Quantitation of *Legionella pneumophila* in One Thousand Commercial and Industrial Cooling Towers

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INTRODUCTION

THERE HAVE BEEN NO EXTENSIVE, long term investigations of the numbers of *Legionella pneumophila* in cooling towers or evaporative condensers and the effectiveness of commonly used methods to mitigate the public health hazard. There is also a lack of published long term information on counts of *Legionella* in towers cleaned by the suggested guidelines published in 1987 by the State of Wisconsin Division of Health, Bureau of Community Health and Prevention.

Cooling towers and evaporative condensers are associated with airborne transmission of the bacterium *Legionella pneumophila*, the agent of Legionnaires' Disease and Pontiac Fever. *Legionella* enter towers by way of the potable, make-up water. After entering the circulating water within the equipment, these bacteria can multiply to numbers of 300,000/ml of tower water.

This paper documents information needed to begin developing logical strategies for dealing with *Legionella* bacteria in towers. This was accomplished by analyzing *Legionella* counts in tower samples collected over the period from January, 1985 through May of 1988.

MATERIALS AND METHODS

SAMPLING PROCEDURE - Tower samples were identified by company location, tower site, and sample day on a tag provided with each sample bottle. Samples were taken from an area on the tower pan, if accessible, which contained sediment and slime. Sediment along with at least 150 ml of water were collected in the sample bottle. The sample b5ttle was transported, when possible, to the laboratory within the same day. Some towers had no access panel or door. Recirculating water in those towers was sampled from a hose bib connected to the recirculating water line. The latter procedure provided reproducible count information when sampling was done at the same location each month.

SAMPLE PROCESSING - Samples arriving at the laboratories in Philadelphia PA and Beltsville MD were logged in and processed by a modification of our previously published direct fluorescent antibody procedure instead of culture methods because we found that viable *Legionella* were often not recovered from tower samples by culture. This finding was recently confirmed by Hussong, et al. and Negron-Alvira et al. Tower sample bottles were shaken vigorously to evenly distribute the sample. The sample was concentrated 50-fold by filtration of 100 ml through a 0.45 µm pore size filter. Vigorous mechanical agitation was used to suspend the filter retentate in 2 ml of tower sample water. Ten µl samples were placed in duplicate, 6 mm areas on masked microscope slides, air dried, heat fixed, and stained with a direct fluorescent monoclonal antibody reagent specific for an outer membrane protein found on all known serogroups of *L. pneumophila*. All fields within each six mm diameter well were counted at 1,000 times magnification with an epifluorescent microscope equipped for fluorescein isothiocyanate stained samples. Positive and negative bacterial controls were stained similiarly. Observed numbers of *Legionella* were recorded and calculated to yield counts per ml of original tower sample. The minimum detectable number of *Legionella* was 10 fluorescent bacteria per ml of original tower sample. This was based on observations of numerous tower samples containing known numbers of *Legionella*.

REPORTING RESULTS - Results were sent by confidential mail to the appropriate individual at each company along with information on how to interpret the result. Companies were contacted by telephone if tower counts were excessive. If no *Legionella* were observed, the count was reported as <l0/ml of tower water sample.

SAMPLE DESCRIPTION - Several geographical and time factors were recorded and evaluated for each tower sample. Samples were taken from towers operated by 209 companies. Some companies had towers located at several geographical sites around the continental U.S., others had only one site. Samples came from towers located in 25 states. Thirty-seven of the towers were sampled from 6 to 34 times over the period of this investigation. They were sampled on differing schedules, but most commonly, every month.

COUNTS BY GEOGRAPHICAL LOCATION - Most tower samples, 75%, were obtained from Pennsylvania, New York, Maryland, and the District of Columbia. The remaining samples were distributed among 22 other states ranging from Florida to California and Washington to Texas. The samples were not evenly distributed by state and therefore no conclusions with respect to geographical location were possible.

ROUTINE BIOCIDE TREATMENTS - Histories of routine biocide treatment were obtained for all but 4 of the 37 most frequently sampled towers. Biocide treatment was generally weekly or biweekly. The most commonly used biocide combination was a quaternary ammonium compound, poly-oxyethylene-dimethyliminio-ethylene-dimethyliminio-ethylene dichloride, in combination with an organo-sulfur compound consisting of sodium dimethyl-dithio-carbamate and di-sodium ethylene-bis-dithio-carbamate. Recommended dosage for the quaternary ammonium biocide was 40 ounces per 1,000 gallons of water. Dosage for the organo-sulfur compound was 44 ounces per 1,000 gallons of water.

TOWER DECONTAMINATIONS -Action by tower owners in response to a trend of increasing numbers of Legionella ranged from addition of more biocide to full scale decontamination. Towers maintained by some contractors were decontaminated when counts reached 200 per ml. The involved towers were receiving routine biocide treatment at the time of the decontamination procedure. In at least two cases, there were accompanying suspected cases of Legionnaires' Disease that may have been associated with the towers. The hypochlorite decontamination protocol used was similar to Cooling Tower Institute method recommended to the Centers for Disease Control in January, 1980 and later modified by the State of Wisconsin Department of Health in 1987. The main difference between the two procedures is that the latter recommends adding biocide before adding dispersant.

DATA ANALYSIS - Data was analyzed using SPSS/PC+ V2.0 (SPSS, Inc., Chicago, IL). Data was first cleaned by inspection of all variables for outliers. Count frequencies, cumulative frequencies, and cumulative percentages were produced for 1336 samples along with measures of central tendency using the Frequencies subprogram.

Seasonal Count Differences - Seasonal variations in counts were explored by use of the Crosstabs and Npar Tests subprograms. Counts were collapsed into 9 groups and cross tabulated by month of year. To more clearly see trends, and because there were similar characteristics among groups of counts, the counts were ultimately collapsed into 3 groups; <l0/ml, 10-199/ml, and 200-100,000/ml. A Chi-Square statistic was obtained for the relationship of month to the 3 count categories as well as for month to all pairs of the 3 count categories with each other. A Kruskal-Wallis one-way nonparametric analysis of variance, a rank order statistic, with month as the independent variable and raw count as the dependent variable, was also calculated. Due to the large number of zero counts, the distributional assumptions of a parametric ANOVA were not met.

Biocide Treatments - The percent of observations with counts equal to or greater than 200 counts/ml were determined for each tower in the investigation which was sampled more than 5 times. All towers in this evaluation received biocide treatment every week or biweekly as described above. The percentages for the weekly treated and biweekly treated towers were calculated and compared.

Tower Decontaminations - Specific cases of tower decontamination were evaluated by the case study method without statistical analysis because only limited information about the decontamination procedure at the site were evaluated by the case study method without statistical analysis because only limited information about the decontamination procedure at the site was available.

RESULTS

OVERALL COUNT FREQUENCIES - 1,336 samples from 472 towers and 209 companies had *Legionella* counts ranging from ~l0/ml up to 100,000 bacteria/ml. Individual towers had counts ranging from <10/ml to 100,000/ml. Fifty-nine percent of the samples had counts of 10/ml or less. Over 90% of the samples had counts at or below 200/ml. About 3% of the tower water samples contained over 1,000 *Legionella*/ml and 46% had no detectable counts at the sensitivity level of the test procedure (Table 1).

TABLE 1 OVERALL COUNT FREQUENCIES

Counts /ml	Cumulative Frequencies*	Cumulative Percent (%)
<10**	612	45.8
10	792	59.3
20	915	68.5
30	954	71.4
40	1,033	77.3
50	1,056	79.0
100	1,145	85.7
200	1,201	89.9
500	1,260	94.3
1,000	1,295	96.9
5,000	1,322	99.0
10,000	1,330	99.6
40,000	1,335	99.9
100,000	1,336	100.0

N = 1336 Median = 10
Mean = 318.5 Mode = <10
* The number of times a count equal to or smaller than the observed count occurs in the sample
** In all computations, zero was used in place of <10.

SEASONAL VARIATIONS IN COUNTS - *Legionella* counts were broken up into 9 categories and crosstabulated with months (Table 2). High counts (>500/ml) occurred during every month of the year. Therefore, routine biocide treatment and Legionella count monitoring should not be restricted to summer months for towers operated all year.

TABLE 2 CROSSTABULATION OF COUNTS BY MONTH*
Observed and Expected Count Frequencies

COUNTS	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	(#/ML)
<10	17** 19***	29 22	24 22	36 34	54 60	56 73	81 87	82 83	109 88	83 80	25 28	16 15	
10- -29	11 9	8 11	11 11	22 17	40 30	39 36	37 43	42 41	40 44	30 40	15 14	8 7	
30- -99	8 7	7 8	4 8	10 12	18 21	28 26	40 31	29 30	25 32	34 29	11 10	4 5	
100- -199	2 2	0 2	4 2	1 4	8 6	10 8	11 9	7 9	4 9	12 8	2 3	2 2	
200- -499	2 2	1 2	2 2	3 3	6 6	12 7	5 9	7 8	9 9	9 8	5 3	0 2	
500- -999	1 1	1 1	3 1	1 2	1 3	4 4	5 4	8 4	3 4	1 4	3 1	0 1	
1000- -4999	0 1	0 1	1 1	0 2	2 3	6 4	11 5	4 5	2 5	5 5	1 2	2 1	
5000- -9999	0 0	0 0	0 0	1 0	0 1	1 1	0 1	1 1	1 1	1 1	0 0	0 0	
10000- -100000	0 0	1 0	0 0	1 1	1 1	4 1	0 1	2 1	0 1	0 1	0 0	0 0	

* In all cases, _.5 was rounded up for expected frequencies.
** Observed frequency.
*** Expected frequency.

Some towers were only operated from May through September, many others were operated throughout the year. Therefore, many more samples were run in the warm months and results can be interpreted only by comparing observed frequencies to expected frequencies. Expected frequencies are the numbers one would see if there were no relationship between the two variables being crosstabulated. A Chi-Square test of significance compares the observed frequencies to the frequencies which would be expected if there were no association between counts and month of the year. A significant result indicates that the likelihood of no association is low. Significance was found when the 3 count categories were crosstabulated with month (Chi-Square, 22 df = 33.7, $p = 0.05$; Table 3).

TABLE 3 CROSSTABULATION OF GROUP COUNTS BY MONTH

Counts per milliliter				
Month		<10	10-199	200-100,000
January	Obs	17-*	21+	3-
	Exp	19	18	4
February	Obs	29(+)	15(-)	3-
	Exp	22	21	5
March	Obs	24+	19-	6+
	Exp	22	21	5
April	Obs	36+	33	6-
	Exp	34	33	8
May	Obs	54(-)	66(+)	10-
	Exp	60	57	14
June	Obs	56(-)	77(+)	27(+)
	Exp	73	70	17
July	Obs	81(-)	88(+)	21+
	Exp	87	83	20
August	Obs	82-	78-	22+
	Exp	83	80	19
September	Obs	109(+)	69(-)	15(-)
	Exp	88	84	20
October	Obs	83+	76-	16-
	Exp	80	77	18
November	Obs	25-	28+	9+
	Exp	28	27	7
December	Obs	16+	14	2-
	Exp	15	14	3
Chi-Square 33.7		D.F. 22	Significance 0.05	
*Pluses and minuses indicate direction of deviation of frequency from expected frequency. Parentheses note a substantial deviation (5 or greater) from expected.				

To determine which count groups accounted for the significance, pairwise Chi-Square tests were calculated for counts of 10-199/ml against counts of 200/ml and greater (Chi-Square, 11 df = 8.2, p = 0.70), counts of <10/ml against 10-199/ml (Chi-Square, 11 df = 19.4 p = 0.05), and counts of <10 against 200/ml and greater (Chi-Square, 11 df = 20.1, p = 0.04). It was clear that the <l0/ml category differed from both the 10-199/ml and the >200/ml and greater group in patterns of occurrence throughout the months of the year. Therefore, the significance was accounted for by the number of negative results versus positive results of any kind.

A close inspection of Table 3, where pluses and minuses indicate whether there were less or more data points in a given category than would be expected, reveals that the most pronounced deviations in the direction of less zeros and more positive counts occurred in June and July. Deviations showing more zeros and less positive counts occurred in February and September. While this would be expected in February, it seems anomalous in September. However, it must be remembered that only the number of such results are counted in the Chi-Square test, not the actual value of the *Legionella* counts.

A Kruskal-Wallis nonparametric analysis of variance was calculated to determine whether the mean rank of the counts was higher in some months than in others. While at least the highest month differs significantly from the lowest month ($p = 0.002$), this does not warrant ignoring tower testing during even the coldest months because high counts were observed in every month.

The same analysis performed on a subset of the data, where all counts of zero were excluded from the data set, yield essentially the same result ($p = 0.06$; Table 4).

TABLE 4 KRUSKAL-WALLIS 1-WAY ANOVA OF COUNT BY MONTH

	All Counts		Zeros Excluded	
Month	Mean Rank*	Cases	Mean Rank	Cases
January	685	41	341	24
February	552	47	336	18
March	661	49	389	25
April	611	75	281	39
May	672	130	320	76
June	759	160	390	104
July	709	190	396	109
August	674	175	363	100
September	585	193	334	84
October	674	175	394	92
November	707	62	365	37
December	628	32	338	16

BIOCIDE TREATMENT AND COUNTS - We often observed that routinely biocide treated towers with counts below detectable levels increased to significant counts, in some instances over 10-to 20-fold, within a 30 day period of time.

Of the sites monitored on a regular basis, for which biocide information was available, 13 sites were treated weekly and 20 sites were treated biweekly. Among these sites, the number of times counts exceeded 200 Legionella/ml equaled 21% for towers treated either weekly or biweekly. No differences between the two treatment schedules, in terms of Legionella counts, could be observed.

TOWER DECONTAMINATION CASES - Several towers were decontaminated during the period of this investigation. Data was not sufficient to permit a statistical evaluation of the observations.

Case One - A tower was decontaminated in October, 1987, by a method similar to that suggested by the Cooling Tower Institute. Two months later, the count was 60/ml and remained low up to the end of this investigation

Case Two - Another tower was decontaminated in June, 1987. The counts following the decontamination were <10/ml and slowly increased to 60 /ml by August and remained around that level until the tower was shut down and drained in October, 1987.

Case Three - Decontamination was performed on another tower when counts reached 20,000/ml in June, 1987. The counts remained around 1,000/ml for the remainder of June (2 additional samplings) and then dropped to the 20 to 40/ml range until the tower was shut down for the season.

Other Cases - Seven towers had counts reduced to <10/ml the month following decontamination, but then increased to over 200/ml two months after decontamination.

CONCLUSIONS

We have established that: 1) *Legionella* monitoring of tower water provided significant, useful information to tower operators; 2) Highest counts were found during June and July and lowest counts were found in September and February; 3) Routine biocide treatment every week or biweekly for control of Legionella did not prevent excessive growth of Legionella in the towers; 4) Tower decontamination using the recommended chlorination protocol, did not prevent regrowth of Legionella within a period of one or two months; and 5) If a tower operator suspects that a tower contains excessive numbers of Legionella, a test of the tower water using a sensitive, specific DFA procedure can prove or disprove the suspected problem before unnecessary full-scale decontamination measures are started.

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PREPARED DISCUSSION OF:

Quantitation of *Legionella pneumophila* in One Thousand Commercial and Industrial Cooling Towers

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MY INITIAL COMMENTS ON the Gilpin et al. paper concern the specificity, sensitivity, and value of their test. Before I address these issues, I should mention that I am very familiar with the polycarbonate filter procedure for concentrating water that they use because it was developed by my laboratory . at the Centers for Disease Control, and I continue to use it in my own private laboratory.

The major concern that I have with the authors' test is their exclusive use of direct immunofluorescence (DFA) microscopy. They do no culturing to confirm the presence of Legionella even in specimens that appear heavily contaminated with it and therefore should be readily culturable.

It must be pointed out that there are many crossing reacting organisms in the environment that can give false positive DFA tests. Sole reliance on DFA analyses will result in the unnecessary hyperchlorination of many cooling towers and other systems and the release of toxic chemicals that are potentially injurious to both humans and the environment.

The sensitivity of the Gilpin et al test may be slightly better than the authors state. It is probably 2 Legionella instead of 10 per milliliter of water. In any case, detection of contamination at 10 Legionella per milliliter should be very adequate for evaluating biocide control and the necessity for hyperchlorination at levels of 10,000 or greater per milliliter. Although the exact concentration at which a tower may become a health hazard is not know, many environmental scientists, including myself, believe that selective amplification of Leqionella should be prevented. Consequently detection of Legionella at 2 cells per milliliter should allow ample latitude in detection of Legionella before it reaches concentrations requiring stronger intervention measures than just fine tuning of existing strategies. Tests having greater sensitivities, in my opinion, such as 1 Legionella per liter of water may have greater sensitivity than necessary for evaluating cooling towers. The benefits of this greater sensitivity are out weighed by the added cost of shipment and analysis for processing 1 liter of water over 100 milliliters of water

My final remarks address the value for testing cooling towers for Legionella. The opinion often heard is "why do any testing because all towers are positive anyway" is strongly challenged by the Gilpin et al study. Their findings are very similar to my own experience and suggest that well-maintained towers can have very low or non-detectable levels of Legionella. This suggests that periodic testing of towers and other systems for biocidal and maintenance efficacy can be helpful in controlling the presence of Legionella in them. However, recommendations for frequency of testing is still very controversial.

AUTHOR'S CLOSURE

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RESPONSE TO COMMENTS

TEST SENSITIVITY -Our conservative estimate of test sensitivity «lO/ml) is based on many tests of tower water containing known numbers of Legionella. With samples containing little or no debris, the test sensitivity may be greater, but we prefer to retain our conservative estimate.

ROUTINE MONITORING -Whether to monitor water routinely should be a decision made by people working with a tower. We found that 90% of the tower samples had 200 or fewer Legionella/ml, but there was no way to predict dramatic increases in counts within periods as short as one month. Also, we found that decontaminated towers had increases in Legionella numbers over 200/ml within 2 months after decontamination. Therefore, routine monitoring was quite useful to tower operators.

TOTAL BACTERIAL MONITORING -We have not found a correlation between total gacterial counts and Legionella cougts and neither did Negron-Alvira et al.

DFA CROSSREACTIONS -We have not observed crossreactivity to other bacteria with the monoclonal antibody reagent used during this investigation. However, we evaluated other reagents and fOUild that they often produced many crossreactions.

REASON FOR MANY TOWERS WITH LOW COUNTS -Previous investigators suggested that all towers had Legionella counts. Two reasons for this difference is that previous investigators tested fewer towers and many of those towers were not routinely treated with biocides.

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