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EFFECTS OF FREEZE–THAW EVENTS ON THE VIABILITY OF *CRYPTOSPORIDIUM PARVUM* OOCYSTS IN SOIL

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ABSTRACT: The effects of freeze–thaw events on the inactivation of *Cryptosporidium parvum* oocysts in soil were examined. Oocysts were inoculated into distilled water in microcentrifuge tubes or into chambers containing soil the water content of which was maintained at 3%, 43%, or 78% of the container capacity. The chambers and tubes were then embedded in 3 soil samples from different aspects of a hillside landscape (Experiments 1 and 2) and in 3 distinct soil types (Experiment 3) and frozen at −10 C. Containers were thawed every 3 days for a period of 24 hr in 1–9 freeze–thaw cycles over 27 days (Experiments 1 and 2) and 2–5 freeze–thaw cycles over 15 days (Experiment 3). Oocyst viability was measured using the fluorescent dyes 4′6-diaminidino-2-phenylindole and propidium iodide. Inactivation rates were greater in soils than in water and greater in dry soil than in moist and wet soils. Soil type showed no effect on inactivation. Oocysts subjected to freeze–thaw cycles had inactivation rates not significantly different from those of oocysts subjected to −10 C under static conditions. The results indicated that 99% of oocysts exposed to soils that are frozen at −10 C will become inactivated within 50 days whether or not freeze–thaw cycles occur.

Large numbers of *Cryptosporidium parvum* oocysts from dairy calves and humans are believed to contaminate watershed environments through leachate from bedding, washing of calving houses, spreading of animal wastes or biosolids on farmland, or irrigation of land with wastewater effluents. Two genotypes of *C. parvum* have been identified, Type I from human sources and Type II from bovine and other ruminant sources (Peng et al., 1997). The Type I genotypes appear to be specific for humans. The Type II genotype is zoonotic, and therefore cattle are considered an important potential source of human infection (Atwill, 1996). Calves can shed billions of oocysts of the Type II genotype into the environment in the first few weeks after birth (O'Handley et al., 1999). The contamination of the environment with oocysts has been blamed as the potential source of oocysts that have been responsible for large outbreaks of gastrointestinal disease such as that which occurred in Milwaukee in 1993 (MacKenzie et al., 1994).

Little is known about the survival of *C. parvum* oocysts once they make their way into the environment. Freeze–thaw events have been suggested to be the events that cause significant inactivation of oocysts (Jenkins et al., 1999). Fayer and Nerad (1996) reported that Type II oocysts in water that were held at −10 C for up to 168 hr remained infectious to mice. At −15 C, oocysts held for 168 hr were not infectious, but oocysts subjected to −15 C for only 8 or 24 hr remained infectious. However, very little is known about how freezing affects oocysts that are in contact with soil. Jenkins et al. (1999) studied the viability of oocysts in field soils and found that increased numbers of *C. parvum* oocysts appeared to have been opened under field conditions by the apparent swelling and shrinking of freeze–thaw events. Mechanical and natural freezing have been used in sludge conditioning (United States Environment Protection Agency, 1979; Vesilind, 1979). Sanin et al. (1994) found that freeze–thaw conditioning coupled with stabilization was effective in reducing pathogenic microorganisms including *Cryptosporidium* oocysts. A reduction in microbial density attributable to freeze–thaw treatment has been reported by Chu et al. (1999).

The purpose of this set of experiments was to ascertain the effects of freezing on oocysts in soils of different types with different water contents. Oocysts were exposed to either single or repeated freeze–thaw events to determine the extent of the effect that the repeated freezing would have on viability. Viability was assayed in these experiments using a dye permeability assay, which is considered a conservative estimate of oocyst potential infectivity. Although this assay has been shown to be of limited value when examining the effects of chemicals (Black et al., 1996), UV irradiation (Clancy et al., 1998; Bukhari et al., 1999), or ozone (Bukhari et al., 2000) on oocyst inactivation, it does appear to be a good indicator of the effects of temperature, natural disinfection, and temporal decay (Jenkins et al., 1997, 1998).

MATERIALS AND METHODS

Cryptosporidium parvum oocysts

Oocysts were obtained from naturally infected, 7- to 14-day-old calves in Tompkins County, New York. A sucrose–Percoll™ (Pharmacia, Uppsala, Sweden) flotation method was used to extract oocysts from calf feces (Jenkins et al., 1997). After extraction, oocysts were stored at 4 C in water containing antibiotics (100 U of penicillin G sodium ml^{-1}, 100 μg of streptomycin sulfate ml^{-1}, and 0.25 μg of amphotericin B per milliliter of suspension) and were used within 2 mo of collection. Viability at the time of soil inoculation ranged from 90% to 95%.

Soil

Soil samples were collected from the top, middle, and bottom of a hillside on an old farm in New York State to determine if soils from different aspects of landscape would effect oocyst inactivation (Experiments 1 and 2). The total clay contents were 15.0% (top-slope soil), 19.3% (mid-slope soil), and 19.5% (toe-slope soil) as determined by the Soils Testing Laboratory, Department of Soils, Crops, and Atmospheric Sciences, Cornell University, Ithaca, New York. Each soil sample was air-dried and sieved (10-mesh U.S. Standard Sieve, 2-mm opening), and then the container capacity was determined (Cassel and Nielsen, 1986). To determine the effect of distinct soil types on oocyst inactivation, a loamy sand (loamy sand, 5% clay), the soil from the top of the hillside (top-slope soil, 15.0% clay), and a silty clay loam soil (silty clay loam, 29.4% clay) were used (Experiment 3). The soils were air-dried and sieved, and their container capacity was determined.

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Containers and chambers

Soils were held in disposable waxed paper containers (9.5 cm in diameter and 5.5 cm in height) with plastic lids. Each container was filled with 230 g of the dried soil. Water was then added to obtain 3%, 43%, and 78% container capacity. The containers were allowed to equilibrate for several days before the chambers or tubes were added. The water contents of the containers were monitored gravimetrically, and water was added as needed. The sentinel chambers developed by Jenkins et al. (1999) were used to place oocysts within the soils. The chambers filled with approximately 0.7 g of the soil of interest were allowed to equilibrate with the bulk soil into which they were to be embedded, and then they were inoculated with 40 μl of water containing 10^7 oocysts. As controls, 2×10^5 oocysts were suspended in distilled water in microcentrifuge tubes that were placed either in the freezer in racks or in the soil containers depending on the experiment.

Oocyst extraction from chambers

Soil from the chambers was put into a 15-ml polypropylene centrifuge tube containing 7 ml of 0.5% 7× detergent (Linbro 7×, Thomas Scientific, Swedesboro, New Jersey) in phosphate-buffered saline (PBS). A small amount of zirconia–silica beads (0.5 mm) were added (BioSpec Products, Inc., Bartlesville, Oklahoma), and the soil, beads, and detergent were mixed for 20 sec using a vortex mixer. The 7-ml mixture was then underlaid with about 7 ml of cold sugar solution (sp. gr. 1.18). After the samples were centrifuged at 1,800 *g* for 20 min at 4 C, about 3 ml of the interface was gently removed and transferred to a second tube. Distilled water (12 ml) was added to the sample; the diluted sample was mixed thoroughly and centrifuged again at 1,800 *g* for 20 min at 4 C. The supernatant was aspirated leaving approximately 0.1 ml in which the pellet was resuspended.

Dye permeability staining and fluorescent labeling

Dye permeability assays using 4′6-diaminidino-2-phenylindole (DAPI) (Sigma, St. Louis, Missouri) and propidium iodide (PI) (Sigma) were performed following the methods described previously (Campbell et al., 1992; Jenkins et al., 1997). Stock solutions of DAPI (2 mg per milliliter distilled water) and PI (1 mg per milliliter 0.1 M PBS) were prepared. Each dye was added to aliquots of recovered oocyst suspensions at the ratio of 1:10 (vol/vol), mixed gently, and incubated for 2 hr at 37 C in the dark. After the oocysts were labeled with DAPI and PI, they were labeled with a monoclonal antibody (Hydrofluor, Strategic Diagnostics, Inc., Newark, Delaware) specific for the oocyst wall. A 1:10 volume of the primary antibody was added, and the samples were incubated for 30 min at 37 C in the dark. Oocysts were washed once using centrifugation in 0.1 M PBS, and then a 1:10 dilution of the secondary antibody was added. The samples were incubated in the dark for 30 min at room temperature. Oocysts were then washed twice in PBS using centrifugation and resuspended in the original volume with 0.3 M 1,4-diazabicyclo [2.2.2] octane in 0.1 M PBS. The dyed and labeled samples were stored at 4 C in the dark until microscopic examination by epifluorescence and differential interference contrast microscopy (Eclipse, E600, Nikon, Japan). Excitation bands at 330–380, 546/10, and 450–490 nm were used. At least 100 oocysts in each sample were examined.

Freeze–thaw treatment

Freezing was done in a non–frost-free freezer (−10 C); temperature was monitored daily. The soils containing oocysts were thawed at room temperature every 3 days for 24 hr. As controls for comparative purpose, oocysts in water also underwent the same freeze–thaw cycles.

Experimental design

Three experiments were performed. A pair of containers was used for each container capacity, and thus 18 soil containers were used in Experiments 1 and 2. In Experiment 3, in which some containers were thawed only on the day they were sampled, a total of 36 soil containers were used. Each container held sufficient chambers to allow for sampling at different cycles. Similarly, paired control samples were analyzed at each sampling time. Thus, in all cases, data represent the mean of 2 samples. Soil containers were randomly placed within the freezer to control the potential effects of position on oocyst inactivation.

Experiment 1: The soil containers contained 3 soil samples taken from different aspects of a sloping landscape. They were maintained at 3 different container capacities and underwent 9 freeze–thaw cycles over a period of 27 days. Each soil had the following soil water contents: top-slope soil had soil water contents of 0.5%, 31.8%, and 59.5%; mid-slope soil had soil water contents of 0.5%, 24.9%, and 46.7%; and toe-slope soil had soil water contents of 0.5%, 36.2%, and 67.8%, at 3%, 43%, and 78% container capacities, respectively. Containers were thawed on days 3, 6, 9, 12, 15, 18, 21, 24, and 27, representing freeze–thaw cycles 1 through 9. In this experiment oocysts were extracted from the soils on days 0, 6, 12, 18, and 27, representing cycles 0, 2, 4, 6, and 9, respectively. One group of control oocysts was in 1.5-ml microcentrifuge tubes in racks placed within the freezer. These oocysts underwent the same freeze–thaw cycles as the oocysts in soil. The other group of oocysts was held at 4 C in the dark and sampled at the same time as all the other samples.

Experiment 2: All aspects of the experiment were the same as those of Experiment 1 with the exception that the control groups (oocysts suspended in distilled water in 1.5-ml microcentrifuge tubes) were placed in each soil container with chambers. Oocysts were sampled at cycles 0, 2, 4, 6, and 9.

Experiment 3: The 3 different soil types in the chambers, which were brought to 3 different container capacities, were placed in containers and underwent 5 freeze–thaw cycles over a period of 15 days. Each soil had the following soil water contents: the top-slope soil had soil water contents of 0.5%, 31.8%, and 59.5% as in Experiments 1 and 2; the loamy sand had soil water contents of 0.5%, 22.8%, and 42.8%; and the silty clay loam soil had soil water contents of 0.5%, 34.4%, and 64.5%, at 3%, 43%, and 78% container capacities, respectively. The containers were thawed on days 3, 6, 9, 12, and 15. Oocysts were extracted from the soils on days 0, 3, 6, 9, 12, and 15, representing cycles 0, 1, 2, 3, 4, and 5, respectively. A second set of containers with chambers was set up such that the containers would be thawed only once at the time of sampling; thus, these oocysts had been in frozen soil for the same period of time as those exposed to freeze–thaw cycles but underwent only a single thawing at the time of their extraction from the soils. The microcentrifuge tubes with the oocysts in water were placed in each container, and the oocysts were sampled at the same time as were the oocysts in the chambers.

Inactivation rates and statistical analysis

The inactivation rates of oocysts were determined with the first-order decay model (Hurst, 1992):

$$P_t = P_0 \times e^{-kt} \quad (1)$$

where P_t is the percentage of viable oocyst at time t, P_0 represents the initial percentage of viable oocysts, k is the coefficient of inactivation, and t is the time (the number of freeze–thaw cycles). On the basis of equation (1), the coefficient of inactivation (k) was determined by regressing $\ln(P_0/P_t)$ against time (t). Each regression was tested for linearity and slope by 1-way analysis of variance, and a mean inactivation rate was determined (Zar, 1999). Confidence intervals (95%) were determined by multiplying the Student's *t* value at appropriate degrees of freedom at an α-level (2-sided) of 0.025 by the standard error of k. The number of freeze–thaw cycles or days required for the oocysts to reach 99% inactivation was determined using:

$$t = \ln(P_0/P_t) \times k^{-1} \quad (2)$$

where t is the number of freeze–thaw cycles or days required to achieve a 99% inactivation rate.

The ANOVA was carried out with Minitab Release 12 (Minitab, Inc., State College, Pennsylvania). Differences in inactivation rates of oocysts in soil and water were determined by Dunnet's test (Zar, 1999). Differences in inactivation rates of oocysts subjected to freeze–thaw cycles and those not subjected to freeze–thaw cycles were determined by the Student's *t*-test (Zar, 1999). Comparisons were made as to the number of viable and nonviable oocysts recovered from the soils. The oocyst extraction method provided good recovery of added oocysts (Fig. 1); however, no attempt was made in this work to determine the percentage of oocysts that might have opened and disintegrated during freezing of the samples. Thus, comparisons were made only as to the relative number of viable oocysts recovered versus the total number of oocysts recovered.

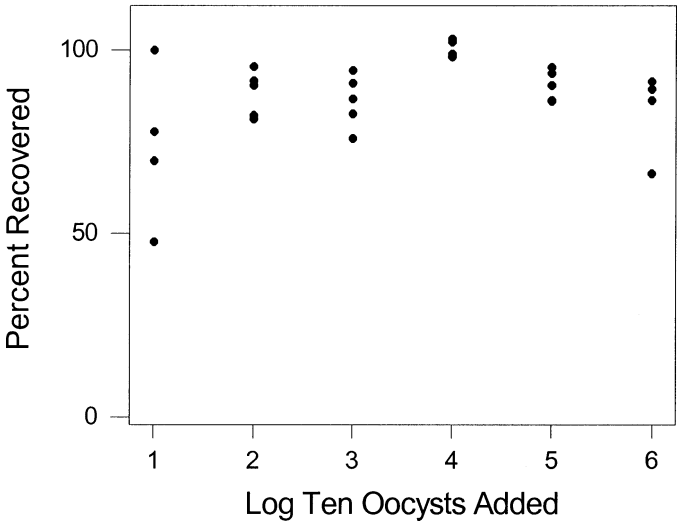

FIGURE 1. Recovery of *Cryptosporidium parvum* oocysts added to soil (15% clay) in chambers. Different numbers of oocysts were added to chambers and extracted using the methods described in the text. Five samples were examined at each concentration, although some data points are superimposed on the graph. The results indicate that around 75–100% of intact oocysts can be extracted using the procedures described.

TABLE I. Mean inactivation rates of *Cryptosporidium parvum* oocysts (k with 95% confidence intervals [CI]) in the 3 soil samples taken from different aspects of a sloping landscape. In a column, means followed by the same letter are not significantly different at $P = 0.05$ based on Dunnet's test.

	Container capacity (%)	k (95% CI) (cycle^{-1})	Cycles required to reach 99% inactivation
Soil			
Top-slope	3	1.07 (0.610 to 1.53) b	4
	43	0.542 (0.257 to 0.827) a	9
	78	0.528 (0.249 to 0.807) a	9
Mid-slope	3	1.10 (0.450 to 1.75) b	4
	43	1.27 (0.884 to 1.66) b	4
	78	1.16 (0.897 to 1.45) b	4
Toe-Slope	3	1.14 (0.626 to 1.65) b	4
	43	1.17 (0.914 to 1.43) b	4
	78	1.16 (0.893 to 1.43) b	4
Water (−10 C)		0.366 (0.223 to 0.509) a	13

RESULTS

Experiments 1 and 2: 9 freeze–thaw cycles over 27 days

The results of Experiments 1 and 2 were combined for regression analysis and for the determination of oocyst inactivation rates (Table I). The differences in the inactivation rates of the oocysts in the different soils were not significantly different based on Tukey's multiple comparison test at a P value of 0.05 (data not shown). The freezing and thawing of oocysts in water generated an inactivation rate significantly less than the inactivation rates associated with the soils at all 3 water contents, except for oocysts in the top-slope soil with container capacities of 43% and 78%. The range of cycles required to reach 99% inactivation for most of the soil treatments was between 4 and 9 compared with the 13 cycles for the water treatment.

Experiment 3: 5 freeze–thaw cycles over 15 days

This experiment compared the effects of a single freeze–thaw event (static) with oocysts exposed to multiple freeze–thaw cycles (cycled) (Table II). Inactivation rates were not significantly different between the cycled and static experiments. Soils at 3% container capacity (dry soils) under both cycled and static conditions had significantly greater inactivation rates than the wetter soil treatments. The inactivation rate for the silty clay loam at 78% container capacity under static conditions was the only exception. Under both cycled and static conditions the inactivation rates of oocysts in soil at 43% and 78% container capacity were not significantly different from those of oocysts in water. On the basis of a Student's *t*-test, the inactivation rate

TABLE II. Mean inactivation rates of *Cryptosporidium parvum* oocysts (k with 95% confidence intervals [CI]) in 3 different soil types (Experiment 3) at 3 levels of water content. One set of replicates was cycled and another not cycled (static). In a column, means followed by the same letter are not different by Dunnet's test at $P = 0.05$.

	Container capacity (%)	Cycled k (95% CI) (cycle^{-1})	Cycles to reach 99% inactivation (cycled)	Static k (95% CI) (cycle^{-1})	Cycles to reach 99% inactivation (static)
Soil					
Loamy sand	3	0.610 (0.430 to 0.790) b	8	0.742 (0.432 to 1.05) b	6
	43	0.365 (0.198 to 0.532) a	13	0.322 (0.159 to 0.485) a	14
	78	0.384 (0.220 to 0.548) a	12	0.311 (0.146 to 0.476) a	15
Top-slope soil	3	0.681 (0.486 to 0.876) b	7	0.605 (0.373 to 0.837) b	8
	43	0.484 (0.299 to 0.669) a	10	0.330 (0.184 to 0.476) a	14
	78	0.337 (0.186 to 0.488) a	14	0.332 (0.178 to 0.486) a	14
Silty clay loam	3	0.759 (0.531 to 0.937) b	6	0.649 (0.415 to 0.883) b	7
	43	0.490 (0.272 to 0.708) a	9	0.437 (0.259 to 0.615) a	11
	78	0.488 (0.324 to 0.652) a	9	0.638 (0.475 to 0.801) b	7
Water (−10 C)		0.390 (0.342 to 0.438) a	12		
Water (−10 C)				0.296 (0.250 to 0.342) a	16

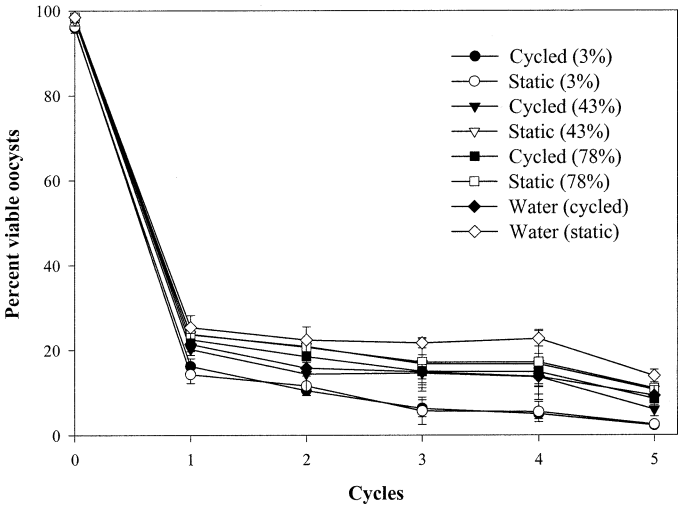

FIGURE 2. Mean percent viable oocyst survival of *Cryptosporidium parvum* oocysts in soil (data from the 3 types of soils in Experiment 3 were grouped together) at the 3 soil water contents and in water under cycled and static conditions.

TABLE III. Mean inactivation rates of *Cryptosporidium parvum* oocysts (k with 95% confidence intervals [CI]) in 3 distinct soil types (Experiment 3) at 3 levels of water content under static conditions and in terms of days. In a column, means followed by the same letter are not significantly different by Dunnet's test at $P = 0.05$.

	Container capacity (%)	k (95% CI) (day^{-1})	Days to reach 99% inactivation
Soil			
Loamy sand	3	0.247 (0.146 to 0.348) b	19
	43	0.111 (0.060 to 0.162) a	44
	78	0.104 (0.049 to 0.159) a	44
Top-slope soil	3	0.202 (0.125 to 0.279) b	23
	43	0.110 (0.062 to 0.158) a	42
	78	0.111 (0.060 to 0.162) a	42
Silty clay loam	3	0.216 (0.139 to 0.293) b	21
	43	0.146 (0.087 to 0.205) a	32
	78	0.213 (0.160 to 0.266) b	22
Water (−10 C)		0.099 (0.084 to 0.114) a	47

of oocysts cycled in water was significantly greater than the inactivation rate of oocysts frozen in water under static conditions. Oocysts survived longer in Experiment 3 than they did in Experiments 1 and 2. As with Experiments 1 and 2, Experiment 3 showed that an initial freeze–thaw event in soil or water resulted in a significant loss in oocyst viability (Fig. 2). In all cases a single event caused the percentage of viable oocysts to drop from 97 to less than 30%. Under both cycled and static conditions the inactivation rates can be determined in terms of days as well as cycles, as illustrated in Table III, which represents the data from the experiments under static conditions. In terms of days (Table III), inactivation rates are much less than those in terms of cycles and indicate that the number of days to reach 99% inactivation can range between 19 and 42 days in soil and 47 days in water.

DISCUSSION

The results of the experiments showed that a significant oocyst inactivation occurs in soil and water after a single freeze–thaw event. Oocysts that were frozen at −10 C for 3 days and then thawed decreased in viability from near 100 to around 25%. The oocysts that survived the initial freezing appeared to survive longer than 7 days as observed by Fayer and Nerad (1996) and continued to undergo a decline in viability because they remained frozen or underwent freeze–thaw cycles.

Experiment 3 demonstrated that in soil the amount of time the oocysts are frozen may be as important as the number of freeze–thaw events relative to oocyst inactivation. Previously, on the basis of field data, Jenkins et al. (1999) suggested that oocysts in soil and water were inactivated by repeated natural freeze–thaw events and showed that oocyst inactivation was greater in soil than in water. On the basis of a large proportion of empty and deformed oocyst walls that were observed, Jenkins et al. (1999) suggested that the physical forces associated with the shrinking and swelling of soil undergoing freeze–thaw cycles accelerated oocyst inactivation. The results of this study suggest that the amount of time that oocysts are frozen within a soil rather than the number of freeze–thaw cycles may have been the main factor in oocyst inactivation.

The data indicated that the soil type may have little or no effect on oocyst inactivation under freezing or freeze–thaw conditions. Oocysts in dry soils appeared to be inactivated more rapidly than oocysts in wetter soils. In water, oocysts were inactivated more rapidly if they underwent repeated freeze–thaw events, but even as in soil, a single freeze–thaw event caused significant oocyst inactivation. Under static conditions oocysts in water appeared to survive better than oocysts in soil, indicating that, if not subjected to freeze–thaw events but to freezing only, oocysts in soil are apparently subjected to different stresses that cause significant inactivation to occur.

The fact that oocysts can be inactivated in soils means that there is a potential for significant decreases in the numbers of infective oocysts to occur if oocysts gain access to soils through the application of either manures or biosolids, or through effluents for irrigation. If the oocysts are transported out of the soil by water, there appears to be considerable likelihood that oocyst survival would increase.

The results of these experiments are important because they indicate that a significant portion of oocysts present in land-applied manures or biosolids would be inactivated by freezing. The land application of animal wastes may cause significant decreases in oocyst viability within a few weeks. The increased inactivation of oocysts by freezing in drier soils would suggest that in those climates where manure or biosolids can be dewatered and frozen by ambient temperatures, the contained oocysts would be inactivated. In biosolids that have been dried in drying beds or dewatered by other methods, the additional process of freezing for extended periods may reduce the numbers of these pathogens by several logs.

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