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Role of predation by zooplankton in transport and fate of protozoan (oo)cysts in granular activated carbon filtration

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ABSTRACT

The significance of zooplankton in the transport and fate of pathogenic organisms in drinking water is poorly understood, although many hints of the role of predation in the persistence of microorganisms through water treatment processes can be found in literature. The objective of this study was to assess the impact of predation by natural zooplankton on the transport and fate of protozoan (oo)cysts in granular activated carbon (GAC) filtration process. UV-irradiated unlabelled *Cryptosporidium parvum* and *Giardia lamblia* (oo)cysts were seeded into two pilot-scale GAC filtration columns operated under full-scale conditions. In a two-week period after seeding, a reduction of free (oo)cysts retained in the filter bed was observed. Zooplankton was isolated from the filter bed and effluent water on a 30 μm net before and during the two-week period after seeding; it was enumerated and identified. Rotifers, which are potential predators of (oo)cysts, accounted for the major part of the isolated zooplankton. Analytical methods were developed to detect (oo)cysts internalized in natural zooplankton isolated from the filter bed and effluent water. Sample sonication was optimized to disrupt zooplankton organisms and release internalized microorganisms. (Oo)cysts released from zooplankton after sonication were isolated by IMS and stained (*EasyStain*™) for microscopic counting. Both *Cryptosporidium* and *Giardia* (oo)cysts were detected in association with zooplankton in the filter bed samples as well as in the effluent of GAC filters. The results of this study suggest that predation by zooplankton can play a role in the remobilization of persistent pathogens such as *Cryptosporidium* and *Giardia* (oo)cysts retained in GAC filter beds, and consequently in the transmission of these pathogens in drinking water.

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1. Introduction

The significance of higher organisms in drinking water is attracting increased scientific attention as we are starting to better understand their capacity to vector waterborne pathogens. Predation on pathogenic organisms by zooplankton was observed in laboratory co-cultures; however the study of waterborne pathogens associated with zooplankton in field

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conditions remains limited to this day. Protists, namely amoebae, are being increasingly described as the Trojan horse of microorganisms; their role as vectors of waterborne pathogens through water systems is becoming more accurately described and highlighted as a potential threat to water safety (Barker and Brown, 1994; Loret et al., 2008). Meanwhile, in some specific cases, the detection of zooplankton in treated water can be paired with an unpredicted persistence of natural indicator bacteria through treatment processes or in distribution systems. For example, the persistence of total coliform bacteria in a distribution system was associated to their transport inside the gut of naturally occurring nematodes, which provided protection to internal bacteria against residual free chlorine (Locas et al., 2007). Such situation raises hypotheses about the role of predation and transport by zooplankton organisms in the exposure to internalized microorganisms in drinking water (Bichai et al., 2008).

Persistent organisms such as *Cryptosporidium* and *Giardia* (oo)cysts have been shown to be internalized by zooplankton in laboratory feeding experiments or co-cultures: *Cryptosporidium* oocysts have been observed within metanauplii of the microcrustacean *Artemia franciscana* (Mendez-Hermida et al., 2007), *Acanthamoeba* sp. subcultured from environmental water isolates (Gómez-Couso et al., 2007), *Acanthamoeba culbertsoni* and ciliates (Stott et al., 2003), as well as nematode *Caenorhabditis elegans* (Huamanchay et al., 2004). Various species of rotifers have been shown to ingest *Cryptosporidium* oocysts (Fayer et al., 2000) and *Giardia* cysts (Trout et al., 2002) in laboratory conditions. Ingestion of both of these protozoan (oo)cysts has also been reported with the cladoceran *Daphnia pulicaria* (Connelly et al., 2007). These studies were all based on direct microscopic methods to visualize the internalized (oo)cysts in pure predator/prey co-cultures. However, rarely have natural environmental zooplankton samples been examined for the recovery of internalized (oo)cysts: *Cryptosporidium* has been detected within rotifers in lake waters (Nowosad et al., 2007) by disrupting the animals before performing FISH detection. To our knowledge, no study has been conducted to date with the objective to detect *Giardia* cysts in environmental zooplankton samples.

The occurrence of higher organisms has been characterized in distribution systems (van Lieverloo et al., 2004) and at some stages of full-scale water treatment plants, mostly in granular material filter effluents (Schreiber et al., 1997, Castaldelli et al., 2005). Characterizing the occurrence of waterborne pathogens and bacterial indicators internalized by zooplankton in field conditions presents important methodological challenges, and was to date only reported by King et al. (1991) and Nowosad et al. (2007) in surface waters, and by Wolmarans et al. (2005) and Thomas et al. (2008) in water treatment plants, the later focusing on amoebal hosts. Yet, the occurrence of internalized (oo)cysts in natural zooplankton samples from drinking water treatment processes has never been assessed. It is not known to which extent predation on (oo)cysts can impact the performance of full-scale drinking water treatment; yet, internalization by zooplankton has been speculated as a possible mechanism involved in the mass reduction of oocysts in slow sand filters seeded with UV-irradiated unstained oocysts of *Cryptosporidium parvum* (Hijnen et al., 2007).

Considering the naturally low concentrations of these organisms in water treatment plants, we propose that the most appropriate way to find evidence for the hypothesized mechanisms of transport of pathogens associated to zooplankton is to monitor environmental zooplankton under controlled conditions of seeding (oo)cysts at pilot-scale. The current study was performed additionally to the study presented by Hijnen et al. (2010), in which two pilot-scale granular activated carbon (GAC) filters operated under full-scale conditions (natural water and filtration conditions), were seeded with UV-irradiated unlabelled (oo)cysts of *C. parvum* and *Giardia lamblia* to establish the elimination capacity for protozoan (oo)cysts. The objective of the present study was to investigate the fate of the retained (oo)cysts and the presence of (oo)cysts internalized by the natural zooplankton present in the GAC filter bed and filtrate. The mass reduction of (oo)cysts in the GAC filter bed during an extended filtration period of two weeks was determined. Simultaneously, zooplankton was isolated from the GAC material and effluent water, and an analytical protocol was developed for both types of samples to be examined for the occurrence of internalized (oo)cysts. To our knowledge, this is the first study to develop and apply a procedure for sample analysis specifically designed to detect both *Cryptosporidium* and *Giardia* (oo)cysts inside a natural population of zooplankton organisms, in an attempt to characterize the role of zooplankton in the ingestion and transport of (oo)cysts through a granular material filter bed and into the effluent water.

2. Material and methods

2.1. GAC filtration seeding test

A pilot plant with two parallel GAC filter columns (15 cm diameter; 1 m deep, 5 m h^{-1} and contact time of 12 min; no back washing) was seeded for 2 h at an influent concentration of $\sim 1.6 \times 10^5$ l^{-1} and 4.8×10^4 l^{-1} of UV-inactivated (unlabelled) *C. parvum* and *G. lamblia* (oo)cysts, respectively. The GAC originated from full-scale filters operated for 40 000 bed volumes of filtering. The complete description of the filtration set-up and seeding experiment is detailed in Hijnen et al. (2010). This seeding test was conducted under full-scale conditions using the filtrate of a conventional treatment (coagulation and rapid sand filtration) supplied with the River Meuse water (the Netherlands) after storage in impoundment reservoirs.

2.2. Experimental protocol

The present study can be described in 3 main experimental parts: (i) To determine the occurrence of a mass reduction over time of the retained (oo)cysts in the filter bed as an hypothesized indication of the presence and activity of predators, GAC samples grabbed at various depths through the filter bed of both columns were analyzed one and three weeks after the seeding test. (ii) Natural zooplankton samples from slow sand filters and from the GAC pilot filters were used to develop the optimal protocol for complete disruption of the zooplankton in order to release and enumerate the internalized (oo)cysts. (iii) Natural zooplankton from the GAC material sampled three weeks after seeding and from the effluent of

the GAC columns sampled one and three weeks after the seeding experiment was isolated, identified and quantified, and zooplankton was treated using the optimized disruption procedure in order to find evidence for the presence of internalized (oo)cyts. The procedures are summarized in Fig. 1. Because of the use of unlabelled (oo)cysts in the seeding experiment direct microscopic evidence of internalized (oo)cysts in the environmental zooplankton could not be obtained in the scope of the current study.

2.3. Analysis of free (oo)cysts retained in the GAC filter bed

One and three weeks after seeding (oo)cysts in the loaded GAC filters, GAC bulk samples were collected throughout the filter bed of both filtration columns according to the following distribution: top layer, 0–5 cm, 5–10 cm, 25–30 cm, 50–55 cm and bottom layer (85–90 cm) The bulk GAC samples were kept in closed jars at 4 °C. The GAC sub-samples from every layer of the columns sampled one week after the seeding test were analyzed separately to measure the number of retained (oo)cysts. From the GAC sub samples taken three weeks after the seeding experiment two composite samples were prepared from the first four layers (0–30 cm) and last two layers (50–90 cm). The GAC sub-samples (5 g) were treated in separate suspensions of 20 ml autoclaved tap water with successively 2 min of hand shaking, 2 min of vortex mixing, 2 min of low energy sonication (LES) and 2 min of high energy sonication (HES) at 45% amplitude. The resulting sub-samples were combined into one suspension for (oo)cyst analysis (Hijnen et al., 2010).

2.4. Isolation of zooplankton from the GAC material

The GAC bulk samples from column 1 and 2 which were used for analysis of the mass reduction were also analyzed to identify and quantify the zooplankton and subsequently to determine the number of zooplankton-associated (oo)cysts. Two composite GAC sub-samples were made for each filtration column by weighting (in 80 ml of sterile tap water i.e. wet weight) 10 g GAC of each filter bed fraction from depth $\leq$30 cm (top layer, 0–5 cm, 5–10 cm, and 25–30 cm depth) and $\geq$50 cm (50–55 cm depth and bottom layer). Composite samples were washed 5 times by vigorous shaking with sterile tap water. All of the washing water was collected and filtered through a 30 μm net to retain the zooplankton. The recovered zooplankton sample was vortexed, filtered 3 additional times and thoroughly rinsed with sterile tap water through the 30 μm net in order to get rid of most free (oo)cysts in the sample and to concentrate the zooplankton.

2.5. Isolation of zooplankton from effluent water

Zooplankton was collected at the outlet of each of two replicate filtration columns using 30 μm plankton net before and

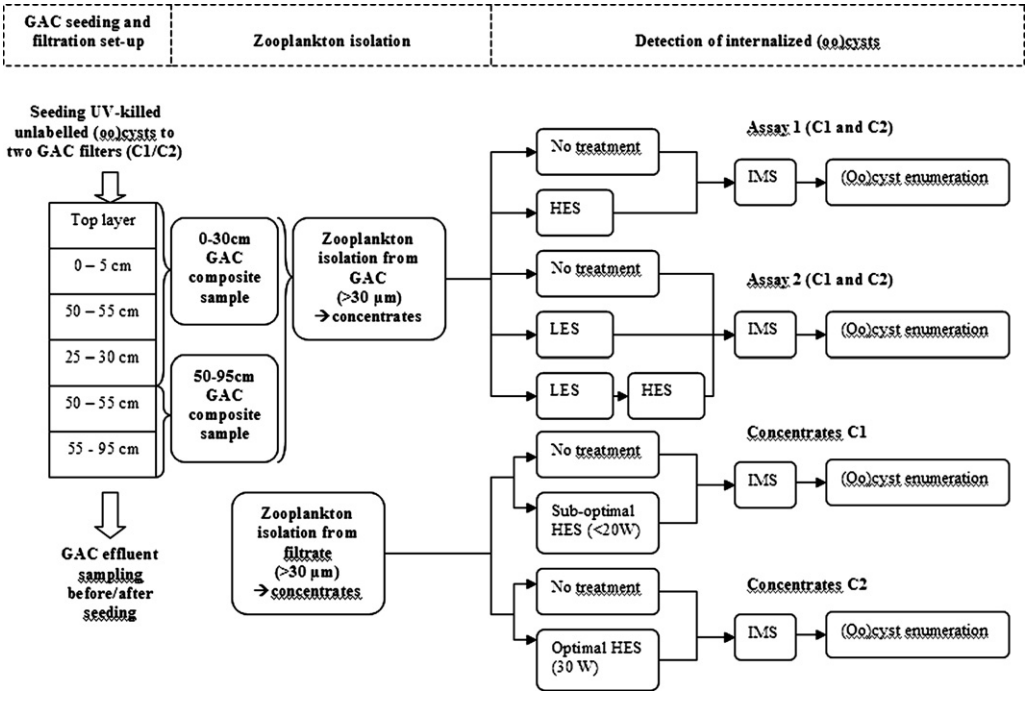

Fig. 1 – Experimental set-up and optimized detection procedures for internalized (oo)cysts in GAC material and effluent water isolated zooplankton. In this seeding experiment, GAC material and effluent water were both sampled for free (oo)cysts and for zooplankton. Zooplankton was collected and concentrated on 30 μm nets and analyzed for detection of internalized (oo)cysts. Zooplankton isolates from GAC filter bed material were analyzed in two assays; Assay 2 included an additional LES step for detachment of free (oo)cysts from carbon fines. Zooplankton isolates from effluent water from column 1 were analyzed through the methodology development phase with suboptimal sonication condition (HES), while isolates from column 2 were analyzed with the optimal protocol. C1: column 1, C2: column 2, LES: Low energy sonication (2 min), HES: High energy sonication (40 s, 65% amplitude, 5 ml suspension in 15 ml tubes, 30 W, unless specified otherwise).

after the seeding experiment. Two effluent volumes of 2100 and 2200 l were filtered through a 30 μm plankton net during a 24 h period one week and 2 days prior to seeding. Then a first sample of 6600–6800 l was collected two days after spiking over a 70 h period, followed by a second volume of 2560 l collected 3 weeks after spiking over a 64 h period. All samples were fixated in 4% formaldehyde and kept at 4 °C until processed and analyzed for (oo)cyst recovery and zooplankton species identification and quantification.

2.6. Zooplankton sample preparation for method development

Natural zooplankton was used to develop the analytical protocol for recovering internalized (oo)cysts. Some fractions of the zooplankton concentrates isolated from the effluent water of the GAC filtration columns prior to the seeding test (unseeded zooplankton) as well as zooplankton samples isolated from the *Schmutzdecke* of slow sand filters were used in order to conduct preliminary tests to determine the optimal sonication conditions to disrupt the animals and extract internalized (oo)cysts. The *Schmutzdecke* from the slow sand filters was scraped from the top 2–3 cm layer of Weesperkarspel pilot-plant (Amsterdam, the Netherlands) while the filter bed was completely saturated with water. The filters had been in operation for several weeks without surface scraping. Subsequently, sand sub-samples were thoroughly washed in sterile (non-chlorinated) tap water 5 times and the rinsing water was filtered through a 30 μm net to collect the zooplankton organisms. Sub-samples of concentrated zooplankton suspensions prepared from slow sand filter samples or GAC effluent water samples (as described above) were put into Petri dish (9 mm diameter) and observed under a binocular (30× magnitude). Animals were picked up individually by using a pipette set to a volume of 5 μl and split into 3 groups of organisms with an expected similar resistance to sonication treatment: animals (in 5 μl drop) were then added to 5 ml of sterile tap water in a 15 ml centrifugation tube until the tube contained 20 organisms of one pre-determined group. The following groups were defined on the basis of a hypothesized difference in resistance to sonication treatment deduced from the presence/absence of a hard shell: (1) nematodes, (2) rotifers with a shell (*Lecane* sp., *Colurella* sp., others), and (3) rotifers without a shell (*Philodina* sp., *Rotaria* sp., others).

2.7. Zooplankton disruption procedure to extract internalized (oo)cysts

High energy sonication (HES) was used to extract the internalized (oo)cysts from the zooplankton. A Branson Sonifier S-250D was used at amplitudes of 45% and 65%, considering that the manufacturer recommends not using amplitude higher than 70% to avoid stress cracking of the microtip. The performance indication of the apparatus for 5 ml samples in 15 ml centrifugation tubes was monitored, and the corresponding power output (W) was determined by using the Sonifier output control curves (Branson Sonifier S-250D, USA). Different sonication times were tested on a standardized volume (5 ml samples) in a standardized container (15 ml centrifugation tubes) kept on ice during the procedure to prevent temperature rise. To test the effect of this HES disruption procedure on the oocyst recovery and staining by *EasyStain*™ (BTF), a suspension of UV-inactivated *C. parvum* oocysts (Waterborne™, $\sim 3 \times 10^4$ oocysts ml^{-1}, in distilled water) was stained either prior to or after sonication (HES). Oocysts were counted on duplicate *Dynal*™ slides using 50 μl volumes of the suspensions (stained/unstained) before and after sonication treatment.

2.8. Elimination of free (oo)cysts from the zooplankton concentrates and recovery tests

In order to focus the analysis on zooplankton-internalized (oo)cysts in this research, efforts were made to exclude free (non-internalized) (oo)cysts possibly present in the zooplankton samples. Two strategies were tested to attempt separating free (oo)cysts from zooplankton: (1) centrifugation and (2) sedimentation. For both methods, a *ColorSeed*™ (BTF, Australia) sample (1 ml, 100 *Cryptosporidium* oocysts and 100 *Giardia* cysts) was added to 15 ml of unseeded effluent zooplankton samples from column 2. Centrifugation was applied at 500 *g* for 2 min. Sedimentation was applied at 4 °C for 24 h. After either separating procedure, the bottom part (pellet) of the sample (where most zooplankton was expected to be found) was divided in 2 fractions, one of which was treated with optimized zooplankton disruption treatment (HES, 40 s at 65% amplitude) and the other remained intact. Then, all fractions of the sample i.e. (i) upper part (supernatant), (ii) lower part sonicated, and (iii) lower part untreated were processed with the IMS method. Positive control slides were also analyzed for staining quality control. Recovery of the *ColorSeed*™ in those tests was assessed in order to determine the recovery associated to the detection method used for (oo)cyst enumeration in the zooplankton samples (GAC material and GAC effluent) in this study.

2.9. Analysis of zooplankton concentrates from the GAC material

Analysis of zooplankton sub-samples for recovery of internalized (oo)cysts was performed twice (Fig. 1). The first time, the analysis allowed to compare (oo)cyst recovery from an untreated fraction of zooplankton concentrates with the (oo)cyst recovery after applying the optimized HES disruption procedure (Assay 1). The second time, a low energy sonication step (LES) was introduced in the handling of the zooplankton isolates in order to detach free (non-internalized) (oo)cysts which from carbon particles observed in the washed zooplankton concentrates (Assay 2). Performing a LES treatment for 2 min in an ultrasonic bath (Branson Model 5510, Danbury, USA) has previously shown to efficiently release (oo)cysts attached to carbon fines in GAC material samples (Hijnen et al., 2010), while not disrupting zooplankton (data not shown). In Assay 1, each of the zooplankton concentrates from GAC composite samples was mixed and divided into 2 sub-samples: (i) 10 ml was untreated; (ii) 10 ml was sonicated (HES) on ice (in 5 ml sub-samples, 40 s at 65% amplitude); in Assay 2, zooplankton concentrates mixed and split into 3 fractions: (i) 10 ml was kept untreated; (ii) 10 ml was treated with LES for 2 min in glass tubes to detach (oo)cysts associated to carbon

fines; and (iii) 10 ml was first treated with LES for 2 min, followed by HES disruption procedure (on ice, in 5 ml sub-samples, 40 s at 65% amplitude).

2.10. Analysis of zooplankton concentrates from the effluent water

Effluent samples from column 1 and column 2 were analyzed for the detection of zooplankton-associated (oo)cysts. Samples from column 1 were all analyzed concomitantly with the method development. All effluent zooplankton samples from column 1 (pre- and post-seeding) were mixed and split into 5 ml volumes, and sonicated on ice for 40 s at 65% amplitude in 50 ml plastic tubes. The sonicated suspension was transferred in a *Dynal*™ tube for further IMS processing. A positive control slide was included as staining quality control. Pre-seeding samples from column 2 were also analyzed throughout the method development phase (see recovery tests described in a previous section). Post-seeding samples from column 2 were analyzed with the optimized procedure (without any additional step to separate free (oo)cysts from internalized ones). The entire post-seeding samples from column 2 were homogenized (vortex) and divided into several 5 ml sub-samples which were sonicated on ice (40 s, 65% amplitude) to disrupt the zooplankton organisms. Finally, the samples were processed with the IMS detection method and further analyzed by fluorescence microscopy for enumeration of recovered (oo)cysts.

2.11. Cryptosporidium, Giardia and zooplankton enumeration

In the treated or untreated sub-samples, *Cryptosporidium* and *Giardia* (oo)cysts were determined by IMS and counted with epifluorescence microscopy (Leica, DM RXA) at a 250× magnitude as previously described (Hijnen et al., 2007). The concentration of internalized (oo)cysts in the GAC material isolated from the zooplankton samples was corrected for the recovery of the analysis as assessed in the current study and expressed in n ml^{-1} using the volume weight of the GAC of 0.66 g ml^{-1}.

For zooplankton enumeration in the GAC material and the GAC effluent, samples of 1 ml to 5 ml of zooplankton concentrate were transferred into a counting plate, allowed to settle for 5 min. The entire counting chamber was scanned and organisms were enumerated with an inverted microscope (Leica, Leitz Labovert FS) (100× magnitude).

3. Results and discussion

3.1. Analysis of free (oo)cysts retained in the GAC filter bed

The concentration of retained (oo)cysts of *C. parvum* and *G. lamblia* in the GAC filters sampled one week and three weeks after the seeding experiment are presented in Fig. 2. An average mass reduction of *Cryptosporidium* oocysts of 66.2% (66.1–66.3%) and 32.1% (−4.6–68.2%) was found in the upper and lower parts of the GAC filter beds, respectively. This mass reduction of retained oocysts is for a minor part (<5%; calculated from water flux and effluent concentrations) due to wash out, and therefore mostly attributed to processes in the filter bed, such as predation. Due to the large variation in *G. lamblia* cyst concentration determined one week after the seeding, the slight mass reduction was not significant for this microorganism.

3.2. Zooplankton enumeration

Fig. 3 shows the average concentration of zooplankton organisms found in the effluent of the GAC columns and in the GAC material sampled from the columns. Concentrations of >300 and >800 organisms/30 g of GAC material were estimated in the filter bed of column 1 and 2, respectively. In the effluent water, concentrations of ~1600 organisms/m^3 and ~2100 organisms/m^3 were calculated for columns 1 and 2, respectively. Although these data are based on limited sample analysis, they indicate a higher zooplankton density in column 2 as compared to column 1. In both columns, zooplankton was found to be distributed throughout the whole depth of the filter bed. Rotifers (mostly *Lecane*, *Philodina* and *Colurella* spp.) were found to be the dominant zooplankton population, followed by nematodes, both in GAC material and effluent water samples. In both columns, rotifers and nematodes were observed to account respectively for ~80% and ~10% of the total number of zooplankton organisms released in effluent waters. Since rotifers are known to internalize (oo)cysts both in laboratory conditions (Fayer et al., 2000, Trout et al., 2002) and in natural aqueous environment (Nowosad et al., 2007), they are suspected to be potential predators of (oo)cysts in granular filters. The important proportion of rotifers observed in the zooplankton populations rejected in the GAC filters effluent suggests that they could also have a role of transport of internalized (oo)cysts into filtered water.

3.3. Zooplankton disruption procedure to extract internalized (oo)cysts

Sonication tests using HES were conducted with the objective to find the optimal conditions (intensity and time) that would result in a complete (and if possible simultaneous) disruption of all zooplankton organisms present in the concentrated samples to release internalized (oo)cysts. Results obtained for increasing sonication durations at amplitudes of 45% or 65% are shown in Table 1. The efficacy of the treatment was calculated as: $(n_{before\_HES} - n_{after\_HES})/n_{before\_HES} \times 100\%$, with $n_{before\_HES} = 20$ zooplankton organisms for all suspensions and all HES conditions tested. The disruption was more effective at a 65% amplitude with a resulting power output of 30 W than at 45% with a power output of 15–20 W, and the higher power output allowed a better synchronization of the disruption of the various zooplankton species present in the samples. After 40 s at 65% amplitude, ≥95% of all tested classes of zooplankton were disrupted. These conditions were selected as the optimized disruption procedure for further treatment of the GAC zooplankton samples. The selected HES treatment was applied to a suspension of *C. parvum* (oo)cysts (in the absence of zooplankton) to determine its impact on the recovery and the staining of free (oo)cysts following IMS. Average recovery rates calculated from: $(n_{before\_HES} - n_{after\_HES})/n_{before\_HES} \times 100\%$

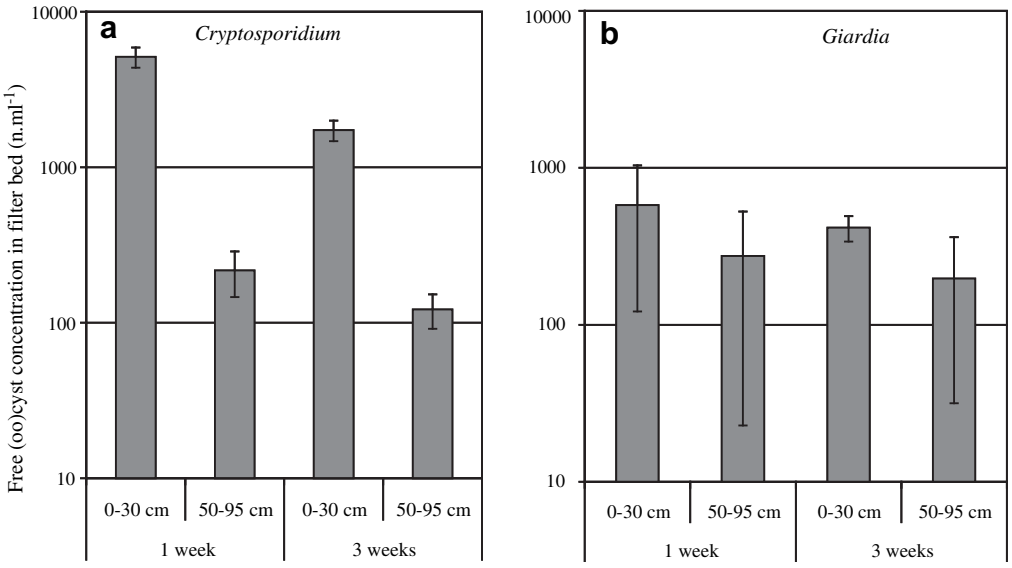

Fig. 2 – Concentrations of free (a) *Cryptosporidium* and (b) *Giardia* (oo)cysts retained in the GAC filter bed 1 and 3 weeks after the seeding test (error bars show the range of data).

(n = number of oocysts) were 42% (±1%; $n = 2$) for oocysts stained prior to HES and 86% (±15%; $n = 2$) for oocysts stained after HES. These results suggest that HES at 65% amplitude (30 W) for 40 s does have a significant impact on the recovery of pre-stained *Cryptosporidium* oocysts. During microscopy enumeration, some oocysts were observed to be damaged due to sonication. However, the recovery of unstained oocysts was not statistically different from 100% ($p = 0.63$), which reproduces the condition of the oocysts seeded in the GAC filtration experiment. This test also allowed concluding that the staining of oocysts by *EasyStain*™ was not, in itself, negatively influenced by sonication. Considering (i) the low impact of the HES disruption on the unstained UV-inactivated oocysts and (ii) the assumption that the internalized (oo)cysts are mostly protected during the HES disruption until they are released from zooplankton hosts, it was concluded that the HES disruption procedure used to release internalized (oo)cysts had a negligible effect on the enumeration of these (oo)cysts.

3.4. *Analysis of zooplankton concentrates from the GAC material*

In the GAC material, free (non-internalized) (oo)cysts are present, as was demonstrated in Fig. 2. Following the zooplankton isolation procedure from the GAC material, part of these free (oo)cysts can remain present in the zooplankton concentrates. Therefore, (oo)cysts in these zooplankton samples were enumerated with and without the HES disruption procedure. The results clearly showed the presence of free (oo)cysts (Fig. 4; untreated samples). In both Assay 1 and 2,

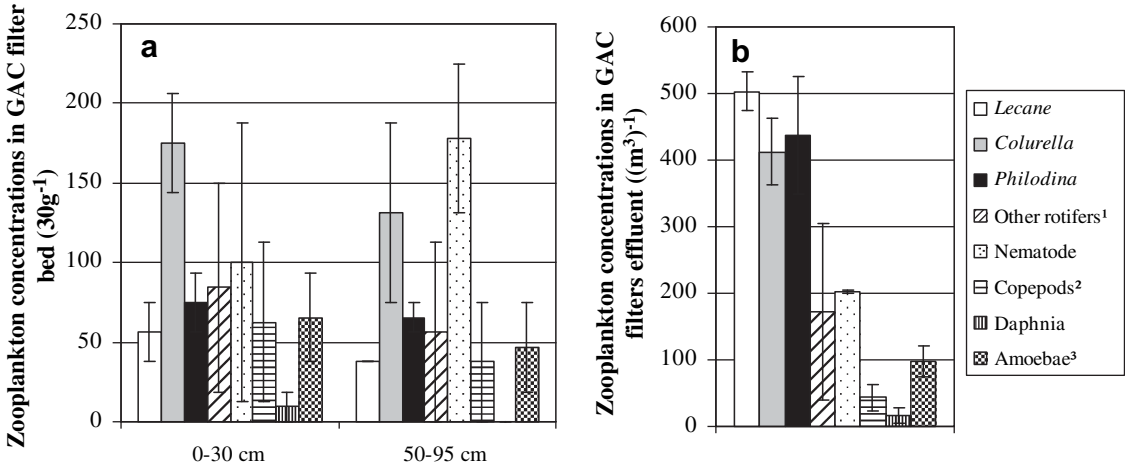

Fig. 3 – Average concentrations of zooplankton organisms (error bars show the range) from column 1 and 2 (a) in the GAC material (30 g^{-1}) in upper and lower parts of the filter bed and (b) in the effluent water ($(m^3)^{-1}$). ([1]*Brachionus*, *Trichocerca*, *Bosmina*, *Polyarthra*, *Asplancha*, *Testudinella*; [2]*Harpaticoida*, nauplii, others; [3]*Testacea*, *Euglypha*).

Table 1 – Sonication (HES) treatment efficiency for zooplankton disruption

Amplitude	45%		65%				
Performance indication	10-15 %		20%				
Power delivered	15-20 W		30 W				
Sonication time (s)	60	90	20	25	30	35	40
Nematodes	70	95	90	100	100	100	nd
Rotifers +	90	90	nd	nd	90	nd	100
Rotifers -	85	100	70	80	nd	95	95
Harpaticoida	nd	nd	nd	nd	nd	nd	100

Treatment efficiency (%) calculated as: $(n_{before\ HES} - n_{after\ HES})/n_{before\ HES}$ x100%
Rotifers +/-: indicates the presence of a hard shell
nd: not determined

after HES disruption of the zooplankton samples, the number of (oo)cysts increased significantly (Fig. 4a,b), especially in the samples from the upper part of the filter. The estimated zooplankton density in this part of the filter bed was also slightly higher for most organisms (Fig. 3). In Assay 2, an additional LES treatment was applied to enumerate (oo)cysts retained on GAC fines present as part of the pellet in these zooplankton samples. Preliminary tests showed that low energy sonication (LES) had no effect on zooplankton organisms when performed for up to 10 min (data not shown). The results showed an increase of (oo)cysts (Fig. 4b) after LES, yet the number of (oo)cysts enumerated after the zooplankton disruption procedure (HES) increased again significantly. These data can be regarded as strong indications for the presence of internalized (oo)cysts from *C. parvum* and *G. lamblia* associated to zooplankton organisms.

3.5. *Analysis of zooplankton concentrates from the effluent water*

The presence of free (oo)cysts in the zooplankton concentrates collected from the carbon filter effluents by filtration on a 30 μm plankton net is not very likely. However, if some free (oo)cysts were to be retained in the 30 μm plankton net when isolating zooplankton, those could not be distinguished from the (oo)cysts extracted from zooplankton. To account for that possibility, although it is thought to be of low probability, two methods were tested to attempt eliminating free (oo)cysts from the zooplankton samples before performing the disruption procedures: centrifugation and sedimentation, both tested by spiking *ColorSeed*™ in unseeded effluent zooplankton samples from column 2. The results of these tests (not presented) showed that separation was not achievable: both (oo)cysts and zooplankton organisms were present in the pellet and the supernatant after both separation procedures. Therefore, for all further analyses, no separation step was applied on the zooplankton concentrates before the HES treatment.

Recovery rates were calculated through those two tests by considering the total recovery of *ColorSeed*™ (oo)cysts (in pellet and supernatant) that had been spiked in the zooplankton concentrates. Recovery rates of 61% and 15% for *Cryptosporidium* oocysts and *Giardia* cysts, respectively, were calculated and were used for correcting the concentrations of internalized (oo)cysts in the filter bed and effluent of GAC columns when interpreting our results. These calculated recovery rates in zooplankton concentrates were higher than those observed for recovery of free (oo)cysts in the GAC effluent during the filtration study by Hijnen et al. (2010), which were determined as 13% for *Cryptosporidium* and 7% for *Giardia*. Those rates were used to correct free (oo)cyst concentrations in the interpretation of our results.

A marked difference was observed in the results of the analyses of zooplankton samples from effluent water of column 1 and 2, which can be explained by the differences in the procedure through which they were handled. Zooplankton concentrates from effluent water of column 1 were analyzed throughout the phase of methodology development and were all found to be negative for the presence of *Cryptosporidium* and *Giardia* (oo)cysts, except for a post-seeding sample in which one *Cryptosporidium* (oo)cyst was found. However, the HES disruption procedure applied on effluent samples from column 1 was different from the optimized procedure subsequently applied to all the other zooplankton concentrates: the HES disruption procedure in the 5 ml subsamples was performed in larger 50 ml centrifugation tubes instead of 15 ml tubes, and the sonifier indicated a low performance (~10%) which corresponds to a power of <20 W being transmitted to the sample. This impacted the disruption efficacy, which was confirmed by the microscopic observation of some intact animals in the treated samples. For all subsequent zooplankton concentrate analyses (all GAC material concentrates from both columns and effluent from column 2), 15 ml centrifugation tubes were used, in which the surface area of the 5 ml concentrate exposed to standard HES sonication is reduced, therefore yielding a higher sonication power.

Zooplankton concentrates from effluent water of column 2 were treated with this optimized HES disruption procedure (40 s at 65% amplitude, >30–35 W). In these disrupted concentrates no intact zooplankton organisms were found, but they did contain numerous (2–125) (oo)cysts (Table 2). The number of (oo)cysts in the zooplankton concentrates collected three weeks after the seeding of (oo)cysts in the GAC filters was terminated were higher than in the concentrates sampled one week after seeding, even though a smaller volume of water was filtered (2.0 vs 6.6 m^3, see Table 2). This is an indication that the detected (oo)cysts had been transported inside of zooplankton organisms rather than free in the effluent water, since free (oo)cyst concentrations would normally have been expected to decrease through time after the seeding was stopped. The increase of the number of internalized (oo)cyst in the filtrate may indicate either a retarded breakthrough of internalized (oo)cysts or an increased grazing efficiency of zooplankton in the filter bed over time. Furthermore, the fact that higher sonication conditions in the analysis of effluent samples from column 2 allowed detecting (oo)cysts as opposed to samples of column 1 (treated with sub-optimal HES procedure) reinforces the demonstration that recovered (oo)cysts from column 2 were in fact internalized and efficiently extracted from zooplankton organisms through the optimized protocol elaborated in this study. Detection of internalized (oo)cyst from effluent water samples is an indication that predation by zooplankton can favor persistence of (oo)cysts in filter beds and act as a vehicle for (oo)cysts to be released into filtered effluents. Although further confirmation is

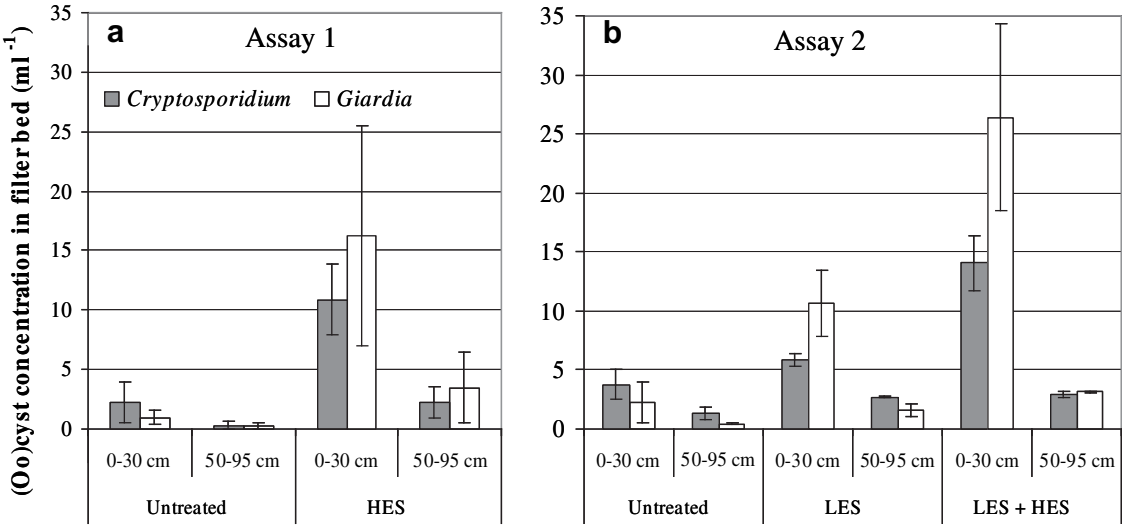

Fig. 4 – Average concentrations of free and internalized (oo)cysts (error bars show the range) from column 1 and 2 in upper and lower parts of the filter bed (m l^{-1}) assuming 100% recovery in (a) Assay 1 and (b) Assay 2. LES: Low energy sonication, HES: High energy sonication.

required, to our knowledge these are the first observations that strongly indicate that predation by natural zooplankton can favor persistence of *Crytosporidium* and *Giardia* (oo)cysts in GAC filter beds and act as a vehicle for (oo)cysts to be released into filtered effluents.

3.6. *Importance of the internalization of (oo)cysts by zooplankton in GAC filtration*

For each part of the filter bed of both columns in Assay 2, the concentration of internalized (oo)cysts was calculated by subtracting the (oo)cyst counts found after LES from the total number obtained after zooplankton disruption (LES + HES). Data from Assay 1 (without LES) were corrected using the results from Assay 2 to estimate the proportion of the raise in (oo)cyst counts following HES which is due to detachment from carbon particles. The average concentrations of internalized (oo)cysts in the upper and lower parts of the filter bed (n ml^{-1}) from column 1 and 2 are shown in Fig. 5 (numbers corrected for recovery as presented before; 61% and 15 % for *Cryptosporidium* and *Giardia* (oo)cysts, respectively) and compared with the concentrations of free (oo)cysts 3 weeks after seeding (data from Fig. 2). From these numbers, we calculate that less than 1% of *Cryptosporidium* oocysts in the filter bed were internalized. For *Giardia*, a higher percentage was estimated (17% in the upper part of the filter bed and 5% in the lower part) but these data are less reliable due to a higher variability in the measured concentrations of free cysts (see Fig. 2). These percentages are based on average values from both columns; when considered separately, higher proportions of internalized (oo)cysts are found in column 2. This level of internalization is expected to be reflected in the amount of internalized (oo)cysts in the zooplankton isolates from the effluent of both columns. Estimated concentrations of internalized (oo)cysts in GAC filters effluent water are presented in Table 2. Numbers are found to reach >100 *Cryptosporidium* oocysts/m^3 and >200 *Giardia* cysts/m^3 in column 2 (most densely populated with zooplankton) 3 weeks after the seeding test. Free (oo)cysts were not measured in the effluent water 3 weeks after seeding, but concentrations would be expected to decrease in the effluent as compared to

Table 2 – Internalized (oo)cysts in zooplankton concentrates and free (oo)cysts from effluent water sampled one week and 3 weeks after the seeding test.

Column/ time after seeding		Sampled effluent volume (l)	Internalized (oo)cysts in zooplankton effluent concentrates			Free (oo)cysts in effluent water	
			C. parvum n; C (n l^{-1})[b]	*G. lamblia* n; C (n l^{-1})[b]	Rotifers (n)[c]	*C. parvum* C (n l^{-1})[d]	*G. lamblia* C (n l^{-1})[d]
C1[a]	Week 1	6700	1; 0.0002	0; <0.001	8900	19.3	0.09
	Week 3	2000	0; <0.0008	0; <0.004	2700	nd	nd
C2	Week 1	6700	9; 0.002	2; 0.002	11 400	17	0.5
	Week 3	2000	125; 0.102	64; 0.246	3500	nd	nd

a Sub-optimal HES disruption (Fig. 1).
b Concentrations corrected for recovery of 61 and 13% for *Cryptosporidium* and *Giardia*.
c Estimated numbers from enumeration data presented in Fig. 3b.
d Corrected for the recovery of the analysis of free (oo)cysts (Hijnen et al., 2010); n = number; C = concentration; nd = not determine.

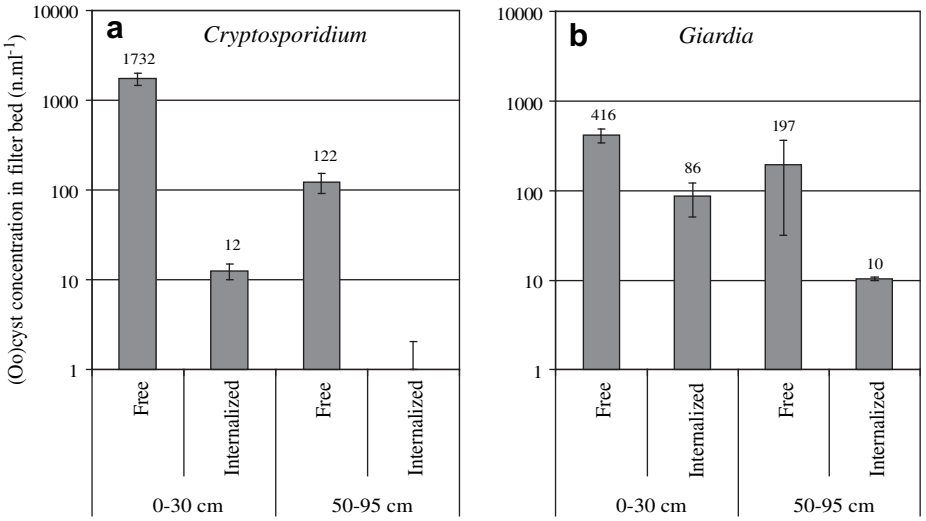

Fig. 5 – Average concentrations of free and internalized (oo)cysts of (a) *Cryptosporidium* and (b) *Giardia* (error bars show the range) from column 1 and 2 in upper and lower parts of the filter bed (m l^{-1}); concentrations were corrected for the assessed recovery percentages (61% and 15% for internalized *Cryptosporidium* and *Giardia* (oo)cysts, respectively, and of 13% and 7% rates for free (oo)cysts of *Cryptosporidium* and *Giardia*, respectively).

concentrations measured 2 weeks earlier, when free *Giardia* cysts were found in a concentration of ~500 cysts/m^3. For effluent water samples, the percentages of internalized (oo)cysts were found to be ≤1% for internalized (oo)cysts and *Cryptosporidium* oocysts, respectively, in effluent water collected 1 week after spiking. However, if calculating the same ratio 3 weeks after seeding, *Giardia* cysts are found to account for nearly 30% of the total cysts in the effluent water of column 2. This calculation is based on the assumption that the concentration of free (oo)cyst in effluent water 3 weeks after seeding would be the same as 2 weeks earlier. This assumption is conservative: it underestimates the calculated ratio of internalized (oo)cysts, since free (oo)cysts can be expected to decrease in the effluent water 3 weeks after stopping the seeding. These results may be an indication that although the process of remobilization of persistent pathogens in filter beds by predation is possible, the level is most likely low as compared to concentrations of free (oo)cysts. Considering the very low targeted concentrations of (oo)cysts in treated water (<1/100 000 l, USEPA, 2006), the assessment of the significance of internalization and transport of (oo)cysts by zooplankton for water safety would need further investigation, which could be undertaken with the perspective of performing a quantitative microbial risk analysis (QMRA).

The current experimental protocol (seeding study) represents a worst-case scenario in which predation on (oo)cysts by natural zooplankton was favored under defined conditions (high concentration of seeded (oo)cysts) in an environmental system. The viability and infectivity of those internalized (oo)cyst remains however undetermined. If (oo)cysts internalized by zooplankton were found to remain infectious, as was demonstrated in the case of the ingestion of *Cryptosporidium* oocysts by the nematode *C. elegans* (Huamanchay et al., 2004), then predation could be seen as increasing the persistence and the transport of these pathogens through filters. However, if (oo)cysts are being degraded by their predators before being naturally rejected in the filter bed or in the effluent water, then predation would be found to be an important mechanism in the removal of protozoan (oo)cysts in biological granular filtration. Connelly et al. (2007) reported a significant decrease in viability of *Giardia* cysts and infectivity of *Cryptosporidium* (oo)cysts due to grazing by the crustacean *D. pulicaria* in lab-scale feeding experiments. On a final thought, our experimental protocol targeted zooplankton larger than 30 μm, therefore excluding the potential impact of most amoebae, while *Acanthamoeba* has been shown to be a potential vehicle for *Cryptosporidium* oocysts (Gómez-Couso et al., 2007). Also, the 30 μm might allow some nematodes to escape due to their small width. The recovery of the zooplankton isolation method is unknown. It is likely that not all zooplankton organisms were extracted from the GAC material samples.

4. Conclusions

Our results suggest that predation by zooplankton population in a GAC filter bed has an effect on the transport and fate of pathogenic protozoan (oo)cysts retained in these filter beds. In two GAC filter columns pre-seeded with UV irradiated unlabelled (oo)cysts of *C. parvum* and *G. lamblia*, a reduction was measured in the retained mass of (oo)cysts during two weeks of filtration. Zooplankton was observed in the GAC material (5–200 organisms per 30 g) and in the filtrates of both columns (10–500 organisms m^{-3}) which consisted for a large part of rotifers. Rotifers are known to ingest *Cryptosporidium* and *Giardia* (oo)cysts under laboratory conditions, and they are thought to be the main potential predator species for (oo)cysts in this study. A high energy sonication (HES) treatment was designed to disrupt the observed zooplankton and to recover internalized *Cryptosporidium* and *Giardia* (oo)cysts. . (Oo)cyst recovery was enhanced in zooplankton concentrates from the GAC filter bed when

disruption of zooplankton organisms was performed by this procedure. In the zooplankton isolates from the GAC filters effluents, an increasing number of (oo)cysts was detected when the proper HES disruption method was applied. These observations are indications for the occurrence of internalized (oo)cysts in the environmental zooplankton present in the GAC filter bed and effluent water. Under the seeding conditions of this study, the ratio of internalized (oo)cysts to the total (oo)cyst concentration (free and internalized) in the filter bed and effluent water were found to be limited. The observed increase in the number of internalized (oo)cysts in the zooplankton concentrates from the effluent 3 weeks after the seeding test can be seen as an indication of increased predation activity over time. The significance of the present findings for the microbiological safety of drinking water requires further research. More quantitative information on predation and remobilization and on the fate of internalized pathogens (viability/infectivity) is required to assess the probability of transmission of internalized infectious (oo)cysts by zooplankton under real environmental conditions, in the perspective to calculate the level of risk arising from these internalized organisms in drinking water.

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