REVIEW / SYNTHÈSE

Protection of waterborne pathogens by higher organisms in drinking water: a review

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Abstract: Higher organisms are ubiquitous in surface waters, and some species can proliferate in granular filters of water treatment plants and colonize distribution systems. Meanwhile, some waterborne pathogens are known to maintain viability inside amoebae or nematodes. The well-documented case of *Legionella* replication within amoebae is only one example of a bacterial pathogen that can be amplified inside the vacuoles of protozoa and then benefit from the protection of a resistant structure that favours its transport and persistence through water systems. Yet the role of most zooplankton organisms (rotifers, copepods, cladocerans) in pathogen transmission through drinking water remains poorly understood, since their capacity to digest waterborne pathogens has not been well characterized to date. This review aims at (*i*) evaluating the scientific observations of diverse associations between superior organisms and pathogenic microorganisms in a drinking water perspective and (*ii*) identifying the missing data that impede the establishment of cause-and-effect relationships that would permit a better appreciation of the sanitary risk arising from such associations. Additional studies are needed to (*i*) document the occurrence of invertebrate-associated pathogens in relevant field conditions, such as distribution systems; (*ii*) assess the fate of microorganisms ingested by higher organisms in terms of viability and (or) infectivity; and (*iii*) study the impact of internalization by zooplankton on pathogen resistance to water disinfection processes, including advanced treatments such as UV disinfection.

Key words: drinking water, pathogen vectors, amoebae, nematodes, zooplankton.

Résumé : Les organismes supérieurs sont omniprésents dans les eaux de surface et certaines espèces peuvent proliférer dans les filtres granulaires des usines de traitement d'eau et coloniser les réseaux de distribution. D'autre part, certains microorganismes pathogènes hydriques peuvent demeurer viables à l'intérieur d'amibes ou de nématodes. Le cas bien connu de la réplication de *Legionella* à l'intérieur d'amibes n'est qu'un exemple de microorganisme pathogène pouvant être amplifié à l'intérieur des vacuoles d'un protozoaire et ainsi, bénéficier de la protection d'une structure résistante favorisant son transport et sa survie à travers les systèmes d'eau potable. Toutefois, le rôle du zooplancton (rotifères, copépodes, cladocères) dans la transmission de microorganismes pathogènes par l'eau potable est méconnu, car leur capacité à digérer les microorganismes pathogènes n'a pas été bien caractérisée jusqu'à présent. Cet revue de littérature a pour but (*i*) d'évaluer les observations scientifiques de diverses associations entre des organismes supérieurs et des microorganismes pathogènes dans un contexte d'eau potable et (*ii*) d'identifier les données manquantes empêcheant d'établir des relations de cause à effet qui pourraient permettre de mieux évaluer le risque sanitaire émergeant de telles associations. Des études supplémentaires sont nécessaires afin de (*i*) documenter l'occurrence des pathogènes associés aux invertébrés dans des conditions de terrain pertinentes comme les réseaux de distribution, (*ii*) évaluer le sort des microorganismes ingérés par les organismes supérieurs en termes de viabilité et/ou d'infectivité, et (*iii*) étudier l'impact de l'internalisation par le zooplancton sur la résistance des microorganismes pathogènes face aux processus de désinfection de l'eau, incluant les traitements avancés comme la désinfection UV.

Mots-clés : eau potable, vecteurs de microorganismes pathogènes, amibes, nématodes, zooplancton.

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Introduction and objectives

The efficiency of disinfection processes in drinking water treatment is influenced to various degrees by the characteristics of the water (temperature, pH, particle counts, etc.) and the physiologic state of the microorganism being targeted. Rather recently, research has focused on the study of microorganisms in their most common natural form, that is as aggregates or as part of a collective structure known as a biofilm, which confers them further resistance to disinfection (Morin et al. 1997; Storey et al. 2004*a*; Mamane-Gravetz and Linden 2005; Matz and Kjelleberg 2005; Mahmud et al. 2006; Chevrefils et al. 2007).

The protection offered to pathogenic microorganisms located inside superior organisms, such as zooplankton organisms (including protozoa) and certain benthic invertebrates, is also a natural protection mechanism used by waterborne pathogens. The protection against disinfectants offered by internalization has not been widely studied. Protection can be the result of symbiotic or parasitic associations between pathogenic microorganisms and higher organisms (Greub and Raoult 2004). The viability of the pathogenic microorganisms that have been ingested without being digested or biodegraded by their predators can be maintained (Barker and Brown 1994). This resistance to digestion has been reported numerous times in amoebae (Barker and Brown 1994; Winiecka-Krusnell and Linder 1999; Greub and Raoult 2004) and nematodes (Chang et al. 1960*a*; Caldwell et al. 2003; Gibbs et al. 2005) but has not been reported for most zooplankton organisms (rotifers, copepods and cladocerans), even though the study of their predation and grazing activities under diverse conditions has been widely documented.

Higher organisms are ubiquitous in natural and man-made water environments. Planktonic species are commonly found in surface waters, where their occurrence is a function of ecological factors, such as seasons, temperature, and depth (Pinel-Alloul et al. 2002), and they are part of a complex trophic network in which feeding habits are influenced by various physical and biological factors. (The reader is referred to Pernthaler (2005) for a global and ecological view of the trophic interactions between the numerous members of the aquatic microfauna.) Although for the most part, invertebrates are intercepted and eliminated during sedimentation, some can reproduce inside the plant and liberate eggs and larvae into the distribution system (Levy et al. 1986). Rotifers and nematodes abundantly colonize granular and biological filters, which constitute ideal media for the proliferation of benthic invertebrates (Lupi et al. 1994; Schreiber et al. 1997; Castaldelli et al. 2005). They are often released into the filter effluents (Matsumoto et al. 2002) and into the distribution systems. Investigations conducted on several drinking water distribution systems have confirmed the abundance of invertebrates (Chang et al. 1960*b*; Van Lieverloo et al. 1998), while amoebae are known to proliferate in the biofilms.

This review discusses the known associations between microorganisms and different groups of higher organisms and presents a critical analysis of research needs, with a specific focus on managing the microbial risk to drinking water. We will present higher organisms in 3 groups based on ecological characteristics. The zooplankton organisms differ from benthic species in that they are found in suspension in surface waters and move themselves more or less passively with the currents. As benthic invertebrates, nematodes are usually the object of separate studies, although they are also considered as a permanent part of the aquatic microfauna in surface waters (Lupi et al. 1995). Zooplankton, as studied by limnologists, is typically subdivided into 4 groups: protists (including protozoa and heterotrophic flagellates), rotifers, copepods, and cladocerans; the 2 last groups being known as crustacean zooplankton (Wetzel 2001). In the context of this review, protists will be presented in the first section as a separate group from the rest of zooplankton organisms — being unicellular organisms, the study of pathogen internalization by protists is of a different nature because it involves intracellular mechanisms, as opposed to the rest of zooplankton organisms, which are pluricellular and possess a more complex digestive system. From a microbiological point of view, we will see that this difference is of major significance, in an attempt to characterize the fate of internalized microorganisms and the microbial risk that they might confer to drinking water. The second part of this review will focus on rotifers and crustacean zooplankton, and the last part will discuss nematodes as pathogens vectors.

Protists: the Trojan Horse of microorganisms

The use of the term "protist" (a unicellular eukaryotic organism) in this section is associated with the expression "Trojan Horse" in the literature; however, here we are referring more specifically to protozoa, i.e., protists behaving as animals (with heterotrophic feeding). In fact, according to the information gathered from the literature, plant protists, for example diatoms, are not included in the group of organisms referred to as the Trojan Horses of microorganisms, even though certain vegetable protists exhibit bacterivorous behaviour, such as flagellate algae (see Nygaard and Tobiesen (1993) for example).

Amoebae

Survival of microorganisms inside amoebae

Protozoa, especially amoebae, have been qualified as the Trojan Horse of the microbial world (Barker and Brown 1994). Amoebae are recognized as being both reservoirs and vehicles of pathogenic microorganisms in the environment, as well as serving as a "crib" (term used by Greub and Raoult (2004)), i.e., an evolutionary incubator that favours adapting to life within human macrophages, and therefore favours pathogenesis. The reader should refer to the Barker and Brown (1994) article on the impact of predation by protozoa on the survival of pathogenic bacteria in the environment and to the article by Greub and Raoult (2004) on amoeba-resistant microorganisms, mainly bacteria but also viruses.

Free-living amoebae generally have 2 stages of development: the trophozoite and the cyst (Greub and Raoult 2004). The trophozoite is the active metabolic stage, feeding on bacteria and multiplying by binary fission. Hostile pH conditions, osmotic pressure, temperature, or even unfulfilled nutritional needs of the amoeba can cause its encystment (Greub and Raoult 2004). Cysts generally have 2 layers, which make their structure very resistant to most

chemical disinfectants, such as chlorine (Greub and Raoult 2004), and which confer them an ability to survive dessication and temperatures between –20 °C and +42 °C (Kahane et al. 2001). When the conditions become favourable once again, there is excystation and a return to active life. Viable bacteria have been observed in trophozoites and in free amoeba cysts (Winiecka-Krusnell and Linder 2001). It is noteworthy to mention that cyst formation is a mechanism common to many protozoa and is not exclusive to amoebae.

In water, free-living amoebae often live in biofilms and in water–earth, water–air, and water–plant interfaces, since feeding in most species occurs in association with surfaces and particulate matter suspended in water (Greub and Raoult 2004). Amoebae, like other protozoa, feed mainly on bacteria, many of which are able to survive following ingestion by amoebae. The most well-known example in the field of drinking water is without a doubt *Legionella pneumophila*, responsible for numerous cases of respiratory illness throughout the world. Survival and transmission of *Legionella pneumophila* to humans is strongly linked to the presence of amoebae in water, since free-living amoebae favour the multiplication of *Legionella pneumophila* in aquatic biofilms and the transport of the bacteria (Greub and Raoult 2004). Intracellular growth inside amoebae was demonstrated to most likely be the only way for *Legionella pneumophila* to proliferate within aquatic biofilms on plasticized polyvinyl chloride in a batch system (Kuiper et al. 2004).

Microorganisms that resist ingestion by amoebae and other protozoa can be divided into 3 groups: those that multiply and cause cellular lysis in amoebae, such as *Legionella* spp. and *Listeria* spp.; those that multiply within the amoeba without causing cellular lysis, such as *Vibrio cholerae*; and those that survive within the amoeba without multiplying, such as certain coliforms and mycobacteria (Barker and Brown 1994). Furthermore, Greub and Raoult (2004) have identified a group of bacteria called LLAP (*Legionella*-like amoebal pathogens), which includes bacteria that are able to cause lysis in the amoeba carrying them in the same manner as *Legionella* spp. do. This group of bacteria is attracting more and more attention due to public health concerns.

Thus, besides predator–prey relationships, cases of parasitism and even endosymbiosis have been observed in certain bacteria or viruses that survive following ingestion by amoeba and avoid being digested. This endosymbiosis can take place initially as a survival strategy adopted by a microorganism facing hostile conditions or physical variations in its environment (Winiecka-Krusnell and Linder 2001). This type of association is not only of considerable importance for the stabilization of infectious agents in the environment but can also increase the potential virulence of bacteria that can evolve to become highly adapted to intracellular growth (Barker and Brown 1994). In fact, certain bacteria, including *Legionella* sp., *Listeria monocytogenes*, or *Mycobacterium avium*, have adapted to living inside human macrophages following exposure to environmental predators such as free-living amoebae (Greub and Raoult 2004). For example, in both macrophages and amoebae, the survival of *Legionella* is characterized by the absence of phagosome–lysosome fusion, which somehow impedes the cell's digestion of the bacterium, and in both cases (macrophages and amoebae), *Legionella* leads to cellular lysis (Greub and Raoult 2004). Hence, it is probable that *Legionella*, like some other intracellular pathogens, has evolved thanks to its association with protozoa in the natural environment in such a way that it has acquired the ability to infect humans or other animals. However, despite survival of bacteria within protozoa often being associated with their pathogenesis, it is noteworthy that protozoa, including amoebae, can also serve as reservoirs of environmental bacteria, such as nonpathogenic coliforms (King et al. 1988), and can protect them from hostile environmental conditions or chlorination.

Literature contains many reports of laboratory experiments in which human pathogenic bacteria were maintained in coculture with various species of *Acanthamoebae*. In most of these studies, bacteria were observed to maintain their viability and to multiply inside the amoeba's feeding vacuoles. For instance, *Helicobacter pylori* was found to preserve its viability and to proliferate inside *Acanthamoebae castellanii* for up to 8 weeks in coculture (Winiecka-Krusnell et al. 2002). Intact and metabolically active bacteria were observed in amoebae vacuoles, and a 2-log increase in *H. pylori* bacterial count was observed after 7 days of coculture with *A. castellanii*. Interestingly, when in coculture for 1 week with various species of *Listeria*, *A. castellanii* was observed to undergo cell lysis and release viable bacteria of *Listeria monocytogenes* and *Listeria seeligeri*, 2 haemolytic species, whereas *Listeria innocua*, a nonpathogenic species, was not freed from the amoeba (Ly and Müller 1990). In similar experiments, amoeba encystment occurred by day 8 of incubation of *Listeria monocytogenes* with *A. castellanii* (Ly and Müller 1990). After 34 days of coculture, almost all amoebae were found to be in cyst form, inside of which *Listeria monocytogenes* had lost its viability. Three serotypes of *Salmonella enterica* (serovar Dublin, Enteritidis, and Typhimurium) were shown to reside and replicate within intracellular vacuoles of *Acanthamoeba rhysodes* (Tezcan-Merdol et al. 2004). A prolonged incubation of the *Salmonella* sp. and *Acanthamoeba* sp. coculture resulted in a gradual change in the morphology of the host cells until they eventually disappeared. *Simkania negevensis*, associated with respiratory illnesses in humans, was reported to infect *Acanthamoeba polyphaga*, survive, and reproduce within the trophozoite vacuoles, as within human cell cultures (Kahane et al. 2001). Furthermore, exposure to hostile conditions caused amoeba encystment, and a certain competition was then observed between the amoeba and the bacteria for survival, resulting in 3 possible behaviours: cysts containing both normal cytoplasm and *S. negevensis*, cysts containing bacteria but without cytoplasm, or finally, the bacteria were found located between the 2 cyst walls. After 79 days at 4 °C, the *S. negevensis* bacteria caught inside the cysts had preserved 56% of their infectivity, whereas free bacteria (the control sample) had not survived 12 days of exposure to the same temperature. In addition, a small proportion of the bacteria (0.3% of the initial infectivity) had survived as long as 21 weeks (148 days) inside the cysts at room temperature. In the drinking water industry, it is most relevant to draw specific attention to those bacteria that show an ability to survive and retain infectivity inside the amoebal cysts, since (oo)cysts are resistant enough to suc-

cessfully penetrate and persist through the various steps of water treatment plants.

The ability of 26 species of water-related mycobacteria to survive inside trophozoites and cysts of *A. polyphaga* was assessed in a recent study (Adékambi et al. 2006). All species studied showed the ability to penetrate into trophozoites and cysts, where they could survive more than 5 or 15 days, respectively. *Campylobacter jejuni* was also shown to infect *A. polyphaga* in vitro at different temperatures typically found in natural waters (Axelsson-Olsson et al. 2005). In fact, aggregation of a great number of motile and active bacteria was observed within vacuoles of the amoeba. The spontaneous rupture of the amoeba allowed the detection of *C. jejuni* by microscopy and by culture. Further studies are required to verify whether (*i*) amoeba infection by *C. jejuni* can occur naturally in the environment and (*ii*) if bacteria that survive in the amoeba are able to infect a vertebrate host (Axelsson-Olsson et al. 2005). Investigation on these aspects was actually found to be lacking from most studies about intracellular replication of bacterial pathogens in amoebae. In fact, these studies are crucial in understanding the ecology of pathogen intracellular survival inside protozoan hosts. They also provide qualitative information on the potential microbial risk. However, very few studies have reported the occurrence of infected amoebae in natural or man-made environments. This information is needed to better discriminate which pathogens are really associated with an increased risk of transmission to humans when amoebae are present in drinking water treatment or distribution systems. Furthermore, with respect to risk quantification, the laboratory experiments are not sufficient, since coculture assays most likely overestimate the number of bacterial pathogens associated with amoebae in comparison with what would be found under field conditions.

Even though the role of amoebae as bacterial reservoirs has long been known, T.J. Robotham was the first researcher, in 1980, to shed light on the role of amoebae as a vector of *Legionella*, which would be favourable for *Legionella* propagation in drinking water systems as well as in humans (Greub and Raoult 2004). Amoebae digestive vacuoles containing bacterial cells following ingestion can be expulsed from the protozoa, which often precedes encystment (Berk et al. 1998). Expelled vacuoles are called vesicles (Brandl et al. 2005). In the case of *Legionella*, a single vesicle can contain up to 10^4 bacteria, according to the calculations of Robotham (1986), while the infectious dose for humans is assumed to be very low. When *A. polyphaga* and *A. castellanii* expelled vesicles that contained viable *Legionella pneumophila* cells, it was found that more than 90% of these vesicles were small enough to be inhaled, i.e., less than 5 μm in diameter (Berk et al. 1998), which supports the hypothesis that humans contaminated with *Legionella* sp. would possibly have inhaled a vesicle that was filled with the bacteria rather than free bacteria (Greub and Raoult 2004). Moreover, these vesicles contained viable bacteria despite a 24 h exposure to biocides used in cooling towers (Berk et al. 1998). The bacteria aggregated together within these vesicles and did not disperse despite freezing and thawing treatments (–70 °C and 35 °C) and ultrasound. The vesicles remained intact following these treatments, contrary to the trophozoites that were completely destroyed under the same conditions (Berk et al. 1998). *Acanthamoeba polyphaga* was shown to produce up to 25 vesicles in 24 h under certain conditions, following *Legionella pneumophila* ingestion (Berk et al. 1998). These vesicles were found to be free in solution. Considering that amoebae, in the trophozoite or cyst stages, usually adhere strongly to a physical substrate, this observation suggests that these vesicles would further facilitate the bacterial dissemination as aerosols rather than the amoebae themselves (Berk et al. 1998).

In summary, these studies reveal many interesting points regarding amoebae as a risk factor in water systems. Amoebae (*i*) favour the replication of human pathogens inside their digestive vacuoles; (*ii*) increase the survival time and resistance to harsh conditions of those protected pathogens; (*iii*) can enhance the potential virulence of those pathogens by favouring adaptation to intracellular survival and growth; (*iv*) favour the transport of pathogens inside vesicles, which are resistant to extreme temperatures and are found free in solutions; and (*v*) favour the transmission of pathogenic bacteria to humans by inhalation of vesicles, considering that a single vesicle can contain the human infectious dose. In terms of risk of transmission to humans, the study of bacterial survival within amoebal cysts and vesicles, considering their higher resistance to harsh external conditions, is more important than the study of their survival within trophozoites and, therefore, deserves more attention in future research work.

Protection of microorganisms ingested by amoebae against water treatment

After having been shown to ingest various species of *Legionella*, 2 species of *Acanthamoeba*, *A. castellanii* and IA (an environmental thermotolerant *Acanthamoebae* isolate), of clinical and environmental origin, respectively, were shown to increase by 1–2 logs the survival of the intracellular bacteria *Legionella erythra* and *Legionella pneumophila* against thermal treatment (with temperatures varying from 40 to 80 °C), as compared with planktonic bacteria under the same conditions (the control sample) (Storey et al. 2004*b*). *Legionella erythra* and *Legionella pneumophila* were found inside the amoebae's vacuoles. However, both species of *Legionella* were more easily disinfected by free and combined chlorine when in contact with *Acanthamoeba* spp. than when in their planktonic state. This surprising result is perhaps due to the ability of *Legionella*, just like other non-spore-forming bacteria to adopt a superior state of resistance in reaction to hostile conditions (as would be the case with the planktonic *Legionella* in the control sample), while the opposite situation could occur with the protected bacteria because their intensified metabolic state within the amoeba could render them more vulnerable to oxidation (Storey et al. 2004*b*). These observations call for more intense investigation, since they contradict the usual conclusions reported in the literature (King et al. 1988; Barker and Brown 1994).

Besides *Legionella* spp., other pathogenic bacteria, such as *Salmonella typhimurium*, *Yersinia enterocolitica*, *Shigella sonnei*, and *Campylobacter jejuni*, as well as environmental coliforms, including *Escherichia coli*, have the ability to survive following ingestion by *Acanthamoeba castellanii.* This association was shown to increase by 30- to 120-fold the re-

sistance of all these bacteria to free-chlorine residual. The ingested bacteria survived exposure to chlorine doses that were well above the dose necessary to inactivate free-living cells of the same bacteria by 2 logs (King et al. 1988). One to 3 bacteria per vacuole were found in most of the amoebae following coculture with each of the bacteria. Furthermore, various species of water-related mycobacteria maintained in coculture with *A. polyphaga* were shown to survive a 24 h exposure to 15 mg/L of free chlorine while protected inside the cysts (Adékambi et al. 2006).

In summary, certain types of interaction between pathogenic bacteria and amoebae offer significant protection to intracellular bacteria against chemical disinfectants, besides favouring their multiplication and transport and increasing their virulence potential. As these interactions have been well documented, additional work is needed to fulfill the lack of quantitative information, which impedes a rigorous quantitative assessment of the risk factors associated with amoebae harbouring pathogens in drinking water systems. An improved risk characterization could potentially influence the disinfection strategies adopted in some drinking water treatment systems. This issue will be further discussed later in the text.

Ciliated protozoa

Survival of microorganisms inside ciliated protozoa

Apart from amoebae, other protists are known to favour the survival of pathogenic microorganisms in the environment, those being mainly the ciliated protozoa of the *Tetrahymena* or *Cyclidium* genera. The relationships are often comparable to those observed in amoebae. *Tetrahymena pyriformis*, an aquatic and bacterivorous ciliated protozoan that feeds by filtration, has been widely used in coculture studies. *Legionella pneumophila* and *Listeria monocytogenes* ingested by *Tetrahymena* multiply within the host and cause cellular lysis (Barker and Brown 1994). After 8–15 days of coculture, *T. pyriformis* lysis led to viable *Listeria monocytogenes* being freed (Ly and Müller 1990). The same phenomenon was observed with *Listeria seeligeri*, while the contrary occurred with the nonpathogenic and nonhaemolytic *Listeria innocua*, where very few host cells underwent lysis (Ly and Müller 1990), despite *T. pyriformis* being just as densely colonized (6×10^3 to 9×10^3 bacteria per cell) as it was with the 2 other species of *Listeria*. A relatively constant coexistence of *Listeria innocua* and *T. pyriformis* populations was observed for 5 weeks, with the majority being intracellular bacteria. Conversely, in the presence of *Listeria monocytogenes* and *Listeria seeligeri*, *T. pyriformis* completely disappeared after about 10 days of incubation, which was followed by the presence of a free *Listeria* population, which declined up until its disappearance at the end of 5 weeks (Ly and Müller 1990). These results suggest that *Listeria monocytogenes* and *Listeria seeligeri* would parasitize *T. pyriformis*, whereas *Listeria innocua* would seem to simply resist digestion by the ciliate without causing its lysis.

Campylobacter was shown to survive inside *T. pyriformis* and *A. castellanii.* When incubated in the presence of both protozoa, the survival of *C. jejuni* was increased by 36 h compared with that of planktonic bacteria (Snelling et al. 2005). However, the presence of *T. pyriformis* did not significantly delay the decline in viability of *Campylobacter coli*, whereas coculture with *A. castellanii* delayed it by about 24 h, suggesting that the relationship between *Campylobacter* spp. and protozoa is species specific.

A comparison of the ingestion of *Salmonella enterica* serovar Thompson and *Listeria monocytogenes* by *Tetrahymena* sp. led to the liberation of numerous vesicles containing viable *Salmonella* cells, whereas ingestion of *Listeria* by the protist resulted in their digestion. The expulsion of vesicles containing *Listeria* was infrequent (Brandl et al. 2005). Up to 50 *Salmonella* cells per vesicle expelled by *Tetrahymena* were counted. This number increased with the initial ratio of bacteria to protozoa cells in coculture. The difference in the ways these 2 bacteria interact with the protozoa was probably not simply due to the difference in the nature of their cell walls (Brandl et al. 2005), since *Enterococcus avium*, a gram-positive bacterium, just like *Listeria monocytogenes*, resisted being digested by *Tetrahymena* sp. The authors suggest that *Salmonella* serovar Thompson is able to alter the normal sequence of events linked to digestion in the *Tetrahymena* digestive vacuoles by stopping the fusion of the phagosome to the lysosome, for example, in the same way as *Legionella* resists digestion inside amoebae and human macrophages.

Various ciliated protozoa were shown, in laboratory conditions, to ingest *Cryptosporidium* oocysts (Stott et al. 2001). Ingestion rates were observed to (*i*) vary in time, (*ii*) generally increase according to prey concentration, and (*iii*) vary significantly from one species of ciliated protozoa to another, the most efficient protozoa studied being *Paramecium caudatum*, which could ingest up to 170 oocysts/h. Furthermore, a relationship between the average number of oocysts ingested and the average size of the protozoa was proposed. Even though the fate of ingested oocysts was not determined and despite the short test time of protozoa feeding (1 h), the authors suggested the possibility of some ingested oocysts being digested or excreted by their predators. In fact, immunofluorescence assays made it possible to detect fragmented oocyst cell walls inside of protozoa digestive vacuoles, and certain *Stylonychia mytilus* were observed to excrete particle debris containing many oocysts whose viability has not been determined. The authors are aware that their laboratory results are probably not representative of natural phenomena, since ingestion of oocysts by protozoa in the environment can depend on their feeding habits, population diversity and density, exposure time, and oocysts distribution. It was observed that when the ciliate *P. caudatum* was exposed to 90 or 9000 *Cryptosporidium* oocysts for 20 min, an individual protozoa ingested 1.38 or 26.7 oocysts on average, respectively (Stott et al. 2003). After 1 h of exposure to the highest oocyst level, the number of oocysts ingested by *P. caudatum* was repeatedly found to be higher than the human infectious dose of 30 oocysts.

Contrary to natural water studies carried out by ecologists, the studies that describe the ingestion ability of zooplankton organisms under artificial conditions have the advantage of supplying precious information on ingestion of waterborne pathogens, which are the main focus of researchers in the field of drinking water treatment, whose priority is to reduce health risks. However, it is important to note that the experimental conditions are often very different from the condi-

tions prevailing in natural aquatic environments. More specifically, the disproportion of microorganism densities in these laboratory tests compared with the natural concentrations in aquatic ecosystems is evident. In addition, none of the reported studies using *Cryptosporidium* as a food source for higher organisms have investigated the viability and infectivity of the oocysts after their ingestion or excretion. Future studies should include such an investigation, since this aspect is significant in assessing the potential associated health risk.

Protection of microorganisms ingested by ciliated protozoa against water treatment

Just like *Acanthamoeba* spp., *T. pyriformis* ingests coliform bacteria as well as the pathogenic bacteria *Salmonella typhimurium, Yersinia enterocolitica*, *Shigella sonnei*, *Legionella gormanii*, and *Campylobacter jejuni*, which survive within the cell where they are protected against chlorination (King et al. 1988). The resistance of all these pathogenic bacteria against chlorination was observed to be more than 50 times higher when ingested by *T. pyriformis.* For the sake of comparison, a freshwater environmental protozoan of the genus *Cyclidium* was isolated and the contact time with chlorine that was necessary for *E. coli* to be inactivated logarithmically by a factor of 2 was even greater than with *T. pyriformis*. It was also found that internalization of *Campylobacter* by *T. pyriformis* and *A. castellanii* significantly increased its resistance to a chemical disinfectant widely used in the poultry industry (Snelling et al. 2005). Coculture of *Salmonella enterica* serovar Thompson with *Tetrahymena* showed that expelled vesicles containing viable bacteria offered a significant protection for *Salmonella* against a free-chlorine treatment of 4.2 (mg·min)/L, as the average proportion of bacteria surviving the treatment when located inside a vesicle was 4.6-fold higher than that of free bacteria (Brandl et al. 2005).

In summary, additional studies are needed to quantify the increase of pathogen resistance to disinfection, including UV treatment, as most studies relied on free chlorine. These studies can be performed in artificial conditions in research laboratories. However, the challenge resides in a proper assessment of the natural occurrence of this phenomenon in surface waters and in distribution systems. This highlights the importance of having ecologists and limnologists collaborating with water engineers on such an issue, so that pathogenic microorganisms receive specific attention when characterizing (quantitatively) trophic relationships in natural aquatic environments.

Rotifers and crustaceans

Survival of microorganisms inside zooplankton

A few rare studies have attempted to characterize the survival of microorganisms ingested by zooplankton organisms other than protists. Even less frequent are such studies performed in natural conditions. Nevertheless, the use of rotifers was suggested as a *Cryptosporidium* oocyst detection tool in Polish lake waters and made it possible to detect viable oocysts contained within rotifers in each of the 3 lakes sampled (Nowosad et al. 2007). This is probably the first record of the ability of *Cryptosporidium* oocysts to survive inside zooplankton organisms in natural waters. However, the oocysts' infectivity has not been verified. We note that in natural conditions, it is common to find densities of 200–300 rotifers per litre and occasionally up to 1000 individuals per litre (Wetzel 2001).

Most studies found in the literature about zooplankton grazing in natural conditions characterize their grazing rates on various species in the microbial community or the impact of a zooplankton species population on other planktonic organism concentrations. However, these studies usually do not provide any information on zooplankton grazing on waterborne pathogens. This is why laboratory experiments are necessary to understand the significance of higher organisms in the context of drinking water. For instance, laboratory experiments were conducted to investigate the fate of *Cryptosporidium parvum* oocysts and *Giardia lamblia* cysts ingested by rotifers and daphnia. Under artificial conditions, 20 000 *C. parvum* oocysts were exposed to populations of 10–20 individuals of 6 rotifer species (Fayer et al. 2000), which were all observed to ingest oocysts. Up to 25 oocysts were found inside *Philodina* rotifers, with the majority containing about 15 oocysts. *Euchlanis triquetra* and *Epiphanes brachionus* rotifers were observed excreting aggregates containing up to 8 oocysts about 15 min after the beginning of exposure of the rotifers to oocysts. (It is known to take between 3 and 20 min, depending on the rotifer species and the environmental conditions, for a particle ingested by a rotifer to move along its entire digestive tract (Wetzel 2001).) Other rotifer species seemed to keep the oocysts internally for the entire duration of the microscopic observation. However, there is no report of oocysts being degraded, digested, or inactivated inside rotifers following ingestion, as it is not known whether or not rotifers have any enzymes that can digest the proteins that form the cell wall of the oocysts (Fayer et al. 2000). The rotifer enzymes identified to date mainly digest carbohydrate substrates. This study consisted of artificially exposing rotifers to *C. parvum* oocysts. The authors did not determine whether or not rotifers ingest oocysts in nature, which, however, has been recently shown by Nowosad et al. (2007). Assays by Stott et al. (2003) also showed that following a 2 h exposure, rotifers ingested an average of 1.6 oocysts per individual, with the maximum observed being 7 oocysts. It is important to note that again, in this study, predators were exposed to oocyst concentrations in the order of 10^4 to 10^6/mL, while typical concentrations in drinking water are less than 0.001 oocyst/mL (Brookes et al. 2004). Oocysts were also seen in rotifer fecal matter after 145 min, but oocyst viability following ingestion was not determined (Stott et al. 2003). No oocyst accumulation inside predators was observed during the assay period. According to the authors, the impact of higher organism predation could potentially reduce the presence of *Cryptosporidium* oocysts in the environment, but zooplankton organisms could also become reservoirs and vectors, therefore favouring transmission of *Cryptosporidium*. When 7 species of rotifers were exposed to high concentrations of *Giardia* cysts, 5 species ingested the cysts in variable quantities. The cysts remained within their bodies for the entire observation period of 20 min (Trout et al. 2002). In general, rotifers ingested smaller quantities of *Giardia* cysts than *Cryptosporidium parvum* oocysts, which the authors hypo-

thetically attribute to *Giardia* cysts being 3–4 times larger than oocysts and their surface possibly having different characteristics. No species of rotifer seems to have excreted *Giardia* cysts. It is not known whether or not the cysts were digested. As these studies were done in artificial conditions, the authors emphasize that it has not been determined whether or not rotifers would ingest *Giardia* cysts in a natural environment. More in-depth studies would be required to (*i*) quantify the probability that rotifers ingest *Cryptosporidium* or *Giardia* (oo)cysts in natural conditions and (*ii*) determine the impact of ingestion (and defecation) on the viability and infectivity of (oo)cysts.

With regard to crustaceans, studies concerning the survival of ingested microorganisms seem to be limited mainly to one single host, which are cladocerans of the genus *Daphnia*. A recent study by Connelly et al. (2007) describes the effect of grazing by *Daphnia pulicaria* on the density, viability, and infectivity of *C. parvum* oocysts and *Giardia lamblia* cysts under artificial conditions. The outer wall of *C. parvum* oocysts was not disrupted or was slightly disrupted in some rare occasions following ingestion and excretion by *D. pulicaria*, whereas the wall of *G. lamblia* cysts was highly disrupted, probably due to their larger size. The authors suggest that repeated ingestion of the (oo)cysts might have occurred during the 24 h grazing period, considering the high concentrations of pathogens and grazers. Distinction was not possible between (oo)cysts that would have been ingested multiple times from those that were never ingested by the daphnia. It was suggested that repeated ingestion and excretion of *G. lamblia* cysts might explain the mechanical damage to the cyst wall, which might have interfered with the measurements of excystation following grazing. In fact, grazing was shown to significantly decrease the viability of *Giardia* cysts (based on standard DAPI-PI vital dye staining techniques) but was shown to increase excystation, which could be attributed to the mechanical disruption of the cysts due to digestion, leading to the release of trophozoites. In the case of *C. parvum* oocysts, the authors reported a significant decrease (87%) in the mean oocyst infectivity due to grazing by *D. pulicaria* in their assay conditions, as measured by in vitro cell culture assays. Although the conditions prevailing in natural systems must be considered when evaluating the impact of zooplankton grazers on human pathogens in water, the authors concluded that *D. pulicaria* can significantly decrease the concentration of infectious (oo)cysts in natural surface waters.

As for the ingestion of bacteria, after exposing *Daphnia carinata* to *Campylobacter jejuni* for 72 h, an average of 33 bacteria was found in association with the surface or the inside of each daphnia, and a grazing rate of 1.75 mL/(individual·h) was calculated (Schallenberg et al. 2005). This value coincides with the typical values found in the literature for natural conditions, since daphnia are known to graze efficiently in lakes and ponds, at rates typically varying between 0.1 and 2.8 mL/(individual·h). Daphnia density for the assays was 40 daphnias/L, which is representative of natural conditions, knowing that the occurrence of daphnia is most often greater than 30 individuals/L and can exceed 100 individuals/L (Schallenberg et al. 2005), while the initial *C. jejuni* density was between 1.4×10^6 and 1.0×10^3/mL, typical of wastewater concentrations. Following 72 h of exposure, *D. carinata* had reduced the *C. jejuni* population by 99% (2 logs) compared with the control (absence of *D. carinata*). Thus, the authors concluded that *C. jejuni* ingestion by *D. carinata* caused the death of the bacteria, and they put forth the hypothesis that daphnia, when present in a high enough density, could reduce the concentration of this pathogenic microorganism in aquatic ecosystems.

Other laboratory assays were carried out to verify the ability of bacteria to survive digestion by *Daphnia ambigua*, a well-known bacterivorous cladoceran that had previously been found to be abundant in natural lake water (King et al. 1991). Cocci-shaped *Staphylococcus* spp., *Alcaligenes* sp., and *Pseudomonas* spp. died following *Daphnia* ingestion, whereas rod-shaped *Corynebacterium* spp. survived digestion. The authors suggest that rod-shaped bacteria survive digestion by *D. ambigua* whereas the coccoidal do not, but they add that a certain allelopathy (or amensalism) could take place in the *D. ambigua* digestive tract, i.e., a type of microbial competition that would be the cause of *Staphylococcus* death rather than the digestion by its host. In fact, they observed that *Staphylococcus* spp. survived 18 h longer after being ingested by *D. ambigua* in the absence of *Corynebacteria* spp., even though it was digested after 19 h. Both *Staphylococcus* and *Corynebacteria* are gram-positive bacteria. The authors put forth the hypothesis that zooplankton could possibly select gram-positive bacteria to feed upon, while it has already been seen that protozoa, whose feeding is characterized by a passive mechanical selectivity (Wetzel 2001), ingest gram-positive bacteria at lower rates than gram-negative bacteria (Pernthaler 2005).

Protection of microorganisms ingested by zooplankton against water treatment

On-site studies were conducted to isolate and identify bacteria associated with zooplankton in Lake Oglethorpe, a stratified eutrophic and shallow lake in the state of Georgia, USA (King et al. 1991). Water samples containing zooplankton were harvested and chlorinated (10 mg/L NaOCl for 5 min) to eliminate planktonic bacteria and conserve only bacteria associated with zooplankton. The ingested bacteria were then freed by putting the zooplankton samples through ultrasound. Bacteria that were cultured from both raw water samples and treated zooplankton samples were assumed to survive zooplankton digestion. Bacteria found in contact with zooplankton, and particularly within the digestive tract, were protected from chlorination, since they could be cultivated following freeing by ultrasound, meanwhile no non-spore-forming bacteria have been reported to date to be able to survive a dose of free chlorine such as the one applied to zooplankton samples. It was also shown that bacteria, such as the coliforms *Enterobacter cloacae* and *Klebsiella pneumoniae*, isolated from drinking water, and *Salmonella livingstone*, isolated from wastewater, could be protected from chlorine and monochloramine disinfection and remain viable inside of the digestive tract of the crustacean amphipod *Hyalella azteca* used as a model invertebrate for these assays (Levy et al. 1986).

From this section, it is important to emphasize the fact that pathogenic protozoa, such as *Cryptosporidium* and

Giardia, which exhibit low infectious dose, could be located inside a zooplankton organism in natural waters. Therefore, it is necessary to assess to what degree this occurrence actually translates into an increased risk of infection, especially in the case of unfiltered waters treated with UV radiation and distributed to consumers. Furthermore, there is evidence that some bacteria can survive within zooplankton organisms, whereas others are mostly digested and biodegraded. Therefore, we stress the need to better understand the ecology of pathogens in natural aquatic environments in order to characterize the fate of pathogens ingested by zooplankton. Surprisingly, there is barely any information in the literature to date about the protection effect of rotifers and crustacean zooplankton against common water treatment processes.

Bacterial colonization of the exoskeleton surface of zooplankton organisms

While there is a lack of information on zooplankton harboring pathogenic microorganisms in water, attachment to planktonic animals is more documented as a vectoring mode for waterborne pathogens. Certain bacteria can attach themselves to the surface of zooplankton organisms where they find a microhabitat that makes it possible for them to persist longer in the environment. To date, the most studied and relevant case in the field of water is that of *V. cholerae*, the bacterium responsible for cholera. For an exhaustive review of *V. cholerae* ecology and microbiology, the readers are invited to refer to Cottingham et al. (2003).

In various species of marine copepods, there is an intestinal flora that includes many types of heterotrophic bacteria and that is dominated by *Vibrio* (Sochard et al. 1979). Copepod defecation was observed as a means of bacterial dispersion in the water and marine sediments, since bacterial counts during copepod digestive tract dissection were lower after defecation. Other bacteria, such as *Pseudomonas* sp. and *Cytophaga* sp. were found to be associated with copepods, without being able to specifically associate them with the digestive tract, however. Considering the sum of the bacteria attached to the surface of copepods and those found in their intestinal flora, it was suggested that there are a greater number of bacteria actually associated with copepods rather than free in the water column. Researchers have therefore studied the association of bacteria with zooplankton organisms; the most documented case being that of *V. cholerae* attachment to the surface of copepods in particular, given its epidemiological importance. Some researchers have studied the relationship between *V. cholerae* and zooplankton in estuary zones (Huq et al. 1983; Tamplin et al. 1990; Huq et al. 1996; Chiavelli et al. 2001; Colwell et al. 2003; Cottingham et al. 2003; Lipp et al. 2003; Huq et al. 2005; Kirn et al. 2005; Alam et al. 2006) and in freshwater (Sarkar et al. 1983) and have suggested a link between episodes of zooplankton abundance and the occurrence of cholera epidemics in certain developing countries, such as Peru and Bangladesh. Up to the beginning of the 1980s, the detection of *V. cholerae* was linked to temporally sporadic events that coincided with epidemics in geographical areas where this bacterium is endemic. However, the existence of a nondetectable state of bacteria, the "viable but not culturable" state, was discovered to be the cause of the latent periods during which *V. cholerae* was not detected, even though it was present in natural waters between 2 epidemics (Xu et al. 1982; Binsztein et al. 2004). This behaviour in *V. cholerae*, as well as its attachment to zooplanktonic organisms, is crucial in understanding its ecology and cholera prevention; for example, Xu et al. (1982) put forth the hypothesis that these viable but not culturable *V. cholerae* cells could survive attached to copepods and then reproduce once optimal conditions arose. Huq et al. (1983) observed that the presence of live copepods increased the survival time of *V. cholerae* in water. The relationship between *V. cholerae* and planktonic copepods could explain the seasonal cholera epidemics — for example in Bangladesh an epidemic begins almost every year in September or October (Huq et al. 1983) shortly after the annual zooplankton bloom in coastal waters. Sanitary concerns are therefore linked to *V. cholerae* colonization of the surface of copepods and other chitinous organisms, considering that a single copepod could support a *V. cholerae* population on its surface sufficient to cause cholera in a human (Huq et al. 1983). Furthermore, bacteria were found to colonize the oral area and egg sac of copepods, where cell division was observed, indicating bacterial multiplication (Huq et al. 1983). The concentrated bacterial adhesion near the crustacean's mouth would suggest that it could serve as food, which could lead to *V. cholerae* being dispersed into the aquatic environment if the bacteria happen to multiply within its host's digestive tract before being excreted in the copepod's fecal matter.

Vibrio cholerae also attaches to many species of cladocerans and rotifers (Tamplin et al. 1990) and to certain species of phytoplankton. Therefore, a simple filtration method on tissue was proposed to extract the bacteria that was attached to plankton in raw waters in developing countries (Huq et al. 1996; Colwell et al. 2003). The method was tested on different strains of *V. cholerae* O1 and O139 originating from many different geographical areas, namely Bangladesh, Brazil, India, and Mexico. The results showed a 99% (2 logs) removal of *V. cholerae* (Huq et al. 1996). A field study in Bangladesh showed effective removal of particles greater than 20 μm and a 48% reduction in cholera cases in the villages that used this filtration method compared with the control villages (Colwell et al. 2003).

The nature of the relationship between *V. cholerae* and copepods has long been unexplained, except for the fact that the chitinous shell of planktonic animals was an adequate environment for the survival and growth of the bacteria. Bacteria of the genus *Vibrio* associated with zooplankton were reported to play an important role in chitin mineralization by bonding to the chitin and using it as an exclusive source of carbon and nitrogen (Heidelberg et al. 2002). Also, the presence of pili on bacterial cells, as is the case with *V. cholerae*, is often associated with the ability to colonize surfaces. Further to being associated with zooplankton, *V. cholerae* is also found in the aggregates of natural biofilms that are either floating or attached to debris (Alam et al. 2006), which can also provide a favourable environment for the persistence and proliferation of *V. cholerae* O1. The formation of biofilms by *V. cholerae* greatly increased their resistance to predation by protozoa compared with planktonic bacteria, notably because bacterial density in biofilms allows, according to the quorum sensing

principle, the production of an exopolysaccharide that inhibits protozoa grazing activity (Matz et al. 2005).

In an attempt to characterize the health risk associated with surface attachment to zooplankton, it is important to note that *V. cholerae* is not the only pathogenic bacterium to adopt such a strategy in aquatic environments. *Helicobacter pylori*, responsible for gastric ulcers in humans, can also attach itself to the surface of cladocerans and copepods, which would suggest that planktonic organisms provide a means of possible transmission of this bacterium to humans (Cellini et al. 2004). The significance of pathogenic bacterial attachment to the surface of zooplankton has never been quantified as a risk factor in microbial risk assessment associated with drinking water. However, the simple filtration technique recommended in developing countries provides evidence that the removal of zooplankton organisms in drinking water can lower the risk of cholera infection in regions where *V. cholerae* is endemic. This reveals the potential sanitary significance of such associations with higher organisms and the relevance of studying them in drinking water. In addition, it is not understood at this time whether or not biofilms forming on biological surfaces, such as zooplankton exoskeleton, provides a significant increase in resistance to disinfection treatments, including chemical and UV disinfection. Further work is therefore necessary to answer those questions.

Nematodes

Nematodes are not generally pathogenic to man. However, the World Health Organization includes them in the list of aesthetic nuisances and indicators of water treatment plant efficiency (Matsumoto et al. 2002). Despite this, certain species of nematodes are human intestinal parasites, such as *Ascaris lumbricoides*, but more frequently, they are vectors of human or animal pathogenic bacteria.

Survival of microorganisms inside nematodes

There are several reports on the ability of nematodes to ingest bacteria, to favour their persistence in the environment, and therefore, to serve as potential vectors for pathogenic organisms. Interestingly, information about this specific problematic can be taken not only from drinking water related studies but also from research in the field of agriculture and food production. In fact, many scenarios in food and agriculture industry can lead to the need to study nematodes as pathogenic bacteria vectors. For instance, certain nematode species pathogenic to plants are controlled with bacteria that are pathogenic to nematodes (Chen et al. 2000), whereas certain nematodes can be beneficial in agriculture as a biological control agent, acting as vectors of bacteria that are pathogenic to other organisms that are harmful to plants (Tan and Grewal 2001).

More importantly in the context of this review, researchers became interested in nematodes as vectors of bacteria that are pathogenic to humans to evaluate the health risk associated with consuming raw fruits and vegetables. These researchers evaluated resistance to disinfectants of bacteria ingested by nematodes, therefore providing information that could be useful in a health risk assessment related to the presence of nematodes in drinking water systems, which information was therefore included in this section.

In the field of drinking water, Chang and his co-workers studied long ago the health significance of nematodes with regard to their ability in protecting bacteria. They found that certain species of nematodes could ingest pathogenic bacteria, such as *Salmonella* sp. and *Shigella* sp. as well as Coxsackie virus and echovirus, and up to 16% of the ingested organisms could survive for 24 h at 25 °C inside their host (Chang et al. 1960*b*). Conventional treatments were observed to be ineffective in terms of removal or inactivation of nematodes, despite their sedimentation being facilitated when they lost their motility, which is possible following chlorination at 180 (mg·min)/L (Chang 1961), a treatment condition too severe to be applied at the head of a treatment plant. Nematodes were observed to survive chlorination as high as 360 (mg·min)/L (Chang 1961).

Nematodes of the *Rhabditidae* family can originate from wastewaters and can therefore transport and protect enteric bacteria and viruses, presenting a potential health risk if found in drinking water systems (Chang et al. 1960*b*). In a study about wastewaters, nematodes isolated from trickling filter effluents were found to contain about 100 viable bacteria per nematode, while an average of 75 viable bacteria per nematode was counted in the effluent of a primary settler in a wastewater treatment plant (Chang and Kabler 1962). Approximately 5%–10% of these bacteria were coliforms, and bacteria such as *E. coli*, *Pseudomonas* sp., *Streptococcus* sp., among others, were identified.

The nematode *Caenorhabditis elegans*, a member of the *Rhabditidae* family that feeds nonselectively, has been used in several studies to investigate host–pathogen interactions. An exhaustive list of known pathogens of *Caenorhabditis elegans*, including various opportunistic and true human pathogens, is found in Sifri et al. (2005). *Caenorhabditis elegans*, used as a model host in agriculture studies, and the nematode *Diploscapter* sp., commonly found in agricultural soil and in compost, can vector various strains of *E. coli* O157:H7, *Salmonella* sp., and *Listeria monocytogenes* (Caldwell et al. 2003), as those nematodes are attracted to the pathogenic bacteria and are able to ingest and transport them in their digestive tract (Gibbs et al. 2005). *Caenorhabditis elegans* has also been shown to transmit bacteriophages from one bacterial colony to another on a Petri dish (Dennehy et al. 2006). Furthermore, when it is pre-exposed to a bacteriophage population, *Caenorhabditis elegans* can better survive in the presence of *Salmonella enteritidis* and *Salmonella pullorum* (Santander and Robeson 2004), suggesting that the phages remain viable and active inside the nematode gut after ingestion.

Caenorhabditis elegans was reported to ingest, transport, and excrete *Cryptosporidium parvum* oocysts (Huamanchay et al. 2004) and, in experimental conditions, 75%–85% of nematodes ingested up to 200 oocysts after 2 h of incubation. The ingested oocysts remained intact, viable, and infectious within the digestive tract of the nematode, with possible excystation and freeing of sporozoite into the gastrointestinal system of the host. Nematodes that contained oocysts and that had been exposed to desiccation for a day were able to cause infection in mice, while oocysts or nematodes alone having undergone the same treatment did

not infect the mice. Furthermore, *C. elegans* containing *C. parvum* oocysts had the ability to infect mice even after having been kept in water for 7 days. However, the nematodes were exposed to unreasonable levels of oocysts (2×10^6 oocysts for 100–200 nematodes) compared with the anticipated conditions in surface water or granular filter water, for example. Our work (F. Bichai, N. Labbé, and B. Barbeau, unpublished results) suggests that *C. elegans* does not spontaneously seek to feed on *C. parvum* oocysts, especially if other particles are available for its feeding, and oocyst ingestion seems to occur rather fortuitously. It would therefore seem highly unlikely to find an oocyst within a nematode in nature. In the perspective of performing a risk assessment, it seems more appropriate to focus on nematodes as vectors of pathogenic bacteria rather than oocysts, since the risk associated with infectious oocysts being carried inside nematodes is likely to be very low.

Protection of ingested microorganisms by nematodes against water treatment

Cocultures of nematodes and bacteria *Salmonella typhi* and *Salmonella wichita* were exposed to 10 mg/L of free chlorine for 15 min, and viable bacteria were released after ingestion and natural defecation by nematodes (Smerda et al. 1970). Chlorine treatment killed all bacteria attached to the surface of nematodes, whereas, depending on the culture medium used after chlorine exposure, the recovery of *Salmonella* varied from 20% to 93.3%. Viable *S. wichita* were freed from nematodes in a tap water solution by defecation, which reflects drinking water conditions and therefore the potential health risk linked to its consumption. Bacterivorous nematodes can excrete from 30% to 60% of bacteria ingested in viable form (Chantanao and Jensen 1969).

Nematodes of the genus *Rhabditis*, which are commonly found in drinking water distribution systems, were shown to provide protection to *E. coli* C600 against free chlorine at doses of 0.5 and 1.0 mg/L (Ding et al. 1995). *Escherichia coli* freed from the nematodes by ultrasound and exposed to chlorine for 15 min were reduced by 6 logs, while bacteria that were protected by nematodes were only reduced by 2.5 logs under the same conditions. It is possible that this 2.5 log reduction is due to bacteria being attached to the surface of nematodes, since after 1 h of exposure to chlorine, the concentration of recovered bacteria was the same as after 15 min. It begs the question of whether or not the ingested *E. coli* had completely resisted the chlorine treatment, while *E. coli* attached to the nematode cuticle had been disinfected almost as easily as bacteria suspended in water. Furthermore, bacteria ingested by nematodes were shown to survive chlorine exposure (2% *v*/*v* or 1050 mg/L) and were subsequently freed by nematode fecal waste, whereas bacteria alone or on the surface of nematodes did not resist the same treatment (Adamo and Gealt 1996).

Nematodes were collected from raw and treated waters of a drinking water plant using surface water (Lupi et al. 1995). Most of them were in larval stage and measured an average of 45 μm in length. These nematodes were exposed to 10 mg/L of free chlorine (NaOCl) for 10 min to kill the bacteria attached to the surface of the nematodes and were then mechanically ground. Heterotrophic bacteria and enterobacteria were recovered from nematodes collected from both raw and treated water, yet in significantly lower quantities in treated water than in raw water (average values were 251 heterotrophic bacteria and 11 enterobacteria per nematode in raw water samples compared with 6.3 heterotrophic bacteria and 2.1 enterobacteria per nematode in clean water). *Caenorhabditis elegans* was observed to disperse the bacteria *E. coli*, *Salmonella typhimurium*, *Listeria welshimeri*, and *Bacillus cereus* by excreting viable cells after ingestion and exposure to 3 mg/L sodium hypochloride for 5–6 min (Anderson et al. 2003). Some chemical disinfectants used in agriculture, including free chlorine (at concentrations of 0.02–0.50 mg/L and contact time of 5 min), can inactivate the bacterium *Salmonella* Poona present on the surface of the nematode *C. elegans*, but not those that had been ingested (Caldwell et al. 2003).

Some species of bacterivorous nematodes can provide protection to potentially pathogenic bacteria in natural and filtered waters as well as in distributed water. Bacteria protected by nematodes seem to be present in treated water in a concentration that is too low to be considered a real risk to human health (Lupi et al. 1995). Even though infectious doses are generally higher for bacteria, it is not definite that this conclusion can be extended to protozoa, such as *Giardia* and *Cryptosporidium*, or to viruses, which have very low infectious doses. Consequently, we suggest that nematodes be considered as an increased risk factor in water systems. Finally, let's note that pathogen protection against UV disinfection within nematodes has not been researched in the literature to this date.

Discussion

The internalization of microorganisms by higher organisms can be considered in various contexts of more or less significance in regards to public health. Ecologists are studying this phenomenon in an attempt to understand aquatic trophic networks and variations in the composition of microbial communities in surface waters, without considering specifically pathogenic microorganisms and without particularly trying to establish a link between these observed natural phenomena and human health. On the contrary, in the agricultural industry, the internalization of microorganisms by higher organisms is clearly related to health issues, offering solutions in certain cases, but causing problems in other cases. Meanwhile, drinking water specialists too often dissociate microorganism inactivation kinetics by disinfection processes from the natural conditions under which they take place. This review aims at (*i*) evaluating the scientific observations of the potential health risk arising from the diverse associations between superior organisms and pathogenic microorganisms in a drinking water perspective and (*ii*) identifying the missing data that impede the establishment of cause-and-effect relationships that would better permit an appreciation of the sanitary risks associated with this phenomenon.

When considering the study of this phenomenon in the specific context of drinking water, the focus, in a perspective of risk analysis, has to be on microorganisms that are pathogenic to humans. The health risks associated with these pathogens being protected by higher organisms in drinking water should be considered at 3 steps in drinking water pro-

duction: (*i*) at the source, especially in the case of unfiltered surface waters; (*ii*) at the effluent of water treatment plant filters, since practically all granular media filters, whether or not they are used in a biological mode, are colonized by invertebrates; and (*iii*) in the water distribution system, as suggested by the well-known case of *Legionella pneumophila*, which proliferates in distribution systems in the presence of amoebae.

The first clue of the significance of higher organisms in water systems can be found in studying their occurrence at those 3 stages of water production. At the source, for instance, concentrations between 2 and 3000 amoebae/L and between 200 and 90 000 amoebae in river water were found during a 3 year investigation in Germany in untreated reservoir water and in rivers used as water supply sources, respectively (Hoffmann and Michel 2001). Densities of 200–300 rotifers/L are common in natural freshwaters and can occasionally reach 1000/L (Wetzel 2001). In terms of filter samples, important densities of nematodes were measured in sand samples taken near the surface of a slow sand filter bed (approx. 570 nematodes in a 30 g sand sample), as well as other types of zooplankton organisms, such as amoebae, rotifers, and copepods (approx. 140 amoebae, a similar quantity of rotifers, and about 60 copepods in a 30 g sand sample) (Hijnen et al. 2007). Invertebrate concentrations, mainly nematodes or rotifers, in the order of several thousands individuals per litre have been reported in the effluent of granular filters (Schreiber et al. 1997; Castaldelli et al. 2005), and concentrations of up to 400 amoebae/L were measured in filtered water from drinking water purification plants (Hoffmann and Michel 2001). As for distribution systems, protozoa are thought to be present in most systems in concentrations between 5×10^4 and 7×10^5/L (Sibille et al. 1998), whereas cladocerans and copepods have been found in concentrations between 600 and 750 organisms/m^3 in samples taken at hydrants (Van Lieverloo et al. 1998).

However, the sanitary significance of higher organisms in water is determined by the presence of internalized waterborne pathogens, whose resistance to primary or secondary disinfection may be enhanced. Invertebrates in distribution systems were found to be colonized by a large variety of bacteria, in numbers ranging from 1 to 10 CFU/copepod and from 10 to 100 CFU/nematode, both inside their digestive system and on their surface where the bacteria could be found individually or in colonies (Levy et al. 1986). Average colony counts reaching up to 4000 bacteria per invertebrate were observed in a drinking water system, approaching the infectious dose for certain of the bacterial species identified, possibly associated with a single superior organism (Wolmarans et al. 2005). Some of the invertebrate-associated bacteria from that system were identified to be frank or opportunistic human pathogens (including *Aeromonas hydrophila*, *Burkholderia cepacia*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Enterococcus faecium*, and *Streptococcus agalactiae*, to name only a few). Moreover, total coliforms, atypical coliforms, and heterotrophic aerobic bacteria were shown to be released from nematodes when they transited through the high-pressure pumps of a drinking water distribution system (Locas et al. 2007), explaining the seasonal recurrence of total coliform bacteria at the volute of pumps (even though free-chlorine residuals were as high as 1 mg Cl_2/L). In that specific case, the free-chlorine residual maintained in the distribution system allowed for the rapid inactivation of the released bacteria, limiting the potential microbial risk. These studies investigating the association of bacteria with invertebrates in distribution pipes, even though most of them do not identify pathogens, are interesting in the way that they report viable bacteria that are really found to be associated with higher organisms in the distribution network, which is the most crucial location in terms of risk of transmission to humans. Of course the unpathogenic bacteria are insignificant in terms of health risk, and there is a need to better identify those invertebrate-associated bacteria to find out in what proportion do pathogens occur in association with invertebrates in field conditions.

Meanwhile, it is estimated that approximately 25% of *Acanthamoeba*, isolated as much from the environment as from humans, carry endosymbionts (Winiecka-Krusnell and Linder 2001). Amoebae infected by bacteria seem to be common in cooling towers (present in 22/40 samples), whereas they seem more rare (or perhaps harder to detect) in natural aquatic environments (present in 3/40 samples), as shown in a study characterizing 40 samples of water, biofilms, and sediments from cooling towers in various American states, as well as 40 samples from various lakes, rivers, creeks, and ponds (Berk et al. 1998). Amoebae were found to be infected mostly by bacteria other than *Legionella pneumophila*, most of which were not culturable outside of an amoeba.

Altogether, these studies lead us to consider the large group of superior organisms as vectors for human pathogenic microorganisms, a reality that we cannot permit ourselves to ignore in the drinking water production industry, considering that a single organism could potentially transmit an infectious dose through a drinking water distribution system. However, very little information is currently available in the scientific literature to quantify the risk associated with this issue. A quantitative microbial risk analysis model was developed for *Legionella erythra* in drinking water distribution systems. This species was used as a substitute for the human pathogen *Legionella pneumophila* (Storey et al. 2004*a*). It was shown that the presence of *Acanthamoebae castellanii* was an important risk factor for Legionnaires' disease, increasing the resistance of the bacterium to free and combined chlorine, as well as to thermal treatment. An increased risk of approximately 2 orders of magnitude was calculated in the presence of amoebae compared with the risk associated with planktonic bacteria exposed to the same thermal treatment conditions or free- or combined-chlorine disinfection conditions. In a similar analysis, the calculated risk was increased by one order of magnitude when considering bacteria associated with biofilms, suggesting that amoebae would be a more important risk factor for *Legionella* in water systems than biofilm attachment. In practice, it is difficult to quantify the risk of Legionnaires' disease caused by inhalation of the bacteria by users of the drinking water distribution system given, on one hand, the obvious lack of available data regarding the dose–response relationship associated with exposure to *Legionella* spp., and on the other hand, the approximation necessary in evaluating volume of water particles inhaled by a user. In this study

(Storey et al. 2004*a*), the maximal risk approach was used, which assumes that exposure to a single pathogenic microorganism results in an infection in the host, which can be justifiable when considering the worst case scenario, which would be that of an immunodeficient individual during a nosocomial contamination episode. All other values entered in the risk analysis model obviously call for estimation, for example, the normal adult respiration rate and the average duration of a shower, which is assumed to be the critical situation for maximum exposure to microorganisms carried in aerosol. Thus, the value of the calculated risk in this model is more relative than absolute. The exercise reported in this study highlights missing information that is necessary to more fairly evaluate the risk associated with the presence of *Legionella* in drinking water distribution systems, but it also allows a comparison of different strategies for the treatment and distribution of water in the context of reducing the risk of Legionnaires' disease while keeping in mind the ecological factors actually occurring in the distribution network. It is important to remember that the risk associated with amoebae harbouring *Legionella* sp. in distribution systems must be considered in terms of inhalation, whereas amoebae could transmit human enteric pathogens such as *Campylobacter* through the usual route of exposure, i.e., by water consumption.

In surface waters, harsh environmental conditions seem to favour the association of some bacterial pathogens with higher organisms. In fact, endosymbiotic relationships between amoebae and bacteria or viruses can take place as a survival strategy used by endosymbionts. Similarly, it is thought that attachment of *V. cholerae* to the surface of planktonic organisms in water can also be seen as a survival mechanism in hostile conditions, since the bacteria can find a rich nutrient source in the chitinous surface of some zooplankton organisms. This can be compared with the formation of biofilms, possibly on a biological surface, known to occur in *V. cholerae*, for instance, in reaction to grazing pressure by protozoa (Matz et al. 2005). Such microbiological behaviours, which enhance the survival time of human pathogens in the environment, are usually not the object of engineering concerns, therefore the contribution of microbiologists, biologists, and ecologists is crucial. Water engineers should, for their part, focus on gathering quantitative information in a risk assessment perspective. One flaw found in the literature in this regard is the lack of information about the resistance of internalized pathogens to various disinfection processes, including UV exposure, of which the efficiency is known to be influenced by physical embedment of microorganisms in water. Specifically, we found that resistance to disinfection of pathogens located inside zooplankton organisms, such as rotifers, copepods, and cladocerans, has not been assessed to date. Such studies can be performed in artificial conditions, using model hosts for instance, since it is difficult to isolate one species of zooplankton organisms from natural water samples. However, it is also important to assess the significance of higher organisms in water systems by performing field experiments. In fact, we are under the impression that laboratory experiments involving feeding higher organisms with pathogens, such as *Cryptosporidium* and *Giardia*, can be considered misleading to some extent or at least incomplete, since artificial conditions of highly improbable occurrence in water systems are necessary to observe ingestion. Such studies can, however, be revealing if they are paired with observations from field experiments of that same host–pathogen association. We therefore suggest that studies such as those performed by King et al. (1991) and Nowosad et al. (2007) on zooplankton harbouring microorganisms and such as that of Berk et al. (1998) on infected amoebae be put forth to detect viable pathogens naturally occurring inside of higher organisms in water samples. To detect internalized human pathogens, it is necessary to sample highly contaminated waters. It is also important to include, in such studies, a relevant assessment of the ability of the recovered pathogens to infect a human host. Additionally, we deem it important to better investigate the occurrence of viable pathogens located inside invertebrates in water distribution systems, similar to the work of Wolmarans et al. (2005), since the abundance of invertebrates in the pipe systems is well known, whereas their sanitary significance remains poorly characterized and addressed.

In practical terms, the time factor is a major challenge in assessing the microbial risk associated with higher organisms as vectors of human pathogens in water. The ingestion of pathogens by some invertebrates can be studied as a removal mechanism, for example in granular filters, if pathogens are digested following ingestion. In fact, many biological processes, like those used in wastewater treatment for instance, rely on higher organisms to digest waterborne pathogens. It is therefore natural to anticipate that the fate of most ingested pathogens is to be eliminated from water. However, studies reported in this review about nematodes vectoring human pathogens, for example, prove that on the contrary, some invertebrates can in some instances become associated (internally or externally) with human pathogens, which are then carried by the invertebrate through the water systems. It is thought that time is an important factor to be considered in such cases, since pathogens may not be digested if water consumption by humans occurs shortly after pathogen ingestion by zooplankton and nematodes. Moreover, most of the invertebrates that are released in the filter effluents are offsprings of the populations growing in the filters and, therefore, are most probably not infected with pathogens from the source water. Altogether, this would suggest that the main risk associated with invertebrates in distribution system would result from the ingestion of pathogens in the distribution mains, a situation that is less probable than in the case of contaminated surface waters. Laboratory experiments have also allowed highlighting the importance of this time factor when bacteria and amoebae are in coculture. In fact, in some cases, a prolonged incubation could lead to the loss of viability in the intracellular bacteria, whereas in other cases, it could result in the destruction of the host cell. The case of bacteria that replicate inside protozoan hosts is probably the most important concern in terms of health risk management for drinking water, since the number of bacteria contained in a single organism can easily exceed the infectious dose for humans. It is also of concern to know that those bacterial pathogens can survive within the resisting form of their host, i.e., the cysts, considering that cysts can resist various extreme conditions and have a prolonged survival time in natural environments

and engineered water systems. We can, however, consider the more specific case of bacterial pathogens replicating inside protozoa separately from the more general case of pathogen ingestion by invertebrates, which most often imply a probability of digestion by the host with time. In fact, when taking this time factor into account, it appears that the sum of conditions that are needed to occur simultaneously at a specific location to observe a significant sanitary risk make this situation more of a coincidence of low probability, except for the case of bacterial replication inside protists. In fact, intracellular pathogens are more likely to persist throughout all steps of the transmission route from the treatment plant to humans in a sufficient number to possibly create an infection in humans after ingestion or inhalation.

It is important to consider the limits of the experimental methods that are used when assessing the survival of microorganisms inside higher organisms and their resistance to disinfection. One of the greatest challenges concerns the necessity to prove whether or not pathogens that survive within a higher organism are able to create an infection in human cells. Microscopic observation is often ambiguous in that aspect and standard culture methods can be misleading, since some bacteria have a viable but not culturable state in which they remain viable. Furthermore, many bacteria found to infect amoebae are not culturable outside of amoebal hosts (Berk et al. 1998). However, it was shown that many bacterial pathogens resist digestion within protozoa by using similar mechanisms as the ones used to infect human macrophages. Therefore, it is interesting to explore whether the use of amoebae could be relevant when assessing the viability of such human pathogens, since replication in amoebae could indicate the ability of bacteria to cause an infection in a human host.

The few cases of pathogenic microorganisms being detected within a host might be clues to a phenomenon that is more widespread than acknowledged by the drinking water treatment industry. The detection of viable *Cryptosporidium* oocysts within rotifers in natural lake waters (Nowosad et al. 2007) could question the validity of the pathogenic microorganism concentrations measured during microbiological characterization of surface waters, which does not usually take into consideration microorganisms that are potentially viable within higher organisms. Some may argue that the occurrence of pathogens protected inside of higher organisms is a rare event in treated waters from a conventional treatment plant. But the objectives in treating drinking water in North America require, in practice, the production of water that contains less than one parasite per 100 000 L (USEPA 2006). In this context, the presence of viable parasites inside higher organisms, even though it is, according to all evidence, a rare phenomenon, could potentially be a non-negligible risk, keeping in mind the protection that this higher organism brings to the pathogenic organisms it harbours. To date, the required information to properly evaluate this risk remains incomplete or missing.

The study of *Legionella* and its mechanisms for resisting digestion by an amoeba, for example, opens the door to the analysis of a more general health risk that might possibly result from the high disinfection resistance of pathogenic bacteria protected by a protozoa or one of its vesicles. Even though predation by higher organisms can be more intense at certain steps in a water treatment system, for example in granular filters where benthic invertebrates such as nematodes proliferate, an evaluation of the importance of the phenomenon of pathogen internalization by higher organisms in raw water is completely absent in the scientific literature. Despite the quantification obviously presenting major methodological challenges, such work would provide valuable knowledge to the water industry. If the concern for public health of drinking water scientists could be combined with the interests of research in freshwater ecology, a significant scientific contribution could arise from such collaboration. The study of the resistance of internalized pathogens to traditional disinfectants as well as to advanced treatments, such as UV disinfection, is imperative to the drinking water industry and to the evaluation of the microbial risk. The emergence of UV disinfection could prove to be an interesting tool if UV rays can successfully inactivate microorganisms harboured by higher organisms, a demonstration that has not yet been done.

While we are starting to understand the resistance mechanisms of microorganisms exposed to disinfection, such as the forming of biofilms, aggregation, and attachment to particles or to surfaces, biological or not, the study of microorganism survival within higher organisms proves to be necessary so that we may no longer ignore the field conditions that characterize natural environments and that are too often excluded from laboratory disinfection assays, which nevertheless determine to this day the disinfection standards for the drinking water industry.

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References

Adamo, J.A., and Gealt, M.A. 1996. A demonstration of bacterial conjugation within the alimentary canal of *Rhabditis* nematodes. FEMS Microbiol. Ecol. **20**: 15–22.

Adékambi, T., Salah, S.B., Khlif, M., Raoult, D., and Drancourt, M. 2006. Survival of environmental mycobacteria in *Acanthamoeba polyphaga*. Appl. Environ. Microbiol. **72**: 5974–5981. doi:10.1128/AEM.03075-05. PMID:16957218.

Alam, M., Sultana, M., Nair, G.B., Sack, R.B., Sack, D.A., Siddique, A.K., et al. 2006. Toxigenic *Vibrio cholerae* in the aquatic environment of Mathbaria, Bangladesh. Appl. Environ. Microbiol. **72**: 2849–2855. doi:10.1128/AEM.72.4.2849-2855.2006. PMID:16597991.

Anderson, G.L., Caldwell, K.N., Beuchat, L.R., and Williams, P.L. 2003. Interaction of a free-living soil nematode, *Caenorhabditis elegans*, with surrogates of foodborne pathogenic bacteria. J. Food Prot. **66**: 1543–1549. PMID:14503703.

Axelsson-Olsson, D., Waldenström, J., Broman, T., Olsen, B., and Holmberg, M. 2005. Protozoan *Acanthamoeba polyphaga* as a potential reservoir for *Campylobacter jejuni*. Appl. Environ.

Microbiol. **71**: 987–992. doi:10.1128/AEM.71.2.987-992.2005. PMID:15691957.

Barker, J., and Brown, M.R.W. 1994. Trojan horses of the microbial world: protozoa and the survival of bacterial pathogens in the environment. Microbiology (Reading, U.K.)**140**: 1253–1259. PMID:8081490.

Berk, S.G., Ting, R.S., Turner, G.W., and Ashburn, R.J. 1998. Production of respirable vesicles containing live *Legionella pneumophila* cells by two *Acanthamoeba* spp. Appl. Environ. Microbiol. **64**: 279–286. PMID:9435080.

Binsztein, N., Costagliola, M.C., Pichel, M., Jurquiza, V., Ramirez, F.C., Akselman, R., et al. 2004. Viable but nonculturable *Vibrio cholerae* O1 in the aquatic environment of Argentina. Appl. Environ. Microbiol. **70**: 7481–7486. doi:10.1128/AEM.70.12.7481-7486.2004. PMID:15574951.

Brandl, M.T., Rosenthal, B.M., Haxo, A.F., and Berk, S.G. 2005. Enhanced survival of *Salmonella enterica* in vesicles released by a soilborne *Tetrahymena* species. Appl. Environ. Microbiol. **71**: 1562–1569. doi:10.1128/AEM.71.3.1562-1569.2005. PMID: 15746361.

Brookes, J.D., Antenucci, J., Hipsey, M., Burch, M.D., Ashbolt, N.J., and Ferguson, C. 2004. Fate and transport of pathogens in lakes and reservoirs. Environ. Int. **30**: 741–759. doi:10.1016/j.envint.2003.11.006. PMID:15051248.

Caldwell, K.N., Adler, B.B., Anderson, G.L., Williams, P.L., and Beuchat, L.R. 2003. Ingestion of *Salmonella enterica* serotype Poona by a free-living nematode, *Caenorhabditis elegans*, and protection against inactivation by produce sanitizers. Appl. Environ. Microbiol. **69**: 4103–4110. doi:10.1128/AEM.69.7.4103-4110.2003. PMID:12839787.

Castaldelli, G., Mantovani, S., Benvenuti, M.R., Rossi, R., and Fano, E.A. 2005. Invertebrate colonization of GAC filters in a potabilisation plant treating groundwater. J. Water Supply Res. Technol. Aqua, **54**: 561–568.

Cellini, L., Del Vecchio, A., Di Candia, M., Di Campli, E., Favaro, M., and Donelli, S. 2004. Detection of free and plankton-associated *Helicobacter pylori* in seawater. J. Appl. Microbiol. **97**: 285–292. doi:10.1111/j.1365-2672.2004.02307.x. PMID:15239694.

Chang, S.L. 1961. Viruses, amoebas, and nematodes and public water supplies. J. Am. Water Works Assoc. **53**: 288–296.

Chang, S.L., and Kabler, P.W. 1962. Free-living nematodes in aerobic treatment plant effluent. J. Water Pollut. Control Fed. **34**: 1256–1261.

Chang, S.L., Berg, G., Clarke, N.A., and Kebler, P.W. 1960*a*. Survival and protection against chlorination of humain enteric pathogens in free-living nematodes isolated from water supplies. Am. J. Trop. Med. Hyg. **9**: 136–142. PMID:13809161.

Chang, S.L., Woodward, R.L., and Kabler, P.W. 1960*b*. Survey of free-living nematodes and amoebas in municipal supplies. J. Am. Water Works Assoc. **52**: 613–618.

Chantanao, A., and Jensen, H.J. 1969. Saprozoic nematodes as carriers and disseminators of plant pathogenic bacteria. J. Nematol. **1**: 216–218.

Chen, S.Y., Charnecki, J., Preston, J.F., and Dickson, D.W. 2000. Extraction and purification of *Pasteuria* spp. endospores. J. Nematol. **32**: 78–84.

Chevrefils, G., Caron, É., Barbeau, B., Payment, P., and Prévost, M. 2007. Blending and filtration effects on UV kinetics of indigenous spores (accepted). J. Am. Water Works Assoc.

Chiavelli, D.A., Marsh, J.W., and Taylor, R.K. 2001. The mannose-sensitive hemagglutinin of *Vibrio cholerae* promotes adherence to zooplankton. Appl. Environ. Microbiol. **67**: 3220–3225. doi:10.1128/AEM.67.7.3220-3225.2001. PMID:11425745.

Colwell, R.R., Huq, A., Islam, M.S., Aziz, K.M.A., Yunus, M., Khan, N.H., et al. 2003. Reduction of cholera in Bangladeshi villages by simple filtration. Proc. Natl. Acad. Sci. U.S.A. **100**: 1051–1055. doi:10.1073/pnas.0237386100. PMID:12529505.

Connelly, S.J., Wolyniak, E.A., Dieter, K.L., Williamson, C.E., and Jellison, K.L. 2007. Impact of zooplankton grazing on the excystation, viability, and infectivity of the protozoan pathogens *Cryptosporidium parvum* and *Giardia Lamblia*. Appl. Environ. Microbiol. **73**: 7277–7282. doi:10.1128/AEM.01206-07. PMID:17873076.

Cottingham, K.L., Chiavelli, D.A., and Taylor, R.K. 2003. Environmental microbe and human pathogen: the ecology and microbiology of *Vibrio cholerae*. Front. Ecol. Environ. **1**: 80–86.

Dennehy, J.J., Friedenberg, N.A., Yang, Y.W., and Turner, P.E. 2006. Bacteriophage migration via nematode vectors: host–parasite–consumer interactions in laboratory microcosms. Appl. Environ. Microbiol. **72**: 1974–1979. doi:10.1128/AEM.72.3.1974-1979.2006. PMID:16517645.

Ding, G., Sugiura, N., Inamori, Y., and Sudo, R. 1995. Effect of disinfection on the survival of *Escherichia coli*, associated with nematoda in drinking water. Water Supply, **13**: 101–106.

Fayer, R., Trout, J.M., Walsh, E., and Cole, R. 2000. Rotifers ingest oocysts of *Cryptosporidium parvum*. J. Eukaryot. Microbiol. **47**: 161–163. doi:10.1111/j.1550-7408.2000.tb00026.x. PMID: 10750844.

Gibbs, D.S., Anderson, G.L., Beuchat, L.R., Carta, L.K., and Williams, P.L. 2005. Potential role of *Diploscapter* sp. strain LKC25, a bacterivorous nematode from soil, as a vector of food-borne pathogenic bacteria to preharvest fruits and vegetables. Appl. Environ. Microbiol. **71**: 2433–2437. doi:10.1128/AEM.71.5.2433-2437.2005. PMID:15870330.

Greub, G., and Raoult, D. 2004. Microorganisms resistant to free-living amoebae. Clin. Microbiol. Rev. **17**: 413–433. doi:10.1128/CMR.17.2.413-433.2004. PMID:15084508.

Heidelberg, J.F., Heidelberg, K.B., and Colwell, R.R. 2002. Bacteria of the γ-subclass *Proteobacteria* associated with zooplankton in Chesapeake Bay. Appl. Environ. Microbiol. **68**: 5498–5507. PMID:12406743.

Hijnen, W.A.M., Dullemont, Y.J., Schijven, J.F., Hanzens-Brouwer, A.J., Rosielle, M., and Medema, G. 2007. Removal and fate of *Cryptosporidium parvum*, *Clostridium perfringens* and small-sized centric diatoms (*Stephanodiscus hantzschii*) in slow sand filters. Water Res. **41**: 2151–2162. doi:10.1016/j.watres.2007.01.056. PMID:17400275.

Hoffmann, R., and Michel, R. 2001. Distribution of free-living amoebae (FLA) during preparation and supply of drinking water. Int. J. Hyg. Environ. Health, **203**: 215–219. doi:10.1078/S1438-4639(04)70031-0. PMID:11279817.

Huamanchay, O., Genzlinger, L., Iglesias, M., and Ortega, Y.R. 2004. Ingestion of *Cryptosporidium* oocysts by *Caenorhabditis elegans*. J. Parasitol. **90**: 1176–1178. doi:10.1645/GE-253R. PMID:15562624.

Huq, A., Small, E.B., West, P.A., Huq, M.I., Rahman, R., and Colwell, R.R. 1983. Ecological relationships between *Vibrio cholerae* and planktonic crustacean copepods. Appl. Environ. Microbiol. **45**: 275–283. PMID:6337551.

Huq, A., Xu, B., Chowdhury, M.A., Islam, M.S., Montilla, R., and Colwell, R.R. 1996. A simple filtration method to remove plankton-associated *Vibrio cholerae* in raw water supplies in developing countries. Appl. Environ. Microbiol. **62**: 2508–2512. PMID:8779590.

Huq, A., Sack, R.B., Nizam, A., Longini, I.M., Nair, G.B., Ali, A., et al. 2005. Critical factors influencing the occurrence of *Vibrio cholerae* in the environment of Bangladesh. Appl. Environ. Microbiol. **71**: 4645–4654. doi:10.1128/AEM.71.8.4645-4654.2005. PMID:16085859.

Kahane, S., Dvoskin, B., Mathias, M., and Friedman, M.G. 2001. Infection of *Acanthamoeba polyphaga* with *Simkania negevensis* and *S. negevensis* survival within amoebal cysts. Appl. Environ. Microbiol. **67**: 4789–4795. doi:10.1128/AEM.67.10.4789-4795.2001. PMID:11571186.

King, C.H., Shotts, E.B., Jr., Wooley, R.E., and Porter, K.G. 1988. Survival of coliforms and bacterial pathogens within protozoa during chlorination. Appl. Environ. Microbiol. **54**: 3023–3033. PMID:3223766.

King, C.H., Sanders, R.W., Shotts, E.B., Jr., and Porter, K.G. 1991. Differential survival of bacteria ingested by zooplankton from a stratified eutrophic lake. Limnol. Oceanogr. **36**: 829–845.

Kirn, T.J., Jude, B.A., and Taylor, R.K. 2005. A colonization factor links *Vibrio cholerae* environmental survival and human infection. Nature, **438**: 863–866. doi:10.1038/nature04249. PMID:16341015.

Kuiper, M.W., Wullings, B.A., Akkermans, A.D., Beumer, R.R., and Van der Kooij, D. 2004. Intracellular prolivferation of *Legionella pneumophila* in *Hartmannella vermiformi*s in aquatic biofilms grown on plasticized polyvinyl chloride. Appl. Environ. Microbiol. **70**: 6826–6833. doi:10.1128/AEM.70.11.6826-6833.2004. PMID:15528550.

Levy, R.V., Hart, F.L., and Cheetham, R.D. 1986. Occurrence and public health significance of invertebrates in drinking water systems. J. Am. Water Works Assoc. **78**: 105–110.

Lipp, E.K., Rivera, I.N.G., Gil, A.I., Espeland, E.M., Choopun, N., Louis, V.R., et al. 2003. Direct detection of *Vibrio cholerae* and *ctxA* in Peruvian coastal water and plankton by PCR. Appl. Environ. Microbiol. **69**: 3676–3680. doi:10.1128/AEM.69.6.3676-3680.2003. PMID:12788781.

Locas, A., Barbeau, B., and Gauthier, V. 2007. Nematodes as a source of total coliforms in a distribution system. Can. J. Microbiol. **53**: 580–585. doi:10.1139/W07-013. PMID:17668016.

Lupi, E., Ricci, V., and Burrini, D. 1994. Occurrence of nematodes in surface water used in a drinking water plant. J. Water Supply Res. Technol. Aqua, **43**: 107–112.

Lupi, E., Ricci, V., and Burrini, D. 1995. Recovery of bacteria in nematodes isolated from a drinking water supply. J. Water Supply Res. Technol. Aqua, **44**: 212–218.

Ly, T.M.C., and Müller, H.E. 1990. Interactions of *Listeria monocytogenes*, *Listeria seeligeri*, and *Listeria innocua* with protozoans. J. Gen. Appl. Microbiol. **36**: 143–150. doi:10.2323/jgam.36.143.

Mahmud, F., Craik, S.A., and Belosevic, M. 2006. The effect of upstream treatment processes on the UV inactivation of *Cryptosporidium parvum*. *In* Proceedings of the American Water Works Association — Water Quality Technology Conference, 5–9 November 2006, American Water Works Association, Denver, Colorado, USA.

Mamane-Gravetz, H., and Linden, K.G. 2005. Relationship between physiochemical properties, aggregation and UV inactivation of isolated indigenous spores in water. J. Appl. Microbiol. **98**: 351–363. doi:10.1111/j.1365-2672.2004.02455.x. PMID:15659190.

Matsumoto, N., Aizawa, T., Ohgaki, S., Hirata, T., Toyooka, K., Kanbayashi, T., Tsutsumi, Y., and Hasegawa, T. 2002. Removal methods of nematoda contained in the effluent of activated carbon. J. Water Supply Water Sci. Technol. **2**: 183–190.

Matz, C., and Kjelleberg, S. 2005. Off the hook — how bacteria survive protozoan grazing. Trends Microbiol. **13**: 302–307. doi:10.1016/j.tim.2005.05.009. PMID:15935676.

Matz, C., McDougald, D., Moreno, A.M., Yung, P.Y., Yildiz, F.H., and Kjelleberg, S. 2005. Biofilm formation and phenotypic variation enhance predation-driven persistence of *Vibrio cholerae*. Proc. Natl. Acad. Sci. U.S.A. **102**: 16819–16824. doi:10.1073/pnas.0505350102. PMID:16267135.

Morin, P., Gauthier, V., Saby, S., and Block, J.-C. 1997. Bacterial resistance to chlorine through attachment to particles and pipe surfaces in drinking water distribution systems. Conference of the Royal Society of Chemistry. Cambridge, United Kingdom.

Nowosad, P., Kuczynska-Kippen, N., Slodkowicz-Kowalska, A., Majewska, A.C., and Graczyk, T.K. 2007. The use of rotifers in detecting protozoan parasite infections in recreational lakes. Aquat. Ecol. **41**: 47–54. doi:10.1007/s10452-006-9043-5.

Nygaard, K., and Tobiesen, A. 1993. Bacterivory in algae: a survival strategy during nutrient limitation. Limnol. Oceanogr. **38**: 273–279.

Pernthaler, J. 2005. Predation on prokaryotes in the water column and its ecological implications. Nat. Rev. Microbiol. **3**: 537–546. doi:10.1038/nrmicro1180. PMID:15953930.

Pinel-Alloul, B., Bourbonnais, N., and Pick, F. 2002. Variations spatiotemporelles des compartiments autotrophes et heterotrophes de la boucle microbienne dans les lacs du sud du Quebec: spatial and temporal variations in autotrophic and heterotrophic compartments of the microbial loop in southern Quebec lakes. Rev. Sci. Eau, **15**: 3–25.

Robotham, T.J. 1986. Current views on the relationships between amoebae, legionellae and man. Isr. J. Med. Sci. **22**: 678–689. PMID:3793451.

Santander, J., and Robeson, J. 2004. Bacteriophage prophylaxis against *Salmonella enteritidis* and *Salmonella pullorum* using *Caenorhabditis elegans* as an assay system. Electron. J. Biotechnol. **7**: 206–209.

Sarkar, B.L., Nair, G.B., Sircar, B.K., and Pal, S.C. 1983. Incidence and level of *Vibrio parahaemolyticus* associated with freshwater plankton. Appl. Environ. Microbiol. **46**: 288–290. PMID:6614907.

Schallenberg, M., Bremer, P.J., Henkel, S., Launhardt, A., and Burns, C.W. 2005. Survival of *Campylobacter jejuni* in water: effect of grazing by the freshwater crustacean *Daphnia carinata* (Cladocera). Appl. Environ. Microbiol. **71**: 5085–5088. doi:10.1128/AEM.71.9.5085-5088.2005. PMID:16151090.

Schreiber, H., Schoenen, D., and Traunspurger, W. 1997. Invertebrate colonization of granular activated carbon filters. Water Res. **31**: 743–748. doi:10.1016/S0043-1354(96)00312-0.

Sibille, I., Sime-Ngando, T., Mathieu, L., and Block, J.C. 1998. Protozoan bacterivory and *Escherichia coli* survival in drinking water distribution systems. Appl. Environ. Microbiol. **64**: 197–202. PMID:9435076.

Sifri, C.D., Begun, J., and Ausubel, F.M. 2005. The worm has turned — microbial virulence modeled in *Caenorhabditis elegans*. Trends Microbiol. **13**: 119–127. doi:10.1016/j.tim.2005.01.003. PMID:15737730.

Smerda, S.M., Jensen, H.J., and Anderson, A.W. 1970. Escape of *Salmonellae* from chlorination during ingestion by *Pristionchus iheritieri* (Nematoda: Diplogasterinae). J. Nematol. **3**: 201–204.

Snelling, W.J., McKenna, J.P., Lecky, D.M., and Dooley, J.S.G. 2005. Survival of *Campylobacter jejuni* in waterborne protozoa. Appl. Environ. Microbiol. **71**: 5560–5571. doi:10.1128/AEM.71.9.5560-5571.2005. PMID:16151149.

Sochard, M.R., Wilson, D.F., Austin, B., and Colwell, R.R. 1979. Bacteria associated with the surface and gut of marine copepods. Appl. Environ. Microbiol. **37**: 750–759. PMID:16345368.

Storey, M.V., Ashbolt, N.J., and Stenström, T.A. 2004*a*. Biofilms, thermophilic amoebae and *Legionella pneumophila* — a quantitative risk assessment for distributed water. Water Sci. Technol. **50**: 77–82. PMID:15318490.

Storey, M.V., Winiecka-Krusnell, J., Ashbolt, N.J., and Stenström,

T.A. 2004*b*. The efficacy of heat and chlorine treatment against thermotolerant *Acanthamoebae* and *Legionellae.* Scand. J. Infect. Dis. **36**: 656–662. PMID:15370652.

Stott, R., May, E., Matsushita, E., and Warren, A. 2001. Protozoan predation as a mechanism for the removal of *Cryptosporidium* oocysts from wastewaters in constructed wetlands. Water Sci. Technol. **44**: 191–198. PMID:11804094.

Stott, R., May, E., Ramirez, E., and Warren, A. 2003. Predation of *Cryptosporidium* oocysts by protozoa and rotifers: implications for water quality and public health. Water Sci. Technol. **47**: 77–83. PMID:12639009.

Tamplin, M.L., Gauzens, A.L., Huq, A., Sack, D.A., and Colwell, R.R. 1990. Attachment of *Vibrio cholerae* serogroup O1 to zooplankton and phytoplankton of Bangladesh waters. Appl. Environ. Microbiol. **56**: 1977–1980. PMID:2383016.

Tan, L., and Grewal, P.S. 2001. Pathogenicity of *Moraxella osloensis*, a bacterium associated with the nematode *Phasmarhabditis hermaphrodita*, to the slug *Deroceras reticulatum*. Appl. Environ. Microbiol. **67**: 5010–5016. doi:10.1128/AEM.67.11.5010-5016.2001. PMID:11679319.

Tezcan-Merdol, D., Ljungström, M., Winiecka-Krusnell, J., Linder, E., Engstrand, L., and Rhen, M. 2004. Uptake and replication of *Salmonella enterica* in *Acanthamoeba rhysodes*. Appl. Environ. Microbiol. **70**: 3706–3714. doi:10.1128/AEM.70.6.3706-3714.2004. PMID:15184177.

Trout, J.M., Walsh, E.J., and Fayer, R. 2002. Rotifers ingest *Giardia* cysts. J. Parasitol. **88**: 1038–1040. PMID:12435156.

USEPA. 2006. Long term 2 enhanced surface water treatment rule (LT2ESWTR). Fed. Regist. **71**: 654–786.

Van Lieverloo, J.H.M., Van Buuren, R., Veenendaal, G., and Van der Kooij, D. 1998. Controlling invertebrates in distribution systems with zero or low disinfectant residual. Water Supply, **16**: 199–204.

Wetzel, R.G. 2001. Limnology, lake and river ecosystems. Elsevier Academic Press, New York.

Winiecka-Krusnell, J., and Linder, E. 1999. Free-living amoebae protecting *Legionella* in water: the tip of an iceberg? Scand. J. Infect. Dis. **31**: 383–385. PMID:10528878.

Winiecka-Krusnell, J., and Linder, E. 2001. Bacterial infections of free-living amoebae. Res. Microbiol. **152**: 613–619. doi:10.1016/S0923-2508(01)01240-2. PMID:11605981.

Winiecka-Krusnell, J., Wreiber, K., von Euler, A., Engstrand, L., and Linder, E. 2002. Free-living amoebae promote growth and survival of *Helicobacter pylori*. Scand. J. Infect. Dis. **34**: 253–256. doi:10.1080/00365540110080052. PMID:12064686.

Wolmarans, E., du Preez, H.H., de Wet, C.M.E., and Venter, S.N. 2005. Significance of bacteria associated with invertebrates in drinking water distribution networks. Water Sci. Technol. **52**: 171–175. PMID:16312964.

Xu, H.-S., Roberts, N., Singleton, F.L., Attwell, R.W., Grimes, D.J., and Colwell, R.R. 1982. Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. Microb. Ecol. **8**: 313–323. doi:10.1007/BF02010671.