Thermal Contribution to the Inactivation of *Cryptosporidium* in Plastic Bottles during Solar Water Disinfection Procedures

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Abstract. To determine the thermal contribution, independent of ultraviolet radiation, on the inactivation of *Cryptosporidium parvum* during solar water disinfection procedures (SODIS), oocysts were exposed for 4, 8, and 12 hours to temperatures recorded in polyethylene terephthalate bottles in previous SODIS studies carried out under field conditions. Inclusion/exclusion of the fluorogenic vital dye propidium iodide, spontaneous excystation, and infectivity studies were used to determine the inactivation of oocysts. There was a significant increase in the percentage of oocysts that took up propidium iodide and in the number of oocysts that excysted spontaneously. There was also a significant decrease in the intensity of infection elicited in suckling mice at the end of all exposure times. The results of the study demonstrate the importance of temperature in the inactivation of *C. parvum* oocysts during application of SODIS under natural conditions.

INTRODUCTION

Protozoan parasites of the genus *Cryptosporidium*, members of the Phylum Apicomplexa, affect a wide variety of vertebrate hosts. In humans, *Cryptosporidium hominis* (anthroponotic origin) and *C. parvum* (zoonotic origin) are responsible in most areas for more than 90% of cases of cryptosporidiosis, a diarrheal disease in which progression and severity are closely linked to the immune status of infected persons.^{1,2} Cryptosporidia are transmitted as infective oocysts, either directly by the fecal-oral route or indirectly by ingestion of contaminated food and water. *Cryptosporidium* oocysts are prevalent in surface waters, extremely resistant to commonly used disinfectants, and generally survive for several months in aquatic environments. *Cryptosporidium* is recognized as a major human waterborne pathogen worldwide.³

In developing countries where the availability of clean drinking water is highly unpredictable for a variety of reasons, the solar water disinfection (SODIS) process is a simple, inexpensive option (because sunlight is freely available) for reducing infant morbidity and mortality associated with waterborne pathogens.^{4,5} The SODIS method involves storing microbiologically contaminated drinking water in transparent containers such as bags or plastic bottles and placing them in direct sunlight for periods of up to 8 hours before consumption.⁶ The biocidal effect of sunlight is caused by optical and thermal processes, and a strongly synergistic effect occurs between the two processes when the temperature exceeds 45°C.^{7,8} In the past 15 years, numerous studies have been carried out under natural and simulated sunlight to evaluate the efficacy of SODIS procedures against a large number of hydric pathogens.^{9–16} *Cryptosporidium parvum* has been demonstrated to be sensitive to this process,^{17,18} and a rapid inactivation of the oocysts in tap water in an experiment conducted on days with different levels of solar insolation (up to 90% inactivation within the first hour) was reported.¹⁹

Much of the published research has focused on the role that solar ultraviolet radiation (UV) plays in solar disinfection of drinking water contaminated with *C. parvum* oocysts.

However, the thermal contribution has not been fully investigated. Taking into account the high temperatures recorded in turbid water samples contained in ordinary transparent polyethylene terephthalate (PET) bottles placed in direct sunlight during SODIS procedures to inactivate *Cryptosporidium*,²⁰ in the present study, we focused on the thermal contribution of incident solar radiation in the inactivation of *C. parvum* oocysts.

MATERIALS AND METHODS

***Cryptosporidium parvum*.** *Cryptosporidium* oocysts were collected from a naturally infected neonatal Friesian-Holstein calf by rectal sampling. Concentration (phosphate-buffered saline [PBS; pH 7.2]/diethyl ether), purification (discontinuous cesium chloride gradients), and quantification (Neubauer hemocytometer) were performed as reported.^{21,22} Oocysts were classified as *C. parvum* by analysis of a fragment of the *Cryptosporidium* oocyst wall protein gene.²³

Experimental design. In previous field studies carried out at the Plataforma Solar de Almería in Spain, PET bottles containing turbid waters contaminated with purified oocysts of *C. parvum* were exposed to full sunlight on the roof of the laboratory for 4, 8, and 12 hours during the daily period of maximum intensity of solar irradiation. The water temperature inside the bottles was measured each hour with a thermometer (model HI 98509-1; Hanna Instruments, S.L., Eibar, Spain) and different temperature profiles were obtained for water turbidity levels of 0, 5, 100 and 300 nephelometric turbidity units and exposure times of 4, 8, and 12 hours.²⁰ The average temperature profile registered for water of different levels of turbidity was simulated in the laboratory in a dry block heat bath fitted with a lid to protect the samples from UV light (Uniequip GmbH, Dresden, Germany). Distilled water (1 mL) and 2×10^6 purified *C. parvum* oocysts were placed in microcentrifuge tubes (1.5 mL). The tubes were maintained at room temperature (18–20°C) for one hour before exposure to the different temperatures for 4, 8, and 12 hours. The temperature was adjusted every 30 minutes in accordance with the average temperature profiles under consideration (Figure 1). After incubation, each sample was centrifuged ($10,000 \times g$ for 5 minutes), the supernatant (0.5 mL) was aspirated, and the sediment was resuspended. In the four-hour

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FIGURE 1. Evolution of the spontaneous excystation, inclusion of the fluorogenic vital dye propidium iodide, and global viability of *Cryptosporidium parvum* oocysts after exposure of samples for 4, 8, and 12 hours to temperatures previously recorded in water samples in 1.5-liters polyethylene terephthalate bottles during solar water disinfection procedures under natural sunlight.

exposure test, the percentage of spontaneous excystation and inclusion/exclusion of the fluorogenic vital dye propidium iodide was determined every hour, whereas for exposure times of 8 and 12 hours, these parameters were evaluated every two hours, in accordance with the protocols described below.

Spontaneous excystation. Aliquots of 10 μ L of the samples were viewed under phase contrast microscopy (400 $\times$ magnification) with an Olympus BH2 microscope (Olympus Optical Co., Ltd., Tokyo, Japan), and the percentage of excystation was calculated.²⁴ The proportions of totally excysted

(empty), partially excysted, and intact oocysts were identified and enumerated in triplicate. A total of 200–400 oocysts were counted for each enumeration, and the percentage of spontaneous excystation was calculated as follows:

$$\text{Excystation (\%)} = \frac{\text{No. empty oocysts} + \text{No. partially excysted oocysts}}{\text{Total No. oocysts}} \times 100$$

The number of oocysts present in the rest of the sediment was recounted in a Neubauer hemocytometer before use in the viability and infectivity assays, as described below.

Viability assays. The viability of *C. parvum* oocysts was determined by inclusion/exclusion of propidium iodide (Sigma, Madrid, Spain),²⁵ and with a further modification that includes an immunofluorescence antibody test to verify oocyst identification.²⁶ Briefly, aliquots of 5×10^4 oocysts were suspended in 100 μ L of Hanks' balanced salt solution (Sigma) and incubated with 10 μ L of a working solution of propidium iodide (1 mg/mL in 0.1 M PBS, pH 7.2) at 37°C for 10 minutes. After staining with propidium iodide, oocysts were washed twice in PBS by centrifugation at $10,000 \times g$, at 4°C for 5 minutes and incubated with 20 μ L of monoclonal antibodies labeled with fluorescein isothiocyanate (FITC) (Aqua-Glo G/C Direct; Waterborne, Inc., New Orleans, LA). Oocysts were first identified under an FITC filter (excitation at 450–480 nm, barrier at 515 nm) before being examined for inclusion/exclusion of propidium iodide under a propidium iodide filter (excitation at 510–550 nm, barrier at 590 nm). Proportions of propidium iodide-positive (dead) and propidium iodide-negative (viable) oocysts were quantified at 400 $\times$ magnification by using an epifluorescence microscope equipped with Nomarski differential interference contrast and FITC and propidium iodide filters (Olympus AX70; Olympus Optical Co., Ltd.). Taking into account that only intact oocysts were considered in the assays involving inclusion/exclusion of propidium iodide and that totally or partially excysted oocysts are not viable, we calculated the global viability of the isolate as follows:

$$\text{Global viability (\%)} = \frac{\% \text{Intact oocysts} \times \% \text{PI-negative oocysts}}{100}$$

Infectivity assays. Litters of CD-1 Swiss mice (10–15 mice per litter, 3–4 days of age, weight = 2.5–3.0 g) were inoculated intragastrically with 0.1 mL of water containing 2.5×10^4 intact oocysts of *C. parvum* at the end of each exposure time (4, 8, and 12 hours). The mice were killed seven days later and the entire small and large intestines were removed and placed in 5 mL of PBS, pH 7.2. The intestines were then homogenized using an Ultra-Turrax® T10 homogenizer (Ika-Werke GmbH and Co., KG, Staufen, Germany), and the oocysts were counted in a Neubauer hemacytometer.²⁷ Infectivity was defined as the percentage of infected mice per litter, and the intensity of infection as the number of oocysts per homogenized intestinal tissue.

Statistical analysis. Differences in excystation, inclusion of propidium iodide, and intensities of infection were compared by pairwise multiple comparison procedures (Student-Newman-Keuls method) and one-way ANOVA, with GraphPad InStat® version 3.05 statistical software

(GraphPad Software, La Jolla, CA). Differences were considered significant at a probability level of $P < 0.05$.

RESULTS

The overall viability of the *C. parvum* isolate used in the assays and calculated as described above was $88.1\% \pm 1.0\%$ (mean $\pm$ SD) (intact oocysts = $92.7\% \pm 1.9\%$, propidium iodide-negative oocysts = $95.0\% \pm 1.2\%$). The infectivity obtained in the murine model was 100% and the mean intensity of infection was $4.6 \pm 1.9 \times 10^6$ oocysts per homogenized intestinal tissue.

Percentages of spontaneous excystation, inclusion of propidium iodide, overall viability, and values of infectivity and intensity of infection obtained for *C. parvum* oocysts after exposure times of 4, 8, and 12 hours to the average temperature profile recorded in 1.5-liters PET bottles during SODIS procedures under natural sunlight are shown in Table 1. There was a significant increase ($P < 0.0001$) in the percentage of spontaneous excystation at 4, 8, and 12 hours compared with the initial isolate. There was also a significant increase ($P = 0.0003$) in the percentage of oocysts that took up propidium iodide. The global viability therefore significantly decreased at the different exposure times ($P < 0.0001$). The results obtained in the neonatal murine model reflected a decrease in oocyst infectivity and a significant decrease ($P < 0.0001$) in the intensity of infection detected at the shortest exposure time (four hours).

The evolution of spontaneous excystation, inclusion of propidium iodide, and global viability in relation to the temperature profile tested at the different exposure times are shown in Figure 1. The spontaneous excystation and inclusion of propidium iodide by *C. parvum* oocysts followed similar patterns throughout all assays. In the 4-hours exposure test, the temperature increased from 22.7°C to 45.7°C. Until an exposure time of three hours, the percentage of spontaneous excysted and the percentage propidium iodide-positive oocysts were similar to those obtained with the original isolate, whereas there was a significant increase ($P < 0.005$) in both parameters, and therefore a significant decrease in the global viability ($P = 0.0002$) at the end of the experiment.

The results obtained in the 8- and 12-hours exposure tests were not significantly different. There was a sharp significant decrease in global viability ($P < 0.0001$) from 6 and 8 hours in the exposure tests of 8 and 12 hours, respectively, which coincided with the maximum temperatures reached in both tests (44.4°C and 43.8°C). The global viability of the oocyst isolate

TABLE 1

Spontaneous excystation, inclusion of the fluorogenic vital dye propidium iodide, global viability, and infectivity and intensity of infection of *Cryptosporidium parvum* oocysts after exposure of samples for 4, 8, and 12 hours to temperatures previously recorded in water samples in 1.5-liters polyethylene terephthalate bottles during solar water disinfection procedures under natural sunlight*

	Exposure time (hours)			
	0	4	8	12
Spontaneous excystation (%)	7.3 ± 1.9	13.5 ± 2.4	28.4 ± 5.2	28.6 ± 5.1
Propidium iodide-positive oocysts (%)	5.0 ± 1.2	11.1 ± 1.2	32.2 ± 7.3	30.6 ± 3.8
Global viability (%)†	88.1 ± 1.0	74.6 ± 2.3	48.4 ± 4.7	49.9 ± 1.3
Infectivity (%)	100.0 ± 0.0	85.0 ± 15.0	63.4 ± 3.4	75.3 ± 15.5
Intensity of infection‡	4.6 ± 1.9	0.6 ± 0.5	0.7 ± 0.5	0.9 ± 0.4

*Values are the mean $\pm$ SD.

†Calculated as described in the Material and Methods.

‡Number of oocysts $\times 10^6$ per homogenized intestinal tissue.

therefore decreased until the end of these tests by approximately 50%.

DISCUSSION

This is the first study that has evaluated the thermal contribution to the inactivation of *C. parvum* oocysts during SODIS procedures, independent of UV radiation, exposing contaminated water samples to temperatures known to be reached inside PET bottles in some regions where this method is used to disinfect drinking water. Exposure times of 4, 8, and 12 hours resulted in a significant reduction in the potential oocyst viability and a significant decrease in the intensity of infection determined in a neonatal murine model. In addition, in light of the results obtained, we can also state that continuous exposure for 12 hours to the temperatures tested did not have a significantly greater effect on viability and infectivity of the *C. parvum* oocysts than an exposure time of 8 hours.

Temperature is always considered a critical factor that determines the survival of *Cryptosporidium* oocysts in the environment. In a recent review of the effect of temperature on the die-off rate for *C. parvum* oocysts in water, soil, and feces,²⁸ the authors concluded that temperature may be the most lethal factor for *Cryptosporidium* in the environment. The studies included in this review were classified into those in which freezing conditions were tested, others in which temperature between 0°C and 37°C were assayed, and those in which temperatures greater than 45°C were used, with exposure times between one and several minutes for high temperatures²⁹ and weeks and even months for freezing and environmental temperatures.³⁰ The novel approach used in the present study was the experimental evaluation of the effect of the variations in temperature recorded in PET bottles during SODIS procedures under field conditions on the survival of *Cryptosporidium*.

In addition, the phenomenon of spontaneous excystation was also recorded, which is based on the fact that in a small percentage of *C. parvum* oocysts, the sporozoites may excyst when incubated at 37°C in the absence of any of the other major host signals, and therefore makes survival of the sporozoites impossible because they are outside the host environment.³¹ In the present study, the temperature exceeded 37°C during approximately 2.5, 6, and 7 hours in the exposure tests of 4, 8, and 12 hours, respectively, and there was a significant increase in the percentages of spontaneous excystation with respect to the initial values. This large increase may be favored not only by the increased temperature, but also by the combination of long periods of exposure. This finding explains the similar percentages of excystation obtained at the end of the 8- and 12-hour tests, which were significantly higher than the percentage excystation observed in the 4-hour test (Table 1). In a previous study,³² when *C. parvum* oocysts were exposed to temperatures between 37°C and 50°C maintained for different periods, a gradual increase in the percentage of spontaneous excystation in samples exposed to 40–48°C as the time of exposure increased was observed, and a maximum value of 53.8% of spontaneous excystation at a constant temperature of 46°C for 12 hours was obtained.

Conversely, in a study on the adaptation of *Cryptosporidium* oocysts to different conditions, temperature was found to be an important signal for excystation in *C. muris* but not in *C. parvum*, and the authors suggested that high temperature by itself does not induce efficient excystation of *C. parvum*

oocysts because such a step would not be essential for intestinal species.³³ However, the results obtained in the present study, carried out with an isolate of *C. parvum*, demonstrate that high temperatures (greater than 40°C) stimulate the process of spontaneous excystation, with values of approximately 30% at the end of the 8- and 12-hour exposure tests.

The results of inclusion/exclusion of propidium iodide demonstrated that the percentage of oocysts that took up this dye (indicative of the integrity of the oocyst wall) was notably higher in the 8- and 12-hour exposure tests than the initial value (5.0%). The assay of the inclusion/exclusion of fluorogenic vital dyes is considered a simple method that can provide useful preliminary data on the effect of environmental pressures, such as temperature, on oocyst survival, although it is generally considered that the technique overestimates oocyst viability in comparison with an animal model and cell culture methods.³⁴ In the present study, the percentage of oocysts that did not take up propidium iodide, and therefore considered potentially viable, was slightly higher than the infectivity values determined in suckling mice.

Thus, although a significant reduction in oocyst infectivity was not achieved, a significant decrease in the intensity of infection in suckling mice has been observed for the shortest exposure time, perhaps because sporozoites lacked sufficient energy to invade cells.³⁵ The ability of *Cryptosporidium* oocysts to initiate infection is linked to finite carbohydrate energy reserves in the form of amylopectin, which is consumed in direct response to environmental temperatures.³⁶ Moreover, a close relationship between oocyst infectivity and ATP content was identified, and it was demonstrated that temperature inactivation at higher temperatures is a function of increased oocyst metabolic activity.³⁰

In conclusion, the present study demonstrates the important effect of temperature on the inactivation of *C. parvum* oocysts during SODIS procedures, independent of UV radiation, which also induces spontaneous excystation and therefore affects oocyst viability and infectivity. Consequently, we suggest that techniques used to measure excystation are not suitable for evaluating the potential viability of oocysts when they are exposed to temperatures greater than 37°C.

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REFERENCES

1. Fayer R, 2004. *Cryptosporidium*: a water-borne zoonotic parasite. *Vet Parasitol* 126: 37–56.
2. Xiao L, Ryan UM, 2004. Cryptosporidiosis: an update in molecular epidemiology. *Curr Opin Infect Dis* 17: 483–490.
3. Clancy JL, Hargy TM, 2007. Waterborne: drinking water. Fayer R, Xiao L, eds. *Cryptosporidium and Cryptosporidiosis*. Second edition. Boca Raton, FL: CRC Press, 305–333.

4. WHO/UNICEF, 2005. *Water for Life: Making It Happen*. Available at: http://www.who.int/water_sanitation_health/monitoring/jmp2005/en/index.html. Accessed May 4, 2009.
5. WHO/UNICEF, 2007. *The State of the World's Children: 2008*. Available at: <http://www.unicef.org/sowc08/report/report.php>. Accessed May 4, 2009.
6. EAWAG, 2008. *Solar Water Disinfection: The Method*. Available at: <http://www.sodis.ch/Text2002/T-TheMethod.htm>. Accessed May 4, 2009.
7. Wegelin M, Canonica S, Mechsner K, Fleischmann T, Pesaro F, Metzler A, 1994. Solar water disinfection: scope of the process and analysis of radiation experiments. *J Water SRT-Aqua* 43: 154–169.
8. McGuigan KG, Joyce TM, Conroy RM, Gillespie JB, Elmore-Meegan M, 1998. Solar disinfection of drinking water contained in transparent plastic bottles: characterizing the bacterial inactivation process. *J Appl Microbiol* 84: 1138–1148.
9. Joyce TM, McGuigan KG, Elmore-Meegan M, Conroy RM, 1996. Inactivation of fecal bacteria in drinking water by solar heating. *Appl Environ Microbiol* 62: 399–402.
10. Smith RJ, Kehoe SC, McGuigan KG, Barer MR, 2000. Effects of simulated solar disinfection of water on infectivity of *Salmonella typhimurium*. *Lett Appl Microbiol* 31: 284–288.
11. Conroy RM, Elmore-Meegan M, Joyce T, McGuigan KG, Barnes J, 2001. Solar disinfection of drinking water protects against cholera in children under 6 years of age. *Arch Dis Child* 85: 293–295.
12. Kehoe SC, Barer MR, Devlin LO, McGuigan KG, 2004. Batch process solar disinfection is an efficient means of disinfecting drinking water contaminated with *Shigella dysenteriae* type I. *Lett Appl Microbiol* 38: 410–414.
13. Lonnen J, Kilvington S, Kehoe SC, Al-Touati F, McGuigan KG, 2005. Solar and photocatalytic disinfection of protozoan, fungal, and bacterial microbes in drinking water. *Water Res* 39: 877–883.
14. Heaselgrave W, Patel N, Kilvington S, Kehoe SC, McGuigan KG, 2006. Solar disinfection of poliovirus and *Acanthamoeba polyphaga* cysts in water: a laboratory study using simulated sunlight. *Lett Appl Microbiol* 43: 125–130.
15. McGuigan KG, Méndez-Hermida F, Castro-Hermida JA, Ares-Mazás E, Kehoe SC, Boyle M, Sichel C, Fernández-Ibáñez P, Meyer BP, Ramalingam S, Meyer EA, 2006. Batch solar disinfection inactivates oocysts of *Cryptosporidium parvum* and cysts of *Giardia muris* in drinking water. *J Appl Microbiol* 101: 453–463.
16. Boyle M, Sichel C, Fernández-Ibáñez P, Arias-Quiroz GB, Iriarte-Puñá M, Mercado A, Ubomba-Jaswa E, McGuigan KG, 2008. Bacterial effect of solar water disinfection under real sunlight conditions. *Appl Environ Microbiol* 74: 2997–3001.
17. Méndez-Hermida F, Castro-Hermida JA, Ares-Mazás E, Kehoe SC, McGuigan KG, 2005. Effect of batch-process solar disinfection on survival of *Cryptosporidium parvum* oocysts in drinking water. *Appl Environ Microbiol* 71: 1653–1654.
18. Méndez-Hermida F, Ares-Mazás E, McGuigan KG, Boyle M, Sichel C, Fernández-Ibáñez P, 2007. Disinfection of drinking water contaminated with *Cryptosporidium parvum* oocysts under natural sunlight and using the photocatalyst TiO₂. *J Photochem Photobiol B* 88: 105–111.
19. King BJ, Hoefel D, Daminato DP, Fanok S, Monis PT, 2008. Solar UV reduces *Cryptosporidium parvum* oocyst infectivity in environmental waters. *J Appl Microbiol* 104: 1311–1323.
20. Gómez-Couso H, Fontán-Saínz M, Sichel C, Fernández-Ibáñez P, Ares-Mazás E, 2009. Efficacy of SODIS method in turbid waters experimentally contaminated with *Cryptosporidium parvum* oocysts under real field conditions. *Trop Med Int Health* 14: 620–627.
21. Kilani RT, Sekla L, 1987. Purification of *Cryptosporidium* oocysts and sporozoites by cesium chloride and Percoll gradients. *Am J Trop Med Hyg* 36: 505–508.
22. Lorenzo-Lorenzo MJ, Ares-Mazás ME, Villacorta-Martínez de Maturana I, Durán-Oreiro D, 1993. Effect of ultraviolet disinfection of drinking water on the viability of *Cryptosporidium parvum* oocysts. *J Parasitol* 79: 67–70.
23. Amar CF, Dear PH, McLauchlin J, 2004. Detection and identification by real time PCR/RFLP analyses of *Cryptosporidium* species from human faeces. *Lett Appl Microbiol* 38: 217–222.
24. Robertson LJ, Campbell AT, Smith HV, 1993. *In vitro* excystation of *Cryptosporidium parvum*. *Parasitol* 106: 13–19.
25. Campbell AT, Robertson LJ, Smith HV, 1992. Viability of *Cryptosporidium parvum* oocysts: correlation of *in vitro* excystation with inclusion or exclusion of fluorogenic vital dyes. *Appl Environ Microbiol* 58: 3488–3493.
26. Dowd SE, Pillai SD, 1997. A rapid viability assay for *Cryptosporidium* oocysts and *Giardia* cysts for use in conjunction with indirect fluorescent antibody detection. *Can J Microbiol* 43: 658–662.
27. Peeters JE, Mazás EA, Masschelein WJ, Villacorta Martínez de Maturana I, Debacker E, 1989. Effect of disinfection of drinking water with ozone or chlorine dioxide on survival of *Cryptosporidium parvum* oocysts. *Appl Environ Microbiol* 55: 1519–1522.
28. Peng X, Murphy T, Holden NM, 2008. Evaluation of the effect of temperature on the die-off rate for *Cryptosporidium parvum* oocysts in water, soils, and feces. *Appl Environ Microbiol* 74: 7101–7107.
29. Fayer R, 1994. Effect of high temperature on infectivity of *Cryptosporidium parvum* oocysts in water. *Appl Environ Microbiol* 60: 2732–2735.
30. King BJ, Keegan AR, Monis PT, Saint CP, 2005. Environmental temperature controls *Cryptosporidium* oocyst metabolic rate and associated retention of infectivity. *Appl Environ Microbiol* 71: 3848–3857.
31. Smith HV, Nichols RA, Grimason AM, 2005. *Cryptosporidium* excystation and invasion: getting to the guts of the matter. *Trends Parasitol* 21: 133–142.
32. Gómez-Couso H, Fontán-Saínz M, Fernández-Alonso J, Ares-Mazás E, 2009. Excystation of *Cryptosporidium parvum* at temperatures that are reached during solar water disinfection. *Parasitol* 136: 393–399.
33. Widmer G, Klein P, Bonilla R, 2007. Adaptation of *Cryptosporidium* oocysts to different excystation conditions. *Parasitol* 134: 1583–1588.
34. Robertson LJ, Gjerde BK, 2007. *Cryptosporidium* oocysts: challenging adversaries? *Trends Parasitol* 23: 344–347.
35. Vetterling JM, Doran DJ, 1969. Storage polysaccharide in coccidial sporozoites after excystation and penetration of cells. *J Protozool* 16: 772–775.
36. Fayer R, Trout JM, Jenkins MC, 1998. Infectivity of *Cryptosporidium parvum* oocysts stored in water at environmental temperatures. *J Parasitol* 84: 1165–1169.