Giardia Cyst Concentrations in River Water

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Water samples from three pristine rivers in the Pacific Northwest were analyzed for *Giardia* cyst presence and concentration using a membrane-filtration–immunofluorescence-assay procedure. A total of 222 samples were collected either monthly or bimonthly over a nine-month period from 17 sampling stations on three rivers and 12 tributaries. Cyst recovery efficiency was monitored using samples seeded with cysts at levels ranging from 0.5 to 50 cysts/L. The recovery efficiency of the procedure averaged 21.8 percent ± 6 percent through 26 sets of samples. *Giardia* cysts were found in 94 (43 percent) of the samples. The corresponding cyst concentrations calculated from the recovery efficiency and the sample volume ranged from 0.1 to 5.2 cysts/L. The distribution of cyst concentrations in positive samples was lognormal. Both the magnitude of cyst concentrations (as indicated by the mean value) and the variability (as indicated by the slope of the distributions) differed among the three rivers. No statistically supportable seasonal variations were found. The principal conclusion was that *Giardia* cysts appear to be continuously present, though at low concentrations, even in relatively pristine rivers.

The presence of *Giardia lamblia* cysts in sources of public water supply has been documented in many areas of the United States and Canada.[1] It has been described as one of the most common etiological agents contributing to outbreaks of waterborne gastroenteritis.[2] Control of *Giardia* cysts in water treatment has been challenging because their removal by filtration requires consistently efficient operation and specialized analytical techniques,[3] and because they are relatively resistant to conventional disinfectants.[4]

Knowledge of *Giardia* cyst concentrations in water is relevant to public health protection for several reasons, yet no data on cyst concentrations in water have been reported. First, the risk of infection through consuming cyst-contaminated water must be directly proportional to cyst concentration, assuming uniform cyst viability and infectivity characteristics. Second, the concentration of cysts in untreated sources of water supply undoubtedly depends on the nature and magnitude of sources (and sinks) throughout the watershed. Examination of surface streams in remote areas has shown that the frequency of cyst discovery is directly related to the intensity of human activity in the watershed.[5] High levels of *Giardia* cysts have been found in municipal sewage treatment plant effluents,[6,7] suggesting that cyst concentrations in streams receiving such effluents would be affected proportionately. Third, the effectiveness of water treatment for *Giardia* cyst removal or inactivation is finite and defined by prior work[3,4] in terms of a percentage of the initial cyst concentration. Accordingly, a treatment plant using a raw water source downstream of a sewage treatment plant discharge would be expected to have a higher cyst concentration in the finished water than would the same facility treating raw water from a watershed with no wastewater sources.

This study was motivated by the importance of cyst concentrations to water quality planning, the current lack of data on cyst concentrations in water, and by preliminary work that indicated the practicality of measuring cyst concentrations in relatively pristine water sources. The work reported here was designed to satisfy four objectives: (1) to demonstrate the characteristics and capabilities of a small-volume, membrane-filtration–immunofluorescence-assay (MF-IFA) procedure for quantification of *Giardia* cysts; (2) to determine the concentration of *Giardia* cysts in water from three Pacific Northwest watersheds with different characteristics; (3) to determine the nature of variations in the *Giardia* cyst concentration as a function of time, both short-term and seasonal; and (4) to examine the influence of watershed characteristics on cyst concentrations.

A nine-month study was conducted, including regular collection and analysis of samples from 17 sampling stations on

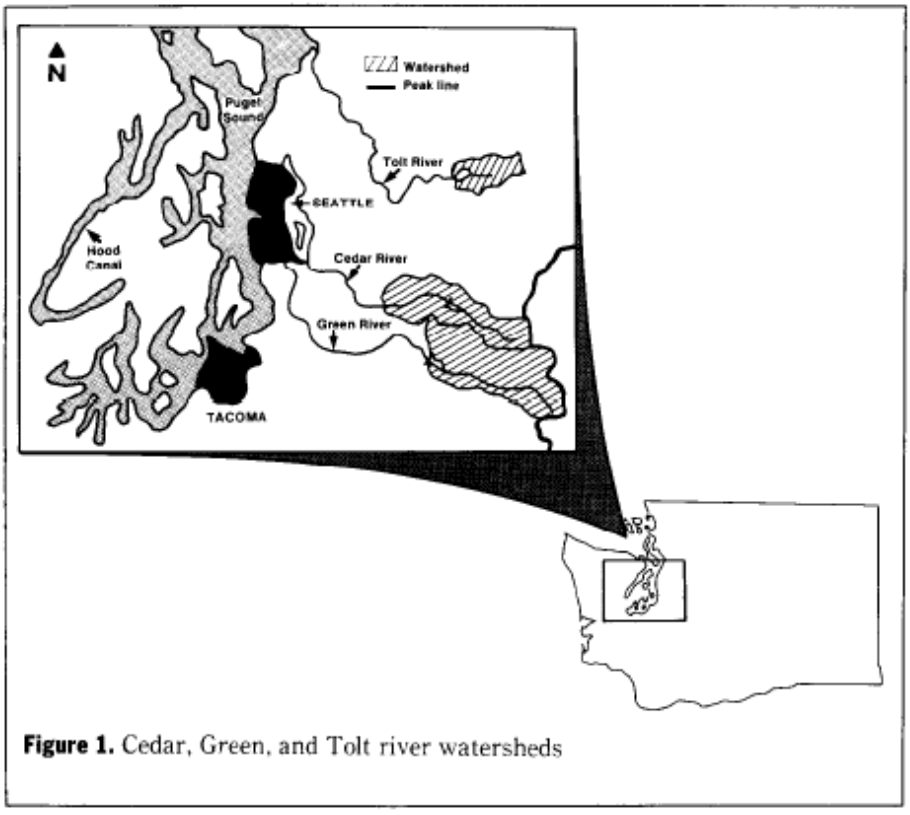

Figure 1. Cedar, Green, and Tolt river watersheds

three rivers and 12 tributaries, to accomplish these objectives. Samples were collected once or twice per month from tributaries or from main-stem stations, respectively. Four sets of consecutive bihourly samples were collected to examine short-term concentration variations over 24-h periods. Sample volumes of 40 L (10 gal) were used for all routine analyses. A few larger samples (120 and 400 L) were analyzed to determine recoveries with larger sample volumes and to check cyst concentrations below the normal limit of detection. All samples were processed in the laboratory using an MF-IFA procedure for recovery, detection, and identification of cysts.

Analysis of more than 200 samples collected over the nine-month period yielded useful information about cyst concentrations in water. *Giardia* cysts were consistently present in the rivers examined, at concentrations ranging from 0.05 to 0.8 cysts/L. The average cyst concentration differed from river to river, although the degree of variability in cyst concentrations was similar from river to river. Sampling above and below stream impoundments with residence times of from 30 to 200 days indicated that cyst concentrations were not reduced by reservoir transit.

Materials and methods

Sample collection. Water samples of approximately 40 L (10 gal) were collected from 17 sampling stations on or tributary to the Cedar, Green, and Tolt rivers, which drain the western slope of the central Cascade Mountains in Washington (Figure 1). Sampling locations were selected to examine cyst concentrations from watersheds with differing characteristics and as a function of transit along the main stem of each river, including passage through an impoundment of substantial size.

The watersheds of each river are controlled by municipal water departments for public water supply. No public access is permitted in the Tolt River watershed, although closely supervised logging and timber management are allowed. In the Cedar River watershed, only very limited public access (such as guided educational tours) is permitted. Other closely supervised activities include logging and timber management, railroad and power transmission rights-of-way maintenance, and research. Public access is permitted in the upper portion of the Green River watershed, extending to a limit of 23 mi above the public water supply diversion. Public activities in the upper Green River watershed include camping, fishing, hunting, snowmobiling, and cross-country skiing. In the lower watershed a limited deer and elk hunt is allowed in the fall. Logging, timber management, and power transmission rights-of-way activities also are allowed under closely controlled conditions in the Green River watershed.

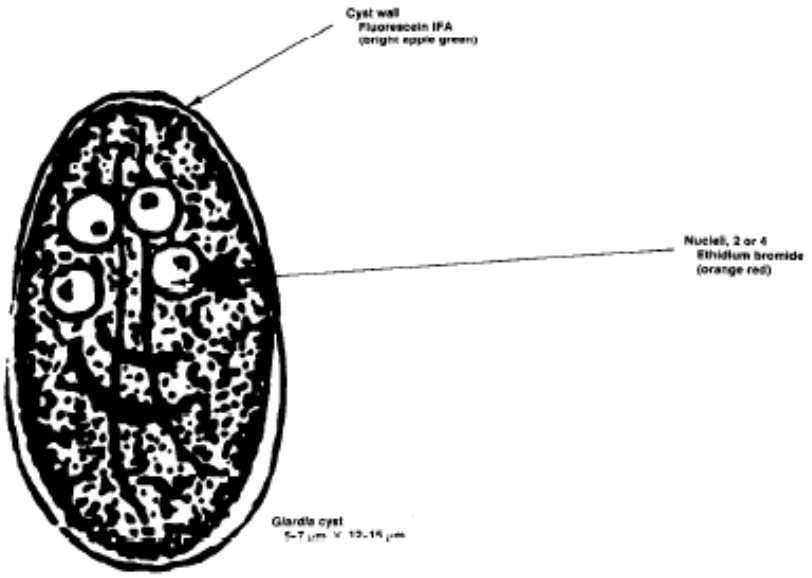

Figure 2. Schematic of *Giardia* cyst stained with fluorescein IFA and ethidium bromide

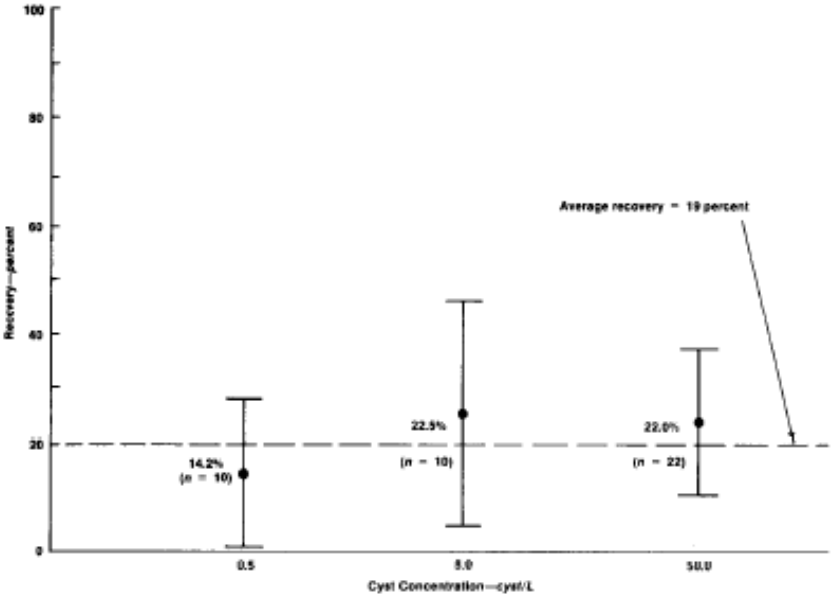

Figure 3. Percentage of cyst recovery versus cyst concentration

TABLE 1

Giardia *cysts concentration summary, including expected values for zero samples for the Cedar, Green, and Tolt rivers*

River	Median Concentration *cysts/L*	Concentration at − 1s	Concentration at + 1s	Coefficent of Variation
Cedar	0.17	0.08	0.54	2.20
Green	0.20	0.08	0.83	3.19
Tolt	0.13	0.06	0.38	1.92

Samples were collected from well-mixed, representative locations and placed in new 20-L disposable containers* that were not reused. Samples were delivered to the laboratory (University of Washington, Seattle) within 4–6 h of collection. Processing was initiated upon receipt of samples and was concluded within 48 h. This article covers the sampling period Jan. 1 to Sept. 30, 1987.

Water sample processing. Sample processing for *Giardia* cyst recovery, identi-

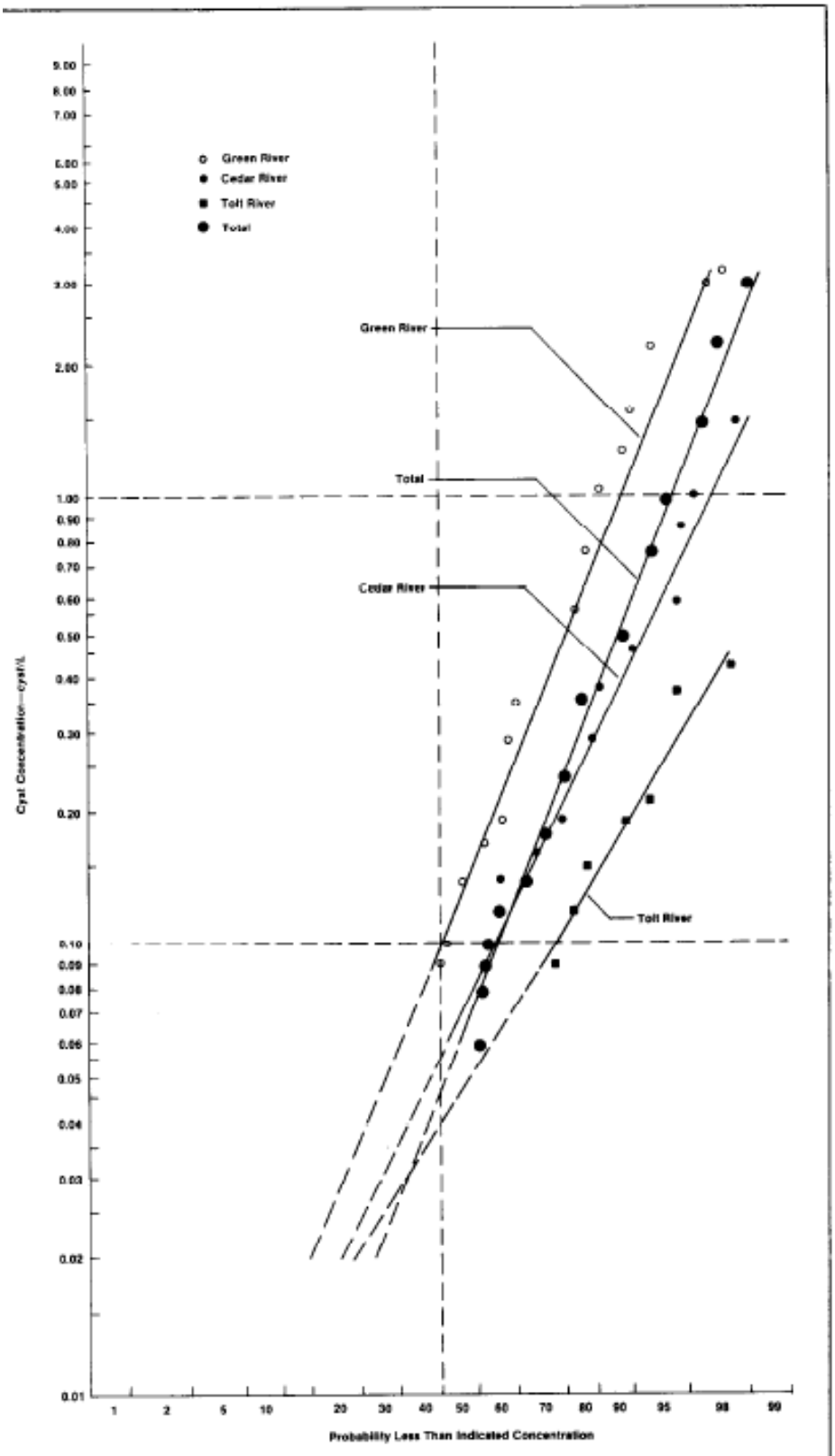

Figure 4. Lognormal probability plot of *Giardia* cyst concentration in the Cedar, Green, and Tolt rivers

fication, and counting was performed in batches of five samples, with negative and positive controls added to each batch for quality control and quantification of cyst recovery efficiency. Samples were batched according to watershed, with negative controls processed between batches and positive controls processed after all other samples to avoid contamination. Positive controls were prepared by adding purified *G. lamblia* cysts to (seeding) 20 L of either 5-μm-filtered distilled water or unfiltered river water to produce a concentration of approximately 50 cysts/L. Cysts for positive controls were of human origin, propagated in gerbils inoculated from axenically cultured trophozoites. Negative controls consisted of 20 L of distilled water, prefiltered by a 5-μm pore size membrane.

Water samples and controls were processed in accordance with the following procedure, and all filtrations were done with polycarbonate membranes.† Samples were mixed thoroughly and filtered through a 293-mm-diameter, 5-μm pore membrane at 250 mm Hg vacuum. A 53-μm monofilament nylon mesh was used as a prefilter. After filtration, the filter membrane was carefully removed and mounted faceup on a 30-cm × 30-cm glass plate. Particles were recovered from the filter surface by alternately rinsing the filter surface from top to bottom with a stream of 0.2-μm-filtered distilled water applied with a wash bottle and by squeegeeing with a 4-cm × 1-mm flexible rubber blade, directing the rinse water into a collection pan. The rinse and squeegee procedures were repeated twice, producing a particle-containing liquid rinse volume of approximately 200 mL. This volume was reduced by centrifuging in 50-mL centrifuge tubes at 650 × g for 15 min and aspirating to 10 percent of original volume. The remaining material was resuspended and combined to form a homogeneous 40–50-mL concentrate. *Giardia* cysts were separated from heavier and lighter debris by layering the concentrate on a gradient of 40 percent potassium citrate as described elsewhere.[8] The cyst-containing particle fraction was harvested from the potassium-citrate-concentrate interface and deposited by vacuum filtration (250 mm Hg) on a 13-mm-diameter, 5-μm pore size membrane contained in an in-line filter holder.‡ The filter was rinsed three times with 5 mL of 0.2-μm-filtered distilled water and kept moist prior to MF-IFA staining.

MF-IFA procedure. The MF-IFA procedure used in this study is similar to that described elsewhere,[8] with modifications to increase processing efficiency. The procedure was applied to particles retained on the 13-mm-diameter, 5-μm pore size filter while in the in-line filter holder.

The first reagent used was a polyclonal rabbit anti-*Giardia* cyst serum prepared by procedures described elsewhere.[8] This serum was diluted 1:150 with phosphate-buffered saline (PBS, pH 7.6) containing 0.1 percent bovine serum albumin (BSA) (essentially fatty-acid-free).§ The MF-IFA was completed using fluorescein-isothiocyanate- (FITC-) labeled goat anti-rabbit serum immunoglobulin G (IgG)

*Cubitainer, VWR Scientific, San Francisco, Calif.
†Nuclepore Corp., Pleasanton, Calif.
‡Model SX00 01300, Millipore Corp., Bedford, Mass.
§Sigma Chemical Co., St. Louis, Mo.

(antiheavy and light chains),* which was diluted 1:40 with PBS containing 0.1 percent BSA.

The MF-IFA reagents were applied to the filters while in the 13-mm filter holders, using antiserum volumes of 0.25 mL. Filter holder ports were stoppered to retain the serum during incubation. Filters were incubated at 37°C for 30 min for both sera. After incubation, each filter was rinsed with three 10-mL volumes of PBS. In the final step, 0.2 mL of ethidium bromide† at 50 μg/mL in PBS was applied to each filter and incubated for 10 min at 5°C. Following a final PBS rinse, filters were mounted on glass slides in elvanol under a glass coverslip as described elsewhere.[8]

Cyst identification and counting. Filters were examined by epifluorescence microscopy at ×250 or ×400 magnification.‡ *Giardia* cysts were identified by the following criteria: size, shape, FITC stain color and distribution, ethidium bromide stain, and brightfield illumination appearance. All features have been described elsewhere,[9] except for the ethidium bromide feature. The addition of ethidium bromide consistently stained the nucleii of *Giardia* cysts processed by membrane filtration (Figure 2). This enabled visualization of the two to four nucleii as red-orange brightly fluorescing objects toward one end of the cyst. This partly overcomes the disadvantage of identifying cysts on polycarbonate filters that cannot be cleared to permit phase contrast examination for internal structure components. Using this procedure, the entire area of each filter was examined. The position of cystlike objects was recorded by the initial examiner for reexamination by a second experienced examiner for independent corroboration.

Statistical analysis and calculations. The number of cysts (n) counted on a filter was consistently a fraction of the actual number of cysts (n_s) occurring in the sample. The n_s was estimated by dividing the number of cysts counted by the recovery percentage (p), measured in positive controls. The actual cyst concentration (c_s) was then estimated by dividing by the sample volume (v):

$$c_s = n / (p \cdot v) \qquad (1)$$

In a significant proportion of samples, no cysts were found. Given a cyst recovery efficiency that was always less than 100 percent, for samples in which no cysts were found the probability that cysts may have been present but escaped detection is finite and can be estimated using a geometric model. This model describes the distribution of the number of independent trials taken before a positive result (whose probability of occurrence is p, in which $0 \leq p \leq 1$) occurs. For this application, the value of the recovery percentage p is the measure of the probability of occurrence p. The geometric distribution of a random variable k is given by:

$$f(k) = p\,(1-p)^k \qquad (2)$$

In this case, k represents the number of cysts that escaped the recovery processes before the first one was caught. Here the assumption must be made that the probability of escape for each cyst $(1 - p)$ was not dependent on the cyst concentration and does not change markedly when the concentration is low. This was a reasonable assumption, as shown by Figure 3. In other words, when there are few cysts in a large sample, there is no reason to believe that the probability of any one cyst being recovered is different from that of any other or that this probability is dependent on whether or not another cyst is recovered. Thus, when no cysts were found, the cyst concentration was computed from the mean of the geometric distribution defined in Eq 2.[10]

$$k_o = E(k) = (1 - p) / p \qquad (3)$$

The cyst concentration was computed by dividing this mean by v, obtaining the following:

$$c_o = k_o / v$$

For example, the cyst concentration for a 40-L sample in which no cysts were found, and from a sample batch for which the measured cyst recovery efficiency was 19 percent, would be:

$$c_o = [(1 - 0.19) / 0.19] / 40\ \text{L}$$

$$c_o = 0.11\ \text{cysts/L}$$

This can be regarded as a worst-case estimate, which will be biased only when there are consistently fewer cysts in a series of samples than the value of this distribution mean.

In order to determine the possibility of seasonal variations in cyst concentrations, seasonal subsets of the data were examined. Histograms of *Giardia* cyst concentration estimates from the entire sampling program and from geographical and time-based subsets were examined to determine their symmetry. Because all were skewed, the nonparametric Kruskal–Wallis (K–W) one-way statistic was applied for one-way group comparisons. Pairwise comparisons of cyst estimates between rivers were also done with a K–W test.[11] When such multiple comparisons were performed, the critical p value, which is usually taken to be 0.05, was divided by the number of comparisons made according to a Bonferroni adjustment factor.[10]

Results

***Giardia* cyst recovery.** The efficiency of *Giardia* cyst recovery in positive controls prepared from 5-μm-prefiltered distilled water, seeded at 50 cysts/L, ranged from 5 to 44 percent with a median value of 21.8 percent. The values were lognormally distributed, with 85 percent of the values lying between 15 and 29 percent (n = 26). In positive controls prepared from seeded 20-L river water samples (Cedar River, station CPR-1, Sept. 1–Oct. 31, 1987) the recovery efficiency averaged 28 percent, $s = \pm 13$ percent, n = 11. In parallel tests using 20-L samples of 5-μm-filtered distilled water seeded at levels of 0.5, 5, and 50 cysts/L, no dependence of recovery efficiency on cyst concentration was found (Figure 3). Based on the median recovery efficiency (22 percent) and a typical sample volume of 40 L, the typical limit of detection of the procedure was 0.11 cysts/L, calculated using Eq 1.

***Giardia* cyst concentrations in river water.** A total of 222 samples collected from the three river systems were examined. Cysts were found in 94 (43 percent) of the samples. The number of cysts found in the positive samples ranged from 1 to 24. Corresponding cyst concentrations calculated from the recovery efficiency and sample volume ranged from 0.1 to 5.2 cysts/L (Figure 4). Overall, and for each of the rivers individually, no cysts were found in half or more of the samples. The distributions of cyst concentrations were all evidently truncated by the limit of detection. Simple extrapolation indicates that the overall median cyst concentration was 0.063/L with a range between ± 1s from 0.017 to 0.24/L. Median concentrations extrapolated for the Cedar, the Green, and the Tolt rivers were 0.04/L, 0.06/L, and 0.003/L, respectively (Figure 4).

An independent check of typical concentrations below the normal limit of detection was made by processing four series of ten 30-gal samples, one series each on the Cedar and the Tolt rivers and two series on the Green River. Concentrations found on the Cedar River averaged 0.07/L. On the Green River, the average concentration was 0.09/L. No cysts were found in the Tolt River samples, in which the extrapolated median concentration (0.003/L) was still significantly below the limit of detection for a 30-gal sample (0.04/L).

A more conservative picture was developed by calculation of an expected (most probable) value of cyst concentration for each zero sample using Eq 4. A comparison of the data processed in this way for each of the rivers showed that although they each had cyst concentrations in the same general range (0.05–0.5/L) and

*Cooper Biochemical, Malvern, Pa.
†Sigma Chemical Co., St. Louis, Mo.
‡Zeiss Model 14, Carl Zeiss Inc., Thornwood, N.Y.

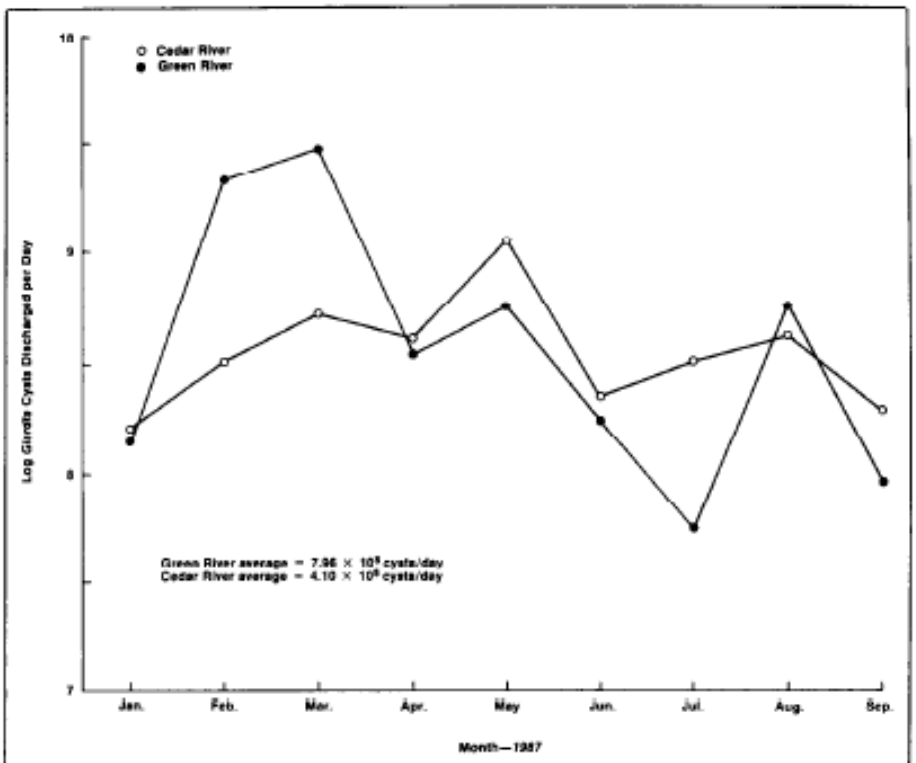

Figure 5. Total *Giardia* cyst discharge by month on the Cedar and Green rivers from January through September 1987

TABLE 2
Characteristics of reservoirs on the Cedar, Green, and Tolt rivers

River	Reservoir Name	Catchment Area *sq mi*	Volume *acre-ft*	Surface Area *acres*	Residence Time *days*
Cedar	C. Morse	143	40,000	1,680	100±
Green	H. Hansen	220	26,000	700	30–50
Tolt	Tolt	19	56,000	850	200±

TABLE 3
Quantiles of Giardia *cyst concentration by season in the Green, Cedar, and Tolt rivers—January–September 1987*

Season	Quantile 0.10	0.25	0.50	0.75	0.90	Number of Samples *n*	Statistical Significance K-W	*p*
Winter	0.09	0.10	0.12	0.37	0.49	48	2.68	0.26
Spring	0.07	0.08	0.11	0.24	0.44	60		
Fall	0.06	0.08	0.10	0.46	0.59	45		

although variations in concentration were similar (similar *s*), differences between them could be distinguished statistically (Table 1).

Comparison of cyst concentrations among the three rivers using the K-W test found that those on the Green River were higher than those on both the Cedar and the Tolt rivers ($p \leq 0.05$) but that the Cedar River cyst concentrations could be distinguished from the Tolt River concentrations only at $p \leq 0.10$. Cyst concentrations in individual tributaries were essentially comparable. They were not statistically distinguishable from each other, river by river. Concentrations at main-stem locations were generally higher than in the tributaries.

Cyst concentrations measured in mainstem rivers were compared for sampling stations above and below a reservoir. The Cedar, Green, and Tolt rivers each have a major impoundment in the reach studied (Table 2). Using the K-W test, the concentrations found above and below each of the three reservoirs could not be distinguished, even at a significance level as low as $p \leq 0.25$. Median cyst concentrations above and below the Cedar, Green, and Tolt reservoirs were 0.12 and 0.22, 0.27 and 0.32, and 0.16 and 0.21 cysts/L, respectively.

Seasonal changes in cyst concentrations were examined by dividing the data for each river into three-month periods corresponding approximately to months of snow cover and freezing temperatures (January–March), cool temperatures and high runoff (April–June), and dry, warm conditions (July–September). The median cyst concentration decreased from 0.12 to 0.10/L from winter to fall; however, by the K-W test the changes were not significant ($p \leq 0.26$), as shown in Table 3. A plot of mass loading of cysts calculated from monthly average cyst concentrations and monthly average river discharges for the farthest downstream sampling points on the Cedar and the Green rivers shows an irregular pattern of variation within a relatively narrow range (Figure 5).

Discussion

This study demonstrated that *Giardia* cysts were continuously present in three rivers in Washington at concentrations ranging from 0.05 to about 1.0 cyst/L during the period from January through September 1987. These observations contrast with the previous impression that *Giardia* cysts were intermittently present in water, an impression probably derived from the intermittent nature of waterborne giardiasis outbreaks.[2]

Several observations regarding temporal variations in cyst concentrations were made. The range of variation found over the nine-month sampling period in each river, i.e., from $-1s$ to $+1s$, is less than a factor of 2 (Figure 4). This is comparable to the concentration variations in dissolved water quality constituents for the same rivers. The data collected at 1–3-h intervals over daily cycles show that cyst concentrations do not vary significantly over time periods of this length. These data should be viewed in light of the likely sources of cysts and location of sampling stations in the watershed. Because human access and activities in the three watersheds are strictly controlled, cyst sources are assumed to be exclusively the animal populations. Cyst shedding by animals ranging from beaver, coyote, and bobcat to deer and elk has been confirmed in these watersheds by examination of fecal samples not reported here.

The cyst concentration levels in the three rivers were statistically distinguishable even though the differences in median concentration were only 10 to 20 percent. Although differences in characteristics of the three watersheds can be described in terms of features that may be relevant to levels and distribution of animal and human activity, the small differences in cyst concentrations among the three watersheds did not justify efforts to identify causal relationships.

The cyst concentrations found in this study may be compared with those reported for the Youghiogheny River at McKeesport, Pa.[6] Analysis of 37 samples of 100 gal each by the US Environmental Protection Agency (USEPA) method[12] resulted in finding cysts in each of the samples with numbers ranging from 1 to 438. Quality control results indicated an average cyst recovery efficiency of 7.7 percent (range from 3 to 13.8 percent). Using the average recovery efficiency, the cyst concentration range for that study was 0.03 to 15/L. The author made a limited comparison (not reported here) of the USEPA method and the method reported here. Samples were taken from a single source at the same time and were processed independently by the two methods. Based on the results, the authors believe that the USEPA method underestimates cyst concentration. In 15 samples processed over a two-month period, no cysts were found in 100-gal samples using the USEPA method. Cysts were found in six of the fifteen 20-gal samples processed by the method reported here; the median concentration was 0.05/L, with the range between ± 1s from 0.03 to 0.10/L. Based on this comparison of limited data, the authors expect that examination will reveal significantly higher cyst concentrations in rivers elsewhere, particularly where wastewater cyst sources are present as described for the Youghiogheny River[6] and where water temperature and physical conditions permit cyst survival.

The procedures described in this article proved effective and practical for finding and quantifying *Giardia* cysts in a project of major magnitude. The method has several advantages. Its applicability to small samples permits processing in the laboratory. It also permits examining the entire particle concentrate with reasonable efficiency. These features lend a degree of control not possible in procedures requiring field application and the processing of large particle volumes, resulting in examination of aliquots that must be assumed representative. Although this procedure has advantages, it is not entirely without problems. Variations in cyst recovery ranged from 7 to 44 percent. These variations could not be accounted for except in terms of features of test materials, including cyst antigen characteristics, stain reactivity, processing solution variations, and variations in the particle composition of the water being processed.

The limit of detection of the procedure used in this study was 0.11 cysts/L for the median recovery efficiency (22 percent) using a 40-L sample. Consistent results were achievable when processing waters with turbidities up to 2.5 ntu. Sample processing and microscopic examination were increasingly difficult for turbidity values greater than 1.5 ntu. Turbidity resulting from silt during periods of runoff was separated efficiently by centrifuging and density gradient sedimentation. High algae counts presented the most interference because their settling velocities are such that they are retained along with the cyst-containing particle fraction. The procedure as described can easily be applied to achieve a lower limit of detection by increasing the sample volume. This may require distributing the final particle concentrate to more than one 13-mm-diameter final filter. The concentration is determined from the total number of cysts identified, the total sample volume, and the cyst recovery efficiency for that batch of samples. An appropriate sample volume can be determined by conducting a pilot study in which 20- to 40-L aliquots of a single large sample (100 gal, for example) are processed along with one or more seeded controls. This will establish the approximate cyst concentration and recovery efficiency.

The time and cost of processing 40-L samples based on examination of a single 13-mm-diameter filter were determined in the course of the project. This can be expressed in terms of time and materials. One experienced technician can process a complete batch of five water samples, plus negative and positive controls, in a 40-h week. Including the cost of labor, chemicals, 293- and 13-mm filters, and amortization of an epi-illumination UV-microscope (at a first cost of $10,000), the total cost per sample was between $250 and $300. This included 25 percent indirect labor costs and overhead costs of 23 percent.

Conclusions and recommendations

Based on application of the small volume MF-IFA method used in this study, the concentration of *Giardia* cysts present in relatively pristine waters was in the range of 0.05 to 1.0/L. Further work is needed to better define factors contributing to variations in cyst recovery using this procedure. Better understanding of this aspect should help to improve the limit of detection.

Giardia cysts should be considered to be present in surface waters at a relatively constant concentration that may depend on the magnitude of sources in the watershed and on factors affecting cyst persistence in water. Further work is required to identify the nature and effect on cyst concentrations of such factors as watershed characteristics, animal population characteristics, and the influence of human activities, including urban runoff and wastewater discharges. Previous work shows that cysts are subject to degradation in the environment, particularly in warmer water (data not reported here). It appears from this study that cysts are not removed by passage through a reservoir, even one of appreciable size. Further work is needed to identify conditions that would contribute to cyst removal or degradation with downstream passage.

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