Parasitol Res (2013) 112:467–471
DOI 10.1007/s00436-012-3155-8

ORIGINAL PAPER

Distribution of *Cryptosporidium* oocysts and *Giardia* cysts in water above and below the normal limit of detection

Jerry E. Ongerth · Frhat M. A. Saaed

Received: 16 August 2012 / Accepted: 27 September 2012 / Published online: 11 October 2012

Abstract Analysis of water samples for *Cryptosporidium* oocysts and *Giardia* cysts is a specialised and demanding pursuit. Understanding and evaluating data resulting from such analyses is equally specialised and complicated by the most common result—not finding any of the target organisms. Coming to an accurate conclusion regarding such monitoring results has been hampered by a lack of pertinent information presented in the context of current monitoring requirements. The work reported here presents laboratory data demonstrating an appropriate skewed distribution model statistical framework. It is shown that the Poisson model provides for understanding how *Cryptosporidium* oocysts and *Giardia* cysts are distributed in water at typical ambient concentrations that are near or most commonly below the limit of detection of the most widely used analytical procedure, USEPA Method 1623. From three to six replicate 50-L volumes of particle-free water were seeded with *Cryptosporidium* oocysts and *Giardia* cysts each at concentrations of ca. 0.2/L, 1–2/L, and 6–8/L. The seeded 50-L volumes were analysed in five 10-L aliquots to determine the number of oocysts and cysts in each. The data conformed to the Poisson distribution. This supports the interpretation that analysis of 10-L surface water samples resulting in not finding any target organisms is the result of their presence below the limit of detection. This interpretation strongly suggests that analysing fewer larger volume samples would provide more useful information.

J. E. Ongerth · F. M. A. Saaed
Civil, Mining and Environmental Engineering,
University of Wollongong,
Wollongong, NSW 2522, Australia

J. E. Ongerth (✉)
University of Wollongong,
CME Bldg 4 EEC,
Wollongong, NSW 2522, Australia
e-mail: jongerth@uow.edu.au

Introduction

Cryptosporidium and *Giardia* are waterborne pathogens of sufficient significance that their monitoring and control for public water supply is required in many parts of the world including Australia, the EU and the USA (National Health & Medical Research Council 2011; European Commission 1998; US Environmental Protection Agency (USEPA) 2006). The procedure specified for monitoring is typically USEPA Method 1623 (USEPA 2005a) based on cartridge filtration, elution, immunomagnetic separation and immunofluorescence assay (IFA) microscopy. The standard volume of water analysed using this procedure is 10 L and the recovery efficiency varies widely between 15–20 and 80–90 % with a typical average ca. 50 % (Federal 2003). The limit of detection of the analytical method for a single oocyst or cyst, the minimum number possible, for conditions of a 10-L sample and 50 % analytical recovery would be 1 (oo)cyst in 5 L or 0.2 (oo)cysts/L.

An outstanding feature of the vast majority of reports on the concentrations of *Cryptosporidium* and *Giardia* in water is that the overwhelming majority of analyses result in not finding any. For example, large-scale surveys of surface water sources of public water supply in the USA, required by USEPA regulations, have resulted in more than 90 % of all analytical results that are zeros. More than 60 % of all systems required to monitor have never reported a positive analytical result (Federal 2003; Crainiceanu et al. 2003; USEPA 2005b). Elsewhere, a major water utility in Australia analysed nearly 5,000 samples in the period from 1998 to 2004 and reported finding only two *Cryptosporidium*-positive samples and four *Giardia*-positive samples among them (O'Keefe 2010). Many if not most literature reports on both *Cryptosporidium* and *Giardia* also refer to findings in terms of the proportion of samples analysed in which these organisms were found (Graczyk et al. 2010; Hsu et al. 2001; Lim et al. 2009; Srisuphanunt et al. 2010). Such predominantly negative results suggest the interpretation that the organisms were

Table 1 *Cryptosporidium* oocysts and *Giardia* cysts recovered in 10-L aliquots of 50 L seeded with ca. 10 oocysts and ca. 10 cysts (u_i=10/50; V_i=10)

Aliquot no.	Sample 1a		Sample 1b		Sample 1c	
	Oocysts	Cysts	Oocysts	Cysts	Oocysts	Cysts
1	1	0	1	0	2	3
2	1	3	1	0	3	1
3	6	8	2	4	2	3
4	3	1	2	1	0	2
5	1	0	3	4	3	1
Total	12	12	9	9	10	10

simply not present at the sampling location at the time of sampling. This interpretation implies intermittency as expressed commonly in the literature. An alternative interpretation is that the organisms were present but below the limit of detection, an interpretation that implies continuous presence but at low concentration. The difference between these two interpretations is of real and significant impact, and in fact has been tested previously (Hansen and Ongerth 1991).

The intent of work reported here was to examine the question of continuous vs. intermittent presence from the standpoint of statistical distribution, to provide laboratory data to illustrate relevant features of the nonuniform distribution of discrete objects at low concentration and to determine if a change in perspective regarding predominantly zero analytical results might be justified.

The approach used here was to seed 50-L volumes of particle-free water with small numbers of *Cryptosporidium* oocysts and *Giardia* cysts at levels both above and below the nominal limit of detection for a 10-L sample and analyse the total volume in 10-L aliquots. The objective was to examine the physical distribution of organisms found in comparison to a theoretical model appropriate to the distribution of discrete objects at low concentration.

Materials and methods

Cryptosporidium parvum oocysts and *Giardia lamblia* cysts were obtained commercially (Waterborne Inc., New Orleans LA, http://www.waterborneinc. com/products3.html). The concentration of oocysts and of cysts in the stock suspensions was estimated by haemocytometer counts; then, working suspensions were prepared by dilution to have a concentration of approximately 10^3 per litre each of oocysts and of cysts. Exact numbers of organisms were determined by a drop counting procedure. Droplets of 5 μL from the stock suspensions containing approximately five organisms were pipetted onto the edge of a microscope slide, then counted using bright light microscopy using ×250 magnification for *Giardia* cysts and ×400 magnification for *Cryptosporidium* oocysts. The counts, replicated from 5 to 10 times, established the exact concentration. A supporting concentration checking procedure consisted of pipetting a precise volume, from 5 to 20 μl as desired, directly onto a 13-mm diameter, 2-μm pore diameter etched-pore polycarbonate filter membrane (Sterlitech, Kent, WA) in an inline filter holder (Millipore, Billerica, MA). Organisms on the filter were IFA stained for *Cryptosporidium* and/or *Giardia* (Waterborne, Inc.) and counted using epifluorescence microscopy as previously described (Hansen and Ongerth 1991; Ongerth and Hutton 2001).

Fifty-litre volumes of particle-free water (MilliQ, Millipore) were placed in a clean 60-L polyethylene container (Nalgene, Rochester, NY). Desired numbers of *Cryptosporidium* oocysts and *Giardia* cysts at levels of approximately 10, 100 and 1,000 were transferred from working suspensions to the water by micropipette (P20, P100, P1,000, Gilson, Middleton, WI). Identical volumes of working suspension at each seeding level, in triplicate, were transferred to 13-mm filters to define the actual numbers of organisms that had been added to the water. After mixing, equal 10-L volumes of the seeded water were processed to determine the number of oocysts and cysts in each 10-L volume. First, each 10-L aliquot was filtered through a 293-mm diameter, 2-μm pore diameter etched-pore polycarbonate membrane (Sterlitech) and particles were recovered by

Table 2 *Cryptosporidium* oocysts and *Giardia* cysts recovered in 10-L aliquots of 50 L seeded with 75–100 oocysts and 70–80 cysts

Aliquot no.	Sample 2a		Sample 2b		Sample 2c		Sample 2d		Sample 2e		Sample 2f	
	Oocysts	Cysts	Oocysts	Cysts	Oocysts	Cysts	Oocysts	Cysts	Oocysts	Cysts	Oocysts	Cysts
1	16	19	25	20	28	25	12	9	18	15	18	28
2	22	20	24	16	10	6	17	15	14	6	15	7
3	8	14	28	17	15	13	13	7	20	24	9	8
4	23	9	15	10	28	17	24	27	15	14	13	14
5	11	17	11	17	13	9	14	19	22	21	20	19
Total	80	77	103	80	94	70	80	77	89	80	75	76

Table 3 *Cryptosporidium* oocysts and *Giardia* cysts recovered in V_i=10 L aliquots of 50 L seeded with 850–900 (3a–c) and 650–750 oocysts (3d–f) and 500–650 cysts

Aliquot no.	Sample 3a		Sample 3b		Sample 3c		Sample 3d		Sample 3e		Sample 3f	
	Oocysts	Cysts	Oocysts	Cysts	Oocysts	Cysts	Oocysts	Cysts	Oocysts	Cysts	Oocysts	Cysts
1	177	140	110	82	133	101	109	99	132	136	151	156
2	279	173	154	99	214	135	99	104	171	126	129	119
3	193	122	182	120	186	107	155	159	136	121	153	125
4	150	124	209	102	164	102	160	139	147	115	96	120
5	105	123	166	103	156	70	132	120	134	105	168	130
Total	904	682	821	506	853	506	655	621	720	603	696	650

sequential rinsing and squeegeeing as previously described (Ongerth 1989; Ongerth and Pecoraro Proctor 1995). Rinse volumes recovered from the filters were centrifuged at 650×*g* for 20 min, then decanted, resuspended in minimal volume and transferred quantitatively with rinsing to 13-mm filters in inline filter holders for IFA staining and enumeration as described above.

The statistical distribution of organism numbers found in the 10-L aliquots was compared to the Poisson distribution,

$$P(x_i) = \exp(-u_i V_i)\frac{(u_i V_i)^{x_i}}{x_i!} \quad (1)$$

expressing the probability of finding exactly x_i organisms in a sample volume V_i in which the average organism is u_i. Statistical analyses were performed using XLSTAT (Addinsoft, Paris).

Results

In three trials of seeding, 50 L of particle-free water with 10 *Cryptosporidium* oocysts and 10 *Giardia* cysts, the number of oocysts recovered in each 10-L aliquot processed ranged from 0 to 6 with averages of 2.4, 1.8 and 2.0 (Table 1, samples 1a–c). The number of cysts recovered ranged from 0 to 8 with averages identical to those of oocysts (Table 1, samples 1a–c).

In six trials, 50 L of particle-free water was seeded with 75–100 *Cryptosporidium* oocysts and 70–80 *Giardia* cysts. The number of oocysts recovered in each 10-L aliquot processed ranged from 8 to 28 with averages ranging from 15 to 21 (Table 2). The number of cysts recovered ranged from 6 to 28 with averages ranging from 14 to 16 (Table 2).

In six trials, 50 L of particle-free water was seeded with 850–900 (samples 3a–c) and 650–750 (samples 3d–f) *Cryptosporidium* oocysts and 500–650 *Giardia* cysts. The number of oocysts recovered in each 10-L aliquot processed ranged from 105 to 279 with averages of 181, 164 and 171 (samples 3a–c) and from 96 to 171 (samples 3d–f) with averages of 131, 144 and 139, respectively (Table 3). The number of cysts recovered ranged from 70 to 173 with averages ranging from 101 to 136 (Table 3).

Discussion

When *Cryptosporidium* oocysts and *Giardia* cysts were seeded to particle-free water at the lowest level, approximately 10 organisms in 50 L, in a proportion of the 10 L (u_iV_i=2.0) aliquots processed, no organisms were found (1/15 for *Cryptosporidium*, 4/15 for *Giardia*). Using the chi-square test, α=0.05, the observed analytical results (Table 1) conform to organism numbers predicted by the Poisson model (Eq. 1, Table 4). The lowest seeding level used in

Table 4 Poisson probability of finding exactly *x* organisms in water having concentrations from 0.02 to 10/L

x (number of organisms)	u_iV_i=0.02 u_i=1/500 L	u_iV_i=0.1 u_i=1/100 L	u_iV_i=0.2 u_i=1/50 L	u_iV_i=1.0 u_i=1/10 L	u_iV_i=2.0 u_i=1/5 L	u_iV_i=10 u_i=1/L
0	0.9802	0.9048	0.8187	0.3679	0.1353	0.0000
1	0.0196	0.0905	0.1637	0.3679	0.2707	0.0005
2	0.0002	0.0045	0.0164	0.1839	0.2707	0.0023
3	0.0000	0.0002	0.0011	0.0613	0.1804	0.0076
4	0.0000	0.0000	0.0001	0.0153	0.0902	0.0189
5	0.0000	0.0000	0.0000	0.0031	0.0361	0.0378

u_i is the average number of organisms per litre of water and V_i is the volume of sample used, 10 L in this example

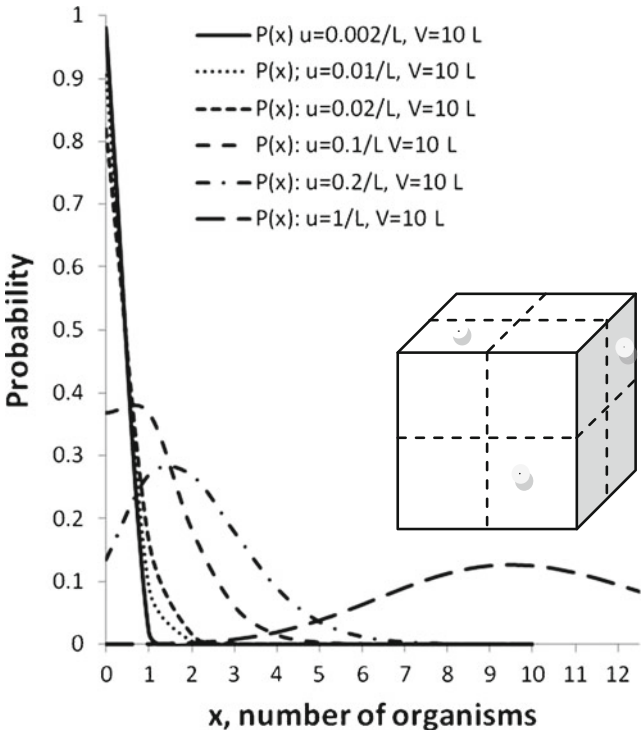

Fig. 1 Predicted Poisson probability (0 to 1) of finding exactly $x=1$ to 12 organisms for average concentrations ranging from 0.02 to 10 organisms per L in 10-L samples

this work, 10 organisms in 50 L or 0.2 per L, was selected as the minimum practical level at which organism numbers could be counted and manipulated (transferred quantitatively to test water volumes) and recovered efficiently by the processing scheme described above. A critical feature of organism concentrations at this level is the fact that on the average only one organism would be present in every 5 L of sample volume. In other words, no organisms would be present in four out of five 1-L volumes. Also, as described by the Poisson model, the distribution of discrete objects (organisms such as *Cryptosporidium* oocysts and *Giardia* cysts) is not symmetrical (Fig. 1). Rather, specifically at low concentration, the distribution is skewed toward numbers lower than the average, resulting in a significant probability of 0. The probability of aliquots having no organisms increases with decreasing concentration, as illustrated by graphical representation of Table 4, Fig. 1. For example, if the concentration of the target organism in the water sampled was truly one organism per 100 L, the probability of not including a single organism in a 10-L sample volume collected at random would be 90.48 % (Table 4, column 3, $x=0$).

Conversely, if an agency analysing water samples for *Cryptosporidium* and *Giardia* finds that no organisms are found in 90 % of its samples, a possible inference would be that the ambient concentration was in the range of 1 in 100 L (0.01/L, not taking recovery efficiency into account. If for example, the recovery efficiency was 50 %, the concentration would be in the range of 0.02/L or 1 in 50 L).

Based on the above reasoning, a logical interpretation of data sets including a preponderance of analytical results of zero organisms found would be that the concentration was below the limit of detection. This is emphatically different from the interpretation that no organisms (e.g. *Cryptosporidium* or *Giardia*) were present in the water at the sampling location at the time of sampling, implying that these organisms are present in that water source only intermittently. Comparison of this interpretation to the analogy of coliform or faecal coliform monitoring of untreated surface water is appropriate. When coliform or faecal coliform analyses show counts of zero in a sample, it is clear that the organisms were present in the source although none were recoverable in the

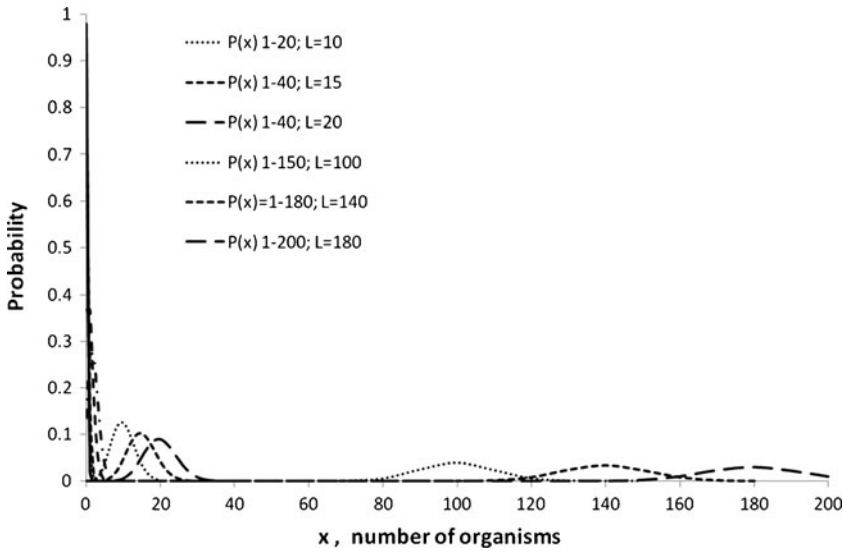

Fig. 2 Predicted Poisson probability (0–1) of finding exactly x organisms for average concentrations ranging from 10 to 20 and 100 to 200 organisms per L

standard 100 mL sample. Which of the two interpretations is correct is a critical issue and is in fact testable by collecting and analysing larger sample volumes. Previous investigations (Hansen and Ongerth 1991; Ongerth 1989) have produced evidence that concentrations of *Cryptosporidium* and *Giardia* are in fact continuous rather than intermittent even though a significant proportion of samples may be below the limit of detection.

As the average number of organisms per unit volume (concentration) increases, the distribution of organisms in aliquots of a large sample becomes more uniform. This is illustrated by the 50-L volumes seeded at higher levels, Tables 2 and 3, and theoretical Poisson distributions at these levels, Fig. 2. Statistical analysis of 10-L sample results at the higher seeding levels indicates that they are better represented by the normal distribution than by the Poisson distribution. However, the average concentrations of *Cryptosporidium* and *Giardia* in the higher ranges have not been reported in environmental samples except in wastewater.

As noted previously, most commonly literature reports of finding and evaluating *Cryptosporidium* and *Giardia* in various environmental media indicate finding these organisms in a proportion of samples. Typically, results derive from single or at most few samples taken from individual sample sites (Graczyk et al. 2010; Hsu et al. 2001; Lim et al. 2009; Srisuphanunt et al. 2010). Inferences based on organism presence vs. absence, particularly when only single or few samples per site were analysed, are most likely to be misleading. This is particularly so when the water quality of samples from different sites was as different as well, tap, canal and ditch (Srisuphanunt et al. 2010), and recovery efficiency was not used to express results as true concentration. Further, quantitative comparisons, for example of the relationship between *Cryptosporidium* and/or *Giardia* concentrations and the concentrations of more easily monitored indicator (Graczyk et al. 2010), will be biased by the skewed distribution of organisms at low concentration.

Understanding the likelihood of negative (zero) findings from application of current monitoring of water supply sources, typically using 10-L samples analysed using USEPA Method 1623, is of unrecognised significance. Each analysis costs ca. $450 (Biovir, Benicia, CA). The value of data obtained when virtually all analytical results are negative is virtually nil. Such data provide no guidance regarding watershed/catchment management. No critical conditions or trends in concentration can be identified. And, no valid estimation of risk or comparison to the relative risk at other sampling points is possible. Although water supply and regulatory agencies may feel comfortable with such results, any sense of security may well be illusory.

The work presented here is offered to help illustrate that the direct cause of negative analytical results is that the limit of detection, dependent only on the sample volume and recovery efficiency of the analysis in the specific water, is above the ambient concentration. Since the recovery efficiency is not readily altered, only processing samples of larger volume can improve data results. Although doubling the sample volume will in many cases double the cost of analysis, producing non-zero results at perhaps half the current sampling frequency would significantly improve the value of the resulting data.

Acknowledgments The authors acknowledge the personal assistance and effort provided by Dr. Long Nghiem as formal supervisor of the doctoral project contributing to this manuscript.

References

Crainiceanu CM, Stedinger J, Ruppert D, Behr C (2003) Modelling the U.S. national distribution of water borne pathogen concentrations with application to *Cryptosporidium parvum*. Water Resour Res 39(9):1235:SWC2-1–SWC2-15

European Commission (1998) European Commission: Council Directive 98/83/EC of 3 November, 1998

Federal Register (2003) Proposed Rules. 68(154) 47655. Monday, August 11, 2003

Graczyk TK, Sunderland D, Awantang GN, Mashinski Y, Luch FE, Graczyk Z, Chomincz L, Breysse B (2010) Relationships among bather density, levels of human waterborne pathogens, and fecal coliform counts in marine recreational beach water. Parasitiol Res 106:1103–1108

Hansen J, Ongerth JE (1991) Effects of time and watershed characteristics on the concentration of *Cryptosporidium* oocysts in river water. Appl Environ Microbiol 57(10):2790–2795

Hsu BM, Huang C, Hsu CLL (2001) Analysis for *Giardia* cysts and *Cryptosporidium* oocysts in water samples from small water systems in Taiwan. Parasit Res 87:163–168

Lim YAL, Ramasame SD, Mahdy MAK, Sulaiman WYW, Smith HV (2009) Detection and molecular characterization of *Giardia* isolated from recreational lake water in Malaysia. Parasit Res 106:289–291

National Health & Medical Research Council (NH&MRC) (2011) Drinking water guidelines, Australia http://www.nhmrc.gov.au/_files_nhmrc/publications/attachments/eh52_aust_drinking_water_guidelines_update_120430.pdf

O'Keefe B (2010) Sydney water inquiry ten year review. http://www.sydneywater.com.au/Publications/FactSheets/McClellan_Review_final_report_100215.pdf

Ongerth JE (1989) *Giardia* cyst concentrations in river water. J Am Water Works Assoc 81(9):81–86

Ongerth JE, Hutton PE (2001) Pilot-scale testing of diatomaceous earth filtration for removal of *Cryptosporidium* oocysts. J Am Water Works Assoc 93(12):54–63

Ongerth JE, Pecoraro Proctor J (1995) Removal of *Cryptosporidium* in rapid sand filtration. J Am Water Works Assoc 87(12):83–89

Srisuphanunt M, Karanis P, Charoenca N, Boonkhao N, Ongerth JE (2010) *Cryptosporidium* and *Giardia* detection in environmental waters of southwest coastal areas of Thailand. Parasit Res 106:1299–1306

U.S. Environmental Protection Agency (USEPA) (2005) *Cryptosporidium* and *Giardia* in Water by Filtration/IMS/FA. EPA-815-R-05-002. Available from: http://www.epa.gov/nerlcwww/online.htm

U.S. Environmental Protection Agency (USEPA) (2005) Occurrence and exposure assessment for the final long term 2 enhanced surface water treatment rule. EPA 815-R-06-002

U.S. Environmental Protection Agency (USEPA) (2006) National primary drinking water regulations: long term 2 enhanced surface water treatment rule. Fed Regist 71(3):654–686