calif.) was used initially, but with this system we could not obtain sample volumes large enough so that they were representative of human respiratory tidal volumes. An Anderson air-sampling device was not used since in this system agar plates are used and the samples obtained with it could not be used for PCR.

PCR. For genetic detection of *Legionella* spp., we used a new improved version of the EnviroAmp kit, which was recently developed by Perkin Elmer, Foster City, Calif., according to the manufacturer's instructions. The level of sensitivity of the new EnviroAmp kit is reported to be 10 cells per ml. In a previous study, Palmer et al. had to modify the kit protocol because of interference from humic or other undefined substances that were present in the samples (14). In this study no further modification or dilution was required with our water samples, and no inhibition was observed. The sample-processing, PCR, and detection steps were carried out as described previously (14). The manufacturer's addition of bovine serum albumin (BSA) to the kit improved the results. In the new protocol, a 0.2% solution of BSA is used to resuspend the pellet after DNA extraction. The results were improved since the BSA enhanced the removal of inhibitory compounds and may assist in stabilizing AmpliTaq.

DFA staining. A 100-ml portion of chlorinated effluent was filtered through a type HVLP membrane filter (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.). The filter was placed into 2 ml of deionized water and vortexed for 30 s to dislodge the cells from the filter. The filter was removed from the vial, and formalin was added to a final concentration of 2%. Then 20 μl of sample was added to an eight-well toxoplasmosis slide (Bellco Biotechnology, Vineland, N.J.). The slide was air dried and heat fixed. A 20-μl portion of one of two fluorescein isothiocyanate-labeled *Legionella* antibody conjugates (SciMedX, Denville, N.J.) was added to each well. The first polyclonal antibody was directed against *L. pneumophila* serogroups 1 through 7. The second polyclonal antibody was species specific and identified *Legionella micdadei*, *Legionella bozemanii*, *Legionella longbeachae* serogroups 1 and 2, *Legionella dumoffii*, *Legionella gormanii*, and *Legionella jordanis*. A monoclonal antibody specific for *L. pneumophila* was also used with samples that gave positive reactions with the polyclonal antisera directed against *L. pneumophila* serogroups 1 through 7 (Genetic Systems, Seattle, Wash.). The slide was incubated for 30 min at 37°C in a humidity chamber, rinsed with phosphate-buffered saline (pH 7.6) and then with deionized water, and then air dried. The number of cells was determined with an epifluorescence microscope (Olympus, Lake Success, N.Y.).

Plate culture method. After concentration as described above for DFA staining, samples were pretreated with acid (1) to eliminate nontarget organisms. Following the acid treatment, 0.1 ml of each sample was spread plated onto buffered charcoal-yeast extract agar containing alpha-ketoglutarate (1.0 g/liter), glycine (3 g/liter), cycloheximide (80 mg/liter), vancomycin (5 mg/liter), and polymyxin B (100 IU/ml). The resulting plates were incubated at 37°C in a humid 4% CO_2 environment for up to 21 days. Presumptive *Legionella* colonies were confirmed by DFA staining. The levels of recovery efficiency for the methods used in this study (the PCR, DFA staining, and plate culture methods) have been reported previously (17).

Seeding experiments. Because we were not able to culture *Legionella* spp. from PCR- and DFA-positive reclaimed water samples, a seeding experiment was performed. Approximately 10^3 cells of *L. pneumophila* ATCC 33215 per ml was inoculated into several 100-ml samples of reclaimed water having average residual total chlorine contents between 4.5 and 5 mg/liter and residual free chlorine contents between 0.50 and 1.5 mg/liter. The exposure times used were 0, 1, 2, 3, and 4 h to stimulate various chlorine contact times in reclaimed water. The water samples were then concentrated and spead plated onto antibiotic-supplemented buffered charcoal-yeast extract medium as described above. Portions of the seeded concentrates were also examined by the PCR and DFA methods. Sterile distilled water was also inoculated and used as a chlorine-free control.

RESULTS

Preliminary screening for *Legionella* spp. with the PCR. At the beginning of this study we performed a preliminary screening experiment with samples obtained from one site in California where reclaimed water was used (site GA) to determine if *Legionella* species were present. Our results indicated that *Legionella* species were detected by the EnviroAmp PCR detection kit in more than 90% of the samples tested (Table 1) at levels of 10^3 cells per ml. Samples obtained from three of five sampling stations contained *L. pneumophila* at levels that were less than 10^3 cells per ml. The total chlorine level in water samples obtained from two of the five sites was greater than 2 mg/liter, while the samples obtained from two other sites had low residual total chlorine contents (<0.3 mg/liter). During this study it was difficult to determine residual chlorine levels at site GA3 since the water was very turbid and the turbidity interfered with the Hach test kit. Since *Legionella* spp. were present, the final two or three samples were tested for the presence of coliform bacteria to see if the presence of these bacteria was correlated with the presence of the *Legionella* spp. The samples obtained from only one site contained coliform bacteria at average levels of 1,600 cells per 100 ml (most probable number).

TABLE 1. Preliminary screening of reclaimed water samples for *Legionella* spp.

Site	% of samples positive as determined by EnviroAmp PCR		Free Cl_2 (mg/liter)	Total Cl_2 (mg/liter)	No. of coliform bacteria (MPN/100 ml)[a]	
	Legionella spp.	*L. pneumophila*			Total	*E. coli*
GA 1	88 (8/9)[b]	33 (3/9)	0.31	2.41	1,600[c]	1,600
GA 2	100 (9/9)	11 (1/9)	0.03	0.08	0[d]	0
GA 3	88 (8/9)	0 (0/0)	ND[e]	ND	0[c]	0
GA 4	100 (9/9)	0 (0/0)	0.38	2.08	0[d]	0
GA 5	88 (8/9)	11 (1/9)	0.15	0.25	0[d]	0

[a] MPN, most probable number.
[b] The numbers in parentheses are the number of positive samples/number of samples tested.
[c] Final two samples tested.
[d] Final three samples tested.
[e] ND, not determined.

Recovery of *Legionella* spp. from reclaimed water samples in California. Because of the high percentage of positive samples obtained in the preliminary survey, the study was expanded to include two other sites in California in addition to the preevaluation site. The EnviroAmp Legionella PCR tests were supplemented with DFA staining and plate culture tests. Our results indicated that *Legionella* species, as determined by the EnviroAmp PCR kit, were always present at sites GA1 through GA5. The DFA staining results confirmed this finding, except that the average numbers were lower. *L. pneumophila* was not detected at sites GA1 through GA5 by the PCR; however, the DFA results indicated that *L. pneumophila* cells were present in most samples but at very low levels (<1 cell per ml). The average total chlorine concentrations were high (>4 mg/liter) at two of the five GA sites, and there appeared to be an inverse relationship between the total chlorine concentration and the presence of *Legionella* spp. since sample sites at which the average total chlorine levels were high (>4 mg/liter) had high numbers of *Legionella* cells ($>10^3$ cells per ml), while samples containing lower total chlorine concentrations (<1 mg/liter) contained low numbers of *Legionella* cells ($<10^3$ cells per ml). Samples in which the free chlorine concentrations were greater than 0.2 mg/liter similarly contained the highest average levels of *Legionella* spp. as detected by the PCR. The numbers of coliform bacteria were below acceptable regulatory levels in all samples.

All of the MS sites were positive for *Legionella* species as determined by the EnviroAmp PCR kit (Table 2). The samples obtained from the three park sprinkler sites contained $>10^3$ cells of *Legionella* species per ml, and one of the five samples obtained from one of these sites contained $<10^3$ *L. pneumophila* cells per ml. The DFA staining results also showed that both *Legionella* species and *L. pneumophila* were present, but the values obtained with the DFA method were at least 1 order of magnitude less than the values obtained with the PCR. The samples obtained from all sprinkler sites were negative for coliform bacteria, but the site MS2 (lake water) sample was positive for both total coliform bacteria and *E. coli*. As observed with the samples obtained from the GA sites, the samples obtained form the MS sites that had the highest total chlorine concentrations (>1.5 mg/liter) and free chlorine contents of more than 0.2 mg/liter contained the highest numbers of *Legionella* cells ($>10^3$ cells per ml).

Like the samples obtained from the two other California

TABLE 2. *Legionella* spp., Cl_2 concentrations, and coliform bacterial levels in samples obtained from three California sites at which reclaimed water is used

Site	No. of samples	Cells/ml as determined by:				Free Cl_2 (mg/liter)	Total Cl_2 (mg/liter)	No. of coliforms (MPN/100 ml)[a]	
		EnviroAmp PCR		DFA staining					
		Legionella spp.	*L. pneumophila*	*Legionella* spp.	*L. pneumophila*			Total	*E. coli*
GA 1	6	$>10^3$	0	22	1	0.63	4.46	0	0
GA 2	6	10^3	0	32	1	0.16	0.39	0	0
GA 3	5	$<10^3$	0	15	<1	0.03	0.61	0	0
GA 4	6	$>10^3$	0	33	<1	0.41	4.13	0	0
GA 5	6	$<10^3$	0	22	<1	0.03	0.05	0	0
MS 1	5	$>10^3$	0	23	1	0.34	3.71	0	0
MS 2	5	$>10^3$	0	9	<1	0.03	0.03	1,068	197
MS 3	5	$>10^3$	$<10^3$	18	<1	0.32	2.12	0	0
MS 4	5	$>10^3$	0	24	1	0.53	1.66	0	0
IR 1	6	10^3	0	28	<1	1.45	>5.0	0	0
IR 2	6	$<10^3$	0	64	13	0.55	1.87	850	0
IR 3	5	10^3	0	11	<1	0.41	4.94	0	0
IR 4	6	10^3	0	8	<1	0.62	2.70	0	0
IR 5	6	10^3	0	8	<1	0.82	2.80	0	0
IR 6	6	10^3	0	11	<1	1.09	4.14	0	0
IR 7	6	10^3	0	15	<1	0.05	0.13	0	0

[a] MPN, most probable number.

TABLE 3. Recovery of *Legionella* spp. from chlorinated effluent and lake water samples in Hawaii

Sample	Cells/ml as determined by:				Free Cl_2 (mg/liter)	Total Cl_2 (mg/liter)
	EnviroAmp PCR		DFA staining			
	Legionella spp.	*L. pneumophila*	*Legionella* spp.	*L. pneumophila*		
Chlorinated effluent samples						
1	$>10^3$	0	ND[a]	ND	2.00	2.50
2	$>10^3$	0	ND	ND	0.52	1.55
3	$>10^3$	0	56	0	2.00	>2.50
4	$>10^3$	0	0	0	0.50	0.90
5	$>10^3$	0	218	0	0.25	1.00
6	0	0	134	5	1.25	1.50
7	$>10^3$	0	92	0	1.50	1.75
8	$>10^3$	0	14	0	0.75	1.50
9	$>10^3$	0	0	0	0.50	1.00
10	$>10^3$	0	0	0	0.75	1.50
Lake water samples						
1	$<10^3$	0	13	0	0	0
2	$>10^3$	0	1	0	0.05	0.10
3	10^3	0	67	0	0	0.09
4	$>10^3$	0	10	0	0	0
5	10^3	0	0	0	0	0.05
6	10^3	0	95	0	0	0
7	$>10^3$	0	4	0	0.05	0.04
8	$>10^3$	0	124	0	0	0
9	10^3	0	0	0	0	0
10	10^3	0	59	0	0	0

[a] ND, not done.

sites, all of samples obtained from site IR were positive for *Legionella* species as determined by both the PCR method and the DFA method, although again, the values obtained with the DFA method were lower. The site IR levels of *Legionella* spp., as determined by the PCR, were lower than the levels detected at the other two sites. Although *L. pneumophila* was not detected with the PCR, low levels of this organism (<1 cell per ml) were detected by DFA staining. The samples obtained from the reservoir site (site IR2) contained the lowest levels of *Legionella* cells detected ($<10^3$ cells per ml). However, the samples obtained from this site contained high levels of total coliform bacteria (average, 850 cells per 100 ml). The chlorine levels in samples collected at this site were not correlated with higher or lower numbers of *Legionella* cells.

Recovery of *Legionella* spp. from reclaimed water in Hawaii. Samples were obtained from the Hawaii site, which included chlorinated effluent and lake water, 10 times over a period of 6 months. The PCR results indicated that *Legionella* spp. were present at levels of $>10^3$ cells ml in all chlorinated effluent samples except one (Table 3). The lake water samples contained *Legionella* spp. at concentrations lower than the concentrations in the chlorinated effluent. The DFA staining results indicated that *Legionella* species were present in five of eight (63%) chlorinated effluent samples and in 8 of 10 (80%) lake water samples. *L. pneumophila* was not detected by the PCR in any of the samples; however, one sample of chlorinated effluent was positive for *L. pneumophila* as determined by DFA staining. The residual total chlorine concentrations were consistently ≥1 mg/liter in the chlorinated effluent samples and negligible in the lake water samples. A coliform analysis was not performed with the samples obtained from these sites.

Recovery of *Legionella* spp. from air samples. Air samples were collected from above the secondary treatment basins at the Hawaii treatment plant. Results obtained with the EnviroAmp PCR detection kit indicated that *Legionella* spp. were present at levels of fewer than 10^3 cells per ml in four of the nine samples tested (44%) (Table 4). When we used the DFA staining method we detected *Legionella* spp. in two of nine samples (22%). *L. pneumophila* was not detected with either method. A plate culture analysis confirmed two of the four samples that were positive as determined by the PCR. One sample, however, was culture positive but was negative as determined by both PCR and DFA analyses. None of the cultured colonies was identified as *L. pneumophila*, but colonies were shown to be *Legionella* spp. by DFA staining.

Recovery of *L. pneumophila* from seeding experiment preparations. When we examined seeded reclaimed water preparations, we observed growth of *L. pneumophila* on buffered charcoal-yeast extract medium on the zero time plates. Growth was not observed on the plates after 1, 2, 3, or 4 h of exposure even though the plates were incubated for 3 weeks. The PCR and DFA staining tests were positive after 0, 1, 2, 3, and 4 h of exposure. When we examined buffered charcoal-yeast extract

TABLE 4. Recovery of *Legionella* spp. from air samples collected above secondary treatment aeration tanks

Sample	Cells/ml as determined by:				Culture CFU/ml
	EnviroAmp PCR		DFA staining		
	Legionella spp.	*L. pneumophila*	*Legionella* spp.	*L. pneumophila*	
1	0	0	0	0	0
2	10^3	0	28	0	0
3	0	0	0	0	10
4	$<10^3$	0	0	0	23
5	0	0	0	0	0
6	$<10^3$	0	12	0	101
7	0	0	0	0	0
8	0	0	0	0	0
9	$<10^3$	0	0	0	0

medium plates streaked with sterile distilled water seeded with *L. pneumophila*, we observed growth after only 4 days of incubation.

DISCUSSION

Water reuse is an environmentally acceptable and feasible option in areas where water is at a premium. Because of long chlorine contact time and high residual chlorine levels, elimination of most microorganisms can be expected. Yanko (24) performed a 10-year study to determine the presence of enteric viruses in reclaimed water and found that only 1 of his 590 samples contained virus. Current California Title 22 regulations govern the microbiological quality of reclaimed water at treatment plants. These regulations state that reclaimed water used to irrigate golf courses, cemeteries, and freeway landscapes cannot contain more than 23 coliform bacterial cells per 100 ml, while reclaimed water used to irrigate parks, playgrounds, and schoolyards cannot contain more than 2.2 coliform bacterial cells per 100 ml (2). Since reclaimed water is often aerosoled in sprinkler systems in areas where there is a high potential for public exposure, regulations state that reclaimed water must be used during off-peak hours to minimize public exposure.

Our results showed that, for two samples obtained during preliminary testing, the reclaimed water samples which we studied met regulatory coliform standards. *Legionella* spp., however, were detected in most reclaimed water samples. This is not surprising since it has been shown that *Legionella* spp. are more resistant to chlorine than coliform bacteria are and, in addition, may be protected by amoebae and/or survive in pipe biofilms. Kuchta et al. (8, 9) reported that a residual free chlorine content of 0.1 mg/liter removed 99% of the *E. coli*, *Staphylococcus aureus*, and *Klebsiella pneumoniae* cells with only 1 min of contact time but that a contact time of 30 to 60 min was required to eliminate *Legionella* spp. If the *Legionella* spp. were sequestered within protozoans, then higher levels of chlorination and longer contact times were required to eliminate the organisms. Other workers have suggested that grazing by other microorganisms that are present in water systems plays a significant role in the control of *Legionella* populations (17, 18). Many organisms, such as *Pseudomonas* and *Aeromonas* spp., produce bactericins that result in significant inhibition of legionellae in treated water (15, 21, 22). Thus, chlorine levels that eliminate microorganisms that control *Legionella* spp. could result in increases in the population of indigenous *Legionella* spp.

In this study, we found that *Legionella* species were detected by PCR and DFA staining methods after tertiary sewage treatment, including chlorination, and were present in water used for reclamation projects in both California and Hawaii. This finding may be explained by the fact that legionellae are ubiqitous in water systems and may become part of the biofilm in pipes. *L. pneumophila*, the species most often associated with disease outbreaks, was detected at three of the five GA sites tested during the preliminary study (Table 1) and in only one of five samples tested at site MS3 (Table 2) during the expanded study. In several cases, water containing high levels of free and residual total chlorine contained the highest numbers of *Legionella* spp. This inverse relationship may be explained by the death of other microorganisms (caused by high residual chlorine contents) that normally graze on *Legionella* spp. or that produce bactericins that inhibit legionellae and keep their populations in check.

Exhaustive efforts to culture legionellae from reclaimed water samples produced negative results. The results of seeding experiments supported this finding since after 1 to 4 h of exposure to reclaimed water, *L. pneumophila* could not be cultured. Chlorine may injure legionellae and may cause them to enter a viable but nonculturable state since the seeded legionellae were easily detected by the PCR and DFA procedures.

The results obtained with air samples collected over the treatment basin indicated that *Legionella* spp. were aerosolized during the treatment process. It should be noted, however, that the air sampling method used in this study was developed to obtain aerosols only 4 ft (ca. 122 cm) from the water surface and does not necessarily simulate the conditions encountered by operators walking near basins. Importantly though, *Legionella* spp. were recovered by standard plate culture methods in 50% of the PCR- and DFA-positive samples, demonstrating that *Legionella* spp. were viable during the nonchlorinated phase of sewage treatment. However, it took more than 2 weeks for most colonies to appear on the media.

Like the results obtained with reclaimed water, the results obtained in the air recovery portion of this study showed that plate culture method was the least sensitive method for recovering *Legionella* spp. However, in one instance, *Legionella* spp. were recovered by the plate culture method but were not detected by the PCR or DFA staining method. This could have been due to the fact that *Legionella* spp. were sequestered within protozoans in the sample, which shielded them from immunofluorescent reagents and the PCR, or the colonies could have represented a *Legionella* species that is not amplified by the PCR primers or detected by the DFAs. With two samples (Table 4, samples 4 and 9) the levels determined by the PCR were low ($<10^3$ cells per ml), but the DFA staining test was negative. This could be explained by the fact that the DFA staining method detects only 7 *Legionella* spp., while the PCR method detects 25 species, or that the potential for cross-reactivity exists with immunofluorescent staining.

Legionella spp. have been detected in almost all water sources that have been examined. We concluded that the PCR method was superior to both the plate culture method and the DFA staining method for detecting *Legionella* spp. in environmental samples. The presence of *Legionella* spp. in the reclaimed water samples evaluated in this study, however, does not suggest that a health problem exists since there have been no reported outbreaks of legionellosis in the areas where the reclaimed water is used. Moreover, the *Legionella* spp. found in this study were detected in reclaimed water samples only by the PCR and DFA staining methods, neither of which indicates the physiological state (culturability) or virulence of organisms. Additional studies on the survival of chlorine-resistant microorganisms, including legionellae, in reclaimed water would be useful to help scientists better understand the complex microbial interactions that occur in this medium.

ACKNOWLEDGMENTS

This work was supported by grant HR-92-06 from the National Water Research Institute. Matching funds were received from County Sanitation Districts of Orange County and the City and County of Honolulu.

We thank the staff at Orange County Sanitation Districts, Orange County Water Districts, Irvine Ranch Water District, and Wahiawa Sanitation District for their assistance, cooperation, and participation in this study. We are most grateful to Claudia Thio for helpful comments and suggestions. We thank Yuang Xin and Mike Okura for technical assistance with the Hawaii samples and Cyndi Mayer for assistance with the seeding experiments. We also thank Patrick McNelly for editing the manuscript and Doreen Borde and Ingrid Ashbaugh for assistance with the tables.

REFERENCES

1. **Bopp, C. A., J. W. Sumner, G. K. Morris, and J. G. Wells.** 1981. Isolation of *Legionella* spp. from environmental water samples by low-pH treatment and use of a selective medium. J. Clin. Microbiol. **13:**714–719.
2. **California Administrative Code.** 1978. Wastewater reclamation criteria. Title 22, Division 4. Environmental health, p. 1,560. California Code of Regulations. Barclays Publishers, South San Francisco.
3. **Dondero, T. J., R. J. Rendtoriff, and G. F. Malluson.** 1980. An outbreak of Legionnaires' disease associated with contaminated air conditioning cooling tower. N. Engl. J. Med. **302:**365–370.
4. **Fang, G. J., V. L. Yu, and R. M. Vickers.** 1989. Disease due to the *Legionellaceae* (other than *L. pneumophila*). Medicine (Baltimore) **68:**116–132.
5. **Fenstersheib, M. D., M. Miller, C. Diggins, S. Liska, L. Detweiler, S. B. Werner, D. Lindquist, W. L. Thacker, and R. F. Benson.** 1990. Outbreak of Pontiac fever due to *Legionella anisa*. Lancet **336:**35–37.
6. **Fliermans, C. B., W. B. Cherry, L. H. Orrison, S. J. Smith, D. L. Tison, and D. H. Pope.** 1981. Ecological distribution of *Legionella pneumophila*. Appl. Environ. Microbiol. **41:**9–16.
7. **King, C. H., E. B. Shotts, Jr., R. E. Wooley, and K. G. Porter.** 1988. Survival of coliforms and bacterial pathogens within protozoa during chlorination. Appl. Environ. Microbiol. **54:**3023–3033.
8. **Kuchta, J. M., J. S. Navratil, M. E. Shepherd, R. M. Wadowsky, J. N. Dowling, S. J. States, and R. B. Yee.** 1993. Impact of chlorine and heat on the survival of *Hartmannella vermiformis* and subsequent growth of *Legionella pneumophila*. Appl. Environ. Microbiol. **59:**4096–4100.
9. **Kuchta, J. M., S. J. States, J. E. McGlaughlin, J. H. Overmeyer, R. M. Wadowsky, A. M. McNamara, R. S. Wolford, and R. B. Yee.** 1985. Enhanced chlorine resistance of tap water-adapted *Legionella pneumophila* as compared with agar medium-passaged strains. Appl. Environ. Microbiol. **50:**21–26.
10. **LaMaire, W., H. Jackson, and L. McFarland.** 1990. Legionnaires' disease outbreak associated with a grocery store mist machine—Louisiana, 1989. Morbid. Mortal. Weekly Rep. **39:**108–110.
11. **Mangione, E. J., R. S. Remis, K. A. Tait, G. W. Gorman, B. B. Wentworth, P. A. Baron, A. W. Hightower, J. M. Barbaree, and C. V. Broome.** 1982. An outbreak of Pontiac fever related to whirlpool use, Michigan. JAMA **253:** 535–539.
12. **Miller, L. A., J. L. Beebe, J. C. Butler, W. Martin, R. Benson, R. E. Hoffman, and B. F. Fields.** 1993. The use of polymerase chain reaction in an epidemiologic investigation of Pontiac fever. J. Infect. Dis. **168:**769–772.
13. **Palmer, C. J., Y. L. Tsai, G. F. Bonilla, L. R. Sangermano, C. Paszko-Kolva, B. Roll, and R. S. Fujioka.** 1993. Detection of *Legionella* species in sewage and ocean water in California and Hawaii, p. 123–131. *In* Proceedings of the Annual Conference of Water and Environment Federations. Water and Environment Federations, Alexandria, Va.
14. **Palmer, C. J., Y. L. Tsai, C. Paszko-Kolva, C. Mayer, and L. R. Sangermano.** 1993. Detection of *Legionella* species in sewage and ocean water by polymerase chain reaction, direct fluorescent antibody, and plate culture methods. Appl. Environ. Microbiol. **59:**3618–3624.
15. **Paszko-Kolva, C., P. A. Hacker, M. Stewart, and R. Wolfe.** 1992. The inhibitory role of heterotrophic bacteria on the cultivation of *Legionella dumoffii*, p. 203–205. *In* J. M. Barbaree, R. F. Breiman, and A. P. Dufour (ed.), *Legionella*. Current status and emerging perspectives. American Society for Microbiology, Washington, D.C.
16. **Paszko-Kolva, C., M. Shahamat, and R. R. Colwell.** 1992. Long-term survival of *Legionella pneumophila* serogroup 1 under low-nutrient conditions and associated morphological changes. FEMS Microbiol. Ecol. **102:**45–55.
17. **Paszko-Kolva, C., M. Shahamat, and R. R. Colwell.** 1993. Effect of temperature on survival of *Legionella pneumophila* in the aquatic environment. Microb. Releases **2:**73–79.
18. **Paszko-Kolva, C., M. Shahamat, H. Yamamoto, T. Sawyer, J. Vives-Rego, and R. R. Colwell.** 1991. Survival of *Legionella pneumophila* in the aquatic environment. Microb. Ecol. **22:**75–83.
19. **Sobsey, M. D.** 1989. Inactivation of health-related microorganisms in water by disinfection processes. Water Sci. Technol. **21:**179.
20. **Tobin, J. O., J. Beare, and M. S. Dunnill.** 1980. Legionnaires' disease in a transplant unit: isolation of the causative agent from shower baths. Lancet **ii:**307–310.
21. **Toze, S., L. Sly, C. Hayward, and J. Fuerst.** 1992. Bactericidal effect of inhibitory non-*Legionella* bacteria on *L. pneumophila* cells in coculture, p. 269–272. *In* J. M. Barbaree, R. F. Breiman, and A. P. Dufour (ed.), *Legionella*. Current status and emerging perspectives. American Society for Microbiology, Washington, D.C.
22. **Toze, S., L. Sly, I. C. MacRae, and J. A. Fuerst.** 1990. Inhibition of growth of *Legionella* spp. by heterotrophic plate count bacteria isolated from chlorinated drinking water. Curr. Microbiol. **21:**139–143.
23. **Winn, W. C., Jr.** 1988. Legionnaires' disease: historical perspective. Clin. Microbiol. Rev. **1:**60–81.
24. **Yanko, B. A.** 1993. Analysis of 10 years of virus monitoring data from Los Angeles County treatment plants meeting California wastewater reclamation criteria. Water Environ. Res. **65:**221–226.