Survival of *Cryptosporidium parvum*, *Escherichia coli*, faecal enterococci and *Clostridium perfringens* in river water

Influence of temperature and autochthonous micro-organisms

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ABSTRACT

Oocysts of *Cryptosporidium parvum* can survive for several months in surface water, one of the main factors determining their success in environmental transmission and thus their health hazard via water. Several factors in the environment like temperature and the presence of other organisms (predators, exo-enzymes) will probably influence oocyst survival. The high persistence of *C. parvum* oocysts may limit the value of the faecal indicator bacteria that are traditionally used to determine the safety of water.
The aim of this study was to determine the rate at which *C.parvum* oocysts, *Escherichia coli,* faecal enterococci and *Clostridium perfringens* spores die in surface water and the effect of temperature and the presence of autochthonous (micro)organisms on the die-off rate.
Microcosms with autoclaved river water were inoculated with *C. parvum* oocysts, *E. coli, Enterococcus faecium* or spores of *Cl. perfringens.* Microcosms with untreated river water were inoculated with concentrated primary effluent containing *E. coli*, faecal enterococci, *Cl. perfringens* and with *C.parvum* oocysts. Microcosms were incubated at 5°C or 15°C at 100 rpm. Viability of oocysts was monitored by *in vitro* excystation and dye-exclusion, viability of the bacteria was determined on appropriate selective media.
When pseudo first-order die-off kinetics were assumed, the die-off rate of *C. parvum* oocysts at 5°C was 0.010 ^{10}log-units per day and 0.006-0.024 ^{10}log-units per day at 15°C. These rates underestimate die-off since oocyst disintegration was not accounted for. Incubation in autoclaved or untreated water did influence the die-off rate of oocysts at 15°C, but not at 5°C. The die-off rate of *E. coli* and faecal enterococci was faster in the non-sterile river water than in autoclaved water at both temperatures. At 15°C, *E. coli* and possibly *Ent. faecium* even multiplied in autoclaved water. In untreated river water, the die-off of *E.coli* and faecal enterococci was approximately ten-fold faster than die-off of oocysts, but die-off rates of *Cl. perfringens* were lower than those of oocysts. As for oocysts, die-off of the bacteria and spores was faster at 15°C than at 5°C. This study showed that oocysts are very persistent in river water: the time required for a 10-fold reduction in viability is 40-160 days at 15°C and 100 days at 5°C. Biological/biochemical activity influenced oocyst survival at 15°C, and survival of both vegetative bacteria at 5 and 15°C. The rapid die-off of *E. coli* and faecal enterococci makes these organisms less suitable as indicators of oocyst presence in water. *Cl. perfringens* survived longer than oocysts in untreated river water, and may therefore prove useful as indicator of the presence of *C. parvum*.

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INTRODUCTION

The protozoan parasite *Cryptosporidium parvum* is able to cause (large) outbreaks of intestinal illness via drinking water (MacKenzie *et al.,*1994) and has been associated with intestinal illness via swimming in surface water (Anonymous, 1987). One of the characteristics of this parasite that enables it to be transmitted by these vehicles is the production of environmentally robust oocysts. These oocysts are reported to survive for 6 months in membrane chambers in river water at ambient temperatures (Robertson, Campbell & Smith, 1992). Chauret *et al.* (1995) reported a viability reduction of 0.99 ^{10}log units after 27 days. They reported that survival in synthetic water was affected by temperature. Once introduced in the environment, the oocysts can be affected by various types of environmental stress: depletion of internal nutrient reservoirs (starvation), predation by zooplankton, structural damage by shear forces, UV radiation from sunlight and exo-enzymes from bacteria or fungi affecting the oocyst wall and chemical damage by free radicals or oxidizing chemicals. It is not clear which of these stresses are determining oocyst survival in surface water. Biological processes may play an important role, especially when oocysts are attached to particles containing other micro-organisms and in sediments. The aim of this study was to determine the rate at which *C. parvum* oocysts die in surface water and the influence of temperature and the presence of biological activity (autochthonous (micro)organisms and/or exo-enzymes) on the die-off rate. The die-off rate of *C. parvum* oocysts was compared to the die-off rate of *Escherichia coli,* faecal enterococci and *Clostridium perfringens,* the indicator-bacteria for faecal contamination.

METHODS

Micro-organisms

Cryptosporidium parvum MRI oocysts were obtained from Moredun Research Institute, Scotland (deer strain, passaged in lambs) and used at an age of 1.5 months.
Escherichia coli WR1, *Enterococcus faecium* WR63 and *Clostridium perfringens* WR62 were all isolated from water and maintained on nutrient-rich media in our laboratory. *Cl. perfringens* was kept in Duncan & Strong medium for 48 hr at 37°C in an anaerobic environment to sporulate and subsequently pasteurized (30', 70°C) to obtain a spore suspension.
Primary effluent from the treatment plant in De Bilt was used as source for environmental *E.coli*, faecal enterococci and *Cl. perfringens*.

Microcosms of autoclaved river water

E.coli and *Ent. faecium* were cultured in liquid, nutrient rich media to early stationary phase. These cultures and the oocyst and spore suspensions were diluted in autoclaved water from the river Meuse to a final density of approximately 10^4 colony forming particles (CFP)/ml and 10^5oocysts/ml. All microcosms contained only one species. The microcosm Erlenmeyers were

placed at 5 or 15°C in the dark at 100 rpm. All microcosm experiments were performed in duplicate.

Microcosms of natural river water
Primary sewage effluent was concentrated by a two-step centrifugation (15', 1050xg) and purified by Percoll-sucrose flotation (specific density 1.10, 15',1080xg). This procedure increased bacterial counts approximately ten-fold. This concentrated and purified primary effluent was diluted 100-fold in river Meuse water. The resulting bacterial densities were 10^{2-4}/ml. MRI oocysts were added to obtain a density of 10^5/ml.

Microbiological analysis
Samples were taken from the microcosms and analysed for:
- *E.coli* on Tryptone Soy agar (4-5h, 37 ± 1°C)/Tryptone Bile agar (19-20h, 44 ± 0.5°C) and confirmed by testing for indole production (Havelaar & During, 1988)
- faecal enterococci on Kenner Faecal agar (48 ± 4h, 37 ± 1°C) (Kenner, 1978)
- *Cl. perfringens* on mCP agar (24 ± 2h, 45°C, anaerobic); yellow colonies were confirmed by testing acid phosphatase activity with ammoniumhydroxide (Bisson & Cabelli, 1979).

Oocyst viability
The percentage of viable oocysts in samples from the microcosms was determined both by *in vitro* excystation and by exclusion of propidium iodide, using the protocols of Campbell, Robertson & Smith (1992). The samples were pretreated in acidified HBSS (1h, 37°C). The oocysts were examined by DIC microscopy and/or by epifluorescence microscopy (1000x). Excystation (%) was calculated as empty oocyst walls (or oocysts with sporozoites protruding)/total number of oocysts counted after excystation times 100% minus percentage of empty oocyst walls before excystation. With PI-exclusion, % viability was calculated as the percentage of oocysts without intracellular PI, but with nuclei staining with DAPI and/or internal sporozoites as determined by DIC microscopy.

Data analysis
The die-off rate was assumed to be logarithmic and could therefore be described as a pseudo first order reaction when the micro-organism densities or percentages were log-transformed. Die-off rates (with 95% confidence intervals) were calculated by linear regression on these log transformed data with Excel 5.0.

RESULTS & DISCUSSION

Viable *Cryptosporidium* oocysts were detected in the microcosms up to day 204 (end of experiment). During these experiments it became apparent that oocyst disintegration occurred, both in autoclaved and natural water. Dead oocysts

that disintegrate were not accounted for in the viability assays, resulting in an underestimation of the die-off rate. Therefore, *Cryptosporidium* die-off rates were calculated from the data of only the first 35 days (Figure 1, Tables 1 & 2). First-order kinetics provided an adequate fit to most experimental data (see 95% Confidence Interval of the inactivation rate). In natural river water, the die-off rate of both *E. coli* and faecal enterococci at 15°C was described by biphasic first-order kinetics (Figure 2, Table 2): a rapid initial die-off in the first two weeks, followed by a slower die-off in the subsequent weeks. It is not clear from these data if this is the reflection of a biological phenomenon, caused by a more persistent or adapted sub-population or a methodological phenomenon, because the densities approached the detection limit.

Both viability assays, excystation and PI exclusion, produced comparable *Cryptosporidium* die-off rates, except at 15°C under sterile conditions.

Already in the first days after introduction in the river water, a proportion of the oocysts attached to the particles present. We did not determine separate die-off rates for free and attached oocysts.

At 5°C, oocyst survival in natural river water equalled survival in autoclaved river water (Tables 1 and 2). At 15°C, oocyst die-off was more rapid in natural than in autoclaved river water. In natural river water, oocyst die-off was also more rapid at 15°C than at 5°C. As this was not the case in autoclaved river water, this temperature effect was probably not a result of increased endogenous metabolism in the oocysts. Since the viability assay was performed on free or attached oocysts, the temperature effect could not be caused by predation by zooplankton that was observed in the natural microcosms, since these oocysts were no longer detectable. A possible cause is the increased biochemical or chemical activity at 15°C.

Table 1. Survival of Cryptosporidium and indicator bacteria in autoclaved river water

Micro-organism	Temp (°C)	Time (days)	Die-off rate (^{10}log organisms/day)	95% Confidence interval
Cryptosporidium	5	35	0.010	-0.042 - 0.062
(excystation)	15	35	0.006	-0.044 - 0.056
Cryptosporidium	5	35	0.010	-0.016 - 0.036
(dye-exclusion)	15	35	0.011	-0.001 - 0.018
Escherichia coli	5	77	0.010	0.004 - 0.021
	15	77	-0.008	-0.001 - 0.018
Enterococcus faecium	5	77	0.014	0.012 - 0.016
	15	77	0.005	0.004 - 0.006
Clostridium perfringens	5	77	0.012	0.007 - 0.016
	15	77	0.027	0.020 - 0.034

In natural river water, the die-off rate of *Cryptosporidium* was approximately tenfold lower than the initial die-off rates of *E. coli* and *Ent. faecium.* In autoclaved river water the die-off rate of *E. coli* and *Ent. faecium* was much lower than in natural river water. *E. coli* WR1 even multiplied at 15°C in autoclaved river water up to a density of 10^5CFP/ml during the first two weeks and remained constant thereafter, up to at least day 77. Die-off of *Ent. faecium* in autoclaved water was slower at 15°C than at 5°C. (Figure 2). Also *Ent. faecium* may be able to grow under these conditions, the die-off rate at 15°C being the net result of die-off and growth in the microcosm.
Clostridium perfringens from sewage was 3 to 4 times more persistent in natural river water than MRI oocysts (Table 2, Figure 1 & 2). The *Cl. perfringens* spore-suspension that was produced in the laboratory and incubated in autoclaved river water died more rapid than oocysts and than the spores from primary effluent in the natural river water microcosms. It is not clear whether this is the result of strain differences or the protocol to prepare *Cl. perfringens* spores, wherein the pasteurisation may have influenced the survivability of these spores.

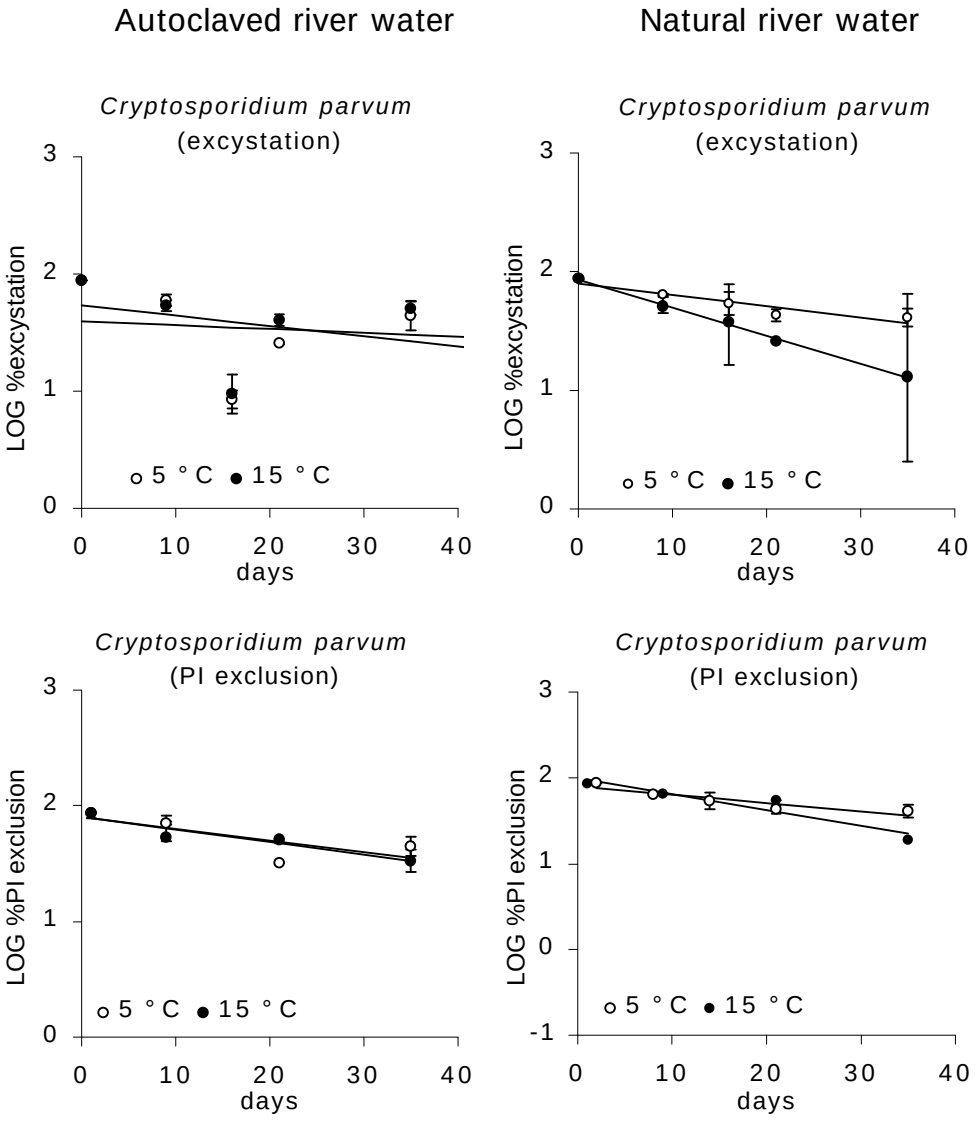

Figure 1. Survival of Cryptosporidium parvum oocysts in autoclaved and natural river water, as assessed with excystation and PI-exclusion.

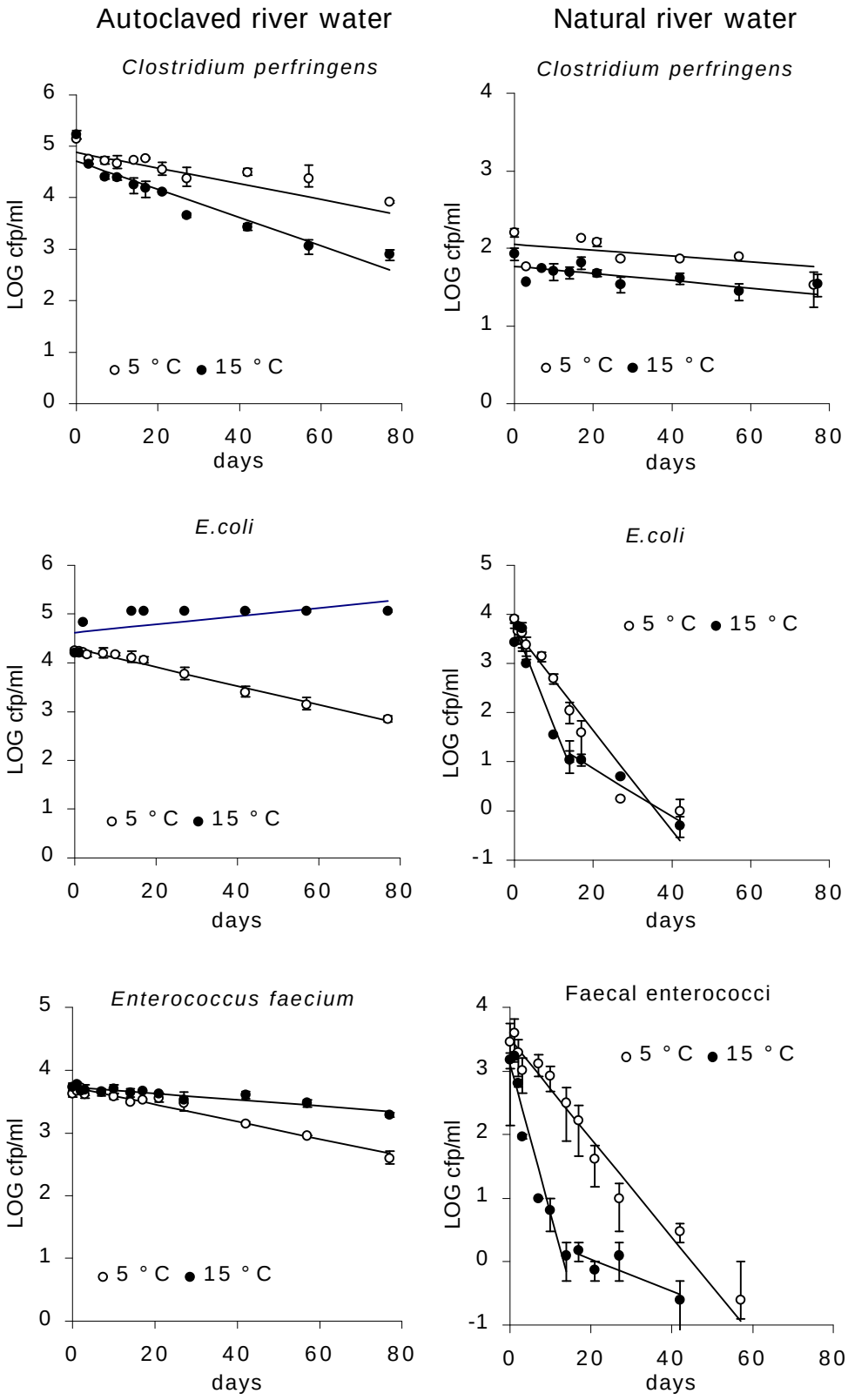

Figure 2. Survival of indicator bacteria in autoclaved and natural river water.

Table 2. Survival of Cryptosporidium and indicator bacteria in natural river water

Micro-organism	Temp (°C)	Time (days)	Die-off rate (^{10}log organisms/day)	95% Confidence interval
Cryptosporidium	5	35	0.010	0.003 - 0.016
(excystation)	15	35	0.024	0.021 - 0.026
Cryptosporidium	5	35	0.010	0.003 - 0.017
(dye-exclusion)	15	35	0.018	0.000 - 0.037
Escherichia coli	5	42	0.102	0.081 - 0.124
	15	*0-14	0.202	0.140 - 0.270
		*14-42	0.049	0.017 - 0.081
Faecal enterococci	5	42	0.077	0.066 - 0.090
	15	*0-14	0.233	0.160 - 0.306
		*14-42	0.025	0.000 - 0.050
Clostridium perfringens	5	42	0.003	-0.011 - 0.018
	15	42	0.005	-0.003 - 0.012

*Biphasic die-off kinetics: phase 1: day 0-14, phase 2 day 14-42.

In conclusion: biological or biochemical activity affects the survival of *C. parvum* oocysts at 15°C, but not at 5°C, while survival of *E coli* and faecal enterococci was affected at both temperatures. In natural river water, a temperature increase had a similar effect on the survival of all micro-organisms: die-off of all micro-organisms was 1.7 to 3 times more rapid at 15°C than at 5°C. At both temperatures, the die-off rate in natural river water for *E. coli* = faecal enterococci > *Cryptosporidium parvum* oocysts > *Cl. perfringens.*
The rapid die-off of *E. coli* and faecal enterococci makes these parameters less suitable as indicators of oocyst presence in water. As *Cl. perfringens* survived longer in natural river water than MRI-oocysts, this parameter may prove useful as indicator of the presence of *C. parvum*.

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