Preliminary study on the occurrence and risk arising from bacteria internalized in zooplankton in drinking water

F. Bichai, W. Hijnen, Eric Baars, M. Rosielle, Y. Dullemont and B. Barbeau

ABSTRACT

In this study, an environmental sampling campaign was conducted to detect internalized *E. coli* and *C. jejuni* bacteria in zooplankton and amoebae samples collected at various stages of three water treatment plants in Amsterdam, the Netherlands. Eight sampling locations were selected and sampling was performed twice, at a two-week interval, at each location. Chlorination was used to inactivate free (external) bacteria in the concentrated zooplankton samples and sonication was used to disrupt zooplankton organisms in order to release and recover internalized bacteria. Zooplankton enumeration was performed by microscopy. No internalized *E. coli* or *C. jejuni* bacteria were recovered from all of the samples analyzed. The occurrence of internalized *E. coli* or *C. jejuni* bacteria in drinking water was estimated to be lower than one internalized bacteria in 10^5 zooplankton organisms, as derived from the detection limit of the sampling campaign. By using the QMRA approach and the Beta-Poisson model, a risk of infection of less than 9.2E-6 and 5.9E-5 was estimated for internalized *E. coli* and *C. jejuni* in drinking water, respectively. This study remains preliminary due to the limited number of samples taken at each location.

Key words | *C. jejuni*, *E. coli*, environmental sampling, internalization, risk assessment, zooplankton

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INTRODUCTION

Evidence has been found that environmental zooplankton organisms can ingest and transport various pathogenic microorganisms in lab-scale or pilot-scale experimental conditions, and protection of internalized microorganisms against water disinfection processes has been documented, mostly in amoebae and nematodes, as reviewed in Bichai *et al.* (2008). Amoebae have been extensively described as vectors of pathogenic microorganisms and were identified in some cases as the cause for enhanced survival and replication of some pathogenic bacteria in environmental and engineered systems (Barker & Brown 1994; Kuiper *et al.* 2004; Loret *et al.* 2008). Various human pathogenic bacteria have been found to infect the gut of *C. elegans* nematodes (Sifri *et al.* 2005), thus suggesting that nematodes could also potentially amplify and vector some waterborne pathogens. Meanwhile, predation and transport by nematodes was found to explain an unpredicted persistence of total coliform bacteria in a full-scale chlorinated distribution system (Locas *et al.* 2007), raising hypotheses about the impact of zooplankton organisms on the microbiological quality of treated drinking water. At pilot-scale, zooplankton has been observed to carry (oo)cysts of *Cryptosporidium* and *Giardia* through GAC filters and into

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the filtered effluent water (Bichai *et al.* 2010b). Recently, a quantitative microbial risk assessment (QMRA) was performed with a focus on protozoan (oo)cysts internalized by rotifers in filtered drinking water. This work suggested a low risk associated to those internalized pathogens when considering their typically low environmental concentrations and removal by water treatment barriers (Bichai *et al.* 2010a). However, in general, the understanding of health risks associated to higher organisms in drinking water remains limited. Literature reports a few cases of detection of zooplankton-associated waterborne pathogens in natural environmental samples (Wolmarans *et al.* 2005; Nowosad *et al.* 2007), but the lack of quantitative information about the occurrence of zooplankton-internalized pathogenic microorganisms constitutes a major gap in the scientific information required to better assess the microbial risk associated to higher organisms in drinking water. In that perspective, the objective of this study is (i) to gather some quantitative indications on the occurrence of health-related microorganisms internalized by natural zooplankton through full-scale drinking water treatment plants, and (ii) to estimate quantitatively the level of risk which can be associated to internalized bacteria in drinking water. To achieve these objectives, an environmental sampling campaign was elaborated to collect natural zooplankton organisms at various locations of three water treatment plants in the Netherlands. An analytical protocol was developed in order to recover internalized bacteria (*Escherichia coli* and *Campylobacter jejuni*) from the isolated zooplankton. *E. coli* and *C. jejuni* were detected during large volume sampling through water treatment, and especially after ozonation (Smeets *et al.* 2006), which raised questions about the association of these bacteria to zooplankton, as a potential explanation for their unexpected persistence through treatment. Risk estimates were computed on the basis of QMRA principles and methodology.

METHODOLOGY

Sampling procedure

E. coli and *C. jejuni* were selected as the two microbial targets for this investigation because they are commonly monitored in the Netherlands, and because of their unexplained persistence through water treatment as reported by (Smeets *et al.* 2006). Natural zooplankton samples were collected in two size-fractions at each sampling points (>30 μm, 10–30 μm); the lower size-fraction was mainly meant to collect the smaller amoebae. Zooplankton and amoebae fractions were isolated at different stages of 3 water treatment plants in Amsterdam (Netherlands): (1) the pretreatment plant at Loenderveen (PLV) of the (2) Weesperkarspel (WPK) plant, and (3) Leiduin treatment plant (PLD). A description of the water treatment trains and the location of the sampling points at each plant are shown in Figure 1. Sampling was performed twice at each location at 2-week intervals. At each

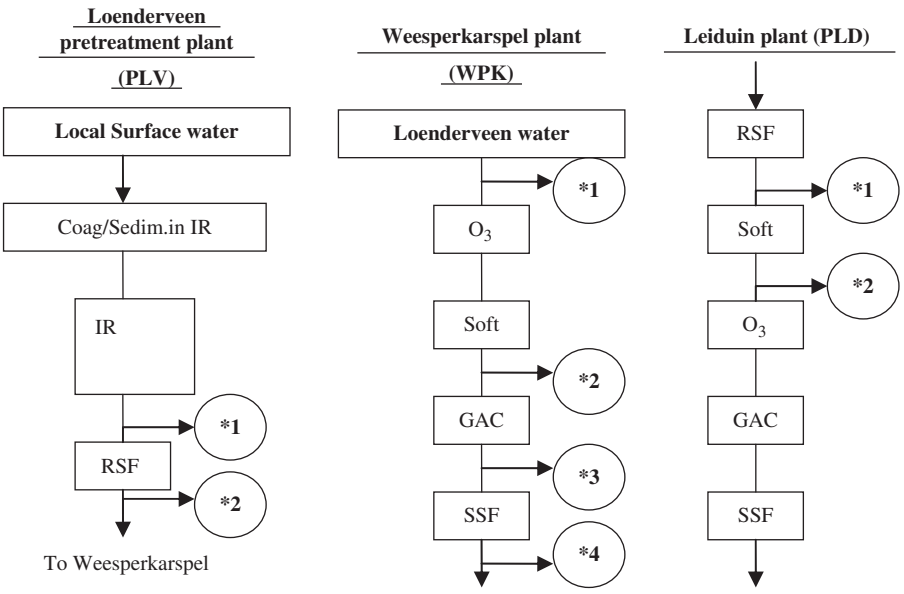

Figure 1 | Sampling points (*) at the three selected treatment plants in the Netherlands. (RSF = Rapid sand filtration; O_3 = Ozonation; Soft = Softening; GAC = Granular activated carbon filtration; SSF = Slow sand filtration; Coag = Coagulation; Sedim = Sedimentation; IR = Impoundment reservoir).

sampling point, a "T"-shaped sampling device (which enables splitting the flow into two parallel samples) was used to collect 2 samples at the same time: (i) one sample (2-hour sampling) was killed and fixed with Lugol for further amoeba and zooplankton identification and quantification; (ii) the other sample (8-hour sampling) was collected using a 30-μm net (zooplankton fraction) followed by a 10-μm net (amoeba fraction), and the 2 size fractions were analyzed separately for the recovery of internalized bacteria. At two sampling points at the end of the treatment before and after the slow sand filtration (WPK-3 and WPK-4), a 10-μm net was used to collect the zooplankton fraction (instead of 30-μm), followed by a second 10-μm net for amoebae, to account for the smaller size of the zooplankton organisms in clean water.

Procedure for enumeration of amoebae and zooplankton

Amoeba samples (10–30 μm) were collected in 3 L bottles (with Lugol) and allowed to settle for 1 week. Then, the supernatant was removed by vacuuming with a sterile pipette. The lower third of the sample was transferred into a 1 L bottle and allowed to settle for 3 days. Then, the supernatant was removed and the sample was reduced to a volume of approximately 50 mL, which was transferred into a 50 mL vial (centrifugation tube) for further settling. After 2 days, the supernatant was removed until a volume of less than 5 ml remained, which was transferred into a counting chamber for enumeration of the organisms under an inverted microscope (Leica, Leitz Labovert FS) at 400× magnification. Amoebae were enumerated as cysts and trophozoites separately. Zooplankton samples (>30 μm) were collected in 50-mL vials (with Lugol) and allowed to settle for 24 hours. Then, the supernatant was removed until a volume of less than 5 ml remained, which was transferred into a counting chamber for enumeration of the organisms under an inverted microscope at 125× magnification. Zooplankton organisms were enumerated as pertaining to the following groups: rotifers, nematodes, copepods, ciliated protozoa. Amoebae found in the >30 μm sample fractions were counted as well. Rotifers were enumerated to the genus level, whereas copepods were identified as calanoid or cyclopoid copepods, and copepod nauplii.

Analytical protocol for recovery of internalized bacteria

An analytical protocol was developed to recover internalized bacteria from the environmental zooplankton and amoebae samples: chlorination (10 mg Cl_2/L, 5 minutes) was applied to each sample in order to inactivate free and surface-attached bacteria. Chlorine residuals were quenched at the end of contact time by adding sodium thiosulfate. All chlorinated samples were split into two parts of equal volumes. One part was kept intact (for ~2 hours) at 4°C until performing bacterial enumeration (non-sonicated sample). The other part was sonicated on ice (Branson Sonifier probe, S-250D) for either 40 seconds at 65% amplitude for zooplankton (>30 μm) or for 30 seconds at 65% amplitude for amoebae (10–30 μm) to break up zooplankton organisms and amoebae and extract the internalized bacteria. Sonication conditions for the two size fractions were selected following optimization tests on natural zooplankton organisms (Bichai *et al.* 2010b) and on a pure suspension of *Acanthamoeba castellanii* (details not shown).

Positive control for the recovery of internalized bacteria protocol

In order to confirm the adequacy of the analytical protocol developed and used in this study to recover internalized *E. coli* and *C. jejuni* bacteria from environmental zooplankton and amoeba samples, environmental samples with a higher bacterial contamination were analyzed with the same analytical protocol as a positive control for the method. In that perspective, GAC filters backwash water samples (~5 L) were collected from the Weesperkarspel treatment plant and sewage water (~12 L) was collected at the effluent of the Houten wastewater treatment plant. The GAC backwash samples were loaded with a high concentration of carbon sediments and were allowed to settle for 48 hours before collecting 200 mL of water at the top of the sediments with a pipet for performing the bacterial analysis. Those contaminated water samples were collected once at each location.

Bacterial enumeration

E. coli were enumerated by standard filtration procedure (0.45 μm) on duplicate LSA agar plates incubated for 24-hour at 37°C. *C. jejuni* was analyzed only for its presence or absence in the sample. Samples were filtered (cellulose nitrate filters, 0.2 μm) in duplicates; each filter was put into 10 mL of Preston broth in 15-mL plastic tubes and incubated at 42°C for 48 hours in semi-anaerobic conditions, using CampyGen™ oxygen catching bags. After incubation, 0.5-mL samples were taken from every tube to perform DNA extraction for PCR confirmation.

Table 1 | Parameters of the Beta-Poisson model

Microorganism	ID_{50}	α	Reference
Escherichia coli	8.3E3	0.22	Petterson *et al.* (2006)
Campylobacter jejuni	896	0.145	Haas & Eisenberg (2001)

Risk calculation

The annual probability of infection in drinking water consumers due to exposure to *E. coli* and *C. jejuni* internalized in zooplankton was calculated using Equation (1):

$$P_{\inf(annual)} = 1 - (1 - R)^n, \text{ with } n = 365 \text{ days}. \quad (1)$$

The daily risk of infection R is calculated by the Beta-Poisson model (Eq. 2):

$$R = 1 - (1 + (D/ID_{50}))^{-\alpha} \quad (2)$$

with the parameters ID_{50} and α described in Table 1.

The daily dose, D, is calculated as the product of the concentration of internalized pathogens in the drinking water (C_{IP_DW}), as determined through the environmental sampling and analytical protocol described above, times the volume of water ingested daily (V_d) (Eq. 3):

$$D = C_{IP\_DW} \times V_d \quad (3)$$

A mean daily consumption of drinking water of 1.6 L/d was used, based on data originating from an epidemiological study in the region of Montreal (Qc, Canada) (Payment *et al.* 1997).

RESULTS AND DISCUSSION

Zooplankton and bacterial enumeration

Figure 2 summarizes the zooplankton concentrations (average from duplicate sampling) found at every sampling point of each of the three water treatment plants selected in this study. Rotifers included members of the following genii: *Colurella, Keratella, Lecane, Polyarthra, Synchaeta, Rotaria, Filinia*, and *Brachionus*.

Bacterial enumeration in all of the environmental zooplankton samples analyzed resulted in no detection of either *E. coli* or *C. jejuni* in the non-sonicated and sonicated samples. This result provides an indication that internalized bacteria are probably occurring only scarcely

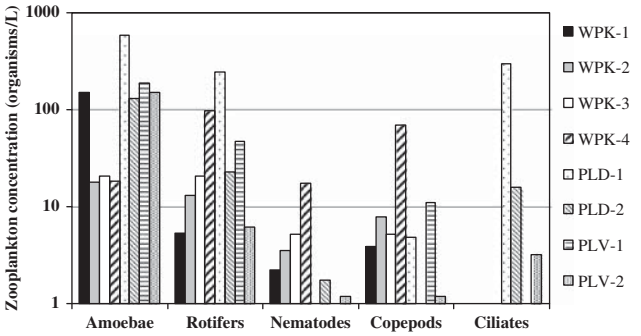

Figure 2 | Zooplankton concentration in environmental samples taken at three water treatment plants in the Netherlands. (WPK = Weesperkarspel; PLD = Leiduin; PLV = Loenderveen; sampling points are numbered accordingly to description in Figure 1). *Note: ciliated protozoa were not enumerated in the Weesperkarspel samples.*

in drinking water treatment plants. The zooplankton enumeration data provided from all samples analysis was used to assess the limit of detection for internalized bacteria in this study. The total number of zooplankton organisms contained in the sum of all water volumes sampled is presented in Table 2. A total number of $\sim 10^5$ zooplankton organisms (including amoebae), isolated from a total sample volume of 7.5 m^3, were disrupted during the sampling campaigns for the recovery of internalized bacteria. This represents an average concentration of ~13 organisms/L, which can be considered to fall within the normal range of values observed for zooplankton organisms in drinking water treatment plants. Typical rotifer concentrations were reported by Schreiber *et al.* (1997) to reach ~5.5 rotifers/L in GAC filtered water, while nematodes were found to occur in concentrations typically below 19 organisms/L, with a highest observed value of 71 nematodes/L. Loret *et al.* (2008) reported concentrations of amoebae ranging from <0.1 to 46 MPN/L in filtered water, with a mean value of ~4 MPN/L, whereas concentrations in the raw water could reach up to 4600 MPN/L in warm water (28.5°C). From the absence of detected bacteria in our samples, internalized *E. coli* and *C. jejuni* were estimated to occur in concentrations of < 1 bacteria in 10^5 zooplankton organisms in full-scale drinking water treatment plants. It is possible though that some smaller amoebae (<10 μm) were excluded from our zooplankton enumeration and from the analysis for internalized bacteria.

Positive control

Highly contaminated water samples (GAC backwash water and sewage) were analyzed with the same disruption protocol as a positive control for the detection of internalized bacteria in

Table 2 | Total estimated number of zooplankton organisms in the total volume of water (7.5 m^3) analyzed for the detection of internalized *E. coli* and *C. jejuni* in the environmental sampling campaign

Amoebae	Rotifers	Copepods	Nematodes	Ciliates	Total zooplankton organisms
3.1×10^4	3.8×10^4	2.3×10^4	7.0×10^3	1.7×10^3	1.0×10^5

zooplankton environmental samples. However, the protocol was found to be hard to apply in such matrices. Both contaminated waters had a high chlorine demand; therefore the chlorination step did not succeed in inactivating all of the external bacteria present in the samples. Therefore, for most samples, a significantly higher *E. coli* count could not be measured in the sonicated sample (disrupted zooplankton) as compared to the non-sonicated sample (intact zooplankton). In only one sewage sample, an indication of the presence of internalized *E. coli* was noted in the amoeba fraction of the sewage water (10–30 μm): *E. coli* was not detected in the chlorinated non-sonicated aliquot (i.e. <1 CFU in $\sim$430 amoebae), whereas low counts of *E. coli* were found in the sonicated aliquot ($\sim$0.005 CFU per amoeba ie. 1 CFU in $\sim$200 amoebae). No *C. jejuni* bacteria were detected in any of the backwash and sewage water samples analyzed. Wastewater and backwash water samples do not seem to be the most appropriate matrix for demonstrating the adequacy of the detection protocol used for internalized bacteria in this study. However, similar analytical protocols have been used in previous studies with conclusive results (King *et al.* 1991; Bichai *et al.* 2010b).

Risk estimates

A point risk estimate was calculated based on the detection limit of <1 internalized bacteria in 10^5 zooplankton organisms derived from our results presented above. Extreme concentrations of up to 71 nematodes/L were reported by Castaldelli *et al.* (2005) in the effluent water of full-scale GAC filters. Such a high zooplankton concentration in water can be considered as a worst-case scenario for the occurrence of internalized bacteria in field conditions. When considering this extreme zooplankton concentration in drinking water, the detection limit reported above leads to a calculation of <1 internalized bacteria in $\sim$1400 L or a concentration $C_{IP\_DW}<0.0007$ internalized bacteria per litre. Using Equation (3) and a drinking water daily consumption of 1.6 L, these numbers lead to a daily dose of 0.001 internalized bacteria. Equations (1)and (2) allow calculating an annual probability of infection of 9.2×10^{-6} and 5.9×10^{-5} associated to internalized *E. coli* and *C. jejuni*, respectively.

Bichai *et al.* (2010a) estimated a mean annual probability of infection of 2.9×10^{-6} and 2.5×10^{-6} associated to internalized *Cryptosporidium* and *Giardia* (oo)cysts in drinking water, respectively, with a possibility of lowering this risk by $\sim$2 orders of magnitude with an additional UV disinfection barrier. (Storey *et al.* 2004) calculated that the risk of infection due to *Legionella* associated to amoebae in drinking water could exceed the USEPA reference risk of 10^{-4} infection annually. However, due to the lack of reliable dose-response data for *Legionella*, this probability of infection was computed on the basis of the maximal risk curve, suggesting that inhalation of one *Legionella* cell by a water consumer would result in one infection. Those risk estimates highlight an important impact of the dose-response parameter used to describe the interaction between the pathogen and the human host.

In the environmental simulations by Bichai *et al.* (2010a), internalized *Cryptosporidium* and *Giardia* (oo)cysts were estimated to be found in filtered water in concentrations corresponding to about one (oo)cyst per 6.4×10^6 and 1.7×10^6 rotifers. Concentrations of internalized bacteria such as *E. coli* and *C. jejuni* can be suspected to be higher than that of internalized (oo)cysts in drinking water, due to typically higher concentrations of these free bacteria in natural waters, and also due to the capacity of virulent strains to multiply inside zooplanktonic hosts, including amoebae (Axelsson-Olsson *et al.* 2005; Alsam *et al.* 2006) and nematodes (Caldwell *et al.* 2003). However, it is important to point out that the concentrations of internalized bacteria used for the risk calculation were based on the detection limit of our environmental sampling protocol. Concentrations of internalized *E. coli* and *C. jejuni* bacteria in full-scale water systems could thus be lower than the concentration used for the risk estimates presented in this study. Additionally, concentrations of higher organisms in water can be thought to vary in a range of more than 3 orders of magnitude, as suggested by the variations between zooplankton concentrations in the effluent water of GAC filters (Schreiber *et al.* 1997), which would also influence the calculation of the risk associated to internalized *E. coli* and *C. jejuni* as presented above. To capture the variability of many of the variables involved in the risk calculation associated to internalized *E. coli* and *C. jejuni*, additional data should be collected and probability

distribution functions could be used to describe each variable in Monte-Carlo simulations, as was done in a formal risk assessment by Bichai *et al.* (2010a) for internalized (oo)cysts.

The possibility remains that some steps of the analytical protocol used to detect internalized bacteria in this study could have negatively impacted the recovery of the microbial targets. In fact, previous testing demonstrated that ultrasonication could partially inactivate free *E. coli* bacteria, with a reduction in bacterial plate counts of ~24% in 30 seconds at 65% amplitude. It is assumed that internalized bacteria are protected against sonication as long as they are inside their host. In fact, sonication was used successfully in previous studies to recover bacteria from the gut of nematodes in laboratory co-cultures (Ding *et al.* 1995; Caldwell *et al.* 2003; Kenney *et al.* 2004, 2005; Bichai *et al.* 2009) and from environmental zooplankton organisms (King *et al.* 1991; Locas *et al.* 2007). However, if some internalized bacteria were released from the hosts before the end of the exposure to ultrasound, they could have been altered and possibly not detected by subsequent culture methods. Assuming relatively low concentrations of *E. coli* and *C. jejuni* in environmental samples, as compared to lab experiments using high concentrations of bacteria fed to zooplankton predators, the effect of sonication on bacterial recovery could potentially explain the undetected internalized bacteria in this study. The fact that the highly contaminated water samples used for a positive control were not well suited for our detection protocol did not allow confirming conclusively the efficacy of the method for enumerating internalized bacteria in environmental samples.

It is recommended that further investigation be undertaken to better characterize the occurrence of internalized pathogenic microorganisms in drinking water in full-scale environmental systems, with a focus on strategic sampling locations, where proliferation of zooplankton and contamination by pathogenic bacteria are suspected. Moreover, we suspect the environmental frequency distribution of internalized bacteria to be normal but skew because of their probable clustering, which would be accentuated in the case of bacterial multiplication inside zooplanktonic hosts. Occurrence of internalized bacteria can also be thought to be impacted by temperature. In this study, all samples were taken in the early spring. For a more reliable view on the occurrence of internalized bacteria, it may be advisable to collect smaller volume samples more frequently over one complete year, with larger volume sampling at critical times. Such a campaign design would increase the chances of detection of the internalization phenomenon, which is most likely rare and unevenly spread. Developing an analytical protocol using microscopy (visual) detection methods for internalized pathogens may reinforce this type of investigation by avoiding the need for mechanical disruption of the zooplankton isolates, therefore probably lowering the risk of damaging the bacteria by sonication. The microbial risk associated to zooplankton-internalized microorganisms in drinking water can be thought to vary greatly among pathogenic species due to their different behaviour inside zooplanktonic hosts and to the variety of dose-response relationships with the human host.

CONCLUSIONS

This study describes environmental sampling activities conducted to detect internalized *E. coli* and *C. jejuni* bacteria in zooplankton and amoebae samples collected at various stages of three water treatment plants in Amsterdam, the Netherlands. No internalized *E. coli* or *C. jejuni* bacteria were recovered from the samples analyzed in this study, corresponding to a total number of $\sim 10^5$ zooplankton organisms isolated from 7.5 m^3 of water. Highly contaminated water samples, such as GAC filters backwash water and sewage, were used to obtain a positive control for our detection protocol. These two water matrices did not prove well suited for the application of our detection protocol due to the high concentration of particles and high chlorine demand. In one sewage sample, an indication of the presence of internalized *E. coli* in amoebae was found. The calculated detection limit of our sampling plan revealed that the occurrence of internalized *E. coli* or *C. jejuni* bacteria in drinking water was lower than one internalized bacteria in 10^5 zooplankton organisms. When considering extreme zooplankton concentrations reported in the literature, risks of infection of 9.2×10^{-6} and 5.9×10^{-5} associated to internalized *E. coli* and *C. jejuni* in drinking water were estimated, respectively. Characterizing the microbial risk associated to higher organisms in drinking water will require further research activities aiming at detecting pathogenic or indicator microorganisms internalized by environmental zooplankton in full-scale water systems.

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