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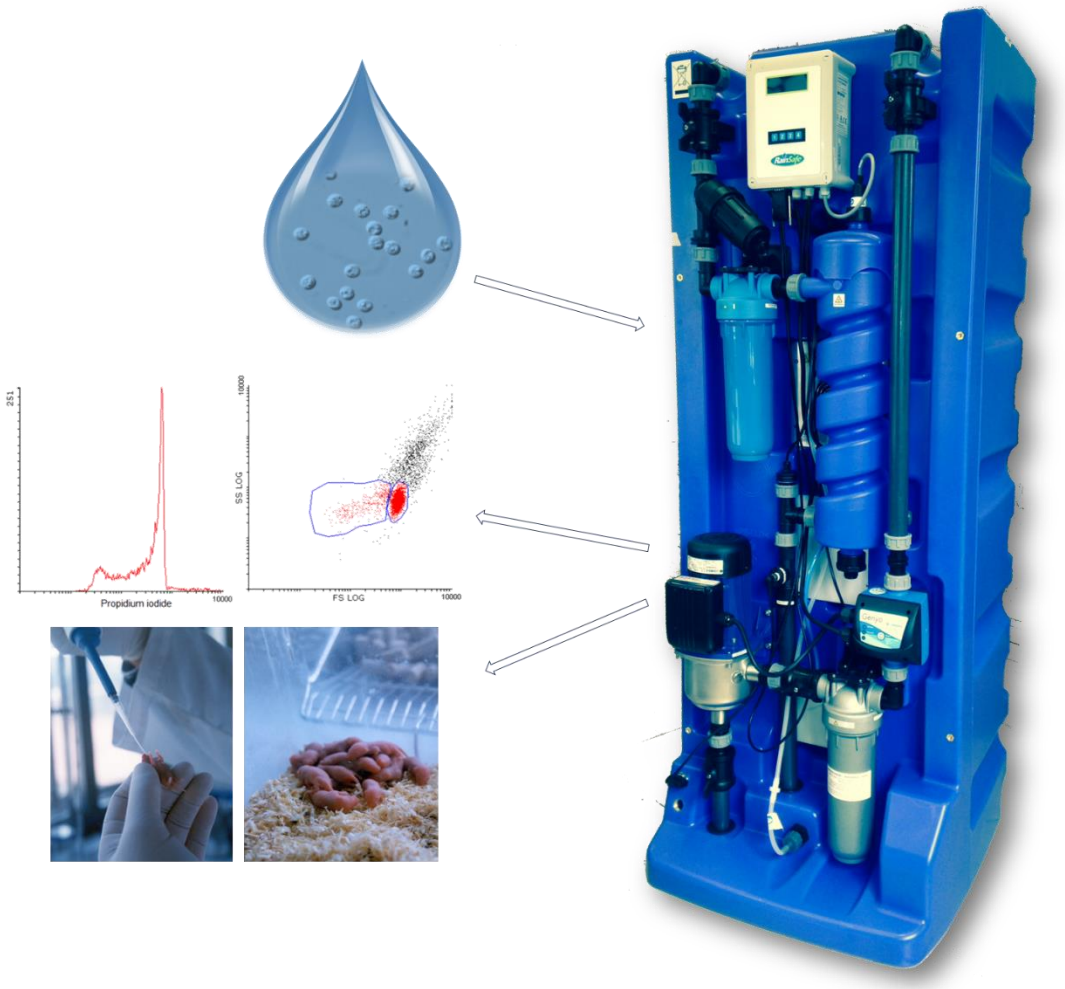
Evaluation of the RainSafe™ equipment

against *Cryptosporidium*



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Acknowledgements

Authors thank to Dr. Rosa Ana Sueiro-Benavides by her assessment in the MPN assay.

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RAINSAFE™ EQUIPMENT

Prototype NM119

TEST WATER

The water employed in the experiments consisted in well water used at household level which physicochemical and microbiological parameters are shown in Table 1.

Table 1. Physicochemical and microbiological parameters of the well water employed in the evaluation of the Rainsafe™ equipment against *Cryptosporidium*.

Parameter	Value	Units
Physical		
Turbidity	0.96	NTU
TDS	90.00	mg/L
Conductivity	190.00	µS/cm
Chemical		
pH	5.60	–
Ammonium	<0.05	mg/L
Sulphates	<25.00	mg/L
Nitrites	0.17	mg/L
Nitrates	23.70	mg/L
Phosphates	0.13	mg/L
Total phosphorus	0.39	mg/L
Combined chlorine	<0.05	mg/L
Free chlorine	<0.05	mg/L
Aluminium	<0.05	mg/L
Iron	<0.05	mg/L
Potassium	2.20	mg/L
Magnesium	10.00	mg/L
Calcium	90.00	mg/L
Water hardness	100.00	mg/L
Microbiological		
Total coliforms at 37 °C	0	UFC/100 mL
Escherichia coli	0	UFC/100 mL
Enterococcus spp.	0	UFC/100 mL

CRYPTOSPORIDIUM OOCYSTS

Cryptosporidium oocysts were collected from a naturally infected neonatal Friesian-Holstein calf. Concentration [0.04 M phosphate-buffered saline (PBS) pH 7.2 and diethyl ether], purification (discontinuous caesium chloride gradients), quantification (improved Neubauer haemacytometer) and molecular characterization were performed as previously reported.[1] Briefly, faeces were collected from a calf by rectal sampling and stored at 5 °C. Faecal material was then homogenized in 10-20 mL of PBS (0.04 M, pH 7.2), filtered through two sieves (mesh sizes 150 and 45 µm), shaken with diethyl ether (2:1, v/v) and concentrated by centrifugation at

2000×*g*, 4 °C, for 15 min. The resulting uppermost three layers were removed carefully and discarded, and the sediment was washed with PBS (0.04 M, pH 7.2) by centrifugation at 2000×*g*, 4 °C, for 15 min. *Cryptosporidium* oocysts were purified on discontinuous caesium chloride gradients of 1.05, 1.10 and 1.40 g/mL by centrifugation at 2000×*g*, 4 °C, for 30 min.[2] Finally, the oocysts were counted in an improved Neubauer haemocytometer using 0.16% malachite green solution as counterstain.[3] The isolate was identified as *Cryptosporidium parvum* by analysis of a ~587-bp fragment of the SSU-rDNA gene.[4]

EXPERIMENTAL DESIGN

Before each experiment, the Rainsafe™ equipment was filled and emptied three times using tap water. Then, the UV-lamp reservoir was filled with well water using a pump (model 1490, Gardena, Husqvarna, Huskvarna, Sweden) fitted to an electric drill (model KR7532K, Black and Decker, Slough, United Kingdom). The equipment was turn off and the water was spiked at the filter bowl with 90×10^6 purified oocysts of *C. parvum*. The machine was turn on and filled at a water flow of 5-6 L/min until a volume of 60 L was shown in the display (approximately, 90 L of total volume, spiking dose of 10^6 oocysts/L). After filling and once the established O_3 level was reached, the equipment remained switched on during 10 min. Then, a second O_3 treatment was forced during 4 min. Samples were taken at the end of this period (t=0 min) and after 15 and 30 min of exposition (sample t=15 min and sample t=30 min) (Figure 1).

In order to recover *C. parvum* oocysts from Rainsafe™ equipment, volumes of treated water were filtered through a nitrocellulose membrane (pore diameter of 2 µm) using the Filta-Max®concentrator tube (IDEXX Laboratories Inc., Westbrook, ME, USA). The membrane was removed using forceps and placed it in resealable polyethylene bag Minigrip® (IDEXX Laboratories Inc.). Then, the membrane was washed by hand with 5 mL of PBS. The washing liquid was transferred to a tube of 15 mL and this step was repeated twice. The tube was centrifuged at 2000×*g*, 4 °C, during 15 min and the supernatant was discarded. The number of oocysts in the sediment was quantified in an improved Neubauer haemocytometer using 0.16% malachite green solution as counterstain.

Moreover, after each disinfection assay, the equipment was washed three times with tap water by filling and emptying it and the number of *C. parvum* oocysts was determined in the washing liquids as described previously (Figure 1).

The water temperature and oxidation reduction potential (ORP) values registered during the experiments were 18-20 °C and 920-1020 mV, respectively.

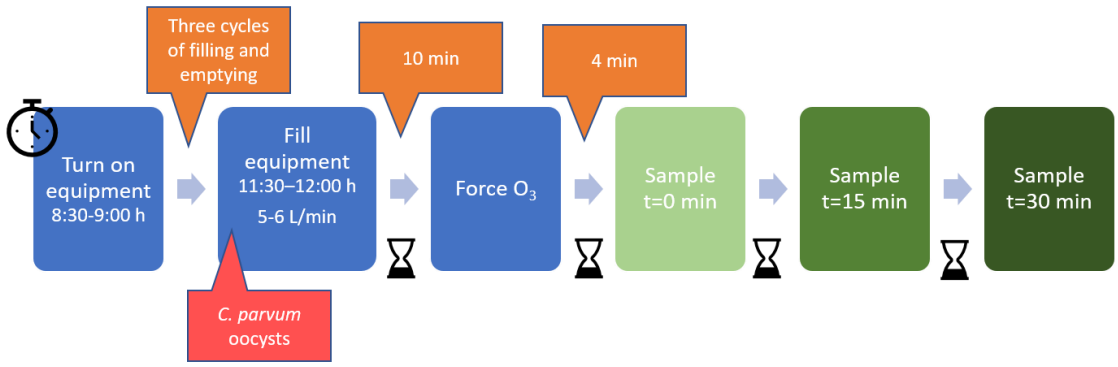


Figure 1. Diagram of the experimental design applied in the evaluation of the Rainsafe™ equipment against *Cryptosporidium*.

ASSESSMENT OF THE *C. PARVUM* OOCYST SURVIVAL

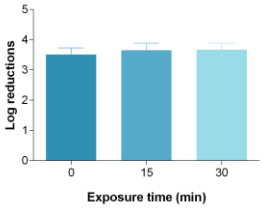
In vitro assays

The viability of *C. parvum* oocysts was determined by inclusion/exclusion of the fluorogenic vital dye propidium iodide (PI) (Sigma-Aldrich, Co., St. Louis, MO, USA) and a further modification that includes an immunofluorescence antibody test to verify oocyst identification.[5,6] Briefly, 200 µL aliquots of sediments were incubated with 2.5 µL of monoclonal antibodies labelled with fluorescein isothiocyanate (Aqua-Glo™ G/C Direct, Waterborne, Inc., New Orleans, LA, USA) and 10 µL of PI working solution [1 mg/mL in PBS (0.1 M, pH 7.2)] at 37 °C for 30 min.[1] The proportions of ruptured (ghost), PI-positive (death) and PI-negative (viable) oocysts were quantified by flow cytometry in a Coulter Epics XL™ Flow Cytometer (Beckman Coulter, Inc., Brea, CA, USA). Data was analyzed using Flowing Software version 2.5.1 (Turku Centre for Biotechnology, University of Turku, Finland). The results are expressed as the percentage of PI- negative (viable) oocysts and log reductions of the viability with respect to *C. parvum* isolate determined for each assay after triplicate counts of more than 10^4 oocysts.

After application of the experimental design showed in Figure 1, **log reductions in oocyst viability of 3.50±0.23; 3.65±0.24 and 3.67±0.22 were obtained for exposure times of 0, 15 and 30 min, respectively**. Therefore, log reductions observed after second O_3 treatment are similar to that obtained at 15 and 30 min of exposure, as not statistically significant differences among the log reduction values were observed (Table 2).

Table 2. Survival of *C. parvum* oocysts after water disinfection treatment in the Rainsafe™ equipment determined by the inclusion/exclusion of the fluorogenic vital dye PI.

Sample	Viability		Viability reductions	
	%	SD	Log	SD
Control	97.333	0.168	-	-
Sample t=0 min	0.036	0.021	3.504	0.233
Sample t=15 min	0.026	0.015	3.652	0.237
Sample t=30 min	0.024	0.013	3.670	0.219



In vivo assays

Litters of Swiss CD-1 mice (10-15 mice/litter) between 2.5 and 3.0 g (aged 3–4 days old) were inoculated intragastrically with 100 µL suspensions containing 10^6; 10^5; 10^4 and 10^3 *C. parvum* oocysts previously exposed to disinfection treatment during 15 min (sample t=15 min). Moreover, 10^3; 5×10^2; 10^2; 50 and 10 unexposed *C. parvum* oocysts (control) were administered to other litters of Swiss CD-1 mice. Seven days post-inoculation, the mice were euthanized by cervical dislocation. The entire small and large intestines were removed, placed in 5 mL of PBS (0.04 M, pH 7.2) and homogenized using an Ultra-Turrax® T25 homogenizer (IKA®-Werke GmbH & Co. KG, Staufen, Germany). Then, 1 mL of the homogenized intestinal tissue was added on 6 mL of PBS, shaken with 3 mL of diethyl ether (2:1, v/v) and concentrated by centrifugation at 2000×*g*, 4 °C, for 15 min. The supernatant was discarded and the sediment was resuspended in 1.5 mL of PBS. The presence and number of *C. parvum* oocysts were determined in 10-100 µL aliquots of the sediment using an immunofluorescence antibody test (Aqua-Glo™ G/C Direct, Waterborne, Inc.) according to the manufacturer's instructions.

The results obtained *in vivo* assays were analysed using two approaches:

1. A most-probable-number (MPN) model for enumeration of infectious *C. parvum* oocysts, as described Slifko et al.[7,8] By application of this evaluation method and using the Information Collection Rule (ICR) Most Probable Number (MPN) Calculator version 2.0 (Copyright ©2009 by the Environmental Protection Agency), a MPN/mL of 3.13 infectious oocysts was obtained for a dose of 10^3 oocysts/mouse (95% confidence

interval, 1.96 to 5.17) *vs* the corresponding value obtained in control litters, 128.80 infectious oocysts of *C. parvum* per mL (95% confidence interval, 77.10 to 195.70). Thus, **the infectivity of *C. parvum* oocysts diminished 1.61 log units after an exposure time of 15 min to disinfection treatment in the Rainsafe™ equipment**.

2. A logit dose-response model, proposed by Finch et al.,[9] used previously in several studies to evaluate *C. parvum* infectivity in mice.[10,11] In this method, the proportion of animals that became infected at each dose is transformed by using the response logit, defined as the natural logarithm of the quotient of the proportion of animals infected divided by one minus the proportion of animals infected [Response logit=ln(P/1-P)]. Least-squares regression of log(dose) against the response logit yields an equation for the straight line. Solving for the response logit obtained after water disinfection treatment and comparison with a dose-response logit equation generated by nonexposed oocysts (control) allows to calculate the level of inactivation. In Table 3, the proportion of mice infected and the corresponding response logit values when mice were inoculated with different dose of control and exposed oocysts are shown.

Table 3. Summary of *C. parvum* doses and infectivity responses in litters of Swiss CD-1 mice.

	Dose	Log(dose)	No. of mice inoculated	No. of mice infected	Proportion of mice infected (P)	Response logit (In P/1-P)
	10^3	3.00	14	14	1.000	∞
	5×10^2	2.70	11	11	1.000	∞
Control	10^2	2.00	29	21	0.724	0.965
oocysts	50	1.70	26	15	0.577	0.310
	10	1.00	26	1	0.038	-3.219
	10^6	6.00	21	21	1.000	∞
Exposed	10^5	5.00	24	24	1.000	∞
oocysts	10^4	4.00	25	23	0.920	2.442
	10^3	3.00	24	9	0.375	-0.511

Using the infectivity results obtained in control litters, the following equation was established:

$$Response\ logit = -7.4399 + 4.3363\ log(dose) \qquad (R^2=0.9772)$$

Solving the equation for the response logit determined after mice inoculation of 10^4 and 10^3 exposed oocysts, log(dose) values of 2.28 and 1.60 were obtained, respectively. Therefore, **log reductions of 1.72 and 1.40 were calculated for doses of 10^4 and 10^3 oocysts**, respectively, **average value of 1.56±0.23 log reductions**, similar to the corresponding value observed using the MPN model.

Finally, considering the intensities of infection, **a reduction of 2.30 log units was determined in litters inoculated with a dose of 10^3 exposed oocysts *vs* control litters** (Table 4).

Table 4. Infectivity and intensity of infection observed in Swiss CD-1 mice inoculated with a dose of 10^3 control and exposed *C. parvum* oocysts.

Group	Infectivity (%)	Intensity of infection	
		Average (min-max)*	Log
Control oocysts	100	3.76×10^5 (7.50×10^2-1.63×10^6)	5.57
Exposed oocysts	37.5	1.87×10^3 (7.50×10-8.55×10^3)	3.27

*Number of oocysts per homogenized intestinal tissue.

CONCLUSIONS

The exposition of *C. parvum* oocysts to UV light and O_3 using the Rainsafe™ equipment showed good levels of oocyst inactivation, higher than 3.5 log reductions determined by the inclusion/exclusion of the fluorogenic vital dye PI. However, *in vivo* assays displayed lower log reductions (1.61; 1.56 and 2.30 units, according to the approach used). These differences could be due to the high sensitiveness of the flow cytometry and the low ID_{50} of the isolate of *C. parvum* employed in this study (51.96 oocysts, dose that infects 50% of the animals). These disinfection assays were carried out at a water flow of 5-6 L/min. Perhaps, lower values of water flow would increase the exposure of *C. parvum* infective forms to UV radiation and would allow to improve the efficacy of Rainsafe™ equipment against *C. parvum*, ensuring higher levels of oocyst inactivation.

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ANNEX. OOCYST RECOVERY

Oocyst recovery data proves that Rainsafe™ equipment is "clean" and "ready" for a new assay without residual oocysts may affect significantly the results of the following treatment. A percentage of 78% of the spiking dose was recovered during sampling and washing steps. The remaining 22% of the oocysts was lost throughout the processing of the samples (washings of the membrane filter, centrifugations…) as we usually observe in our laboratory when water samples are analyzed (Figure 2).

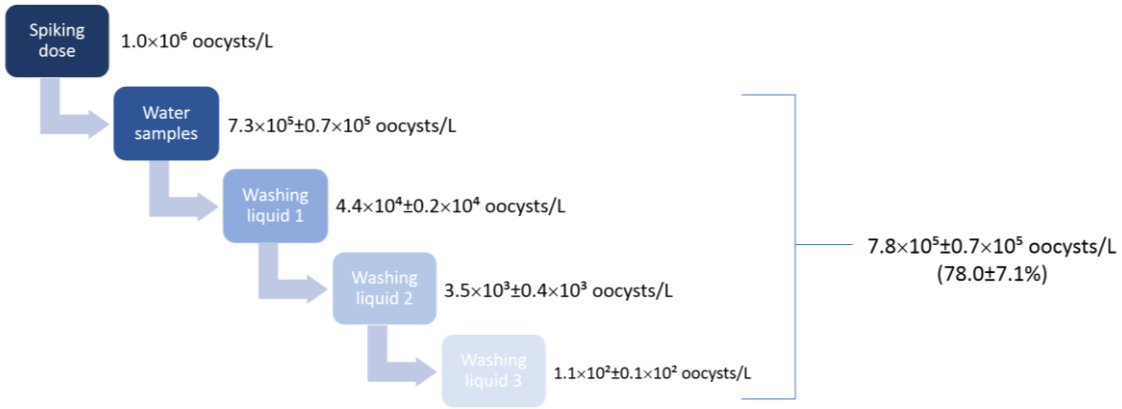

Figure 2. Reduction in *C. parvum* oocyst load of water after sampling and subsequent washes of the Rainsafe™ equipment.