Cryptosporidium and *Giardia*: new challenges to the water industry

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

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Cryptosporidium and *Giardia*: new challenges to the water industry
- Medema G.J. - 1999

Thesis Universiteit Utrecht. – With ref. –
With summaries in English and Dutch
Subject headings: intestinal protozoa / drinking water / water treatment

ISBN 90 – 393 – 2120 – 5

Cover:
Foreground: Cryptosporidium oocysts and *Giardia* cysts stained with a MAb-FITC conjugate or with DAPI.
Background: Giardia trophozoites stained with Evans blue
Photography and digital image processing: Ciska Schets, Leonard Bik, Annette Daems, Erik Zeegers

Printed by Ipskamp, Amsterdam

Cryptosporidium and *Giardia*: new challenges to the water industry

***Cryptosporidium* en *Giardia*:**
nieuwe uitdagingen voor de waterleidingbedrijven

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor
aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. dr. H.O. Voorma
ingevolge het besluit van het College voor Promoties
in het openbaar te verdedigen
op donderdag 29 april 1999 des namiddags te 4.15 uur

door

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geboren op 28 februari 1962 te Zoetermeer

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Het onderzoek in dit proefschrift is uitgevoerd bij het Rijksinstituut voor Volksgezondheid en Milieu en bij Kiwa Onderzoek & Advies en is gefinancierd door het Directoraat Generaal Milieu van het Ministerie van Volksgezondheid, Ruimtelijke Ordening en Milieu en het gezamenlijke onderzoeksprogramma van de waterleidingbedrijven.

Aan mijn vader en moeder

Aan Marjolein, Merel, Joost, Jasmijn en Madelief

Anthonie van Leeuwenhoek was the first to describe *Giardia* trophozoites, already in 1681. He saw 'animalcules, a-moving very prettily' through his self-made microscope when he examined his own diarrhoeic stool. He published his observations in his letter of 4 November 1681 to the Royal Society in London.

This is part of this letter of van Leeuwenhoek, as it was translated by C. Dobell, who made it perceptible that the animalcules were indeed *Giardia*:

'*I weigh about 160 pounds, and have been of very nigh the same weight for some 30 years, and I have ordinarily of a morning a well-formed stool; but now and then hitherto I have had a looseness, at intervals of 2,3, or 4 weeks, when I went to stool some 2,3, or 4 times per day. But this summer this befell me very often, and especially when I partook of hot smoked beef, that was a bit fat, or ham, which food I am very fond of; indeed, it persisted once for three days running, and whatever food I took, I kept in my body not much above 4 hours; and I imagined (for divers reasons) that I could get myself well again by drinking uncommon hot tea, as hath happened many a time before.*
My excrement being so thin, I was at divers times persuaded to examine it; and each time I kept in mind what food I had eaten and what drink I had drunk, and what I found afterwards: but to tell all my observations here would make all too long a story. I will only say that I have generally seen, in my excrement, many irregular particles of sundry sizes, most of them tending to a round figure which are very clear and of a yellow colour: these were the ones that make the whole material look yellow to our eye. And there were also, besides, such like particles that were very bright and clear without one being able to discern any colour in them (…)
All the particles aforesaid lay in a clear, transparent medium, wherein I have sometimes also seen animalcules a-moving very prettily; some of 'em a bit bigger, others a bit less, than a blood-globule, but all of one and the same make. Their bodies were somewhat longer than broad, and their belly which was flatlike, furnisht with sundry little paws, wherewith they made such a stir in the clear medium and among the globules, that you might e'en fancy that you saw a pissabed running up against a wall; and albeit they made a quick motion with their paws, yet for all they made but slow progress. Of these animalcules I saw at one time only one in a particle of matter as big as a sand-grain; and anon, at other times some 4,5 or even 6 or 8.'

Dobell, C. (1932). Antony van Leeuwenhoek and his "little animals". Swetz & Zeitlinger, Amsterdam, The Netherlands

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Protozoan parasites *(Cryptosporidium, Giardia, Cyclospora)*

With C.R. Fricker[1] and H.V. Smith[2]

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The authors wish to thank Morteza Abbaszadegan, Jamie Bartram, Phil Berger, Joe Cotruvo, David Cunliffe, dr. Feuerpfeil, Arie Havelaar, Henk Ketelaars, Mark LeChevallier, Yasumoto Magara, Ynez Ortega, E. Pozio, Stig Regli, Joan Rose, Steven Schaub, Ciska Schets, Susan Shaw and all the participants of the meeting on the Rolling Revision of the WHO Guidelines for Drinking-water quality in Medmenham, 17 to 21 March 1998 for critical reading of the manuscript. Their useful comments have improved the comprehensiveness of the description of the current knowledge of these parasites and of the means to control waterborne transmission.

This paper will be published as addendum to the Guidelines for Drinking-water Quality of the World Health Organisation, Geneva, Switzerland.

WATERBORNE PROTOZOAN PARASITES

Several species of parasitic protozoa are transmitted through water. Of these, *Giardia intestinalis* and *Entamoeba histolytica/dispar* are long recognised as the most common intestinal parasites throughout the world. Morbidity and, in particular for *E.histolytica/dispar,* mortality rates are high, especially in non-industrialised countries. More information on *Entamoeba* can be found in volume 2 of the WHO Guidelines for Drinking Water Quality and in the informal consultation document on enteric protozoa (1994).
A wide variety of free-living amoebae is capable of multiplication in (drinking) water, but only few species have been identified as pathogenic for man. These are *Naegleria fowleri* and *Acanthamoeba spp*. *Naegleria fowleri* can be present in thermally polluted waters and sporadically causes lethal primary amoebic meningoencephalitis. Only one outbreak has been related to a drinking water supply system (Marshall *et al.,*1997). *Acanthamoeba* can be found in the entire aquatic environment. It sporadically causes keratitis in contact lens wearers after exposure to contaminated recreational water and contact lens cleaning fluids (Marshall *et al.,* 1997). Drinking water taps were identified as the source of contamination of home-made lens cleaning solutions containing *Acanthamoeba* (Seal *et al.,* 1995). *Acanthamoeba* has also been suggested as a vehicle for environmental transmission of *Legionella* bacteria (Campbell *et al.,* 1995).
The increasing population of severely immunocompromised people, due to the AIDS epidemic, cancer chemotherapy and organ transplants, has increased the prevalence of opportunistic infections and has led to the recognition of the disease causing potential of other intestinal protozoan parasites, such as *Cryptosporidium parvum, Cyclospora* sp. and Microsporidia as human pathogens.

The first human cases of cryptosporidiosis were reported in 1976 (Meisel *et al.,* 1976; Nime *et al.,* 1976) and *Cryptosporidium* was first thought to be an opportunistic pathogen of immunocompromised persons. The recognition of frequent cases in immunocompetent individuals and a number of waterborne outbreaks have changed this image. *C. parvum* is now recognised as one of the most commonly identified intestinal pathogens throughout the world. It's relative occurrence is dependent on factors such as age and other demographic characteristics of the study population and season. In children at the age of 1-5 with diarrhoea, it can be the most frequently found pathogen (Palmer, 1990).
Cyclospora sp. has recently been recognised as a waterborne pathogen. Initially, it was referred to as cyanobacterium-like bodies but is now classified as *Cyclospora* sp. (Bendall *et al.,* 1993; Ortega *et al.,* 1993). It has been associated with several waterborne outbreaks world-wide.
Microsporidia are a large group (almost 1000) of species and are widely distributed in nature (Casemore, 1996). Although they were recognised as pathogens in fish, birds and some mammals, several species have recently been identified as cause of disease in severely immunocompromised humans. These species have primarily been associated with infections of the intestinal tract, but

dissemination to the biliary, urinary and respiratory tract may occur. Some species have been implicated in ocular infections in immunocompetent persons. The mode of transmission is still unclear, but a faecal-oral route is likely. Waterborne transmission has not been demonstrated, but their persistence in water, resistance to disinfection and small size (some as small as 1-2 μm) suggest that this must be considered possible, especially for immunocompromised individuals.
Toxoplasma gondii is a coccidian parasite that has long been recognised as human pathogen. It is an intracellular parasite with felines as the definitive host. These are infected primarily by the consumption of infected mammals and birds, that act as secondary hosts. In these secondary hosts, the parasite settles itself as tissue cysts in muscle and brain tissue. Only felines carry the parasite in their intestinal tract and shed oocysts that sporulate in the environment. The oocysts are 10-12 μm and can survive in water and moist soils for long periods of time. Consumption of undercooked meats and raw milk and contact with feline faeces (cat litter, sand boxes) are the primary sources of *Toxoplasma* infections in humans (Guy, 1996). Two waterborne outbreaks have been reported. Both were believed to have been derived from contamination of water by cat faeces.

This review focuses on *Cryptosporidium parvum, Giardia intestinalis* and *Cyclospora* sp.*,* since these are the parasites of primary concern to drinking water supply and a large amount of information on waterborne transmission has accumulated from recent research.

Significance of *Cryptosporidium* and *Giardia* as waterborne pathogens
Oocysts of *Cryptosporidium* and cysts of *Giardia* are ubiquitously present in the aquatic environment. They have been found in most surface waters, their concentration being related to the level of faecal pollution or human use of the surface water (Hansen & Ongerth, 1991; LeChevallier *et al.,* 1991).The environmentally robust (oo)cysts are very persistent in water (DeReignier *et al.,* 1989; Robertson *et al.,* 1992; Chauret *et al.,* 1995) and are extremely resistant to the disinfectants commonly used in drinking water treatment (Hibler *et al.*, 1987; Korich *et al.,* 1990; Finch *et al*., 1993[a.b]). These characteristics, coupled with the low numbers of (oo)cysts required for an infection (Rendtorff, 1954; Dupont *et al.,* 1995; Okhuysen *et al.,* 1998) make these organisms the most critical pathogens for the production of safe drinking water from surface water with disinfection and for post-treatment contamination. Well protected groundwaters, that are not under the influence of surface water or other sources of contamination, are free of these (and other) enteropathogens. If abstraction, treatment and distribution of these waters are properly designed and operated, the risk of faecal contamination is very low and they will not be a source of waterborne transmission of parasitic protozoa. Groundwaters that are under the influence of surface water or other contamination sources (surface run-off) can be contaminated with, low levels of, *Cryptosporidium* and *Giardia* (Hancock *et al.,* 1997) and cause waterborne illness (Craun *et al.,* 1998). Treatment of these waters with disinfection alone offers no protection against

Cryptosporidium and only limited protection against *Giardia*. Hence, filtration of these waters is necessary to produce safe drinking water.
Many waterborne outbreaks of giardiasis and cryptosporidiosis have been reported in industrialised countries (Craun, 1990; MacKenzie *et al.,* 1994; Craun *et al.,* 1998). In these outbreaks, (oo)cysts have entered the drinking water because of surface water treatment failure, (increased) contamination of the source water and leakage into the distribution system.
In a significant number of these outbreaks, the drinking water that was implicated as the cause of the outbreak complied with the WHO-guidelines for *Escherichia coli* levels and turbidity (Craun 1990; Craun *et al.,* 1998). In most outbreaks, deviations from normal raw water quality or treatment operation could be identified. However, in a drinking waterborne outbreak in Las Vegas, no abnormalities in operation or water quality (raw or treated) were detected (Goldstein *et al.,* 1996).
The occurrence of outbreaks in the absence of a warning signal from the routine water quality monitoring for coliforms that the water may be contaminated is a severe shortcoming of the coliforms as indicator for microbiologically safe drinking water, which calls for additional means to safeguard drinking water.

THE PARASITES AND THE DISEASE

Cryptosporidium parvum

Taxonomy

Members of the genus *Cryptosporidium* (Apicomplexa, Cryptosporidiidae) are small coccidian protozoan parasites that infect the microvillous region of epithelial cells in the digestive and respiratory tract of vertebrates. Several species of *Cryptosporidium* have been described. These species appear to be specific for a class of vertebrates: *C. parvum, C. muris, C. felis* and *C. wrairi* infect mammals, *C. baileyi* and *C. meleagridis* infect birds, *C. serpentis* infects reptiles and *C. nasorum* tropical fish. Infections in humans are almost exclusively caused by *C. parvum.* This species is also frequently found in infections of cattle and sheep and causes infections in a wide range of other mammal species.

Life-cycle

Infected hosts shed oocysts, the environmentally resistant transmission stage of the parasite, with their faeces (Fayer & Ungar,1986, Fayer *et al.,* 1997). These oocysts are immediately infectious and may remain in the environment for very long periods without losing their infectivity, due to a very robust oocyst wall that protects the four sporozoites against physical and chemical damage. When the oocyst is ingested by a new host, the suture in the oocyst wall opens (excystation), triggered by the body temperature and the interaction with stomach acid and bile salts. Four motile sporozoites are released that infect the epithelial cells of the small intestine, mainly in the jejunum and ileum. The parasite infects the apex of the epithelial cells and resides beneath the cell

membrane of the epithelial cells but outside of the cytoplasm. The sporozoites transform into several life stages in an asexual (merogony) and a sexual reproduction cycle (gametogony). The oocysts are the result of the sexual reproduction cycle. Oocysts of *C. parvum* are spherical with a diameter of 4-6 μm. Thick- and thin-walled oocysts are formed. The thin-walled oocysts may excyst within the same host and start a new life cycle (autoinfection). This may lead to a heavily infected epithelium of the small intestine, resulting in malabsorptive or secretory diarrhoea. The thick-walled oocyst is excreted with the faeces and is environmentally robust.

Pathogenicity

Infection studies with healthy human volunteers show a very good relation between probability of infection and the ingested oocyst dose of a bovine *C. parvum* strain (Dupont *et al.,* 1995). At the lowest dose (30 oocysts) the probability of infection was 20%. This probability increased to 100% at 1000 oocysts. When the dose-response data are fitted with an exponential model, the probability of infection (P_i) is described by:
$P_i = 1-e^{-r \times dose}$, where r, the dose-response parameter, is 0.004005 (95% CI 0.00205 - 0.00723) for this *C. parvum* strain (Teunis *et al.,* 1996). This approach assumes that ingestion of even a single oocyst results in a distinct probability of infection (of 0.5%). Although there was a clear dose-response relation for infection, occurrence of symptoms of intestinal illness in the volunteers was not dose related. Recent studies indicate that the relation between oocyst dose and probability of infection and illness varies between *C. parvum* strains (Chappell, pers. comm)

The disease

The average incubation period is around 7 days, but shows a strong variation (Ungar, 1990; Dupont *et al.,* 1995). Watery diarrhoea is the most prominent symptom of an intestinal infection with *C. parvum* (Fayer & Ungar, 1986; Ungar, 1990). Frequent and voluminous bowel movements can cause dehydration and weight loss (Arrowood, 1997). Other symptoms are nausea, abdominal cramps, vomiting and mild fever. MacKenzie *et al*. (1994) compared clinical data from cases detected by (passive) laboratory surveillance with cases detected by (active) telephone surveys during the 1993 Milwaukee waterborne outbreak, which involved 400 000 patients. Patients who submitted a stool sample for laboratory diagnosis suffered more serious disease, as manifest from the higher prevalence of the following complaints in these patients: fatigue, loss of appetite, nausea, fever, chills and sweats, and vomiting.
In immunocompetent persons, the infection is limited by the immune response that eventually clears the host of the parasite. The occurrence of persistent and heavy infections in patients with deficiencies in the cellular (AIDS, chemotherapy, congenital) or humoral (congenital hypogammaglobulinaemia) immune responses suggests that both types of immune response are needed to limit and clear the infection. Several animal studies suggest that the immune response results in protection against re-infection (Zu *et al.,* 1992). Protective immunity in humans is suggested by the high rates of asymptomatic carriage in

countries with a high prevalence of cryptosporidiosis. Also, infected volunteers that were rechallenged with the same strain one year after the initial infection were significantly less sensitive to (re)infection (Okhuysen *et al.,* 1998). However, the rates of diarrhoea were similar in both exposures, but the illness was less intense in the re-infected volunteers, which indicates some degree of protective immunity.

The duration of the infection is generally 7-14 days for the immunocompetent, but also 23-32 days have been reported as median duration of the infection (van Asperen *et al.,* 1996). The peak intensity of oocysts shedding, with an average concentration of 10^6/g, coincides with the peak intensity of clinical symptoms. Oocyst shedding lasts for at least 2 weeks in 82% of the infected persons, 42% shed oocysts for at least 3 weeks and 21% for at least 4 weeks (Baxby *et al.,* 1985). Again, there is a difference between cases from laboratory surveillance (duration 2-4 weeks) and cases in the general population (duration typically 3-6 days). Relapses of diarrhoea are commonly seen, both population based (outbreak) studies and in volunteer experiments report 1-5 additional episodes in 40-70% of patients. This phenomenon considerably increases the mean duration of disease and its variability.
The mortality in immunocompetent patients is generally low. In immunodeficient persons however, the infection can be persistent and severe (Ungar, 1990) resulting in very profuse diarrhoea that leads to severe dehydration. Severe infections have been reported in patients with concurrent infections (AIDS, but also measles, chicken pox), persons with congenital immune deficiencies, persons receiving immunosuppresive drugs (for cancer therapy, transplants or skin lesions) and malnourished persons (Fayer *et al.,* 1997). Also, pregnancy may predispose to *Cryptosporidium* infection (Ungar, 1990). The prevalence of cryptosporidiosis in AIDS patients in industrialised countries is around 10-20% (Current & Garcia, 1991). In the absence of an effective immune response, the infection may spread throughout the entire intestinal tract and to other parts of the body (gall bladder, pancreas, respiratory tract). Despite extensive effort, no consistently effective therapeutic agent has been found (Blagburn & Soave, 1997). Immunotherapy with monoclonal antibodies or hyperimmune bovine colostrum have been reported to resolve diarrhoea in AIDS patients at least temporarily (Riggs, 1997). Similar findings were reported for several chemotherapeutic agents (azithromycin, paromomycin) (Blagburn & Soave, 1997).

The severe dehydration, the spread of the infection and the lack of an effective therapy lead to a high mortality in immunodeficient patients. Accurate data are lacking. In one study in the UK, 19% of the AIDS patients with cryptosporidiosis were suspected to have died from the infection (Connolly *et al.,* 1988). A compilation of case reports of cryptosporidiosis resulted in a mortality rate of 46% in AIDS patients and 29% in patients with other immunodeficiencies (Fayer & Ungar, 1986).

Prevalence

In stool surveys of patients with gastro-enteritis, the reported prevalence of *Cryptosporidium* is 1-4% in Europe and North America and 3-20% in Africa, Asia, Australia, South and Central America (Current & Garcia, 1991). Peaks in the prevalence in developed countries are observed in the late summer (van Asperen *et al.,* 1996) and in spring (Casemore, 1990).
Asymptomatic carriage, as determined by stool surveys, generally occurs at very low rates in industrialised countries (< 1%) (Current & Garcia, 1991), although in day care centres higher rates have been reported (Lacroix *et al.,* 1987; Crawford & Vermund, 1988; Garcia-Rodriguez *et al.,* 1989). Routine bile endoscopy suggests a higher asymptomatic prevalence: 13% of non-diarrhoeic patients were shown to carry *Cryptosporidium* oocysts (Roberts *et al.,* 1989). High rates of asymptomatic carriage (10-30%) are common in non-industrialised countries (Current & Garcia, 1991). Seroprevalence rates are generally higher than faecal carriage rates, from 25-35% in industrialised countries up to 95% in South America (Casemore *et al,* 1997). Seroprevalence rates increase with increasing age (Zu *et al.,* 1992; Kuhls *et al.,* 1994) and are relatively high in dairy farmers (Lengerich *et al.,* 1993) and day care centre attendants (Kuhls *et al.,* 1994).

Routes of transmission
A major route of exposure is person-to-person transmission, as illustrated by outbreaks in day-care centres (Fayer & Ungar, 1986; Casemore, 1990; Cordell & Addiss, 1994) and the spread of these outbreaks in the households of the attending children. Also sexual practices that imply oro-anal contact yield a high risk for exposure to *Cryptosporidium.* Transmission from animals (mammals) to man occurs, especially from newborn animals. Many infections have been derived from contact with infected calves and lambs (Casemore, 1990). Also pet animals can be infected with oocysts, but appear to be no important source of human infection (Casemore *et al.,* 1997; Glaser *et al.,* 1998). Indirect person-to-person or zoonotic transmission may occur by contamination of water used for recreation (swimming pools) or drinking or by food (raw milk and meat, farm-made apple cider)(Casemore *et al.,* 1997).
Waterborne outbreaks of cryptosporidiosis have been attributed to contaminated drinking water, both from surface and ground water sources (Craun, 1990; Mackenzie *et al.,* 1994; de Jong & Andersson, 1997), and to recreational water and swimming pools (Joce *et al.,* 1991; MacKenzie *et al*., 1995; van Asperen *et al.,* 1996; Anon, 1998; Kramer *et al.,* 1998).
Drinking-waterborne outbreaks have been caused by contamination of the source water due to heavy rainfall or melting snow (Richardson *et al.,* 1991; Pett *et al.,* 1993; MacKenzie *et al.,* 1994) or to sewage contamination of wells (d'Antonio *et al.,* 1985; Kramer *et al.,* 1996), inadequate treatment practices (Richardson *et al.,* 1991; Craun *et al.,* 1998) or treatment deficiencies (Anon., 1990; Leland *et al.,* 1993; Craun *et al.,* 1998) or combinations of these factors (MacKenzie *et al.,* 1994). Also, leakage or cross-connections in the distribution system have caused outbreaks of cryptosporidiosis (Craun, 1990; de Jong & Andersson, 1997; Craun *et al.,* 1998). The number of people affected by a

cryptosporidiosis outbreak through drinking water ranges from several up to 400.000.
During several of these outbreaks, oocysts were detected in the drinking water in a wide range of concentrations (Haas & Rose, 1995). Examination of drinking water during outbreaks is usually too late to determine the concentrations that triggered the outbreak. To obtain 'historical' data on the occurrence of oocysts in drinking water, researchers have attempted to detect oocysts in ice (MacKenzie *et al.,* 1994), in in-line filters (van Asperen *et al.,* 1996) and in sediments of water storage tanks (Pozio *et. al.*, 1997). The detected concentrations are probably an underestimation of the concentrations that led to the outbreak, although Haas & Rose (1994) showed for the Milwaukee outbreak that, with some assumptions, the measured concentration in drinking water was close to the predicted concentration on the basis of the attack rate, water consumption and dose-response relation.
Low oocyst concentrations in drinking water have also been found in situations where no evidence for the occurrence an outbreak was present (LeChevallier *et al.,* 1991; Karanis & Seitz, 1996; Rose *et al.,* 1997; McClellan, 1998). Current detection methods do not allow the determination of pathogenicity of oocysts in water, which makes it difficult to determine the significance of low oocyst levels in drinking water. Given this uncertainty, detection of oocysts in treated water should always lead to the use of additional tests to confirm the presence of (viable) *C. parvum* oocysts and a thorough examination of other water quality parameters that may indicate a faecal contamination event. If these additional tests indicate the presence of *C. parvum* oocysts, this should lead to an epidemiological study to determine if significant waterborne transmission occurs and careful examination of the source of the contamination and the installation of control measures (improved source protection and/or water treatment).

Giardia intestinalis

Taxonomy

Giardia is a flagellated protozoan. The taxonomy and host specificity of this organism have been and still are matter of much debate. *Giardia* has been found in more than 40 animal species (Meyer, 1994). Nowadays, five species of *Giardia* are established in the scientific literature: including the three species proposed by Filice (1952): *G. muris* in rodents, birds and reptiles, *G. intestinalis* (syn: *duodenalis,* syn: *lamblia)* in mammals (including man), rodents, reptiles and possibly in birds, *G. agilis* in amphibians, *G. ardae* in the Great Blue Heron (Erlandsen *et al.,* 1990) and *G. psittaci* in the budgerigar (Erlandsen & Bemrick, 1987). Recently, a morphologically distinct *Giardia* was isolated from the straw-necked ibis (Forshaw *et al.,* 1992), that was later suggested to be a distinct strain of *G. ardae* (McRoberts *et al.,* 1996).

Giardia is thought to be predominantly asexual, which makes the species concept difficult to apply.

A high degree of genetic heterogeneity is found in human and animal isolates (Nash *et al.,* 1985; Andrews *et al.,* 1989; Meloni *et al.,* 1989; Morgan *et al.,* 1994) which makes speciation uncertain and suggests that it is a clonal parasite (Tibayrenic, 1994). *G. intestinalis* can be subdivided by several techniques into two groups (Homan *et al.,* 1992, 1994). It is still uncertain if and how this heterogeneity is related to host specificity and pathogenicity of *Giardia.*

Life-cycle

Giardia has a simple life cycle (Feely *et al.,* 1990; Meyer, 1994). As with *Cryptosporidium*, the parasite is shed with the faeces as environmentally robust cyst, that is transmitted to a new host. In the duodenum of the new host, the trophozoite emerges from the cysts and completes a mitotic division to produce two trophozoites that attach to the epithelial cells by their adhesive disc and feed on the epithelial cell. The trophozoites detach from the epithelial cells, probably because these cells have a rapid turnover (72 hours) and undergo mitotic division in the intestinal lumen. During periods of diarrhoea, these trophozoites may be transported with the intestinal contents and are excreted. The trophozoites do not survive long outside the host. During the passage through the intestine, part of the trophozoites begin to encyst and leave the host with the faeces as cysts. Cysts are more often encountered in formed stools. *Giardia intestinalis* cysts are elliptical and 8-12 μm long and 7-10 μm wide. The cyst wall is 0.3-0.5 μm thick and has a fibrillous structure. In the cyst, two to four nuclei can be found together with axonemes of the flagella of the trophozoite.

Pathogenicity

Human feeding studies with *G. intestinalis* cysts produced a dose response relation between the probability of infection and the ingested cyst dose (Rendtorff, 1954). No data on the viability of the ingested cysts were provided. A dose of 10 cysts resulted in an infection in 100% (2/2) of the volunteers. The dose-infection relation could be described with an exponential model (Rose *et al.,* 1991[b]): $P_i = 1\text{-}e^{-0.0199*dose}$ (95% CI of r: 0.0044-0.0566). Although overall 53% of the volunteers became infected in this feeding study, and changes in bowel motions were observed, none of the volunteers developed symptoms of giardiasis. The infection-to-illness ratio varies between different isolates, as shown by the different response to two different isolates from symptomatic human infections in the volunteer study of Nash *et al.* (1987). Also host factors (age, nutritional status, predisposing illness, and previous exposure) determine the outcome of an infection (Flannagan, 1992). Asymptomatic carriage appears to be the most common form of infection with *Giardia* (Farthing, 1994), ranging from 16-86% of the infected individuals. The mechanism by which *Giardia* causes diarrhoea and malabsorption is still unclear. *Giardia* could act as physical barrier, but the area covered by trophozoites is probably too small for affecting the absorption of nutrients. No evidence has been found for the production of toxins (Buret, 1994). *Giardia* infections appears to affect gut enzyme (lactase, disaccharidase) activities and damage the mucosal surface (shortening of crypts

and villi) and give rise to overgrowth of the small intestine by bacteria (Tomkins *et al.,* 1978) or yeasts (Naik *et al.,* 1978).

The disease

The time between infection and the occurrence of *Giardia* cysts in the stool is 12 to19 days (Jokipii *et al,* 1985). The incubation period for the occurrence of symptoms varies between 1-75 days, but is generally between 6-15 days, and coincides with the occurrence of *Giardia* in stool (Rendtorff, 1954; Brodsky *et al.,* 1974). The most prominent symptoms are diarrhoea (fatty, yellowish) weakness, weight loss and abdominal pain and to a lesser extent nausea, vomiting, flatulence and fever. In the majority of cases, the infection is acute and self-limiting within 2-4 weeks. A significant proportion of the infected population will go on to have chronic infection with intermittent diarrhoea (estimated at 30-50%)(Farthing, 1994). Weight loss can be profound (10-20%) in this group. The ability of *Giardia* to change the surface epitopes of the trophozoites during infection (Nash, 1992), may play a role in the occurrence of chronic infections. There is evidence that infection with *Giardia* results in 'failure to thrive' in children, by impairment of the uptake of nutrients (especially fats and vitamin A and B12) (Farthing, 1994; Hall, 1994). Excretion of cysts varies between 10^6-10^8 per gram of stool, as determined in positive stool samples (Tsuchiya, 1931), but a significant proportion of the stool samples does not show *Giardia* in detectable levels. Excretion patterns vary between hosts and isolates.

Prevalence

Giardia infections are very common in children in developing countries (Rabbani & Islam, 1994; Farthing, 1994). Also in developed countries, the prevalence peaks at the age of 1-4 years (Flannagan, 1992); a second peak is observed at the 20-40 age group, partly due to the care for the young children and partly due to travelling. In developing countries, the prevalence of giardiasis in patients with diarrhoea lies around 20%, ranging from 5-43% (Islam 1990). In developed countries, this prevalence varies from 3% (Hoogenboom-Verdegaal *et al,* 1989; Adam, 1991; Farthing, 1994; Kortbeek *et al.,* 1994) to 7% (Quinn, 1971).
As a reaction to infection with *Giardia,* both a humoral and cellular immune response is generated by the host. Secretory IgA and IgM appear to play a role in clearance of the intestinal infection, by reducing the mobility of trophozoites and preventing their adhesion to the mucosa (Farthing and Goka, 1987). The immune response can also be seen in the serum antibodies. The immune response can give some degree of protection against reinfection, as indicated by lower attack rates in chronically exposed populations (Istre *et al.,* 1984; Rabbani & Islam, 1994). This protection is limited however, recurrence of symptomatic infections, even after several infections, is common (Gilman *et al.,* 1988; Wolfe 1992; Hall, 1994), which may be related to the antigenic variation shown by *Giardia* (Nash, 1992).
Giardiasis can be treated with nitroimidazoles, quinacrine and furazolidone (Boreham, 1994). For patients with persistent giardiasis several approaches can

be taken, such as increasing duration and dose of drug admission, administering an alternate drug or a combination of drugs.

Routes of transmission

Person-to-person faecal-oral transfer of *Giardia* cysts is the major route of transmission of giardiasis, as indicated by the high prevalence in situations with poor hygienic conditions in developing countries, in day-care centres and nurseries (Black *et al.,* 1977; Pickering & Engelkirk, 1990; van de Bosch, 1991) and secondary spread to the house-hold in day care centre outbreaks (Black *et al.,* 1977). Foodborne outbreaks have been the result of contamination of food by infected workers or household members (Osterholm *et al.,* 1981; Islam, 1990; Thompson *et al.,* 1990).

The role of animals in the transmission of human giardiasis is still controversial. Although *Giardia* commonly occurs in pet, farm and wild mammals, there is no unequivocal evidence that these *Giardia* have caused infections in humans (Erlandsen, 1994). *Giardia intestinalis* isolates from animals and man may be morphologically indistinguishable (Flannagan, 1992) and this has led to many reports on animal sources of human giardiasis, including waterborne cases caused by *Giardia* cysts from beavers and muskrats (Moore *et al.,* 1969; Dykes *et al.,* 1980). However, the genetic diversity within and between human and animal isolates (Thompson *et al.*, 1988) is too high to draw definite conclusions regarding host specificity. Cross-transmission studies have not been well controlled and the results have been contradicting (Davies & Hibler, 1979; Hewlett *et al.,* 1982; Belosevic *et al.,* 1984; Kirkpatrick & Green, 1985; Woo & Patterson, 1986).

Waterborne outbreaks of giardiasis have been reported for almost 30 years (Moore *et al.,* 1969; Brodsky *et al.,* 1974; Craun, 1990). In the US, *Giardia* is the most commonly identified pathogen with more than 100 waterborne outbreaks, based on epidemiological evidence (Craun, 1990). Waterborne outbreaks have also been reported from Canada, Australia, New Zealand, United Kingdom and Sweden. These outbreaks have been linked to consumption of untreated surface water that was contaminated by human sewage (Craun, 1990) or by wild rodents (Moore *et al.,* 1969; Dykes *et al.,* 1980), to ground water that was contaminated by human sewage or contaminated surface water, to surface water systems receiving only disinfection (Craun, 1984; Kent *et al.,* 1988) or ineffective filtration (Dykes *et al.*, 1980; Craun, 1990) and by cross-connections or damage in the distribution system (Craun, 1986).

Cyclospora sp.

Taxonomy

Cyclospora was first described by Eimer in 1870 from the intestines of moles, and is related taxonomically to other protozoan parasites such as *Cryptosporidium* and *Toxoplasma*. The first likely observation of this parasite as a pathogen for human beings was by Ashford (1979). Confirmation of the coccidian identity and genus was made in 1993 (Ashford *et al*., 1993; Ortega *et al.,* 1993). *Cyclospora* is a

member of the subphylum Apicomplexa, class Sporozoasida, subclass Coccidiasina, family Eimeriidae. Organisms of the genus *Cyclospora* have an oocyst with two sporocysts, each of which contains two sporozoites (Levine, 1973) and have been found in snakes, insectivores and rodents. Molecular phylogenetic analysis suggests that the genus *Cyclospora* is closely related to the genus *Eimeria* (Relman *et al*., 1996).

Life-cycle

Many of the details of the life cycle of *Cyclospora* in human beings are not yet known. *Cyclospora* completes its life cycle within one host (monoxenous). Ortega *et al* (1993) proposed that *Cyclospora* that are infective to human beings should be designated *Cyclospora cayetanensis* on the basis of the development of the oocyst *in vitro.* However, Ashford *et al*., (1993) question the species name and Bendall *et al* (1993) preferred the use of the term CLB (denoting *Cyclospora*-like body) until further information is forthcoming regarding the biology of this coccidian parasite. In this review, *Cyclospora* sp. will be the nomenclature used to describe those organisms infective to man.

The endogenous stages of *Cyclospora* sp. are intra-cytoplasmic and contained within a vacuole (Bendall *et al*., 1993), and the transmissive stage, the oocyst, is excreted in the stool. The life cycle of *Cyclospora* sp. may complete within enterocytes (Sun *et al*., 1996). *Cyclospora* sp. oocysts are spherical, measuring 8-10 µm in diameter. They are excreted unsporulated in the stool and sporulate to infectivity in the environment. Unsporulated oocysts contain a central morula-like structure consisting of a variable number of inclusions whilst sporulated oocysts contain two ovoid sporocysts. Within each sporocyst reside two sporozoites. Each sporozoite measures 1.2 x 9 µm.

Pathogenicity

Cyclospora sp. infects enterocytes of the small bowel and can produce disease (Bendall *et al.,* 1993). Both symptomatic and asymptomatic states have been described. A moderate to marked erythema of the distal duodenum can occur with varying degrees of villous atrophy and crypt hyperplasia (Connor *et al.,* 1993). However, little is known of the pathogenic mechanisms. As yet, no virulence factors have been described for *Cyclospora* sp. No animal or human feeding studies have been undertaken. As for *Giardia* and *Cryptosporidium*, it is assumed that the organisms are highly infectious, and that doses less than 100 sporulated oocysts may lead to a high probability of infection.

The disease

Symptoms include watery diarrhoea, fatigue, abdominal cramping, anorexia, weight loss, vomiting, low-grade fever and nausea which can last several weeks with bouts of remittance and relapse. The incubation period is between 2 and 11 days (Soave, 1996) with moderate numbers of unsporulated oocysts being excreted for up to 60 days or more. Illness may last for weeks and episodes of watery diarrhoea may alternate with constipation (Soave, 1996). In immunocompetent individuals the symptoms are self-limiting and oocyst

excretion is associated with clinical illness (Shlim *et al*., 1991), whereas in immunocompromised individuals diarrhoea may be prolonged.

Prevalence

Cyclospora sp. oocysts have been isolated from the stools of children, immunocompetent and immunocompromised adults. Oocysts have been described in the stools of residents in, and travellers returning from, developing nations, and in association with diarrhoeal illness in individuals from North, Central and South America, the Caribbean, the Indian sub-continent, Southeast Asia, Australia and Europe. Outbreaks of cyclosporiasis have been reported from Nepal and North and South America. In north America and Europe cyclosporiasis is associated with overseas travel and travellers' diarrhoea. Point source outbreaks have been reported in the USA and Nepal. In 1996, a total of 1465 cases were reported in the USA and Canada, with around half of them occurring following events at which raspberries had been served (Anon., 1996; Herwaldt *et al*., 1997). Most cases occurred during spring and summer. Sporadic cases of cyclosporiasis have been reported from many countries and *Cyclospora* sp. oocysts are increasingly being identified in stools from immunocompetent individuals without foreign travel histories. Studies in Nepal, Peru and Tanzania seek to address *Cyclospora* sp. epidemiology, life cycle and pathology.

Cyclospora sp. oocysts were detected in faecal samples from 11% of Haitians with chronic diarrhoea who were seropositive for human immunodeficiency virus (HIV) (Pape *et al*., 1994). Apart from HIV, *Cyclospora* sp. oocysts were the sole pathogen identified in many of these patients. Whilst clinical disease can resolve without treatment, trimethoprim-sulphamethoxazole (TMP-SMZ) is the drug of choice.

Routes of transmission

Epidemiology indicates that *Cyclospora* sp. is transmitted by water and food (Hoge *et al*., 1993; Anon., 1996; Herwaldt *et al*., 1997). An outbreak amongst house staff and employees in a hospital dormitory in Chicago occurred following the failure of the dormitory's water pump. Illness was associated with the ingestion of water in the 24 hours after the pump failure and *Cyclospora* sp. oocysts were detected in the stools of 11 of 21 persons who developed diarrhoea (Anon, 1991; Wurtz, 1994).

An outbreak occurred amongst British soldiers and dependants stationed in a small military detachment in Nepal and 12 of 14 persons developed diarrhoea. *Cyclospora* sp. oocysts were detected in stool samples from 6 of 8 patients. Oocysts were also detected microscopically in a concentrate from a 2 litre water sample. Drinking water for the camp consisted of a mixture of river water and chlorinated municipal water. Chlorine residuals of 0.3 to 0.8 ppm were measured before and during the outbreak. No coliforms were detected in the drinking water (Rabold *et al*., 1994).

DETECTION METHODS

Cryptosporidium and *Giardia*

The methodology required for the detection of *Cryptosporidium* oocysts and *Giardia* cysts in water is completely different from that traditionally used in the water industry. The methods that are currently available are at best tentative because of their low and variable recovery and the inability to differentiate viable oocysts of strains that are infectious to humans. The overall procedure consists of several stages, namely: sample collection and concentration, separation of (oo)cysts from contaminating debris and detection of (oo)cysts. Many factors, such as water quality and age of the (oo)cysts, can have significant effect on the overall recovery efficiency and thus it is almost impossible to compare the effectiveness of two methods that have been performed in different laboratories unless these factors are standardised. Furthermore, there is considerable interest in determining if (oo)cysts are viable and potentially infectious. Thus methods have been and are currently being developed to assess the viability of (oo)cysts in the environment.

Quality assurance

Microscope counts

Care must be taken to ensure that the particles being counted are (oo)cysts, whether or not they contain sporozoites, and that algae and yeast cells are excluded from any counts that are made. The criteria used for determining that a particle is in fact a *Cryptosporidium* oocyst or *Giardia* cyst vary between laboratories. Some workers use only the fact that (oo)cysts fluoresce when labelled with a fluorescein isothiocyanate tagged anti-*Cryptosporidium* or anti-*Giardia* monoclonal antibody and that it is in the proper size range that a particle is a cyst or oocyst, whilst others will additionally use differential interference contrast microscopy or nucleic acid stains to determine if the particles that are counted are indeed (oo)cysts. This more detailed analysis allows the confirmation of the counted particles as presumptive (oo)cysts.
Many factors influence the microscope counts: the amount of background debris and background fluorescence, experience and alertness of the counting technician, fluorescence intensity after staining with the monoclonal antibody and the quality of the microscope. QA protocols should define how these factors are addressed.

Recovery efficiency

Given the low and variable recovery efficiency of the methods that are used for environmental monitoring for *Cryptosporidium* and *Giardia*, it is essential that laboratories collect their own data on the recovery efficiency of their method in the different water types they monitor. This can be achieved by seeding a second water sample with a known number of cysts and oocysts and determine which percentage of these (oo)cysts is recovered by the total protocol for sampling, processing and counting of environmental samples.
This assay is influenced by the number, age and storage conditions of the (oo)cysts used for seeding. These have to be standardised (at least within a laboratory) to collect meaningful recovery data. The recovery efficiency should be assessed sufficiently frequent to be able to determine how the variation in

the recovery efficiency influences the uncertainty of the monitoring data. This is essential for the interpretation of environmental monitoring.

Concentration techniques

Cartridge filtration

The initial methodology to detect *Giardia* and *Cryptosporidium* in water used polypropylene cartridge filters with a nominal pore size of 1 μm, through which large volumes of water (100-1000 litres) are passed at a flow rate of 1-5 litres per minute. Trapped material is then eluted by cutting the filter open and washing either by hand or by stomaching using a dilute detergent solution. The resulting washings from these cartridges sometimes totals three or four litres and they must then be further concentrated by centrifugation. The ability to recover *Cryptosporidium* oocysts by this technique was originally reported to be in the range of 14-44% (Musial *et al.*, 1987) although lower recovery efficiencies (< 1-30%) have often been reported since (Ongerth & Stibbs, 1987; Clancy *et al.,* 1994; Shepherd & Wyn-Jones, 1996). Differences in reported recovery rates may be due to a number of factors including water quality, laboratory efficiency and oocyst age.

Membrane filtration

A method described by Ongerth & Stibbs (1987) utilised large (142 or 293 mm diameter) 2 μm absolute, flat bed membranes for the concentration of oocysts from water samples and many workers have now adopted this procedure. Water is pumped through the membranes and the concentrated materials are recovered by 'scraping' the surface of the membrane together with washing with dilute detergent followed by further concentration using centrifugation. However, whilst with low turbidity water, it is relatively easy to filter 10-40 litres, with some high turbidity waters, it is only possible to filter 1-2 litres. As with cartridge filtration, a range of recovery efficiencies has been reported for flat bed membranes. Nieminski *et al.* (1995) reported an average recovery of 9% for *Cryptosporidium* and 49% for *Giardia*. In a study of the efficiencies of several different membranes for recovering both *Cryptosporidium* oocysts and *Giardia* cysts, Shepherd & Wyn-Jones (1996) suggested that 1.2 μm cellulose acetate membranes gave higher recovery (30-40% and 50-67% respectively) than the 2 μm polycarbonate membranes (22-36% and 41-49% respectively) preferred by Ongerth & Stibbs (1987).

Flocculation

Another established procedure for concentrating (oo)cysts is the calcium carbonate flocculation procedure developed by Vesey *et al.* (1993[b]). A fine precipitate of calcium carbonate ($CaCO_3$) is formed in a water sample by the addition of calcium chloride and sodium bicarbonate, followed by adjusting the pH to 10.0 with sodium hydroxide. After allowing the precipitate to settle, the supernatant fluid is aspirated off and the sedimented material resuspended after dissolving the calcium carbonate with sulphamic acid. Recovery efficiencies using this method have been reported to be as high as 70% for both *Cryptosporidium* and *Giardia* (Campbell *et al.*, 1994; Vesey *et al.*, 1993[b]; Vesey

et al., 1994; Shepherd & Wyn-Jones, 1996). More recent work has demonstrated that this is the upper limit of the detection efficiency and that lower recoveries are usually encountered. Use of aged oocysts for seeding experiments together with leaving the oocysts in contact with water for a few days prior to analysis showed that recovery rates of 30-40% were more normally seen. The viability of the oocysts is affected by this concentration (Campbell *et al.,* 1995). Flocculation with aluminium sulphate ($Al_2(SO_4)_3$) did not affect the viability of oocysts, while the recovery efficiency was comparable to the $CaCO_3$ flocculation (Schwartzbrod, pers. comm.).

New methods

The search for new methods for concentrating water samples to detect the presence of protozoan parasites continues and many methods have been evaluated, including cross-flow filtration, continuous flow centrifugation and vortex flow filtration (Whitmore, 1994). Methods which are currently receiving attention include vortex flow filtration (Fricker *et al.,* 1997), the Gelman envirochek filters (Clancy *et al.*, 1997) and the Genera filter system (Sartory, pers. comm.), amongst others.

There continues to be much debate over which method is most appropriate. Realistically there is no one single method which is most suitable for all situations. The choice of method should be made with due regard to a number of factors, including the purpose of sampling, the water quality and the facilities in the laboratory which will perform the analysis. Ideally, the method chosen should efficiently concentrate as large a sample as possible and yield a concentrate which can be examined easily. Many workers prefer to concentrate only a small volume of water initially and to examine the entire concentrate, whilst others take large samples and examine only a fraction of the final concentrate. Either approach is defensible, but the methods used to concentrate small volumes (e.g. 10-20 l) tend to be easier to perform and generally have a higher recovery efficiency and so it is often preferable to take a large number of low volume samples and to examine all of the concentrate. Other factors which may affect the choice of concentration method include the site of sample collection and the distance which samples must be transported.

Separation techniques

Since the concentration of *Cryptosporidium* oocysts and *Giardia* cysts is based almost exclusively on particle size, the techniques are not specific and a large amount of extraneous material is concentrated as well. This material may interfere with the successful detection of (oo)cysts, either by increasing the total volume which needs to be examined, or by obscuring or mimicking (oo)cysts during examination. Some form of separation technology is therefore normally required to reduce the time taken to examine a sample and to prevent (oo)cysts being missed.

Density centrifugation

Density centrifugation is used by many workers to separate (oo)cysts from background debris and thus reduce the amount of material to be examined. Several workers use sucrose density centrifugation to separate parasites from faecal material in clinical samples. This basic technique has been adopted for use with environmental samples, although some workers prefer to use Percoll-sucrose or Percoll-percoll gradients. Whatever flotation method is used, several groups have demonstrated that this is an inefficient procedure when trying to detect protozoan parasites in water concentrates. Of particular interest was the finding of Bukhari & Smith (1996) that sucrose density centrifugation selectively concentrated viable, intact *Cryptosporidium* oocysts. Fricker (1995) demonstrated that the recovery of oocysts from water samples could be affected by the length of time that they were in contact with the water concentrate but that this was only the case when sucrose flotation was performed. Spiked samples which are examined directly without density centrifugation gave similar recovery efficiencies, irrespective of whether they were examined immediately after seeding or after 48 hrs contact with the concentrate. However, when sucrose flotation was used, the recovery of (oo)cysts in raw water fell from a mean of 55% to 18% after the same period of contact. This reduction in recovery efficiency was also seen with concentrates of reservoir water (67 to 23%) and fully treated water (80 to 52%).

Immunomagnetic separation

Autofluorescing algae, which may not be completely removed by the density gradient centrifugation, can cause severe problems when examining slides for protozoa by epifluorescence microscopy. More efficient methods for separation of (oo)cysts from other particulates have been sought. Many workers have attempted the use of immunomagnetic separation (IMS). The principles behind this technology involve the attachment of specific antibodies to magnetisable particles and efficient mixing of the particles in the sample. The (oo)cysts attach to the magnetisable particles and are isolated from this debris with a strong magnet. The technique is very simple, but there are several sources of failure. An important source is the quality and specificity data of the monoclonal antibodies which are available. Most of the commercially available monoclonal antibodies to *Cryptosporidium* or *Giardia* are of the IgM type, and are therefore of low affinity since they have not undergone affinity maturation or isotype switching. When IMS is used and beads are mixed with water concentrates, the immunoglobulin-(oo)cyst-bonds are subjected to shear forces and therefore the stronger the bond, the more likely the bead is to remain in contact with the (oo)cyst. The way in which the antibody is attached to the bead may also have an effect on recovery efficiency, since if the attachment between the bead and the antibody is not strong, the antibody may detach and the oocyst will not be recovered. The turbidity of the water concentrate appears to be the most critical factor associated with the recovery efficiency of IMS. Oocysts seeded into relatively clean suspensions are recovered efficiently, with recoveries of over 90% being reported (Campbell *et al.,* 1997[a,b]). However, the real benefit of a good separation technique is with samples which have yielded a highly turbid

concentrate and it is in these samples that IMS does not appear to perform as efficiently. The use of antibodies of higher affinity may serve to improve the recovery efficiency of oocysts from high turbidity samples. Although this technique is also able to separate *Giardia* cysts, not much effort has been put into testing the recovery efficiency of these cysts by IMS.

Flow cytometry

Workers in the United Kingdom attempted to use flow cytometry with environmental samples in order to detect *Cryptosporidium* oocysts, but found that the sensitivity of these instruments was not high enough to distinguish oocysts from background noise (Vesey *et al.,* 1991). Incorporation of a cell sorting facility onto flow cytometers enabled oocysts to be sorted efficiently from background material (Vesey *et al.,* 1993[a]). This technique is shown to work equally efficient for *Giardia* cysts (Vesey *et al.,* 1994; Medema *et al.,* 1998[a]). Water concentrates are stained in suspension with FITC-labelled antibodies and passed through the fluorescence activated cell sorter (FACS). Particles with the fluorescence and light scatter characteristics of (oo)cysts are sorted from the sample stream and collected on a microscope slide or membrane filter, that is examined by epifluorescence microscopy to confirm the presence of (oo)cysts. The FACS procedure is not specific and sensitive enough to enable the count of sorted particles as a definitive number of (oo)cysts present, since other organisms/particles of similar size may cross-react with the monoclonal antibody and have similar fluorescence characteristics. In addition, some water samples contain high numbers of autofluorescent algae which may also mimic (oo)cysts and therefore lead to incorrect conclusions if the FACS is used directly to produce (oo)cyst counts. However, the confirmation by epifluorescence microscopy can be performed much easier and more reliably than direct microscopy of non-sorted samples. Several researchers from the United States, France and the Netherlands have confirmed the benefits of FACS when examining water samples for the presence of (oo)cysts (Danielson *et al.,* 1995; Compagnon *et al.*, 1997; Medema *et al.,* 1998[a]). FACS is widely used in the United Kingdom for water analysis and is becoming more and more adopted in other parts of Europe, in Australia and in South-Africa.

Detection

Immunofluorsecence microscopy

Routine detection of *Cryptosporidium* oocysts and *Giardia* cysts relies on the use of epifluorescence microscopy which may be applied to examine material deposited on multiwell slides or membrane filters. The (oo)cysts are specifically stained with monoclonal antibodies which have been labelled directly with FITC or are labelled during staining with an FITC-labelled anti-mouse antibody. There have been no definitive studies to compare the efficiency of these procedures, but the tendency now is towards staining with a directly labelled antibody. This tends to give less non-specific binding and can make preparations easier to examine. Several anti-*Cryptosporidium* antibodies and anti-*Giardia* antibodies are commercially available and whilst most workers have their preferences, there does not appear to be a single antibody which is preferred for all purposes. One

specific failing of the commercially available antibodies is that they all apparently cross-react with other members of the genera and therefore cannot be used to specifically identify *C. parvum* or *G. intestinalis*.

A number of other detection techniques have been tried by various workers in order to improve the ease of identification of both *Cryptosporidium* oocysts and *Giardia* cysts.

FISH
Fluorescence In-Situ Hybridisation (FISH) has been suggested as a tool for the specific detection of *Cryptosporidium parvum* (Vesey *et al.,* 1997; Lindquist, 1997). Vesey *et al.* (1997) also showed that the stainability of oocysts with the FISH-method correlated with excystation. This FISH method could be combined with the IFA method. However, the intensity of the FISH-fluorescence signal is relatively weak, which makes microscopic interpretation difficult.

PCR
Perhaps one of the most extensively tested procedures is the use of the polymerase chain reaction (PCR) to detect specific sequences of nucleic acids which may be species or genus specific. Clearly, the ability to distinguish between *C. parvum* and other morphologically similar members of the genus is useful and nucleic acid based techniques may prove useful for this.
However, despite the exquisite specificity and sensitivity which PCR can offer, difficulties have been experienced with the application of PCR to water concentrates. This has largely been due to inhibition of the DNA amplification process. PCR is sensitive to the concentration of many compounds within the reaction mixture and those of particular concern to researchers working with water concentrates are divalent cations and humic and fulvic acids, which are compounds frequently found in water and which can cause a high degree of inhibition. Nonetheless many workers have described protocols for the detection of *Cryptosporidium* oocysts by PCR and a wide variety of primers have been described. These primers have been designed from various regions of the genome and some which have apparent specificity include those from regions coding for the 18 S rRNA (Johnson *et al.,* 1995), or mRNA coding for the *Cryptosporidium* heat shock protein *Hsp70* (Stinear *et al.,* 1996, Kaucner & Stinear, 1998), in combination with cell culture (Rochelle *et al.,* 1996, 1997). Abbaszadegan *et al.* (1997) first reported the application of PCR primers from gene sequences coding for inducible heat shock proteins to specifically detect *Giardia* cysts. The sensitivity of the standard PCR was reported to be one cyst in water samples. They also reported that amplification of heat shock-induced mRNA utilising the same HSP primers was indicative of viable *Giardia* cysts.
The use of PCR for the detection of cysts and oocysts in water concentrates offers some advantages over that of direct microscopical examination, since the process can largely be automated and thus several samples can be processed simultaneously. Furthermore, the technique is theoretically sensitive down to a level of a single (oo)cyst and recent developments have suggested that it may be possible to distinguish viable from non-viable (oo)cysts. Some workers claim

to be able to detect a single oocyst in a water concentrate by using a procedure involving reverse transcription (RT) PCR where the target sequence codes for the *Cryptosporidium* heat shock protein *Hsp 70* (Stinear *et al.,* 1997). The data presented showed that a single viable oocyst could be detected by this procedure, even in the presence of PCR inhibitors. Such a method would be of considerable value to the water industry, facilitating rapid screening of samples although as yet it is not quantitative and thus may be of limited value in some circumstances.
The use of RT-PCR against induced mRNA, a nucleic acid with a short half-life, overcomes the concern that "false positive" results could be obtained either from non-viable oocysts or from free DNA. Many researchers still favour a holistic approach, where the intact organism can be viewed directly. A combined approach may be used whereby molecular techniques are used as a screening tool on a portion of a water concentrate and that where positive results are generated, other approaches which involve microscopical examination are used.

Methods for determining oocyst viability
The significance of finding oocysts in treated and to a lesser extent raw waters is not always clear, since some of the organisms which are detected may be non-viable and thus pose no threat to public health. Therefore, there has been considerable interest in developing *in vitro* methods which can determine oocyst viability.

Excystation
The most widely accepted *in vitro* procedure for determining oocyst viability, excystation, has not been used in combination with the IFA method, because excystation is difficult to incorporate in the IFA protocol. Excystation has been used in combination with PCR to detect the presence of viable *Cryptosporidium* oocysts (Filkorn *et al.,* 1994; Wiedenmann *et al.,* 1997). The sensitivity of this method in environmental samples needs further research. Excystation has been used in survival and disinfection studies. In the latter, this technique appears to yield a lower inactivation rate than the neonatal mouse infectivity assay (Finch *et al.,* 1993[a]; Clancy *et al.,* 1998).

Vital dyes
The ability of *Giardia* cysts to stain with the vital exclusion dye propidium iodide (PI) has been shown by various workers to correlate with the inability to excyst or infect animals (Schupp & Erlandsen, 1987; Smith & Smith, 1989). PI can therefore be used as indicator of cell death for *Giardia* cysts.
Campbell *et al.* (1992) developed a procedure based on the exclusion of PI for *Cryptosporidium* oocysts, using 4'6-diamidino-2-phenyl indole (DAPI) as supporting stain, which gave a good correlation with *in vitro* excystation. Four classes of oocysts can be identified using the assay: those which are viable and include DAPI but exclude PI, those which are non-viable and include both DAPI and PI and two classes which include neither DAPI or PI, those with internal contents (sporozoites) and therefore potentially viable, and those without and

therefore non-viable, as determined by DIC. microscopy. The DAPI/PI procedure is simple to perform and whilst some workers have expressed some reservations over its' applicability, it can be used for routine environmental work. The incorporation of DAPI into the nucleic acid acts as a further criterion for determining if a particle is an oocyst or not.
An alternative to the DAPI/PI approach to determine viability has been suggested by Belosevic and Finch (1997) who have used new nucleic acid stains to differentiate between viable and non-viable oocysts. Two new stains have been identified, SYTO9 which stains non-viable oocysts green or bright yellow, while viable oocysts have a green halo surrounding the cell whilst the interior remains unstained and MPR71059 which stains non-viable oocysts red whilst viable oocysts remain unstained. These approaches have not been widely tested although Belosevic and Finch (1997) have demonstrated that the results obtained with these dyes, correlate well with mouse infectivity using an outbred CD-1 neonatal mouse model. Since these vital stain-assays are apparently simple and quick to perform, they may be suitable for incorporation into the methods for the detection of oocysts in water samples, but this has yet to be proven.

Cell culture
Attempts have been made to develop *in vitro* models of infectivity using tissue culture (Upton *et al,* 1994, Rochelle *et al,* 1996; Slifko *et al.,* 1997). For these assays, water samples are concentrated by normal procedures and bacteria may be removed by exposure of the concentrate to concentrations of chlorine which are lethal to bacterial cells but which are thought not to effect oocysts. The concentrates are then inoculated onto the tissue culture monolayer, left in contact for a period to allow potentially infectious oocysts to infect cells before the remaining debris is washed away. The monolayer is then left for 24-48 hours before being examined for the presence of intracellular parasite antigen or nucleic acid. Immunofluorescent techniques have been used to identify cells which have become infected. This offers a way in which infection may potentially be quantified. However, it is not clear if the presence of a single infectious oocyst will lead to one or more infected cells. In theory one might expect that an oocyst which excysts successfully would produce four infected tissue culture cells, but initial results have not demonstrated that this can be consistently achieved. Other workers (Rochelle *et al.,* 1996) have adopted a somewhat different approach whereby they detect the presence of *Cryptosporidium* nucleic acids using PCR. Whilst the cell culture method cannot be used to directly enumerate the oocysts present in any given sample, it can be applied in a "most probable number" format to give an estimation of the number of oocysts present in a water concentrate.

Molecular methods
The RT-PCR methods that amplify induced mRNA that codes for heat shock proteins also indicate viability of *Giardia* cysts (Abbaszadegan *et al.,* 1997) and *Cryptosporidium* oocysts (Stinear *et al.,* 1997; Kaucner & Stinear, 1998). In

combination with the reported sensitivity and specificity (see Detection), these methods may prove to be very valuable for the water industry.

Typing methods
With the current detection techniques, it is not possible to identify the origin of (oo)cysts in a water sample. Several typing methods are available for both *Cryptosporidium* and *Giardia* and these are able to discriminate between human and animal *C. parvum* strains (Ogunkadale *et al.,* 1993; Bonnin *et al.,* 1996; Deng & Cliver, 1998), but these are not yet applicable to surface water samples.

Cyclospora

Detection methods for stool samples
No methods have been developed for the detection of *Cyclospora* in environmental samples. Therefore, the information on detection of this parasite in stool samples is given as guidance.
Identification of *Cyclospora* in stool samples is based upon the appearance of the oocyst either in direct or concentrated wet films. Concentration either by the formalin-ether (formalin-ethyl acetate) method or sucrose flotation is effective. Oocysts have also been reported from jejunal aspirates (Bendall *et al*., 1993). Organisms seen in stool samples are normally the unsporulated oocysts of *Cyclospora* sp. In wet mounts, oocyst walls appear as well-defined non-refractile spheres measuring 8-10 µm in diameter by bright field microscopy, and within an oocyst is a central morula-like structure containing a variable number of inclusions. At higher (x 400) magnification, the inclusions appear refractile, exhibiting a greenish tinge. Oocysts are remarkably uniform in size (Ashford, 1979; Long *et al*., 1991). Occasionally, oocysts which either have collapsed into crescents or are empty are encountered. Under UV illumination (330-380 nm) the oocyst wall autofluoresces causing the organisms to appear as blue circles. Organisms do not stain with Lugol's iodine. Staining of air-dried faecal smears with acid fast stains can aid identification, and, according to Wurtz (1994), the rapid dimethyl sulphoxide-modified acid fast staining method is more effective than either the Kinyouin or the modified Ziehl-Neelsen method. Oocysts stain variably with acid fast stains ranging from deep red to unstained. A modified safranin method (microwaving followed by safranin staining) stains oocysts a brilliant reddish orange (Visvesvara *et al*., 1997).
Sporulated oocysts contain two sporocysts and each sporocyst contains two crescentic sporozoites. In instances where excystation *in vitro* have been successful, exposure of oocysts/sporocysts to an excystation medium at 37°C for up to 40 minutes causes the emergence of two crescentic sporozoites from each sporocyst.

Concentration techniques for environmental samples
As mentioned earlier, no method has been developed specifically for the detection of *Cyclospora* sp. in environmental samples, but because *Cyclospora*

sp. oocysts are larger than *C. parvum* oocysts and smaller that *G. intestinalis* cysts, it is assumed that methods developed for *Cryptosporidium* and *Giardia* will prove effective for sampling and recovering *Cyclospora* sp. oocysts from water concentrates.

Detection techniques for use in environmental samples

There are no *in vitro* culture methods for increasing the numbers of *Cyclospora* sp. oocysts nor have any *in vivo* amplification models been described. A proportion of oocysts stored in faeces, water or 2.5% potassium dichromate at temperatures between 22°C and 37°C for up to 14 days in the laboratory will sporulate (Ortega *et al*., 1993; Smith *et al*., 1997). No commercially available polyclonal or monoclonal antibody with specificity to exposed epitopes on *Cyclospora* sp. oocysts is available currently. Therefore, the autofluorescent properties of the oocyst wall under UV illumination have been used in an attempt to detect oocysts in a variety of food and water concentrates. The primers identified by Relman *et al*. (1996), which amplify the small subunit rRNA coding region, have been used to amplify the *Cyclospora*-specific sequence from nucleic acid liberated from berries (strawberries and raspberries) implicated in a series of outbreaks in the USA in 1996. However, to date, no positive results have been reported.

CONTROL OF WATERBORNE TRANSMISSION

Cryptosporidium and *Giardia* are ubiquitous in surface waters throughout the world. Reported concentrations generally range from 0.01-100 per litre. These concentration data are not corrected for the (low) recovery of the detection method, so the actual concentrations may be more than tenfold higher. Higher concentrations are found in urbanised or agricultural waters than in pristine waters (LeChevallier *et al.,* 1991; Rose *et al.,* 1991[a]).

Sources of surface water contamination are the discharge of untreated and treated sewage, run-off of manure and wildlife. The relative significance of these sources may differ between watersheds. Large rivers and lakes often receive both agricultural run-off and treated and untreated domestic wastewater and their relative contribution has not been quantified.

Wildlife may be an important contamination source in pristine watersheds and has been implicated as the source of waterborne giardiasis, although this is still a matter of much controversy.

Oocysts and cysts can survive for months in surface water (DeReignier *et al.,* 1989; Robertson *et al.,* 1992; Chauret *et al.,* 1995; Medema *et al.,* 1997[a]). Under natural conditions, the die-off rate of *Cryptosporidium* oocysts in water is 0.005-0.037 ^{10}log-units per day. For *Giardia*, the die-off rate is higher and (more) temperature dependant: from 0.015 ^{10}log units per day at 1°C to 0.28 ^{10}log-units per day at 23°C (DeReignier *et al.,* 1989).

Although the state in which (oo)cysts occur in water (suspended or attached to particles) is relevant for water treatment (sedimentation, filtration), and cysts and oocysts readily attach to particles (Medema *et al.,* 1998[b]), little information

is available as yet on the significance of these factors in the environmental ecology of (oo)cysts.
Recent information shows that overall 12% of groundwater supplies in the US were contaminated with *Cryptosporidium* and/or *Giardia* (Hancock *et al.*, 1997), mostly in infiltration galleries and horizontal wells. No data on the level of protection and travel time and distance of these groundwater sources were given.

Prevention of the transmission of protozoan parasites through drinking water requires a multiple barrier approach: protection of watersheds used for drinking water production to contamination with protozoa and the installation of adequate treatment coupled with verification that the treatment works effectively by monitoring of water quality and operational parameters.

Watershed protection
The major sources of surface water contamination with *Cryptosporidium* and *Giardia* are discharges of treated or untreated sewage (stormwater overflows), run-off or discharges of manure from agricultural lands and, in more pristine waters, wildlife. One of the most important aspects of watershed protection is the recognition of the local sources of contamination with *Cryptosporidium* and *Giardia* and to control the contamination as much as possible, by diversion or treatment of discharges, reduction of direct input of faeces, especially in otherwise pristine waters, by man, farm animals, wildlife or manure.
Treatment of sewage in activated sludge systems or waste stabilisation ponds is an important barrier against environmental transmission. Both types of processes remove 90-99.7% of the cysts and oocysts (Sykora *et al.,* 1991; Grimason *et al.,* 1992).
Treatment of agricultural wastes before land application also reduces the number and viability of *Cryptosporidium* oocysts: aerobic treatment of cattle slurry at increased temperatures and ammonia concentrations rapidly inactivates oocyst (Svoboda *et al.,* 1997) and also composting of bedding reduces the viability of oocysts.
Storm runoff and snowmelt from unprotected watersheds have been implicated as source of peak contamination of source water (Stewart *et al.,* 1997; Atherholt *et al.,* 1998), and may result in a treatment overload and the contamination of drinking water with (oo)cysts. Knowledge of the characteristics of the plume of contamination from watershed sources can be used to locate and design abstraction points. The importance of this is illustrated by the fact that the intake of the southern plant of Milwaukee in Lake Michigan proved to be exactly in the plume of the Milwaukee river. The turbidity in the raw water peaked and this coincided with treatment failure resulting in the breakthrough of turbidity and oocysts in the Milwaukee drinking water leading to the massive outbreak (MacKenzie *et al.,* 1994).
Installation of pretreatment storage reservoirs flattens peak contaminations (Ketelaars *et al.,* 1995) and, because of the storage capacity, it is possible to stop the intake of surface water temporarily during high contamination events.

Since the protozoa are typically related to faecal contamination of surface water, several studies have tried to determine the use of indicator bacteria to predict high protozoa levels. No consistent relation is observed, however, between indicator bacteria (thermotolerant coliform) levels and concentration of *Giardia* or *Cryptosporidium.* The low and varying recovery of the protozoa detection methods may be an important confounder in detecting these relationships. As (oo)cysts are much more persistent than coliforms and enterococci in water, it is likely that these bacteria are not valid indicators, especially if the contamination source is distant. More persistent bacteria (spores of *Clostridium perfringens*) may prove useful indicators for these persistent protozoa (Payment & Franco,
1993; Hijnen *et al.,* 1997). Since no valid surrogates are available, watershed monitoring to determine local sources of contamination and to define the amount of treatment necessary should therefore include monitoring for protozoa.
Development of transport and fate models for predicting the (oo)cyst concentrations based on data on the sources may help identify important sources or environmental events that determine protozoa levels at abstraction points (Medema *et al.,* 1997[b]).

Currently, neither the number of species of *Cyclospora* infective to human beings is known nor is it known whether human-derived oocysts are infectious to non-human hosts. However, the primary sources of pollution will be human faeces contaminated with oocysts. As *Cyclospora* sp. oocysts are larger than *C. parvum* oocysts but smaller than *G. intestinalis* cysts, it is likely that they will be discharged with final effluents from waste stabilisation ponds and sewage treatment works. Oocysts take up to 14 days to mature (sporulate) in the laboratory, sporulating more rapidly at higher (up to 37°C) temperatures. Sporulation time in the environment will depend upon ambient temperature and sporulated oocysts may be found distant from the pollution source in the aquatic environment. Sources of pollution with unsporulated oocysts are likely to be effluent discharges from sewage treatment and waste stabilisation ponds with detention times of less than 1 week.
Like *C. parvum* oocysts and *G. intestinalis* cysts, oocysts of *Cyclospora* sp. are likely to survive longer at lower temperatures when suspended in water. *Cyclospora* sp. oocysts stored 4°C do not appear to sporulate (Smith *et al*., 1997). A proportion of oocysts stored at 4°C for up to 2 months will sporulate when subsequently incubated at temperatures between 22°C and 37°C. No data are available regarding survival and transport in soil.

Adequate treatment

Filtration

The principal barrier for these resistant protozoa is physical removal by filtration. The smaller size of *Cryptosporidium* oocysts makes them more difficult to remove than *Giardia* cysts. Rapid sand filtration is a common treatment process used to remove particles and when operating efficiently is theoretically capable of 3 log removal of *Cryptosporidium* oocysts (Ives, 1990). Other investigations

have given a range of removal rates including 91% (Rose *et al.,* 1986) and greater than 99.999% (Hall *et al.,* 1994[b]) with the higher removal rates being achievable when coagulant dosing has been applied to the water prior to filtration.
Diatomaceous earth filtration has been reported to achieve >99% removal of *Giardia* (Jakubowski, 1990) and even up to 4-6 log-units for *Cryptosporidium* under laboratory conditions (Ongerth & Hutton, 1997).
Conventional treatment (coagulation, sedimentation, filtration), direct filtration (with chemical pretreatment) and high-rate filtration can remove 99% of the (oo)cysts, when properly designed and operated (LeChevallier *et al.,* 1991; Nieminski, 1994; West *et al.*, 1994). Typically the chemicals used are ferric or aluminium salts and there appears to be no real difference in the effectiveness of aluminium sulphate, polyaluminium chloride, ferric sulphate and ferric chloride in removing oocysts and similarly sized particles (Ives, 1990).
If filters are backwashed, the backwash water may contain high levels of (oo)cysts (Richardson *et al.,* 1991). If this backwash water is recycled, treatment with coagulation and sedimentation or microfiltration will reduce re-contamination of the water with (oo)cysts. If this is not feasible, it is recommended that the recycled water is returned at a constant, low rate (Rose *et al.,* 1997).
Slow sand filtration can efficiently remove (oo)cysts, but the efficiency reduces at lower temperatures. No data are available for removal of oocysts in full scale plants but a number of pilot scale studies have been completed where the removal efficiencies were generally good. Hall *et al.* (1994) demonstrated removals of greater than 99.95%. In another study using surface water, heat-inactivated oocysts were added at a concentration of 4000 per litre and no oocysts were found in the filtrate. At the end of the study, intact oocysts were found only in the upper 2.5 cm of the sand filter (Timms *et al.,* 1995).
Micro- and ultrafiltration can remove over 99.99% (Jacangelo *et al.,* 1991; Adham *et al*., 1994; Drozd & Schwartzbrod, 1997) as long as the integrity of the system is maintained.

Soil passage
Soil passage, used in bank filtration and infiltration, is probably an effective physical barrier against (oo)cysts. It's effectiveness depends on travel time and distance and composition of the soil (Mawdsley *et al.,* 1996).

Pretreatment reservoirs
Storage in reservoirs with a residence time of 5 months can reduce the (oo)cyst concentration by 99% (Ketelaars *et al.,* 1995). Experimental evidence suggests that sedimentation of *Cryptosporidium* oocysts and *Giardia* cysts is unlikely to have a significant effect on their removal from a body of water unless they are attached to other particles (Medema *et al.*, 1998[b]).

Disinfection
Disinfection with chlorine has always been an important barrier for waterborne pathogens. The high resistance of especially *Cryptosporidium* oocysts against

chlorine disinfection (Korich *et al.,* 1990; Smith *et al.,* 1990; Ransome *et al.,* 1993) renders this process ineffective for oocyst inactivation in drinking water treatment. Chlorine dioxide is slightly more effective, but still requires a high CT product (measure of disinfectant dose: (residual) concentration of disinfectant C x contact time T) of 78 mg.min l^{-1} for 90% inactivation of oocysts (Korich *et al.,* 1990). *Giardia* is less resistant against chlorine: 99.99% reduction can be achieved with a CT of 180-530, depending on temperature and pH of the water (Hibler *et al.,* 1987). Chlorine dioxide gives 99% reduction at CT values of 4.7-28 (Leahy *et al.,* 1987; Rubin, 1988).
Ozone is the most potent (oo)cysticide: at 20°C, the CT for 99% inactivation of *C. parvum* oocysts is 3.5 mg.min.l^{-1} (Finch *et al.,* 1993[a]) and for *G. intestinalis* cysts 0.6 mg.min.l^{-1} (Finch *et al.,* 1993[b]). The effectiveness of ozone reduces at lower temperatures. Peeters *et al.*(1989) found that 0.4 mg.l^{-1} residual ozone for six minutes was sufficient to kill 10 000 oocysts ml^{-1} whilst Korich *et al.* (1990) demonstrated that 1 mg.l^{-1} for ten minutes at 25°C would result in a reduction in viability of 99%. Parker *et. al.* (1993) found that 3 mg.l^{-1} for ten minutes was required to kill all oocysts and similar high figures were quoted by Ransome *et al.* (1993). Hence, the CT values required for inactivation of cysts and oocysts are high. CT values are limited, however, since high CT's can give rise to formation of high concentrations of (geno)toxic by-products. Exposure of *Cryptosporidium* oocysts to multiple disinfectants has been shown to be more effective than was to be expected from both disinfectants alone (Finch *et al.,* 1994; Liyanage *et al.,* 1997) and synergism between environmental stress during sand filtration has also been observed (Parker *et al.,* 1993). The multiple stresses that (oo)cysts encounter in the environment and during treatment might limit the infectivity of (oo)cysts.
Conventional UV systems have a limited effect on *Cryptosporidium* and *Giardia* viability. UV doses of 110-120 mJ/cm^2 result in 99% inactivation of *C. parvum* oocysts (Ransome *et al.,* 1993), as assayed with in vitro viability methods and 97% of *G. intestinalis* cysts (Rice & Hoff, 1981). A recent study of Clancy *et al.* (1998), using animal infectivity, showed that pulsed and advanced UV are much more effective against *Cryptosporidium*; they obtained 99.98% inactivation at UV-doses as low as 19 mJ/cm^2. The results of laboratory disinfection experiments should be translated with caution to the full scale treatment of environmental (oo)cysts. In surface water treatment, (oo)cysts may be protected against the disinfectant because they are attached to colloids. On the other hand, (oo)cysts that have been exposed to environmental stressors may be more susceptible to disinfectants (Parker *et al.,* 1993). Moreover, the design and operation of full-scale treatment systems will, in general, be less optimal for inactivation than the laboratory setting.
The removal of *Cryptosporidium* oocysts and *Giardia* cysts by well designed, maintained and operated treatment processes are summarised in Table 1.

There is little information available regarding the ability of water treatment processes to remove or inactivate *Cyclospora* sp. oocysts. As *Cyclospora* sp. oocysts (8-10 µm diameter) are larger than *C. parvum* oocysts but smaller than *G. intestinalis* cysts, it is likely that physical removal will be similar to that

obtained with *Giardia* and *Cryptosporidium*. In an outbreak in Nepal, filtration and chlorination did not affect the integrity of the oocysts (Rabold *et al*., 1994). Although chlorine residuals remained at acceptable levels (0.3 - 0.8 ppm) and no coliform indicator bacteria were detected, *Cyclospora* sp. oocysts were found in the drinking water supply. Little is known about survival of the oocysts in different environments or what treatment can effectively inactivate the oocysts.

Risk assessment to design adequate treatment

One of the key issues in treatment is to determine what level of treatment is adequate. This requires maximum acceptable concentrations in drinking water. In analogy to toxic compounds, these could be derived from a maximum acceptable risk and the dose response relation of these parasites. An infection risk of 10^{-4}

Table 1. Removal of Cryptosporidium *oocysts and* Giardia *cysts by treatment processes.*

Type of process	Removal efficiency (^{10}log-units)		Most important efficiency-determining parameters
	Crypto-sporidium	*Giardia*	
Disinfection processes			
Chlorine	0	0 - 2	dose, contact time, installation design,
Chloramines	0	0 - 2	disinfectant-demand, temperature, pH (esp
Chlorine dioxide	0	0 - 2	chlorine), formation of toxic by-products, synergism of
Ozone	0 - 2	1 - 4	multiple disinfectants
UV	0 - 4	0 - 4?	dose at 254 nm, turbidity, solutes, system design
Filtration processes			
Rapid sand filtration	0 - 1	0 - 1	filtration rate, recycling of backwash water
Slow sand filtration	1.2 -> 3.7	1.2 -> 3.7	presence of “Schmutzdecke”, filter depth, filtration rate,
Diatomaceous earth	2 - 6	2 - 6	filtration rate, filter depth, pore size, precoat thickness,
Membrane filtration	2 - > 4	2 - > 4	system integrity, membrane type
Coagulation/filtration	2 - 2.5	2 - 2.5	coagulant dose, pH, temperature, installation
Other processes			
Soil passage	> 2 - > 5	> 2 - > 5	soil composition, residence time, travel distance,
Reservoir storage	0.5 - 2	0.5 - 2	residence time, reservoir design, temperature

per year has been suggested as acceptable for pathogens in drinking water (Regli *et al.,* 1991). The maximum concentrations of viable (oo)cysts in drinking water to meet this risk level are very low (Rose *et al.,* 1991[b]; 1997). Current techniques do not allow an evaluation of compliance with these concentrations. Therefore, safe-guarding of drinking water with respect to protozoan parasites should be done by a quantitative description of the protozoa concentrations in the source water and the removal efficiency of the treatment steps. Surface

water utilities and groundwater utilities that may be influenced by surface water or other sources of contamination should monitor their source water for protozoa and determine the rate of protozoa removal and inactivation achieved in the treatment plant, in order to determine if acceptable concentrations of protozoa in drinking water have been achieved.

Verification of efficiency of parasite removal

For routine monitoring, water quality and process parameters are required to verify treatment performance. Several parameters have been suggested as a surrogate for (oo)cyst removal by filtration processes: turbidity, particle counts (LeChevallier & Norton*.,* 1992; Hall & Croll, 1997), clostridial spores (Payment & Franco, 1993; Hijnen *et al,* 1997) or aerobic spores (Nieminski, 1997) and particulate matter (Anon., 1997). Although turbidity or particle counts of filtered water depend on both the levels in raw water and filter performance, in general, a turbidity of 0.1 to 0.5 NTU or counts of particles >3 μm below 50 per ml are indicative of good quality water. On-line monitoring of turbidity or particle counts gives direct and continuous information on (individual) filter performance and are very valuable tools for optimising treatment efficiency for (oo)cyst removal.

Critical moments in the filter cycle are just after backwash or, in case of slow sand filtration, scraping of the clogged top-layer from the filterbed. A slow increase in filtration rate or filtering-to-waste minimises the risk of (oo)cyst breakthrough.

For disinfection processes, disinfectant dose, contact time, residual disinfectant concentration after this contact time, pH and temperature are commonly used to monitor the disinfection performance. The most critical conditions for disinfection processes are low temperatures and high turbidity of the water that is to be disinfected.

CONCLUSIONS AND RECOMMENDATIONS

Health risk assessment

The abundance and size of drinking waterborne outbreaks in developed countries show that transmission of *Giardia* and *Cryptosporidium* by drinking water is a significant risk. In the case of *Cryptosporidium*, the absence of an adequate cure for immunocompromised patients increases the problem. Although the outbreaks receive most attention, low-level transmission of these protozoa through drinking water is very likely to occur in developed countries and in developing countries alike (Fraser & Cooke, 1991; Isaac-Renton *et al*., 1996). Cysts and oocysts are regularly found in drinking water (Isaac-Renton *et al.,* 1996; Karanis & Seitz, 1996; Rose *et al,* 1997), although only a small proportion may be viable and infectious to man. A major drawback for the determination of the health significance of (oo)cysts in (drinking) water is that methods for a sensitive and specific detection of infectious (oo)cysts, with a consistently high recovery are not available.

Risk management

Cryptosporidium poses a serious health risk to immunocompromised persons, especially AIDS patients. An important step forward in the reduction of the consequences of waterborne outbreaks and other cases of cryptosporidiosis would be the definition of an adequate therapy for the immunocompromised patients. Currently, prevention of exposure to (potentially) contaminated water is a means to reduce the risk. Especially the immunocompromised population should be informed about the risk and means to prevent exposure. Boiling of tap water, use of mineral or bottled water, not swimming in surface water or pools are some of the options. Local considerations play a major role and public health authorities are encouraged to provide guidance on the safety of drinking water for the immunocompromised and on applicable means to reduce exposure (Anon., 1995[a.b]; Juranek, 1995). In outbreak situations, rapid investigation of size and source of the outbreak and installation of control measures to prevent further transmission is required. Useful guidance on management of waterborne outbreaks can be found in the report of the UK group of experts (Anon., 1990) and the CDC Guidance Manual (Juranek, 1995) and a workshop report (Anon., 1995).

The protozoa, and to a lesser extent the viruses, have changed the philosophy in the developed countries towards safe-guarding of drinking water from monitoring of the 'end-product' drinking water to monitoring raw water and the efficiency of the treatment. Furthermore, the extreme resistance of these organisms implies that a "zero-risk" is no longer achievable. Treatments should be designed to reduce the (oo)cyst concentrations in the raw water as far as possible and preferably include filtration step(s). This implies that information on the parasite concentrations in the raw water is necessary, as well as information on the removal efficiency of the treatment. Quantitative risk assessment provides a tool for the combination of information on raw water quality (concentrations detected, recovery of the detection method, viability) and treatment efficiency (removal by different steps in the treatment) (Teunis *et al.,* 1997). The current detection methods are generally sensitive enough to determine the concentrations of *Cryptosporidium* and *Giardia* in surface water, but are in many cases not sensitive enough for an accurate description of removal efficiency. For the latter description, data from laboratory studies and (seeded) pilot plant studies may provide additional information. Another approach is to determine if an adequate surrogate parameter can be found for the description of removal efficiency for *Cryptosporidium* (as *Giardia* is easier to eliminate both with disinfection and filtration, the description of treatment efficiency should be targeted on *Cryptosporidium*). Several parameters have been evaluated on a limited scale as surrogates for protozoa removal: aerobic spores, clostridial spores, particles and algae, but a broader evaluation is necessary to determine the value of these parameters.

The definition of maximum acceptable concentrations of pathogens in drinking water based on a maximum acceptable (infection) risk level has become possible by the availability of volunteer data and dose-response models (Haas, 1983; Dupont *et al*., 1995; Teunis *et al.,* 1996).

An annual infection risk level of 10^{-4}, as proposed by the US EPA, is currently used in the US (Rose *et al.,* 1997), Canada (Wallis *et al.,* 1995) and the Netherlands (Medema *et al.,* 1995) as the basis to determine the appropriate removal efficiency of surface water treatment systems. Although there are still questions about the significance of (oo)cyst occurrence in drinking water, this is the way forward. The implementation of guideline levels is still hampered, however, by the difficulty to determine source water quality and treatment efficiency accurately.

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Cryptosporidium and *Giardia* - the Dutch perspective

This paper is based on Medema, G.J., Hijnen, W.A.M., Nobel, P.J. and van der Kooij, D. (1997). *Cryptosporidium* and *Giardia* – the Dutch perspective. Chartered Institution of Water and Environmental Management, Technical papers from the CIWEM symposium, 4 December 1997, CIWEM, United

Kingdom

INTRODUCTION

A number of waterborne outbreaks of cryptosporidiosis and giardiasis in the USA, Canada and the UK have shown that water can be an important transmission route for *Cryptosporidium parvum* and *Giardia intestinalis.* In the USA, *Giardia* is the most frequent cause of waterborne outbreaks of intestinal illness with over 95 outbreaks (18% of all outbreaks) in the past 25 years, and a total of over 25000 cases of disease (Craun, 1990). 'Waterborne' *Cryptosporidium parvum* outbreaks are less frequent (1% of all outbreaks), but the number of cases that is involved is relatively large, especially in the Milwaukee outbreak with an estimated 403,000 cases (Craun *et al.,* 1998). The waterborne outbreaks of giardiasis and cryptosporidiosis that occurred in the US and UK raised concern over the safety of Dutch drinking water with regard to *Cryptosporidium* and *Giardia*. The cause of drinking water contamination with these parasites that led to the reported outbreaks was not limited to obvious treatment inadequacies or post treatment contamination, but also occurred in apparently well-treated water (Craun *et al.,* 1990, 1998). Moreover, coliforms, the warning-parameter used to determine the microbiological safety of the drinking water, were not detected in many of the drinking waters that were the suspected cause of an outbreak.

Several characteristics of the parasites facilitate their waterborne transmission: they are shed in high numbers by infected persons or animals, they are very resistant to environmental stress and to chemical disinfection, they can be transmitted from livestock and wildlife to man and their infectivity is high: even a dose of 1 (oo)cyst gives a discrete probability of infection. These findings make *Cryptosporidium* and *Giardia* critical pathogens for the safety of drinking water. Hence, the drinking water companies and government agencies (Directorate of the Environment, Inspectorate of Environmental Hygiene) in the Netherlands need information on the occurrence of these parasites in water to be able to determine the (im)probability of transmission of *Cryptosporidium* and *Giardia* through drinking water.

PUBLIC HEALTH DATA

Data on the occurrence of *Cryptosporidium* and *Giardia* in the general population are scarce in the Netherlands. Several incidental surveys have been conducted to determine the prevalence of cryptosporidiosis and giardiasis. Bänffer (1990) could isolate *Cryptosporidium* in 1.2% of 2000 stool samples of patients with diarrhoea and van Knapen *et al.* (1984) found *Cryptosporidium* in 0.88% and *Giardia* in 7.9% of diarrhoeal stools. A study in patients that visited the general practitioner with gastro-enteritis indicated a prevalence of 1-2% for *Cryptosporidium* and 3% for *Giardia* (Hoogenboom-Verdegaal *et al.* 1989). A study with random samples of the human population from four different regions in the Netherlands showed that *Cryptosporidium* was present in stools of patients with diarrhoea at a rate of 1.6%. The isolation frequency was highest

in young children and in the age group of 25-35 years. Asymptomatic carriage was rare. *Giardia* on the other hand, was found in 3-5% of the stool samples of both symptomatic and asymptomatic persons of all ages (Kortbeek *et al.,* 1994). *Giardia* has been reported as the cause of an outbreak of gastro-enteritis in a day-care centre in Tilburg (Bosch, 1991) and is probably the cause of more, unreported outbreaks (Kortbeek, pers. comm.).
These isolation frequencies are similar to the frequencies reported in other industrialised countries. The isolation frequency of *Cryptosporidium* ranges from 1 to 3% and is also relatively high (4%) in children from 1 to 15 (Casemore, 1990, 1997). For *Giardia*, the isolation frequency in all age groups ranges from 2 to 15% and rises to 8-39% in young children, with day-care attendance as an important risk factor (Healy, 1979).
Recent data are available from (only) one of the Dutch regional health laboratories (Stichting Artsenlaboratorium Haarlem) that routinely monitors stool samples of patients with diarrhoea for the presence of *Cryptosporidium* and *Giardia* (Mank, 1997). Other laboratories only monitor for these parasites on specific request, which mainly occurs when the symptoms are persistent and no other pathogens can be found. The monitoring data from the Artsenlaboraratorium Haarlem show that *Cryptosporidium* could be isolated from 3.3% of patients with persistent (> 7 days) diarrhoea and 5.4% in patients with acute (1-5 days) diarrhoea. Both groups were comprised of patients that had visited a General Practitioner for their symptoms. Asymptomatic carriage was reported in 0.5% of the persons tested (Mank, 1997). *Giardia intestinalis* was found in 14.6% of the patients with persistent diarrhoea, in 1.8% of patients with acute diarrhoea and in 2.0% of the stools of asymptomatic persons (Mank, 1997). Symptomatic infections with both parasites were most prevalent in the age group of 0-14 years.
The prevalence data obtained by Mank (1997) are high compared to other studies in west-European countries. This may be caused by the selection of patients with persistent diarrhoea and by the optimised protocol that was used for protozoa detection in stool samples.
Seasonality was observed in the isolation of *Cryptosporidium* with relatively high frequencies in August and September and to a lesser extend in March/April. Also *Giardia* was most frequently isolated in late summer and autumn. Mank (1997) suggested that the increased prevalence of both *Cryptosporidium* and *Giardia* in summer is related to exposure to these parasites in recreational water.
In the summer of 1995, a sudden increase in the isolation frequency of *Cryptosporidium* (up to 17%) was noted in the west of the Netherlands (Asperen *et al.,*1996). A case control study indicated that the major risk factors were a household member with diarrhoea and a visit to a swimming pool. No particular pool was implicated. Drinking water, surface water recreation and holidays abroad were not associated to this epidemic. The data from the Artsenlaboratorium Haarlem showed that an increase of isolation frequency of *Cryptosporidium* occurs each summer (Mank, pers. comm.).

Although no survey-data are published, cryptosporidiosis is known to cause severe infections in immunocompromised individuals (patients with aids or chemotherapy) in the Netherlands. The effect of various therapeutic strategies is tested on these patients. No specific warning is given to this group with respect to prevention of waterborne infection with *Cryptosporidium* and *Giardia.*

VETERINARY DATA

Numerous reports show that *Cryptosporidium* and *Giardia* infection is prevalent in farm, pet and wild animals (Panciera *et al.,* 1971; Davies & Hibler, 1979; Mann *et al.,* 1986; Erlandsen & Bemrick, 1988; Angus, 1990; Casemore, 1990; Xiao, 1994). Both parasites can commonly be isolated from cattle and sheep, especially new-born and young animals. Pigs, goats and horses are also frequently infected. Few data are available on the occurrence of *Cryptosporidium* and *Giardia* in farm, pet or wild animals in the Netherlands. A study in 11 herds showed that 55% of the 375 diarrhoeic calves sampled excreted *Cryptosporidium* oocysts (Leeuw *et al.,* 1984). *Cryptosporidium* is commonly seen in stool from diarrhoeic calves that are presented to the Faculty of Veterinary Health of the Utrecht University (Breukink, pers. comm.) and has been isolated in low numbers from 50% of 44 young foals (Göhring, 1993) and 100% of adult horses (Medema, pers. obs.). No *Cryptosporidium* oocysts were found in a survey of 20 lambs (Feberwee & Wipkink, 1992).
Cryptosporidium baileyi is also identified as the cause of respiratory infections in commercially raised chickens in the Netherlands (Heijmans, pers com.).
No data on the prevalence of *Giardia* infections in animals in the Netherlands are present.

ENVIRONMENTAL DATA

In 1991, ubiquitous presence of *Cryptosporidium* and *Giardia* in domestic waste water and in surface water had been shown in the USA (Rose, 1988; Rose *et al.,* 1991[a]; LeChevallier *et al.,* 1991), Canada (Hansen & Ongerth, 1991; Ongerth & Stibbs, 1987), the UK (Smith *et al.,* 1991; Gilmour *et al.,* 1991; Badenoch, 1990; Poulton *et al.,* 1991) and Germany (Exner & Gornik, 1990). In a first survey of the occurrence in surface water that was initiated by the water industry together with government agencies in the Netherlands in 1991, *Giardia* was detected in only 2 of 12 surface water samples and none of the post-treatment samples. No *Cryptosporidium* was detected in any of the samples. The low isolation frequency was partly due to the insensitivity and poor recovery efficiency of the methods used at that time (Medema, 1992). A subsequent, more detailed study in 1993 at the abstraction point of the Water Storage Company Brabantse Biesbosch in the river Meuse and at the outlet of the Biesbosch reservoirs showed the presence of *Cryptosporidium* in river water in 12% of 52 samples and *Giardia* in 60% of 52 samples. High levels were observed during the winter months. Reservoir storage with an average residence

time of 5 months markedly reduced the levels of both parasites, but both were detected occasionally and in low numbers at the outlet of the reservoirs (Ketelaars *et al.,* 1995).

POTENTIAL FOR WATERBORNE TRANSMISSION

The first inventory of the available knowledge in the early 1990's showed that *Giardia* and *Cryptosporidium* are present in the human and farm animal population in the Netherlands. Isolation frequencies were comparable to other industrialised countries. They are the causative agents in a proportion of the cases of gastro-enteritis that occur in the human population. With the reported isolation frequencies and the estimated annual number of all cases of gastro-enteritis in the Netherlands (2×10^6, Hoogenboom-Verdegaal *et al.,* 1989, 1990), an estimated number of 20,000-30,000 symptomatic C*ryptosporidium* infections and 60,000-100,000 symptomatic *Giardia* infections occur annually in the human population in the Netherlands.
The Netherlands is a country with a high population density, both in humans and in livestock, and *Cryptosporidium* and *Giardia* are present in these populations. Hence, the sources of environmental contamination are present. In addition, both protozoa have been detected in surface water.
The combination of the available information led to the conclusion that there is a clear potential for waterborne transmission of *Cryptosporidium* and *Giardia* in the Netherlands. This potential is largest for water recreation, because of the direct contact with surface water. If *Cryptosporidium* and *Giardia* can be transmitted through drinking water depends on the concentration of (oo)cysts found in source water and the efficiency of the treatment systems. Both need to be evaluated.

PREVENTION OF TRANSMISSION THROUGH DRINKING WATER

No outbreaks or cases of cryptosporidiosis or giardiasis through drinking water have been reported in the Netherlands. The absence of active surveillance of these parasites in stool samples makes outbreaks difficult to detect, so no firm information on the safety of drinking water can be derived from the absence of cases. The attention is primarily focussed on prevention of waterborne transmission. Several barriers in the water route reduce the risk of waterborne transmission:

- 98% of the domestic waste water is biologically treated before it is discharged into surface water. However, the river Meuse receives a high quantity of untreated sewage in Belgium, before it enters the Netherlands,
- 84% of the drinking water is produced from ground water or by soil passage of surface water (bank filtration, dune infiltration),
- multiple barriers for pathogens are installed in surface water treatment systems,
- strict hygienic procedures are used for distribution system maintenance and repair.

To be able to evaluate the effectiveness of these preventive measures, information is needed on the sources of surface water contamination, the occurrence and fate of *Cryptosporidium* and *Giardia* in surface water and the elimination capacity of the treatment systems.

DRINKING WATER TREATMENT – MULTIPLE BARRIERS

In the Netherlands, 64% of the drinking water is produced from (deep) ground water. This is mainly located in the north, south and east of the country. In the west, the sodium chloride-concentration in groundwater is too high for the production of drinking water, because of the intrusion of sea water. Abstracted groundwater is generally free from faecal contamination. No chemical disinfection is applied. The groundwater is only treated with aeration and filtration to remove methane, ammonium, iron and manganese.
Four basic treatment schemes are used for the production of drinking water from surface water in the Netherlands (Table 1). The most commonly used schemes are 1) storage in open reservoirs, followed by coagulation, filtration (rapid sand, dual media, GAC) and disinfection (chlorine, ozone) and 2) dune infiltration. In this latter scheme, water is pre-treated by coagulation and filtration, transported to the dunes and artificially recharged into the sand dunes along the coastal area in almost all of the west of the Netherlands. The water is pumped through the dune-sand and recovered from the dunes. The average travel time through the dune-sand is two months, but there is pressure to reduce the residence times since this would reduce the area needed for drinking water production. After soil passage, the water is either treated by filtration followed by UV or stored in an open reservoir and treated more extensively by rapid filtration (with ozone and GAC filtration) and slow sand filtration.
A small but increasing percentage of surface water is treated by bank filtration along the river Meuse and Rhine. Also here, residence times are usually two months or more. After soil passage, water is treated with filtration (sand, dual media, GAC) and in some cases ozonation or UV. Another small percentage of the surface water treatments use a direct treatment with coagulation/filtration or ozonation, both followed by GAC filtration and slow sand filtration.
In total, 50% of the Dutch surface water supplies use passage of two months through the soil (fine-grained sand, loamy sand), which is very effective in removing pathogenic micro-organisms.

ASSESSMENT OF THE SAFETY OF DRINKING WATER

Current approach: the use of indicator bacteria

The current approach to check the microbiological quality of drinking water leaving surface waterworks is monitoring for the presence of total and thermotolerant coliforms, faecal enterococci and spores of sulphite reducing bacteria. The frequency and volume of sampling are regulated in the Dutch

Table 1. Surface water treatment in the Netherlands

Treatment	Volume (Mm^3/yr)	Percentage of total
Bank filtration	80	6
Artificial recharge (dunes)	180	14
Storage reservoirs	160	12
Direct treatment	50	4
Total	**470**	**36**

Drinking Water Decree (1984). The standards for indicator bacteria in this Decree (Table 2) are more stringent than the standards in the current European Drinking Water Directive (1980), particularly for the spores of sulphite reducing clostridia. A stricter standard in terms of frequency and volume was felt necessary, since this parameter could indicate the breakthrough of pathogenic micro-organisms that were more resistant to oxidation processes than coliforms and faecal streptococci, such as viruses and protozoa. It was also feasible, since only a very small fraction of 1 litre samples of treated water contained these spores (Havelaar, 1981). In the current revision of the EU-Directive, the proposed new standard for *Clostridium perfringens* is now similar to the Dutch standard for clostridial spores.

Table 2. Dutch and EU standards for indicator bacteria in treated water leaving the treatment facility.

	Dutch Drinking Water Decree		**EU Drinking Water Directive**	
Parameter	MAC[a]	Minimum sampling frequency	MAC[a]	Minimum sampling frequency
Total coliforms	< 1/300 ml	daily[b]	0/100 ml	Daily
Thermotolerant Coliforms	< 1/300 ml	daily[b]	0/100 ml	Daily
Faecal Enterococci	< 1/100 ml	weekly[c]	0/100 ml	Undefined
Spores of sulph. red. clostridia	< 1/100 ml	weekly[c]	0/20 ml	Undefined

[a] MAC: Maximum Acceptable Concentration
[b] Weekly for ground water supplies
[c] Not defined for ground water supplies

New approach: guidelines for pathogens?

The occurrence of outbreaks of waterborne disease through drinking water that complies with the coliform standard, implies that this standard is inadequate to safeguard the microbiological quality of drinking water under all circumstances.

The development of dose-response assessments for microbial pathogens has made it possible to design a risk-based approach, analogous to the approach taken against the risk of toxic chemicals in drinking water (Medema & Havelaar, 1992; van der Kooij *et al.*, 1995). Maximum acceptable concentrations of pathogens in drinking water can be determined based on a maximum acceptable risk level, in much the same way as maximum acceptable concentrations are developed for toxic compounds. The Netherlands is in the process of adopting the risk strategy that was developed by the researchers in the US (Haas, 1983; Rose *et al.,* 1991[b], Regli *et al.,* 1991) in conjunction with the US Environmental Protection Agency. In this approach, a risk level of one infection per 10.000 persons per year is regarded as maximum acceptable for pathogens in drinking water. The Ministry of the Environment has issued draft guidelines for maximum acceptable pathogen concentrations in drinking water, based on the 10^{-4} infection risk level, the exposure of consumers to unheated drinking water (average: 0.25 l/day) and a safety factor of 10 for interspecies variation in both pathogen virulence and host susceptibility to infection (Table 3).

Table 3. Maximum acceptable mean concentration (MAMC) of pathogens in drinking water.

Pathogen*	MAMC (n/l)	MAMC (absence in m^3)
Cryptosporidium	2.6×10^{-5}	38
Giardia	5.5×10^{-6}	180

* MAMC values for other pathogens (bacteria, viruses) are issued, but not presented here.

These maximum acceptable mean concentrations (MAMC) are way below the lower detection limit of the protozoa methods, so monitoring for the presence of protozoa in drinking water cannot be used to show compliance with these MAMC-values. Compliance with the MAMC-values can be assessed by combining data on source water quality with the elimination capacity of treatment systems. Or, in other words, the difference between the concentration in the source water and the MAMC-value is the required elimination capacity of the treatment processes. Currently, this approach is used to evaluate the microbiological safety of newly designed treatment systems.

RESEARCH NEEDS

Sources of surface water contamination

The design of surface water treatment systems is determined, at least in part, by the concentration of *Cryptosporidium* and *Giardia* in source water. Hence, the cost of the production of drinking water from surface water is related to the (oo)cyst concentration level in source water: the lower the concentration, the lower the costs for installation and operation of an adequate treatment system.

Reduction of the environmental load of (oo)cysts by installing source water protection measures would reduce the costs of water treatment.
Similarly, these measures would reduce the health risk of water recreation.
Potentially important sources for contamination of surface water with *Cryptosporidium* oocysts and *Giardia* cysts are treated and untreated sewage discharges, run-off from agricultural lands and, in more pristine waters, wildlife.
Hence, the occurrence of *Cryptosporidium* and *Giardia* in several domestic waste waters and in effluents from activated sludge systems for sewage treatment was monitored. These data were used to determine the relative significance of treated and untreated sewage discharges. Alos, the applicability of an emission model to determine the relative significance of treated and untreated discharges was determined (chapter 3).
Since most surface waters in the Netherlands are influenced by the international rivers Rhine and Meuse, it was important to determine the *Cryptosporidium* and *Giardia* load presented by these rivers (chapter 3).
The significance of wildlife and especially waterfowl for the contamination of water in pre-treatment reservoirs was determined by measuring the occurrence of (oo)cysts in animal faeces and calculating the load of (oo)cysts to reservoir water, using information on prevalence of the animals on and around the reservoirs (chapter 4).

Environmental ecology
To determine the probability that (oo)cysts that are discharged into rivers and streams arrive at an abstraction point for drinking water production or at a bathing area, information is needed on the fate of (oo)cysts in the aquatic environment. Important processes that determine this fate are die-off and sedimentation. Therefore, both the kinetics of survival of *Cryptosporidium* oocysts in surface water under natural conditions (chapter 5) and the kinetics of sedimentation of free and attached *Cryptosporidium* oocysts and *Giardia* cysts in water (chapter 6) were assessed.
This information is integrated into a quantitative descriptive model of the emission and dispersion of *Cryptosporidium* and *Giardia* in surface water in the Netherlands (chapter 3), identifying the significance of the different (oo)cyst sources and processes in surface water (transport and survival) in determining the concentration of *Cryptosporidium* and *Giardia* in surface waters.

Quantitative description of protozoa-occurrence in source waters
The new approach towards microbiologically safe drinking water requires quantitative information on the occurrence of *Cryptosporidium* oocysts and *Giardia* cysts at the sites where water is abstracted for the production of drinking water. These were determined with the emission and dispersion model, but also by monitoring of the concentration *Cryptosporidium* oocysts and *Giardia* cysts at several abstraction sites (chapter 3). The largest abstraction site was intensively monitored for both protozoa to determine the variation in source water quality and the relation of protozoa occurrence with other water quality variables (chapter 7).

Sensitive detection methods
The recovery efficiency of the methods to concentrate and enumerate (oo)cysts of *Cryptosporidium* and *Giardia* in water was very low. The methods that are used to concentrate cysts and oocysts from water also concentrate the majority of other biological particles > 1 μm in the water sample (i.e. algae). These particles interfered with the detection of (oo)cysts by immunofluorescence microscopy. Flow cytometry with cell sorting was applied to facilitate sensitive detection by isolating *Giardia* cysts and *Cryptosporidium* oocysts from other particles in the water concentrates and to facilitate application of vital dyes to evaluate the viability of *Cryptosporidium* oocysts and *Giardia* cysts in these samples was studied (chapter 8).

Quantitative description of treatment efficiency
To determine if treatment is adequate, methods are needed to assess treatment efficiency. Monitoring the concentration of *Cryptosporidium* oocysts and *Giardia* cysts before and after the treatment process is a method that can be used only to assess the removal capacity of the initial treatment processes (chapter 5). After the initial treatment, the concentration of (oo)cysts in the water is too low to be able to assess the removal in subsequent treatment processes. Since spores of sulphite-reducing clostridia are persistent in water, very resistant to chemical oxidising agents and small (approximately 1 μm), they could be a useful surrogate parameter to determine treatment efficiency for *Cryptosporidium* oocysts and *Giardia* cysts (Payment & Franco, 1993; Hijnen *et al.,* 1997). If clostridial spores and (oo)cysts behave similarly during treatment, adequate removal of spores would imply adequate removal of (oo)cysts. In chapter five, these spores are applied to describe the removal capacity of subsequent treatment processes (coagulation/filtration, disinfection). Since both the concentration of *Cryptosporidium* and *Giardia* in source water and the efficiency of spore-removal by treatment processes showed considerable variation, statistical methods had to be developed to describe the survey data.

Risk assessment
The ultimate goal, description of the safety of the drinking water treatment, can be achieved by performing a risk assessment. This requires quantitative knowledge of all the factors that contribute to the health risk of consumption of drinking water from a surface water source: concentration of *Cryptosporidium* oocysts and *Giardia* cysts in source water, the recovery efficiency of the detection method, the viability of (oo)cysts in water, the removal or inactivation of (oo)cysts by water treatment, the consumption of (unheated) drinking water by the community and the dose-response relation of *Cryptosporidium* and *Giardia*. Chapter nine describes the use of data on all these factors to analyse the risk of infection by *Cryptosporidium* and *Giardia* in drinking water from a surface water source and the contribution of the uncertainty in these data-sets to the overall uncertainty of the risk estimate. The latter is useful information to focus future research efforts to reduce both the health risk itself and the uncertainty of the risk estimate.

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Modelling the discharge of *Cryptosporidium* and *Giardia* by domestic sewage and their dispersion in surface water

With J.F. Schijven

ABSTRACT

Modelling the discharge of parasitic protozoa into surface water and the dispersion in rivers and streams gives insight into the contribution of the different sources of environmental contamination and in the transmission of these organisms from the point of discharge to drinking water abstraction points and bathing sites. We tested the applicability of emission (PROMISE) and dispersion (WATNAT) models developed for chemical pollutants to describe the environmental behaviour of *Cryptosporidium* and *Giardia* in the Netherlands. The annual load of *Cryptosporidium* and *Giardia* in domestic wastewater was 3.2×10^{13} and 3.8×10^{14} respectively. The majority (85%) of the *Cryptosporidium* oocysts was discharged with effluent of wastewater treatment plants, while the majority (82%) of the *Giardia* cysts was discharged with untreated wastewater discharges and sewer overflows. The calculated concentration of *Cryptosporidium* and *Giardia* in surface water was largely determined by the import of (oo)cysts through the international rivers Rhine and Meuse. The estimated annual import through the river Rhine and Meuse was 3.2×10^{14} *Cryptosporidium* oocysts and 2.1×10^{15} *Giardia* cysts, of which the river Rhine contributed 66 to 87%. This outweighed the total load of the discharges of treated and untreated wastewater in the Netherlands.
The combination of PROMISE and WATNAT predicted concentrations of *Cryptosporidium* and *Giardia* in surface water that were in the same order of magnitude as the concentrations that were observed at 5 of the 6 sites compared. At one site, the models predicted concentrations that were 7 to 37 times lower than the observed concentrations. The major contamination source at this site was not domestic wastewater but agricultural run-off, that is not yet included in the model.
This is a first step in the direction of a quantitative description of the transmission cycle of *Cryptosporidium* and *Giardia* through water. The use of these models combines observational occurrence data from different water types and surface water sites and experimental data from laboratory survival studies into a single integrated description. The description needs further improvement by incorporation of agricultural run-off and increasing the number and time frame of input monitoring data.

This research was carried out on behalf of the Directorate General for the Environment, Ministry of Housing, Physical Planning and the Environment. The authors wish to thank A. de Nijs, M. ‘t Hart and G. Elzenga for their skilfull assistance with the application of the models for protozoa discharge and dispersion and L. Kruidenier, G.B. Engels and H.A.M. de Bruin and the samplers from Water Storage Company Brabantse Biesbosch and NV PWN Water Company North Holland for their contribution to the input and validation data on protozoa in waste and surface water.

This paper has been submitted for publication.

INTRODUCTION

The protozoan parasites *Cryptosporidium parvum* and *Giardia intestinalis* have caused many outbreaks of gastro-intestinal illness through water (Craun *et al.,* 1998; Lisle & Rose, 1995). The (oo)cyst stage of these parasites is excreted by infected hosts and is environmentally robust. It can survive long in surface water *(*DeReignier *et al.,*1989; Robertson *et al*, 1992; Chauret *et al.,* 1995*,* 1998; Medema *et al.,*1997) and is resistant to chemical disinfection (Finch *et al.,* 1993[a,b]). Low numbers of (oo)cysts are sufficient to establish infection in a susceptible host. Extrapolation from data from a dose-response experiment in human volunteers (Dupont *et al.,*1995; Rendtorff, 1954) with the exponential model indicates that even a dose of one single (oo)cyst results in a probability of infection: for *Cryptosporidium* of 0.4% and for *Giardia* of 2% (Teunis *et al.,*1996). Although these are extrapolations of the experimental data, it is feasible that a successful survival strategy of these parasites depends on the infectivity of single (oo)cysts. The persistence of (oo)cysts in the aquatic environment, their resistance to chemical disinfection and infectivity make the (oo)cysts of *Cryptosporidium* and *Giardia* critical pathogens for drinking water production from surface water sources and for surface water recreation.
Many studies have shown the presence of *Cryptosporidium* an *Giardia* in surface water at abstraction points for drinking water production (LeChevallier *et al.,*1991, Atherholt *et al.,*1998; Rose *et al.,* 1991; Carrington & Smith, 1995; Hutton *et al.,* 1995; Armon *et al.,* 1995; Karanis *et al.,* 1996; Medema *et al.,* 1999). Surprisingly few surveys have been conducted in bathing areas, but the few available studies show the presence of *Cryptosporidium* and *Giardia* in both fresh (Ruiter *et al.,* 1998) and marine (Johnson *et al.*1995; Genthe *et al.,*1998) bathing waters.
Important sources of *Cryptosporidium* and *Giardia* contamination of surface water are discharges of untreated and treated domestic sewage and agricultural run-off. High concentrations of both *Cryptosporidium* and *Giardia* have been demonstrated in untreated and treated domestic wastewater (Sykora *et al.,*1991; Rose *et al.*, 1986; Madore *et al.,* 1987) and in livestock manure (Svoboda *et al.,*1997; Xiao *et al.,*1994; Casemore *et al.*, 1997). An increase in the parasite concentration in surface water is observed downstream of agricultural areas (Hansen & Ongerth, 1991; Ong *et al.,* 1996) or discharges of untreated and treated domestic wastewater. Furthermore, wildlife is suggested as a significant source in pristine waters (Hansen & Ongerth, 1991; Craun, 1990).
The relative significance of these different sources for the occurrence of (oo)cysts at a specific surface water site is determined by a combination of factors: i) the contamination level of these sources, ii) the magnitude of the sources (load), iii) transport of the (oo)cysts from the source to surface water, iv) transport of (oo)cysts in surface water from the contamination source to the specific site and v) survival of (oo)cysts. (Oo)cysts have been shown to survive for several months in faeces and water (DeReignier *et al.,*1989; Robertson *et al*, 1992; Chauret *et al,*1995,1998*;* Medema *et al.,*1997) Although little is known about the mechanisms that govern the transport of (oo)cysts in river water, it

seems likely that transport by the flow of the water is the major determinant. (Oo)cyst concentrations have been shown to be positively correlated to water flow and turbidity levels (Atherholt *et al.,*1998; Medema *et al.,* 1999). In these studies, *Giardia* concentrations decreased with increasing water temperature, while *Cryptosporidium* concentrations were not temperature related at temperatures below 20°C. The transport of (oo)cysts to and in watersheds can be rapid, as illustrated by the high concentrations found in watersheds in the 'first flush' after storm events (Stewart *et al.,*1997).
A quantitative description of the discharge and fate of (oo)cysts in surface water gives insight in the significance of the different sources and of the processes that determine the probability of (oo)cysts being transported from the point of discharge to drinking water abstraction sites or bathing sites.
In the Netherlands, models have been developed for the description of emission of chemical pollutants to surface water and the subsequent dispersion of these pollutants in surface water. We studied the applicability of these models to describe the emission of *Cryptosporidium* oocysts and *Giardia* cysts to surface water from treated and untreated sewage discharges and the dispersion of the discharged (oo)cysts through surface waters in the Netherlands. This information can be used to design an effective source water protection strategy.

METHODS

Modelling the emission by domestic wastewater.

Model
The emission of *Cryptosporidium* oocysts and *Giardia* cysts by discharges of treated and untreated domestic wastewater was calculated by PROMISE version 2.5.0. PROMISE was developed to describe the emission of nitrogen, phosphorus, heavy metals and polycyclic aromatic compounds (Quarles van Ufford & Ros, 1991). PROMISE holds the regional information on locations and amounts of sewage discharges, treated (biologically) and untreated (direct discharges and sewer overflows). The data on the discharge situation in 1993 were used (Coppoolse *et al.,* 1993).

Input data
For description of the emission of parasites, the model needed input on the average excretion of (oo)cysts per inhabitant per year, the total number of inhabitants and the removal of *Cryptosporidium* and *Giardia* by biological wastewater treatment.
The average excretion was calculated from the average concentration of (oo)cysts in domestic wastewater and the average wastewater production per person per year.
The concentration of *Cryptosporidium* and *Giardia* in domestic wastewater and their removal by sewage treatment was determined by sampling 10-50 l settled influent and 100 l secondary effluent at 5 biological wastewater treatment plants. Samples were taken with the cartridge filtration method using CUNO

Microwynd DPPPY 1µm at 4 l/min, filters were cut and fibres washed with 0.1 % Tween 20 in PBS. The 2 l eluate was concentrated by two subsequent centrifugation steps of 15 min, at 900xg for *Giardia* and at 1200xg for *Cryptosporidium.* Pellets were purified by sucrose flotation and analysed by immunofluorescence microscopy, as described in Medema *et al.* (1999). Six bimonthly samples were taken at each site from May 1994 to May 1995. At each site, one influent sample and one effluent sample was seeded with (oo)cysts to determine the recovery efficiency of the detection method.
The average production of domestic wastewater per person per year (122.3 m^3) was calculated from the total population of the Netherlands in 1994 (15.4 x 10^6 (Anon., 1994)) and the total volume of domestic wastewater produced in 1994 (1876 x 10^6 m^3 (Coppoolse *et al.,*1993)).

Modelling the dispersion in surface water

Model
The dispersion of *Cryptosporidium* and *Giardia* in surface water was calculated with WATNAT version 2.05 (Schijven *et al.,* 1996). WATNAT is a hydrological model that consists of a hydrodynamic transport module for the network of waterways in the Netherlands, based on a schematisation that divides the Netherlands in 85 water districts. The districts are connected to the main water courses by a total of 109 “nodes” along these courses. A second module describes processes that affect water quality (such as die-off) in each part of the network.
The parasites were considered to be transported in surface water by advection, like other small particles. For every district and node, the transport of protozoa was calculated from the input of protozoa by discharges of domestic wastewater (output from PROMISE), the input fluxes of protozoa from nodes upstream and the output fluxes to the nodes downstream. In the case of the districts and nodes directly adjacent to the border at the point of entry of waterways into the Netherlands, the load of protozoa from abroad was needed as input.

Input data
To calculate parasite dispersion, WATNAT required input from discharges from treated and untreated domestic wastewater and the concentration of *Cryptosporidium* and *Giardia* in raw and treated wastewater. The output data from PROMISE on the discharges of (oo)cysts from domestic wastewater per district and node were fed into WATNAT.
The Netherlands is the delta of two large international rivers (Rhine and Meuse). These rivers carry the load of *Cryptosporidium* and *Giardia* from agricultural and wastewater discharges into these rivers in the countries upstream. Most of the surface waters in the Netherlands are branches of these rivers. Hence, the contribution of these rivers, both in water flow and parasite load are important input data for WATNAT. The concentration of *Cryptosporidium* and *Giardia* at the point of entry of the Rhine and Meuse into the Netherlands was estimated

by collecting 100 l samples at each site in the autumn of 1995, by the method described by Medema *et al.,*1998[a]. For the model calculations it was assumed that the load of (oo)cysts (that is the number of (oo)cysts entering the Netherlands per time unit) remained constant.
To determine the impact of the concentrations at the point of entry on the calculated concentrations in surface water throughout the Netherlands, the surface water concentration calculations were performed both with and without these input data.
Data on survival of *Cryptosporidium* oocysts in water of Medema *et al*. (1997), Robertson *et al.* (1992) and Chauret *et al.* (1995) and on survival of *Giardia* cysts of DeReignier *et al.* (1989) were also incorporated in this model. From the reported experimental data, inactivation rates were calculated as the slope of the linear regression line of the log-linear plot of the concentration of viable (oo)cysts versus time. Data on sedimentation rates indicate that sedimentation of unattached (oo)cysts is not a significant factor in the transport of (oo)cysts in surface water (Ives 1990; Medema *et al.*, 1998[b]). Sedimentation of attached (oo)cysts can be significant (Medema *et al.*, 1998[b]), but was not incorporated in the model, because too few data were available.

Calculated versus observed parasite concentrations
To validate the outcome of the model calculations, they were compared to observed concentrations at six surface water sites throughout the Netherlands. Two sites were the point of entry of the river Rhine and Meuse into the Netherlands and the other four points were abstraction points for drinking water production. The latter were located on the river Meuse, approximately 215 km downstream of the point of entry, in two branches of the river Rhine 80 and 105 km downstream of the point of entry and in a regional stream in an agricultural area and was not influenced by either Rhine or Meuse. The concentration of *Cryptosporidium* and *Giardia* at these sites was determined by collecting and assaying 5 100 l samples at each site during the autumn of 1995 by the method described by Medema *et al.,* 1998[a].

RESULTS

Cryptosporidium and *Giardia* in raw and treated domestic wastewater

Giardia was found in all samples of primary and secondary effluent, while *Cryptosporidium* was detected in 20/31 (65%) of the primary effluent samples and in 14/31 (45%) of the secondary effluent samples. To determine the actual concentrations of these parasites in sewage, the observed concentrations were corrected for the average recovery efficiency of the detection method. The average recovery efficiency in influent samples was 0.36% for *Cryptosporidium* oocysts and 10% for *Giardia* cysts. The corrected *Cryptosporidium* concentration in primary effluent ranged from < 1 to 3.9×10^5 per litre (Figure 1). Most of the samples contained < 1 to 100 oocysts per litre, but occasional peak concentrations up to 10^4 to 3.9×10^5 per litre were observed in August,

September, November and February. The *Giardia* concentrations ranged from 21 to 2600 per litre. No strong seasonal variation was observed; the concentrations were relatively low in August and in April. *Giardia* concentrations were generally higher than the *Cryptosporidium* concentrations, but less variable.

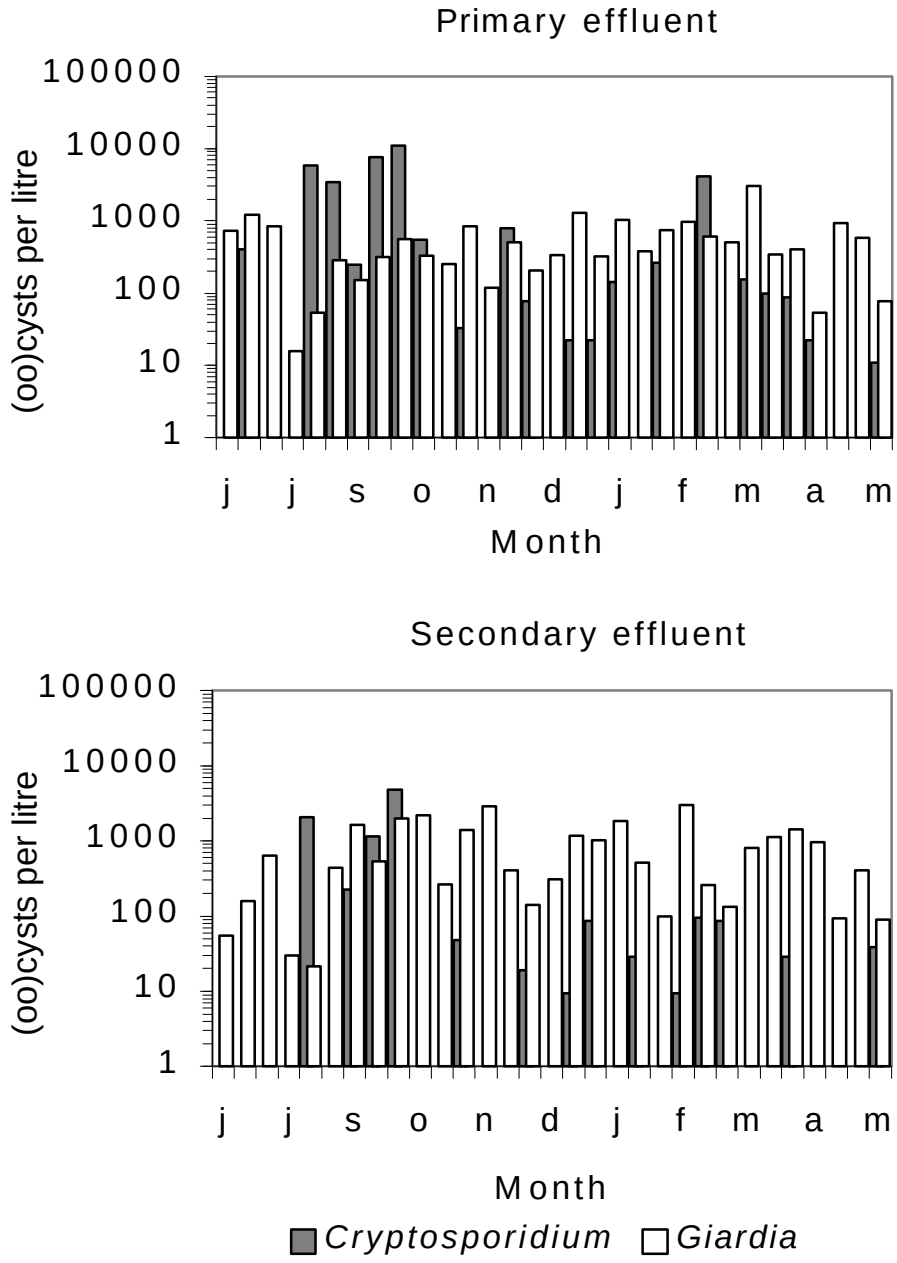

Figure 1. Concentration of Cryptosporidium *oocysts and* Giardia *cysts in primary and secondary effluent of five sewage treatment plants (data corrected for the average recovery of the detection method)*

Emission model input

To determine the average excretion per inhabitant per year, the median of the concentration in primary effluent was used, corrected for the average recovery efficiency of the detection method: 17 oocysts per litre for *Cryptosporidium* and

200 cysts per litre for *Giardia.* The total domestic wastewater production by 1.53×10^7 inhabitants was 1.87×10^{12} litre per year and 97.6% of the inhabitants was connected to a sewer (data 1993). Most of the wastewater is treated in biological treatment plants before discharge to surface water, but a small proportion is discharged untreated, because no treatment is installed or overflow of sewers occurs during heavy rainfall.
The removal efficiency of biological treatment was determined from the difference in the average influent and effluent concentration. For *Cryptosporidium,* this resulted in a removal efficiency of 75%. Similar removal efficiencies are also reported by Rose *et al.*(1986) and Madore *et al.* (1987) for biological wastewater treatment. For *Giardia*, a negative removal efficiency was calculated, because the corrected average concentration in effluent was higher than in influent. Using the uncorrected median concentration, the removal efficiency for *Giardia* cysts would be 93%. Analysing the data of Sykora *et al.*(1991) on *Giardia* concentrations in in- and effluent of 11 biological wastewater treatment plants yielded a removal efficiency of 99.6%. Robertson *et al.,* (1995) reported a removal of 89-97% of *Giardia* cysts in wastewater by biological treatment. For PROMISE calculations, we used an intermediate removal efficiency of 99%.

Emission model output
Combination of the average concentration of (oo)cysts in domestic wastewater and the total volume of domestic wastewater produced in the Netherlands provided the annual load of (oo)cysts through this route in 1994: 3.2×10^{13} *Cryptosporidium* oocysts and 3.8×10^{14} *Giardia* cysts (Table 1). Division of this load by the population number yielded the average excretion per inhabitant per year: 2.1×10^6 *Cryptosporidium* oocysts and 2.5×10^7 *Giardia* cysts.
The model calculations showed that the vast majority (85%) of the *Cryptosporidium* oocysts that were discharged into surface water came from the discharge of biologically treated wastewater (Table 1). For *Giardia* cysts, this is

Table 1. Estimated total emission of Cryptosporidium oocysts and Giardia cysts from domestic wastewater in the Netherlands in 1994.

	Cryptosporidium	*Giardia*
Excretion		
Average excretion/inhabitant/year	2.1×10^6	2.5×10^7
Total excretion	3.2×10^{13}	3.8×10^{14}
Removal		
Removal in biological treatment	75%	99%
Discharge		
Treated sewage	7.6×10^{12} (85%)	3.6×10^{12} (18%)
Overflow	2.6×10^{11} (3%)	3.2×10^{12} (16%)
Not treated	$1.1.x.10^{12}$ (12%)	1.3×10^{13} (66%)

only 18%. As the removal efficiency of sewage treatment for *Giardia* that was entered in the model was higher, the majority of cysts (82%) enter surface water through discharge of untreated sewage (Table 1).

Cryptosporidium and *Giardia* in rivers at point of entry

The concentration of *Cryptosporidium* and *Giardia* in the rivers Meuse and Rhine at the point of entry in the Netherlands is displayed in Table 2. The average concentrations, corrected for the recovery efficiency of the detection method, and the yearly volume of river water flowing into the Netherlands were used to calculate the annual load of (oo)cysts into the Netherlands through these rivers.

Table 2. Concentration of Cryptosporidium and Giardia in the river Rhine and Meuse as they enter the Netherlands, autumn 1995.

	Cryptosporidium	*Giardia*
Rhine (Lobith)		
Number of samples	5	5
% positive	100	100
Average concentration (l^{-1})*	4.5	22
Concentration range (l^{-1})*	0.6 –9.4	7.5 –33
Annual volume of river water (m^3)	6.2×10^{10}	
Parasite load ((oo)cysts per year)	2.8×10^{14}	1.4×10^{15}
Meuse (Eijsden)		
Number of samples	4	4
% positive	100	100
Average concentration (l^{-1})*	5.4	95
Concentration range (l^{-1})*	1.9 - 13	38 –210
Annual volume of river water (m^3)	7.0×10^{9}	
Parasite load ((oo)cysts per year)	3.8×10^{13}	6.7×10^{14}

*Corrected for the average recovery efficiency of the detection method in surface water samples (16% for *Cryptosporidium* and 12% for *Giardia*)

Dispersion model input

The output data from PROMISE on the discharges of (oo)cysts by raw and treated domestic wastewater to districts and nodes were used as input for WATNAT. Also, the load of (oo)cysts flowing into the Netherlands through the river Rhine and Meuse were input data for WATNAT.

To incorporate survival in the model, inactivation rates of (oo)cysts in water were abstracted from literature. For *Cryptosporidium* oocysts, the inactivation

rate was based on survival studies of Medema *et al* (1997), Robertson *et al* (1992) and Chauret *et al.* (1995). Medema *et al.* (1997) found inactivation rates of 0.01 – 0.024 ^{10}log-units per day and no differences in inactivation rate between 5 and 15°C. From the data of Robertson *et al.* (1992) and Chauret *et al.* (1995) inactivation rates of 0.0022 to 0.0027 ^{10}log-units per day were calculated. Chauret *et al.* (1995) found that the inactivation rate was independent of water temperature up to 20°C. From these studies, we deduced a conservative, temperature-independent inactivation rate of 0.003 ^{10}log-unit day^{-1}. For *Giardia,* a temperature-dependent inactivation rate of 0.01 x t (^{o}C) was used, based on the study of DeReignier *et al.* (1989). Their survival data were clustered into periods with similar average water temperatures. The slope of the regression analysis of the ^{10}log of the surviving fraction and the temperature (0.01 day^{-1} °C^{-1}) was used as the temperature-dependent inactivation constant. The daily temperature of surface water in the Netherlands throughout the year was abstracted from Nijs & Burns (1990).

Dispersion model output

15 surface water sites (mainly abstraction points) were selected (Figure 2) and the average and range of the *Cryptosporidium* and *Giardia* concentration was calculated (Table 3). Dispersion modelling resulted in average concentrations of

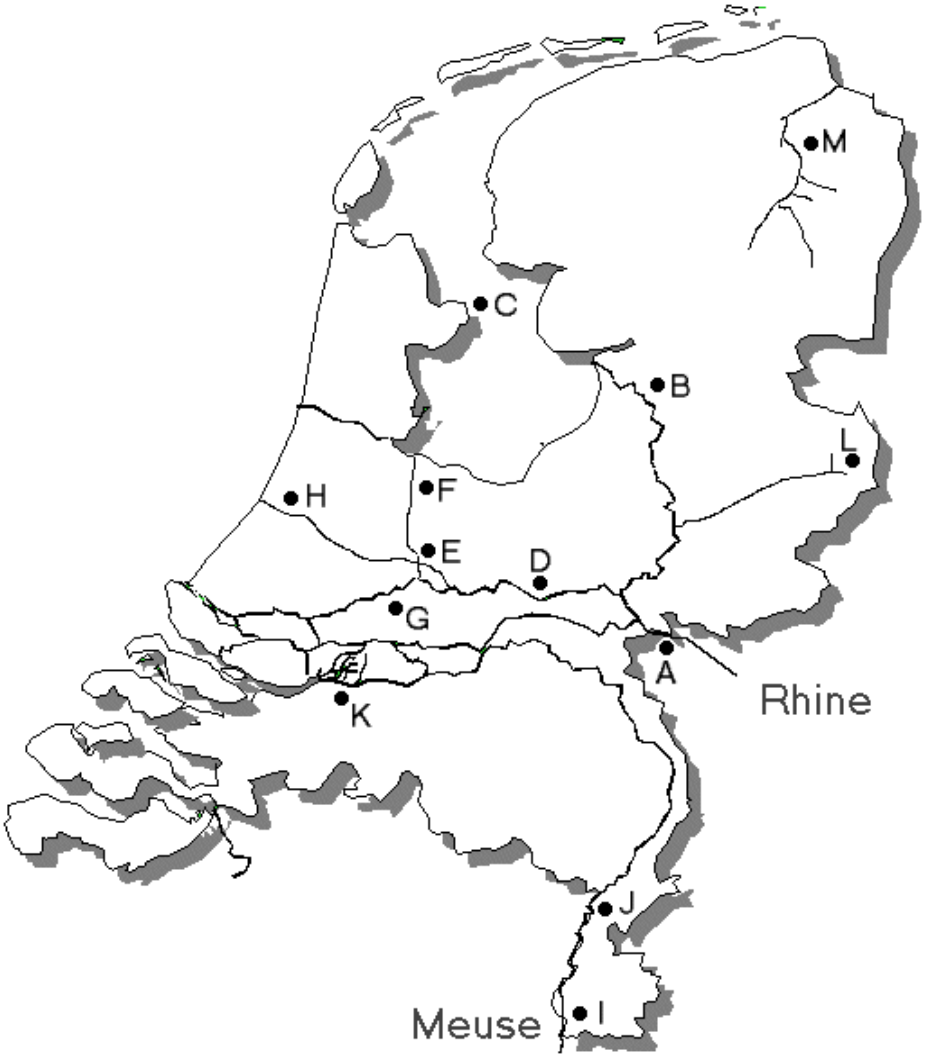

Figure 2. Surface water sites at which the concentration of Cryptosporidium and Giardia were calculated with

PROMISE and WATNAT.

0.30 to 0.38 *Cryptosporidium* oocysts per litre and 0.013 to 0.09 *Giardia* cysts per litre in streams with little or no influence from the international rivers, to 1.9 to 7.6 oocysts and 0.6 to 57 cysts per litre in surface water under the direct influence of the river Rhine or Meuse. To investigate the contribution of the load of (oo)cysts from abroad, concentrations were calculated with and without this load. The import of (oo)cysts by Rhine and Meuse had a strong effect on the calculated concentrations. At the sites that were under the direct influence of these rivers (B to G, J and K) the calculated *Cryptosporidium* concentrations were 4.4 to 200 times lower if the import through Rhine and Meuse was neglected; for *Giardia* the concentrations without import were even 4.2 to 4100 times lower. Evidently, at the sites that were not under the influence of these

Table 3. Calculated average concentrations of Cryptosporidium *and* Giardia *at various surface water sites, with and without the input of the (oo)cysts that enter the Netherlands with the Rhine and Meuse.*

Site	With import		Without import	
	Cryptosporidium (oocysts . l^{-1})	*Giardia* (cysts . l^{-1})	*Cryptosporidium* (oocysts . l^{-1})	*Giardia* (cysts . l^{-1})
Rhine/IJssel				
A	5.1	23	0.001	0.001
B	4.9	15	0.057	0.024
C	1.9	0.4	0.051	0.001
Rhine/Lek/ARK				
A	5.1	23	0.001	0.001
D	5.0	18	0.025	0.009
E	4.2	5	0.090	0.020
F	3.1	0.6	0.69	0.029
Rhine/Lek/Oude Rijn				
A	5.1	23	0.001	0.001
D	5.0	15	0.025	0.009
G	4.6	11	0.063	0.015
H	1.3	0.05	1.0	0.040
Meuse				
I	7.6	57	0.048	0.007
J	7.6	45	0.063	0.011
K	5.0	14.4	0.22	0.025
National waters				
L	0.30	0.09	0.30	0.09

M	0.38	0.013	0.38	0.013

rivers (L and M), or only slightly (H), the concentrations with and without import were similar.
In the calculations with import, the concentration of *Cryptosporidium* and *Giardia* decreased with residence time of the water in the Netherlands (Table 3: compare C to B and A; F to E, D and A; K to J and I; H to G, D and A). In the calculations without import, the reverse was observed. So, the longer the residence time of the water in the Netherlands, the more important the local discharges. The contribution of the discharges of domestic wastewater in The Netherlands accounted for 0.5 to 23% of the calculated average *Cryptosporidium* concentration at the sites that were under the direct influence of the Rhine or Meuse, and even less than 5% of the average *Giardia* concentration at these sites.

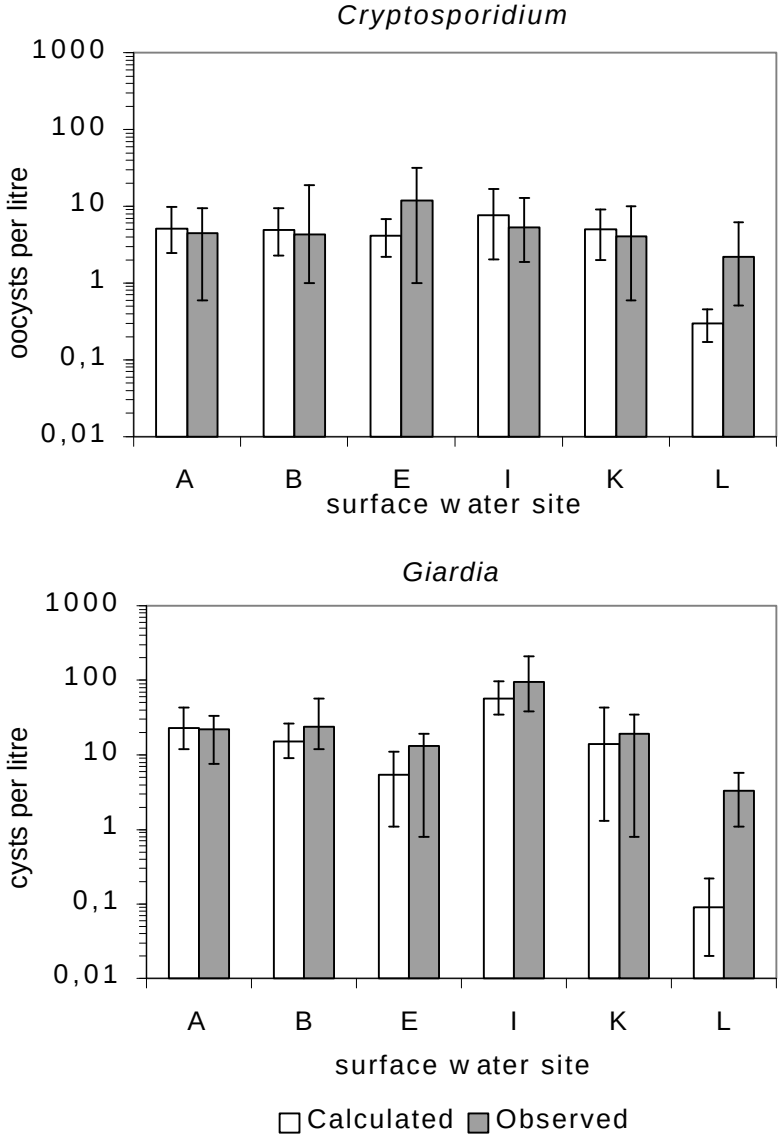

Figure 3. Calculated versus observed average, minimum and maximum concentrations of Cryptosporidium and Giardia at six surface water sites.

Calculated versus observed concentrations

Data on the occurrence of *Cryptosporidium* and *Giardia* in surface water were present for 6 sites. Comparison of these data to the emission and dispersion model calculations showed that the differences between the calculated and observed concentrations of *Cryptosporidium* and *Giardia* in surface water were less than a factor 2 to 3 in most cases (Figure 3). At site L, the calculated concentration of *Cryptosporidium* was a factor 7 lower than the observed concentration. For *Giardia*, the calculated average concentration was even a factor 37 lower than the observed average concentration. This site was upstream of domestic wastewater discharges and downstream of an agricultural area with cattle, sheep and application of manure for soil fertilisation, indicating that the main source of faecal pollution was run-off from this area, whereas the calculated concentrations were determined by the calculated discharge of domestic wastewater. Furthermore, this site was not under the influence of the river Rhine or Meuse.

At one site in the river Meuse (K), data on the occurrence of *Cryptosporidium* and *Giardia* from weekly to biweekly sampling throughout the year were available (Medema *et al.,*1999). The daily model output at this site was compared to the observed concentrations (Figure 4). For *Cryptosporidium,* the model calculated concentrations that were lower than the observed concentrations during winter but higher in spring and summer. The model calculated *Giardia* concentrations that were close to the observed concentrations during late summer and fall, but relatively high in winter, spring and early summer.

DISCUSSION

Model validation

This study has shown that the models PROMISE and WATNAT, that were developed for the description of the emission and dispersion of chemical pollutants, can be applied to approximate the emission of *Cryptosporidium* and *Giardia* by domestic wastewater and their dispersion in surface water. The concentration in surface water that is calculated by the combination of these models was close to the observed concentration at five of the six surface water sites tested. The site where the model predicted lower concentrations than were observed (L) was mainly influenced by agricultural pollution, that is not yet incorporated in the model. This shows the relevance of the inclusion of agricultural pollution in the model calculations. For that purpose, data are needed on the occurrence of *Cryptosporidium* and *Giardia* in manure from livestock in the Netherlands and on the routes of transport of (oo)cysts from manure to surface water (run-off, movement through soil). This information is now emerging (Mawdsley *et al.*, 1996; Svoboda *et al.,* 1997).

Based on the concentration of (oo)cysts in domestic wastewater and the total wastewater production, the total annual excretion of *Cryptosporidium* oocysts

and *Giardia* cysts in the Netherlands was estimated to be 3.2 x 10^{13} and 3.8 x 10^{14} respectively, and an average annual excretion of 2.1 x 10^{6} oocysts and 2.5

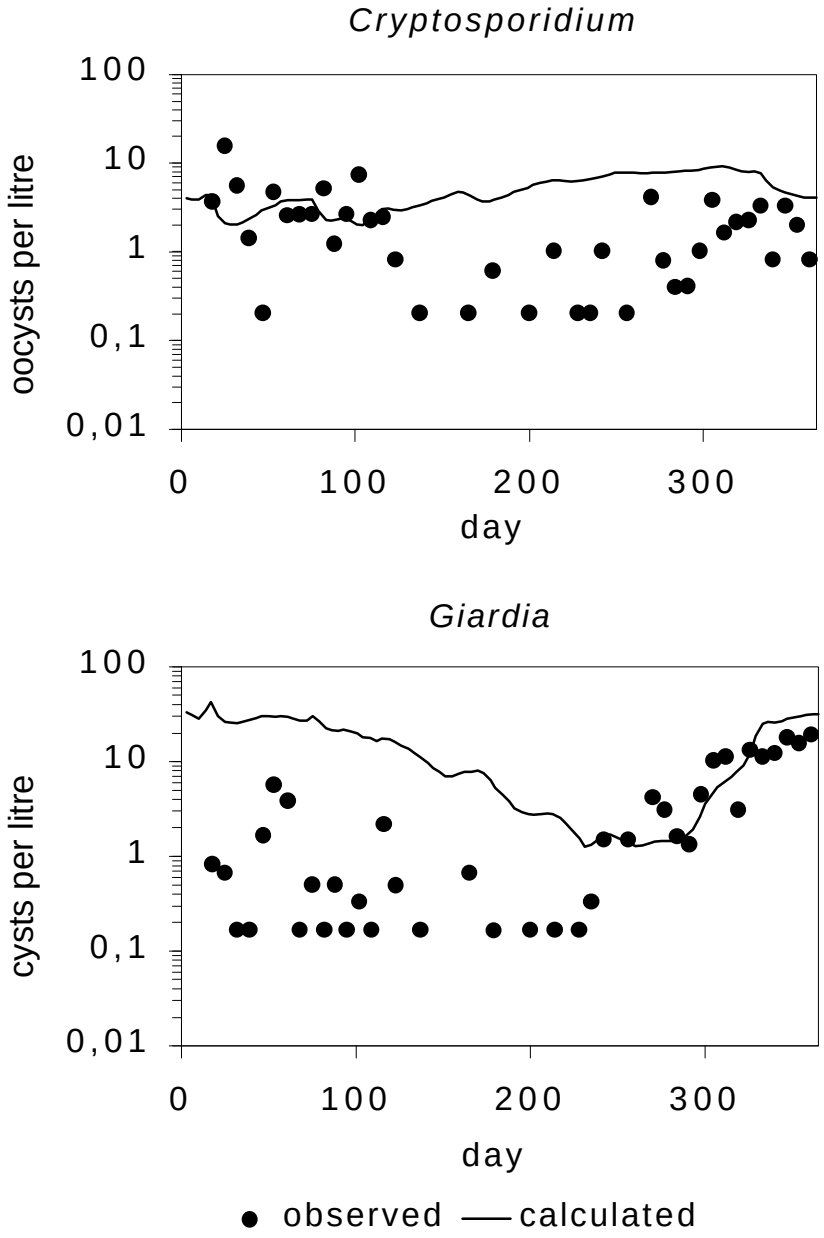

Figure 4. Calculated versus observed concentration of Cryptosporidium *and* Giardia *in the river Meuse at Keizersveer (site K) throughout 1994.*

x 10^{7} cysts per inhabitant were calculated. Alternatively, combination of these data with data on the prevalence of cryptosporidiosis and giardiosis in the Netherlands results in the estimated excretion of (oo)cysts per case. A population study by Hoogenboom *et al.* (1989), indicated that the total number of cases of gastro-enteritis in the Netherlands was 2 x 10^{6} per year. *Cryptosporidium* and *Giardia* were detected in stool samples of 1.6 and 3 to 5% of these cases, respectively. The prevalence of asymptomatic *Giardia* infections in the Netherlands was also 3 to 5%, while asymptomatic carriage of *Cryptosporidium* was rare (Kortbeek *et al.*, 1994). This indicates that approximately 32,000 cases of cryptosporidiosis occur annually in the

Netherlands. For *Giardia*, the prevalence data amount to 60,000 - 100,000 cases of symptomatic infections and 500,000 - 800,000 asymptomatic infections annually.
To account for the total number of oocysts in domestic wastewater, the total excretion per case of cryptosporidiosis should be approximately 10^9 oocysts. Chappell *et al.* (1996) infected human volunteers with *C.parvum* oocysts in different doses. They determined that the median oocyst excretion in infected persons that contracted diarrhoea was 3×10^7 per case, ranging from 5×10^5 – 3×10^9. This indicates that the average excretion per case in natural infections is higher than reported for the healthy adult volunteers or that other sources, such as cattle slurry, contribute significantly to the contamination of domestic wastewater.
For *Giardia*, this would amount to a total excretion of approximately 4×10^9 – 6×10^9 cysts per gastro-enteritis patient with *Giardia* or to 5×10^8 – 8×10^8 per infected person, if also asymptomatic carriage is included. Data on two infected individuals reveal large fluctuations in the daily excretion of cysts (Tsuchiya, 1931), ranging from 3 – 700×10^6 cysts per day. The total number of cysts excreted amounted to 10^9 – 10^{10} in both individuals. If these data are representative for the excretion of *Giardia* by patients with giardiasis in the Netherlands, these are well within range of the calculated total excretion on the basis of the cyst concentration in sewage.
The comparison of the calculated and observed protozoa concentrations throughout the year at the one site in the river Meuse where annual data were available showed that the observed concentrations were lower than the calculated concentrations of *Cryptosporidium* and *Giardia* during spring and summer and for *Cryptosporidium* also in early autumn. This corresponded with water temperatures of more than 15°C. A possible explanation for this discrepancy is that the inactivation rate of cysts and oocysts is higher under natural conditions than the rates calculated from the laboratory experiments. This does, however, not explain the discrepancy between the calculated and observed *Giardia* concentrations during winter.

Model results and implications
The calculation of the number of (oo)cysts discharged into surface water through sewer overflows, untreated and treated domestic wastewater discharges makes it possible to select the most appropriate preventive measures for source water protection, both on a national and on a regional (district) scale. Since the majority (85%) of the *Cryptosporidium* oocysts are discharged with treated wastewater, enhancing removal of *Cryptosporidium* oocysts by wastewater treatment will result in the most significant reduction of the discharge to surface water For *Giardia*, this will not be an effective strategy, since 82% is discharged with untreated wastewater or sewer overflows. Hence, increasing the percentage of households that is connected to sewers (1994: 98%) and the percentage of wastewater that is treated (1994: 97,6%) and prevention of overflows (1994: 0.87% of total volume of domestic wastewater) will be more effective to reduce the discharge of *Giardia*.

From the WATNAT-calculations, it is clear that the import of (oo)cysts through the rivers Meuse and especially the Rhine is very significant for the contamination of the majority of the surface waters in the Netherlands. The total load of (oo)cysts through these rivers into the Netherlands outweighed the total discharge of (oo)cysts from treated and untreated domestic wastewater by approximately 1 order of magnitude (Table 1 and 2). The calculated concentrations in surface water that were influenced by these rivers were also much lower when the import of (oo)cysts through Rhine and Meuse was set to zero (Table 3).
The influence of the import reduced with increasing residence time of the water in the Netherlands. Two processes may contribute to this reduction: dilution and inactivation. Dilution would equally affect the concentrations of both protozoa. Since the concentrations of *Giardia* were reduced to a larger degree than *Cryptosporidium* (Table 3), and the inactivation constant for *Giardia* was higher than for *Cryptosporidium,* it is likely that inactivation is a more important process than dilution in determining the *Cryptosporidium* and *Giardia* concentrations at the study-sites.
The impact of the (oo)cyst import on the concentrations in surface waters in many parts of the Netherlands indicates that source water protection is an international issue and that preventive measures on a national scale should be balanced against source water protection measures taken in countries upstream the river Rhine and Meuse.

Improvements in input data and model
The calculated discharges of *Cryptosporidium* and *Giardia* to surface water were based on point estimates of the concentration of (oo)cysts in sewage and the removal efficiency of wastewater treatment. Both were estimated from the median concentrations measured before and after the treatment. From the data it was obvious that both the concentrations in domestic wastewater and the removal efficiency showed considerable variation. Another source of variation is the recovery efficiency of the detection method. More data and an improved detection methodology are required for an accurate description of the discharge of (oo)cysts through domestic wastewater and inclusion of temporal variations in the (oo)cyst concentration in domestic wastewater.
Likewise, the data on the occurrence of *Cryptosporidium* and *Giardia* in surface water that were used either as input for the model or to verify the model output were limited in number and in time frame. Improvement of the model can be obtained by collection of a more elaborate data set that covers all seasons of the year, both at the sites that the rivers enter the Netherlands as input for WATNAT and at several selected surface water sites to verify the model outcome. This also allows verification of the seasonality that is observed in the measured data set with relatively low concentrations of *Cryptosporidium* and *Giardia* in summer.
The dominance of the international rivers may have masked discrepancies between calculated and observed concentrations. Hence, inclusion of one or more sites that are not influenced by the international rivers and receive

domestic wastewater would allow a better validation of the model calculations, but no data were available.
In PROMISE, combined sewer overflows are modelled as a constant discharge of a small percentage of raw sewage (0.87% in 1994). However, the nature of these discharges is not constant but follows periods of heavy rainfall, leading to short spikes in the concentration of (oo)cysts in the receiving water (Stewart *et al,* 1997; Atherholt *et al.,* 1998). These spikes are significant, since they may overload drinking water treatment systems and result in outbreaks of disease (Craun *et al.,*1998). It is possible to incorporate the short term variations rainfall and water flow in discharge and dispersion models. This extension would allow predictions of the occurrence of spikes in the (oo)cyst concentration at abstraction points.

This study is a first step in the direction of a quantitative description of the transmission cycle of *Cryptosporidium* and *Giardia* through water. The use of these models combines observational occurrence data from different water types and surface water sites and experimental data from laboratory survival studies into a single integrated description. The description needs further improvement: incorporation of agricultural run-off and increasing the number and time frame of concentration data in sewage, effluent and points of entry of the Rhine and Meuse. In addition, improvement of the recovery efficiency of the detection method would improve the accuracy of the input data.

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The significance of wildlife as source of *Cryptosporidium* and *Giardia* in pre-treatment reservoirs

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ABSTRACT

Cryptosporidium parvum and *Giardia* are widespread in mammals. Several outbreaks of giardiasis and cryptosporidiosis have been associated with contact with infected mammals, both directly or through contamination of water. Although zoonotic transmission of especially *Giardia* is still controversial, mammals, both domestic and wild, should be regarded as a reservoir of (oo)cysts of *Cryptosporidium* and *Giardia* that may cause human infections. In addition, birds, although they are natural host to parasite species that are not infectious to humans, may act as vectors for the transmission of human (oo)cysts.
The significance of wildlife to contamination of pre-treatment storage reservoirs of river water was determined by a collecting information on the occurrence of *Cryptosporidium* spp. and *Giardia* spp. in wildlife on and around the reservoirs, animal population densities and daily faeces production of these animals. The load of (oo)cysts from animal faeces was used to calculate the expected concentration in reservoir water and compared to the measured concentrations in these reservoirs to assess the contribution of (oo)cysts from wildlife to the observed (oo)cyst concentrations in the water.
Cryptosporidium spp. oocysts were detected in several bird species and in sevaral mammals. *Giardia* spp. was also detected in and in one of the mammals. Calculation of the concentration of (oo)cysts in reservoir water from the (oo)cyst load from wildlife showed that the gadwall can significantly (0.5 – 9.4%) contribute to the concentration of oocysts of *Cryptosporidium* spp. measured in reservoir water. Similarly, both the mallard and eurasian wigeon can be significant sources of *Giardia* spp. cysts in reservoir water. Also the great cormorant, tufted duck and cattle contributed to the *Cryptosporidium* spp. contamination in the reservoir and the gadwall to contamination with *Giardia* spp, but their contribution was limited to 0.5 - 8.2% (maximum estimate). The overall calculated contribution of waterfowl to the contamination of reservoir water with *Cryptosporidium* is 1.0 – 16% and 4 - 67% for *Giardia*.
This quantitative study showed that waterfowl is not only a potential source of (oo)cysts but can be a significant source of (oo)cysts in pre-treatment reservoirs.

The authors wish to express their appreciation to all the bird watchers from the Natuur- en Vogelwacht Dordrecht for their efforts to perform bird counts and collect faecal samples and the personnel of Water Storage Company Brabantse Biesbosch for sample transport.
This research was funded by the General Directorate of the Environment of the Ministry of Housing, Physical Planning and the Environment.

This paper has been submitted for publication.

INTRODUCTION

Although the major sources of contamination of surface water with *Cryptosporidium* and *Giardia* are sewage discharges (Rose *et al.,* 1986; Madore *et al.,*1987; Sykora *et al.,* 1991; Medema & Schijven, 1998) and run-off from agricultural lands (Atherholt *et al.,* 1998), wildlife has been recognised as a potentially significant source of water contamination in more pristine waters (Dykes *et al.,* 1980; Erlandsen *et al.,* 1991; Smith *et al.,* 1993). Wildlife has even been suggested as sources of outbreaks of waterborne giardiasis (Dykes *et al.,*1980).

Zoonotic transmission of cryptosporidiosis is well documented. There are various reports of outbreaks or cases of cryptosporidiosis in school children or students after exposure to calves or lambs (Casemore, 1997). Occupational exposure to infected animals (mainly calves) has also resulted in human infection (Current, 1994; Casemore, 1997). Companion animals, particularly dogs and cats have been implicated as likely sources of cryptosporidiosis, since they are occasionally infected with *Cryptosporidium parvum* (Casemore, 1997). However, despite the close contact with these animals, associations with human infection are rare. In these cases, it is not possible to verify if the animals have infected their owners or vice versa (Ungar, 1990). Indirect evidence indicates that contact with horses and contact with horse manure are risk factors for cryptosporidiosis (Casemore, 1990). Several outbreaks of waterborne cryptosporidiosis in the US, Canada and UK have been associated with contamination of the water by run-off from livestock (Craun *et al.,* 1998), like the large outbreak in Milwaukee (MacKenzie *et al.,* 1994). However, subsequent genotyping of this outbreak strain suggests that the oocysts were not of animal but of human origin.

C. parvum has been identified in over 80 species of mammals (Casemore, 1997), many of which are quite prevalent in the Netherlands on or around pre-treatment reservoirs. Ruminants (deer, cattle, sheep) are frequently infected with *C. parvum* (Angus, 1990). Symptomatic infection occurs primarily in neonatal animals, that shed high numbers of oocysts (neonatal calves: 10^{5-7}per gram faeces (Current, 1985)) at the peak of the infection. The main symptom is diarrhoea, more severe symptoms are rare. Infection has also been demonstrated in adult animals, but their faeces contains much lower concentrations of oocysts (Fayer *et al.,* 1997). *C. parvum* has been detected in horses (mainly in foals), pigs (mainly piglets), cats, dogs, foxes, and a variety of rodents (rabbits, squirrels, rats, mouse, vole, hamster)(Kim, 1990; Perryman, 1990; Lindsay & Blagburn, 1990; Fayer *et al.,* 1997) and a wide variety of other mammals that are not endemic in the Netherlands. Cross transmission has been demonstrated between a variety of these mammalian hosts (Fayer *et al.,* 1990). Hence, wild animals are a reservoir for *C. parvum* and may spread the infection within the population of wild animals or to livestock and humans, either directly or through contamination of surface water that is used for drinking water.

Other species of *Cryptosporidium* are identified in birds, reptiles, amphibians and fish (Fayer *et al,* 1990, 1997), but these are not associated with human infections, except for a single case of an immunocompromised person infected

with *C. baileyi* (Ditrich *et al.,* 1991). Recent reports indicate that waterfowl may shed of viable oocysts of *C. parvum* after ingestion of these oocysts (Grazcyk *et al.,* 1996). Moreover, naturally infected Canada geese were shown to carry and shed the zoonotic strain of *C. parvum*, as demonstrated by their infectivity to neonatal mice and genotyping of TRAP C2 and beta-tubulin genes (Grazcyk *et al.,* 1998). Hence, birds that feed on sewage sludge or agricultural lands may ingest *C. parvum* oocysts and are a potential source of water contamination and zoonotic transmission of *C. parvum*.

Evidence for the zoonotic transmission of *Giardia* is primarily circumstantial and subject to debate (Erlandsen, 1994). *Giardia* has been identified in many species of animals (Kulda & Nohynkova, 1987; Erlandsen *et al.,* 1991; Erlandsen, 1994). In many cases, the *Giardia* isolates from animals are morphologically indistinghuisable from the human *Giardia* isolates (Healy, 1990). This had lead to the implication of animals as the source of human infection (Dykes *et al.,* 1980; Jakubowski, 1988; Erlandsen, 1994). In addition, reports of waterborne outbreaks of giardiasis emerged and several of the implicated watersheds were without obvious human faecal contamination. Dykes *et al.* (1980) reported an outbreak of giardiasis in a town that was supplied with water from a mountainous area in Washington, USA. No evidence of human faecal contamination of the watershed was found, but *Giardia* cysts were found in trapped beavers. The cysts isolated from the beavers were shown to be infectious to specific pathogen-free beagle puppies. Beavers were frequent carriers of *Giardia* with prevalence rates of 10-18% (Healy, 1990). Several waterborne outbreaks have subsequently been related to contamination of water by beavers (Lopez *et al.,* 1980; Lippy, 1981; Wilson *et al.,* 1982) and later also by muskrats, another aquatic mammal with an even higher prevalence of *Giardia* (33-95%; Healy, 1990; Erlandsen, 1994). These reports have been criticised, as the provided evidence was only circumstantial (Woo, 1984, Erlandsen, 1994). Infection of humans with *Giardia* cysts derived from beavers appears to be possible (Davies & Hibler, 1979) and also human derived cysts can infect beavers and muskrats (Erlandsen *et al.,* 1988). However, the trophozoites from 9 out of 11 naturally infected beavers were distinct from trophozoites seen in human infections and the binary cysts seen in naturally infected muskrats are also distinct from *G. intestinalis* (Erlandsen & Bemrick, 1989). In any case, the cysts found in naturally infected beavers and the cross-transmission of human *Giardia* to beavers and muskrats indicates that these animals may be infected with human derived *Giardia* cysts and act as a source of contamination of surface water with *Giardia* cysts that are infectious to humans.
Another observation on the potential for zoonotic transmission of *Giardia* is that *Giardia* is quite prevalent in companion animals (dogs, cats) and farm animals (cattle, sheep, pig, goats), but, even though human contact with these animals is close, as yet no natural human infections attributable to these animals have been reported (Healy, 1990; Erlandsen, 1994).
Giardia is also present in birds and they too have been suggested to play a role in the transmission of human giardiasis, based on the similarity of the cysts found in birds (great blue heron (Georgi *et al.,* 1986) and parakeet (Box, 1981)).

Erlandsen *et al.* (1991[a]) examined 370 samples of 74 bird species for the presence of *Giardia.* They found a high prevalence in herons (Ciconiformes) of 55% and in budgerigars (Psittaciformes) of 47%. *Giardia* was also found occasionally in other bird species. They considered it doubtful that the *Giardia* from these birds could lead to human infections, since the *Giardia* found in herons and budgerigars both appear to be distinct species (*G. ardae* in herons and *G. psittaci* in budgerigars) and cross-transmission studies with these cysts in mammalian animal models have not resulted in any detectable infections (Erlandsen *et al.,* 1991[b]). They suggested that contamination of water with avian cysts would only be a confusing factor, since the current methods for *Giardia* cyst detection in water cannot distinguish between the different species. *Giardia* sp. was also found in the *C. parvum* infected Canada geese (Grazcyk *et al.,* 1998), but these cysts were not examined for infectivity in a mammalian animal model.
So, wildlife is a reservoir of *Cryptosporidium* spp. and *Giardia* spp. that may give rise to contamination of surface water with cysts and oocysts. *C. parvum* oocysts that are shed by mammals are a potential source for human infection and at least one bird species sheds *Cryptosporidium* oocysts that are potentially infectious to humans
Giardia cysts that are shed by beavers and muskrats are also potentially infectious to humans, while avian cysts are not.

In the Netherlands, most of the surface waters are contaminated by sewage discharges and agricultural run-off. Hence, contamination of (oo)cysts from wildlife is potentially significant in only a few watersheds that are used for drinking water production. These are the open pre-treatment storage reservoirs of river water and reservoirs with water that is recollected after dune-infiltration. These reservoirs are particularly vulnerable to contamination from wildlife (Medema & Schets, 1994), especially waterfowl, during cold winters, when these reservoirs may be the only unfrozen surface water in the area and animal densities can be high.
The objective of this study was to determine the significance of wildlife as a source of contamination of reservoirs of river water with (oo)cysts of *Cryptosporidium* spp. and *Giardia* spp. Therefore, information was collected on the occurrence of *Cryptosporidium* spp. and *Giardia* spp. in stool samples of the animals living on and around the reservoirs, animal population densities and daily faeces production of these animals. This information was used to calculate the expected concentration in reservoir water from the load of (oo)cysts from animal faeces and compared to the measured concentrations in these reservoirs.

MATERIALS AND METHODS

Reservoirs

The Biesbosch reservoirs are three reservoirs of 40, 33 and 13 Mm^3 and a surface area of 305, 210, and 100 hectares. Water from the river Meuse is fed into the first reservoir and flows through this and the two subsequent reservoirs with an average retention time of 5 months. The reservoirs serve as a first step in the treatment of river water to drinking water. The water quality is improved by several processes (die-off of pathogenic micro-organisms, nitrification, photolysis of micropollutants, sedimentation) in the reservoirs (Oskam, 1995) and peak contamination events in the river are either excluded by selective intake or equalised during the flow through the reservoirs. The reservoir water is transported to several water treatment plants for further treatment to drinking water for approximately 2.9 Million people.

The reservoirs are man-made and the banks are lined with tarmac. In comparison with natural lakes in the Netherlands, the reservoirs are deep (15-25m) and have low algal concentrations, due to forced mixing and grazing by zooplankton (Breemen & Ketelaars, 1995). The reservoirs are surrounded by a national forest with many creeks. The water and quietude attract many birds that rest and feed on the reservoir water or banks. The number of birds on the reservoirs may rise up to 24.000 during winter (Bruijn, 1996). The hard surface of the reservoir banks makes the reservoirs unsuitable for aquatic mammals, like (musk)rats, so these will only occasionally visit the reservoirs. The banks and surrounding area harbour small mammals, but also deer and cattle are present in the vicinity of the reservoirs. Hence, contamination of water by wildlife will occur primarily from the birds that reside on the reservoir water or banks and to a lesser extend from mammals that visit the reservoir banks.

Bird counts

From February 1994 to February 1995, the number of birds that were present on the posterior reservoir of the Biesbosch reservoirs or its banks were counted. Counting and speciation was performed by experienced bird watchers. Counting started around 9am and was continued until 3pm. Four evening-counts were carried out to assess which bird species were continuously present on the reservoirs and which left the reservoirs to forage in the surrounding area.

Sampling

Fresh faecal pellets were collected from waterfowl that resided on the banks of the Biesbosch reservoirs. By continuous observation of bird behaviour it was possible to collect faecal pellets fresh. The pellets were collected from places on the banks that had just been visited by a homogeneous group of birds, to be certain of the origin of the fresh faeces. From every bird group a number of pellets was collected and combined into one sample bag (Minigrip) and transported to the laboratory. In the laboratory, the part or all of the pellet was weighed and 1:1 suspended in 5% potassium bichromate in a 50 ml centrifuge tube to prevent growth of bacteria or molds. Fixed pellets were stored at 2-8°C.

Processing
The pellet was suspended in the bichromate solution and an aliquot of approximately 1 g was weighed and diluted in 10 ml PBS 0.01 M, pH7.2 with 0.1% Tween 80 and 0.1% SDS. The pellet was suspended by vortexing. The suspension was filtered through a 35µm filter. The centrifuge tube and filter were washed with 40 ml of PBS with Tween/SDS. The 50 ml suspension was centrifuged at 1050 x g for 10 minutes. 45 ml supernatant was aspirated and the pellet was resuspended and washed again in PBS with Tween/SDS and 10 ml pellet was retained. This pellet was underlayed with 10 ml cold sucrose solution with a specific density of 1.18 and centrifuged for 15 min. at 1050 x g without brakes. The upper 15 ml were collected, washed in demineralised water and filtered over a 1.2 µm, 25 mm polycarbonate filter. The filter was stained with FITC-labelled monoclonal antibodies against *Giardia* (Cellabs, Australia) and *Cryptosporidium* (Shield Diagnostics, UK) at 37°C for 30-45 min. The filters were washed with PBS and embedded in a DABCO/glycerol solution of pH 7 (Medema *et al,* 1998) on a microscope slide and sealed with nail polish.

Analysis
The filters were screened with a Zeiss Axioskop with epifluorescence at a magnification of 250x. Potential (oo)cysts were confirmed at a magnification of 1000x using fluorescence and size and shape criteria (Medema *et al,* 1998[a]). Where possible, confirmation with DIC-optics was employed.

RESULTS AND DISCUSSION

Cryptosporidium spp. oocysts were detected in several bird species: common shelduck (*Tadorna tadorna*), gadwall (*Anas strepera*), eurasian wigeon (*Anas penelope*), great cormorant (*Phalacrocorax carbo*), mute swan (*Cygnus olor*), black-headed gull (*Larus ridibundus*) and tufted duck (*Aythya fuligula*) (Table 1) and in sevaral mammals: muskrat (*Ondorata zibethicus*), hedgehog (*Erinaceus europaeus*), rabbit (*Oryctolagus cuniculus*) and cow (*Bos taurus*) (Table 1). No oocysts of *Cryptosporidium* spp. were detected in faecal samples from mallard ducks. O'Donoghue *et al.* (1987) reported the occurrence of *Cryptosporidium* in colonic and bursal contents of mallard ducks that were submitted to their laboratory for post-mortem analysis. Since all infected ducks showed clinical symptoms of infection of the respiratory tract, these specimens formed a selection of ill mallard ducks and not of the total mallard population.
Giardia spp. was also detected in birds: mallard (*Anas plathyrhynchos*), gadwall, eurasian wigeon and mute swan) and in one of the mammals (muskrat). As far as the authors could trace, this is the first report of the occurrence of *Cryptosporidium* spp. in the common shelduck, eurasian wigeon, great cormorant, mute swan and tufted duck and of *Giardia* spp. in the mallard, gadwall, eurasian wigeon and mute swan. Erlandsen *et al.* (1991[a]) have examined faecal samples of 16 mallards, but found no *Giardia* cysts or trophozoites.

Table 1. Occurrence of Cryptosporidium spp. and Giardia spp. In birds and mammals living on and around the Biesbosch reservoirs

Species		Animals sampled	Samples (number)	Positive samples (%)		Positive animals (%)		Average concentration ((oo)cysts/g)*	
				Cryptosporidium	*Giardia*	*Cryptosporidium*	*Giardia*	*Cryptosporidium*	*Giardia*
Birds									
Common shelduck	*Tadorna tadorna*	44	8	25	0	2,3	0	45,6	0
Mallard	*Anas platyrhynchos*	168	17	0	6	0	24	0	444
Gadwall	*Anas strepera*	43	8	13	13	14	4,7	274	19
Tufted duck	*Aythya fuligula*	18	5	20	0	11	0	2,9	0
Eurasian wigeon	*Anas penelope*	66	4	25	50	9,1	83	0,36	184
Common coot	*Fulica atra*	5	1	0	0	0	0	0	0
Greylag goose	*Anser anser*	133	6	0	0	0	0	0	0
Great cormorant	*Phalacrocorax carbo*	10	5	20	0	10	0	64	0
Mute swan	*Cygnus olor*	5	4	25	25	20	20	5,2	26
Oystercatcher	*Haematopus ostralegus*	2	1	0	0	0	0	0	0
Northern shoveler	*Anas clypeata*	2	1	0	0	0	0	0	0
Blue heron	*Ardea cinerea*	1	1	0	0	0	0	0	0
Common phaesant	*Phasianus colchicus*	2	1	0	0	0	0	0	0
Black-headed gull	*Larus ridibundus*	1	1	100	0	100	0	8	0
Common buzzard	*Buteo buteo*	1	1	0	0	0	0	0	0
Mammals									
Beaver	*Castor canadensis*	8	8	0	0	0	0	0	0
Polecat	*Putorius putorius*	2	1	0	0	0	0	0	0
Coypu	*Myocastor coypus*	1	1	0	0	0	0	0	0
Muskrat	*Ondarata zibethicus*	2	2	50	50	50	50	2490	360
Rabbit	*Oryctolagus cuniculus*	31	4	25	0	6,5	0	1,2	0
Hedgehog	*Erinaceus europaeus*	13	2	50	0	38	0	38500	0
Bank vole	*Clethrionomys glareolus*	1	1	0	0	0	0	0	0
Red fox	*Vulpes vulpes*	2	1	0	0	0	0	0	0
Roe deer	*Capreolus capreolus*	2	1	0	0	0	0	0	0
Cow	*Bos taurus*	7	7	29	0	29	0	2870	0

The average concentration per animal is calculated from the concentration of (oo)cysts in the positive faecal samples x the fraction of animals contributing to the positive samples

In all animals, finding of (oo)cysts in faecal samples was occasional: most samples were negative (Table 1). Because most of the samples were composite pellets of more than one animal, it was not possible to calculate the actual prevalence. A rough estimate of the prevalence was calculated from the percentage of animals that contributed to positive samples (Table 1). For several animals, this estimate is relatively accurate, since pellets of many animals were tested and the number of animals in positive samples are low (common shelduck, gadwall, great cormorant, tufted duck, rabbit, cow). In these cases the calculated prevalence in birds and rabbits is low, ranging from 2.3 – 14%. Only cows show a higher prevalence of 29%.
For other positive animals, the estimated prevalence is less accurate, since only a small number of pellets was tested (mute swan, black-headed gull, muskrat, hedgehog) or the number of animals in positive samples was high (mallard, eurasian wigeon).
To estimate the load, the average concentration of (oo)cysts in faecal pellets was calculated from the concentration of (oo)cysts in the positive samples and the fraction of the birds that contributed to the positive samples (Table 1).

The monoclonal antibodies that were used in this study were not specific to *C. parvum* and *Giardia intestinalis.* Hence, the detected (oo)cysts were regarded as *Cryptosporidium* spp. and *Giardia* spp. The same or similar antibodies are used for monitoring the presence of *Cryptosporidium* and *Giardia* in water throughout the world. So, none of these surveys specifically indicate the presence of (oo)cysts that are infectious to humans, but only their potential presence. Of particular interest was the observation that biological particles in several stool samples showed specific fluorescence but were too large ($\geq$ 20 μm) to be *Cryptosporidium* oocysts or *Giardia* cysts. Avian infections of *Eimeria*, a coccidian protozoon that is related to *Cryptosporidium* and produces oocysts in the observed size range, are common. Cross-reaction with the monoclonal antibodies was not tested. However, these and other observations (Rodgers *et al.,* 1995) show that the antibodies are not specific to *Cryptosporidium* and *Giardia* and more specific or confirmation methods are necessary.

The water in the reservoirs is contaminated with *Cryptosporidium* oocysts and *Giardia* cysts (Medema *et al.,* 1998[b]). The source of (oo)cysts may be the (oo)cysts that are present in the river water at the inlet (Medema *et al.,* 1998[b]) or in the population of waterfowl and other animals. To determine if the shedding of (oo)cysts by animals living on or around the posterior Biesbosch reservoir (Petrusplaat) may present a significant contribution to the (oo)cyst concentration in reservoir water, the daily (oo)cyst load from wildlife was estimated and compared to the concentration of *Cryptosporidium* and *Giardia* found in the reservoir water (Medema *et al*, 1998[b]). The daily (oo)cyst load from wildlife was calculated from:
1. the average number of animals on and around the reservoir
2. the average weight of a pellet of faeces from one animal,
3. the estimated number of faecal pellets shed daily per animal and

4. the percentage of the faecal pellets that would end up in reservoir water. For waterfowl and mammals that could find their food in or around the reservoir, and were present in/on the water for the main part of the day, this was estimated to be 50-100%. For waterfowl that used the reservoirs only as a resting place this was estimated to be 40-80% and for birds and mammals that were infrequent visitors of the banks of the reservoir this was estimated to be < 1%.
5. the average concentration of (oo)cysts in the faecal pellets.

Assuming a constant load from animal faeces and a perfectly mixed reservoir, the concentration of (oo)cysts in the reservoir water and in water leaving the reservoir can be described as function of load and time:

$C_r = L/V_s * (1 - e^{-t/\tau})$

In which:
C_r = the concentration of (oo)cysts in reservoir water ((oo)cysts per litre)
L = the daily load of (oo)cysts from animal faeces ((oo)cysts/day)
V_s = the volume of water supplied daily to the reservoir (4.55×10^8 litres)
t = time (days)
τ = retention time of water in the reservoir (28.6 days)

If the time that the contamination from animal droppings occurs is larger than the water retention time, the concentration of (oo)cysts in the reservoir approaches L/V_s.
The bird counts showed that the birds that shed cysts or oocysts all stayed on the reservoir for a minimum of three months. Therefore, the expected concentrations of (oo)cysts in reservoir water were calculated by division of the cyst or oocyst load by the water volume that was supplied into the reservoir daily (Table 2).
The average concentrations that were measured in the reservoirs in 1994 (corrected for the median recovery efficiency of the detection method) were 0.049 oocysts per litre and 0.064 cysts per litre (Medema *et al.,* 1998[b]). These concentrations were much (> 100-fold) higher than the maximum estimate for many animals (Table 2). However, the estimated *Cryptosporidium* concentration by contamination from the gadwall and the estimated *Giardia* concentration from contamination by the mallard and eurasian wigeon indicate that these birds can significantly contribute to the (oo)cyst concentration in reservoir water. The maximum estimate of several other animals indicate that also these may contribute to the contamination of reservoir water, although less prominently. These are the great cormorant, tufted duck and cattle for *Cryptosporidium* and the gadwall for *Giardia*. The overall contribution of waterfowl to the contamination of reservoir water with *Cryptosporidium* is 1.0 to 16% (24% if also cattle is included) and 4 to 67% for *Giardia*.

For the calculations of the expected concentrations it was assumed that the faecal contamination by wildlife was homogeneously distributed over the total

Table 2. (Oo)cyst load from wildlife on the posterior reservoir (Petrusplaat)

Animal species	Average number of animals	Average weight of faecal pellet (gram)	Number of pellets per day	Percentage of pellet(s) entering reservoir water (%)	(oo)cysts per gram	Estimated daily (oo)cyst load		Estimated concentration in reservoir water		Contribution to concentration in reservoir water (%)	
						Minimum	Maximum	Minimum	Maximum	Minimum	Maximum
Cryptosporidium											
Common Shelduck	10	8.0	*5-50*	40 - 80	45.6	7.3×10^{3}	1.5×10^{5}	1.6×10^{-5}	3.2×10^{-4}	0.03	0.7
Gadwall	40	4.8	*5-50*	40 - 80	274	1.1×10^{5}	2.1×10^{6}	2.3×10^{-4}	4.6×10^{-3}	0.5	9.4
Eurasian Wigeon	234	3.3	*5-50*	40 - 80	0.36	5.6×10^{2}	1.1×10^{4}	1.2×10^{-6}	2.5×10^{-5}	0.002	0.05
Great Cormorant	55	28.9	*2-8*	50 - 100	64	1.0×10^{5}	8.1×10^{5}	2.2×10^{-4}	1.8×10^{-3}	0.5	3.7
Mute Swan	1.2	126	*2-8*	50 - 100	5.2	7.9×10^{2}	6.3×10^{3}	1.7×10^{-6}	1.4×10^{-5}	0	0.03
Black-headed Gull	68	5.0	*5-50*	20 - 60	8	5.4×10^{3}	1.1×10^{5}	1.2×10^{-5}	2.4×10^{-4}	0.02	0.5
Tufted Duck	411	4.8	*5-50*	50 - 100	2.9	1.4×10^{4}	2.9×10^{5}	3.1×10^{-5}	6.3×10^{-4}	0.06	1.3
Muskrat	*10*	30	*2-8*	*< 1*	2490	0	6.0×10^{4}	0	1.3×10^{-4}	0	0.3
Rabbit	*50*	1.3	*15-60*	*< 1*	1.2	0	4.7×10^{1}	0	1.0×10^{-7}	0	0
Hedgehog	*10*	1.8	*2-8*	*< 1*	38500	0	5.5×10^{4}	0	1.2×10^{-4}	0	0.2
Cow	*10*	797	*2-8*	*< 1*	2870	0	1.8×10^{6}	0	4.0×10^{-3}	0	8.2
Giardia											
Mallard	107	7.1	*5-50*	40 - 80	444	6.7×10^{5}	1.3×10^{7}	1.5×10^{-3}	3.0×10^{-2}	2.3	46
Gadwall	40	4.8	*5-50*	40 - 80	19	7.3×10^{3}	1.5×10^{5}	1.6×10^{-5}	3.2×10^{-4}	0.03	0.5
Eurasian Wigeon	234	3.3	*5-50*	40 - 80	184	2.8×10^{5}	5.7×10^{6}	6.2×10^{-4}	1.2×10^{-2}	1.0	20
Mute Swan	1.2	126	*2-8*	50 - 100	26	3.9×10^{3}	3.1×10^{4}	8.6×10^{-6}	6.4×10^{-5}	0.01	0.1
Muskrat	*10*	*30*	*2-8*	*< 1*	360	0	8.6×10^{3}	0	1.9×10^{-5}	0	0.03

Regular script: data based on observations, *Italic script: data based on assumptions*

water volume of the reservoir. Although this was useful to determine the relative significance of wildlife as a source of (oo)cysts, the distribution of faeces will be far from homogeneous. Animals that sheds (oo)cysts into the reservoir water close to the reservoir outlet may give rise to a very short peak in the concentration of (oo)cysts in the water leaving the reservoirs to the treatment system. In some months the number of birds on the reservoir is 2-5 fold higher than the annual average number that was used to calculate the average load. In these months, the load and thereby the contribution may be higher.

In conclusion, this quantitative approach indicates that waterfowl is not only a potential (Erlandsen *et al.,* 1991[a], Grazcyk *et al.,* 1998) but also a potentially significant source of surface water contamination with oocysts of *Cryptosporidium* spp. and cysts of *Giardia* spp.

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

Influence of temperature and autochthonous micro-organisms

With Mahdieh Bahar and Ciska Schets

ABSTRACT

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

This research has been carried out on behalf of the General Directorate for the Environment (DGM).

This chapter has been published as: Medema, G.J., Bahar, M., Schets, F.M. (1997). Survival of *Cryptosporidium parvum*, *Escherichia coli,* faecal enterococci and *Clostridium perfringens* in river water. Influence of temperature and autoch-thonous micro-organisms. *Water, Science and Technology*, 35(11):249-252.

INTRODUCTION

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

METHODS

Micro-organisms

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

Microcosms of autoclaved river water

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

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

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

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

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

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

RESULTS & DISCUSSION

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

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

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

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

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

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

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

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

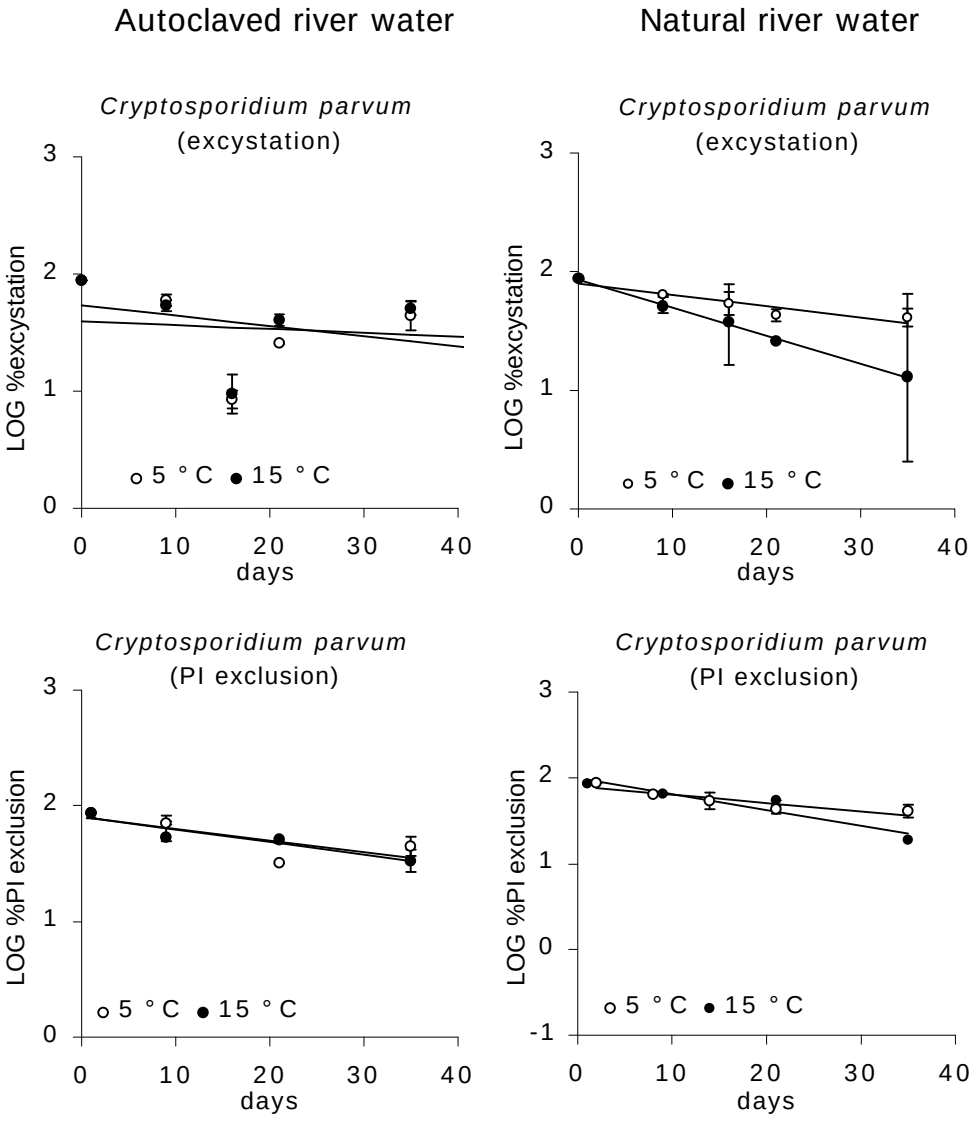

Figure 1. Survival of Cryptosporidium parvum oocysts in autoclaved and natural river water, as assessed with excystation and PI-exclusion.

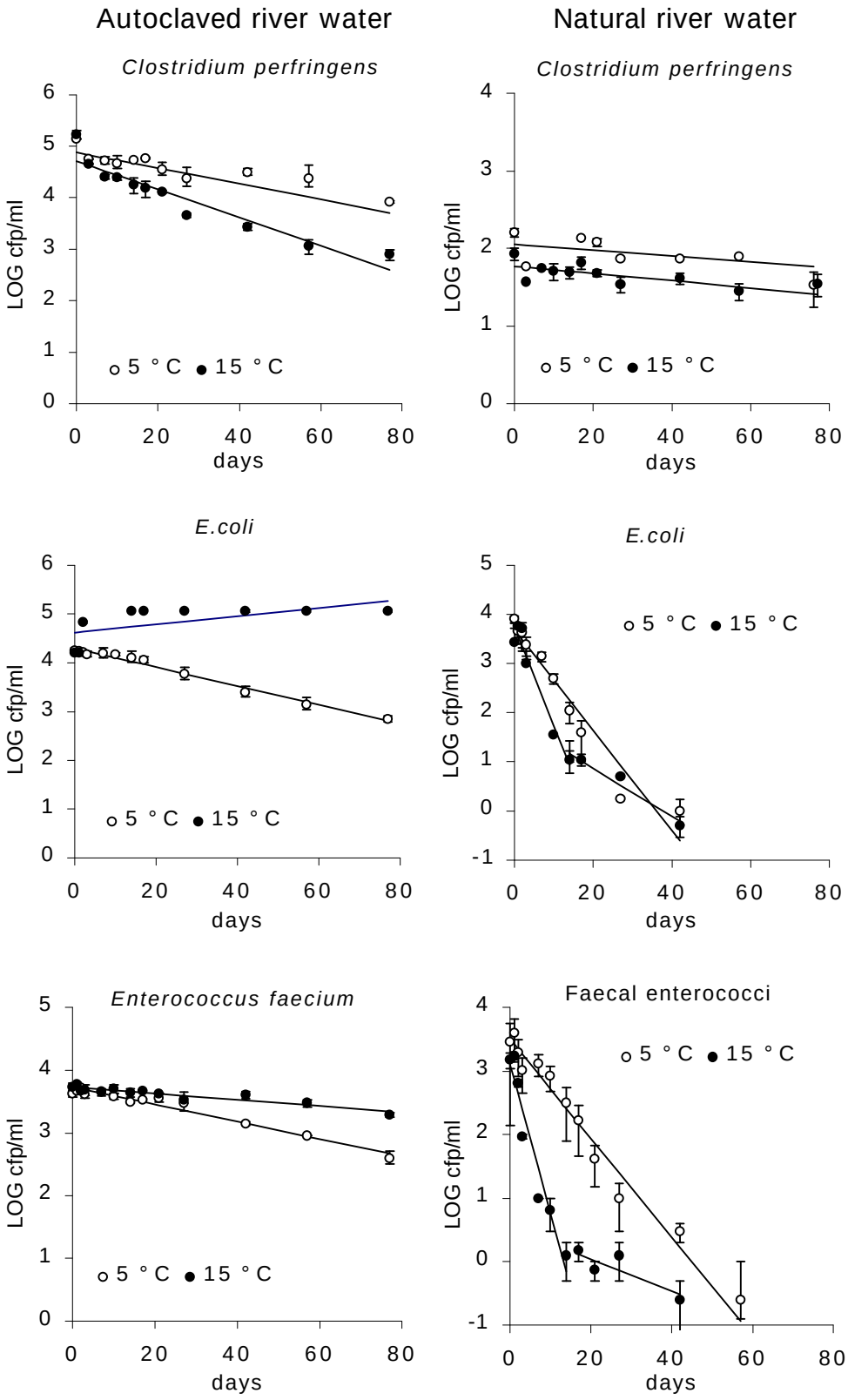

Figure 2. Survival of indicator bacteria in autoclaved and natural river water.

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

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

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

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

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Sedimentation of free and attached *Cryptosporidium* oocysts and *Giardia* cysts in water

With Ciska Schets, Peter Teunis and Arie Havelaar

ABSTRACT

Experimental analysis of the sedimentation velocity of *Cryptosporidium parvum* oocysts and *Giardia lamblia* cysts was compared with mathematical description of their sedimentation velocities by using measurements of (oo)cyst size and density and the density and viscosity of the sedimentation medium to determine if the sedimentation kinetics of freely suspended oocysts of *C. parvum* and cysts of *G. lamblia* can be described by Stokes' law. The theoretically calculated sedimentation kinetics showed a good agreement with the experimentally observed kinetics. Both showed a decline in sedimentation velocity over time, caused primarily by variation in (oo)cyst density. The initial apparent sedimentation velocities in Hanks balanced salt solution at 23°C was 0.35 $\mu m.s^{-1}$ for oocysts and 1.4 $\mu m.s^{-1}$ for cysts.
(Oo)cysts that enter the surface water environment by discharges of biologically treated sewage may be attached to sewage particles, and this will affect their sedimentation kinetics. Therefore, (oo)cysts were mixed with settled secondary effluent. (Oo)cysts readily attached to the (biological) particles in effluent; 30% of both cysts and oocysts attached during the first minutes of mixing and this fraction increased to approximately 75% after 24 h. The sedimentation velocity of (oo)cysts attached to secondary effluent particles increased with particle size and was, already in the smallest size fraction (1 to 40 μm) determined by the sedimentation kinetics of the effluent particles.
The observed sedimentation velocities of freely suspended (oo)cysts are probably too low to cause significant sedimentation in surface water or reservoirs. However, since a significant proportion of both cysts and oocysts attached readily to organic biological particles in secondary effluent, sedimentation of attached (oo)cysts after discharge into surface water will probably be a significant factor in the environmental ecology of *C. parvum* and *G. lamblia.* Attachment to particles influences not only sedimentation of (oo)cysts in surface water but also their behaviour in drinking water treatment processes.

This work was funded by the Ministry of Housing, Physical planning and the Environment.
The authors are grateful for the supply of purified *C. parvum* oocysts by dr. J. Peeters and dr. D. de Graaf of the National Institute of Veterinary Research in Brussels, Belgium and for advice on fluid mechanics by L.C. Rietveld of the Department of Civil Engineering at the Technical University of Delft.

This paper has been published as: Medema, G.J., Schets, F.M., Teunis, P.F.M., Havelaar, A.H. (1998). Sedimentation of free and attached *Cryptosporidium* oocysts and *Giardia* cysts in water. *Applied and Environmental Microbiology,* 64(11):4460-4466.

INTRODUCTION

The protozoan parasites *Cryptosporidium* and *Giardia* have been implicated as the causative agents of many outbreaks of waterborne intestinal illness (2,11). Water appears to be an important vehicle for the transmission of these parasites, along with direct contact with infected persons or animals. Both parasites produce a robust (oo)cyst to be able to endure environmental stress and to make the probability of reaching a new, susceptible host as high as possible. The (oo)cysts are shed by infected persons or animals and enter surface water through direct faecal input, discharge of treated and untreated sewage, and run-off from agricultural lands. Transport of infectious (oo)cysts from the source of surface water contamination to areas where exposure of potentially new hosts to surface water occurs, such as bathing areas, abstraction points for drinking water production, and drenching areas for livestock or wildlife, is governed by several hydrodynamical, chemical and biological factors, i.e.: water flow, attachment of freely suspended (oo)cysts to particles, sedimentation and resuspension of free and attached (oo)cysts and survival of (oo)cysts.

Water flow is the driving force for the transport of the (oo)cysts. Free (oo)cysts will probably behave as particles that are transported by advection. It is likely that a part of the (oo)cysts is attached to larger particles and that hydrodynamic behavior will be influenced by the characteristics of these particles.

(Oo)cysts can survive for months in surface water. The inactivation rate depends on the presence of an autochthonous microflora (predation or structural or metabolic injury by exo-enzymes), temperature and sunlight-intensity (1a,8,10).

In stagnant waters, (oo)cysts will slowly settle to the sediment. This may occur in natural environments in which the water flow is low (lakes and slow-flowing streams and rivers in summer) and in man-made environments such as reservoirs for drinking water production (5), settling tanks in sewage treatment or drinking water treatment and in sewers. Sedimentation of particles in water follows Stokes' law which implies that sedimentation velocity depends on particle size, difference in density between particle and water and the viscosity of water. Theoretical calculations by Ives (4) indicate that the sedimentation velocities of single *Cryptosporidium* oocysts and *Giardia* cysts in stagnant water are low: 0.5 and 5.5 $\mu m.s^{-1}$ respectively. In natural waters, the (oo)cysts may occur in a freely suspended form, but, as particle concentrations in the waste water and surface water environments are high, a proportion of the (oo)cysts in surface water will probably be attached to particles such as clay, sand, plankton, algae and (bio)flocs. The sedimentation velocity of attached (oo)cysts will be affected by the characteristics of the particle (size, density). The sampling techniques (filtration, flocculation) used in previous studies of the presence of *Cryptosporidium* and *Giardia* in water (6,9,12,13) have not allowed discrimination between free and attached (oo)cysts, therefore no data on the occurrence of attached (oo)cysts exist. Since (oo)cysts have a high survivability, settling of (oo)cysts may result in accumulation of (oo)cysts in aquatic sediments. Disturbance of these sediments, for instance by bathers,

ships or increased water flow, may give rise to high concentration peaks in the water, yielding a relatively high risk of exposure of bathers or breakthrough through drinking water treatment systems.
This paper describes the experimental analysis of the sedimentation velocity of free *Cryptosporidium* oocysts and *Giardia* cysts and the mathematics of the sedimentation kinetics by Stokes' law, as well as the experimental analysis of the kinetics of attachment of (oo)cysts to particles in the effluent of a biological wastewater treatment plant and the sedimentation velocities of (oo)cysts attached to these particles. Wastewater treatment effluent was selected to mimic natural conditions after discharge of treated sewage, since this is one of the major sources of contamination of surface water with *Cryptosporidium* and *Giardia* and contains a high level of mainly biological particles. A proportion of the (oo)cysts entering surface water by this route may be expected to be attached to such particles.

MATERIAL AND METHODS

Protozoa

A bovine strain of *C. parvum* was obtained from naturally infected calves. Oocysts were purified by cesium density centrifugation and stored in 5% potassium dichromate at 2-8°C. Oocysts of a single batch were used at an age of 2-8 months. *Giardia lamblia* cysts were obtained from PRL Dynagenics (Phoenix, Arizona, USA). This was a human isolate (CH3) that was maintained in gerbils. Cysts were purified from a faecal pellet suspension by zinc sulphate flotation at a specific density of 1.09 and subsequent centrifugation. Cysts were recovered by membrane filtration, fixed in formalin, and washed and stored in distilled water.

Internal characteristics

The presence of internal characteristics in cysts and oocysts were determined by differential interference contrast (DIC) microscopy at the onset of the experiments. Totals of 300 cysts and 300 oocysts were assayed. 298 of 300 (99.3%) of the *C. parvum* oocysts showed internal contents. Also 298 of 300 (99.3%) of the *G. lamblia* cysts showed internal contents and 78% had a viable type morphology by the criteria of Sauch (12).

Preparation of suspensions of free (oo)cysts

Sedimentation experiments were performed with suspensions of 1000-2000 (oo)cysts of both parasites in 10 ml of Hanks' balanced salt solution (Gibco BRL, Life Technologies, model no. 24020-091), which was filtered through 0.2 μm pore size filters to remove interfering particles ($HBSS_f$).
The more homogeneous the distribution of (oo)cysts in the suspension prior to sedimentation, the more accurate the determination of the sedimentation kinetics. The homogeneity of the (oo)cyst suspensions was tested by taking 10 1-ml aliquots of the 10 ml suspension. These aliquots were filtered through 25 mm diameter polycarbonate filters (Millipore RTTP 02500) with a pore size of

1.2 μm. The filters were covered with a mixture of monoclonal antibody-FITC conjugates specific for *Cryptosporidium* (Detect-IF, Shield diagnostics, Dundee, UK) and for *Giardia* (Giardia-Cel, Cellabs, Brookvale, Australia) and incubated for 30 to 45 min at 37°C. Monoclonal antibodies were prefiltered through 0.2 μm pore size filters to remove interfering particles. After staining, the filters were washed with $HBSS_f$ and embedded in Dabco (1,4-diazabicyclo [2,2,2]-octane)-glycerol mounting medium (adjusted to pH 7 with 1 M HCl). Filters were screened for (oo)cysts of *Cryptosporidium* and *Giardia* with a Zeiss Axioskop epifluorescence microscope fitted with x25 and x100 Plan Neofluar objectives, with FITC (filter set 09) dichroic mirror and filters.

Preparation of suspensions of (oo)cysts attached to particles

To test the sedimentation characteristics of attached (oo)cysts, the protozoa were allowed to attach to particles in secondary effluent (effluent of the sedimentation basin after activated sludge treatment) of the biological domestic wastewater treatment plant of Utrecht. Effluent characteristics were as follows: turbidity 2.4 NTU; pH 7.4; dissolved organic carbon 6.4 mg of C/liter; and specific density 1.001 g/ml at 20.5°C. The majority (> 99%) of the particles in the secondary effluent sample were < 10 μm; only a small fraction of the particles was larger, as large as > 400 μm. The larger particles were open, floc-like structures that consisted mainly of micro-organisms.

(Oo)cysts were added to effluent to a concentration of 3000-6000 per 10 ml and were allowed to attach for 24 h at 15°C on a rotary shaker table (experiment 1) or for 96 h at 15°C without agitation (attachment during/after settling), after which the settled effluent was aspirated and the pellet was supplemented with unseeded effluent up to the initial volume (experiment 2).

In the sedimentation experiments, the particles with attached (oo)cysts were divided into different size categories: 1 to 40 μm; 40 to 80 μm; 80 to 120 μm; 120 to 200 μm and > 200 μm, as determined with an ocular micrometer. Depending on the concentration of the particles, a certain proportion of free (oo)cysts would be filtered onto the same area of the membrane surface as a particle, and would be hidden or would be apparently attached to the particle. The proportion of apparently attached (oo)cysts can be estimated by determining the surface area of the membrane that is covered with particles. In the worst case (all particles from 10 ml of effluent appear on the membrane), only 7.6% of the membrane was covered with particles. Therefore, no attempts were made to correct for apparent attachment.

Sedimentation experiments

Sedimentation experiments were performed with 10 ml volumes in glass tubes of 10 by 1.5 cm. The height of the sedimentation column was 65 to 67 mm and the diameter of the column was 14.0 ± 0.06 mm. Hindered settling (reduction of the sedimentation velocity by the upward flow of displaced fluid) does not significantly (< 5%) influence the sedimentation velocity of particles smaller than approximately 1/45 of the column diameter (3). Hence, sedimentation of particles up to approximately 300 μm can be determined in these columns. The volumetric fraction of the suspended (oo)cysts and of the effluent particles was

(much) smaller than 0.003, which was too low to cause a significant reduction in sedimentation velocity.
The columns were placed in a temperature-controlled room at 23°C on a vibration-free surface. At time zero, 10 1-ml samples were taken from the first column to determine the initial (oo)cyst concentration and distribution. The sedimentation suspensions were allowed to settle and after predetermined time intervals, 9 ml samples were taken from subsequent columns. These samples were taken with a 1 ml pipette with an internal diameter of 3.15 ± 0.04 mm that was attached to the tube of a stereomicroscope-stand. With the focusing screws of the microscope stand, the pipette tip was lowered very gently until just below the meniscus of the sedimentation solution. The solution was pumped from the column by a peristaltic pump (101 U/R, Watson Marlow, Falmouth, United Kingdom) at a rate of 1 to 1.5 ml.min^{-1}. During sampling, the pipette tip was lowered with the focus-screw to follow the descending meniscus. After 9 ml was sampled, the pipette tip was taken out of the column and the sample was pumped at maximum speed through the tubing into a collector tube. The sample was filtered, stained, and examined as described above.

Theoretical sedimentation velocity
The sedimentation velocity of particles in liquids follows Stokes' law, which is described by the following equation:

$$V_s = g/18 \times d^2 \times (r_p - r_l) \times h^{-1} \qquad (1)$$

where:
V_s = sedimentation velocity (m s^{-1})
g = gravitational acceleration (9.81 m s^{-2})
d = particle diameter (m)
r_p = specific density of particle (kg m^{-3})
r_l = specific density of liquid (kg m^{-3})
h = dynamic viscosity of liquid (kg m^{-1} s^{-1})

By measuring particle size and density and the density and viscosity of the liquid, the theoretical sedimentation velocity can be calculated and compared with the experimentally determined sedimentation velocity.

(Oo)cyst size
The size distribution of the protozoan (oo)cysts was determined by measuring the lengths and widths of 100 (oo)cysts at a magnification of x1,000 with a calibrated micrometer.

(Oo)cyst density
The densities of *Cryptosporidium* oocysts and *Giardia* cysts were determined by density gradient centrifugation in Percoll-0.15 M NaCl. The gradient was made by layering Percoll-0.15 M NaCl solutions of decreasing specific density (1.100, 1.090, and 1.080 to 1.010 g.ml^{-1}). The gradients were loaded with 100 µl of

oocyst or cyst suspension in $HBSS_f$ and with dextran Density Marker Beads (Pharmacia DMB 1-7) prepared in 0.15 M NaCl according to the manufacturers instructions (30 µl of each density). The gradient was centrifuged at 390xg for 15 min and allowed to come to a standstill without braking. After centrifugation, marked bands of the density marker beads were observed, but the (oo)cysts did not show up as a visible band. Therefore, 0.5 ml aliquots were gently taken from the gradient, by the same procedure used to take the upper 9 ml sample in the sedimentation experiments. The beads and oocysts in these aliquots were pelleted at 13,000 x g for 30 seconds and wet mounts of 10 µl of these pellets were made. The number of oocysts or cysts was counted, and the presence of density marker beads was recorded.

Liquid density
The specific densities of $HBSS_f$ and secondary effluent were measured at 23°C with a calibrated densitometer in the range of 1,000-1,100 $kg.m^{-3}$.

Viscosity
The kinematic viscosities of $HBSS_f$ and of the secondary effluent were determined with a calibrated BS/U-tube viscometer for a viscosity range of 0.3-3 mm^2s^{-1} according to ISO 3104 (1). The viscosity measurements were performed at 23°C.
The dynamic viscosity η was calculated from the kinematic viscosity ν by:

$$h = n \times r \qquad (2)$$

where:
η = dynamic viscosity (kg m^{-1} s^{-1})
ρ = density of the medium (kg m^{-3})
ν = kinematic viscosity (m^2s^{-1})

RESULTS

Homogeneity of suspensions of free (oo)cysts

In the first experiment, the sedimentation suspension was prepared by mixing a small volume (10 to 120 µl) of cyst and oocyst stock suspension with a large volume (100 to 150 ml) of $HBSS_f$ by gentle shaking (20x) followed by 15 min of sonification to break any (oo)cyst clumps. 10 ml volumes were pipetted into the sedimentation tubes. To determine the homogeneity of the sedimentation suspension, (oo)cysts concentrations of ten 1-ml samples of the 10 ml were counted. A random distribution of the relatively small number of low (oo)cysts would result in Poisson distributed counts, where the variance σ^2 in the counts equals the mean m ($\sigma^2/\mu = 1$). At 9 df and $\alpha = 0.05$, the counts can be regarded as homogeneously distributed when $\sigma^2/\mu < 1.9$. The experimental σ^2/μ was 18.4 to 20.3. Hence, this mixing protocol did not result in a homogeneous distribution of (oo)cysts.

A different mixing procedure was used in the second experiment: (oo)cyst suspensions were made by mixing $HBSS_f$ with the stock (oo)cyst suspension on a 1:1 volume basis, and by consecutive mixing (1:1) with $HBSS_f$ until the required volume was reached. The resulting σ^2/μ values of the sedimentation suspensions in the second experiment were 2.8 for *Giardia* and 2.5 for *Cryptosporidium.*
The fraction of the (oo)cysts in the sedimentation suspensions that were clumped was < 1%.

Sedimentation experiments with free (oo)cysts
The results of the sedimentation experiments are plotted in Fig. 1A and B. Experiments 1 (Fig. 1A) and 2 (Fig. 1B) were duplicate experiments, that differed only in the homogeneity of the (oo)cyst suspension (see above). The higher degree of homogeneity in experiment 2 appeared to yield less variation in the *C. parvum* counts, especially in the first ten hours of the sedimentation experiment.
of their size difference, higher for *Giardia* cysts than the rate for *Cryptosporidium*

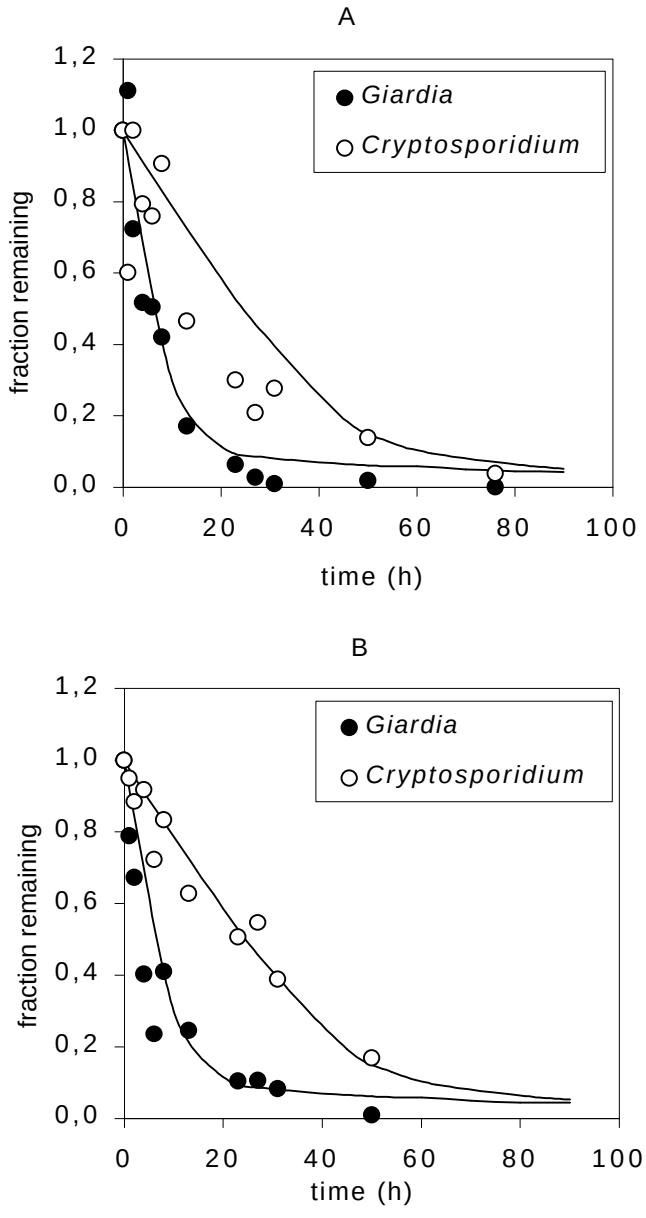


Figure 1.
DC/Dt plot of the duplicate sedimentation experiments A (A) and B (B) with C. parvum oocysts (O) and G. lamblia cysts (●).
The concentration at time t is calculated as fraction of the initial concentration (= C_t/C_0). The lines represent the Monte Carlo simulation of sedimentation of (oo)cysts according to Stokes' Law.

See text for details.

oocysts. The rate at which the concentration of (oo)cysts decreases is a function of the sedimentation velocity of the (oo)cysts. This rate was, as expected on the basis For both protozoa, the rate at which the (oo)cysts settled appeared to decline over time, indicating that a proportion of the (oo)cysts settled relatively slowly

Theoretical sedimentation velocity

To compare the observed decline in (oo)cyst concentration with the decline that was expected from Stokes' equation, the values of the Stokes parameters and their variations were determined for the conditions used in the experiments.

(Oo)cyst size.

C. parvum oocysts were spherical with a mean diameter of 4.9 μm. The distribution of the oocyst sizes (Fig. 2) was fitted to a lognormal distribution. The parameters of the fitted distribution were $\mu^ = 1.5932$ and $\sigma^ = 0.06276$. *G. lamblia* cysts were elliptical, both the mean largest (length = 12.2 μm) and mean smallest diameter (width = 9.3 μm) are listed in Table 1, together with the mean eccentricity (ratio between length and width) of the cysts: 1.3. The eccentricity was correlated to the length of the cysts: longer cysts were thinner and shorter cysts were rounder (Fig. 3). To allow for this correlation, cyst lengths and widths were fitted with a bivariate normal distribution. The parameters that describe this distribution are the location vector [$l^$ = 12.2 μm; $w^$ = 9.3 μm] and the covariance matrix [cov(w,w) = 1.185; cov(w,l) = cov(l,w) = -0.307; cov(l,l) = 0.590], where l = length, w = width and cov = covariance.

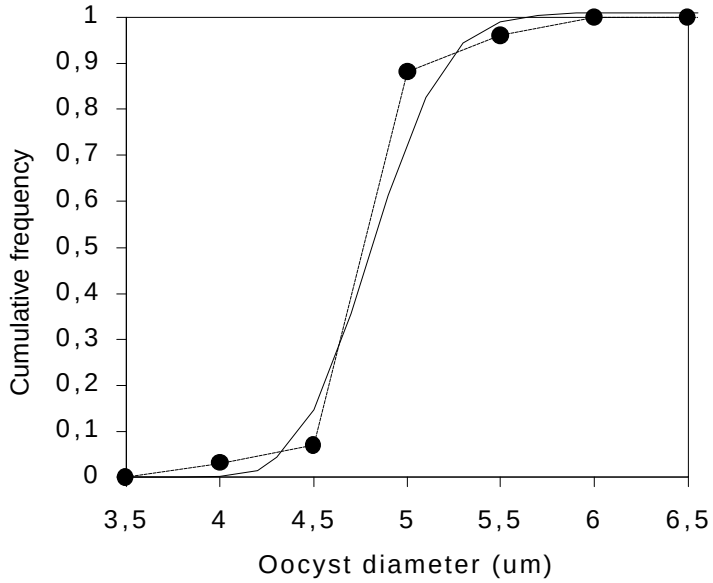


Figure 2. Cumulative frequency distribution of the diameter of the spherical C. parvum oocysts. The data-points and dashed line represent the experimental size measurements; the solid line is the best fitting lognormal probability density function.

(Oo)cyst density

The densities of *C. parvum* oocysts were determined by duplicate density gradient centrifugation experiments. The density of *G. lamblia* cysts was determined by the same procedure, but in separate experiments. Student's t test showed for both protozoa that the hypothesis that the duplicate data sets

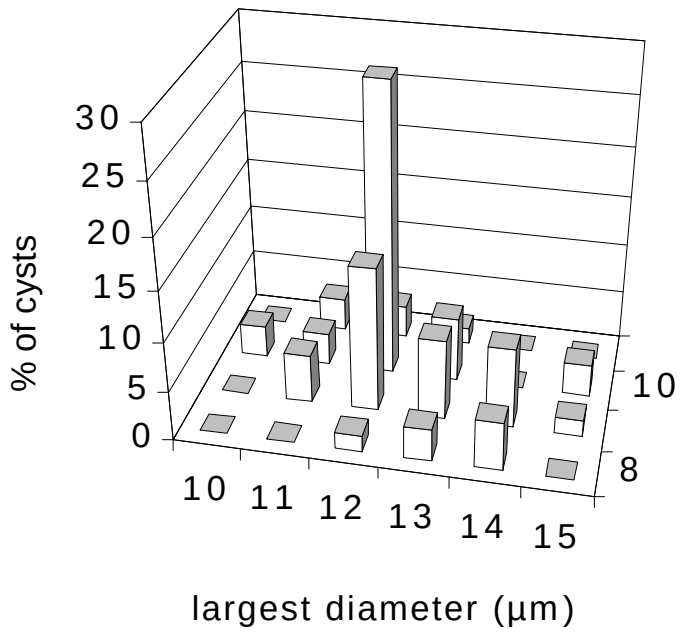


Figure 3. Distribution of the largest and smallest diameter of G. lamblia cysts.

originated from the same distribution was acceptable (data not shown). The duplicate data sets were therefore pooled. The distribution of densities of the cysts and oocysts are shown in figure 4A to B. Since the distribution appeared slightly skewed, a lognormal distribution was fitted to the data: for *C. parvum* oocysts the maximum likelihood values for the distribution parameters were $\mu\hat{} = 6.952$ and $\sigma\hat{} = 0.01624$, and for *G. lamblia* cysts these values were $\mu\hat{} = 6.943$ and $\sigma\hat{} = 0.01599$. The geometric mean density of oocysts was 1,045.4 $kg.m^{-3}$, for cysts the geometric mean density was 1,036.2 $kg.m^{-3}$.

Table 1. Sizes of C. parvum oocysts and G. lamblia cysts

Parameter	Size (µm)		
	Mean (± standard deviation)	Minimum	Maximum
Cryptosporidium oocysts diam.	4.9 (0.3)	3.9	5.9
Giardia cysts			
Largest diam.	12.2 (1.1)	9.8	14.7

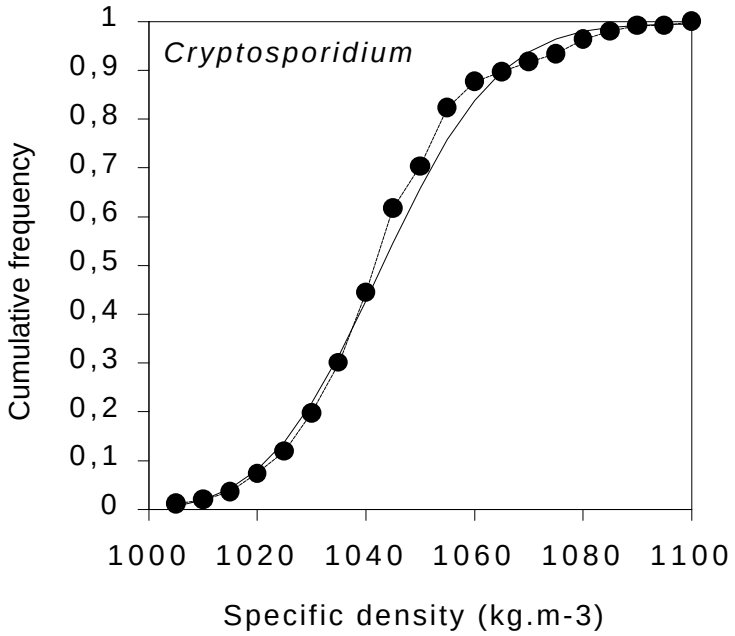
Smallest diam.	9.3 (0.8)	7.8	10.8
Eccentricity	1.3 (0.2)	1.0	1.8

Liquid density

The specific density of $HBSS_f$ at 22.5°C was 1,005 $kg.m^{-3}$.

Viscosity

Duplicate measurements of the kinematic viscosity gave a mean of 0.9505 $mm^2.s^{-1}$ (standard deviation 0.0006 $mm^2.s^{-1}$) for $HBSS_f$ at 23.3°C. The dynamic viscosity was calculated as 0.000955 Pa.s at 23.3°C. Corrected for the average temperature during the sedimentation experiments, the mean dynamic viscosity was 0.000944 Pa.s.



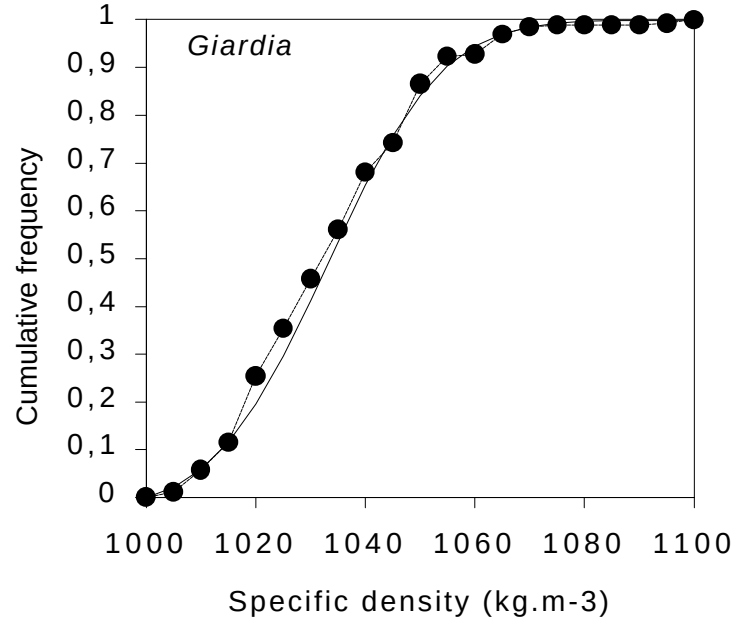


Figure 4. Cumulative frequency distribution of the density of C. parvum oocysts (A) and G. lamblia cysts (B). The data-points and dashed lines represent the experimental density measurements; the solid lines are the best fitting lognormal probability density functions.

Sedimentation velocity

To estimate the distribution in sedimentation velocity of cysts and oocysts on the basis of these parameter values, a Monte Carlo simulation of the sedimentation experiment was performed. In this simulation, a sedimentation column of 9 ml, with the same dimensions as those for the experimental column, was assumed to contain a homogeneously distributed suspension of cysts and oocysts, both in a concentration of 100 ml^{-1}. A total of 1,000 random samples were drawn from the lognormal density distribution and the lognormal or multinormal size distribution. For *Giardia*, the average of length and width was used in Stokes' equation, since Brownian motion will probably result in a randomization of the orientation of the cysts during settling. The resulting parameter values were entered in Stokes' equation, along with the mean values for the dynamic viscosity and density of $HBSS_f$. This resulted in a Stokes' velocity of 1,000 cysts and oocysts at each time interval. By giving each of the 1,000 (oo)cysts a random initial position in the sedimentation column (by random sampling from a uniform distribution for the height of the column), the fraction of (oo)cysts that remained in the upper 9 ml could be calculated for 96 time intervals of 1 h. The resulting decline of the (oo)cyst concentration is plotted in Fig. 1, together with the experimental data. The plots show that, as with the experimental sedimentation, the theoretically calculated sedimentation velocities decrease over time for both cysts and oocysts. The initial apparent sedimentation velocities were 0.35 $\mu m.s^{-1}$ for oocysts and 1.4 $\mu m.s^{-1}$ for cysts. Similar Monte Carlo simulations with the mean density (fixed) and random samples from the size distributions and with mean size (fixed) and random samples from the density distribution showed that the sedimentation kinetics were determined primarily by the variation in density for both parasites.

Attachment of (oo)cysts

To determine the kinetics of attachment, (oo)cysts were added to secondary effluent, mixed, and incubated at 15°C under continuous mixing. The percentage

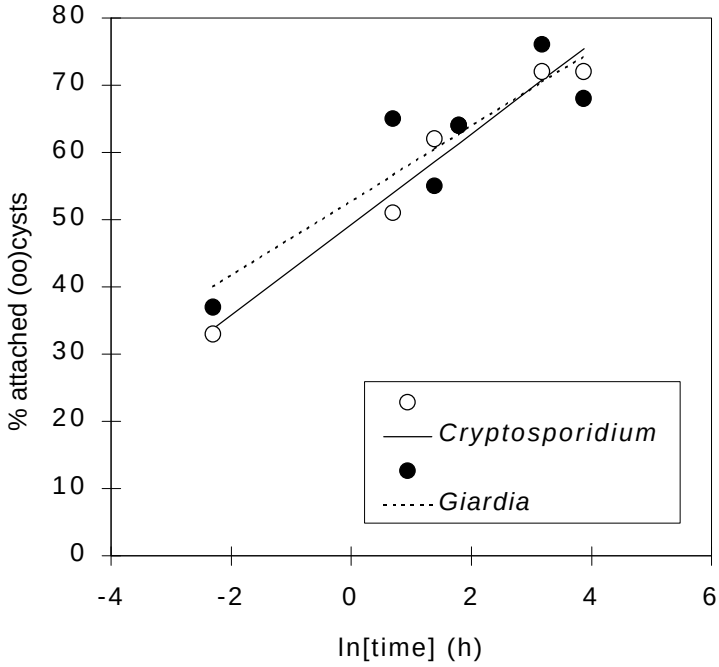


Figure 5. Attachment rate of Cryptosporidium oocysts and Giardia cysts to

particles from biologically treated sewage effluent.

of *C. parvum* oocysts and *G. lamblia* cysts that attached to the particles in secondary effluent was determined as a function of time. Attachment kinetics were assumed to follow an exponential relation; the percentage attachment was therefore plotted against the natural logarithm of the incubation time (Fig. 5). Although no (oo)cysts were attached before the suspensions were prepared, approximately one third of both cysts and oocysts was attached to particles from secondary effluent at the time the first sample was taken, which was immediately after mixing. Apparently, during the preparation of the suspension, the (oo)cysts attach readily to the particles. The percentage attachment appeared to reach a maximum after approximately 24 h; prolonged incubation (48 h) did not result in a higher percentage of attached (oo)cysts. This may be due to detachment of attached (oo)cysts caused by shear forces by the fluid movement.

Sedimentation experiments with attached (oo)cysts

Also in the sedimentation experiments with attached (oo)cysts, the homogeneity of the (overall; i.e. suspended and attached) counts was tested. The σ^2/μ-values of the counts of the ten 1-ml aliquots were 2.2 for oocysts and 6.4 for cysts (caused by 1 low count) in the first experiment and 3.3 for oocysts and 1.3 for cysts in the second experiment. The sedimentation velocity of free (oo)cysts and

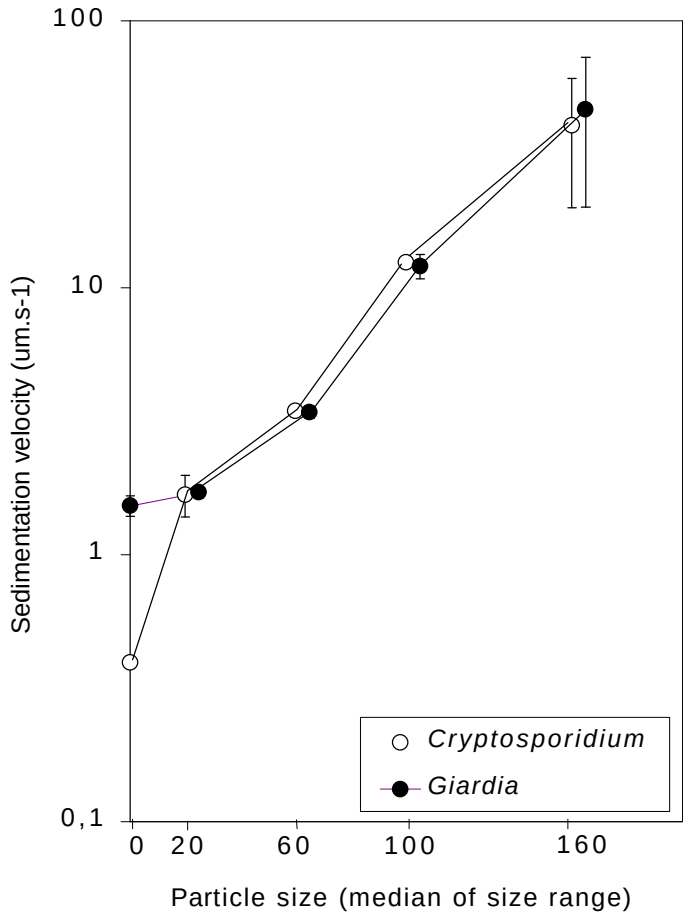


Figure 6. Sedimentation velocity of Cryptosporidium oocysts and Giardia cysts attached to particles in biologically treated sewage effluent of different size-ranges. Error bars represent the minimum and maximum sedimentation velocity in duplicate experiments.

(oo)cysts attached to particles in the different size categories was calculated by linear regression of the concentration decrease in the upper 9 ml of the sedimentation column over time. The linear area of the concentration-time curve was determined optically from a concentration-time plot. The observed sedimentation velocities of the free cysts and oocysts in this system were 1.5 $\mu m.s^{-1}$ and 0.40 $\mu m.s^{-1}$ respectively, which is comparable to the sedimentation velocity of free (oo)cysts in $HBSS_f$.
When (oo)cysts were attached to effluent particles, the sedimentation velocity increased with particle size (Fig. 6). Attached *C. parvum* oocysts and *G. lamblia* cysts had similar sedimentation velocities. The sedimentation velocity of the (oo)cysts appeared, already in the smallest size fraction (1 to 40 μm), to be determined by the sedimentation velocity of the particles.

DISCUSSION

The results of this study show that the sedimentation kinetics of free *G. lamblia* cysts and *C. parvum* oocysts can be adequately described by the size and density of the (oo)cysts and the density and viscosity of the sedimentation medium. The theoretical sedimentation kinetics, which were calculated from independent measurements of the (oo)cyst and medium characteristics as used in these experiments, provide an adequate fit with the sedimentation kinetics observed in the experiments. This fit was better for *C. parvum* when a more homogeneous initial oocyst suspension was used in the sedimentation experiments.
Both the experimental and the theoretical sedimentation kinetics show a decline in apparent sedimentation velocity over time. The density measurements showed that the (oo)cyst population in the sedimentation experiments consisted of cysts and oocysts with various densities. According to Stokes' law, (oo)cysts with a high density have a higher sedimentation velocity than that of (oo)cysts with a low density. At the onset of the sedimentation experiment, the observed sedimentation velocity would be the net result of the sedimentation of all (oo)cysts. As time progressed, the denser (oo)cysts settled and the observed sedimentation velocity was composed more and more by the sedimentation velocity of (oo)cysts with a relatively low density. The simulated experiments with (oo)cysts with a constant size or density indicated that the observed variation in the sedimentation velocity was determined primarily by the variation in density.
The variation in (oo)cyst density was not apparent from the (oo)cyst morphology as observed by DIC microscopy: 99.3% of both cysts and oocysts showed internal contents. Further differentiation in high- or low density (oo)cysts was not possible by DIC microscopy. A possible effect of experimental handling on (oo)cyst morphology was not tested. However, handling of (oo)cysts was mild and the observed sedimentation kinetics compared favourably with the calculated sedimentation kinetics using the

measured density distribution of the (oo)cysts used in the experiments. The similarity between the calculated and the measured sedimentation kinetics provides validation of both the sedimentation experiments and the measurements of the (oo)cyst size and density distribution.
In aquatic environments, variation in density is likely to occur due to variations in age (depletion of internal resources) and exposure to stressors. In our experiments, all (oo)cysts were all of the same age and had encountered similar conditions in the laboratory. Density variation may also affect the recovery efficiency of centrifugation and flotation protocols that are used in the methods to detect *C. parvum* and *G. lamblia* in water (6,9). The (oo)cysts in the low density range will probably be less efficiently recovered by centrifugation techniques, while the denser (oo)cysts are less efficiently recovered by flotation techniques. Since the flotation medium (Percoll-sucrose) is used at a density of 1,090-1,100 $kg.m^{-3}$, this will probably not result in significant losses of (oo)cysts, since more than 95% of the (oo)cysts have a density below 1,090 $kg.m^{-3}$ (Fig. 4). However, LeChevallier *et al.* (7) showed that increasing the Percoll-sucrose density to 1,150 $kg.m^{-3}$ improved the recovery efficiency.
Mixing of small volumes with large volumes is common practice in the preparation of stock suspensions of (oo)cysts for experiments to determine recovery efficiency of detection methods. As we have shown, this protocol does not result in homogeneous suspensions and may give rise to a high degree of variation in the (oo)cyst concentration measurements, as observed in the initial hours of the first sedimentation experiment (Fig. 1). The mixing protocol using consecutive 1:1 dilutions until the required volume is obtained consistently gave a homogeneity that was close to random distribution, and less variation between counts was observed in the second sedimentation experiment (Fig. 1). This latter protocol is therefore to be preferred for the preparation of (oo)cyst suspensions. Although the obtained distribution in our experiments was not completely homogeneous, the variation in counts was small enough to allow determination of the sedimentation velocities (Fig. 1). These observed sedimentation kinetics are validated by the calculated sedimentation velocities, using independently obtained size, density and viscosity data of (oo)cysts and sedimentation liquid.
The initial apparent sedimentation velocity in HBSS at 23°C was 0.35 $\mu m.s^{-1}$ for oocysts and 1.4 $\mu m.s^{-1}$ for cysts. These are lower than the sedimentation velocities that were calculated by Ives (4) in water of 20°C using Stokes' law and point estimates of (oo)cyst size and density. The estimated density of cysts and oocysts (1,050 $kg.m^{-3}$) used by Ives were higher than the observed densities in this study. Also, the 'diameter' of *Giardia* cysts that Ives used (14 μm) is considerably larger than that observed in this study. Both result in higher calculated sedimentation velocities. The most important difference between the sedimentation velocities calculated by Ives (4) and those in this study is that our sedimentation velocities are not based on point estimates of (oo)cyst size and density but on the observed distribution of size and density in our (oo)cyst population. Since size and especially density showed a considerable variation, this latter approach results in more accurate estimates of the sedimentation velocity.

The observed sedimentation velocities are very low and will probably not result in significant sedimentation in natural aquatic habitats. Turbulence caused by water flow, wind, temperature and movement of aquatic organisms is more likely to influence the movements of (oo)cysts in water than gravitational settling.
Discharges from activated sludge treatment systems of sewage are an important source of surface water contamination with *Giardia* and *Cryptosporidium*. *Giardia* cysts and *Cryptosporidium* oocysts were shown to rapidly attach to particles from secondary effluent of a biological wastewater treatment plant. In the protocol used in this study, approximately 35% of both cysts and oocysts almost instantly attached and up to approximately 70% attachment was attained after 24 h. Attachment kinetics depend on the probability of particle collision, the probability of attachment of colliding particles and the firmness of the attachment. The attachment probability partly depends on the surface characteristics of cysts and oocysts. These may have been altered by the purification and storage procedures. The observed attachment kinetics are therefore valid only for the experimental conditions.
The sedimentation characteristics of (oo)cysts that were attached to effluent particles were, already in the smallest size fraction (1 to 40 μm), governed by the sedimentation characteristics of the particles. The particles were mainly bioflocs, with an irregular and open structure. The nature of the particles and the sample location (after secondary settling of activated sludge) imply that the density of these particles is relatively low. The density of the effluent particles can be deduced from Stokes' equation: assuming their density is not related to their size, the sedimentation velocity is a function of d^2. The function $V = c * d^2$, where d is the median of the size ranges, yielded an adequate fit ($r = 0.99$, by nonlinear regression by the Levenberg-Marquardt method) to the data of particles from the size ranges from 0 to 200 μm. From the constant c, the viscosity and the specific density of effluent, the apparent specific density of the effluent particles can be calculated to be 1,003 to 1,004 $kg.m^{-3}$, which was only slightly denser than the effluent (1,001 $kg.m^{-3}$). This also explains why, although attachment of *Giardia* cysts to the smallest size fraction resulted in a particle with more than double the diameter of free cysts, the sedimentation velocity increased from only 1.5 to 1.7 $\mu m.s^{-1}$.
A significant proportion of the (oo)cysts attached (rapidly) to biological particles in secondary effluent and the sedimentation kinetics of the attached (oo)cysts were determined by the sedimentation characteristics of the particles. The sedimentation velocity of free (oo)cysts is probably too low to cause significant sedimentation in natural aquatic habitats. These observations imply that attachment is an important factor in the environmental ecology of *Cryptosporidium* and *Giardia* that needs further research. Attachment to particles will influence not only sedimentation of (oo)cysts in surface water environments, but probably also survival of (oo)cysts and their removal during drinking water treatment by filtration, soil passage and disinfection processes.

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Frequency distributions of *Cryptosporidium* and *Giardia* in raw water and elimination capacity of water treatment

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ABSTRACT

Fluctuations in raw water quality and in treatment efficiency are important, since the risk of (oo)cyst breakthrough to drinking water is relatively high, both in the event of a high concentration in raw water or a low treatment efficiency, and especially high when both events coincide. The aim of this study was to incorporate the variation in raw water quality and treatment efficiency in a description of the concentrations of *Cryptosporidium* and *Giardia* in drinking water. The variation in raw water quality was determined by intensive monitoring of a surface water source for *Cryptosporidium* and *Giardia.* The variation in the efficiency of a treatment system was determined with data on removal of spores of sulphite-reducing clostridia by a full-scale treatment plant. This parameter was regarded as a surrogate for removal of *Cryptosporidium* and *Giardia.* Different statistical probability distributions were compared to be able to select the best method to describe raw water quality and treatment efficiency.
The negative binomial distribution provided a better fit to the protozoa concentration data in river water than the Poisson-with-added-zero's distribution. For stored water, with a high percentage of zero counts, the fit of both distributions was comparable.
The recovery efficiency of the detection method was regarded as a stochastic process and was described with the beta-binomial distribution.
The differences between the statistical methods that were used to describe the variation in treatment processes increased with increasing percentage of non-detects after treatment. For data-sets with approximately 50% or more zero counts after treatment, replacement of zero counts with the average was considered the best option for the description of treatment variation. This description renders the least underestimation of treatment efficiency, but is still conservative.
The variation in individual treatment processes was not independent, but correlated to the performance of previous processes. Therefore, an accurate description of the efficiency of micro-organism removal by a chain of treatment processes can only be made by accurate concentration measurements in raw and treated water. More sensitive methods are needed for enumerating spores of sulphite-reducing clostridia in large volumes of water to be able to assess the efficiency of the overall treatment process.

The research on the water quality of the river Meuse and the Biesbosch reservoirs was carried out on behalf of the General Directorate of the Environment and Water Storage Company Brabantse Biesbosch. The authors appreciate the efforts of the research technicians of Brabantse Biesbosch for the analysis of the water quality parameters.
The research on treatment performance was carried out as part of the Collective Research Programme of the water companies in the Netherlands. Data on the occurrence of spores of sulphite-reducing clostridia after successive treatment steps were kindly provided by the collaborating water companies and the research was supervised by the task group "Verwijdering van Micro-organismen".

INTRODUCTION

The protozoan parasites *Cryptosporidium parvum* and *Giardia lamblia* have emerged as significant waterborne pathogens over the past decades. Many outbreaks of waterborne cryptosporidiosis and giardiasis have been recorded (Craun, 1990; Craun *et al.,* 1998), and also endemic waterborne transmission is likely to occur. The resulting disease consists of a self-limiting diarrhoea that lasts for several days (Fayer *et al.,* 1997) in the majority of cases, but disease burden and mortality are high in the immunocompromised part of the infected population.
Several outbreaks of *Cryptosporidium* and *Giardia* related diarrhoea have been related to drinking water that was produced by treatment of surface water. These outbreaks have been the result of a peak contamination event in the raw water or treatment inadequacies (Badenoch, 1990; Richardson *et al.,* 1991; Craun, 1990; Dykes, 1980), or a combination of both (MacKenzie *et al.,* 1994). Surveys of surface water show that these parasites are ubiquitously present in the aquatic environment (Rose *et al.,* 1997), even in pristine environments. Hence, all surface water treatment systems have to deal with these protozoa. In several waterborne outbreaks, the coliforms, the parameter that was used to demonstrate the microbiological safety of drinking water, did not warn against parasite breakthrough through the treatment (Craun, 1990; Craun *et al.,* 1998), particularly because the coliforms were more efficiently eliminated by disinfection than both parasites. Therefore, a different approach was needed to demonstrate the safety of drinking water. The approach that has been taken is to determine the concentrations of *Cryptosporidium* and *Giardia* in raw water and to demonstrate the efficiency of the treatment system in eliminating these parasites (Badenoch, 1990; van der Kooij *et al.,* 1995; Teunis *et al.,* 1997; Hijnen *et al.,* 1997; LeChevallier *et al*, 1997; Karanis & Seitz*,* 1996). Most studies using this approach acknowledge the fact that the concentration of the parasites in raw water at a single site shows considerable variation, with maximum concentrations of up to tenfold the average concentration (Atherholt *et al.,* 1998; Stewart *et al.,* 1997). On the other hand, most studies regard the efficiency with which a treatment process eliminates micro-organisms as a constant. However, also the removal efficiency of a treatment process shows considerable variation (LeChevallier & Norton, 1992; van der Kooij *et al.,* 1995; Hijnen *et al.,* 1997), due to variations in many different process factors and fluctuations in feed water quality. It is important to consider both the variation in raw water quality and in treatment efficiency, since the risk of (oo)cyst breakthrough to drinking water is relatively high, both in the event of a high concentration in raw water or a low treatment efficiency, and especially high when both events coincide.
Hence, for treatment design and operation it is essential to know the probability that these events occur, or, in other words, to determine the probability distribution of the (oo)cyst concentration in raw water and the probability distribution of the removal efficiency of the treatment system.

The latter can also be used as a basis for studies to define the factors that determine treatment efficiency and thus optimisation of the treatment for protozoa removal.
The aim of this study was to incorporate the variation in raw water quality and treatment efficiency in a description of the concentrations of *Cryptosporidium* and *Giardia* in drinking water. The variation in raw water quality was determined by intensive monitoring of a surface water source for *Cryptosporidium* and *Giardia.* The variation in the efficiency of a treatment system was determined with data on removal of spores of sulphite-reducing clostridia by a full-scale treatment plant. This parameter was regarded as a surrogate for removal of *Cryptosporidium* and *Giardia.* Different statistical probability distributions were compared to be able to select the best method to describe raw water quality and treatment efficiency.

METHODS

Surface water treatment

Source water – The river Meuse is a large international river with a length of 900 km, that originates in France and flows through France, Belgium and the Netherlands to the North Sea. Its catchment area is 33.000 km^2 of mainly agricultural and urbanised land. Faecal pollution enters the river from run-off of the agricultural lands and the numerous discharges of treated and untreated sewage.
Treatment - Water from this river is fed into three successive reservoirs of 40 Mm^3, 33 Mm^3 and 13 Mm^3 and an average total residence time of five months. After quality improvement during reservoir storage (Oskam, 1995), this water is transported to several treatment plants in the southwestern part of the Netherlands. In one of these plants, water that is received from the reservoirs is filtered through microstrainers and is supplemented with ferric sulphate to induce floc formation. Flocs are produced in four compartments and are removed by lamella sedimentation. At low water temperatures, WISPRO is added as coagulant-aid. The water is subsequently treated with ozone with CT_{10} values of 1.1-1.2 $mg.min.l^{-1}$ followed by a dual media filtration with a filtration rate of 12-14 $m.h^{-1}$ and a GAC filtration with 10 min contact time. After addition of chlorine at a dose of 0.3-0.4 mg free chlorine/l, the water is distributed to the consumers. This plant is properly designed and operated and during the time of data collection, no extraordinary events occurred in raw water quality or treatment performance.

Water quality assessment

Sample sites – Samples were collected from the river Meuse at a sampling pier 8 km upstream of the inlet of the reservoirs from January to December 1994 at weekly intervals, except during May – September, when samples were collected at biweekly intervals. In the same period, samples were collected weekly at the outlet of the last reservoir.
At the treatment plant, samples were collected after microstraining, after lamella sedimentation, and after ozonation weekly for a period of three years.

Cryptosporidium and *Giardia* – Water volumes of 200 litre (river) or 2000 litre (reservoir) were filtered through wound polypropylene filters (Cuno Super Microwynd DPPPY) with a nominal porosity of 1 μm, at a flow rate of 3 litre.min^{-1}. Before sampling, sample equipment was flushed with at least 100 litres of sample water. After sampling, each filter was stored on ice, along with the water that remained in the housing after sampling, and was processed within 3 days. For processing, filters were cut longitudinally and the filter material was divided into an outer, middle and inner layer. The fibres of each layer were teased apart and washed for 15 min in deionized water containing 0.1% Tween 80 and 0.1% sodium dodecyl sulphate (SDS) in a Stomacher 3500 lab blender. The material that was washed of the fibres was concentrated in two subsequent centrifugation steps, at 900xg for *Giardia* and 1200xg for *Cryptosporidium*. *Cryptosporidium* oocysts and *Giardia* cysts were purified from the concentrate with sucrose flotation at a specific density of 1.100. Purified concentrates were distributed onto one or more polycarbonate membrane filters with a pore size of 1.2 μm (Poretics) and labelled with fluorescent monoclonal antibodies specific for *Cryptosporidium* (Shield Diagnostics, UK) and *Giardia* (Cellabs, Australia). The complete filter was monitored at 250x magnification with a Zeiss Axioskop, equipped with epifluorescence. Suspected fluorescent particles were confirmed at 1000x magnification, using the size, shape and fluorescence criteria for (oo)cysts.

Spores of sulphite reducing clostridia (SSRC) – Samples were pasteurised for 30 min. at 70°C and filtered through a 47 mm diameter membrane filter, pore size 0.45 μm. The filter was embedded in sulphite agar with cycloserin and incubated for 48 ± 4 hr at 37 ± 1°C and black colonies were counted.

Spores of Clostridium perfringens (SCP) – In the river Meuse and at the outlet of the reservoirs, samples were also analysed for the presence of spores of *Clostridium perfringens.* Samples were pasteurised for 30 min. at 60 °C and filtered through a 47 mm diameter membrane filter, pore size 0.45 μm. The filter was placed on mCP (Bisson & Cabelli, Armon & Payment) and incubated for 24 ± 4 hr at 45 ± 0.5°C. Yellow colonies were marked and all yellow colonies turning red after the filter was placed on a filterpad embedded in NH_4OH were counted.

Coliforms – Samples were filtered filtered through a 47 mm diameter membrane filter, pore size 0.45 μm. The filter was put onto Lauryl Sulphate agar and incubated for 18 ± 2 h at 37 ± 1°C for enumeration of total coliforms (COLI37) or at 44 ± 0.5°C for enumeration of thermotolerant coliforms (COLI44). At least five colonies from each sample were confirmed in brilliant green bile lactose broth at 37 or 44°C.

Faecal streptococci - Samples were filtered through a 47 mm diameter membrane filter, pore size 0.45 μm. The filter was put onto Kenner faecal agar and incubated for 48 ± 4 hr at 37 ± 1°C for enumeration of faecal streptococci (FSTREP).

Chlorophyll A – the chlorophyll A concentration was measured spectrophotometrically.

Physical and chemical parameters – turbidity, flow and temperature were measured with continuous monitors.

Statistical analysis of the data
Descriptive statistics were calculated with Microsoft Excel97. The product-moment correlation coefficient between variables was also calculated with Excel97; microbial counts were log-transformed to approach normality.

Distribution of micro-organisms
Two statistical distributions were fitted to the microbial concentration data. These were the Poisson distribution and the negative binomial distribution. Both distributions have been reported to provide an adequate fit to microbial concentration data in water (El-Shaarawi *et al.*, 1981, 1985, Pipes *et al.* 1977; Gale *et. al.*, 1997, Haas & Rose, 1996). For the Poisson distribution, the modification for added zero count (Poisson plus added zero's) of El-Shaarawi (1985) was used.
The Poisson distribution is described by:

$$P(k \mid l) = \frac{e^{-l}\, l^{k}}{k!} \qquad (1)$$

in which P(*k*) is the probability of *k* micro-organisms being present and l is the Poisson parameter that is both the mean and variance of the distribution.
For the added zero's this is modified to:

$$k > 0 : P(k \mid l, q) = (1-q)\frac{e^{-l}\, l^{k}}{k!} \qquad (2)$$
$$k = 0 : P(k \mid l, q) = q + (1-q)e^{-l}$$

The Poisson parameters l is now the mean of the non-zero counts ($k > 0$) and the added parameter q is the fraction of zero counts ($k = 0$).
The negative binomial distribution can be described with:

$$P(k \mid p, r) = \binom{h + r - 1}{r - 1} p^{r} (1-p)^{k} \qquad (3)$$

in which P(*k*) is again the probability that *k* micro-organisms are present, *p* is the binomial probability and *r* is the cluster parameter, indicating the level of clustering of micro-organisms.
The distributions were fitted to the data sets using a maximum likelihood procedure and the goodness-of-fit of these distributions to the data was compared.

Recovery efficiency
At regular intervals during the collection of samples for protozoa-analysis, additional samples were taken and seeded with *Cryptosporidium* oocysts and *Giardia* cysts during sample collection by injecting the seed suspension in the inlet tube of the filter housing. The seed suspension was counted using the filtermethod described above. The seeded sample was processed as a normal

sample, using separate materials to avoid cross-contamination. The count of the seed suspension and the count of the seeded sample were used, after correction for the (oo)cyst count in the unseeded sample, to calculate the ration of the numbers of (oo)cysts detected to the number of (oo)cysts seeded.
The recovery efficiency was described by the beta-binomial distribution (Teunis *et al.,* 1997), assuming that each of the seeded (oo)cysts has a certain probability *p* of being recovered, and that this probability is not constant but varies due to variations in many factors that affect the recovery efficiency. This variation is assumed to be beta-distributed.

The use of spores of sulphite-reducing clostridia to assess treatment efficiency
No data on the concentration of *Cryptosporidium* or *Giardia* were collected after any of the treatment processes, because the detection method was not sensitive enough to allow an accurate assessment of the protozoa concentrations at these sites. Instead, data were collected on the concentration of spores of sulphite-reducing clostridia after the individual treatment processes. These spores are, like (oo)cysts, very persistent in water and resistant to chemical disinfection. They are smaller than (oo)cysts and may therefore be conservative indicators for the removal of (oo)cysts by filtration. Little is known, however, of the comparability of the absorption of spores and (oo)cysts to surfaces. Several studies have suggested the use of these spores as a surrogate for (oo)cyst removal (Payment & Franco, 1993; Hijnen *et al.,* 1997).
Samples of 100-200ml were collected before coagulation/sedimentation, before ozonation, after ozonation and in treated water and analysed for the presence of spores of sulphite-reducing clostridia by the method described above. Samples were collected weekly for a period of three years.

Description of treatment efficiency
To describe the efficiency of treatment processes in eliminating micro-organisms, the concentration of micro-organisms after the treatment process is compared to the concentration before the process. The simplest and most widely used approach is to determine the average concentration of micro-organisms before and after the treatment process and to calculate the elimination capacity as the quotient of these two averages. The elimination capacity of a process is expressed as percentage elimination or, using the log-transformed average concentrations, as decimal elimination. The decimal elimination capacity (DEC) will be used in this paper (method A) and is defined by:

$$DEC = \log \overline{C}_{in} - \log \overline{C}_{out} = \log\left(\frac{\overline{C}_{in}}{\overline{C}_{out}}\right) \qquad (4)$$

The use of the average concentrations before and after the treatment process is a robust approach, in the sense that it can be used with most data-sets, regardless of the percentage of zero counts. However, a major drawback is that information on variation in the process efficiency, that can be substantial, is lost. Another approach that can be taken is, provided the concentration measurements before and after the process can be coupled, to calculate an

elimination capacity from all the individual concentration measurements before and after the process. This yields a series of decimal elimination capacities from which the average decimal elimination capacity and the standard deviation can be calculated.
In formula:

$$\overline{DEC} = \frac{\sum_{i=0}^{i=n} \log\left(\frac{C_{i,out}}{C_{i,in}}\right)}{n} \qquad (5)$$

in which n is the number of coupled concentration measurements before and after the treatment process.
This approach allows for the description of the variation in treatment efficiency. Many data sets on removal of micro-organisms contain zero counts, especially after the treatment process. When the effluent count is zero, the treatment efficiency cannot be determined accurately, indicating data-pairs with zero counts should be discarded. This method was used to calculate the DEC- (method B). The bias in this approach is small if the zero effluent count is the result of an influent count that is too low to determine the treatment efficiency, but it the bias is high if the influent count is high, since a zero count after the process indicates a relatively high treatment efficiency and discarding will result in an underestimation of the treatment efficiency. For these data pairs, replacement with the lower limit of the detection method is a more valid approach. Hence, a lower threshold level was set on the influent count to make sure the influent count was high enough to determine treatment efficiency. The threshold level was calculated as the minimum influent count that would result in detectable levels in the effluent at the average treatment efficiency, as described by the DEC. Data-pairs with zero counts in the effluent and influent counts lower than the threshold level were discarded from the data set. For all the data-pairs influent counts higher than the threshold level, zero effluent counts were replaced by the lower detection limit to calculate the DEC- (method C). Two other methods to deal with zero counts were used: replacement of all zero counts with the detection limit (method D), or with the average concentration before or after the treatment process (method E).
A different approach to determine the treatment efficiency is to regard the treatment efficiency as a stochastic process (Teunis *et al.,* 1997). The efficiency is described as the probability *p* that an organism that enters the process passes through the process. As the treatment efficiency of a process varies, the probability *p* is also allowed to vary according to a beta distribution, a distribution with two parameters a and b. The number of organisms that pass through the process will follow a beta-binomial distribution (Stuart and Ord, 1987):

$$P(k \mid n, a, b) = \binom{n}{k} \frac{B(k+a, n-k+b)}{B(a, b)} \qquad (6)$$

with *n* as the micro-organism count before the process, *k* as the count after the process and *p* as probability of passing the process that is beta-distributed with parameters a and b.
The beta-binomial distribution was fitted to the coupled concentration measurements before and after the treatment process using a maximum likelihood procedure to determine the values of a and b. The average treatment efficiency can be expressed as the median of the probability density function of beta-distributed *p* and the variation with the 5 and 95% percentile of this function. (method F). This approach can handle zero counts after the process, although the uncertainty in *p* increases as the percentage of zero counts increases.
The data-sets also contained a small fraction of data pairs where the count before treatment was slightly higher than after treatment. Since multiplication of spores is very unlikely in the processes incorporated in this study, this was considered to be the result of the short-term inhomogeneity of the spore counts in the water before and after treatment. These data-pairs should not be discarded, since they indicate poor removal. To be able to incorporate these data-pairs in the beta-binomial distribution and in the DEC-distributions, the count before treatment was equated to the count after treatment (no removal).
To compare the six methods to describe the treatment efficiency, descriptive statistics of the data-set were calculated and, where appropriate, the cumulative frequency distribution of the DEC-values were plotted and the median and 5% and 95% percentile were calculated to see how the different methods described the variation of the removal of SSRC by the treatment process.

RESULTS & DISCUSSION

Occurrence of (oo)cysts in river and reservoir water

Descriptive statistics of the occurrence of *Cryptosporidium* oocysts and *Giardia* cysts in the river Meuse at the inlet of the reservoirs and in the water at the outlet of the reservoirs are given in table 1.

Repeatability

Duplicate samples were taken from river water to analyse the repeatability of the sampling, processing and counting procedure. The individual counts per 100 litre and their average are depicted in figure 1. If the variation in counts is due to random error in sampling, processing and counting, the counts will follow the Poisson distribution. A χ^2-test showed that the observed variation in 24 of 30 duplicate *Cryptosporidium* counts and 22 of 26 duplicate *Giardia* counts are not significantly different from Poisson variation. Although this test is not very powerful with one set of duplicate counts, the combination of the χ^2-tests of the 30 ‘replicates’ gives an indication of how well the variation within duplicate counts follows the Poisson distribution. The average of the χ^2 value of the 30 duplicate *Cryptosporidium* counts is 2.21 and of the 26 duplicate *Giardia* counts

Table 1. Descriptive statistics of the concentration of Cryptosporidium oocysts and Giardia cysts in river and reservoir water.

	River Meuse		After reservoir storage	
	Cryptosporidium	*Giardia*	*Cryptosporidium*	*Giardia*
Number of samples	39	39	50	50
% Positive samples	90	85	28	20
Range (n/100 l)	0 – 38	0 – 58	0 – 1.0	0 – 0.4
Mean (n/100 l)	5.7	11.5	0.064	0.032
Standard deviation	6.8	16.7	0.161	0.077

is 2.92. Both do not differ from Poisson ($p = 0.14$ and $p = 0.09$, respectively). The mean difference between duplicate samples is a factor 2.6 (1.0 - 12) for oocysts and 2.1 (1.0 - 5.7) for cysts.
Calculation of the correlation coefficient between the (oo)cyst count and the χ^2-value, showed that the precision of the method was not related to the oocyst count in the sample ($r = 0.29$, $p > 0.1$), but was negatively related to the cyst count ($r = 0.56$, $p < 0.01$). The precision of the method was not sample-related, since small differences in *Cryptosporidium* counts concurred with large differences in *Giardia* counts and vice versa, and there was no correlation between the χ^2-value of the *Cryptosporidium* and *Giardia* counts.

Seasonal variation
As the level of variation in the average protozoa counts over the year is higher than the Poisson-variation in the duplicate samples, the seasonal variation is not an artefact of the detection method, but a reflection of differences in the presence of (oo)cysts in the water during the seasons.
In the river Meuse, highest *Cryptosporidium* concentrations were observed between January and April and from October to December (Fig. 1A). The concentration of *Giardia* showed a peak in February and in April, and rose in September and October to consistently high concentrations in November – December (Fig. 1B). At the reservoir outlet, neither the *Cryptosporidium* nor the *Giardia* concentrations showed a clear seasonality, although they were most frequently found in the second half of the year (Fig. 1).
The *Cryptosporidium* concentrations in river water exceeded *Giardia* concentrations in January – April. This may be a reflection of the presence of young farm animals, especially lambs, during this time of year. From May to

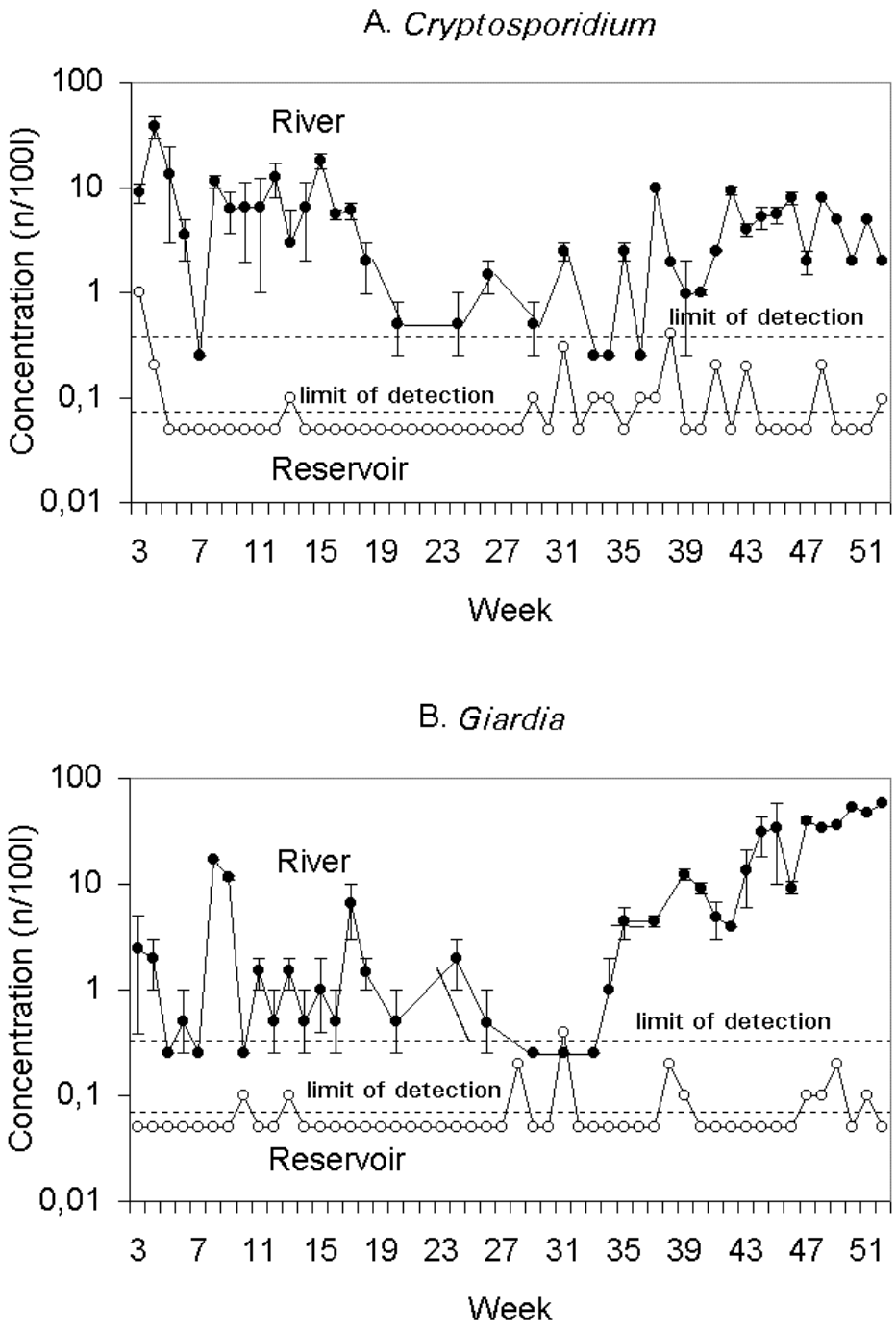

Figure 1. Concentration of Cryptosporidium (A) and Giardia (B) in river and reservoir water over the period of one year. Error bars in the river water data set show the results of duplicate samples.

August, concentrations of both protozoa were in the same order of magnitude. Starting in September, the concentration of *Cryptosporidium* and *Giardia* increased simultaneously, suggesting a common source. From September to December, the *Giardia* concentrations were higher than the *Cryptosporidium* concentrations.

The predominance of *Giardia* in raw and treated domestic wastewater (Medema *et al.,* 1997[b]) indicates that discharges of treated or untreated domestic wastewater are the principal source of faecal contamination in the second half of the year, while the predominance of *Cryptosporidium* in agricultural wastes (mainly from calves; Schijven *et al.,* 1999) and the relatively high

Cryptosporidium counts in winter and spring indicate that this is the principal source in the first half of the year.

Distribution of (oo)cyst concentrations

The frequency distribution of the *Cryptosporidium* and *Giardia* concentrations in river and stored river water are depicted in figure 2. The maximum likelihood ℓ

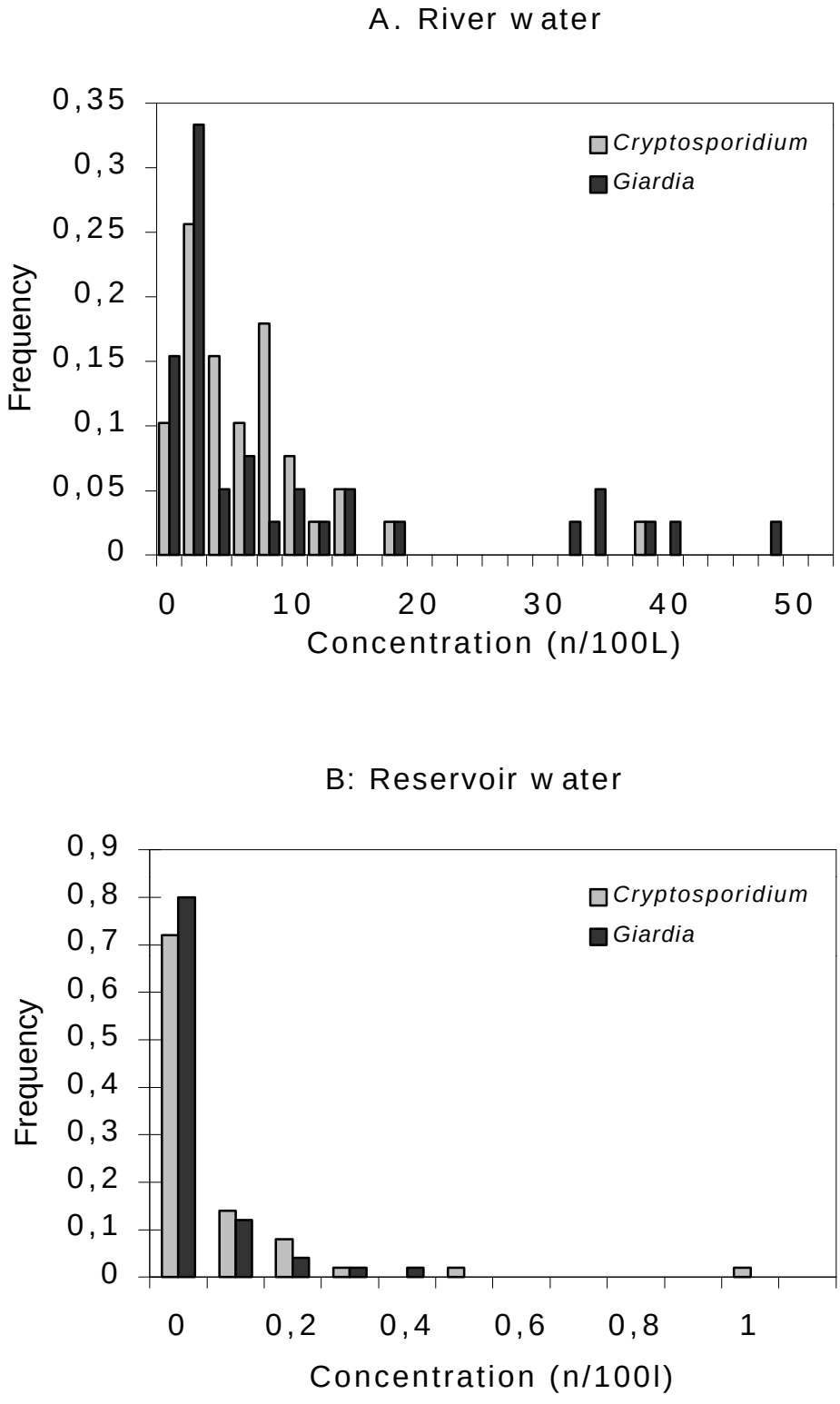

Figure 2. Frequency distribution of Cryptosporidium and Giardia concentrations in river (A) and reservoir (B) water over the period of one year.

values indicated that the negative binomial distribution provided a better fit to the protozoa concentration data in river water than the Poisson-with-added-zero's distribution (Table 2). For stored water, with a high percentage of zero counts, the fit of both distributions was comparable.

Table 2. Parameter values for the maximum likelihood fit for the negative binomial and Poisson-with-added-zero's distribution. To compare the goodness-of-fit of the two distributions, the minimum value for -2ℓ is given (smaller value indicates better fit).

	negative binomial			Poisson-with-added-zero's		
	ρ	p	-2ℓ	λ	θ	-2ℓ
River water						
Cryptosporidium	0.905	0.0736	270.9	12.69	0.102	531.6
Giardia	0.399	0.0171	302.3	27.15	0.154	1437
Stored water						
Cryptosporidium	0.301	0.314	105.4	1.86	0.644	116.5
Giardia	0.338	0.513	71.4	1.03	0.688	71.4

Recovery of the (oo)cyst concentration and detection method

The overall recovery efficiency of the method for sampling, concentration and detection of cysts and oocysts was low. The recovery efficiency might have been influenced by the amount of debris and other small organisms, such as algae, in the sample. However, no significant correlation was observed between turbidity or chlorophyll A – content and the recovery efficiency of cysts or oocysts. Also, the number of (oo)cysts added (600 - 10600) to the samples to determine the recovery was not significantly correlated with the recovery efficiency. Hence, the recovery efficiency of the concentration and detection method was independent of these factors and was regarded as a stochastic process: every (oo)cyst had a certain probability of being recovered. The variation that was observed in the measured recovery efficiencies was regarded as a reflection of the variation in the probability.

The parameter values of the best fit of the beta-binomial distribution to the recovery measurements from this study and the shape of the beta-distribution are given in Teunis *et al*. (1997). The median and 90%-range of the recovery efficiency were 0.029 (0.017 - 0.046) for *Cryptosporidium* and 0.029 (0.008 - 0.074) for *Giardia* in river water and 0.013 (0.004 - 0.029) for *Cryptosporidium* and 0.005 (0.004 - 0.011) for *Giardia* in stored water. The addition of calcium hydroxide to the reservoir water for softening produced a precipitate of calcium carbonate that was clearly visible in the concentrated reservoir water, and may have been the cause of the lower recovery efficiency in reservoir water.

Correlation of *Cryptosporidium* and *Giardia* with other water quality parameters in river water

Cryptosporidium concentrations in river water were positively correlated to the concentration of all faecal indicator bacteria and to river flow and turbidity (Table 3). Several, but not all, of the peak concentrations of *Cryptosporidium* concurred with a peak turbidity (Figure 3, week 3,4,5, week 12, week 15, week 44 and week 50). The strongest correlation was observed with spores of sulphite-reducing clostridia and *Cl. perfringens*. The concentration of *Giardia* cysts in the river Meuse was only negatively correlated to the chlorophyll concentration. Interestingly, if only the second half of the year, in which the *Giardia* concentrations were high, was tested, *Giardia* concentrations

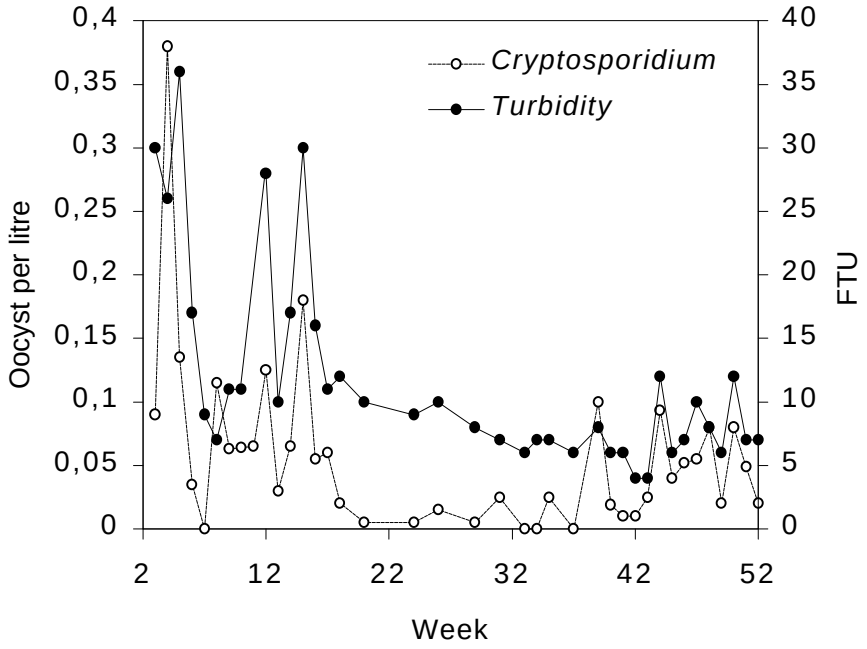

Figure 3. Coincidence of peaks in turbidity measurements with peaks in Cryptosporidium counts in river water in week 4, 12, 44 and 50, not in week 8 and 39.

were correlated to many more variables (Table 3), with the strongest correlation with spores and river flow (positive) and temperature (negative), despite the lower number of observations. The exclusion of the first half of the year did not change the correlation of the *Cryptosporidium* concentrations with most other variables. However, the correlation coefficient with the coliforms and temperature fell below the 5% significance level, while chlorophyll A was now negatively correlated to *Cryptosporidium.* In this second half of the year, a positive correlation was also observed between both protozoa.

The low number of positive protozoa samples in stored water did not allow an accurate calculation of the correlation between the protozoa and other water quality parameters in stored water.

The Meuse is a typical rain-river, with high flows and high turbidities in winter and spring, due to rainfall and melting snow. At this time, the level of faecal pollution, as judged by the concentration of indicator bacteria, is also relatively high and the water temperature is low. The concentration of *Cryptosporidium* and *Giardia* (at higher *Giardia* concentrations) was positively correlated with flow

and turbidity, and with faecal indicator bacteria, especially with clostridial spores. Spores and (oo)cysts are both very persistent in the aquatic environment (Medema *et al.,* 1997[a]), and it may be this feature that is the basis for the superior correlation between (oo)cyst and spore-concentrations. Correlation of the concentration of *Cryptosporidium* and *Giardia* with the faecal indicators and turbidity has been reported by other authors, although not consistently (Rose *et al.,* 1991, LeChevallier *et al.,* 1991, Atherholt *et al.,* 1998).

Table 3. Correlation between Cryptosporidium and Giardia concentrations with other water quality parameters in river and stored water (product-moment correlation coefficients).

Parameter	River water (week 1-53)		River water (week 26-53)	
	Crypto.	*Giardia*	*Crypto.*	*Giardia*
Giardia	-0.00	-	0.55**	-
SSRC	0.75***	0.15	0.55	0.70*
SCP	0.76***	0.03	0.72*	0.66*
COLI37	0.51**	-0.03	0.43	0.05
COLI44	0.58***	-0.15	0.49	0.32
FSTREP	0.57***	-0.11	0.60*	0.52*
Temperature	-0.44**	-0.23	-0.33	-0.84***
Chlorophyll A	-0.27	-0.56***	-0.81***	-0.44*
Discharge	0.61***	-0.02	0.71*	0.68*
Turbidity	0.66***	-0.28	0.65**	0.44*
NH_4^+	0.38*	0.20	0.63**	0.59**

* significant at the 0.05 level
** significant at the 0.01 level
*** significant at the 0.001 level

Removal of (oo)cysts by reservoir storage

The average removal of (oo)cysts during storage of the river water in the three successive reservoirs with a total average residence time of 5 months, can be described by calculating the DEC from the average (oo)cysts concentration at the in- and outlet of the reservoirs (Table 4). Due to the long residence time in the reservoirs and the dispersion of the residence time, it was not feasible to define coupled data-pairs to calculate the variation in the DEC.

The concentration data of *Cryptosporidium* and *Giardia* were corrected for the median of the best fitting beta-binomial distribution of the recovery efficiency data before and after reservoir storage. Since the recovery efficiency in river water was higher than in stored water, this correction reduced the DEC for both protozoa.

Removal of indicators by reservoir storage

The average removal of bacteria that are most indicative of faecal contamination (COLI44, FSTREP and SCP) was more efficient than the removal of *Giardia* cysts and especially of *Cryptosporidium* oocysts. The DEC of the reservoirs for SSRC and of COLI37 was comparable to the DEC for *Cryptosporidium* and *Giardia,* suggesting these could be useful indicators for protozoa removal by reservoir storage. However, the ratio between SSRC and SCP and between COLI37 and COLI44 in stored water increased markedly during the season with high water temperatures, suggesting that growth or recontamination of SSRC and COLI37 occurred in the reservoirs during this time. This reduced the DEC for these two parameters to a level that was comparable to the DEC of the protozoa, but, since no multiplication of (oo)cysts occurs in water, these parameters were not suitable to describe the fate of (oo)cysts during reservoir storage.

Table 4. Removal of Cryptosporidium oocysts, Giardia cysts, indicator bacteria, ammonium and turbidity by storage of river water in three successive reservoirs with an average residence time of 5 months.

Variable	Unit	C_{in}	C_{out}	DEC
Cryptosporidium	oocysts.l^{-1}	1.97*	0.049*	1.6*
Giardia	cysts.l^{-1}	3.97*	0.064*	1.9*
SSRC	cfp.l^{-1}	2462	85	1.5
SCP	cfp.l^{-1}	3016	14	2.3
COLI37	cfp.l^{-1}	34624	746	1.7
COLI44	cfp.l^{-1}	9988	24	2.6
FSTREP	cfp.l^{-1}	3771	8	2.7
NH_4^+	mg.l^{-1}	0.205	0.068	0.48
Turbidity	FTU	11.7	2.2	0.73

* After correction of the average concentration with the median of the best fitting beta-binomial distribution for the recovery efficiency

Also turbidity and ammonium were no suitable as surrogate parameters, since they were less efficiently removed than the (oo)cysts.

A concurrent study has shown that waterfowl can be a significant source of (oo)cysts in these reservoirs (Medema *et al.,* in prep.). Even though these birds will also contaminate the water with indicator bacteria, it is likely that the ratio between (oo)cysts and indicator bacteria will be affected, thereby limiting the suitability of the indicators for predicting (oo)cyst presence at the outlet of the reservoirs.

Treatment efficiency

The data on the removal of SSRC by the coagulation/sedimentation, by ozone and by filtration are presented in figure 4. The concentration of SSRC at the inlet

of the processes varied considerably, showing a seasonal fluctuation with high concentrations in summer and low concentrations in winter. Descriptive statistics of the data are given in table 5. The data before and after coagulation/sedimentation contained few (< 5%) zero counts. After ozone treatment, 51.9% of the counts were zero and in treated water, the vast majority (94.1%) of the counts was negative.

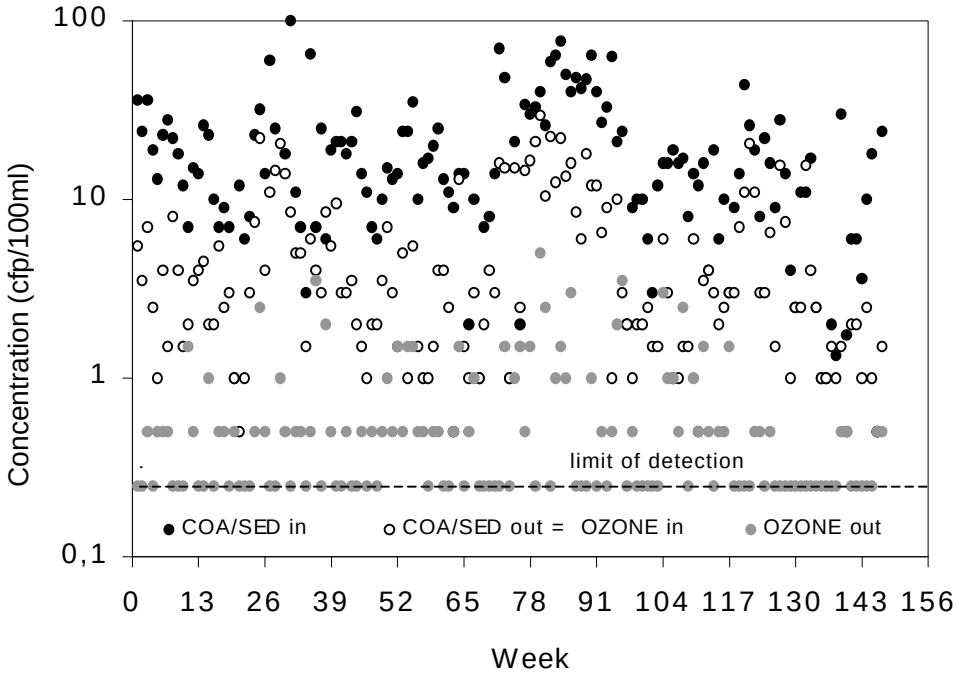

Figure 4. Concentration of spores of sulphite reducing clostridia at the in- and outlet of a coagulation/ sedimentation step and an ozonation step in a surface water treatment system over the period of three years.

To determine if the treatment affected the distribution of the concentration of spores, as shown by Gale *et al.* (1997), the Poisson and the negative binomial distribution were fitted to the concentration data. The negative binomial distribution fitted the concentration of spores in the water entering the

Table 5. Descriptive statistics of the data of the concentration of spores of sulphite-reducing clostridia before and after the treatment processes

	Inlet coa/sed	Outlet coa/sed	Outlet ozone	Treated water
Number of samples	156	156	156	153
% positive samples	98.1	95.5	48.1	5.9
Mean (cfp.100ml^{-1})	19.27	5.68	0.48	0.14
Standard deviation	18.4	5.64	0.77	0.78
Minimum	0	0	0	0
5-percentile	2	0.5	0	0
Median	14	3	0	0
95-percentile	60.8	16.6	2	1
Maximum	110	29.5	5	7

treatment system adequately (Table 6), and also the subsequent data sets, although the spore-concentrations after coagulation were more dispersed. The Poisson distribution did not fit to any of the data sets. The cluster parameter of the negative binomial distribution (*k*) indicated that the level of clustering increased after every treatment process.

Table 6. Parameter values of the best (maximum likelihood) fit of the Poisson and negative binomial distribution to the data on the concentration of spores of sulphite-reducing clostridia before and after treatment.

Sampling site	Poisson		negative binomial		
	μ^	*p* (G-test)	μ^	k^	*p* (G-test)
Before treatment (cfp/100ml)	19.31	0.000	19.31	1.417	0.69
After coagulation/sedimentation (cfp/200ml)	10.35	0.000	10.35	1.088	0.05
After ozonation (cfp/200ml)	0.962	0.006	0.962	0.780	0.28
Treated water (cfp/100ml)	0.143	0.000	0.143	0.039	1.00

These data sets did not allow us to determine if the type of clustering is the same as the clustering reported by Gale *et al.* (1997). The data sets in this study represent the concentration of spores before and after treatment processes over time, while Gale *et al.* reported spatial clustering. This spatial clustering may be present in our data sets, but cannot be discriminated from temporal clustering. Nonetheless, the level of clustering increased after treatment. This may also occur with the pathogenic protozoa and other pathogenic micro-organisms during treatment. This implies that the micro-organisms are not randomly distributed in water after treatment, but that for short periods treated water contains a relatively high concentration, while most of the time the water contains no pathogenic micro-organisms.

Using these data to calculate the removal of SSRC by the individual treatment processes and by the combined treatment, based on the average concentration before and after the treatment process(es), showed that the average removal by coagulation/sedimentation was 0.57 ^{10}log-units, ozone eliminated 1.03 ^{10}log-units and removal by the filtration steps was 0.52 ^{10}log-units. The overall removal was in this case equal to the sum of the removal by the unit processes: 2.12 ^{10}log-units.

Figure 4 shows that the elimination of SSRC by the treatment processes can vary considerably. To describe this variation, the individual data-pairs of the concentration of spores before and after each treatment process on the same day were used to calculate the DEC-values, using method B-E. The same data-pairs were used to determine the maximum likelihood estimates of the beta-binomial distribution of *p*, the probability of a spore to pass through the

treatment process. To be able to compare *p* to the DEC-values, $-^{10}\log(p)$ was calculated. The individual DEC-values and the distribution of $-^{10}\log(p)$ were used to determine the frequency distribution of the treatment efficiency (Figure 5).

Coagulation/sedimentation

For the coagulation/sedimentation process (and the data set with < 5% zero counts), the method used to calculate the distribution of the DEC-values had little effect. The median DEC of the coagulation/sedimentation process was 0.6 ^{10}log-units. The elimination capacity of the process varied from 0 to 1.7. The 5-percentile was still 0 and the 95 percentile was 1.3; the distribution was very slightly skewed towards the higher DEC-values. The beta-binomial function (α = 0.9741, β = 1.9444) yielded a very similar distribution; with a median of 0.53, and 5- and 95-percentiles of 0.11 and 1.6 respectively. Also this distribution was slightly skewed towards the higher $-^{10}\log(p)$-values.

Ozonation

For the ozone process (and the data-set with 51.9% zero counts after treatment), the different methods for calculating the DEC-values resulted in different distributions (Fig. 5). The median of all methods that were used to deal with the zero counts (method B to E) was lower than the DEC of 1.03 ^{10}log-units based on the average concentration before and after the ozone process (method A). As most of the zero counts after ozonation indicate that the process was effectively inactivating the spores at the time of sampling, it was not surprising that methods that discard these data-pairs (method B) or replace the zero with a positive number (detection limit (method C,D) or average concentration (method E)) produced a lower median DEC. The beta-binomial function (α = 0.2874, β = 1.3467) has not altered or discarded the zero counts, resulting in the highest median DEC ($-^{10}\log(p)$) of 1.22 ^{10}log-units, with a wide 90% interval of 0.14 – 4.7.

Filtration

The data-set on the spore-concentration in treated water contained very few (5.9%) positive counts, while the data-set before the filtration contained 48.9% zero counts and the positive counts were generally low (P95 is 4 spores per 200 ml). These data sets were not suitable to make an accurate description of the variation of spore-removal by the filtration steps. The different methods to calculate the variation resulted in very different frequency distributions (Fig. 5). If all data-pairs with zero counts are discarded (method B), only four data pairs remain, all with equal counts after and before filtration (DEC = 0, Fig. 5). Replacing zero's with the detection limit result in very many data pairs where 1 spore entered the processes and 1 spore came out again (DEC = 0). This gave a very steep frequency distribution around the median DEC = 0. Discarding all data-pairs where the influent concentration was below the threshold of 3.3, left 12 data-pairs with a median DEC of 0.6 ^{10}log-units. Replacing zero counts with the average of the series gave a median DEC-value similar to the DEC-value of the average concentrations (0.54 ^{10}log-units; method E), because many of the data-pairs were set equal to the average concentrations. The beta-binomial

function could not be calculated accurately; the a and b were very low (0.000115 and 0.000907 respectively).

Combination of treatment processes

If the distribution of DEC-values of the individual treatment steps is known, the elimination capacity of the combined treatment processes can be calculated by combining the distributions. Alternatively, the distribution of the DEC-values of the combined treatment can be calculated from the individual data-pairs of the spore concentration before and after the combined treatment processes, under the assumption that the data collected before and after the combined treatment processes on the same day could still be regarded as coupled data.
Comparing these two alternatives showed that combining the distributions from the individual processes yielded a higher level of variation in the combined processes than calculating the DEC-values from the data before and after the combined processes. The results were consistent, irrespective of the method used for calculating the DEC-distribution. The data on the elimination of spores by the coagulation/sedimentation in combination with the ozonation, as calculated with method E (replace zero counts with the average of the series) are given as example: the median DEC of the processes were 0.58 and 0.72 ^{10}log-units, respectively. The level of variation of these data sets, expressed as the 90% interval, was 0 – 1.3 and 0 – 1.5 ^{10}log-units respectively. Combination of these two data-sets to describe the elimination of coagulation/sedimentation and ozonation together resulted in a median DEC of 1.37 ^{10}log-units with a 90%-interval of 0.39 – 2.4 ^{10}log-units. The DEC-distribution based on the data-pairs before coagulation/sedimentation and after ozonation had a median DEC of 1.36 ^{10}log-units with a smaller 90%-interval of 0.58 – 2.1 ^{10}log-units.
Hence, the variation of the combination of treatment processes is smaller than would be expected on the basis of the variation of the individual treatment processes. In other words, the variation in the individual processes is reduced by ‘counter-variation’ in subsequent processes. There was indeed a negative correlation between the DEC of the coagulation/sedimentation process and the DEC of the ozone process (Figure 6). This correlation was primarily determined by the low DEC-values of the ozone process at the time that the coagulation process yielded high DEC-values. Although low DEC-values of the ozonation occurred throughout the year, the majority (71%) of the ozone DEC-values below 0.5 ^{10}log-units occurred in the winter months when the temperature was low. Similarly, the high DEC-values of the coagulation process occurred during low temperatures. Under these conditions WISPRO was added to the water to enhance floc formation, which resulted in improved removal of micro-organisms. Describing the variation in these combined processes with the different methods to calculate the DEC resulted in similar frequency distributions, despite the relatively high percentage of zero counts after ozone (Fig. 5). The median DEC from method B to E was 1.30 - 1.45 ^{10}log-units and 95-percentiles of 2.07 – 2.11. The largest differences were observed in the 5-percentiles; from 0.30 for method D (replace zero with detection limit) to 0.58 for method E (replace zero with average).

The beta-binomial distribution (a = 0.4908, b = 12.794) predicted overall a more efficient elimination (Fig. 5) with a median of 1.8 ^{10}log-units and a 90% interval of 0.85 - 3.8.
The elimination capacity of the overall treatment system for spores of sulphite-reducing clostridia, as determined by method A (average concentration in raw and treated water), is 2.12 ^{10}log-units. The high percentage of zero counts in treated water did not allow an accurate description of the variation in the overall treatment. All methods resulted in a median DEC of approx. 1.2 ^{10}log-units, substantially lower than 2.12 ^{10}log-units, except for method E (replace zero with the average of the series) that gave a median DEC of 2.00 ^{10}log-units. Again, all methods replace the zero counts with a positive number, thereby reducing the DEC-value. Here, 94.1% of the data-pairs contained a zero count in treated water. Hence, every method underestimated the true average elimination capacity of the overall treatment. Method C gave the highest median DEC value and 90%-interval (1.2 – 2.6 ^{10}log-units) and was considered the closest conservative approximation of the true distribution of the DEC-values of the overall treatment.
The beta-binomial function (α = 0.01547; β = 0.97889) yielded a very high median DEC-value of 20 ^{10}log-units. The distribution of –log(p)-values was very wide (Fig. 5), with a 90% interval from 1.4 – 84 ^{10}log-units.

Selection of methods

From the comparisons between the different methods to describe the variation in treatment processes it is obvious that with spore-counts before and after treatment with < 5% zero counts, the difference between the methods were very small and also the best fitting beta-binomial distribution gave a very similar distribution of $-^{10}\log(p)$. For the data-set with approximately 50% zero counts after treatment, the beta-binomial distribution attributed overall a higher elimination capacity to the treatment than the DEC-frequency distributions, and resulted therefore in a less conservative description of the treatment efficiency. Within the DEC-distributions, all methods underestimated the treatment efficiency with these data-sets. Method B (discard zero counts) and D (replace zero counts with the detection limit) consistently resulted in the lowest median values and were therefore considered less appropriate. With this type of data-sets, method C (threshold) and E (replace zero counts with the average) were considered the best options for the description of treatment variation. These descriptions renders the least underestimation of treatment efficiency, but are still conservative.
In the data-set with > 95% zero counts after treatment, this effect was very pronounced and the beta-binomial distribution was inappropriate. From the DEC-methods, only method E is appropriate, but it is obvious that with this type of data-sets the description of variation in treatment is inaccurate.

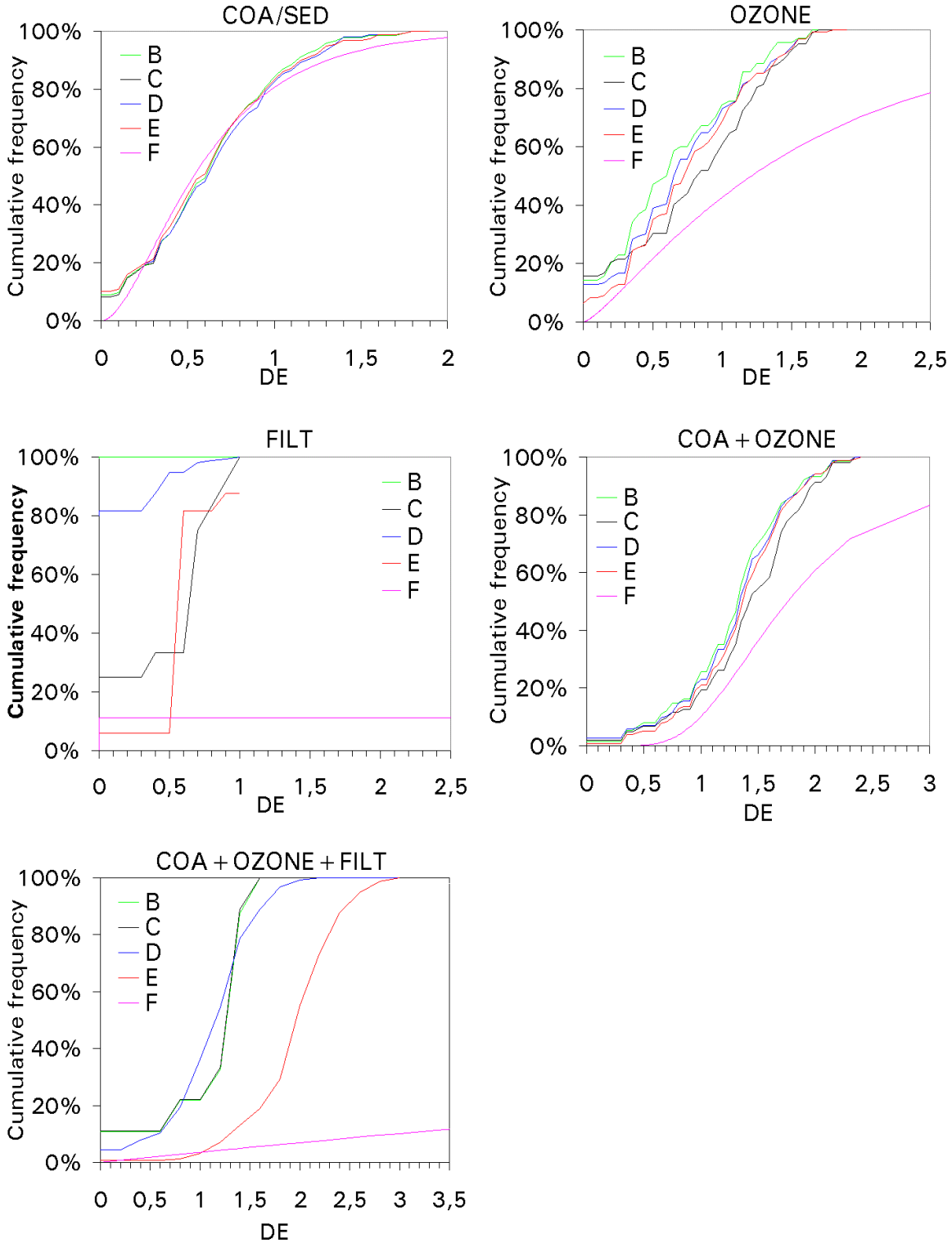

Figure 5.
Frequency distributions of the Decimal Elimination Capacity (DEC) of spores of sulphite reducing clostridia by coagulation/sedimentation, ozonation, filtration and the combination of coagulation/sedimentation and ozonation and of the overall treatment process. The frequency distributions are calculated from the coupled data of the concentration of spores before and after the treatment processes according to different methods (see text for details).

Sensitivity improvement of spore quantification

From these data sets it is clear that these well-designed and operated treatment processes showed a considerable amount of variation. These data sets also showed that the variation in individual treatment processes was not independent, but correlated to the performance of previous processes. Therefore, an accurate description of the efficiency of micro-organism removal by a chain of treatment processes can only be made by accurate concentration measurements in raw and treated water. The current methods for the spores of sulphite-reducing clostridia are appropriate for the measurements in most raw waters but are not sensitive enough for treated water measurements. Therefore, we have recently developed an *in* situ method for the isolation of indicator bacteria from large volumes of treated water. This method is an upscale of the currently used membrane filtration method and is a 100-1000-fold more sensitive (Hijnen *et al*., in prep.). This method is currently used to perform concentration measurements in finished water, to be able to describe the performance of the overall treatment process.

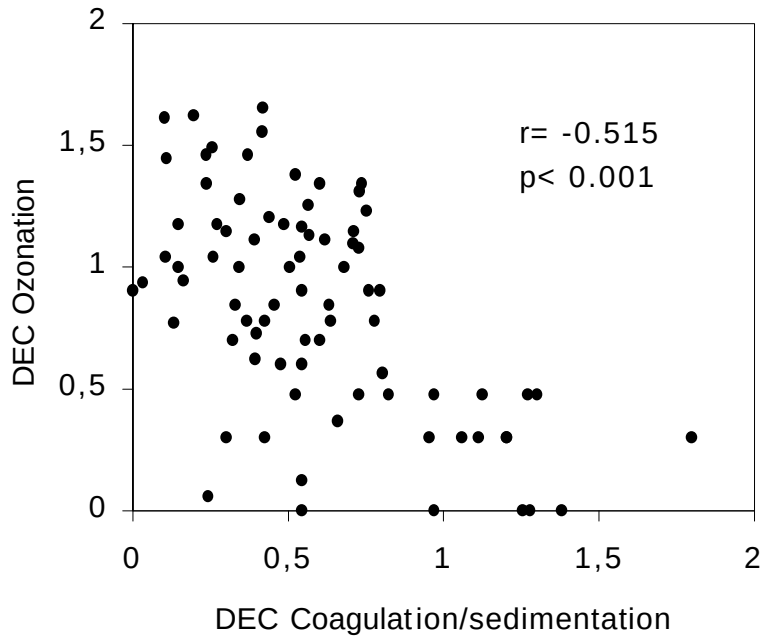

Figure 6. Correlation of the elimination of spores by coagulation/sedimentation and ozonation.

Spores in treated water

Even though the elimination capacity of the overall treatment could not be accurately described, it was clear that in a small percentage of the samples (5.9%), spores were detected in treated water. No obvious treatment failures were recorded at or just before the dates of the positive treated water samples. Two of these occasions coincided with relatively high counts in raw water at higher water temperatures. The other seven occasions occurred in October-November and March-April, the period in which the addition of coagulant aid to the coagulation process was started and terminated respectively. Under similar conditions, however, the spores were reduced to below 1/100 ml. There were no obvious reasons for the presence of spores in treated water. A more detailed examination of the influence of source water quality and treatment parameters

will yield the factors that primarily determine the elimination of micro-organisms by treatment systems and the critical control points for optimising treatment performance.

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Improved detection and vital staining of *Cryptosporidium* and *Giardia* with flow cytometry

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Dot-plot of the FACSort flow cytometer showing the position of *Giardia* cysts and *Cryptosporidium* oocysts in the sort gate

ABSTRACT

Flow cytometry with fluorescence activated cell sorting (FACS) was used to purify *Cryptosporidium* oocysts and *Giardia* cysts from water. With this purification step *Cryptosporidium* and *Giardia* were found in a higher percentage of the samples and significantly higher *Giardia* concentrations were detected in these positive samples. Because FACS removed most of the debris from the concentrated water samples, the microscopic preparations could be examined more rapidly for the presence of (oo)cysts and the morphological characteristics of the (oo)cysts could be interpreted more unambiguously than with the conventional immunofluorescence microscopy method. The use of FACS made it possible to apply PI-staining on environmental samples to determine the fraction of dead (oo)cysts. Sample processing did not appear to influence the PI-staining characteristics of the *Cryptosporidium* oocysts, but did increase the percentage of PI-positive *Giardia* cysts. This suggests that this protocol can be used for determining the percentage of dead *Cryptosporidium* oocysts in environmental samples. Analysis of environmental samples suggests that reservoir storage increases the percentage of PI-positive (dead) oocysts.

This research has been carried out on behalf of the General Directorate for the Environment (DGM). The authors thank L. Kruidenier and G.B. Engels for sampling and sample processing and A.H. Havelaar for critical reading of the manuscript.

This chapter has been published as: Medema, G.J., Schets, F.M., Ketelaars, H.A.M., Boschman, G. (1998). Improved detection and vital staining of *Cryptosporidium* and *Giardia* with flow cytometry. *Water Science and Technology*, 38(12):61-66.

INTRODUCTION

Current detection techniques for *Cryptosporidium* oocysts and *Giardia* cysts in water samples consist of the filtration of large volumes of water, elution of particles and (oo)cysts from the filter material, concentration of (oo)cysts by centrifugation and flotation and staining of the (oo)cysts with monoclonal antibodies specific to the (oo)cyst wall, labelled with fluorescein. The stained samples are examined with epifluorescence microscopy for particles that have the morphological and fluorescence characteristics of (oo)cysts. The recovery of the filtration/centrifugation/flotation method is low (Clancey *et. al.*, 1994) and the microscopic examination is hampered by the presence of debris, other micro-organisms (algae) and autofluorescing particles in water samples. The identification of (oo)cysts is therefore labour-intensive and inaccurate. Flow cytometry with fluorescence-activated cell sorting (FACS) can facilitate this step of the detection method (Vesey *et. al.,*1994). The flow cytometer can discriminate MAb-stained (oo)cyst-like particles on the basis of light-scatter and fluorescence characteristics and separate these from the debris particles prior to microscopic confirmation. This results in purified water concentrates in which it could also be possible to use vital staining assays on the concentrated (oo)cysts. Uptake of propidium iodide (PI) has been reported to correlate with the inability to excyst *in vitro* for both *Giardia* cysts (Smith and Smith, 1989) and *Cryptosporidium* oocysts (Campbell *et. al.,* 1992). For *Giardia* cysts, the uptake of PI also correlated with the inability to infect mice (Schupp and Erlandsen, 1987). Exclusion of PI, on the other hand, did not correlate with the ability of *Giardia* to excyst (Smith and Smith, 1989). So, uptake of PI can be used as an indicator of cell death for *Cryptosporidium* oocysts and *Giardia* cysts. PI (and DAPI: di-amidino-phenyl-indole, used as help-stain for the identification of *Cryptosporidium* oocysts) are however non-specific stains and cannot be used in the conventional assay, since most biological particles in water concentrates stain with both fluorochromes, resulting in microscopic preparations with very high background fluorescence. The aim of this study was to evaluate the use of the incorporation of a flow cytometer with FACS in the processing of water samples for the detection and vital staining of *Cryptosporidium* oocysts and *Giardia* cysts.

MATERIALS AND METHODS

Protozoa

Purified oocysts of a (cervine/)ovine *Cryptosporidium parvum* strain were obtained from Moredun Animal Health Ltd, Edinburgh, Scotland (MRI). Purified *Giardia lamblia* cysts, of a human isolate maintained in gerbils, were obtained from PRL Dynagenics, Phoenix, USA.

Sampling and processing

Samples of 10-100 l of sewage and secondary effluent, 200 l of river water or 1000-2000 l reservoir water were collected and processed as described by LeChevallier *et. al.* (1991). The final concentrate was siphoned down to approximately 2 ml, gently suspended, filtered through a 25 mm polycarbonate membrane filter (1.2 µm pore size) and stained with monoclonal antibodies specific for *Giardia* (Giardia-Cel IF test;

Cellabs Diagnostics, Brookvale, Australia) and *Cryptosporidium* (Detect IF Cryptosporidium; Shield Diagnostics, Dundee, UK).

Recovery efficiency

To determine the recovery efficiency of the detection methods, water samples were seeded by injection of a *Cryptosporidium* oocyst suspension and a *Giardia* cyst suspension in the filter inlet hose during sampling. The concentration of cysts and oocysts in these suspensions was determined before every seeding experiment.

Sample processing for flow cytometry

Samples for flow cytometry analysis were processed identically up to the final concentration. The final concentrates were siphoned down to approximately 300 µl and gently resuspended. 100 µl of each undiluted monoclonal antibody was added to the samples, and the mixture was incubated at 37 °C for 30-45 min. After staining the samples were diluted to 10 ml with phosphate buffered saline (PBS: 0.01 M, pH 7.2) with 0.1% (w/v) sodium dodecyl sulphate (SDS) and 0.1% (v/v) Tween 80 and centrifuged for 10 min at 1080 x g. Samples were siphoned down to approximately 300 µl and resuspended. Samples were then filtered through 35 µm mesh filters to remove sand and other large particles.

Flow cytometry and cell sorting

Sample analysis was performed on a Becton Dickinson FACSort flow cytometer. The FACSort was equipped with a 15 mW Argon ion laser operating at 488 nm, two light scatter detectors (forward and side (90^{o}) angle), a fluorescence detector with a bandpass filter of 530/30 nm and electromechanical, aerosol-free sorting with a catcher-tube that captured particles from the flow stream. The concentrates were analysed at a flow rate of 60 µl min^{-1}. The catcher tube is capable of catching particles from the flow stream at a rate of 300 sec^{-1}. Even at an analysis speed as high as 20,000 - 30,000 cells per second, which is often required for the concentrates with huge amounts of debris particles, the FACSort was able to sort all particles specified by the sort region if put in the "recovery" sort mode. The sheath fluid consisted of isotonic saline (FACSFlow; Becton Dickinson cat.no. 342003). The signals used for sorting were forward angle light scatter (FSC) and FITC fluorescence (FL1), because this signal combination gave the best discrimination between stained (oo)cysts and debris. Instrument controls (photomultiplier tube voltage, amplifier gains) were optimised so that stained oocysts and cysts appeared at the top of a FSC-FL1 (log-log) dot plot. These instrument settings were stored and recalled in subsequent analysis. In the FSC-FITC dot plot a sort region was defined that included both oocysts and cysts. All particles with fluorescence and light scatter characteristics of oocysts and cysts were sorted directly onto1.2 µm 25 mm polycarbonate membrane filters. Filters were placed on microscope slides, embedded and examined for the presence of (oo)cysts. Before each use, the instrument alignment, settings and performance were checked by running CaliBRITE FITC Flow Cytometer Beads (Becton Dickinson cat. no. 349502) and a positive control sample with stained *Cryptosporidium* oocysts. The beads were to give narrow peaks in a FSC and a FL1 histogram at a fixed signal level and the oocysts were to appear as a tight cluster at fixed signal levels in a FL1-FSC dot plot.

PI/DAPI staining

After processing, prior to cell sorting with the flow cytometer, the sample concentrates were pre-treated in acidified Hanks' Balanced Salt solution and stained with PI (1 $g.l^{-1}$) and DAPI (2 $g.l^{-1}$) for 2 hours as described by Campbell *et. al.* (1992). MAb-FITC conjugate was added in the last 30-45 min. of this incubation time.

Microscopy

Microscopic preparations were examined using a Zeiss Axioskop epifluorescence microscope fitted with x 10 eyepieces and x 25 oil and x 100 oil Plan Neofluar objectives, with dichroic mirror and filters for FITC (blue 09), PI (green 15) and DAPI (UV 02). Slides were examined for the presence of (oo)cysts. *Cryptosporidium* oocysts were defined as spherical shapes with an apple green fluorescence of the oocyst wall and a diameter of 3-7 µm. *Giardia* cysts were defined as oval to spherical shapes with an apple green fluorescence of the cyst wall and a size of 5-15 x 8-18 µm.

Statistical analysis

To evaluate differences between concentrations obtained with the direct method and the method with the FACSort, an analysis of variance was performed on ^{10}log transformed concentrations using Microsoft Excel 7.

RESULTS

The appearance of the microscopic preparations made with and without the FACSort was very different. While filters made with the direct method contained large amounts of particles (micro-organisms and other colloids) and background fluorescence, filters made with the FACSort showed a much lower number of particles that could be individually assayed since they were not obscured by other particles.

In unseeded samples, the FACSort method yielded more positive samples: cysts were detected in 16/17 samples and oocysts in 10/17 samples, while the direct method yielded only 14/17 and 8/17 positive samples for cysts and oocysts respectively.

The use of the FACSort also resulted in higher concentrations: for *Cryptosporidium* oocysts in seeded river and seeded sewage samples especially (Fig. 1A), and for *Giardia* cysts in all water types tested, both with the seeded and natural samples. For *Giardia* cysts the sensitivity improvement was most profound at low concentrations of cysts in natural river water samples and in sewage and seeded sewage samples: the FACSort method detected up to 84 times more cysts than the direct method (Fig. 1B). Using all samples that were positive to compare both methods in an analysis of variance, the *Giardia*-concentrations found with the FACSort method were overall significantly higher ($P=0.03$) than with the direct method. For *Cryptosporidium* this difference was not significant ($P=0.32$).

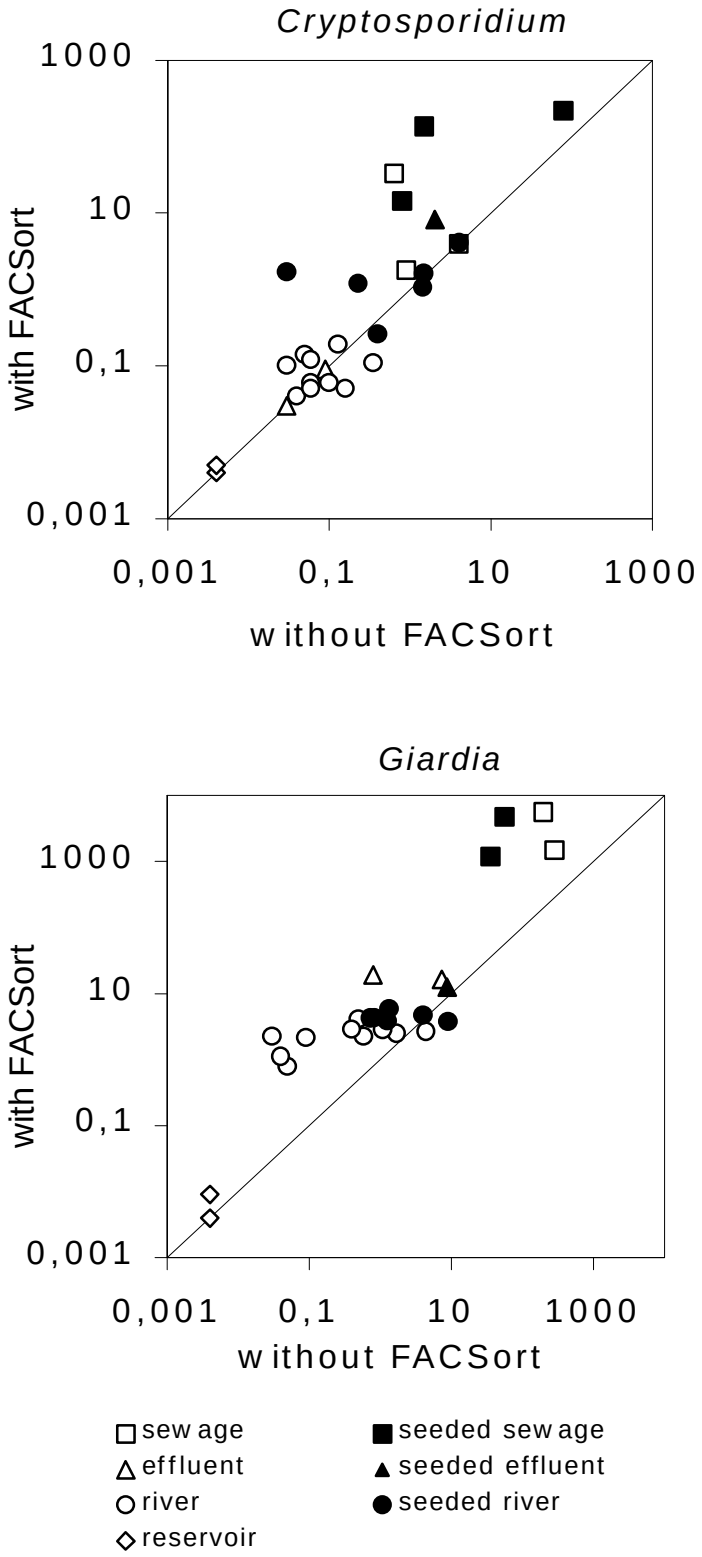

Figure 1.
Concentration of Cryptosporidium (A) and Giardia (B) in seeded and unseeded water samples from several types of water, as determined by the method with (y-axis) and without (x-axis) purification with a FACSort flow cytometer.

Staining of the water concentrates with PI and DAPI prior to cell sorting with the FACSort resulted in filters on which the staining characteristics of the individual (oo)cysts could be examined without interference from other particles. PI could therefore be used to determine the fraction of dead cells in environmental samples. However, during sample processing, the (oo)cysts are exposed to various steps that may affect their viability. To determine if any of

the processing steps had an effect on the PI-staining characteristics of (oo)cysts, seeding experiments were performed on every sample-processing step independently. Settled sewage was processed by centrifugation, sonification, flotation and washing with PBS/Tween/SDS. Before each processing step, *Giardia* cyst and *Cryptosporidium* (oo)cyst suspensions with known PI-staining characteristics were seeded to a fraction of the sample. The percentage of *Cryptosporidium* oocysts that stained with PI was not affected by centrifugation, sonification, flotation or washing (Fig. 2). The percentage of *Giardia* cysts staining with PI (nonviable cysts), however, was increased after sonification and, to a lesser extend, after flotation or washing of the sample. PI-staining of *Giardia* cysts in processed water samples would therefore not reflect the staining characteristics of the cysts in the sampled water. Sorting of *Cryptosporidium* oocysts that are stained with PI and the MAb-FITC conjugate with the FACSort did not influence the percentage of PI-positive oocysts.
The percentage of PI-positive oocysts in natural surface water samples was determined at two sites: at the inlet (river water) and outlet of three subsequent storage reservoirs with a residence time of five to six months. Thirteen samples were taken at both sites from March-August 1995.
As the oocyst number per sample was low, data from all samples were pooled. Nine of 23 oocysts (39%) detected in the river water samples were PI-positive. After reservoir storage, 19 of 25 oocysts (76%) were PI-positive.

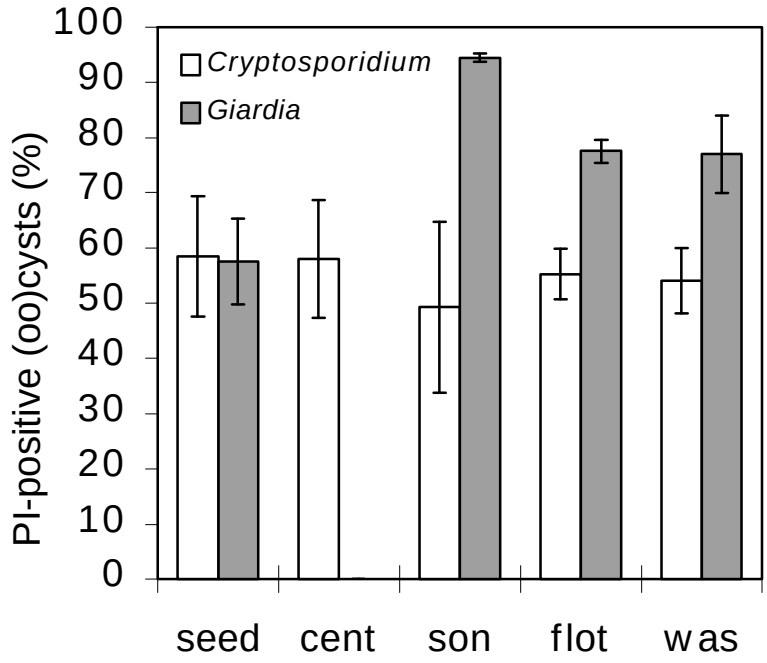

Figure 2. Percentage of PI-positive oocysts before (seed) and after sample processing by centrifugation (cent), sonification (son), Percoll-sucrose flotation (flot) and washing in PBS with Tween and SDS (was).

DISCUSSION

The results clearly demonstrate that incorporation of fluorescence-activated cell sorting improves the sensitivity of the immunofluorescence detection method for *Giardia* and *Cryptosporidium* in water samples: the FACSort method yielded more positive samples for both protozoa and higher *Giardia* concentrations than the direct microscopic method. The FACSort could separate fluorescing (oo)cysts from most

other particles in concentrated water samples. The microscopic preparations were much more easily and rapidly screened for the presence of the parasites. The removal of most of the debris also made it easier to evaluate the morphological features of the (oo)cysts, and thereby to discriminate between characteristic and non-characteristic fluorescing particles. The difference between the methods for *Cryptosporidium* detection was not as distinct as for *Giardia* and was most pronounced in seeded samples. Seeded samples contained brightly fluorescing oocysts, while naturally contaminated samples also contained oocysts with a weak fluorescence. Weakly fluorescing oocysts may have fallen below the lower FL1-treshold level of the sort region, even though the FACSort sort region is set broadly around the cluster of the Moredun MRI *Cryptosporidium* oocysts. A weak fluorescence of these oocysts is probably caused by a reduced number of epitopes that can react with the monoclonal antibody. This can be the result of strain differences in the reactivity with the monoclonal antibody or by changes in the oocyst wall when oocysts are exposed to environmental stress. In environmental surveillance, these weakly fluorescing oocysts may go undetected by both methods.
This study showed that the FACSort made it possible to apply PI-staining on environmental samples to determine the fraction of dead (oo)cysts. Sample processing did not appear to influence the PI-staining characteristics of the *Cryptosporidium* oocysts, but did increase the percentage of PI-positive *Giardia* cysts. This suggests that the combination of FACSort and this protocol can be used for determining the percentage of dead *Cryptosporidium* oocysts in environmental samples, but not for *Giardia* cysts. This type of data is essential for determining the validity of viability assays on environmental (oo)cysts, and should preferably include also various strains and ages of (oo)cysts.
Although the total number of oocysts detected was low, the data on environmental samples suggest that reservoir storage increases the percentage of PI-positive (dead) oocysts.
The purification with flow cytometry may also prove useful as pre-treatment for cell culture and PCR assays to concentrate the (oo)cysts and remove toxic or inhibitory compounds in the concentrated water samples.

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Assessment of the risk of infection by *Cryptosporidium* or *Giardia* in drinking water from a surface water source

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October 13, 1998

Abstract

The significance of the presence in drinking water of the protozoan microparasites *Cryptosporidium parvum* and *Giardia lamblia* for public health may be analyzed by means of risk assessment. This requires quantitative knowledge of all the contributing factors, from the concentration of these organisms in the source water to the dose response relation for the probability of infection or disease in a human host.

The major contributing factors are: the concentration of cysts or oocysts in raw water, the recovery of the detection method, the viability of recovered cysts or oocysts, the removal of organisms in the treatment process, and the daily consumption of unboiled tap water. To enable analysis of the uncertainty in the calculated risk of infection, each of these factors is treated as a stochastic variable, for which a suitable distribution is proposed. A frequency distribution for the probability of infection is then constructed with standard sampling techniques.

This first evaluation of the calculation of the risk of infection due to exposure to *Cryptosporidium* oocysts and *Giardia* cysts via drinking water, shows that the uncertainty in the estimated removal efficiency of the treatment process dominates over uncertainties in other contributing factors.

Short title drinking water risk assessment

Keywords drinking water, water treatment, infection, risk assessment, uncertainty analysis ,*Cryptosporidium*, *Giardia*

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List of symbols

C	Concentration of pathogenic microorganisms
R	Recovery of the detection method
I	Fraction of the organisms capable of infection (viability)
DR	Decimal reduction of the treatment process
V	Daily individual consumption of unboiled drinking water
P^*_{inf}	Unconditional probability of infection
r	Infectivity parameter of the exponential dose response model
ρ	Cluster parameter of the negative binomial distribution
p	Binomial probability of success
n, k	Numbers of independent (Bernouilli) trials and of successes
λ	Poisson parameter (mean number of organisms in a sample)
θ	Fraction of zero's in Poisson–with–added–zero's distribution
α, β	Parameters of the Beta distribution
$\hat{\alpha}, \hat{\beta}$	Maximum likelihood estimates of α and β
ℓ	Log–likelihood function
$\hat{\ell}$	Maximum value of ℓ for a given model and a given set of observations
$\chi^2_{df,p}$	Upper percentile p of the χ^2 distribution with df degrees of freedom

Introduction

Drinking water has since long been recognized as a potential vector for the transmission of communicable diseases (Andersson and Stenstrøm, 1986; Galbraith *et al.*, 1987; Benton *et al.*, 1989; Craun, 1991). Although concentrations of pathogenic microorganisms may be quite low, drinking water reaches almost every member of a population, so that even a quite low risk of infection may affect a significant number of consumers. Since drinking water is also a homogeneous medium, estimation of the concentration of pathogenic micro-organisms that eventually reach a tap in a household is feasible. This greatly facilitates estimation of the ingested dose. For many food products this information would be much more complicated to get by.

In this paper, we will focus on the assessment of the risk of infection by *Cryptosporidium* and *Giardia* via drinking water from a surface water supply. Infection by either of these organisms may lead to gastro-enteritis with watery diarrhea, vomiting, and abdominal pain. In healthy individuals cryptosporidiosis is usually self–limiting, with a duration of less than one month. Giardiasis is often asymptomatic, but infection may also be chronic. Immunodeficient persons (young children, elderly persons, those receiving immunosuppressive treatment, AIDS–patients) may have difficulty in clearing the parasites, leading to prolonged diarrhea, which may be life–threatening (Benenson, 1990).

Both *Cryptosporidium* and *Giardia* are currently considered of major importance for drinking water safety, both because of their high infectivity and because of their resistance to chemical disinfection. Concentrations of *Cryptosporidium* at levels below the detection limit of current methods have been reported to lead to waterborne outbreaks (Goldstein *et al.*, 1996). The outbreak of waterborne cryptosporidiosis in 1993 in Milwaukee, WI, which affected over 400,000 people, resulting in many deaths, has had a profound impact on risk awareness in the drinking water community (Mackenzie *et al.*, 1994).

Recent developments in dose-response assessment for pathogenic microorganisms have opened opportunities to analyze the impact of waterborne pathogens on public health in a quantitative manner (Regli *et al.*, 1991; Gerba and Rose, 1993; Rose *et al.*, 1993). Knowledge of dose-response relations enables characterization of the significance of an organism for public health, by means of one or more of its effects in humans (Haas, 1983; Haas *et al.*, 1993). This approach offers promising opportunities for public health management, like the interpretation of pathogen concentrations in terms of health risks, or a rational approach to weighing of risk factors.

In order to obtain reliable risk estimates, the individual dose must be estimated. Furthermore, the response, i.e. the proportion of subjects showing effects when exposed to this dose, must be estimated from dose-response

information.

For a given surface water supply, the individual daily dose may be calculated as:

$$\text{Dose} = \text{C} \times 1/\text{R} \times \text{I} \times 10^{-\text{DR}} \times \text{V}$$

C Concentration of pathogenic micro-organisms in untreated (surface) water (or partially treated water, if data are available)

R Recovery of the detection method

I Fraction of the detected pathogens that is capable of infection (viability)

DR Removal or inactivation efficiency of the treatment process, expressed as its Decimal Reduction factor (DR = 0 when concentrations in drinking water are used)

V Daily individual consumption of unboiled drinking water

When the dose is known, the probability of infection by *Cryptosporidium* oocysts or *Giardia* cysts may be calculated with the dose-response relation of the exponential model (Haas, 1983; Haas *et al.*, 1993):

$$P^*_{\text{inf}} = 1 - e^{-r \times \text{Dose}}$$

$P^*_{\textbf{inf}}$ probability of infection

r dose response parameter

Or, in low-dose approximation:

$$P^*_{\text{inf}} \approx r \times \text{Dose}$$

In the present paper we specifically address some of the factors that contribute to the dose estimate, and attempts are made to evaluate the uncertainty in each of these factors. This will be illustrated with data on two protozoan parasites, well known for their transmission via the drinking water route: *Cryptosporidium parvum* (Mackenzie *et al.*, 1994) and *Giardia lamblia* (IsaacRenton *et al.*, 1994).

For both organisms a dose-response relation for infection in humans has been published (Haas, 1983; Regli *et al.*, 1991; Haas and Rose, 1994). This enables the translation of the calculated doses into probabilities of infection. With the risk of infection, the effects of both organisms (transmitted via the drinking water route) on public health may be assessed.

For this exercise, we have used data from different, nonconnected locations for the estimation of the factors contributing to the dose, with emphasis on the quality of the data sets for statistical analysis. Therefore, we feel that the results are indicative for a typical situation of a consumer drinking water produced from a surface water source, rather than an evaluation of the situation at a specific, existing location.

Methods

We started with looking for suitable data sets for the occurrence of *Cryptosporidium* and *Giardia* in the source water. In order to enable quantitative conclusions, a sufficient number of positive (i.e. nonzero) samples must be available. Since recoveries of the available standard detection procedures are often quite low (Medema *et al.*, in prep.), measurements enabling estimation of the recovery must also be present, preferably for the same experimenter, performing under the same circumstances with exactly the same procedures for concentration and detection as used for the source water samples.

The data used here were collected at the outlet of the Biesbosch (NL) storage reservoirs. Water from the river Meuse (NL) is stored in these reservoirs (mean storage time 5 months) to be used for drinking water production, by several treatment plants, for some major centers of population in the Netherlands (Knoppert and Vreedenburgh, 1982). During a 1-year period, concentrations of *Cryptosporidium* and *Giardia*, as well as a number of relevant additional parameters (microbiological and chemical) have been measured at the inlet (the river Meuse at Keizersveer (NL)) and at the outlet of the storage system. The results of this study, and their interpretation (estimated removal of protozoan cysts or oocysts by storage) are reported in a separate paper (Medema *et al.*, in prep.).

The outlet of the storage reservoirs is the last stage for which (oo)cyst numbers may be directly determined. At later stages of the treatment chain, their concentrations are below the limit of detection. Therefore, the numbers of cysts or oocysts in this water are taken as the basis for our risk calculation.

Data Collection

Source water

The occurrence of *Giardia* cysts and *Cryptosporidium* oocysts in water at the exit of the storage reservoirs has been monitored over a complete one year period (Medema *et al.*, in prep.). Sufficiently large sample volumes have been taken, to obtain a considerable proportion of positive (i.e. nonzero) organism counts. The sample volume typically was 2,000 liters (1,000 liters for each species). Protozoan cysts or oocysts were concentrated with a standard procedure involving centrifugation, flotation, filtration, labeling with monoclonals, and microscopic analysis (Medema *et al.*, in prep.).

Recovery

During the data collection period, the recovery of the method, used for concentration and detection of cysts and oocysts, was determined at regular intervals. Recoveries of the detection method were estimated by means of

spike experiments. A sample from a stock suspension containing a known number of cysts or oocysts per unit volume were added to the water before passage through the filter unit (1,000 - 10,000 cysts or oocysts added). This water sample was then treated as usual. The counts from such a filter permit calculation of the ratio of numbers of cysts or oocysts detected to numbers of cysts or oocysts seeded: the recovery (Medema *et al.*, in prep.).

Viability

After prolonged exposure to all kinds of environmental factors during storage (like radiation, mechanical agitation, or microbial digestion), not every oocyst or cyst will retain the ability to excystate. One measure for the amount of damage inflicted upon the organisms, is the presence of a normal or "viable type " morphology, as observed with Nomarski DIC (differential interference contrast) optics.

Published data on the viability of both *Cryptosporidium* oocysts and *Giardia* cysts from surface waters based on morphological criteria have been used as an example in this paper (LeChevallier *et al.*, 1991).

Treatment efficiency

In the finished water, direct detection of pathogenic organisms is virtually impossible (Regli *et al.*, 1991). Therefore, a "surrogate" variable is needed, to estimate the removal of *Cryptosporidium* oocysts and *Giardia* cysts in a treatment process. Spores of sulphite reducing clostridia (SSRC) are presumed to be suitable candidates for this purpose, because of their morphological similarity to protozoan cysts or oocysts, and their environmental robustness. These spores may be detected and enumerated much easier than their protozoan counterparts. Data on numbers of SSRC in raw and treated water of a plant using stored water from the Biesbosch reservoirs, have been provided for the "Braakman" plant, by N.V. Delta Nutsbedrijven. In this plant, the outgoing water of the storage reservoirs is treated in a coagulation/flotation/filtration process. The data set we used was collected in 1988, the first year the plant was in function. For our purpose, this is a suitable dataset, because the SSRC counts in the source water were fairly high (up to 425 / 100 ml), thereby facilitating estimation of their removal. In later years the source water quality has improved ($<$ 42 SSRC / 100ml in the past year). This limits the estimation of removal properties to rather low values (approximately 2.3 log-units with standard sample volumes).

Consumption

When the concentration of pathogenic microorganisms in the drinking water is known, the dose (the amount an individual is exposed to) is determined

by the volume of unboiled water that is consumed. In previous publications dealing with microbiological risk via drinking water, a daily consumption of 2 liters has been used (Regli *et al.*, 1991). A detailed U.S. survey on water consumption has been published (Roseberry and Burmaster, 1992), indicating a median consumption of 0.96 liters daily. A less detailed report for the Netherlands indicates a much smaller daily individual consumption of about 0.25 liters (Haring *et al.*, 1979). We had the opportunity to add a question on the consumption of unboiled water to a food intake population survey in a medium sized city in the Netherlands. Full results will be published elsewhere, but preliminary analysis confirms the smaller amount of water consumed daily, as compared to the U.S.

Dose response

Calculation of the risk of infection from the ingested dose requires dose-response information. For both *Cryptosporidium* and *Giardia* the dose-response relation for infection has been investigated in human feeding studies (Rendtorff, 1954a; DuPont *et al.*, 1995).

Data analysis

Source water

Since the Poisson distribution appeared not to provide a satisfactory fit to the data on the occurrence of *Cryptosporidium* oocysts and *Giardia* cysts in the stored river water, frequency distributions for numbers of organisms per sample volume (k) were fitted with the negative binomial distribution (El-Shaarawi *et al.*, 1981; Pipes *et al.*, 1977):

$$P(k \mid p, \rho) = \left(\begin{array}{c} k+\rho-1 \\ \rho-1 \end{array} \right) p^{\rho}(1-p)^{k}$$

with cluster parameter ρ and binomial probability p. Parameter values were estimated with a maximum likelihood procedure.

In addition to this, the Poisson–with–added–zero's distribution was used (El-Shaarawi, 1985), because of the considerable amount of samples with zero counts.

$$\begin{aligned} k = 0: &\quad P(k \mid \lambda, \theta) = \theta + (1-\theta)e^{-\lambda} \\ k > 0: &\quad P(k \mid \lambda, \theta) = (1-\theta)\frac{e^{-\lambda}\lambda^{k}}{k!} \end{aligned}$$

With θ the fraction of zero's, and λ the Poisson parameter (mean of the nonzero counts). Goodness of fit for the negative binomial distribution and the Poisson with added zero's distribution were compared using the likelihood ratio.

The data seemed to show seasonal fluctuations: in the summer numbers of organisms were lower than in winter (Medema *et al.*, in prep.). For this reason we divided the data set into two: winter (weeks 1-12 and 36-52) and summer (weeks 13-35) samples. These were subjected to the same treatment as the complete data set.

Recovery

For the recovery, a data set consisting of pairs of counts —numbers seeded and numbers recovered— was available. Each organism in the seeded sample is considered to have a probability p of being recovered. Between pairs, this probability is allowed to change, so that each trial is governed by a different probability of detection (i.e. recovery). Assuming this probability is Beta distributed, the number of detected organisms will follow a BetaBinomial distribution (Stuart and Ord, 1987):

$$P(k \mid n, \alpha, \beta) = \left(\begin{array}{c} n \\ k \end{array} \right) \frac{B(k+\alpha, n-k+\beta)}{B(\alpha, \beta)} \tag{1}$$

Each trial consists of a number n of added organisms, a number k recovered, and a Beta-distributed binomial probability p of success (i.e. being recovered), with parameter α and β. This approach has a few advantages over standard methods using the quotient of recovered and added numbers. Most important: instead of assuming a single fixed recovery, variation between experiments is allowed to take place. This may be more realistic in the real world, where it is often impossible to keep all factors influencing the recovery constant. In addition, this approach directly leads to a probability distribution for the recovery. Methodological aspects are treated in some more detail in a separate paper (Teunis and Slob, subm.).

Viability

The data for the viability —defined for the moment as the fraction of cysts or oocysts showing a "viable type morphology"— also consist of pairs of observations: total numbers found (n), and numbers having the desired morphological features (k). Hence, these data may also be analyzed as a BetaBinomial distribution. Relative to the standard procedure, this removes a lot of noise: the numbers involved are quite small, and when one first calculates the fractions, a fraction calculated for a small number of organisms is weighed equally to one that is derived from a much larger number of observed organisms (1 out of 2 is 50 % , as is 10 out of 20). The BetaBinomial approach does not suffer from such effects.

Treatment efficiency

Like recovery and viability, the removal of cysts or oocysts in a treatment process may be viewed as a stochastic process (Teunis and Slob, subm.). The probability that an organism that has entered the process will be present at the exit, then represents the removal efficiency. Therefore, the BetaBinomial approach may be employed here, likewise. An additional advantage of this method is that zero's in the recovered numbers are not a principal problem, so there is no need to use threshold values when no organisms have been detected in a sample.

Dose response

For both *Cryptosporidium* and *Giardia*, the experimental data are fitted well by the simple exponential dose-response model, rather than the more elaborate beta-poisson model (Haas, 1983; Haas *et al.*, 1993; Haas and Rose, 1994). In the low dose approximation, the probability of infection is proportional to the dose, with the parameter of the exponential model as scale factor. For both organisms, a distribution of parameter values was constructed via (5,000) bootstrap replications of the original data set (Haas *et al.*, 1993).

Uncertainty Analysis

When a frequency distribution has been determined for each of the contributing factors, the distribution of the daily dose may be computed by means of random sampling from these distributions (Monte Carlo approach). Typically, 5,000 random samples have been drawn from the probability distributions for (oo)cyst concentration, recovery, viability, removal by treatment, and daily water consumption. All factors have been assumed to be statistically independent.

The source water concentration has been determined in a sample volume of 1,000 liters. At the low prevailing concentrations, this results in a high proportion of zero counts in such a volume. In a simple multiplicative calculation, as mentioned in the introduction, this would produce a high number of doses equalling zero.

In order to avoid truncation, we therefore proceeded as follows: a sample from the Negative Binomial distribution describing the number of organisms detected in 1,000 liters of stored water is drawn. Then a probability of recovery is drawn from the Beta distribution in question (figure 2). From these two samples, a corrected number of organisms was estimated, by considering the uncorrected number as the number of successes k and the probability p as the probability of success in an unknown number n of Bernouilli trials.

A realization for n may be obtained by sampling from the binomial distribution with n trials, probability p, and k successes.

$$P(k \mid n, p) = \left(\begin{array}{c} n \\ k \end{array} \right) p^k (1-p)^{n-k}$$

with probabilities of success p, and numbers of successes k sampled from the distributions for the numbers of detected cysts or oocysts sampled from the distributions derived in the previous sections (see sections: results). For every p and k, this produces a sample number of cysts or oocysts present in 1,000 liters of water, n. These have been used for subsequent calculations leading to the daily dose.

The resulting corrected numbers of organisms per 1,000 liters may then be adjusted for viability, in a similar procedure. Now the number of organisms represents the number of trials, n, and the number of viables is the number of successes k. The probability of success (i.e. finding a viable type morphology), p, is drawn from the appropriate Beta distribution (figure 3).

At this point, we have a collection of samples representing numbers of viable type cysts or oocysts per 1,000 liters of water. From these, the actual dose has been calculated by simple multiplication with sampled probabilities of removal (or $10^{-\mathrm{DR}}$), and consumed volumes.

For the construction of a sample of dose-response parameter estimates, bootstrap replications of the dose response were used (Haas *et al.*, 1993). With these, the probability of infection may be calculated in a straightforward manner, with the low dose approximation:

$$P^*_{\mathrm{inf}} \approx r.Dose$$

with dose-response parameter r (Haas, 1983; Regli *et al.*, 1991).

Results

Oocysts and cysts in the stored water

Counted numbers in the stored water appeared to fit well with a negative binomial distribution, both for oocysts of *Cryptosporidium* and for cysts of *Giardia*.

The maximum likelihood $\hat{\ell}$) values for the Poisson–with–added–zero's (PZ) distribution did not point to a much better fit than the negative binomial distribution to data sets with many zero's, after storage (see table 1. For reasons of simplicity, we have dropped this PZ distribution, and used only samples drawn from the negative binomial distribution for further (uncertainty) analysis.

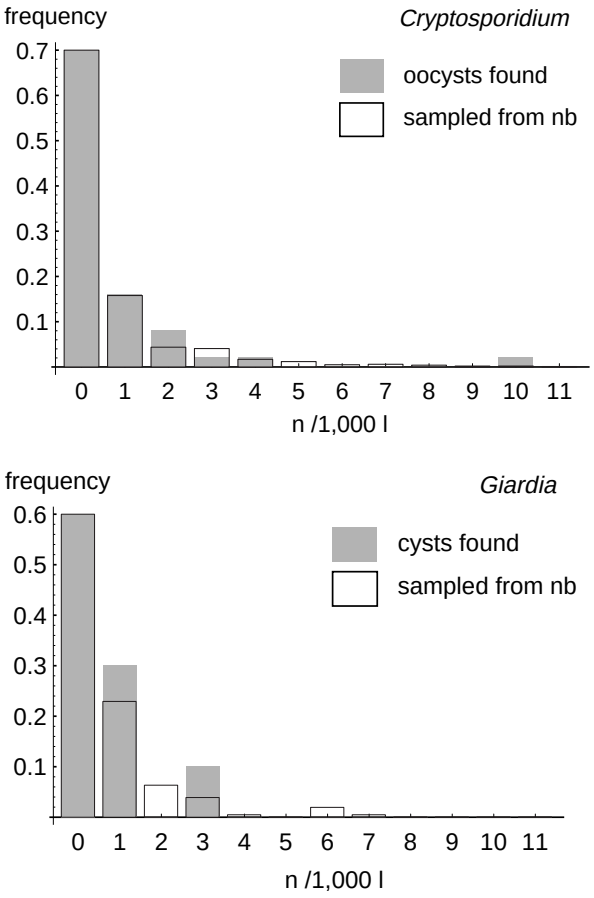

Figure 1: Histograms of samples drawn from the negative binomial distribution (nb) fitted to source water data (counted numbers of oocysts or cysts in 1,000 liters, also shown, as grey bars) for the complete one year sampling period. Parameter values are shown in table 1).

Cryptosporidium parvum						
	Negative Binomial			Poisson with Zero's		
period	$\hat{r}$	$\hat{p}$	$\hat{\ell}$	$\hat{\lambda}$	$\hat{\theta}$	$\hat{\ell}$
year	0.301	0.314	105.4	1.86	0.644	116.5
summer	1.348	0.789	39.3	0.526	0.315	39.6
winter	0.204	0.176	57.8	3.144	0.695	61.8

Giardia lamblia						
	Negative Binomial			Poisson with Zero's		
period	$\hat{r}$	$\hat{p}$	$\hat{\ell}$	$\hat{\lambda}$	$\hat{\theta}$	$\hat{\ell}$
year	0.338	0.513	71.4	1.027	0.688	71.4
summer	0.161	0.334	33.2	1.594	0.799	33.2
winter	1.346	0.802	36.0	0.606	0.450	35.6

Table 1: Parameter values for best (maximum likelihood) fit, for Negative Binomial and Poisson–with–added–Zero's distribution. The minimum value of -2 times the log-likelihood $\hat{\ell}$ is given for comparison of the two distributions. Results are shown for the complete dataset, –a one year period– and for summer and winter separately.

Differences between summer and winter data may be judged by comparing the sum of $\hat{\ell}$-values for summer and winter with the $\hat{\ell}$-value for the complete sampling period. The difference between the two likelihoods may be compared with the χ^2 with 4-2=2 degrees of freedom. The difference appears to be significant for *Cryptosporidium* ($\Delta\hat{\ell} = 8.3$, $p > 0.99$), but not for *Giardia* ($\Delta\hat{\ell} = 2.2$, $p < 0.70$).

Recovery

Usually, the recovery of a detection method is calculated as the ratio of cysts or oocysts found to cysts or oocysts expected. As explained above (section: methods for data analysis), we have used a different approach here, by treating the loss of cysts or oocysts as a stochastic process: an (oo)cyst may show up under the microscope, or it may fail to do so. This leads to a Binomial-Beta distribution for the recovery. The maximum likelihood estimates for the two parameters $\hat{\alpha}$ and $\hat{\beta}$ are given in table 2

The difference in $\hat{\ell}$-values, the (log-) likelihood ratio, may be compared with χ^2_{2-1}. The difference between simple Binomial (fixed recovery) and BetaBinomial (recovery changes with each experiment) then appears to be significant (more than 99.99% level) for *Cryptosporidium*, while it is not (below 90% level) for *Giardia*. Hence, for *Cryptosporidium* the hypothesis that the recovery varies between experiments, is not rejected, while for *Giardia* it is. The smaller variability in the fitted Beta distribution for *Giardia* (figure 2) is in agreement with this conclusion.

Recovery								
	Binomial	Beta Binomial		Fit		Range		
species	$\hat{p}$	$\hat{\alpha}$	$\hat{\beta}$	$\Delta\hat{\ell}$	sign.	P_5	P_{50}	P_{95}
Cryptosporidium	0.0127	2.749	181.4	28.0	+	0.004	0.013	0.032
Giardia	0.00619	9.206	1455.6	2.39	-	0.003	0.006	0.010

Table 2: Parameter values for best (maximum likelihood) fit for measurements of the recovery of the detection methods for *Cryptosporidium* and *Giardia*. Binomial model: fixed probability of recovery ($\hat{p}$, with likelihood $\hat{\ell}$), Beta Binomial: Beta distributed probability of recovery, with parameters $\hat{\alpha}$ and $\hat{\beta}$. Goodness of fit: compare the difference in $\hat{\ell}$, $\Delta\hat{\ell}$, with $\chi^2_{2-1,0.95}$=3.841 (sign.=+ $\rightarrow$ significance). Range: median and 90% range for recovery estimates, from the best fitting BetaBinomial distribution (percentiles at 5, 50 and 95% are given).

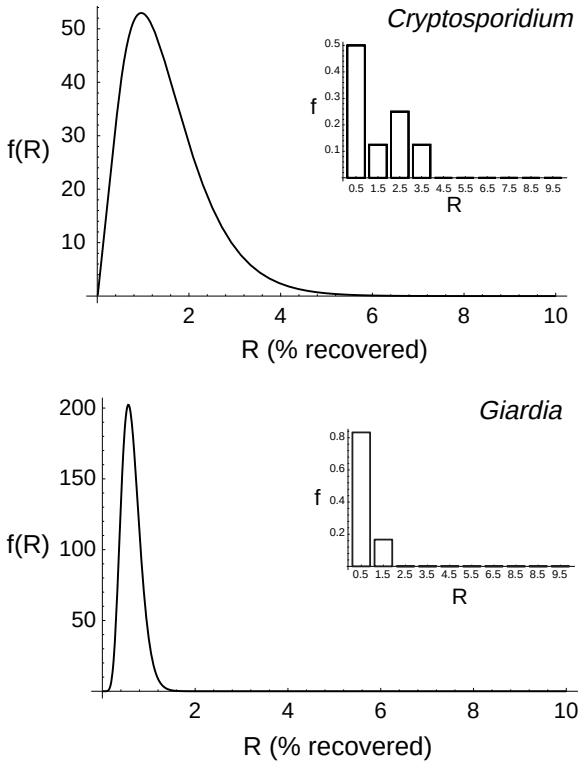

Figure 2: Recovery of the detection method for *Cryptosporidium* oocysts and *Giardia* cysts in the stored water: shape of the best fitting Beta distribution for the binomial probability of detecting an (oo)cyst (see table 2). Insets show histograms of the recovered fractions, calculated from data pairs.

Viability								
	Binomial	Beta Binomial		Fit		Range		
species	$\hat{p}$	$\hat{\alpha}$	$\hat{\beta}$	$\Delta\hat{\ell}$	sign.	P_5	P_{50}	P_{95}
Cryptosporidium	0.384	1.65	2.46	16.3	+	0.08	0.39	0.78
Giardia	0.129	2.93	17.4	9.3	+	0.04	0.13	0.29

Table 3: Parameter values for best fit (MLE) of fixed Binomial probability and Beta Binomial parameters to viability data of (LeChevallier *et al.*, 1991). Likelihood ratio test for improvement in fit: compare the difference in $\hat{\ell}$ for fixed and beta distributed binomial p with χ^2_{2-1}. Range: median and 90% range for viability estimates, from the best fitting BetaBinomial distribution (percentiles at 5, 50 and 95% are given).

In figure 2 the shape of the Beta distribution for the probability p of an (oo)cyst being recovered is shown, for the maximum likelihood parameter values $\hat{\alpha}$ and $\hat{\beta}$. The median, and the 5% and 95% percentiles are given in table 2

Viability

For the estimation of the fraction viable cysts or oocysts, we have used published data from (LeChevallier *et al.*, 1991). This type of data may be summarized as percentages viable organisms. This has the disadvantage that each pair of counts (total versus viable) receives the same weight, regardless of the total number of organisms that were found. The insets in figure 3 illustrate how this may lead to overrepresentation of 0, 50, and 100% values. Here the use of a binomial distribution with beta distributed probability of success, i.e. having the right morphology, provides a better alternative. Compared to a fixed probability of success, the beta-binomial distribution provides a significantly better fit (better than 99.99 % significance level judged by likelihood ratio). See table 3 for details. The shapes of the best fitting Beta distributions are given in figure 3, table 3 lists median values and boundaries of a 90% interval.

Decimal Reduction

As yet, we do not have measurements on the removal efficiency of protozoan cysts or oocysts in drinking water production facilities in the Netherlands. As mentioned above (section: data collection), spores of sulphite reducing clostridia (SSRC) are considered indicators for the (oo)cyst stages of protozoan parasites, because of similarities in size and shape, and because these spores are quite persistent in the environment. If we consider this a valid assumption, the removal efficiency for protozoan cysts or oocysts may be estimated by using the reduction in SSRC counts.

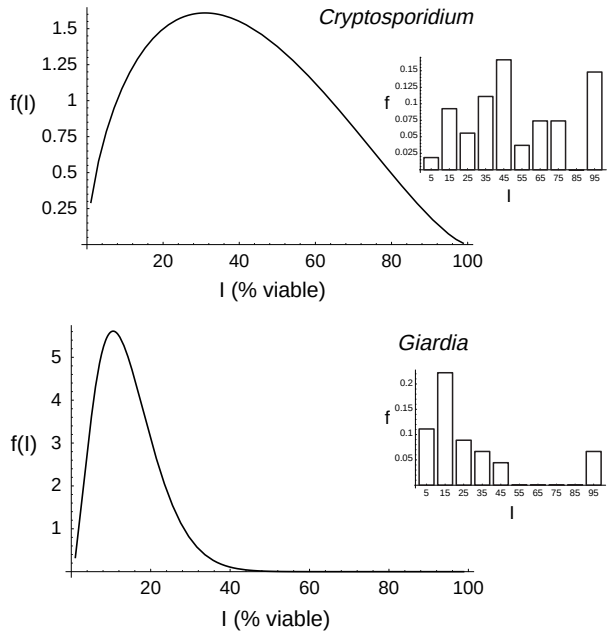

Figure 3: Best fitting Beta distribution for probability of finding an (oo)cyst with viable type morphology (see table 3). Insets show histograms of viable type fractions, calculated from data pairs.

Removal								
	Binomial	Beta Binomial		Fit		Range		
species	$\hat{p}$	$\hat{\alpha}$	$\hat{\beta}$	$\Delta\hat{\ell}$	sign.	P_5	P_{50}	P_{95}
SSRC	0.0016	0.0255	4.107	174.55	+	1.64	12.6	51.8

Table 4: Parameter values for best fit (MLE) of Binomial probability and Beta Binomial parameters to SSRC data for the Braakman treatment plant. The probability that a spore succeeds in passing the process, is p. When this probability is Beta distributed, with parameters α and β, a significantly better fit is possible. Range: median and 90% range for estimated removal of spores from sulphite reducing clostridia, from the best fitting BetaBinomial distribution (percentiles at 5, 50 and 95% are given). The removal of spores is expressed here as their decimal reduction, or $-^{10}\log(p)$.

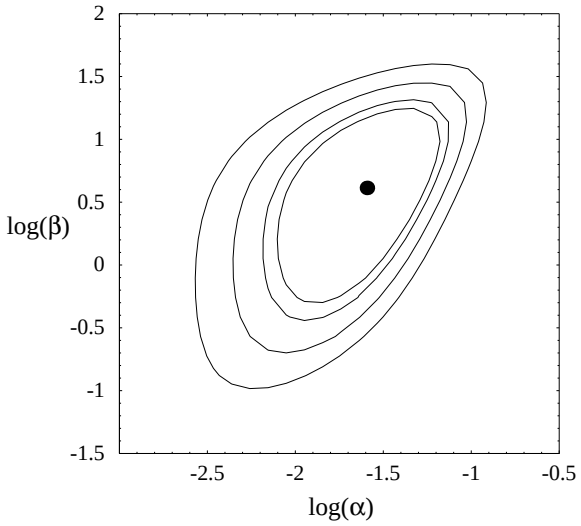

Figure 4: Confidence interval for the parameters α and β of the Beta Binomial distribution, fitted to the data on removal of SSRC (see also table 4). The dot marks the position of the MLE (maximum likelihood estimate), the concentric area's show 99.9, 99, 95, and 90 % confidence regions, respectively, outward from the MLE-point.

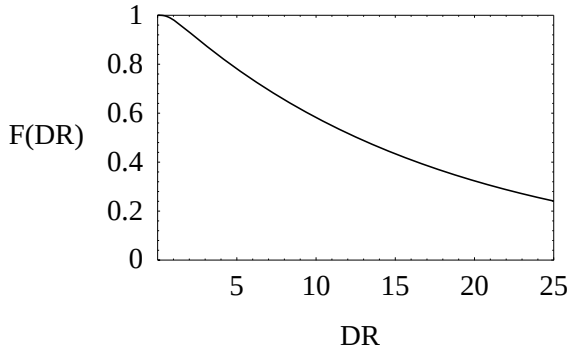

Figure 5: Decimal reduction for spores of sulphite reducing clostridia (SSRC): shape of the best fitting Beta distribution for the binomial probability of an (oo)cyst leaving the treatment process when it has entered (see table 4).

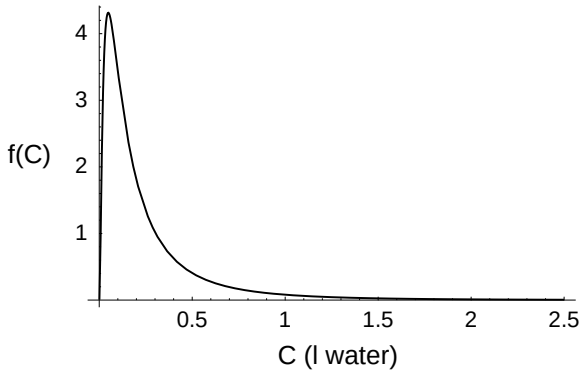

Figure 6: Shape of the Lognormal distribution fitted to the data from the population survey in the Netherlands.

The probability of a spore passing the treatment process may be assessed in two ways: as a fixed binomial probability of success, leading to a single estimated value $\hat{p}$. The assumption that the performance of the process is constant may not be very realistic, however. When p is allowed to vary between measurements, a significant improvement in goodness of fit is the result (compare log-likelihood values $\hat{\ell}$ in table 4). Confidence regions for the parameters (α, β) of the Beta distribution for p indicate a regularly shaped likelihood plane with no strong correlation between $alpha$ and β (figure 4).

Removal of micro-organisms may also be expressed as decimal reduction. These may be easily calculated from p: DR=-$^{10}\log(p)$. The best fitting Beta distribution appears to be quite wide on a log scale, as may be appreciated from table 4 and figure 5.

Consumption

A maximum likelihood procedure was used to fit a lognormal distribution to the observed consumption data. The Kolmogorov-Smirnov test indicated acceptable fit ($\alpha = 1 - P(D_n \leq d) > 0.20$) (Hogg and Tanis, 1988). Published data for the U.S. also appeared to fit well with a lognormal distribution (Roseberry and Burmaster, 1992). The median amount of consumed water is lower, however: in the Netherlands 0.153 liters/day vs. 0.963 liters/day in the U.S. (figure 6).

The dispersion in the daily consumed water volume exceeds that of the U.S. population study. The width of the lognormal distribution may be conveniently expressed by means of an uncertainty factor (Slob, 1994), e.g. the ratio between median and lower 2.5% quantile (which equals the ratio between upper 97.5% quantile and median). The distribution fitted to the Dutch data yields an uncertainty factor of 8.2 ; for the U.S. survey this un-

certainty factor is 3.1 .

Estimation of the daily dose

The information on (oo)cyst concentrations, recoveries, treatment efficiencies, viabilities, and the consumption of unboiled drinking water may now be used to calculate daily doses for both *Cryptosporidium* and *Giardia*. To this end, samples are drawn from the fitted probability distributions for each of these factors. With each of these consecutive samples a daily (oo)cyst dose may be calculated, with the procedure described in the section on uncertainty analysis.

This produces a collection of doses, the frequency distribution is given in figure 7.

Only dose estimates based on the concentrations pooled over the complete observation period are presented here. The uncertainty in the performance of the treatment process (over 50 log-units, see figure 5) dominates over all other factors, so that the frequency distributions for summer and winter separately appear to be quite similar to those in figure 7).

Risk Estimation

Sample distributions of probabilities of infection are shown in figure 8 for *Cryptosporidium* and *Giardia*.

Probabilities of infection have been calculated separately for summer and winter data, as well as for the pooled data set. Since the differences between summer and winter estimates appear to be quite small (data not shown), subsequent calculations are made only for pooled data.

Having estimated the risk of infection with both *Cryptosporidium* and *Giardia*, it is tempting to calculate the combined risk from these pathogens. Assuming independence, the risk of becoming infected with either organism or both may be calculated by considering the probability of not becoming infected at all.

$$P_{\text{inf}} = 1 - (1 - P_{cr})(1 - P_{gi}) = P_{cr} + P_{gi} - P_{cr}P_{gi}$$

Where P_{cr} and P_{gi} represent the probabilities of infection with *Cryptosporidium* and *Giardia*, respectively.

Figure 9 shows the probabilities of infection for repeated exposures. These have been calculated by resampling from the Monte Carlo distribution of daily (= single exposure) risks. This would presuppose a kind of ergodicity for the consumption of unboiled drinking water: averaging over an ensemble (the population) produces the same results as averaging over time for a single consumer. For instance, the contribution due to heavy consumers, that is: persons consuming a quantity in the upper region of the

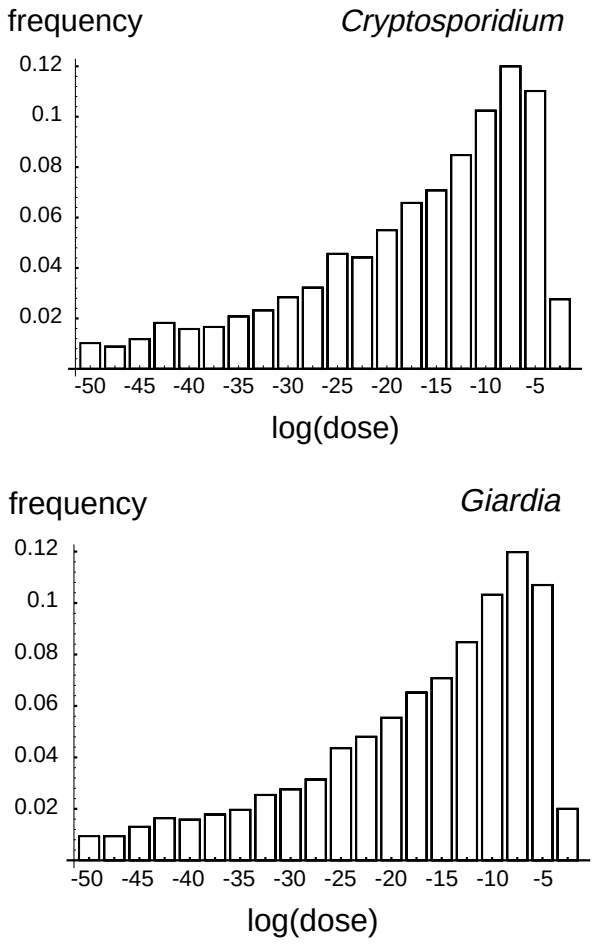

Figure 7: Distribution of estimated daily dose of *Cryptosporidium* oocysts and *Giardia* cysts, obtained by calculation from 5,000 Monte Carlo estimates of each of the constituting factors.

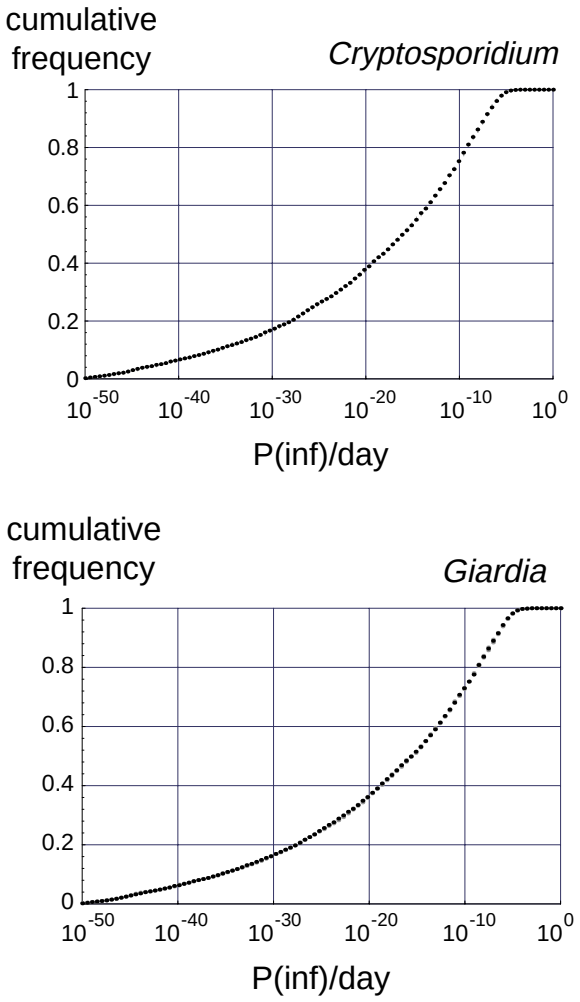

Figure 8: Cumulative distribution of estimated daily risks of infection with *Cryptosporidium* oocysts and *Giardia* cysts, based on the doses in figure 7.

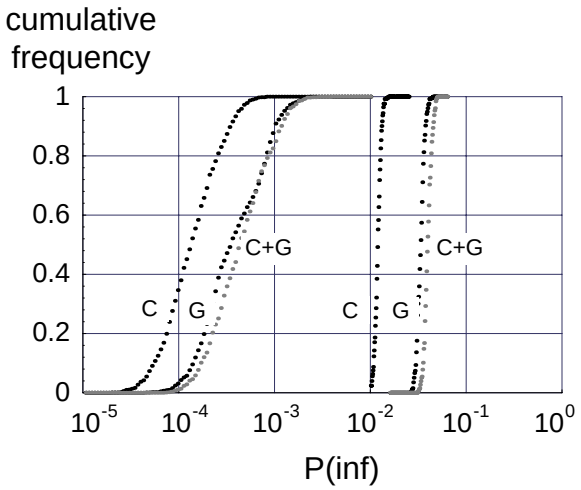

Figure 9: Cumulative distribution of estimated yearly risks of infection (365 exposures) with *Cryptosporidium* oocysts (C), or *Giardia* cysts (G), or both (C+G). Rightmost curves: lifetime risk (70 years) for *Cryptosporidium* oocysts (C), *Giardia* cysts (G), or both (C+G).

frequency distribution for the water consumption on any occasion during prolonged periods, would be neglected this way.

Discussion

In the present paper we have aimed at evaluating the complete chain of factors that leads from contaminated surface water to risk of infection via drinking water . The two protozoan parasites *Cryptosporidium parvum* and *Giardia lamblia* are interesting subjects for such an exercise, because of their significance for public health, and because their removal to acceptable concentrations is critical for many surface water treatment plants, especially when a generous safety margin is desired. In this perspective, the work described here should be regarded as a preliminary study, mainly to investigate problem areas and to test the feasibility of the chosen approach.

Occurrence

Surface water is an important source for the production of drinking water in the Netherlands. The high population density and intensive agriculture lead to potentially high levels of contamination in these waters, with microorganisms that are pathogenic to humans. The Biesbosch storage reservoirs are situated near the mouth of the river Meuse, which at this point has received (untreated) waste water from a number of major population centers.

Close to these reservoirs, a large proportion of the borders of the river Meuse are used for grazing cattle. Dairy calves are a well known non-human reservoir for *Cryptosporidium*. This is reflected in the numbers of cysts or oocysts that have been found in the inlet water of the storage reservoirs (Medema *et al.*, in prep.). The main function of the reservoirs is storage of river water so that transient contaminant peaks are buffered, and a reserve is available when the inlet has to be closed due to unacceptable contamination with hazardous substances (Knoppert and Vreedenburgh, 1982). At the same time, storage of the river water for a prolonged period acts to remove and/or inactivate pathogenic microorganisms entering with the river water. In the case of protozoan cysts or oocysts this leaves a minor fraction of the organisms suspended in the outlet water.

For the purposes of the present work, we were not only interested in characteristics like mean concentration and its standard deviation, but rather in the shape of the frequency distribution, and the value(s) of its parameter(s). Therefore, it is explicitly important to have access to original counts, rather than derived characteristics, like mean concentrations. The numbers actually counted are reported only rarely, thus rendering most literature data not clearly interpretable.

In outlet water, both the Poisson distribution with added zeros and the negative binomial distribution fit significantly better than the Poisson distribution (Medema *et al.*, in prep.). This is due to the large proportion of low counts and zero's. The *Cryptosporidium* data are fitted slightly better with the negative binomial distribution, while the *Giardia* data are fitted better with the Poisson distribution with added zero's, albeit to a slight degree (table 1).

Recovery

The low percentages recovered with the detection method for *Cryptosporidium* and *Giardia* cause a high proportion of zero counts in the source water samples. Both for *Cryptosporidium* and *Giardia*, the recovery appears to be quite constant, however, presumably due to careful experimentation with standardized methods. When an organism is Poisson distributed in the environment, and it is detected with a method that has a constant recovery, the counts will still be Poisson distributed, with a smaller mean (Fisher and van Belle, 1993). This remark holds as long as low recovery is not due to a high threshold, but rather to a low probability of detection, as proposed in the section on data analysis. The Negative Binomial distribution may be constructed from the Poisson distribution by assuming its parameter to be Gamma distributed (El-Shaarawi *et al.*, 1981). A constant probability of recovery would simply change the value of the scale parameter (p) of the Negative Binomial distribution. Therefore, when organisms are Negative Binomially

distributed in the source water, numbers in the recovered sample will also follow the Negative Binomial distribution, with same shape parameter (r) and transformed scale parameter (p).

Viability

Correction of the estimated concentrations for percentage viables has been based on viable type morphology (LeChevallier *et al.*, 1991). This is probably an overestimation of the actual viability of the cysts or oocysts to cause infection. A more reliable measure for the viability may be obtained examining vital dye exclusion, or by allowing the recovered cysts or oocysts to excystate *in vitro*, or even to assay their infectivity in an animal model. At present, no data are available to allow the analysis of such a more elaborate data set with the method presented here.

Water treatment

The drinking water plants that obtain their water from the Biesbosch storage reservoirs do not (yet) have data concerning the removal of pathogenic protozoa. Therefore, we had to resort to surrogate data. The distribution in figure 5 has been obtained by fitting a Beta Binomial distribution to a data set containing repeated measurements of the numbers of spores from sulphite reducing clostridia (SSRC) in a conventional treatment plant, using a coagulation/flotation/filtration process.

As stated earlier, our interpretation of the data from the treatment process allows for fluctuations in the efficiency of the process. The fitted Beta distribution may be interpreted as a frequency distribution of the probability that an organism penetrates the treatment process. This latter probability may be expressed in log-units, i.e. as a decimal reduction. On a log-scale, the probability distribution for the decimal reduction (figure 5) appears to extend over a quite wide range. In fact, the lower limit of the decimal reduction (table 4) is determined largely by the few non-zero measurements of SSRC in the finished water. Calculation of the decimal reduction from the mean of source water concentrations over the mean of finished water concentrations is equivalent to calculating the fixed binomial probability of passage ($\hat{p}$ in table 4). Expressed in log-units, this would amount to a decimal reduction of 2.8 . The median decimal reduction estimated from the fitted Beta-binomial distribution, is 12.6 , not a surprising result, with the highly skewed distribution in figure 5.

Inspection of the data set reveals that in only a few occasions, a non-zero count has been found in the finished water, whereas at the same time, source water counts were never zero. In most occasions, the process appears to work quite efficiently. Relatively rare are the occasions where failure occurs,

with a markedly decreased removal efficiency. Although these episodes of decreased removal efficiency occur with a low rate (once, or a few times a year), they have a profound influence on the decimal reduction. This leads to the conclusion, that this is a process with a good nominal performance. Infrequently, this performance degrades, and the process has a much lower efficiency. Nonetheless, these relatively rare occasions have a large influence on the risk of infection, due to the low background risk when the removal efficiency is at its nominal value.

With respect to risk analysis, it seems that the probability of occurrence of episodes of decreased removal efficiency should receive more attention. As treatment processes are improving more and more, their mean performance, according to design specifications, is likely to be well above that which is necessary to stay below specified risk standards (e.g. 1 infection in 10,000 per individual per year). If, however, due to some internal (mismanagement, instrument failure) or external (poor source water quality, excessive pathogen load, low temperature) cause, the performance degrades transiently, such an event may by itself determine the ensuing health risks almost entirely. Such an analysis would call for a completely different approach to the characterization of treatment processes for risk analysis.

We have not accounted for the addition of chemical disinfectants. In the treatment plant that produced the data used here, ozonation is presently used as a final treatment stage. For *Cryptosporidium* this may be of little importance, but *Giardia* cysts may experience additional inactivation when disinfectants are applied (up to 2 log-units (Finch *et al.*, 1993b; Finch *et al.*, 1993a)).

Dose response

Dose response data for infection with *Giardia lamblia* in humans were reported 40 years ago (Rendtorff, 1954b). The reported results clearly indicated high infectivity, i.e. a high proportion of subjects shedding newly formed cysts after a low ingested dose (3 - 10 cysts), but not one of the infected persons developed gastroenteric symptoms. Although infection with *Giardia* is usually asymptomatic in humans (Benenson, 1990), this seems to be at odds with the generally accepted significance of this organism as a major agent for waterborne gastro-enteritis. We follow the approach of Haas et al (Haas, 1983; Haas *et al.*, 1993; Regli *et al.*, 1991), who note that infection is a prerequisite for the development of illness. Therefore, risk estimates based on infection as an endpoint may overestimate the number of illness cases, and thus constitute a worst case.

Dose response data for infection with *Cryptosporidium parvum* in human volunteers have been published recently (DuPont *et al.*, 1995). The low dose infectivity of *Cryptosporidium parvum* appears to be about 5 times

(4.975×) lower than that of *Giardia lamblia* (Haas and Rose, 1994).

Both dose response data sets appear to be fitted well by the exponential dose response curve (Haas, 1983), rather than the more elaborate Beta-Poisson curve. This is a somewhat intriguing feature of dose response data for both protozoan parasites, since the Beta-Poisson model allows for variation in the probability of causing infection, once the organism has entered the host. Such variation may result from differences in infectivity between individual parasites, or variation in the degree of host resistance. It would seem that this variation does not show itself with these protozoan parasites, for some reason as yet unknown.

Risk of infection

Our estimate of the mean annual individual risk of infection for this (hypothetical) can be read from figure 9. The median individual probability of infection with *Cryptosporidium* is seen to equal just over 10^{-4}, for *Giardia* this is approximately 5×10^{-4}. Because the risk of infection with *Giardia* is higher that by *Cryptosporidium*, the annual individual probability of becoming infected with either organism (or both) is only slightly above that for *Giardia*.

Assuming mutual independence of all factors, the lifetime risk, for a person living 70 years, is easily calculated (figure 9). For *Cryptosporidium* this probability of becoming infected at least once equals just over 1 % . For *Giardia* this would be near 5 % .

The infection risks in figure 9 must be considered to represent a worst case scenario. If ozonation decreases the risk of infection with *Giardia* by, say 2 log-units, while the risk of infection with *Cryptosporidium* remains the same, then the annual risk of infection with *Giardia* would stay well below the 10^{-4} limit, and the risk of infection with both would be largely determined by that of *Cryptosporidium*.

Would such a risk of infection be acceptable, if it represented the real situation? If a person would drink water of this quality during his or her complete lifetime (70 years), the probability of experiencing infection with a waterborne protozoan parasite at least once would be almost 1 % .

The calculation of risk with repeated exposures may not be accurate, because we assumed that any person drinks a random volume of water, sampled from one and the same distribution. It is conceivable that in fact this distribution has arisen because a population consists of different individuals, each with their own typical consumption pattern. Tall adult persons may be expected to drink larger volumes than newborn infants. Considering those who drink a lot as the most vulnerable subpopulation (drinking large volumes of water on doctor's prescription, e.g. with a kidney disorder), our method of sampling may underestimate the health risks for such a subpopu-

lation.

Dose response information for both *Cryptosporidium* and *Giardia* is based on healthy adult hosts. From a public health perspective, this is not the most important group. Compared to newborns, elderly persons, and other risk groups, the risk of infection calculated with these data may be underestimated. On the other hand, this is only so when the probability of becoming infected is different for persons in a risk group. It is also conceivable that these persons at risk are just more likely to develop symptoms, once they have become infected. In that case, the assertion that the infection risk is by itself a conservative measure, is valid.

The main contribution to uncertainty in our calculations is the removal efficiency for spores of sulphite reducing clostridia in the treatment process. Without reliable data on the removal of *Cryptosporidium* oocysts and *Giardia* cysts in a drinking water production process, the value of calculations like these remains theoretical.

In a preliminary exercise, we used published data on the decimal reduction in a single treatment plant (plant 307) (LeChevallier *et al.*, 1991). This was the only occasion known to us where repeated measurements were reported for one and the same treatment plant, and for both protozoan parasites. No counts of organisms were reported, only processed data as decimal reductions expressed in log-units. This appeared to be a process with mean removal of only about 1 log-unit. Calculation of probabilities of infection led to daily, rather than annual risks of infection in the order of magnitude of 10^{-4} . Other publications report mean decimal reduction values of 5 or more (Payment and Franco, 1993).

Without any information on the frequency of occurrence of such removal efficiencies, the reliability of risk assessments based on these numbers remains unknown. Analysis of the removal of micro-organisms in a treatment process as a stochastic process, with each organism that enters having a certain probability of passing the process, easily leads to such information. We have demonstrated the usefulness of this approach (Teunis and Slob, subm.) by applying it to data from an existing treatment plant. The same method is also suited to quantify the fraction viable type organisms in a sample, or to quantify the recovery of a detection method.

The analysis of data on indicator organisms (SSRC) presented here, indicates that it may be the frequency with which a process of high specification may fail, that determines the health risks, rather than the mean (or median) decimal reduction for which the process was designed. Therefore, quantitative analysis of the properties of treatment processes should include the probability of failure, due to human factors (process operation) or non-human factors (instrument failure, excessive load, etc.).

Acknowledgements

This work was carried out on behalf of the Directorate General for the Environment, Ministry of Housing, Physical Planning and the Environment, The Hague, The Netherlands.

The data on reduction in numbers of spores of sulphite reducing clostridia in the “Braakman” plant were kindly provided by the laboratory of N.V. Delta Nutsbedrijven in Terneuzen, via the assistance of Dr. G.M.H. Suylen and H. Castelijns.

The Public Health Department of the Region “IJssel-Vecht” has granted the authors access to a public health survey (MORGEN project), resulting in valuable information on the consumption of drinking water in a medium-sized Dutch city. Their assistance is gratefully acknowledged, especially the help of Dr A. Baltissen.

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General discussion

At the onset of our research many reports on waterborne transmission of the parasitic protozoa *Cryptosporidium parvum* and *Giardia intestinalis* in the US and the UK had been reported, both through drinking water and through recreational waters. The principal question of our research was: are these protozoa a significant threat to the safety of drinking water in the Netherlands?
To answer these questions, the research was focussed on:

- the methodology to detect *Cryptosporidium* and *Giardia* in water
- the significance of the different sources of surface water contamination with *Cryptosporidium* and *Giardia*
- the fate of discharged *Cryptosporidium* oocysts and *Giardia* cysts in the surface water environment
- the presence and concentration of *Cryptosporidium* and *Giardia* in surface water at abstraction points for drinking water production
- the efficiency of surface water treatment in eliminating *Cryptosporidium* and *Giardia*
- the assessment of the risk of *Cryptosporidium* and *Giardia* in drinking water to public health

SOURCES OF *CRYPTOSPORIDIUM* AND *GIARDIA*

From published work it was clear that *Cryptosporidium* and *Giardia* could be present in domestic waste water, in farm animals and in wildlife. These were also considered potential sources of surface water contamination with these protozoa. However, very few quantitative data were available to determine the actual (oo)cyst load from these sources and thereby the relative significance of these sources. To obtain this information, we monitored the occurrence of protozoa in domestic waste water and effluent and used this as input for a discharge model. *Giardia* was consistently present in domestic waste water and effluent of sewage treatment plants, while *Cryptosporidium* was present in 65% of the settled sewage samples with occasional peak concentrations (up to 3.8×10^5 oocysts per litre). With the discharge model, PROMISE, the (oo)cyst discharges through treated and untreated domestic waste water could be estimated both on a national and a regional scale. On a national scale, treated waste water was responsible for 85% of the *Cryptosporidium* discharge to surface water, while 82% of the *Giardia* cysts was discharged with untreated waste water and sewer overflows. Although the load of cysts and oocysts through these discharges is significant, combined results from protozoa monitoring in surface water and a hydrodynamic dispersion model showed that these loads are outweighed by the import of *Cryptosporidium* and *Giardia* through the two large international rivers, Rhine and Meuse. This means that source water protection requires an international approach. Optimisation of pathogen removal by sewage treatment in the Netherlands (i.e. by ultrafiltration) will have effect on the microbial quality of surface water only on a local or regional scale. This may still be worthwhile, since it will reduce the pathogen loads to recreational waters and abstraction points for drinking water production that are under the direct influence of the sewage discharge, and thereby the health risk of bathing at these sites and the costs of drinking water treatment. However, most of the points of surface water abstraction are in waters that are under the influence of Rhine or Meuse. Reduction of the

pathogen concentrations at these sites, and thereby the costs of drinking water treatment, will require measures to install and improve sewage treatment both in the Netherlands and the countries upstream.

In this thesis we showed that wildlife, especially waterfowl, in the Netherlands infrequently carries *Cryptosporidium* and *Giardia* and can be a significant source of protozoa in pristine waters, such as water in pre-treatment reservoirs or reservoirs that recollect water after artificial recharge in the dunes. Although many of the cysts and oocysts that are shed by waterfowl will be non-pathogenic to man, the reported shedding of *C. parvum* oocysts that are infectious to mammals by Canadian geese indicates that the possibility that waterfowl is a source of *Cryptosporidium* oocysts that are pathogenic to man cannot be neglected. This stresses the need for development of methods to discriminate between pathogenic and non-pathogenic cysts and oocysts in environmental samples. So, on the one hand wildlife has the potential to contaminate pristine waters with pathogens such as *Cryptosporidium* and *Giardia*, but also *Campylobacter* and other bacterial and possibly viral zoonosis. On the other hand, the water companies have invested in recent years to improve the ecological values of their watersheds, like improvement of bank vegetation and introduction of grazers. This has resulted in an increase in the species and number of animals that visit or live in or around these waters. From the viewpoint of hygiene, the lower the numbers of wildlife around pristine watersheds, the lower the load of zoonotic pathogens. This appears not to comply with a “green image”, but there are some relatively simple measures to reduce peak loadings such as scraping of droppings from banks and especially from an ice cover of reservoirs or the temporary exclusion of new-born ruminants from the zone adjacent to the watersheds.

FATE OF *CRYPTOSPORIDIUM* AND *GIARDIA* IN THE SURFACE WATER ENVIRONMENT

Several authors had shown that *Giardia* cysts and especially *Cryptosporidium* oocysts can survive for several months in water. These studies were done, however, under laboratory conditions in controlled environments without the presence of the microflora that is normally present in surface water. Our experiments in both sterile and natural water showed that the presence of the autochtonous microflora increased the inactivation rate of *Cryptosporidium* oocysts at a water temperature of 15°C, but not at 5°C, when biological activity is low. But even in the presence of the microflora, the inactivation of oocysts is slow: 0.01 ^{10}log per day at 5°C and 0.018 – 0.024 ^{10}log per day at 15°C, and will result only in significant die-off in aquatic habitats with a residence time of more than 3 months (pre-treatment reservoirs, sediment in rivers and streams, soil passage).
The sedimentation experiments showed that the sedimentation rate of free, suspended *Giardia* cysts and *Cryptosporidium* oocysts was too low to affect the transport of (oo)cysts through the aquatic environment significantly. However, the same studies showed that cysts and oocysts rapidly attach to particles in sewage effluent and that the sedimentation rate is determined by the characteristics of the particles to which the (oo)cysts attach. This indicates that sedimentation does occur in rivers, lakes and reservoirs and that the sediment may be a reservoir of

Cryptosporidium and *Giardia.* Resuspension of the sediment by activity of bathers or animals, ships, wind or changes in water flow may give rise to short peaks in the concentration of (oo)cysts in the water.

CRYPTOSPORIDIUM AND *GIARDIA* IN SOURCE WATERS

The main question of the water industry at the onset of this work was: are *Cryptosporidium* and *Giardia* present in our source water and in what concentrations? We have studied the occurrence of *Cryptosporidium* and *Giardia* at the largest abstraction point for drinking water production in the Netherlands (chapter 7) and have shown that the concentrations are relatively high in winter and early spring and low in summer. At this study site, there was a strong correlation between the concentration of *Cryptosporidium* and water temperature, river flow, turbidity and the concentration of faecal indicator bacteria. For *Giardia*, this correlation could be demonstrated for the second half of the year only. The concentration of *Cryptosporidium* showed short peaks and most of these peaks coincided with a peak in turbidity. This information can be used for selective intake of river water into the reservoirs: stopping the intake at periods of high turbidity will reduce the *Cryptosporidium* load into the reservoirs.
Several other abstraction sites in the Netherlands have been monitored for the presence of *Cryptosporidium* and *Giardia*. We have linked the concentration at these sites to the discharges of treated and untreated sewage and import through the large rivers with the emission/dispersion models. This allowed us to determine the most important sources of contamination for the individual abstraction sites and to determine the effect of source protection measures (improved sewage treatment, removal of sewer overflows) on the water quality at the individual sites.

EFFICIENCY OF WATER TREATMENT

With the data on the presence of *Cryptosporidium* and *Giardia* in source waters available, the next question was: is our treatment system equipped to eliminate these protozoa to negligible risk levels?
In this work, we used spores of sulphite-reducing clostridia as a surrogate for describing the removal of persistent micro-organisms by treatment. The data on the elimination of spores under full scale conditions showed that the elimination capacity of a treatment process varies considerably. We showed that, fortunate-ly, the variation in one unit-process is in part counteracted by the variation in the next unit-process. Hence, the variation in a treatment system with multiple barriers is smaller than expected from the variation in the individual processes.
This means that treatment efficiency and the variation therein is best assessed by monitoring the concentration of the surrogate parameter (i.e. spores) in both source and treated water

RISK ASSESSMENT

To enable us to determine the risk of infection with *Cryptosporidium* or *Giardia* through drinking water, we have developed a risk assessment strategy. Exposure assessment was performed by integrating information on protozoa concentrations in source water, the recovery efficiency of the detection method, the viability of (oo)cysts in natural waters, the treatment efficiency and the consumption of unboiled drinking water. The combination of exposure data with dose-response models for *Cryptosporidium* and *Giardia* yielded an estimate of the risk of infection through drinking water at a specific treatment plant. The probabilistic approach that was taken allowed us to determine the uncertainty of the risk estimate and the contribution of the uncertainty of all the individual factors to the overall uncertainty of the risk estimate. The uncertainty in the treatment efficiency dominated over the uncertainty in other factors. This uncertainty is partly due to the variation of the treatment system itself, but mainly to the poor data to describe the elimination of micro-organisms by treatment processes.

DETECTION METHODOLOGY

The presence of debris, algae and other biological particles in the microscopic preparations of water concentrates, hindered the microscopic examination. This made identification of *Cryptosporidium* oocysts and *Giardia* cysts inaccurate and labour-intensive and thus costly. The incorporation of a flow cytometer that could selectively sort (oo)cyst-like particles onto a membrane resulted in much ‘cleaner’ preparations, in which the features of the cysts and oocysts could be interpreted more unambiguously. We have developed this application for a FACSort flow cytometer, resulting in a reduction of the time needed for microscopic analysis and an improved accuracy of the counts. The preparations were clean enough to be able to use vital dyes to test the viability of the (oo)cysts. Sample processing did not appear to alter the PI-staining characteristics of oocysts, so this protocol can be used to determine the presence of potentially viable and dead oocysts. Application of this method to environmental samples showed that a significant fraction (39%) of the oocysts in river water is dead. This percentage increased after reservoir storage to 76%, but, in both cases, the number of oocysts assayed was low (23-25).
Since sample processing increased the percentage of PI-positive (dead) *Giardia* cysts, the viability of cysts could not be assessed in this manner.

PRACTICAL IMPLICATIONS FOR THE WATER INDUSTRY AND GOVERNMENT AGENCIES

At the onset of our work, no data were available on the presence of *Cryptosporidium* and *Giardia* in source waters. Now, most of the abstraction points have been monitored and the presence of these protozoa is demonstrated at all sites: in the international rivers and in regional surface waters and even in more pristine watersheds. So, for most surface water treatment systems data on the concentration of *Cryptosporidium* oocysts and *Giardia* cysts in their source water are available. The

difference between these concentrations and the guideline levels in drinking water (chapter 2) yields the required elimination of (oo)cysts by the treatment processes (treatment goal). Protozoa monitoring is not suitable to determine if a treatment meets the treatment goal; protozoa concentrations are too low and the methods too insensitive and expensive. The use of surrogate parameters, such as spores of sulphite-reducing clostridia or particles, is a cheap alternative that allows the description of treatment efficiency and of the variation in treatment processes.
In order to deal with the variation in protozoa concentrations in source waters and the variation in treatment efficiency, statistical methods have been developed. With these methods, the data can be translated into an estimate of the concentration of *Cryptosporidium* oocysts and *Giardia* cysts after treatment, and in the health risk of these concentrations.
A water company (or Inspectorate) that wants to evaluate the microbiological safety of a pilot or full scale treatment system has to monitor its source water for protozoa (especially in winter, when concentrations are highest), determine its treatment goal and design a monitoring strategy for spores of sulphite-reducing clostridia (and possibly other surrogates), that allows (at least) the demonstration of the overall treatment efficiency, but preferably also of the contribution of the unit-processes to the overall treatment efficiency.

FUTURE RESEARCH

The most important need is a proper method for the enumeration of *Cryptosporidium parvum* and *Giardia intestinalis* in water samples with a high and reproducible recovery efficiency. The currently available methods have a low and variable recovery efficiency. This severely hampers the interpretation of the results of environmental monitoring.
Although many promising new or improved methods have been reported, the reproducibility of these reports is generally low. There is a strong need for a uniform approach to determine the performance characteristics of concentration and detection methods, to make all the research efforts done to develop a better method comparable. Also, the specificity of the detection methods needs to be improved to allow for the specific detection of cysts of *G. intestinalis* and oocysts of *C. parvum,* since these represent a health risk. Similarly, methods are needed to discriminate between live and dead (oo)cysts, like the dye-exclusion method for *Cryptosporidium* oocysts described in this thesis. Specific detection of viable (oo)cysts of the species that are pathogenic to man will allow us to set treatment goals that are optimal in terms of safety and costs.
The current developments in tissue culture of *C. parvum* oocysts, in conjunction with molecular methods such as PCR, or direct molecular methods, such as FISH or RT-PCR on heat shock mRNA, are promising developments. However, all detection methods depend on the recovery efficiency of the concentration methods. If less than 10% of the (oo)cysts that are present in environmental samples are available after concentration, it will be difficult to interpret the results of viability tests and specific methods, since the vast majority of the (oo)cysts in the water sample go undetected.

In our approach, we use surrogate parameters to describe the treatment efficiency for elimination of *Cryptosporidium* and *Giardia.* This needs to be validated by studies in which the elimination of the surrogate and (oo)cysts by a treatment process is compared, to ensure that if we conclude that treatment is adequate on the basis of elimination of surrogates, it is also eliminating pathogens adequately.

The data on the efficiency of water treatment systems that are described in this thesis show that all treatment processes show a considerable variation in the efficiency of micro-organism removal. If the process factors that determine the efficiency of micro-organism removal can be elucidated, this will yield control measures for optimising treatment design and operation with respect to micro-organism removal.

The safety of drinking water is currently monitored by assessing the absence of coliforms, faecal enterococci and spores of sulphite-reducing clostridia in finished water by frequent sampling of small volumes (300ml). This gives only very little information on treatment performance. An optimised monitoring strategy of raw, treated and finished water in increasing sample volumes will render both types of information and hence a better indication of the safety of drinking water.

The level of exposure of the inhabitants of the Netherlands to *Cryptosporidium* and *Giardia* through recreational waters is probably more significant than exposure through drinking water. Preliminar studies have shown the presence of *Cryptosporidium* and *Giardia* in recreational waters in the Netherlands in concentrations that may give rise to a health risk to bathers. More extensive monitoring of recreational waters is required to assess the level of this risk.

The epidemiological investigations on the outbreak of cryptosporidiosis in the summer of 1995 pointed towards chlorinated swimming pools as a source of *Cryptosporidium* infections. Also in other countries, swimming pools have recently been identified as a source of outbreaks of cryptosporidiosis. The high resistance of oocysts makes the free chlorine in pool water ineffective. This calls for an extensive and detailed study on the transmission of *Cryptosporidium* in swimming pools and measures to control this transmission.

In our studies, no data were collected on the occurrence of *Cryptosporidium* and *Giardia* in farm animals. The high density of farm animals in the Netherlands and the prevalence of these protozoa in farm animals, especially newborn calves and lambs, that is reported in other countries imply that this may be a significant reservoir of *Cryptosporidium* and *Giardia.* The significance of farm animals as a source of surface water contamination can be established by determining the percentage of animals shedding (oo)cysts and the concentrations of (oo)cysts in manure, and the fraction of the (oo)cysts in manure that is transported to surface water.

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The abundance and size of drinking waterborne outbreaks in developed countries show that transmission of *Giardia* and *Cryptosporidium* by drinking water is a significant risk. In the case of *Cryptosporidium*, the absence of an adequate cure for immunocompromised patients increases the problem. Although the outbreaks receive most attention, low-level transmission of these protozoa through drinking water is very likely to occur. Cysts and oocysts are regularly found in drinking water, although only a small proportion may be viable and infectious to man. A major drawback for the determination of the health significance of (oo)cysts in (drinking) water is that methods for a sensitive and specific detection of infectious (oo)cysts, with a consistently high recovery are not available.

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variation in the efficiency of a treatment system was determined with data on removal of spores of sulphite-reducing clostridia by a full-scale treatment plant. This parameter was regarded as a surrogate for removal of *Cryptosporidium* and *Giardia.* Different statistical probability distributions were compared to be able to select the best method to describe raw water quality and treatment efficiency. The negative binomial distribution provided a better fit to the protozoa concentration data in river water than the Poisson-with-added-zero's distribution. For stored water, with a high percentage of zero counts, the fit of both distributions was comparable.
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Risk assessment
The significance of the presence in drinking water of the protozoan micro-parasites *Cryptosporidium parvum* and *Giardia lamblia* for public health may be analysed by means of risk assessment. This procedure is described in chapter 9. Quantitative risk assessment requires quantitative knowledge of all the contributing factors: the concentration of cysts or oocysts in raw water, the recovery of the detection method, the viability of recovered cysts or oocysts, the removal of organisms in the treatment process, and the daily consumption of unboiled tap water. To enable analysis of the uncertainty in the calculated risk of infection, each of these factors was treated as a stochastic variable, for which a suitable distribution is proposed. A frequency distribution for the probability of infection is then constructed with standard sampling techniques. This first evaluation of the calculation of the risk of infection due to exposure to *Cryptosporidium* oocysts and *Giardia* cysts via drinking water, shows that the uncertainty in the estimated removal efficiency of the treatment process dominates over uncertainties in other contributing factors.

The thesis concludes with a **General discussion**. This chapter gives a brief summary of the most important findings of this thesis. Their practical implications for the water industry and government agencies are discussed. Finally, the most important gaps in the current knowledge are identified.

Practical implications for the water industry and government agencies
For most of the surface water treatment systems in the Netherlands, data on the concentration of *Cryptosporidium* oocysts and *Giardia* cysts in their source water are available. The difference between these concentrations and the guideline levels in drinking water (chapter 2) yields the required elimination of (oo)cysts by the treatment processes (treatment goal). Protozoa monitoring is not suitable to determine if a treatment meets the treatment goal; protozoa concentrations are too low and the methods too insensitive and expensive. The use of surrogate parameters, such as spores of sulphite-reducing clostridia or particles, is a cheap alternative that allows the description of treatment efficiency and of the variation in treatment processes (chapter 7).
In order to deal with the variation in protozoa concentrations in source waters and the variation in treatment efficiency, statistical methods have been developed. With these methods, the data can be translated into an estimate of the concentration of *Cryptosporidium* oocysts and *Giardia* cysts after treatment and in the health risk of these concentrations.
A water company (or Inspectorate) that wants to evaluate the microbiological safety of a pilot or full scale treatment system has to monitor its source water for protozoa (especially in winter, when concentrations are highest), determine its treatment goal and design a monitoring strategy for spores of sulphite-reducing clostridia (and possibly other surrogates), that allows (at least) the demonstration of the overall treatment efficiency, but preferably also of the contribution of the unit-processes to the overall treatment efficiency.

Research needs

- The most important need is improvement of the method for the enumeration of *Cryptosporidium parvum* and *Giardia intestinalis* in water samples with a high and reproducible recovery efficiency. There is a strong need for a uniform approach to determine the performance characteristics of concentration and detection methods to make all the research efforts done to develop a better method comparable. Another need for method improvement is specific detection of viable (oo)cysts of the species that are pathogenic to man will allow us to set treatment goals that are optimal in terms of safety and costs.
- Ensure that the spores of sulphite-reducing clostridia are adequate surrogate parameters for evaluating elimination of protozoa by treatment processes.
- Optimisation of the monitoring strategy to determine if treatment is adequate and drinking water is safe.
- Optimisation of treatment systems for protozoa removal.
- Monitoring of recreational waters is required to assess the level of risk due to water recreation.
- Assessment of the risk of transmission of *Cryptosporidium* in swimming pools and measures to control this transmission.
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Chapter 8 describes the use of a flow cytometer with fluorescence activated cell sorting (FACS) to purify *Cryptosporidium* oocysts and *Giardia* cysts from water. With this purification step *Cryptosporidium* and *Giardia* were found in a higher percentage of the samples and significantly higher *Giardia* concentrations were detected in these positive samples. Because FACS removed most of the debris from the concentrated water samples, the microscopic preparations could be examined more rapidly for the presence of (oo)cysts and the morphological characteristics of the (oo)cysts could be interpreted more unambiguously than with the conventional immunofluorescence microscopy method. The use of FACS made it possible to apply PI-staining on environmental samples to determine the fraction of dead (oo)cysts. Sample processing did not appear to influence the PI-staining characteristics of the *Cryptosporidium* oocysts, but did increase the percentage of PI-positive *Giardia* cysts. This suggests that this protocol can be used for determining the percentage of dead *Cryptosporidium* oocysts in environmental samples. Analysis of environmental samples suggests that reservoir storage increases the percentage of PI-positive (dead) oocysts.

Risk assessment
The significance of the presence in drinking water of the protozoan micro-parasites *Cryptosporidium parvum* and *Giardia lamblia* for public health may be analysed by means of risk assessment. This procedure is described in chapter 9. Quantitative risk assessment requires quantitative knowledge of all the contributing factors: the concentration of cysts or oocysts in raw water, the recovery of the detection method, the viability of recovered cysts or oocysts, the removal of organisms in the treatment process, and the daily consumption of unboiled tap water. To enable analysis of the uncertainty in the calculated risk of infection, each of these factors was treated as a stochastic variable, for which a suitable distribution is proposed. A frequency distribution for the probability of infection is then constructed with standard sampling techniques. This first evaluation of the calculation of the risk of infection due to exposure to *Cryptosporidium* oocysts and *Giardia* cysts via drinking water, shows that the uncertainty in the estimated removal efficiency of the treatment process dominates over uncertainties in other contributing factors.

The thesis concludes with a **General discussion**. This chapter gives a brief summary of the most important findings of this thesis. Their practical implications for the water industry and government agencies are discussed. Finally, the most important gaps in the current knowledge are identified.

Practical implications for the water industry and government agencies
For most of the surface water treatment systems in the Netherlands, data on the concentration of *Cryptosporidium* oocysts and *Giardia* cysts in their source water are available. The difference between these concentrations and the guideline levels in drinking water (chapter 2) yields the required elimination of (oo)cysts by the treatment processes (treatment goal). Protozoa monitoring is not suitable to determine if a treatment meets the treatment goal; protozoa concentrations are too low and the methods too insensitive and expensive. The use of surrogate parameters, such as spores of sulphite-reducing clostridia or particles, is a cheap alternative that allows the description of treatment efficiency and of the variation in treatment processes (chapter 7).
In order to deal with the variation in protozoa concentrations in source waters and the variation in treatment efficiency, statistical methods have been developed. With these methods, the data can be translated into an estimate of the concentration of *Cryptosporidium* oocysts and *Giardia* cysts after treatment and in the health risk of these concentrations.
A water company (or Inspectorate) that wants to evaluate the microbiological safety of a pilot or full scale treatment system has to monitor its source water for protozoa (especially in winter, when concentrations are highest), determine its treatment goal and design a monitoring strategy for spores of sulphite-reducing clostridia (and possibly other surrogates), that allows (at least) the demonstration of the overall treatment efficiency, but preferably also of the contribution of the unit-processes to the overall treatment efficiency.

Research needs

- The most important need is improvement of the method for the enumeration of *Cryptosporidium parvum* and *Giardia intestinalis* in water samples with a high and reproducible recovery efficiency. There is a strong need for a uniform approach to determine the performance characteristics of concentration and detection methods to make all the research efforts done to develop a better method comparable. Another need for method improvement is specific detection of viable (oo)cysts of the species that are pathogenic to man will allow us to set treatment goals that are optimal in terms of safety and costs.
- Ensure that the spores of sulphite-reducing clostridia are adequate surrogate parameters for evaluating elimination of protozoa by treatment processes.
- Optimisation of the monitoring strategy to determine if treatment is adequate and drinking water is safe.
- Optimisation of treatment systems for protozoa removal.
- Monitoring of recreational waters is required to assess the level of risk due to water recreation.
- Assessment of the risk of transmission of *Cryptosporidium* in swimming pools and measures to control this transmission.
- Assessment of the percentage of animals shedding (oo)cysts and the concentrations of (oo)cysts in manure, and the fraction of the (oo)cysts in manure that is transported to surface water.

Samenvatting

Aanleiding

De afgelopen decennia is duidelijk geworden dat de protozoa *Cryptosporidium parvum* en *Giardia intestinalis* via water overgedragen kunnen worden. In de Verenigde Staten en in Engeland hebben beide parasieten een groot aantal epidemieën van darminfecties veroorzaakt. Hoofdstuk 1 geeft een overzicht over de beschikbare kennis over de parasieten, de ziekte die ze veroorzaken, de overdracht via water en de maatregelen om deze overdracht te voorkomen.

De belangrijkste ziekte-symptomen zijn diarree en gewichtsverlies. Bij personen met een goed ontwikkelde afweer duren deze symptomen een aantal dagen. Het afweersysteem werkt de infectie weer weg. Bij mensen met een ernstig verzwakte afweer kunnen de symptomen ernstiger en langduriger worden en uiteindelijk de dood tot gevolg hebben.

De parasieten hebben een aantal eigenschappen die de overdracht via water vergemakkelijkt: ze overleven lang in water, ze zijn heel resistent tegen desinfectiemiddelen en ze zijn heel infectieus; het binnenkrijgen van 1 (oo)cyste (het overlevingsstadium) geeft al een discrete kans op een infectie.

Het grote aantal epidemieën via drinkwater in geïndustrialiseerde landen, en de grote omvang van enkele van deze epidemieën, laten zien dat overdracht van *Cryptosporidium* en *Giardia* via drinkwater een reëel risico kan zijn. Deze epidemieën hebben de meeste aandacht gekregen, maar zeer waarschijnlijk vindt ook op een endemisch niveau overdracht van deze parasieten via drinkwater plaats. Cysten en oöcysten zijn in veel geïndustrialiseerde landen in drinkwater aangetroffen, waarbij er zeker niet altijd aanwijzingen zijn dat die ook tot het optreden van ziektegevallen leiden. Doordat er geen methoden beschikbaar zijn waarmee de menspathogene (oö)cysten specifiek, gevoelig en met een consistent hoog rendement kunnen worden aangetoond, is niet duidelijk vast te stellen of de (oö)cysten in drinkwater daadwerkelijk ziekte kunnen verwekken bij de mens.

Hoofdstuk 2 geeft een overzicht van de problemen die in geïndustrialiseerde landen zijn opgetreden met overdracht van *Cryptosporidium* en *Giardia* via drinkwater en evalueert de betekenis hiervan voor de Nederlandse situatie. In een aantal epidemieën die in de Verenigde Staten en Engeland zijn opgetreden via drinkwater was de oorzaak van de besmetting van het drinkwater met *Cryptosporidium* en *Giardia* een duidelijke storing in de zuivering of besmetting van het leidingnet. Een ander deel van de epidemieën trad op via drinkwater waarbij geen aanwijzingen waren dat er met zuivering of distributie iets mis was. Dat werd nog versterkt doordat de parameter die werd gebruikt om de microbiologische veiligheid te beoordelen (bacteriën van de coligroep) geen waarschuwing gaf dat er parasieten in het drinkwater aanwezig waren, omdat deze bacteriën beter werden geëlimineerd door de zuivering dan de parasieten.

Onderzoek naar het voorkomen van *Cryptosporidium* en *Giardia* in buitenlands oppervlaktewater hebben laten zien dat deze parasieten zeer algemeen voorkomen, zelfs in water dat niet direct door rioollozingen of afspoeling van mest wordt verontreinigd.
Ook de Nederlandse oppervlaktewaterbedrijven hebben dus met deze parasieten te maken. Deze ontwikkelingen waren aanleiding voor de Nederlandse waterleidingbedrijven en overheid om een onderzoeksprogramma te starten om de (on)waarschijnlijkheid van overdracht van *Cryptosporidium* en *Giardia* via Nederlands drinkwater vast te stellen.

De aanpak voor het veiligstellen van drinkwater is door de protozoa verschoven van eind-productbewaking naar controle van de grondstof en het aantonen van het rendement van de zuivering. De hoge resistentie en infectiviteit van deze parasieten betekenen dat een “nul-risico” in de praktijk niet meer te realiseren is. De zuivering moet zodanig worden ontworpen en bedreven dat het risico zo laag mogelijk is, doordat de concentraties die in oppervlaktewater voorkomen zo ver mogelijk worden verlaagd, bij voorkeur met behulp van fysische barrières. Dat betekent dat zowel informatie nodig is over het voorkomen van *Cryptosporidium* en *Giardia* in de grondstof (concentraties, rendement meetmethode, levensvatbaarheid) en over de mate waarin zuiveringsprocessen deze parasieten elimineren. Deze gegevens kunnen middels een kwantitatieve risico-analyse worden gecombineerd tot een schatting van het infectierisico via drinkwater.

Doel van dit onderzoek
De belangrijkste onderzoeksvragen waren:
- Wat zijn de belangrijkste bronnen voor de verontreiniging van oppervlaktewater met deze parasieten?
- Wat gebeurt er met de parasieten in oppervlaktewater?
- In welke concentraties komen ze voor bij innamepunten voor drinkwaterproductie?
- Hoe goed worden de parasieten verwijderd door de zuivering?
- Hoe kan de detectiemethode voor het aantonen van *Cryptosporidium* en *Giardia* in water worden verbeterd?
- Hoe kan worden aangetoond dat drinkwater veilig is?

Protozoa in huishoudelijk afvalwater en de grote rivieren
Hoofdstuk 3 beschrijft de resultaten van een onderzoek naar het voorkomen van *Cryptosporidium* en *Giardia* in rioolwater, effluent van rioolwaterzuiveringsinstallaties en in oppervlaktewater. Beide parasieten zijn op alle onderzochte locaties aangetroffen in vrijwel alle monsters. Vanuit de hoek van de chemische oppervlaktewaterverontreiniging waren emissie- en verspreidingsmodellen beschikbaar. Onderzocht is of deze toepasbaar waren voor het beschrijven van de emissie van de parasieten

vanuit huishoudelijk afvalwater naar oppervlaktewater en de verdere verspreiding van de parasieten in oppervlaktewater. Door deze modellen te gebruiken konden gegevens van beschrijvend onderzoek naar de concentratie van *Cryptosporidium* en *Giardia* op individuele meetlocaties worden geïntegreerd met gegevens van experimenteel onderzoek naar het gedrag van deze parasieten tot een goede beschrijving van het voorkomen in oppervlaktewater dat wordt verontreinigd door rioollozingen.
Met deze modellen is berekend dat jaarlijks 8,0 x 10^{12} *Cryptosporidium* oöcysten en 3,8 x 10^{12} *Giardia* cysten vanuit gezuiverde en ongezuiverde lozingen van huishoudelijk afvalwater op het oppervlaktewater worden geloosd. Het grootste deel (85%) van de *Cryptosporidium* oöcysten werd geloosd via effluent van rioolwaterzuiveringsinstallaties, terwijl het grootste deel (82%) van de *Giardia* cysten via riooloverstorten en ongezuiverde lozingen in het oppervlaktewater werden gebracht.
De concentraties *Cryptosporidium* en *Giardia* die werden berekend met deze modellen, werden voor het grootste deel bepaald door de aanvoer vanuit het buitenland via de Rijn en Maas. De jaarlijkse 'import' via deze routes werd geschat op 3,2 x 10^{14} *Cryptosporidium* oöcysten en 2,1 x 10^{15} *Giardia* cysten, waarvan de Rijn respectievelijk 66 en 87% voor haar rekening nam. Deze vracht was groter dan de totale hoeveelheid die via rioollozingen in Nederland werd geloosd.
Met de combinatie van het emissiemodel (PROMISE) en het verspreidingsmodel (WATNAT) werden de concentraties *Cryptosporidium* en *Giardia* in oppervlaktewater bij innamepunten voor de drinkwaterbereiding berekend. Voor 6 van deze locaties waren meetgegevens beschikbaar om de modelberekeningen te kunnen valideren. Op 5 van de 6 locaties lagen de gemeten en berekende concentraties zeer dicht bij elkaar. Op de overgebleven locatie waren de voorspelde concentraties 7 tot 37 keer lager dan de gemeten concentraties. Deze locatie werd ook niet of nauwelijks verontreinigd door rioollozingen, maar voornamelijk door agrarische bronnen, die nog niet in het model zijn opgenomen.
De modelmatige beschrijving is dus goed te gebruiken voor oppervlaktewater dat door rioollozingen wordt verontreinigd.

De natuurlijke fauna als bron van *Cryptosporidium* en *Giardia*
In hoofdstuk 4 wordt een studie beschreven naar de betekenis van in het wild levende dieren, met name watervogels, voor de besmetting van opslagbekkens met *Cryptosporidium parvum* en *Giardia intestinalis.* Van beide parasieten is bekend dat zij bij veel zoogdieren voorkomen. Meerdere epidemieën van giardiase en cryptoporidiose zijn geassocieerd met contact met besmette dieren, zowel direct als via besmetting van water. Hoewel zoönotische transmissie van *Giardia* nog steeds controversieel is, moeten zoogdieren worden beschouwd als reservoir van *Cryptosporidium* en *Giardia* (oö)cysten die de mens kunnen infecteren. Voor watervogels is dit minder waarschijnlijk, maar deze kunnen wel

drager (en dus uitscheider) zijn van voor de mens pathogene *Cryptosporidium* oöcysten en waarschijnlijk ook *Giardia* cysten.
In deze studie werd informatie verzameld over het voorkomen van *Cryptosporidium* spp. en *Giardia* spp. in mest van de natuurlijke fauna op en rond de opslagbekkens van de Brabantse Biesbosch, over het aantal dieren dat op en rond de bekkens voorkwam en hun dagelijkse productie van faeces. De geschatte (oö)cyste-vracht vanuit deze dierfaeces werd vergeleken met de gemeten concentraties *Cryptosporidium* en *Giardia* in het bekkenwater.
Cryptosporidium spp. oöcysten werden in faeces van diverse vogelsoorten en zoogdieren aangetroffen. Ook *Giardia* spp. cysten werden bij een aantal vogelsoorten aangetroffen en bij een zoogdiersoort (de muskusrat). Uit de vergelijking van de vracht vanuit de dierfaeces en de gemeten concentraties in bekkenwater bleek dat de krakeend, aalscholver, kuifeend en runderen een significante bijdrage (0,5 – 9,4%) kunnen leveren aan de verontreiniging van bekkenwater met *Cryptosporidium* oöcysten. De wilde eend, de smient en de krakeend kunnen significante bronnen zijn voor *Giardia* cysten. De bijdrage van alle diersoorten op en rond de bekkens aan de besmetting van het bekkenwater was 1 tot 16% voor *Cryptosporidium* en 4 tot 67% voor *Giardia*.
Deze kwantitatieve studie heeft aangetoond dat watervogels niet alleen in potentie, maar ook in werkelijkheid een significante bijdrage kunnen leveren aan de besmetting van bekkenwater.

Overleving
Hoofdstuk 5 beschrijft een studie naar de overleving van *Cryptosporidium parvum* oocysten in oppervlaktewater. Doel was het vaststellen van de afstervingssnelheid en de invloed van de temperatuur en de aanwezigheid van autochtone micro-organismen daarop. De afstervingssnelheid werd vergeleken met indicatorbacterien voor faecale verontreiniging: *Escherichia coli*, faecale enterococcen en sporen van *Clostridium perfringens*.
Oöcysten blijken zeer persistent in water: de tijd die nodig is voor een tienvoudige reductie van hun aantal is 40-160 dagen bij 15°C en ongeveer 100 dagen bij 5°C. Biologische of biochemische activiteit in het water beïnvloedde de overleving van oöcysten bij 15°C en van de vegetatieve bacteriën (*E. coli*, enterococcen) bij zowel 5 als 15°C.
De snelle afsterving van *E. coli* en faecale enterococcen maken deze organismen minder geschikt als indicator voor de aanwezigheid van *Cryptosporidium* oöcysten. *Cl. perfringens* was het meest persistent en overleefde zelfs langer dan oöcysten. Daarmee zou dit een geschikte indicator kunnen zijn voor de aanwezigheid van *C. parvum*.

Sedimentatie
In hoofdstuk 6 wordt een onderzoek beschreven naar de sedimentatiesnelheid van (oö)cysten van *Cryptosporidium parvum* en *Giardia intestinalis.* Daarbij worden metingen van de sedimentatiesnelheid

vergeleken met een mathematische beschrijving van de snelheid met de wet van Stokes. Om deze berekeningen uit te kunnen voeren zijn de karakteristieken van de (oö)cysten en het sedimentatiemedium gemeten. De berekende sedimentatie-kinetiek vertoonde een goede overeenkomst met de kinetiek die in de experimenten werd waargenomen. Beide lieten zien dat de sedimentatiesnelheid afnam met de tijd, hetgeen voornamelijk werd veroorzaakt door verschillen in de dichtheid van de (oö)cysten. De initiële sedimentatiesnelheid in Hanks balanced salt solution van 23°C was 0,35 $\mu m.s^{-1}$ voor oöcysten en 1,4 $\mu m.s^{-1}$ voor cysten.
Als (oö)cysten op oppervlaktewater worden geloosd via een biologische rioolwaterzuivering, kunnen de (oö)cysten mogelijk hechten aan deeltjes in het rioolwater. Deze hechting zal de sedimentatie-kinetiek beïnvloeden. (Oö)cysten die werden gemengd met effluent van een rioolwater-zuiveringsinstallatie bleken snel te hechten aan de (biologische) deeltjes in het effluent: 30% van de cysten en van de oöcysten hechtte al binnen enkele minuten aan deze deeltjes en het hechtingspercentage steeg tot ca. 75% na 24 uur. De sedimentatiesnelheid van deze gehechte (oö)cysten werd geheel bepaald door de sedimentatie-kinetiek van de deeltjes, zelfs bij de kleinste deeltjes (1 tot 40 μm).
De waargenomen sedimentatiesnelheden van de vrij gesuspendeerde cysten en oöcysten was waarschijnlijk te laag om onder natuurlijke omstandigheden een rol te spelen. Omdat een groot deel van de (oö)cysten echter snel kan hechten aan biologische deeltjes in secundair effluent, is sedimentatie van gehechte (oö)cysten waarschijnlijk wel relevant voor de verspreiding van *Cryptosporidium* en *Giardia* in water. Daarnaast beïnvloedt hechting ook het gedrag van deze (oö)cysten in de drinkwaterzuivering.

Protozoa in ruw water en het rendement van de zuivering
Fluctuaties in de ruw water kwaliteit en het zuiveringsrendement zijn belangrijk, omdat het risico op doorbraak van (oö)cysten naar drinkwater hoog is als hun concentratie in de grondstof hoog of het zuiverings-rendement laag is, en met name wanneer deze beide momenten samenvallen. Hoofdstuk 7 is de beschrijving van een studie die tot doel had de variatie in de kwaliteit van het ruwe water en van het zuiveringsrendement mee te nemen in een schatting van de concentratie van *Cryptosporidium* en *Giardia* in drinkwater.
De variatie in de ruw water kwaliteit werd bepaald door een intensief monitoringsprogramma uit te voeren bij een innamepunt in de Maas. De variatie van het zuiveringsrendement werd bepaald aan de hand van bedrijfsgegevens over de verwijdering van sporen van sulfietreducerende clostridia. Deze parameter werd beschouwd als een surrogaat voor de verwijdering van (oö)cysten.
Verschillende statistische methoden zijn vergeleken op hun vermogen om de ruw water kwaliteit en het zuiveringsrendement te beschrijven.
De negatief binomiale verdeling gaf de beste beschrijving van de protozoa concentraties in de Maas. Voor de data-set uit water na bekkenopslag,

met veel nullen, beschreven de negatief binomiale verdeling en de Poisson-with-added-zero's de metingen even goed.
De opbrengst van de meetmethode werd beschouwd als een stochastische variabele, die kon worden beschreven met de beta-binomiale verdeling.
Voor het beschrijven van de eliminatie van sporen van sulfietreducerende clostridia door de zuivering zijn verschillende statistische methoden met elkaar vergeleken. De praktijkgegevens van de concentratie sporen in de zuivering bevatte een aantal metingen waarin geen sporen werden aangetroffen (nul-metingen). Dit aantal werd groter naarmate het water verder werd gezuiverd. Voor de data-sets met een gering percentage nul-metingen gaven alle statistische methoden een vergelijkbare beschrijving. Voor de data-sets met 50% nul-metingen of meer werden duidelijke verschillen zichtbaar. De meest geschikte methode om met deze data-sets om te gaan is het vervangen van de nul-metingen door het gemiddelde van de meetreeks. Deze methode onderschat het verwijderingsrendement, het minst, maar is dus nog steeds conservatief. Het is duidelijk dat de beschrijving van het zuiveringsrendement moet verbeteren door gebruik te maken van de gevoelige methoden om sporen aan te tonen. Deze is nu ontwikkeld.
Door de beschrijving van het rendement van individuele processen te vergelijken met het rendement van dezelfde processen in combinatie, bleek dat het rendement van een zuiveringsproces afhankelijk is van het rendement van voorafgaande processen. De variatie in het rendement werd niet groter naarmate het water door meerdere processen werd gezuiverd. Dit impliceert dat de variatie in het rendement van een proces wordt 'opgevangen' door de variatie van het volgende proces. Dat betekent ook dat het rendement van de totale zuivering alleen adequaat kan worden beschreven door (gevoelige) metingen in ruw en rein water.

Verbeterde detectie en levensvatbaarheidskleuringen met flow cytometrie
Hoofdstuk 8 beschrijft het gebruik van een flow cytometer die fluorescerende cellen kan sorteren om *Cryptosporidium* oöcysten en *Giardia* cysten uit waterconcentraten te zuiveren. Door gebruik te maken van de flow cytometer werden beide parasieten in meer monsters water aangetroffen. Omdat de flow cytometer het grootste deel van het debris uit een waterconcentraat verwijderd, worden de microscopische preparaten beter, makkelijker en sneller te beoordelen dan met de conventionele methode. De zuiverheid van de preparaten maakte het ook mogelijk om een kleuring met PI toe te passen, om te bepalen welk aandeel van de (oö)cysten in oppervlaktewater dood is. Het protocol om de monsters te concentreren en op te werken leek geen invloed te hebben op de levensvatbaarheid van *Cryptosporidium* oöcysten. Het percentage PI + (= dode) *Giardia* cysten nam echter toe als gevolg van de bewerkingen tijdens opwerken, met name door de sonificatie-stap. Dit geeft aan dat het protocol kan worden gebruikt om het percentage dode

Cryptosporidium oöcysten in water monsters te bepalen, maar niet geschikt is voor het bepalen van de levensvatbaarheid van *Giardia*. Toepassing van dit protocol op monsters voor en na opslag in voorzuiveringsreservoirs met een verblijftijd van vijf maanden gaf aan dat de levensvatbaarheid van de oöcysten tijdens deze opslag terugloopt van 61% naar 24%.

Risico analyse

Het risico op aanwezigheid van *Cryptosporidium* en *Giardia* in drinkwater, en het gezondheidsrisico daarvan, kan worden onderzocht aan de hand van een kwantitatieve risico analyse. Deze procedure wordt beschreven in hoofdstuk 9. Hiervoor is kwantitatieve informatie nodig over alle factoren die het risico bepalen: de concentratie *Cryptosporidium* en *Giardia* in de grondstof, de opbrengst van de detectiemethode, de levensvatbaarheid/infectiviteit van de (oö)cysten, de verwijdering van (oö)cysten bij de drinkwaterzuivering en de dagelijkse consumptie van ongekookt drinkwater. Om ook de onzekerheid van het berekende risico te analyseren werd elk van deze factoren beschouwd als stochastische variabele waarvoor een geschikte kansverdeling is geselecteerd. Uit de kansverdelingen van alle factoren is met een Monte Carlo analyse de verdeling van het infectierisico door beide parasieten via drinkwater van een bepaalde locatie geschat. Deze analyse heeft laten zien dat de onzekerheid in het berekende infectierisico grotendeels wordt bepaald door de onzekerheid in het zuiveringsrendement.

Dit proefschrift eindigt met een algemene discussie, waarin een kort overzicht van de belangrijkste resultaten wordt gegeven en hun implicaties voor waterleidingbedrijven en overheid. Als laatste worden de lacunes in de huidige kennis geïdentificeerd.

Betekenis van dit onderzoek voor de waterleidingbedrijven en overheid

De meeste oppervlaktewaterbedrijven in Nederland kennen nu de concentratie *Cryptosporidium* en *Giardia* in hun grondstof. Het verschil tussen de deze concentraties en de voorlopige richtwaarden in drinkwater (hoofdstuk 2) is het benodigde zuiveringsrendement. Het zuiveringsrendement kan niet vastgesteld worden door metingen aan *Cryptosporidium* en *Giardia*. Daarvoor zijn de concentraties te laag en de methoden te duur en te ongevoelig. Een goedkoop alternatief is het gebruik van surrogaatparameters, zoals de sporen van sulfietreducerende clostridia. Dat maakt het mogelijk het zuiveringsrendement en de variatie daarin te beschrijven (hoofdstuk 7).
De concentratie protozoa in de grondstof en het rendement van de zuivering vertonen beide aanzienlijke fluctuaties. Daarom zijn statistische methoden ontwikkeld waarmee een schatting gemaakt kan worden van de concentratie *Cryptosporidium* en *Giardia* in drinkwater en het gezondheidsrisico daarvan.

Een waterleidingbedrijf kan de veiligheid van een zuiveringssysteem demonstreren door:

1. het meten van de concentratie protozoa in de grondstof (en dan met name in de winter, als de protozoa concentraties het hoogst zijn),
2. op basis van deze metingen vaststellen wat het benodigde zuiveringsrendement is,
3. het uitvoeren van een meetprogramma waarmee aan de hand van sporen van sulfietreducerende clostridia, of andere surrogaatparameters, het werkelijke zuiveringsrendement kan worden aangetoond en vergeleken met het benodigde rendement. Dat meetprogramma zou in ieder geval het rendement van de totale zuivering moeten aangeven, maar bij voorkeur ook de bijdrage van de individuele processen daaraan.

Onderzoeksbehoefte

- Verbetering van de (reproduceerbaarheid van de) opbrengst van de detectiemethoden is momenteel de belangrijkste behoefte, alsmede het ontwikkelen van methoden waarmee de levensvatbaarheid van (oö)cysten in water kan worden vastgesteld en waarmee de menspathogene soorten specifiek kunnen worden aangetoond.
- Vaststellen van de procesfactoren die verantwoordelijk zijn voor de variatie in het zuiveringsrendement, zodat de zuivering geoptimaliseerd kan worden voor de verwijdering van micro-organismen.
- Vaststellen dat het gebruik van sporen van sulfietreducerende clostridia een geschikte surrogaatparameter is voor het beschrijven van de eliminatie van *Cryptosporidium* en *Giardia* door zuiverings-processen.
- Optimalisatie van de meetstrategie waarmee de werking van de zuivering en veiligheid van drinkwater wordt vastgesteld.
- Onderzoek naar het voorkomen van *Cryptosporidium* en *Giardia* in recreatiewater.
- Onderzoek naar het voorkomen van *Cryptosporidium* en *Giardia* in zwembaden en naar methoden om ze te bestrijden.
- Onderzoek naar het voorkomen van *Cryptosporidium* en *Giardia* bij landbouwhuisdieren; de concentraties in mest en de fractie die daarvan in oppervlaktewater terecht komt.

DANKWOORD

Waar je mee omgaat, word je mee besmet!
In mijn geval zijn dat twee parasieten, *Cryptosporidium* en *Giardia*. Ik geloof niet dat ik het 'genoegen' heb gehad om letterlijk met een van beide besmet te zijn, maar naarmate het proefschrift vorderde ben ik me wel steeds meer als parasiet gaan gedragen. Het voordeel van parasitair gedrag is dat het heel doelgericht is, het nadeel is dat het vereenzaamd. Beide aspecten komen het best tot uiting in dit proefschrift zelf: het ligt er (yes!) en er staat maar één naam op de kaft.
Ik wil hier graag de namen noemen van iedereen die, in welke vorm ook, heeft bijgedragen aan de totstandkoming van dit proefschrift. Allemaal bedankt voor jullie bijdrage!

Die dank begint bij Arie Havelaar, die aan de basis heeft gestaan voor mijn promotie-onderzoek. Arie, vanaf het moment dat ik het RIVM binnenstapte heb je me je vertrouwen gegeven en zowel de ruimte als de begeleiding gegeven waardoor ik mezelf kon ontwikkelen. Je kennis van de watermicrobiologie, je contacten, je kritische, maar altijd positieve, benadering vormden de ideale niche voor mij. De spiegelende wijze waarop je in ons overleg met mij omging hebben me zelfvertrouwen gegeven. Dat had ik nodig voor mijn promotie-onderzoek, maar ook lang daarna zal ik daar van profiteren. Bedankt ook voor je opmerkingen en suggesties voor verbetering van de verhalen als co-promotor van dit onderzoek.

Frans van Knapen, jij begeleidde mijn onderzoek op afstand, eerst vanuit het Laboratorium voor Parasitologie van het RIVM en later, als promotor, vanuit de Vakgroep voor Voedingsmiddelen van Dierlijke Oorsprong van de Faculteit Diergeneeskunde van de Universiteit Utrecht. Je hebt een aantal tips gegeven die op cruciale momenten het onderzoek richting hebben gegeven en er toe hebben geleid dat dit proefschrift nu afgerond is. Onze gesprekken voerden steevast van organisatiepolitiek, langs zoonosen in het algemeen naar *Cryptosporidium* en *Giardia* in het bijzonder. Ik heb gebruik kunnen maken van je brede kennis op het gebied van de parasitologie en daarbuiten. Bedankt daarvoor!

Een heel groot deel van dit proefschrift is voortgevloeid uit het werk dat ik samen met Ciska Schets heb gedaan. Ciska, hier ligt het dan! De constructieve manier waarop wij konden samenwerken, je zorgvuldigheid, kritische blik (en tong, soms), je veelzijdigheid en brede interesse voor alles wat met methodiekontwikkeling te maken had, je enthousiasme en je inzet hebben dit proefschrift mogelijk gemaakt. We hebben de parasieten meermalen verwenst vanwege hun grenzeloze vermogen om niet-reproduceerbare resultaten op te leveren. Naarmate het onderzoek vorderde hebben we ze toch meer onder de knie gekregen, zodat we uiteindelijk een onderzoek zo konden opzetten als het sedimentatie-onderzoek. Ook de minutieuze wijze waarop je het manuscript van de afzonderlijke verhalen en uiteindelijk van het proefschrift hebt doorgenomen en van commentaar hebt voorzien hebben me geholpen het beste te maken van dit proefschrift.

George Engels, Ria de Bruin, Mahdieh Bahar en Laurens Kruidenier, jullie hebben stuk voor stuk heel, heel veel uren achter de microscoop doorgebracht in onze

speurtocht naar de ‘appelgroen fluorescerende, ronde tot ovaalronde bolletjes’ in rioolwater, mest en oppervlaktewater. De gegevens die jullie daarmee hebben verzameld zijn zo goed als allemaal in dit proefschrift verwerkt. Bedankt voor jullie inzet en de plezierige manier waarop we het onderzoek hebben gedaan.

Jack Schijven, je veelzijdigheid, de manier waarop je je vastbijt in een onderwerp en de consciëntieuze wijze waarop je met gegevens omgaat waren de beste basis voor het modelleerwerk dat we hebben gedaan. Deze eigenschappen zullen je ook met glans door je promotie-onderzoek loodsen. Ik vind het leuk dat ik dat nog steeds van redelijk dichtbij kan meemaken.

Peter Teunis, ik heb nog nooit iemand zo charismatisch over wiskunde en statistiek horen praten. Tijdens mijn studie Biologie waren dit typische hakken-over-de-sloot-vakken. Jij hebt me boven de formules uitgetild en laten zien dat het gaat om de concepten/hypotheses/modellen die erachter zitten en waarmee je kunt proberen de werkelijkheid te beschrijven. Door jou inbreng is dit proefschrift meer geworden dan een beschriving van experimenteel onderzoek. Ik denk met veel plezier terug aan onze discussies, zowel over werk als daarbuiten en hoop dat er nog meer zullen volgen.

Kirsten Mooijman, Reina van de Heide en Willemijn Lodder, mede door jullie was er binnen Watermicrobiologie een plezierige en betrokken sfeer, waarin niet gauw wat teveel was.

Alle medewerkers van het Laboratorium voor Water- en Levensmiddelen-microbiologie en later het Microbiologisch Laboratorium voor Gezondheidsbescherming, oftewel alle LWL/MGB-ers, bedankt voor de plezierige werksfeer. Ik heb jullie allemaal als heel collegiaal ervaren. Veel van jullie hebben je betrokkenheid laten zien in discussies over mijn onderzoek, maar ook door mee te gaan het veld in tijdens de zwemwaterstudie. Ik bewaar goede herinneringen aan mijn tijd bij jullie!

Ook op mijn nieuwe werkplek bij Kiwa was er betrokkenheid bij mijn promotie-onderzoek en vooral bij mijn welbevinden tijdens het afronden van het proefschrift. In de sectie Biologie hebben jullie, Bart Wullings, Hans Vrouwenvelder, Hein van Lieverloo, Pieter Nobel en Wim Hijnen, de totstandkoming van het drukwerk met interesse gevolgd en hebben jullie, zeker de laatste weken, mijn kuren en humeur doorstaan. Die waren ook in het Laboratorium voor Microbiologie te merken. Harm Veenendaal, Alexandra de Savornin Lohman, Anke Brouwer, Remco Voogt, Marijan Aarts, Daan van der Klift, Ellen van der Speld, Ton Braat en Wilma Tolsma, jullie hebben met me meegeleefd. Ik was er lang niet vaak genoeg voor jullie, maar dat werd vaak met de mantel van ‘hij is met zijn proefschrift bezig’ afgedekt.

Dick van der Kooij, jij vond het van begin af aan (en eigenlijk al daarvoor) vanzelfsprekend dat ik mijn promotie zou afronden, nadat ik bij Kiwa kwam. Bedankt voor je morele steun. Ik heb veel geleerd van je heldere kijk op de betekenis van *Cryptosporidium* en *Giardia* voor de waterleidingbedrijven en op een boel andere aspecten. Je enthousiasme voor het vak is net zo charismatisch als het vak zelf!

Wim, Pieter, Bart, Hans, Harm, Dick en ook Johan Groennou, onze discussies over de verschillende aspecten van de protozoa (methoden, verwijdering, statistiek, betekenis) geven me het uitdagende gevoel dat, hoewel dit proefschrift er nu ligt, er nog veel te doen is. Aan het werk dus!

Bedankt ook alle secretaresses die hebben allerhande hand-en-span diensten hebben verricht: Elise Nijkamp, Klara Draaisma, Loes van Dijk, Jeanette Wouters en Sylvia Leeman van het RIVM en Marieke ten Broeke, Hanneke van Driel en Jaqueline Hoogstraten van Kiwa.

Zowel het RIVM, in de personen van Arie Havelaar en later Andre Henken, als Kiwa, in de personen van Dick van der Kooij en Myriam van Rooij, wil ik graag bedanken voor de gelegenheid die me is geboden om mijn promotie-onderzoek uit te voeren en af te ronden.

A special thanks to Huw Smith and the crew at the Scottish Parasite Diagnostic Laboratory, Andy Campbell, Zia Bukhari, Jerry Parker, Tony Grimason and Lucy Robertson, for the view in the kitchen of detection of *Cryptosporidium* and *Giardia* in water, viability techniques and general parasitological lab-techniques and the discussions on various aspects of waterborne transmission of these parasites. For me, this was the starting point of the work that has resulted in this thesis.

De grote interesse die de waterleidingbedrijven hebben getoond voor mijn werk heb ik altijd als erg stimulerend ervaren. Ik heb veel geleerd over de wijze waarop deze problematiek voor de waterleidingbedrijven speelt van de discussies in de werkgroep Microbiologie en later de werkgroep Verwijdering van Micro-organismen. Lambert van Breemen, Henk Castelijns, Jochem Evendijk, Jan Heijnen, Peter Hiemstra, Ton Hoekstra, Frans Houtepen, Peter Nuhn, Paul Ooms, Marcel Rutte, Arie de Ruijter, Jon Schellart, Guus Soppe, Trudy Suylen, Bram van der Veer, Jannie Willemsen-Zwaagstra, Henk van der Zouwen bedankt voor jullie inbreng, betrokkenheid en het beschikbaar stellen van gegevens. Deze dank geldt in het bijzonder voor Waterwinningbedijf Brabantse Biesbosch, dat vanaf het eerste uur direct betrokken zijn geweest met het onderzoek, met name in de persoon van Henk Ketelaars. Henk, we hebben veel gediscussieerd over de betekenis van de parasieten en vooral over het wel en wee van de detectiemethode. Je ervaart nu zelf dat daar veel wee in zit. Je grote betrokkenheid bij dit onderzoek heb ik altijd heel inspirerend gevonden. Je kennis over de protozoa en je streven naar perfectie hebben in grote mate bijgedragen aan de inhoud en ook aan de vorm van dit proefschrift. Ik vind het plezierig dat we ook in de toekomst samen aan deze parasieten zullen werken.
Ook Wim Hoogenboezem van NV PWN Waterleidingbedrijf Noord-Holland, bedankt voor je inbreng in het ‘Crypto-werk’. Ook jouw betrokkenheid en inzicht hebben me geholpen bij de totstandkoming van dit proefschrift. In Glasgow bleek dat onze gezamenlijke achtergrond en interesse breder was dan alleen de watermicrobiologie. Ik kan nu eindelijk aan het boek over evolutie beginnen.

Op dezelfde wijze als ik veel heb opgestoken van de contacten met de waterleidingbedrijven, waren ook de contacten met de Inspectie Milieuhygiene en de

beleidsdirectie Drinkwater, Water, Landbouw van VROM prettig en leerzaam. Maup Oversluizen, Frans Wetsteijn, Monze Joppen, Frans Swinkels, Arno van Breemen, Wennemar Cramer en Bernard Haring, via jullie heb ik gezien hoe beleid en wetgeving wordt gevormd en toezicht wordt gehouden op naleving daarvan. Ik heb veel waardering voor de constructieve wijze waarop jullie dat met de drinkwatersector vormgeven en vind het leuk om daar nu 'van de andere kant' aan bij te dragen.

Gert Boschman van Becton Dickinson, bedankt voor het vertrouwen dat je in ons had voor de ontwikkeling van de *Cryptosporidium* en *Giardia* - applicatie voor de FACSort en de ondersteuning die je daarbij hebt gegeven.

Daytona Daytrippers en The Dice, bij jullie heb ik genoten van de muziek die we samen hebben kunnen maken de afgelopen jaren. Voor mij waren dit ook telkens momenten om lekker 'uit te waaien'.

Marjolein, jou ben ik het meest dankbaar. Je was letterlijk mijn belangrijkste steun en toeverlaat. Mijn steun omdat je in doen en laten altijd achter me stond, en liters koffie en thee naast me neerzette terwijl ik met koptelefoon op naar het beeldscherm staarde. Mijn toeverlaat was je vooral doordat je het reilen en zeilen van ons gezin voor het grootste deel voor je rekening nam, zowel tijdens de weekeinden en vrije dagen dat ik boven zat, als tijdens de 'een-ouder-vakanties'. Na twee jaar vrijwel geheelonthouding verheug ik me er erg op dat we weer met zijn allen op vakantie kunnen.

Zo, nu kan ik eindelijk *echt* van het gekraai van mijn jongste dochter gaan genieten en kunnen de andere kinderen ook weer eens bij de computer, want het 'schriftje' van pappa is af!

CURRICULUM VITAE

The author of this thesis, Gertjan Medema, was born February 28, 1962 in Zoetermeer, The Netherlands. From 1973-1979, he attended the Erasmus College high school in Zoetermeer. He started to study Biology at the University of Leiden in 1979, where his interest in Microbiology started in his first year with “All is everywhere, the environment selects” (Beijerinck), which resulted in a MSc project on the interaction between *Rhizobium* and plant root hairs (Botanical Laboratory, Dr. Jan Kijne, Prof. Quispel, Prof. Lugtenberg) and on methanogenic bacteria in anaerobic treatment of wastewater from the Heineken breweries (Dr. D. Klijnhout, Dr. C. Wijffelman). He also obtained his certificate as high school teacher in Biology. He graduated as MSc in Biology in 1985.

From 1986-1987, he studied the mixotrophic growth of *Thiospaera pantotropha*, a versatile bacterium isolated from a microbial denitrification reactor for wastewater treatment at the Faculty of Chemistry of the Technical University of Delft (Prof. J. Gijs Kuenen, Dr. L. Robertson), as a substitute for his military service.

Now captured by microbes and water, he started at the National Institute of Public Health and the Environment in 1988 at the Department of Water Microbiology of the Laboratory of Water and Food Microbiology (Dr. ir. Arie H. Havelaar) where he worked on disinfection of *Aeromonas* in drinking water with chlorine dioxide (1988-1989), the infectivity of ‘viable, non-culturable *Campylobacter jejuni´* in water (1989-1991), methods for *Campylobacter* detection in water and wildlife as a source of *Campylobacter* in reservoirs (1990-1992) and *Plesiomonas shigelloides* in Dutch bathing waters (1990-1992). He was principal investigator in a large study on the relation between faecal contamination of recreational waters and intestinal health effects after bathing (1991-1996).

His first contact with the parasitic protozoa *Cryptosporidium* and *Giardia* was in 1991. He performed a number of studies on the sources, occurrence and behaviour of these parasites in water. The studies on *Cryptosporidium* and *Giardia* that are described in this thesis were carried out between 1994 and 1998.

Since 1997, he is working at Kiwa, the research institute of the drinking water companies in The Netherlands, where he studies the occurrence of *Cryptosporidium* and *Giardia* and other pathogens in source waters and the efficiency of water treatment in removing these micro-organisms.

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