Global Water Research Coalition

Waterborne Pathogens

A review of current knowledge on waterborne pathogens
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Prepared by:
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Suez Environnement - CIRSEE

March 2006
GWRC is a non-profit organization that serves as a collaborative mechanism for water research. The benefits that the GWRC offers its members are water research information and knowledge. The Coalition focuses on water supply and wastewater issues and renewable water resources: the urban water cycle.

The members of the GWRC are: the Awwa Research Foundation (US), CRC Water Quality and Treatment (Australia), EAWAG (Switzerland), Kiwa (Netherlands), Suez Environment- CIRSEE (France), Stowa - Foundation for Applied Water Research (Netherlands), DVGW – TZW Water Technology Center (Germany), UK Water Industry Research (UK), Veolia- Anjou Recherché (France), Water Environment Research Foundation (US), Water Research Commission (South Africa), WateReuse Foundation (US), and the Water Services Association of Australia.

These organizations have national research programs addressing different parts of the water cycle. They provide the impetus, credibility, and funding for the GWRC. Each member brings a unique set of skills and knowledge to the Coalition. Through its member organizations GWRC represents the interests and needs of 500 million consumers.

GWRC was officially formed in April 2002 with the signing of a partnership agreement at the International Water Association 3rd World Water Congress in Melbourne. A partnership agreement was signed with the U.S. Environmental Protection Agency in July 2003. GWRC is affiliated with the International Water Association (IWA).
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Infectious diseases caused by pathogenic bacteria, viruses and protozoa, are the most common and widespread health risk associated with drinking water. Most waterborne pathogens are introduced into drinking water supplies by human or animal faeces (enteric pathogens) but they can also exist naturally in water environments as indigenous aquatic micro-organisms.

Controlling the risks related to these pathogens is a permanent challenge for the water industry. In addition to the constantly evolving range of pathogens to consider, assessing and managing such risks requires the integration of information issued by a wide range of disciplines. The necessary knowledge is however still sketchy and incomplete for most pathogens, and research efforts are necessary to fill the remaining gaps of knowledge.

The purpose of this study is to provide an updated, comprehensive review of current knowledge on a selection of pathogens of interest for the drinking water industry, and to identify the remaining gaps of knowledge and thus the necessary research to be conducted. This review is based on the literature published up to 2005. Emphasis has been laid on the information needed to assess and manage the risks related to each of these pathogens in drinking water production and distribution.

The pathogens considered in this review were selected on the basis of:

− Their recognized or highly suspected transmission by drinking water through ingestion, inhalation, or contact
− Their recognized pathogenic character for humans
− The severity of their health effects.

Due to the similarity of these selection criteria to those used by the USEPA for the establishment of the drinking water Contaminant Candidate List (CCL), the micro-organisms listed in the two versions of the CCL (USEPA 1998, USEPA 2005) were automatically included in this study.

The micro-organisms identified as the etiologic agents in waterborne disease outbreaks in Europe, Canada, and the USA (Risebro 2006, Lee 2002, Blackburn 2004, and for Legionella in Europe: http://www.ewgli.org), were also considered in this study. Burkholderia was added to the list because of its implication in outbreaks in Australia and southeast Asia (Inglis 2000), and hepatitis E virus and Cyclospora because of their recent recognition as emerging water-related pathogens (WHO 2003).

As a result of this selection, the micro-organisms addressed in this study include enteric bacteria: Campylobacter spp., Escherichia coli O157:H7, Helicobacter pylori, Salmonella spp., Shigella spp., Yersinia spp., aquatic bacteria: Aeromonas spp., Burkholderia pseudomallei, Cyanobacteria, Legionella spp., non-tuberculosis mycobacteria, enteric viruses: adenovirus, calicivirus, enterovirus (coxsackievirus and echovirus), hepatitis A virus (HAV), hepatitis E virus (HEV), rotavirus, enteric protozoa: Cryptosporidium, Giardia, Cyclospora, Microsporidia and the aquatic protozoan, Acanthamoeba spp.

This list is not exhaustive and should evolve in the future, since the number of known pathogens for which water is a transmission route continues to increase and new pathogens continue to be discovered.

Each micro-organism in this study is described in a summary fact sheet composed of the following items: microbiology, human health effects, geographical distribution, epidemiology, ecology, inactivation/removal, surrogates, environmental detection and research needs.

Since much remains unknown about many aspects that are common to viruses, the study features a general section on « virus research needs ».

GWRC members have conducted (or supported) a great number of studies on waterborne pathogens, and produced a considerable number of reports. It appears from these reports that most of their research efforts have been dedicated to risk management. A special section summarizes the research areas covered by these studies.

Finally, the conclusion gives an overview of the main gaps in current knowledge on these waterborne pathogens, but does not propose any priority for research, since this would require to take into account financial and political considerations which are not in the scope of this study.
Bacteria
**Campylobacter**

<table>
<thead>
<tr>
<th>Health significance</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
<td>Important animal source</td>
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</tr>
</tbody>
</table>

Adapted from table 7.1 "Waterborne pathogens and their significance in water supplies" (WHO 2004).

**Microbiology**

*General description*

Since the late 1970s, *Campylobacter* spp. have become one of the most important causes of acute human gastroenteritis worldwide (Hunter 1997). Campylobacters are slender, spirally curved, motile, Gram-negative bacteria. They are strictly microaerophilic, non-saccharolytic and oxidase positive.

*Classification*

There are 16 species and six subspecies assigned to the genus *Campylobacter*, of which the most frequently reported in human disease are *C. jejuni* (formerly known as *Campylobacter fetus subsp. Jejuni*) and *C. coli*. These species are also called thermophilic campylobacters in that they are able to grow at 43°C.

**Human health effects**

*Clinical features*

Campylobacteriosis is an infection of the gastrointestinal tract. Symptoms of the infection include diarrhoea (often including the presence of mucus and blood), abdominal pain, malaise, fever, nausea and vomiting. The illness usually lasts 2 to 5 days but may be prolonged by relapses, especially in adults. Many of those infected show no symptoms. In some individuals reactive arthritis (painful inflammation of the joints) can occur. Rare complications include seizures due to high fever or neurological disorders such as Guillain-Barre syndrome or meningitis. Death from campylobacteriosis is rare and is more likely in the very young, the very old, or those already suffering from a serious disease such as AIDS. The pathogenic mechanisms of *C. jejuni* are still not completely understood, but it does produce a heat-labile toxin that may cause diarrhea. *C. jejuni* may also be an invasive organism.

*Dose – response data*

The infective dose is low: it has been estimated that approximately 500 cells of *C jejuni* can cause human illness (Black 1988). Different β-Poisson dose-response models based on experimentation on human volunteers have been described (Medema 1996). More recently, a model fitting the dose - response relationship observed from both the human feeding study and outbreaks data has been described (Teunis 2005). This model more appropriately fits the results observed with low doses.

*Target populations*

Although anyone can suffer from *Campylobacter* infection, children under 5 years and young adults (15-29) are more frequently afflicted than other age groups.

*Diagnosis*

Diagnosis is performed by culture of faeces on a selective agar, usually a commercial campylobacter medium containing special supplements. Incubation has to be carried out in a microaerophilic atmosphere (5% oxygen).
**Treatment**

As with all diarrhoeal illnesses, treatment involves rehydration therapy plus antibiotic therapy for those with severe infection.

**Geographical distribution**

Worldwide.

**Epidemiology**

Approximately 5%-14% of all diarrhoea worldwide is believed to be caused by *Campylobacter*. In almost all developed countries, the incidence of human campylobacter infections has been steadily increasing for several years. The reasons for this are unknown (WHO 2004).

In addition the emergence of antibiotic-resistant strains of Campylobacter all over the world has been observed.

**Transmission routes**

Transmitted by the faecal-oral route.

Campylobacteriosis is considered to be a zoonosis, a disease transmitted to humans from animals (mainly poultry) or animal products. In animals, campylobacters seldom cause disease. People are exposed to the bacteria after consuming contaminated food such as undercooked meats, contaminated water, or raw milk and by direct or indirect contact with animals. Contact with raw poultry and ingestion of undercooked poultry are the major sources of human infections in the USA (Tauxe 1988). Person to person transmission is relatively uncommon (Hunter 1997).

**Waterborne outbreaks**

Although the majority of cases are sporadic, numerous outbreaks have been related to drinking untreated or inadequately chlorinated water, especially in Nordic-European countries (Koenraad, Rombouts et al. 1997) (Nygard 2003) (Melby 1991) (Miettinen 2001) (Rautelin 1990) (Kuusi 2004), in the USA (Craun 1996), in England and Wales (Said 2003) and also in Canada (Alary 1990) and in New Zealand (Stehr-Green 1991).

Two outbreaks involving both *Campylobacter* and *E coli 0157* infection associated with contamination of a drinking water supply have been reported: the first occurred in 1996 in a Fife village affecting 711 residents (Jones 1996) and the second occurred in May 2000 in Walkerton, Ontario, affecting over 2,000 persons and killing seven (Holme 2003).

Recently, one study suggested that swimming in natural sources of water was a novel risk factor (Schonberg-Norio 2004).

**Ecology**

**Regrowth in distribution systems/biofilms**

Cannot multiply in water.

However, one study (Buswell 1998) suggested that integration into aquatic biofilms can enhance *Campylobacter* survival.

**Occurrence**

Campylobacters have frequently been isolated from surface water, such as streams, rivers, and lakes, due to discharges from wastewater treatment plants, runoff from pastures after rain, and direct contamination by wild birds (Brennhovd 1992) (Jones 2001) (Diergaardt 2004) (Hörman, Rimhanen-Finne et al. 2004) (Arvanitidou 1995) (Koenraad 1997) most frequently at a concentration of less than 10 MPN (most probable number) per 100ml. They have occasionally been isolated from ground-water (Stanley...
1998) and drinking water (Moore 2001) (Arvanitidou 1994) but the numbers detected were low (maximum 0.3 MPN in 100ml) (Savill 2001). Studies have shown that sewage and sewage sludge contain campylobacters in concentrations of $10^2$ to $10^5$ CFU/100 ml and $10^1$ to $10^3$ CFU/100 ml, respectively (Jones 1990).

**Reservoirs**

The bacteria are widely distributed and found in most warm-blooded domestic and wild animals. They are common in food animals such as poultry, cattle, pigs, sheep, ostriches, and shellfish and in pets including cats and dogs. Recently Axelsson-Olsson et al. (Axelsson-Olsson 2005) suggested that amoebae *Acanthamoeba polyphaga* may serve as a nonvertebrate reservoir for *C. jejuni* in the environment.

**Survival**

Several studies reported the persistence of *Campylobacter* spp. in water in a viable but non-cultivable form (VBNC) which is thought to be capable of causing infection (Jones 1991) (Rollins and Colwell 1986) (Talibart 2000). However the role of this form as a survival mechanism or simply a degenerative form remains controversial. *C. jejuni* and *C. coli* survive in cold water (at temperatures below 10°C) much longer (for up to 4 months) (Rollins and Colwell 1986) than they survive in water at temperatures higher than 18°C (Korhonen 1991) (Korhonen 1991) (Thomas 1999). In water microcosm experiments, factors such as temperature, oxygenation, anaerobic conditions, nutrient and biofilms influenced their survival. Survival was enhanced by the presence of autochthonous microflora and nutrients, and decreasing temperature and anaerobic conditions (Thomas 1999) (Leclerc 2004). Buswell et al. (Buswell 1998) suggested that the integration into aquatic biofilms can enhance *Campylobacter* survival. Recently one study showed that *C. jejuni* cells survived longer when cocultured with amoebae than when cultured alone (Axelsson-Olsson, 2005). It has been shown that the origin of the strain is a determining factor for the survival of *C. jejuni* in drinking water at 4°C: poultry isolates showed a prolonged survival, which could be an indication that these strains could play an important role in the transmission of campylobacteriosis through water (Cools 2003).

**Inactivation/removal**

Published data on inactivation are summarized in annex 1. Blaser at al. (Blaser 1986) suggested that disinfection procedures (using chlorine and monochloramine) commonly used for treatment of drinking water to remove coliform bacteria are adequate to eliminate *C. jejuni*. However, ingestion of *C. jejuni* by protozoans has been shown to protect against subsequent disinfection (King 1988).

**Surrogates**

As *Campylobacter* spp. are faecally borne pathogens and are not particularly resistant to disinfection, *E coli* is an appropriate indicator for the presence/absence of *Campylobacter* spp. in drinking water supplies (WHO 2004).

**Environmental detection**

Traditional culture methods are available but they are time-consuming (4 to 5 days) and laborious, requiring prolonged incubation, selective enrichment and biochemical identification (Fricker 1987). Moreover, they fail to detect viable but not culturable states.

Different molecular methods have been developed to detect *Campylobacter* in water. Direct PCR assays have been developed for *Campylobacter* spp. (Moore 2001) (Moreno 2003) or specifically for *C. jejuni* and *C. coli* (Oyofo 1993) (Kirk 1994). These tests are rapid and sensitive but cannot distinguish viable and dead microorganisms.

Several approaches have been described to only detect viable campylobacters in water:

- PCR or seminested PCR assays combined with enrichment culture (Hernandez 1995) (Waage 1999); a PCR enzyme-linked immunosorbent assay (ELISA) combined with 48H of enrichment culture to detect viable *C. jejuni* and *E. coli* (Sails 2002); an in situ hybridization assay to detect viable...
thermotolerant Campylobacter species in river water and sewage (Moreno 2003); a reverse transcriptase polymerase chain reaction assay targeting mRNA (Sails 1998).

- A real-time PCR assay for the quantitative detection of Campylobacter jejuni in food after 48H of enrichment culture was developed (Sails 2003), but to date, no rapid quantitative method has been developed to detect viable Campylobacter in water.
- A wide range of genotyping methods have been used in molecular epidemiology and taxonomy studies: ribotypes (Hernandez 1996), RAPD fingerprinting (Hernandez 1995) (Karita 2003) (Yakoob 2000) (Hernandez 1996), PCR and sequencing (Lu 2002), PCR-RFLP (restriction fragment length polymorphism) and AFLP (amplified fragment length polymorphism) (Ronner 2004) (Moreno 2002) or by pulsed field gel electrophoresis (Steele 1998) (Hanninen 1998). Currently all these available alternative molecular methods are at the experimental stage and validated protocols do not yet exist. In the future evaluation studies and standardization of these new methods will be necessary.

Research needs

Partly for lack of rapid and specific methods to detect this microorganism in environmental water, much remains unknown about the epidemiology, ecology and resistance to treatment.

Analytical methods

It is necessary to develop and validate molecular methods for rapid, specific, and quantitative detection of viable C. jejuni and C. coli in environmental water. Evaluation studies including comparative studies with the reference culture method and standardization of these new methods will also be necessary.

Route of transmission

There is a lack of knowledge concerning the role of various environments in the transmission of Campylobacter. One of the major gaps in our knowledge at present is the relative contribution of each of the known and unknown sources to the overall burden of disease. Research needs should include the percent contribution from drinking water as opposed to other exposure routes associated with human campylobacteriosis infections.

Occurrence

Occurrence needs to be more documented with levels of contamination on various types of water, including groundwater and treated drinking water.

Reservoirs

Studies on potential new reservoirs for campylobacters need to continue.

Environmental survival

Further studies should investigate the ability of Campylobacter to enter the VBNC state, and the role of the VBNC state in this organism.

The impact of the majority of environmental variables on Campylobacter strains survival and the risk that this poses to human health remain unclear.

Biofilms: the survival of the pathogen in potable water biofilms and the role of biofilm as a reservoir of Campylobacter infections need to be further investigated. How are Campylobacter's infectivity, exposure route and resistance to treatment altered by association with biofilms and by intracellular survival within protozoa?

Inactivation / removal

Specific data on Campylobacter removal and inactivation by conventional clarification and disinfection processes are lacking.
**E coli O 157:H7**

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Adapted from table 7.1 *“Waterborne pathogens and their significance in water supplies”* (WHO 2004).

**Microbiology**

**General description**

Whereas *E coli* bacteria are usually symbiotic as part of the normal intestinal flora of animals and humans, some strains are capable of causing serious diarrhoeal infections in humans. Pathogenic *E coli* are divided into six groups based on serological and virulence characteristics: enterohemorrhagic (EHEC), enterotoxigenic (ETEC), enteropathogenic (EPEC), enteroinvasive (EIEC), enteroaggregative (EAggEC), and diffuse adherence (DAEC).

The most prominent representative of the EHEC, previously called VTEC (Verotoxin-producing *E coli*) or STEC (shiga toxin-producing *E coli*) is *E coli O157:H7*, first recognized as a human pathogen in 1982 (Riley 1983). Its pathogenicity has been attributed in part to the production of a shiga-like toxin that is cytotoxic for Vero cells.

**Classification**

*E. coli* O157:H7 is a specific serotype of *E. coli*. The combination of letters and numbers in the name of the bacterium refers to the specific markers found on its surface and distinguishes it from other types of *E. coli*.

**Human health effects**

**Clinical features**

*E. coli* O157:H7 causes acute bloody diarrhoea and abdominal cramps with little or no fever; sometimes the infection causes nonbloody diarrhoea or no symptoms. The incubation period is 3-4 days, and usually the illness resolves in 5 to 10 days.

In some persons, particularly children under 5 years of age and the elderly, the infection can also cause a complication called hemolytic uremic syndrome (HUS), in which the red blood cells are destroyed and the kidneys fail. About 2%-7% of infections lead to this complication (Mead 1998).

Except in the case of humans with a severe infection, the number of *E.coli O157* in faecal material will be several orders of magnitude lower than the concentration of other *E.coli* species.

**Dose – response data**

The infectious dose is estimated at less than 100 bacteria (Griffin 1991) (Keene 1994). Due to its high virulence, no human dose-response study has been conducted with this microorganism. However, a $\beta$-Poisson model based on animal studies has been shown to be applicable to man (Haas 2000). Application of $\beta$-Poisson models developed for *Shigella dysenteriae* has also been proposed (Powell 2000, Teunis 2004).

**Target populations**

All persons. Children under 5 years old and the elderly are more likely to develop serious complications.
**Diagnosis**

Diagnosis is based on clinical presentation and culture of faeces using a modified sorbitol MacConkey agar. A positive isolation is confirmed by biochemical tests and agglutination by specific O157 antiserum (Hunter 1997). The O157:H7 strain is distinguished microbiologically from other *E. coli* by its inability to ferment sorbitol and, most importantly, by its production of "shiga-like" toxins.

**Treatment**

Most persons recover in 5-10 days without antibiotics or any other specific treatment. Hemolytic uremic syndrome is a life-threatening condition usually treated in an intensive care unit. Blood transfusions and kidney dialysis are often required. With intensive care, the death rate for hemolytic uremic syndrome is 3%-5%.

**Geographical distribution**

Worldwide. Since the first description of this illness in 1982, infections have been reported from more than 30 countries.

**Epidemiology**

**Transmission routes**

*E coli O157:H7* is mainly transmitted by food. The major source is ground beef; other sources include unpasteurized milk and juice, sprouts, lettuce, and salami. Transmission via drinking water and recreational water, person-to-person contact as well as direct animal-to-man contact has also been documented (WHO 2004). Contamination of produce may occur after using contaminated water for irrigation or processing.

**Waterborne outbreaks**

In recent years, several outbreaks of *Escherichia coli* O157:H7 have been associated with consumption of contaminated municipal water, well water, and contact with recreational waters (Bopp 2003) (Swerdlow 1992) (Chalmers 2000) (Centers for Disease Control 2000) (Dev 1991) (Bruce 2003) (Isaacson 1993) (Keene 1994). Two outbreaks involving both *Campylobacter* spp. and *E coli 0157* coinfection associated with contamination of a drinking water supply have been reported: the first occurred in 1996 in a Fife village affecting 711 residents (Jones 1996) and the second occurred in May 2000 in Walkerton, Ontario, affecting over 2,000 persons and killing seven (Holme 2003).

Despite the potential for large contamination of the environment, waterborne infection is relatively rare because *E coli O157* is as susceptible to chlorination as bacterial indicators. In most outbreaks associated with drinking water the cause was the contamination of the water supply after treatment and during distribution.

**Ecology**

**Regrowth in distribution systems/ biofilms**

Not extensively documented. The persistence of *E coli O157:H7* in drinking water biofilms has not yet been studied.

**Occurrence**

Sources of environmental contaminations are thought to be animal and human faeces or sewage. *E coli O157* have been detected in a variety of water environments: in raw water (Gannon 2004) and surface water (Johnson 2003) (Kogure 1997). It is commonly present in animal and human wastewaters (Garcia-Aljaro 2005).
Levels of contamination and distribution are not well documented, particularly in developing countries.

**Reservoirs**

Man, livestock, such as healthy cattle and sheep (Chapman 1993) and, to a lesser extent, goats, pigs, chickens and wild birds are a major source of EHEC strains. These sources can be regarded as threats to the quality of water supplies, particularly private supplies in which adequate treatment is not guaranteed.

**Survival**

A limited number of studies has evaluated the ability of *E. coli* O157:H7 to persist in environmental water. Limited observations, however, reported that *E. coli* O157:H7 is particularly well adapted for surviving in the aquatic environment (Kogure 1997) (Maule 2000). It survives for long periods of time in water, especially at cold temperatures (<10°C) (Wang 1998) (Porter 1997). *E. coli* O157:H7 survived in inoculated water for up to > 300 days, depending on the type of water (Warburton 1998).

Kerr et al. confirmed that *E coli* O157:H7 can survive for long periods in the nutrient-starved conditions offered by bottled mineral water (Kerr 1999).

Artz et al. reported that wells with higher water quality as assessed using the European Union Drinking Water Directive standards may allow survival of *E. coli* O157:H7 for long periods (Artz 2002). *E. coli* O157:H7 in soil persisted for over 5 months after application of contaminated compost or irrigation water (Islam 2004).

Several reports indicate that *E. coli* O157 becomes nonculturable during prolonged storage in water (Mizunoe 1999) (Rigsbee 1997) (Wang 1998) (Kolling and Matthews 2001) but the fate of *E coli* O157 as viable but not culturable bacteria (VBNC) has not been investigated.

As for other agents (*Legionella, Mycobacteria...*) Acanthamoeba may serve as a host for *E. coli* serotype O157 survival and growth in water and may protect them (Barker 1999) against chlorination and other disinfection measures.

One study reported that *Escherichia coli* O157:H7, *Salmonella* spp., and *Vibrio cholerae* can grow in nutrient-limited, reconditioned wastewater over the temperature range of 4 to 46 degrees C when the biological oxygen demand of this water is <2 (Rajkowski 2001).

**Inactivation/ removal**

Published data on removal and inactivation are summarized in annex 1. Water treatment processes of coagulation, filtration and disinfection are effective in removing enteric bacteria. The incidence of *E.coli* O157 in treated drinking water is likely to be a problem principally if there has been a failure of the chlorination process at the treatment works or contamination of the treated water in supply.

Chlorine levels typically maintained in water systems are sufficient to inactivate these organisms. (Rice 1999). But one study demonstrated that *E. coli* O157:H7 adapts to starvation conditions by developing a chlorine resistance phenotype (Lisle 1998). Further studies should be carried out on this topic.

**Surrogates**

There is no indication that the response of *E coli* O157 to water treatment and disinfection procedures differs from that of other *E coli*. Hence conventional testing for *E coli* (or, alternatively, thermotolerant coliform bacteria) provides an appropriate index for the presence of *E coli* O157 in drinking water (WHO 2004).

**Environmental detection**

Concentration: the most widely used filtration method for recovering bacteria is membrane filtration using microporous membranes typically composed of cellulose esters. Following filtration, the cells recovered on the membrane filter can be directly assayed or cultured.
Traditional culture methods rely upon a preliminary enrichment step followed by plating on selective media, such as Sorbitol MacConkey agar and screening negative sorbitol-fermenting colonies followed by confirmation by biochemical and serological tests: they are laborious and time-consuming (at least 3 days) and cannot detect injured cells nor the viable but non culturable state.

A variety of immunological methods have been developed.
The use of enrichment in conjunction with immunological detection has been reported to detect viable \textit{E. coli O157:H7} in surface water (Shelton 2004).
An approach combining fluorescent antibody and tetrazolium dye reduction, which detect respiratory activity, has been used to enumerate viable \textit{E. coli O157:H7} in water (Pyle 1995).
Another approach has been described which combines immunomagnetic separation with cyanoditolyl tetrazolium chloride (CTC) incubation to determine respiratory activity and fluorescent antibodies for enumeration by either fluorescence microscopy or laser cytometry (Pyle 1999).
Recently one study (Bukhari 2004) reported the rapid detection (8H) of \textit{E. coli O157:H7} using commercial immunological lateral diffusion assays.
The combined use of an immunomagnetic separation method and immunoblotting has been described for the enumeration and isolation of \textit{Escherichia coli O157} in wastewater (Garcia-Aljaro 2005).
For several years, PCR-based detection has been increasingly used because it is highly sensitive and rapid and it allows the detection of bacteria in a VBNC state.
Campbell et al. (Campbell 2001) described a multiplex PCR assay in combination with an enrichment step for sensitive detection of \textit{E. coli O157:H7} in water. Another study (Tims 2003) reported the detection of viable \textit{E. coli O157:H7} by enrichment and PCR after rapid biosensor detection. Direct PCR has also been used for rapid detection (Fode-Vaughan 2003) (Jothikumar 2002).
Some studies reported the simultaneous detection of several bacterial pathogens in addition to \textit{E. coli O157:H7}. Simultaneous detection of \textit{Escherichia coli O157:H7}, \textit{Listeria monocytogenes} and \textit{Salmonella} strains by real-time PCR (Bhagwat 2003); Morin et al. (Morin 2004) developed a reverse transcription-multiplex PCR assay for simultaneous detection of viable \textit{Escherichia coli O157:H7}, \textit{Vibrio cholerae O1}, and \textit{Salmonella Typhi} by targeting mRNA.
Only two studies (Ibekwe 2002) (Ibekwe 2003) reported quantitative detection in environmental samples based on real-time PCR. This promising method needs to be optimized, validated and compared with the standard culture method.
Commercial PCR kits are available for screening food and environmental samples: the Probelia kit from Diagnostic Pasteur or the Bax® system from DupontQualicon. But these kits are not suitable for the detection of \textit{E. coli O157} in water samples with low contamination.
Genotyping \textit{E. coli O157} strains by pulsed-field gel electrophoresis (PFGE) has been commonly used to track sources of \textit{E. coli O157} outbreaks (Preston 2000) (Bender 1997) (Bopp 2003) as well as to study the genetic diversity of strains from range cattle environments (Renter 2003).

Research needs
To date, research has focused on agricultural sources of \textit{E. coli O157} and how it enters the food chain. Not much work has been done on \textit{E. coli O157} in water although it is an increasing concern to the water industry. Much remains unclear about the ecology of this microorganism and its resistance to water treatment.

\textbf{Analytical methods}
Numerous PCR-based methods have been described for the detection of \textit{E. coli O157:H7} in water, but optimisation and validation is still needed in order to use them as an alternative to the traditional culture method.
Need to validate the existing PCR-based methods.

\textbf{Ecology}
Need to clarify the source, distribution, and fate of \textit{E. coli O157} in environmental water.
The different animal reservoirs need to be identified and their relative importance clarified.

**Environmental survival**

Insufficient data is available on the survival of *E. coli O157* in water. Many environmental factors including temperature, nutrient availability, predation and sunlight should be evaluated. Need to determine whether *E. coli O157* exhibits similar decay rates in natural waters as faecal indicator organisms. This would demonstrate whether or not monitoring microbiological quality using standard parameters such as coliforms and *E. coli* could be an adequate indication of the potential presence of *E. coli O157*.

Long–term survival of non-culturable yet viable *E coli O157* may have implications for the transmission of the disease and should be studied further.

Does *E.coli O157* survive long enough on land to contaminate surface or groundwater sources, via surface runoff?

**Treatment**

Future research must be carried out to ensure that *E coli O157* is removed by normal water treatment processes as other *E coli*. Furthermore, research must be carried out to confirm whether *E coli O157* in VBNC state may develop a chlorine resistance phenotype.
Helicobacter Pylori

<table>
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<th>Health significance</th>
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<tr>
<td>Resistance to chlorine</td>
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</tr>
<tr>
<td>Relative infectivity</td>
<td>Unknown</td>
</tr>
<tr>
<td>Important animal source</td>
<td>No</td>
</tr>
</tbody>
</table>

Microbiology

**General description**

*Helicobacter pylori* was recognized as a human pathogen in 1983 (Marshall 1984) and was subsequently identified as a human carcinogen by the International Agency for Research on Cancers (IARC). It is included in the Contaminant Candidate List (CCL) (U.S. Environmental Protection Agency. 2005) (U.S. Environmental Protection Agency. 1998).

*H. pylori* is a Gram-negative microaerophilic, spiral-shaped, motile bacterium. Two morphologically distinct forms, a spiral shape and a coccoid form have been identified. The spiral shape is routinely cultured from clinical samples. To date, the coccoid form has never been cultured but it remains metabolically active (Hunter 1997). Transformation from the spiral-shaped bacterium to the coccoid form is thought to result from variations in the environment such as oxygen stress, temperature changes, the presence of antibiotics and other stress-inducing conditions (Engstrand 2001). It has been suggested that the coccoid form is responsible for transmission in the environment (Hulten 1998).

**Classification**

When it was first described in 1983, the species was placed into the genus *Campylobacter*, but it was given its own genus later in 1989. There are at least 14 species of *Helicobacter*, but only *H. pylori* has been identified as a human pathogen.

Human health effects

**Clinical features**

*H pylori* is found in the stomach; although most infections are asymptomatic, the organism is the causative agent of gastritis and duodenal ulcers and plays a role in the development of gastric cancer. Infections are more prevalent in developing countries and are associated with overcrowded living conditions (WHO 2004). It has been estimated that more than half of the world's population is infected with this organism (Lambert 1995).

**Dose – response data**

The infectious dose is unknown.

**Target populations**

All persons but the majority of *H pylori* infections are initiated in childhood and are chronic without treatment.

**Diagnosis**

Diagnosis can be confirmed by endoscopy followed by microscopy or culture of biopsy material onto appropriate media. A serological blood test and a urease breath test using radiolabelled carbon meals are also available.
Treatment

Therapy for H. pylori infection consists of 10 days to 2 weeks of one or two effective antibiotics, such as amoxicillin plus metronidazole.

Geographical distribution

H pylori infection is widespread throughout the world (Goodwin 1997)

Epidemiology

The epidemiology of H Pylori is still unclear. Studies on prevalence or seroprevalence suggest that drinking water might play some role in infection with H pylori (McKeown 1999) which has not been confirmed by other work (Begue 1998) (Teh 1994).

Transmission routes

The mode of transmission remains unclear (Stone 1999) but four different pathways have been suggested (Leclerc 2002): (1) by the faecal-oral route, the bacterium excreted by faeces, might colonize water sources, becoming available to be transmitted to humans; (2) by oral-oral route, H pylori which colonizes dental plaque and saliva, may be transmitted by saliva to other individuals (person to person transmission); (3) by gastric-oral route, the typical modality of transmission in childhood, by contaminated vomitus; (4) by gastric-gastric route, the bacterium be transmitted by endoscopic procedures. Recently several studies suggested that water may be a vector of H. pylori in its faecal-oral route (Horiuchi 2001) (Rolle-Kampczyk 2004) (Queralt 2005) (Watson 2004) (Karita 2003).

Waterborne outbreaks

Not documented.

Ecology

Very little is known about the ecology of H pylori.

Regrowth in distribution systems/biofilms

Cannot multiply in water. However, several studies have shown the presence of H. pylori in biofilms in water distribution systems (Park 2001) (Azevedo 2003) (Bunn 2002). Recently, Watson et al. (Watson 2004) suggested that biofilms in water distribution systems may act either as sites for the passive accumulation of helicobacters or as potentially important reservoirs of infection.

Occurrence

H. pylori has not been isolated from environmental waters samples by traditional culture techniques except in one study (Lu 2002) which isolated H pylori from untreated wastewater. Using molecular techniques (PCR), H. pylori specific DNA was detected in sewage (Vincent 1995), surface water (Hegarty 1999) (Queralt 2005), and water supplies (Watson 2004) (Hulten 1998) (Hulten 1996) (Horiuchi 2001) including biofilms (Park 2001) (Bunn 2002). Recently one study (Cellini 2004) reported the presence of free and plankton-associated H. pylori in seawater.

The health significance of finding Helicobacter spp. DNA in drinking water still remains unknown. Occurrence of Helicobacter pylori in surface, ground and finished waters needs to be further studied. Levels of contamination are unknown.
**Reservoirs**

Not determined precisely. Until recently the natural reservoir for *H. pylori* was thought to be the human gastrointestinal tract (Axon 1996). However, the association of *Helicobacter* with nonhuman sources, such as livestock (Vaira 1992), domestic cats (Bode 1998) and vegetables (Hopkins 1993) suggested potential environmental reservoirs.

**Survival**

Data on the environmental survival of *H. pylori* is limited. *H. pylori* in the environment transforms into a nonculturable, coccoid form, (Adams 2003) which frequently results in the failure to detect this bacterium in environmental samples by conventional culture techniques. It is unclear whether the coccoid form, suggested to be similar to the viable but non culturable (VBNC) state of waterborne bacteria, could revert to the spiral form and therefore be able to infect humans (Engstrand 2001). Only one study has shown a successful reversion of coccoid *H. pylori* to the rod shaped culturable form. This study was performed in mice (Wang 1997) but contradictory results had been reported in pigs (Eaton 1995).

The non cultivable coccoid form may persist up to 20 to 30 days in water as well as in food (Leclerc 2002). It can survive for up to 10 days at 4°C in tap water (Fan 1998). One study showed that free-living amoebae promote growth and survival of *Helicobacter pylori* under experimental conditions (Winiecka-Krusnell 2002). The putative dependence of *H. pylori* on free-living amoebae in nature could be important with respect to transmission and prevalence and needs to be studied.

**Inactivation/removal**

Published data on inactivation are summarized in annex 1. Readily inactivated by free chlorine (Johnson 1997). However, Baker et al. argued later that *H. pylori* could tolerate disinfectants better than the classical faecal indicator, *Escherichia coli* (Baker 2002).

**Surrogates**

*E. coli* (or alternatively, thermotolerant coliforms) cannot be used as an index for the presence/absence of *Helicobacter pylori* (Hegarty 1999) (WHO 2004).

**Environmental detection**

To date, there is no standard culture method for detecting *H. pylori* in water samples. Traditional media may be too nutrient rich to recover potentially stressed *H. pylori* from water (Azevedo 2004). One study reported the development of a plating medium for selection of *Helicobacter pylori* from water samples in seven days at 37°C (Degnan 2003). But the performance of this culture method has not yet been determined.

Immunological methods have been developed (Hegarty 1999), but they are limited because *H. pylori*-monoclonal antibodies may cross-react to closely related bacterial species or may not react with the non culturable coccoid form which may have a different antigenicity (Enroth 1995).

Different molecular methods have been developed to detect *H pylori* in water: Development of fluorescent in situ hybridization (FISH) to be applied in river and wastewater (Moreno 2003). A direct PCR assay (Benson 2004), two PCR-based techniques for detecting helical and coccoid forms of *Helicobacter pylori* (Shahamat 2004) and an IMS-PCR (Enroth 1995).

Molecular typing methods have been used in epidemiological studies: RAPD fingerprinting (Karita 2003) (Yakoob 2000), PCR and sequencing (Lu 2002) and PCR- RAPD.
Currently all these available methods are at the experimental stage and validated protocols do not yet exist. As no reference method exists, it is difficult to compare the performance of existing methods.

Research needs

Much remains unknown about this newly described bacteria. There are obvious research needs about the pathogenesis of human disease which is still poorly understood, including therapeutic drugs and infective dose. But research priorities for water utilities concern the epidemiology of *H. pylori*, in particular its possible waterborne transmission as well as the ecology which has not yet been extensively studied.

**Analytical methods**

Develop a reference culture method capable of detecting viable and culturable *H pylori* in potable waters.

Develop and validate molecular methods for rapid, specific, and quantitative detection of *H. pylori* in environmental water.

**Route of transmission**

The waterborne transmission route has been suggested by a large number of studies but there is still no evidence of transmission by this route. More epidemiological studies, including molecular methodologies for microbial source tracking, should be carried out, in order to assess the risk of waterborne transmission.

It would be important to estimate the relative importance of waterborne as opposed to other forms of transmission in both developed and developing countries.

**Occurrence**

Large studies on occurrence including treated, municipal drinking water and groundwater need to be carried out. The health significance of finding *Helicobacter spp*. DNA in drinking water needs to be documented.

**Reservoirs**

Studies on potential reservoirs for *H pylori* infections need to continue.

**Environmental survival**

Further studies should investigate the ability of *H. pylori* to enter the VBNC state, whether the coccoid form is viable and infectious and the role of the VBNC state in this organism.

The survival and development of the pathogen in potable water biofilms and the role of biofilm as a reservoir of *H pylori* infections need to be further investigated.

**Inactivation**

Efficacy of different inactivation treatment processes (disinfectants, UV...) must be evaluated, with a special attention to the capability of the organism to be present as aggregates.
Salmonella

<table>
<thead>
<tr>
<th></th>
<th>S. typhi</th>
<th>Other salmonellae</th>
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<tbody>
<tr>
<td>Health significance</td>
<td>High</td>
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</tr>
<tr>
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<td>May multiply</td>
</tr>
<tr>
<td>Resistance to chlorine</td>
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<tr>
<td>Relative infectivity</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Important animal source</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Adapted from table 7.1 "Waterborne pathogens and their significance in water supplies" (WHO 2004).

Microbiology

**General description**

Salmonella spp. are considered one of the most important causal agents of foodborne illness in developed countries.

The genus Salmonella is comprised mostly of facultatively anaerobic, oxidase-negative, catalase-positive, Gram-negative, rod-shaped bacteria. Most strains are motile and ferment glucose with production of both acid and gas.

**Classification**

The genus Salmonella belongs to the family Enterobacteriaceae. Salmonella nomenclature is complex, and scientists use different systems to refer to and communicate about this genus. The nomenclature for the genus Salmonella has evolved from the initial one serotype-one species concept proposed by Kauffmann (Kauffmann 1966) on the basis of the serologic identification of O (somatic) and H (flagellar) antigens. In one nomenclature system (Le Minor 1982), there is only one species recognized, Salmonella cholerasuis, divided into seven subspecies. In 1987, Le Minor and Popoff (Le Minor 1987) requested that Salmonella enterica (type strain LT2) be the type and the only species of the genus Salmonella. Recently Tindall et al. provided a clear interpretation of the nomenclature and taxonomy of the genus Salmonella (Tindall 2005). According to the CDC system (essentially based on the recommendations established by the WHO Collaborating Centre), the genus Salmonella contains two species, each of which contains multiple serovars. The two species are S. enterica - the type species which is divided into six subspecies - and S. bongori. Currently, most of the journals all over the world have adopted this system.

The genus comprises more than 2,500 serovars, most of which are considered potential human pathogens, but only a reduced number of serovars have been associated with human infections. Both serovars, S. typhi and S. paratyphi, cause enteric fever only in humans and in higher primates. Salmonella enteriditis and Salmonella typhimurium have a broad host-spectrum and constitute the two most important serovars for salmonellosis transmitted from animals to humans.

Human health effects

**Clinical features**

Salmonella are the cause of two enteric diseases called salmonellosis: enteric fever (typhoid), resulting from bacterial invasion of the bloodstream, and acute gastroenteritis, resulting from a foodborne infection/intoxication.

Symptoms of gastrointestinal disease include diarrhoea, fever, and abdominal cramps that develop 12 to 72 hours after infection. The illness usually lasts 4 to 7 days, and most persons recover without treatment. A few may develop a chronic condition called Reiter's syndrome. This syndrome can last for months or years and can lead to arthritis.

The incubation period for typhoidal disease is usually 10-14 days (range 5-23 days). Typhoid fever is a more severe illness and can be fatal. Although typhoid is uncommon in areas with good sanitary systems, it is still prevalent elsewhere, and there are many millions of cases each year.
**Dose – response data**

The infectious dose is variable according to the pathogenic *Salmonella* strains. It is estimated typically at over $10^5$ for non-typhoidal salmonellosis (Kothary and Babu 2001). As few as $10^3$ organisms can cause a typhoid infection (Hunter 1997). Different β-Poisson dose-response models based on experiments on humans have been described (Haas 1999, Teunis 1999, FAO/WHO 2002).

**Target populations**

Anyone can get salmonellosis, but it occurs more often in young children, the elderly, and the immunocompromised.

**Diagnosis**

Diagnosis of both gastrointestinal and typhoidal diseases is based on the culture of the responsible pathogen from faeces or blood culture. Confirmation of identity is based on biochemical and serological agglutination reactions (Hunter 1997).

**Treatment**

Persons with severe diarrhoea may require rehydration, often with intravenous fluids. Antibiotics are not usually necessary unless the infection spreads from the intestines. The antibiotic drugs of choice for typhoid are now ciprofloxacin and chloramphenicol. There are several vaccines which are effective against typhoid (Hunter 1997).

**Geographical distribution**

Worldwide, but according to the CDC, most cases of *Salmonella* illness have been reported in North America and Europe.

**Epidemiology**

Multidrug-resistant (MDR) strains of *Salmonella* are now encountered frequently and the rates of multidrug resistance have increased considerably in recent years, probably in response to antimicrobial usage in food animals. In the 1990s, an antibiotic-resistant strain of *S. typhimurium*, called Definitive Type 104 (DT104), was first found in the United Kingdom (Baggesen 2000), in Western Europe, and recently in the United-States. It is the second most common strain (after *S. enteritidis*) of *Salmonella* found in humans. This strain poses a major threat because it is resistant to several antibiotics normally used to treat people with *Salmonella* infections.

**Transmission routes**

*Salmoneella* is spread by the faecal-oral route. Infections with non-typhoidal serovars are primarily associated with person-to-person contact, the consumption of a variety of contaminated foods (mainly contaminated raw meat, poultry and poultry-derived products) and exposure to animals, including domestic animals such as cats and dogs. Infection by typhoid serovars (*S. typhi* and *S. paratyphi*) is associated with ingestion of contaminated water or food, direct person-to-person transmission being uncommon (WHO 2004).

**Waterborne outbreaks**

In the early twentieth century, typhoid was the most common known cause of waterborne outbreaks in both Europe and the USA. Typhoid and paratyphoid are now uncommon in the western world but are still a major problem in many less affluent countries. In 2001, however, an explosive waterborne outbreak caused by multidrug-resistant (MDR) *Salmonella Typhi* in a non-endemic community with otherwise good sanitation was reported (Swaddiwudhipong 2001). Despite their widespread distribution in the water environment, non-typhoidal salmonellae rarely cause waterborne outbreaks (Hunter 1997).
Ecology

Regrowth in distribution systems/biofilms

The ability of *Salmonella* strains to persist/multiply in the biofilm of distribution systems remains poorly documented.

Occurrence

*Salmonellae* are disseminated in the natural environment (water, soil, sometimes plants used as food) and through human or animal excretion. Humans and animals (either wild or domesticated) can excrete *Salmonella* either when clinically diseased or after having had salmonellosis, if they remain carriers.

*Salmonella* species are often detected in sewage, river and irrigation water, freshwater, marine coastal water and ground water (Ho and Tam 2000) (Fewtrell 1994) (Baudart 2000) (Morinigo 1986) (Catalao Dionisio 2000) (Gannon 2004). One study reported the isolation of *Salmonella typhimurium* from drinking bottled water (Radhakrishna 2003).

*S typhi* and *paratyphi* only colonize humans. They are disseminated by human sewage. *S typhi* has occasionally been isolated from water and sewage but has never been detected in drinking water (Hunter 1997).

Reservoirs

The only reservoir for *S typhi* and *S. paratyphi* is human.

A large number of serovars, including *S typhimurium* and *S enteritidis*, infect humans and also a wide range of animals, including poultry, cows, pigs, sheep, birds and even reptiles which constitute important reservoirs for *Salmonella*.

Survival

The persistence of *Salmonella* strains in water in a viable but non-cultivable form (VBNC) has been reported (Roszak 1984) but the health risk posed by such VBNC forms remains unclear (Santo Domingo 2000). Caro et al. (Caro 1999) demonstrated that *S typhimurium* in the VBNC state was not pathogenic but further studies should investigate if VBNC cells can be resuscitated and regain pathogenicity and virulence.

Cho et al. (Cho 1999) reported that *S. typhi* survived longer in groundwater than in pond water in both a culturable and a VBNC state.

Environmental factors such as temperature, pH, and water activity affect pathogen survival. *Salmonella* grows in foods within the range of 7-54°C. The optimum temperature for growth is 37°C. Recently, one study showed that sunlight was the most important inactivating factor of *E. coli* and *S. typhimurium* in tropical estuarine water and that *E. coli* cells under all test conditions showed better survival capacity in comparison to *S. typhimurium* (Chandran 2005).

One study reported that *Escherichia coli* O157:H7, *Salmonella* spp., and *Vibrio cholerae* can grow in nutrient-limited, reconditioned wastewater over the temperature range of 4 to 46 degrees C when biological oxygen demand of this water is <2 (Rajkowski 2001).

Inactivation/removal

Published data on inactivation are summarized in annex 1. Disinfection procedures commonly used for treatment of drinking water to remove coliform bacteria are adequate to eliminate *Salmonella* (WHO 2004). However, ingestion of *S. typhimurium* by predatory protozoa increases resistance to free chlorine residuals (King 1988).

Surrogates

*Escherichia coli* (or thermotolerant coliforms) is a generally reliable index for *Salmonella* spp. in drinking water supplies (WHO 2004).
Environmental detection

Standard culture methods followed by conventional biochemical methods are available for identification of *Salmonella* spp. in environmental water samples (Morinigo 1986). Conventional typing methods such as serotyping and phage typing are used for the identification of *Salmonella* strains (serovars). But conventional identification is laborious, time-consuming (4-5 days) and cannot detect VBNC bacteria.

In the ten past years, a large number of PCR-based detection methods have been developed mainly for food and clinical samples (Aabo 1993) (Way 1993) (Kwang 1996) (Vazquez-Novelle 2005). A combination of immunomagnetic separation and real-time PCR or PCR assays has also been reported (Fluit 1991) (Mercanoglu 2005). Some PCR assays are specific of *Salmonella* serovars (Lin 2004), *S. enteritidis* (Lampel 1996) or *S. typhi* (Sharma 1995). Recently, real-time PCR has been increasingly used for rapid screening of food samples (Malomy 2004) or quantification of RNA targets belonging to *Salmonella* spp. in water (Fey 2004).

Morin et al. (Morin 2004) described a rapid and reliable technique for the simultaneous detection of three viable pathogens *Escherichia coli O157:H7*, *Vibrio cholerae O1*, and *Salmonella Typhi* by targeting mRNA. Kong et al. described the rapid detection of six types of bacterial pathogens including *Salmonella typhimurium* in marine waters by multiplex PCR (Kong 2002).

At the present time, all these available alternative molecular methods are at the experimental stage and none has yet been optimized and validated on water samples.

Commercial PCR-based kits are available for screening food samples or environmental samples. In particular the Probelia kit from Sanofi Diagnostic Pasteur or the Bax® system from DupontQualicon; both are AFNOR (French standardization agency) certified. But these kits are not suitable for water samples with low contamination levels.

Molecular typing of *Salmonella* strains is increasingly used with various techniques, such as pulsed-field gel electrophoresis (Baudart 2000), RAPD (Laconcha 1998), PCR ribotyping (Lagatolla 1996) and microarray technology (Alvarez 2003).

Research needs

*Salmonella* constitutes a major public health concern for the food industry. But waterborne transmission is scarce in developed countries. Waterborne outbreaks associated to *S typhi* and *paratyphi* are now uncommon in the western world. Nevertheless the disease is still very common elsewhere, affecting an estimated 12.5 million people each year (Hunter 1997).

Because it is mainly a foodborne pathogen, the environmental ecology of *Salmonella* strains has been poorly investigated, particularly as regards the diversity and survival of strains in natural ecosystems. The role of biofilms in the survival of pathogenic *Salmonella* strains remains unknown. Transfer of antibiotics resistance and virulence factors in drinking water biofilms could be an interesting area of future research.
Shigella

<table>
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<td>Relative infectivity</td>
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<tr>
<td>Important animal source</td>
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</tr>
</tbody>
</table>

Adapted from table 7.1 "Waterborne pathogens and their significance in water supplies" (WHO 2004).

Microbiology

*General description*

Shigellosis is a global human health problem. Worldwide, shigella infections probably affect over 2 million people and cause the death of approximately 600,000 each year, predominantly in developing countries (Niyogi 2005) (WHO 2004).

*Shigella* spp. are Gram-negative, non-spore-forming, non-motile bacilli, which grow in the presence or absence of oxygen. Through plasmid transfer these bacteria can resist a large spectrum of antibiotics, which currently leads to a resurgence of shigellosis.

*Classification*

*Shigella* spp. are members of the family Enterobacteriaceae. The genus comprises four species: *Sh. dysenteriae*, *Sh. flexneri*, *Sh. boydii*, and *Sh. sonnei*. These species are subdivided into serotypes on the basis of their somatic O antigens, many of which are shared with other enteric bacilli, including *E coli*.

Human health effects

*Clinical features*

*Shigella* spp. are known to cause disease only in humans and primates. The symptoms of shigellosis include diarrhoea and/or dysentery with frequent mucoid bloody stools, abdominal cramps and tenesmus. The incubation period is usually 24-72 hours. All species can produce severe disease, but illness due to *Sh. sonnei* is usually relatively mild and self-limiting. *Shigella dysenteriae* type 1 (previously known as *Sh. shigae*) is the causative agent of the most severe form of bacillary dysentery, at the origin of outbreaks in many developing countries.

*Dose – response data*

The infectious dose is low, ranging from less than 10 organisms (Kothary and Babu 2001) to 200 viable organisms (DuPont 1989). Several β-Poisson models have been developed for *Shigella flexneri* and *S. dysenteriae* (Haas 1983, Crocket 1996).

*Target populations*

Most cases of shigella infection occur in children under 10 years of age.

*Diagnosis*

Laboratory diagnosis is performed by culturing stool samples using selective/differential agar media. Enzyme-linked immuno-sorbent assays (ELISA) have been developed to screen stool samples for *Shigella* species. Several PCR protocols have also been developed for detection of *Shigella* spp. in faeces (Li 2004), in particular (Islam 1992) described an IMS-PCR to detect *Shigella dysenteriae* type 1 and *Shigella flexneri* in faeces.
Treatment

Antimicrobial agents are the mainstay of therapy of all cases of shigellosis. Due to the global emergence of drug resistance, the choice of antimicrobial agents for treating shigellosis is limited.

Currently, no vaccines against *Shigella* infection exist. Both live and subunit parenteral vaccine candidates are under development (Niyogi 2005).

Geographical distribution

Worldwide.
In industrialized countries, *Sh. sonnei* predominates. In tropical countries the various serotypes of *Sh flexneri* and *Sh dysenteriae* predominate, although *Sh. boydii* and *Sh. sonnei* are also found (Hunter 1997).

Epidemiology

Transmission routes

*Shigella* spp. are enteric pathogens predominantly transmitted by the faecal-oral route through person-to-person contact, contaminated food and water. Flies have also been identified as a transmission vector from contaminated faecal waste (WHO 2004). *Shigella* infections can also be venereally transmitted by oral-anal contact in male homosexuals (Hunter 1997).

Waterborne outbreaks

Numerous waterborne outbreaks associated with drinking water have been recorded worldwide mainly due to *Sh. sonnei* in developed countries (Levy 1998) (CDC. 2000; CDC. 2001) (Alamanos 2000) (Egoz 1991) (Chen 2001). In 1998, a waterborne outbreak involving a small round structured virus, *Campylobacter* and *Shigella* co-infections occurred in Switzerland, 1998 (Maurer 2000). In most cases, the drinking water was not treated or was improperly treated prior to consumption. Outbreaks associated with swimming in unchlorinated surface water were also reported (Blostein 1991) (Fleming 2000) (Keene 1994).

Ecology

Regrowth in distribution systems/biofilms

Not proved to replicate in water.
The role of biofilm in the survival of *Shigella* spp. remains unknown.

Occurrence

Data are limited. Available data on prevalence in water supplies may be underestimated, because detection techniques generally used can have relatively low sensitivity and reliability (WHO 2004). The distribution of *Shigella* spp. in the environment reflects human faecal contamination. *Shigella* spp. have been isolated from some water supply systems in tropical countries (Khalil 1994). *Sh. dysenteriae* type 1 and *Sh. flexneri* strains were isolated from surface waters in Bangladesh (Faruque 2002). Faruque et al. suggested that the rapid and simple detection of phages specific for *Shigella* strains may be a useful tool for predicting the presence of *Shigella* in environmental waters (Faruque 2003).

Reservoirs

Humans and other higher primates appear to be the only natural hosts for shigellae.
Survival

Studies on the survival of *Shigella* spp. have given differing results. One study (Popovitch and Bondarenko 1982) reported survival of *Sh. sonnei* and *Sh. flexneri* of up to about 50 days at 25°C in river water. By contrast another study found survival of *Sh flexneri* for only 4-7 days at 25-35°C (El-sharkwai 1989). A recent study found that in laboratory conditions *S. dysenteriae* type 1 survived for a mean duration of 3.33 days and *S. flexneri* for a mean 11.167 days in field water samples: concentration of bacteria and water temperatures were positively correlated with the duration of survival (Ghosh 2001).

The comparative microbial survival in seawater was studied: *Sh. typhi* and *Sh. sonnei* showed similar or greater survival than enteric viruses and *E. coli*. There was no clear association between microbial survival and water temperature (Wait 2001).

In addition, *Shigella* spp. did not grow but survived for >28 days at 4 to 25 degrees in reconditioned wastewater (Rajkowski 2001).

The survival of *Shigella* strains (*Sh. sonnei* and *Sh. dysenteriae*) in water in a viable but non-cultivable form (VBNC) has been reported (Islam 1993) (Colwell 1985) but the health risk posed by such VBNC form needs to be further investigated.

Inactivation/removal

Published data on inactivation are summarized in annex 1. Disinfection procedures commonly used for treatment of drinking water to remove coliform bacteria are adequate to eliminate *Shigella* spp (WHO 2004).

However, ingestion of *Shigella sonnei* by predatory protozoa has been shown to increase resistance to free chlorine (King 1988). The authors suggested that this is a mechanism allowing survival of fastidious bacteria in dilute and inhospitable aquatic environments.

Surrogates

*Esherichia coli* (or thermotolerant coliforms) is a generally reliable index for *Shigella* spp. in drinking water supplies (WHO 2004).

Environmental detection

The standard method is based on filtration of large volumes of water (2-5l) through membranes, then isolation of *Shigella* by selective culture media followed by identification by biochemical tests and serotyping (June 1993). This process may take 48-72 h or even longer and cannot detect VBNC bacteria.

A colony blot immunoassay to detect enteroinvasive *Escherichia coli* and *Shigella* in water samples has been reported (Szakal 2001), but this approach is not very sensitive because of interaction with background bacteria.

Few PCR-based detection methods have been developed to specifically detect *Shigella* species in water (Bej 1991) (Islam 1993) (Theron 2001), but no validated protocol is available.

Techniques for the multidetection of pathogens have emerged in the recent years:

Kong et al. described the rapid detection of six types of bacterial pathogens including *Shigella flexneri* in marine waters by multiplex PCR(Kong 2002). A duplex real-time PCR assay for detection of 17 species of food- or waterborne pathogens including *Shigella* spp in stools has been developed (Fukushima 2003). There are promising approaches which are at the experimental stage and need to be optimized and validated on water samples.

Molecular typing techniques such as RAPD (Yamada S 1999) (Bando 1998), pulsed-field gel electrophoresis, enterobacterial repetitive intergenic consensus sequence-based PCR typing (Surdeanu 2003) and ribotypes (Faruque 2003), are often used in epidemiological studies.
Research needs

The resurgence of Shigellosis in particular in the USA due to the emergence of drug resistant *Shigella* strains requires research on the virulence characteristics of environmental *Shigella* isolates in order to understand their pathogenic potential. The drug resistance patterns of the environmental and clinical *Shigella* strains should be evaluated.

The microbial ecology of *Shigella* species remains poorly known. In particular, very little is known about the presence and the survival of pathogenic strains in the aquatic environment, their interaction with biofilms and the role of protozoa. Transfer of antibiotics resistance and virulence factors in drinking water biofilms should be investigated in the future.

Few PCR-based methods are available for rapid and specific detection of *Shigella* species in water, but viability detection and quantitation are still lacking.
Yersinia

<table>
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<td>Relative infectivity</td>
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<tr>
<td>Important animal source</td>
<td>yes</td>
</tr>
</tbody>
</table>

Adapted from table 7.1 "Waterborne pathogens and their significance in water supplies" (WHO 2004).

Microbiology

**General description**

*Yersinia* spp. are Gram-negative, facultatively anaerobic, non-spore forming bacilli. They are motile at 25°C but not at 37°C.

**Classification**

The genus *Yersinia* is classified in the family Enterobacteriaceae and features seven species. The species *Y. pestis*, *Y. pseudotuberculosis* and certain serotypes of *Y. enterolitica* are pathogens for humans. *Y. pestis* is the causative agent of plague through contact with rodents and their fleas. It is considered as a potential bio-terrorism agent. Both *Y. pseudotuberculosis* and *Y. enterocolitica* species are potential waterborne pathogens and will be addressed. Six serotypes and four sub-types of *Y. pseudotuberculosis* and more than 50 serotypes of *Y. enterocolitica* have been described.

**Human health effects**

**Clinical features**

*Yersinia*osis causes fever, diarrhoea and abdominal pain, which lasts for about one to three weeks. Other clinical manifestations include greatly enlarged painful lymph nodes referred to as "buboes". *Y. pseudotuberculosis* most commonly causes a mesenteric adenitis, which can be indistinguishable from acute appendicitis. The incubation period for both species is between 3 and 7 days (Hunter 1997).

**Dose – response data**

The infectious dose is probably high, up to $10^9$ organisms (Hunter 1997).

**Target populations**

*Y. enterocolitica* mainly affects children under five years old.

**Diagnosis**

The diagnosis may be made by culture from faeces, mesenteric lymph nodes, blood culture. Positive culture is confirmed by biochemical and serological investigations. Because it is uncommon, many laboratories do not routinely perform the identification of *Yersinia* spp. from faeces.

**Treatment**

Most cases recover on their own without treatment. Those with severe symptoms or bloodstream infections are generally treated with antibiotics.
Geographical distribution

Worldwide.

Epidemiology

Only *Y. enterocolitica* strains that possess virulent factors are pathogenic for humans. Environmental strains of *Y. enterocolitica* of serotypes O:3, O:9, O:5, O:27 and O:8 are the most frequently associated with human infections in Europe, Japan, Canada and the USA (Schieman 1990).

**Transmission routes**

Transmitted by the faecal-oral route, with the major source of infection considered to be food, particularly meat and meat products, milk and dairy products. Ingestion of contaminated water is also a potential source of infection as well as contact with an infected person or animal (WHO 2004).

**Waterborne outbreaks**

Epidemiological data concerning waterborne yersiniosis are scarce (Schieman 1990) involving only a small number of individuals (Thompson 1986) (Inoue 1988) (Han 2003). A large waterborne outbreak of yersiniosis was reported in Montana in 1974 (Eden 1977), but many of the strains isolated from water sources were later identified as non-pathogenic, leaving the cause of this outbreak uncertain (Hunter 1997).

Ecology

**Regrowth in distribution systems/biofilms**

Not extensively documented. Some strains may persist and/or multiply in water. The role of biofilms in the persistence of *Yersinia* spp. has not yet been investigated.

**Occurrence**

*Yersinia enterocolitica* are ubiquitous, being isolated frequently from soil, water, animals, and a variety of foods. *Y. pseudotuberculosis* is less ubiquitous than *Y. enterocolitica*, and although frequently associated with animals, has only rarely been isolated from soil, water, and food.

Various authors have described the isolation of *Yersinia* spp. from surface water samples. *Y. pseudotuberculosis* were isolated from river water in Japan (Inoue 1988) (Fukushima 1995). Aleksic et al. reported the isolation of *Yersinia* spp. strains from well water and drinking water plants in Germany but none of the isolates was positive in virulence tests (Aleksic 1988). The occurrence of non-pathogenic strains of *Yersinia* in surface water sources was also reported in Norway (Brennhovd 1992), in Argentina (Escudero 1994), in Italy (Massa 1988) and in Greece (Arvanitidou 1995). Pathogenic strains of *Y. enterocolitica* were isolated in sewage and polluted surface waters (Sandery 1996) (Falcao 2004). But *Y. enterocolitica* strains detected in drinking water are more probably non-pathogenic strains of probable environmental origin (Arvanitidou 1994) (Romano 1997) (WHO 2004).

**Reservoirs**

Domestic and wild animals are the principal reservoir for *Yersinia* spp. Pigs are the major reservoir of pathogenic *Y. enterocolitica*; rodents are the major reservoir of *Y. pseudotuberculosis*.

**Survival**

*Yersinia enterocolitica* is a psychrotropic organism, which can grow in refrigerated foods. Some species and strains of *Yersinia* may replicate in water environments under the conditions of minimum nutrition and low temperature (4°C) (Buzoleva 2000) (WHO 2004).
The lengthy survival of *Y. enterocolitica* in water was noted by (Karapinar 1991), who detected survivors in sterile spring water after 64 weeks at 4°C. Liao et al. showed the long-term survival of *Yersinia enterocolitica* in sterile water (several years) at room temperature (Liao 2003). Other works reported the long-term survival of *Y. pseudotuberculosis*, *Y. enterocolitica* and other *Yersinia* species at reduce temperatures (4-6 degrees) (Terzieva 1991) (Kuznetsov 1998). Chao et al. (Chao 1988) found that survival was greatly reduced with increasing temperatures. In addition, the longevity of *Y. enterocolitica* was chiefly regulated by predators and toxin producers.

The persistence of *Yersinia* strains in water in a viable but non-cultivable form (VBNC) has not yet been investigated.

**Inactivation/removal**

Published data on inactivation are summarized in annex 1. *Yersinia* spp. are sensitive to disinfection processes (Korol 1995) (WHO 2004). However, ingestion of *Yersinia enterocolitica* by protozoans has been shown to protect them against subsequent disinfection (King 1988). Control measures that can be used to minimize the presence of pathogenic *Yersinia* spp. in drinking water supplies include protection of raw water supplies from human and animal waste, adequate disinfection and protection of water during distribution (WHO 2004).

**Surrogates**

Due to the long survival and/or growth of some strains of *Yersinia* spp. in water, *E. coli* (or, alternatively, thermotolerant coliforms) is not a suitable index for the presence/absence of these organisms in drinking water.

**Environmental detection**

The currently recommended method for isolation of *Yersinia* spp. is based on membrane filtration followed by enrichment and isolation on selective media. Confirmation of typical colonies is by the demonstration of motility and biochemical reactions. This method is time-consuming (2 weeks). For *Y. enterocolitica*, the subsequent differentiation between pathogenic and non-pathogenic strains can be difficult and unreliable. API 20E has been reported to be the system of choice for identifying pathogenic *Yersinia* isolates (Neubauer 1998).

Several PCR-based methods have been described for the detection of pathogenic *Yersinia enterocolitica* in environmental water samples (Kapperud 1995) (Sandery 1996) (Waage 1999). Alexandrino et al. (Alexandrino 2004) reported a PCR protocol for rapid detection of *Yersinia enterocolitica* serovar 0:3 in wastewater samples. Currently all these available alternative molecular methods are at the experimental stage for research purposes and validated protocols do not yet exist.

The multidetection of pathogens has emerged for several years: Kong et al. described the rapid detection of six types of bacterial pathogens including *Yersinia enterocolitica* in marine waters by multiplex PCR (Kong 2002). A duplex real-time PCR assay for the detection of 17 species of food- or waterborne pathogens including *Yersinia* spp in stools has been developed (Fukushima 2003). There are promising approaches which are at the experimental stage and need to be optimized and validated on water samples.

Molecular typing of *Yersinia enterocolitica* in epidemiology investigations has been reported using restriction endonuclease analysis (Kapperud 1991) (Han 2003) and pulsed-field gel electrophoresis (PFGE) (Marranzano 2003).

**Research needs**

The epidemiology and the ecology of the microorganism require further research.
Transmission route

More epidemiological studies, including molecular methodologies for microbial source tracking, should be carried out in order to document the role of waterborne transmission of *Yersinia enterocolitica* and *Y. pseudotuberculosis*.

Occurrence and distribution

The presence and distribution in water of these pathogens together with their survival capacity should be investigated in greater depth. In particular the impacts of microbial interactions (including biofilms/aggregates and protozoan) on pathogen survival require special attention. The health significance of *Y enterocolitica* in environmental water remains controversial and needs to be further studied.

Survival

The determination of the effects of various environmental factors (for example, ultraviolet irradiation) on the survival and growth of pathogenic *Yersinia* remains unknown.

Analytical methods

In order to carry out the research work described above there is a need to develop and validate additional PCR-based methods to detect and quantify 1) pathogenic strains of *Y enterocolitica* and 2) *Y. pseudotuberculosis* in surface and treated waters.
Aeromonas

<table>
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<td>Relative infectivity</td>
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<tr>
<td>Important animal source</td>
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</tr>
</tbody>
</table>

Microbiology

**General description**

*Aeromonas* species are Gram negative, non-spore-forming, rod-shaped, facultatively anaerobic bacteria that are ubiquitous and autochthonous in aquatic environments. Morphologically, aeromonads are indistinguishable from members of the Enterobacteriaceae family from which they primarily differ by being oxidase-positive (WHO 2002).

*Aeromonas hydrophila* is included in the Contaminant Candidate List (CCL) (U.S. Environmental Protection Agency. 2005) (U.S. Environmental Protection Agency. 1998).

**Classification**

The taxonomy of the genus is still debated. Although historically in the family Vibrionaceae, they have been placed into their own family, the Aeromonadaceae (Bergey’s Manual 2005). The genus is divided into two groups. The group of psychrophilic non motile aeromonads consists of only one species, *A. salmonicida*, an obligate fish pathogen that is not considered further here. The group of mesophilic motile aeromonads is considered of potential human health significance; the main species are: *A. hydrophila*, *A. caviae*, *A. veronii* subsp. *sobria* (WHO 2002).

**Human health effects**

**Clinical features**

*Aeromonas* spp. can cause infections in humans, including sepsicaemia, particularly in immunocompromised patients, wound infections and respiratory tract infections. There have been some claims that the species *A. hydrophila*, *A. caviae* and *A. veronii* subsp. *sobria* can cause gastrointestinal illness but epidemiological evidence is yet not consistent (WHO 2002). The main putative virulence factors are: exotoxins, endotoxin (LPS), presence of S-layers, fimbriae or adhesins and the capacity to form capsules (Merino 1995). The key virulence factors of *Aeromonas* have been investigated (Albert 2000) (Rahim 2004) (Erova 2006).

**Dose – response data**

The infectious dose is unknown.

**Target populations**

Individuals at the greatest risk of infection are children, the elderly and the immunocompromised. (Merino 1995).

**Diagnosis**

*Aeromonas* can be isolated by culture of faeces on ampicillin containing blood agar. Confirmation is by biochemical tests (Hunter 1997).
Treatment

Treatment for infection with *Aeromonas* is generally not necessary for gastrointestinal illness. But for other pathologies, antibiotic therapy is usually implemented.

Geographical distribution

Worldwide.

Epidemiology

The health significance of detecting mesophilic aeromonads in public water supplies is not well understood. Although reports from Australia have suggested an association between gastroenteritis and aeromonads in drinking water (Burke 1984) (Burke 1984), other recent works have shown little similarity between the majority of water-related *Aeromonas* strains and diarrhoea-associated isolates (Havelaar 1992) (Borchardt 2003) (Szczuka 2004). A consensus would be that only certain strains isolated from drinking water supplies are likely human enteric pathogens.

Transmission routes

The common routes of *Aeromonas* infections may be the ingestion of contaminated water or food or contact of the organism with a break in the skin through water related activities, such as swimming, diving, boating and fishing (Schubert 1991).

Waterborne outbreaks

No waterborne outbreak linked to either drinking water or recreational water contact has ever been reported (Hunter 1997). To date there is no firm evidence that direct transmission occurs via drinking water (WHO 2002).

Ecology

Regrowth in distribution systems/biofilms

*Aeromonas* has shown potential to grow in water distribution systems, especially in biofilms (Chauret 2001), where it may be resistant to chlorination. Biofilm production could explain the persistence of this gram-negative pathogen organism in chlorinated tap water. (Fernandez 2000).

Growth of *Aeromonas* generally occurred in the peripheral parts of distribution systems and was associated particularly with drinking-water derived from anaerobic groundwater containing methane (WHO 2002). *Aeromonas* densities usually showed a seasonal pattern, with peak values occurring in summer (WHO 2002). It has been recently reported that the isolation of *Aeromonas* in natural mineral water was the consequence of a localized development of a biofilm, with no exogenous contamination of the aquifer (Villari 2003).

The factors that affect the occurrence of *Aeromonas* in water distribution systems are not fully understood, but organic content, temperature, the residence time of water in the distribution network and the presence of residual chlorine have been shown to influence population sizes (WHO 2002).

Occurrence

*Aeromonas* species are commonly found in soil and water.

They have been isolated in lakes, rivers, marine waters, sewage effluents as well as drinking waters (LeChevallier 1982) (Kühn 1997) (Chauret 2001) (Sen 2004) (Legnani 1998) and mineral and thermal waters (Hunter 1993) (Biscardi 2002). Reported densities in clean rivers, lakes and storage reservoir water range from 1 to 10^5 cfu/ml, in groundwater fewer than 1cfu/ml, in post-treated drinking water from 0 to 10^2 cfu/ml and in drinking water distribution systems up to 10^2 to 10^3 cfu/ml attributed to growth in biofilms (Nichols 1996) (WHO 2002). *Aeromonas* have also been found in high numbers in raw sewage (10^6 to 10^8 cfu/ml) and in sewage effluents (10^4 to 10^5 cfu/ml) pollution (Theron 2002).
Since relatively little is known about the pathogenic mechanisms of *Aeromonas* species, several studies have demonstrated that many mesophilic aeromonads, mainly *A. hydrophila*, isolated from surface and drinking water can exhibit toxigenic factors and can thus pose a public health risk. (Handfield 1996) (Kühn 1997) (Ormen 2001).

Recently the rare species *Aeromonas culicicola*, so far only known in mosquitoes in India, has been surprisingly isolated from a drinking water supply in Spain (Figueras 2005), but since the taxonomy of *A. culicicola* is still controversial, these data will have to be confirmed.

**Reservoirs**

Environmental water sources.

**Survival**

Not extensively documented.

Temperature has an influence on the survival ability of the bacteria in nutrient-poor water (Sautour 2003). Aeromonads have been found to grow between 5°C and 45°C with an optimal growth range of 22-28°C. Brandi et al. (Brandi 1999) reported that the survival of *Aeromonas* spp. varies considerably, depending on species and water type. Kühn et al. (Kühn 1997) reported that potentially pathogenic *Aeromonas* strains could persist for several months in drinking water.

Additionally Aeromonads readily multiply in domestic or industrial wastewater and are found in siphons, sinks and drainage systems (Schubert 1991).

**Inactivation/removal**

Published data on removal and inactivation are summarized in annex 1. It appears that if free cells of *Aeromonas* are relatively susceptible to common chlorine-based disinfectants (Knochel 1991) (Medema 1991), populations associated with biofilms may survive high chlorine levels (WHO 2002). Treatment can significantly reduce levels of *Aeromonas* in finished drinking water. But it appears difficult to control its growth in biofilms (Chauret 2001) (WHO 2002).

**Surrogates**

*E coli* (or alternatively, thermotolerant coliforms) cannot be used as an index for the presence/absence of *Aeromonas* spp.

**Environmental detection**

Reference culture methods include membrane filtration followed by incubation on ampicillin-dextrin agar (Havelaar 1987) (Handfield 1996) (HMSO 1994) typically at 28-30°C for 24-48 hours. Pre-enrichment with alkaline peptone water has proved successful for recovery of small numbers of *Aeromonas* from water (Moyer 1992). But identification to phenospecies or genospecies levels using classical biochemical testing can be problematic.

A large number of phylogenetic studies on the genus *Aeromonas* have been published based on 16S rDNA RFLP analysis (Figueras 2000) or sequence analysis of gyrB and rpoD genes (Yanez 2003) (Soler 2004). Recently Ormen et al. (Ormen 2005) reported a lack of agreement between biochemical and genetic identification of *Aeromonas* spp.

As yet, few alternative immunological or molecular methods have been reported to detect and identify *Aeromonas* species in environmental water samples. Some PCR assays have been described: the detection of *Aeromonas caviae* and *Aeromonas trota* by polymerase chain reaction in seafood and water samples (Khan 1997); the detection of *Aeromonas* spp in environmental water using 16S rDNA targeted oligonucleotide primers (Dorsch 1994), and recently the PCR identification of virulence factors of *Aeromonas* strains isolated from US drinking water utilities (Sen 2004). Recently, one study (Sen 2005) reported the development of a rapid identification method for *Aeromonas* species by multiplex PCR. Kong et al. described the rapid detection of six types of bacterial pathogens including *Aeromonas hydrophila* in marine waters by multiplex PCR (Kong 2002).
To date, there is no rapid and specific molecular method for the detection and quantification in environmental water samples of the most important human pathogen species, *A. hydrophila*, *A. caviae*, *A. veronii* subsp. *sobria*. In epidemiological studies, molecular typing methods are widely used such as RAPD, repetitive extragenic palindromic PCR (REP-PCR) (Szczuka 2004), pulsed-field gel electrophoresis (Borchardt 2003) (Villari 2003) and ribotyping (Moyer 1992).

Research needs

The pathogenicity, ecology and epidemiology of *Aeromonas* spp. are not yet fully understood and require further studies.

- At first, there is a need to develop rapid and specific PCR methods to detect and quantify the main pathogen species in water *A. hydrophila*, *A. caviae*, *A. veronii* subsp. *sobria*.

- Waterborne transmission has not yet been well established. The health significance of detecting mesophilic aeromonads in public water supplies is not well understood: the occurrence and characterization of the virulent strains in drinking water should be investigated. The study currently conducted under the AWWARF project n°2957 ("Characterization of waterborne *Aeromonas* species for their virulence potential") should address this need. Genetic diversity and stability of *Aeromonas* strains in water need to be studied, and further epidemiological studies should be carried out.

- The role of biofilm in the survival and growth of putative pathogenic *Aeromonas* strains in potable water should be investigated. The results of epidemiological studies may suggest that some water supplies are colonized with strains that are pathogenic to humans while others are not (Hunter 1997). Future research could help determine which factors in a water supply influence the strains types likely to colonize it. Transfer of antibiotics resistance and virulence factors in drinking water biofilms should be an area of future research using aeromonas as a model organism.

- Further work is needed to understand the clinical significance of enteric isolates of *Aeromonas*, to clarify the pathogenic mechanisms of *Aeromonas* spp and substantiate the causative role of these organisms in gastroenteritis. There is also a need for reliable data on the human infective dose for enteropathogenic aeromonad strains. Host susceptibility and bacterial virulence factor relationships should be further investigated.

- Treatment methods and/or operations for controlling *Aeromonas* regrowth in distribution systems are needed.
Burkholderia pseudomallei

<table>
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<tr>
<td>Important animal source</td>
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</tr>
</tbody>
</table>

Adapted from table 7.1 “Waterborne pathogens and their significance in water supplies” (WHO 2004).

Microbiology

**General description**

*Burkholderia pseudomallei* is an environmental organism commonly found in soil and muddy water; it is a strictly aerobic, non-spore-forming, oxidase and catalase positive, Gram-negative bacillus. *B. pseudomallei* is the aetiological agent of melioidosis, a life threatening septic infection prevalent in SE Asia and Northern Australia. *B. pseudomallei* is an organism that has been considered as a potential agent for biological warfare and biological terrorism (Dance 2002).

**Classification**

Until recently this species was classified as belonging to the genus *Pseudomonas*.

Human health effects

**Clinical features**

Illness from melioidosis can be categorized as acute or localized infection, acute pulmonary infection, acute bloodstream infection, and chronic suppurative infection. Unapparent infections are also possible. The incubation period (time between exposure and appearance of clinical symptoms) is not clearly defined, but may range from 2 days to as long as 26 years (Hunter 1997). Whether exposure to *B. pseudomallei* will result in disease, probably depends on the balance between the virulence of the strain, the immune status of the host (e.g. diabetes mellitus) and the size of the inoculum (Dance 2000).

**Dose – response data**

The infectious dose is unknown.

**Target populations**

Although melioidosis can occur in healthy children and adults, it occurs mainly in people whose defence mechanisms against infection are impaired by underlying conditions or poor nutrition or living conditions.

**Diagnosis**

Melioidosis is diagnosed by isolating *Burkholderia pseudomallei* from the blood, urine, sputum, or skin lesions. Various serological tests, such as agglutination and complement fixation tests, are available.

**Treatment**

Most cases of melioidosis can be treated with appropriate antibiotics.
Geographical distribution

Tropical and subtropical areas of Southeast Asia and Northern Australia (Dance 1999). Melioidosis is endemic in Southeast Asia, with the greatest concentration of cases reported in Vietnam, Cambodia, Laos, Thailand, Malaysia, Myanmar (Burma), and northern Australia.

Epidemiology

Transmission routes

Although the epidemiology of melioidosis is not yet fully understood, humans and animals are believed to acquire the infection by inhalation of dust, ingestion of contaminated water, and contact with contaminated soil and water, especially through skin abrasions, and by contamination of war wounds in the case of military troops. Person to person transmission can occur by contact with the blood and body fluids of an infected person (Godoy 2003).

Waterborne outbreaks

Not extensively documented. In two Australian outbreaks of melioidosis, indistinguishable isolates, typed by pulsed-field gel electrophoresis, were isolated from cases and the drinking water supply (Inglis 2000) (Currie 2001).

Ecology

Regrowth in distribution systems/biofilms

No yet documented. May survive and multiply within biofilms.

Occurrence

Burkholderia pseudomallei is a widely distributed environmental saprophyte (Dance 2000). It can multiply in soil and water. The organism can predominantly be found in tropical regions, typically in soil or surface-accumulated muddy water, from where it may reach raw water sources and also drinking-water supplies. (WHO 2004). Isolation rates are higher during the rainy season and in still water rather than running water. Some studies reported its isolation from drinking water supplies (Zanetti 2000) (Inglis 2000) (Currie 2001).

Reservoirs

The main reservoir is believed to be environmental water and soil in tropical areas (Dance 2000). Besides humans, many animal species are susceptible to melioidosis. These include sheep, goats, horses, swine, cattle, dogs, and cats.

Survival

Little is known about the climate, physical, chemical and biological factors which control the proliferation and survival of Burkholderia spp. in the environment. The optimal temperature and pH value for B. pseudomallei were 24 C to 32 C and 5 to 8, respectively (Tong 1996). But Chen et al. reported that this bacterium can still grow at 4 degrees C (Chen 2003), which was suggested to be related with the occurrence of melioidosis in some cold areas.

Inactivation/removal

Data are limited. Two studies reported the effect of chlorine disinfection in potable water (Howard 2003) (Howard 2005). The most recent study has shown that chlorine proved the most effective disinfectant with a 99.99% reduction of a 10(6) CFU/mL pure bacterial culture followed by 99.9% reduction by
monochloramine and 99% reduction by UV and suggested that amoeba can enhance the survival of the bacteria (Howard 2005). The published inactivation data are summarized in annex 1.

Surrogates

Because of the environmental occurrence of *B. pseudomallei*, *E. coli* (or, alternatively, thermotolerant coliforms) is not a suitable index for the presence/absence of this organism.

Environmental detection

A reference culture method is available but it is time consuming: it includes filtration through a 22µm filter, then incubation on a selective broth at 40°C (Ashdown 1979) for seven days, followed by biochemical identification.

Some PCR assays have been developed to rapidly detect *B. pseudomallei* in soil (Brook 1997) or clinical samples (Lew 1994). Recently, a real-time PCR assay using fluorescent hybridization probes has been used in blood samples (Tomaso 2005).

However, no rapid molecular method has yet been developed for water samples.

In molecular epidemiology studies, molecular typing methods have been used, such as pulsed-field gel electrophoresis (Inglis 2000) (Currie 2001), ribotypes (Trakulsomboon 1997) or restriction endonuclease analysis (Yap 1995).

Research needs

The epidemiology and ecology of *B. pseudomallei* are still poorly understood. In particular, more research is needed to investigate the role of amoeba and biofilms in the survival of *B. pseudomallei* in potable water.

To document the occurrence of this organism in water, there is a need for a rapid, sensitive and specific molecular method to detect and quantify *B. pseudomallei* in environmental water samples.

More epidemiological studies, including molecular methodologies for microbial source tracking, should be carried out, investigating the role of waterborne transmission.
Cyanobacteria

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<td>Important animal source</td>
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</tr>
</tbody>
</table>

Microbiology

General description

Cyanobacteria, otherwise known as blue-green algae, are among the most ancient species known on this planet. They are single-celled organisms that live in fresh, brackish, and marine water. A feature of cyanobacteria is that because they are planktonic organisms and need to float, they possess gas vacuoles, which act as buoyancy aids (Hunter 1997). Depending upon the species and environmental conditions, colonies may form filaments, sheets or even hollow balls. Cyanobacteria are photosynthetic prokaryotes of significant ecological and biotechnological interest, since they strongly contribute to primary production and are a rich source of bioactive compounds. In eutrophic fresh and brackish waters, their mass occurrences (water blooms) are often toxic and constitute a high potential risk for human health. Of the cyanobacterial genera which include toxing-forming species, those of particular concern when mass populations occur include Microcystis, Anabaena, Planktothrix (formerly known as Oscillatoria), Aphanizomenon, Cylindrospermopsis, Phormidium, Nostoc, Anabaenopsis and Nodularia.

Cyanobacteria are included in the Contaminant Candidate List (CCL) (U.S. Environmental Protection Agency. 2005) (U.S. Environmental Protection Agency 1998). Guideline values for drinking water were recently introduced by the World Health Organization (WHO 1998), with a recommended limit of 1 µg of microcystin-LR equivalents per liter.

Classification

Despite being called algae, cyanobacteria are true prokaryote bacteria. They belong to the class Photobacteria, photosynthetic bacteria that contain chlorophyll. The phylogeny of the cyanobacteria is poorly understood at present. Most classification schemes are organized by cell or colony shape, but recent efforts may soon provide a truly evolutionary scheme.

Human health effects

The cause

Most illnesses related to cyanobacteria are thought to be mediated by toxins. Cyanobacterial toxins are classified according to the way in which they affect the human body. Hepatotoxins (which affect the liver) are produced by some strains of the cyanobacteria Microcystis, Anabaena, Oscillatoria, Nodularia, Nostoc, Cylindrospermopsis and Umezakia. Hepatotoxins are the most widely distributed types of cyanobacterial toxins in aquatic environments; of these, the most commonly encountered in freshwaters are microcystins, which have also been identified as potent liver carcinogens (Nishiwaki-Matsuhima 1992). Neurotoxins (which affect the nervous system) are produced by some strains of Aphanizomenon and Oscillatoria. Cyanobacteria from the species Cylindrospermopsis raciborski may also produce toxic alkaloids, causing gastrointestinal symptoms or kidney disease in humans (WHO 1989).

Understanding of the true range of cyanobacterial toxins, of their occurrence and health significance is still incomplete.

The cyanotoxins of highest concern to the water industry are:
- neurotoxins: Anatoxin-a, Anatoxin-a(s), Saxitoxins, Neosaxitoxins including the new neurotoxic B-methylamino-L-alanine (BMAA) which may be associated with neurodegenerative diseases such as Alzheimer's disease (Cox 2003);
- hepatotoxins: Microcystins, Nodularins, Cylindrospermopsins;
- dermatotoxins: Aplysiatoxins and Lyngbiatoxins (which also affect the gastro-intestinal tract);
- Lipopolysaccharide (LPS) endotoxins which can affect any exposed tissue (WHO 1999).

**Clinical features**

Disease due to cyanobacterial toxins varies according to the type of toxin and the type of water or water-related exposure (drinking, skin contact, etc.). Adverse health effects associated with exposure to high concentrations of cyanobacterial toxins include stomach and intestinal illness; breathing disorders; allergic responses; skin irritation; liver damage; and neurotoxic reactions, such as tingling fingers and toes. Scientists are exploring the health effects associated with long-term exposure to low levels of cyanobacterial toxins. Some studies have suggested that such exposure could be associated with chronic illnesses, such as liver cancer and digestive-system cancer. Microcystins and nodularins are potent toxins, which are also tumor promoters (Falconer 1989). Low doses can disrupt the enzymes that control cell division, contributing to cancer (Ito 1997). The causes of the high prevalence of liver cancer in parts of China may be associated with repeated exposure to cyanobacterial microcystins in drinking water (Yu 1989).

Animals, birds, and fish can also be poisoned by high levels of toxin-producing cyanobacteria.

**Dose – response data**

The toxicity of known cyanobacterial toxins by various administration routes has been determined (Kuiper-Goodman 1999): the lethal dose 50 (LD50) for microcystins by oral administration has been estimated to be between 5000-10900µg/kg, and a provisional guideline value of 1 µg/L for microcystin-LR in drinking water has been proposed by the WHO (WHO 2004).

**Target populations**

Children are at higher risk than adults for illness from CyanoHABs because of their low weight, relatively to the dose of toxin they can receive.

**Diagnosis**

Diagnosis is based primarily on history (recent contact with an algal bloom), signs of poisoning, and necropsy findings. It may be possible to identify cyanobacterial cells in the stomach content or faeces. In people who develop allergic-type symptoms, a skin test may be useful.

**Treatment**

No specific treatment is available. However, intense supportive therapy may be needed in some cases.

**Geographical distribution**

Cyanobacteria are found worldwide, including North and South America, Africa, Australia, Europe, Scandinavia and China. In warmer climates, these organisms can grow year-round.

**Epidemiology**

**Transmission routes**

Humans can be exposed to cyanobacterial toxins by drinking water that contains toxins (from a lake, reservoir or untreated water), by direct skin contact in particular by swimming in water that contains high concentrations of cyanobacterial cells, or by inhaling water droplets from irrigation or water-related recreational activities. Transmission through consumption of filter-feeding shellfish such as mussels or fish may occur (Hunter 1991).
**Waterborne outbreaks**

Human populations have been adversely affected by the ingestion of water containing cyanobacterial cells and their toxins and by recreational and occupational skin contacts (Carmichael 2001) (Falconer 1983) (Hunter 1997). The most recent and serious known human poisoning episode attributed to cyanobacterial toxins occurred at Caruaru, Brazil in 1996, causing the death of over 50 patients. An outbreak of acute liver failure occurred at a dialysis center due to human intoxication by microcystins during renal dialysis treatment. It was estimated that 19.5 µg/l microcystin was in the water used for dialysis treatments, i.e. 19.5 times the level set as a guideline for safe drinking water supplies by the World Health (Carmichael 2001). Several studies suggested that the high prevalence of liver cancer in parts of China may be associated to repeated exposure to cyanobacterial microcystins in drinking water (Yu 1989).

**Ecology**

**Regrowth in distribution systems/biofilms**

Toxic algae may grow in open-air finished water reservoirs (Lippy 1976).

**Occurrence**

Cyanobacteria are ubiquitous in aquatic environments. They are common in freshwater lakes and reservoirs and the intertidal zone of seashores, and can also be found on damp rocks, salt marshes, river beds and tree trunks (Hunter 1997). Toxic cyanobacterial populations have been reported in freshwaters in over 45 countries, and in numerous brackish, coastal, and marine environments (Codd 2005) as well as in European freshwater bodies (Via-Ordorika 2004). Hoeger et al. (Hoeger 2005) confirmed that toxin-producing cyanobacteria (blue-green algae) are abundant in surface waters used as drinking water resources. One recent study (Vieira 2005) reported the detection of toxic cyanobacteria at a concentration level up to of 20,000 cells/ml as well as Microcystins at concentrations of up to 1.25µg/l in public water supply reservoirs in Brazil.

**Reservoirs**

Aquatic environments. Specific reservoirs have not been identified.

**Survival/ growth**

Most toxic cyanobacteria grow best in warm, clear, eutrophic or hypertrophic waters that are not turbulent. Explosive overgrowths of cyanobacteria are encouraged by excess nutrients in the water—primarily phosphorus and nitrogen—from sources such as agriculture, urban and rural runoff water, and wastewater. Maximum growth rates are attained by most cyanobacteria at temperatures above 25 °C. Conditions limiting growth include low temperature (<10°C), low phosphate and nitrogen concentrations and high turbulence.

**Inactivation/removal**

Comprehensive information on management strategies for toxic blue-green algae is presented in a recent GWRC report by House *et al.* (House 2004). Chlorine has been shown to successfully remove a range of cyanotoxins including microcystins. Each cyanotoxin requires specific treatment parameters, particularly solution pH and free chlorine residual (Senogles-Derham 2003). Chlorination was ineffective in removing anatoxin-a (Nicholson 1994). The most effective process for destroying microcystins and nodularins seems to be ozonation (WHO1999). Microcystins and anatoxin-a would be destroyed under conditions usually utilised for ozonation prior to granular activated carbon (GAC) filtration. The saxitoxin class of compounds was very resistant to oxidation by ozone and would require further treatment such as GAC filtration (Rosinato 2001). Newcombe *et al.* confirmed that ozonation of saxitoxin at moderate doses was found to be ineffective.
(Newcombe 2002). One study investigated the potential limitation of a water treatment system involving ozonation coupled with several filtration steps, when faced with various bloom conditions (Hoeger 2002).

Surrogates

As Cyanobacteria are common environmental organisms, *E. coli* (or alternatively, thermotolerant coliforms) cannot be used as an index for the presence/absence of Cyanobacteria.

Environmental detection

- **Methods of detection of Cyanobacterial toxins:**
  Concentration of cyanobacterial toxins from water samples may be required and can be performed using concentration procedures such as Solid phase extraction (SPE) (Environment Agency 1998) or using more recent immunoaffinity methods (McElhinney 2002) (Arando-Rodriguez 2003). Most methods for the detection of cyanobacterial toxins in water samples have been directed at microcystin. The traditional method for detection and quantification of Cyanobacterial toxins is the mouse bioassay. Sensitive biological assays have been proposed as alternatives to the mouse assay, such as the protein phosphatase inhibition assay (Heresztyn 2001). A rapid bioluminescence assay has also been developed (Lawton 1990). A wide range of chromatographic methods is being used. Currently routine analysis of microcystins is most commonly carried out using high-performance liquid chromatography with photodiode array detection (HPLC-PDA) (McElhinney 2005) (Environment Agency 1998). The gold standard method for cyanotoxin analysis uses liquid chromatography (LC) coupled with mass spectrometry (MS), but these methods are laborious and time-consuming. A promising alternative for microcystin detection is the use of immunological assays (Metcalfe 2003). But one drawback is that these methods do not provide a direct indication of the toxicity, which can be possible with in vitro enzyme inhibition assays.

- **Cyanobacteria detection methods:**
  Detection and identification of cyanobacteria are traditionally done by microscopic examination of water samples. But the morphological characteristics do not allow differentiation between toxic and non toxic cyanobacteria.
  Genetic methods such as PCR are increasingly being developed to detect the presence of genes responsible for cyanobacterial toxin production (Hisbergues 2003) (Pan 2002) (Fergusson and Saint 2003) (Burns 2004). Other articles reported the development of species-specific PCR assays using specific sequences in the RNA polymerase gene (rpoC1) to detect the toxic cyanobacterium *Anabaena circinalis* (Fergusson and Saint 2000) and *Cylindrospermopsis raciborskii* (Wilson 2000). Baker et al. reported the development of PCR assay followed by RFLP or sequencing of the PCR products for identification of cyanobacteria in environmental samples (Baker 2001). Other studies developed molecular probes for the detection of toxigenicity in species capable of producing microcystins (Tillett 2001). Baker et al. described the analysis of a cyanobacterial bloom by use of a PCR-based methods for direct detection and identification of strains present and determination of their toxigenicity (Baker 2002). A multiplex PCR assay for the rapid identification of potentially toxic environmental Microcystis from environmental samples has recently been described (Ouahid 2005). Several studies used a real-time PCR approach to quantify microcystin-producing cyanobacteria (Foulds 2002) (Kurmayer 2003).
  In addition, improved molecular approaches to study cyanobacterial diversity at the strain level have been widely described, including PCR- RFLP (Neilan 1995), multiplex- Randomly Amplified Polymorphic DNA (Neilan 1995) and PCR- denaturing gradient gel electrophoresis (DGGE) (Zwart 2005) (Lyautey 2005). Although a large number of molecular methods have been developed to detect and identify potential toxigenic cyanobacteria in water, all these methods are at the experimental stage and no validated protocol yet exist. It is impossible to compare results obtained by using different methods. Further evaluation and comparative studies need to be carried out in order to validate such alternative methods.

Research needs

Because toxigenic Cyanobacteria can grow in drinking water reservoirs and recreational water, the USEPA has added cyanobacteria in the CCL and considers that this organism is a priority for investigation. Substantial research programs need to be conducted on the following themes:
At first, there is a need to develop and validate molecular techniques for the detection and quantification of toxigenic cyanobacteria in water samples in order to acquire data on the occurrence and epidemiology of these organisms.

It is also necessary to further develop accurate, sensitive and specific detection methods - including the sample treatment step - for individual toxins.

Yet few studies have explored the links between harmful cyanobacterial algal blooms and human health. Particularly, the potential human health impact associated with the consumption of treated water from source waters with toxic blue-green algae remains unknown. Information on health effects is needed to document episodic and low dose exposure to cyanotoxins via recreational and drinking waters.

Further research programs should be carried out in order to investigate the conditions favoring the occurrence of potentially toxic cyanobacteria and cyanotoxins, understand the dynamics of cyanobacterial bloom events and associated problems, and thus facilitate the improved management of fresh water resources.

In order to assess the health risk related to cyanotoxins more data are needed on their occurrence and distribution as well as on their persistence and degradation in aquatic environments.

Additional research is required to determine whether standard and advanced water-treatment practices successfully remove cyanobacteria and their toxins from drinking-water sources.

Finally, risk management strategies for cyanobacterial cells and toxins in waterbodies need to be developed.
Legionella

<table>
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<td>Multiply</td>
</tr>
<tr>
<td>Resistance to chlorine</td>
<td>Low</td>
</tr>
<tr>
<td>Relative infectivity</td>
<td>Moderate</td>
</tr>
<tr>
<td>Important animal source</td>
<td>No</td>
</tr>
</tbody>
</table>

Adapted from table 7.1 “Waterborne pathogens and their significance in water supplies” (WHO 2004).

Microbiology

**General description**

Legionellae were first recognized as human pathogens in 1976 after an outbreak of pneumonia among veterans attending a convention in Philadelphia (Fraser 1977). *Legionella* spp. are heterotrophic bacteria found in a wide range of water environments; they can proliferate at temperatures above 25°C. They are Gram-negative, rod-shaped, non-spore forming bacteria that require L-cysteine for growth and primary isolation.

**Classification**

The genus *Legionella*, a member of the family *Legionellaceae*, has at least 48 species of which 19 have been associated with disease in humans (Muder 2002). The type species is *Legionella pneumophila*. Some species can be further differentiated into serotypes: there are at least 15 for *L pneumophila* but so far no more than two for any other species.

**Human health effects**

**Clinical features**

*Legionella* infections can lead to two forms of disease, namely legionellosis or Legionnaires’ disease, an acute purulent pneumonia with an incubation of 3-6 days, and Pontiac fever, a self-limiting non-pneumonic disease with high attack rate and onset (5 h to 3 days), consisting of fever, headache, nausea, vomiting, aching muscles and coughing.

*L. pneumophila* serogroup 1 is most frequently related to human disease. A study revealed that *L. pneumophila* serogroup 1 is specifically present in the Paris area (Lawrence 1999). *L micdadei* is the second most frequent species causing legionellosis in the United-States and *L longbeachae* infections are the most frequent in Australasia including New-Zealand (WHO 2002). To date legionellosis has been detected almost exclusively in humans, but some animals (guinea-pigs, rats, mice, marmosets, and monkeys) are susceptible to experimental infection (WHO 2002).

**Dose – response data**

The infectious dose is unknown, but it is supposed to depend on the susceptibility of the exposed population. Infectivity may be substantially enhanced if amoebae are inhaled. Vacuoles in infected amoebae may contain many hundreds of *Legionella* cells providing a large inoculum in a restricted area of the respiratory tract (WHO 2002).

A model to qualitatively assess the risk of legionellosis has been proposed in which legionellosis risk can be described in terms of three factors: pathogen proliferation potential, exposure potential and population susceptibility (Cooper 2004) (McCoy 2004).

A simplistic quantitative microbial risk assessment (QMRA) based on the maximum risk curve has been recently developed for *Legionella* within a water distribution system (Storey 2004). An exponential dose-response model for humans, derived from experiments on Guinea pigs, has also been proposed (Ambroise 2003).
**Target populations**

Host factors influence the likelihood of Legionnaires’ disease: males are more frequently affected than females, and most cases occur in the 40-70-year age group. Risk factors include smoking, alcohol abuse, cancer, diabetes, chronic respiratory or kidney disease and immunosuppression, as in transplant recipients. The disease is extremely rare among children under 15 years.

**Diagnosis**

Different laboratory tests can be used in the diagnosis of *Legionella* infection: culture on selective media, urinary antigen test, direct fluorescent antibody (DFA) stain of sputum or other samples from lung and antibody testing (serology). PCR detection and other molecular methods have been increasingly used for clinical diagnosis of legionellosis.

**Treatment**

Several antibiotics are effective *in vivo* against *Legionella*. The newer macrolides and quinolones are considered drugs of choice. Pontiac fever patients recover without specific therapy.

**Geographical distribution**

Worldwide.

**Epidemiology**

**Transmission routes**

Inhalation of *Legionella*-containing aerosols is considered as the usual mode of infection. Aspiration following ingestion of contaminated water is another route of infection. There is no evidence of person-to-person transmission (Leclerc 2002).

**Waterborne outbreaks**

As legionellosis is a notifiable disease in most developed countries, a lot of epidemiological data are available. Legionellosis outbreaks are regularly reported and the per annum incidence rate of the disease is currently of 10 cases per million persons in Europe (http://www.ewgli.org). Although legionellosis is mostly sporadic and frequent in hospitals, recent large outbreaks of Legionnaires’ disease in Australia and Europe have been responsible for the resurgence in interest in Legionnaires’ disease. *Legionella* outbreaks and single cases have been traced to cooling towers, evaporative condensers, hot water from showers, spas and whirlpools, nebulizers and other aerosol-producing medical devices (Leclerc 2004). The information available suggests that the number of legionellae in the water cooling systems associated with outbreaks is always above $10^5$ and usually greater than $10^6$ cfu/l when determined by culture.

The most spectacular outbreak of legionellosis occurred in July 2001 in Murcia, Spain with 449 confirmed cases and six deaths. The source of the outbreak was proven to be improperly controlled contaminated hospital cooling towers in the north of the city (Garcia-Fulgueiras 2003). Previously, an outbreak occurred in April 2000 in Victoria, Australia, causing 93 confirmed cases and three deaths. The cause was an improperly treated cooling tower at the newly opened Melbourne Aquarium (McCoy 2004). This was the largest outbreak of legionnaire’s disease ever recorded in Australia.

In 1999, an outbreak of Legionnaires’ disease affected many visitors to a flower show in the Netherlands causing 21 deaths. The source was a contaminated whirlpool spa on exhibition at the flower show (Den Boer 2002).

In France, during the World Soccer cup in June 1998, 19 cases of legionellosis were identified among visitors to Paris. Three patients died.

Recently in May 2005, an outbreak of 34 cases of Legionnaires’ disease was detected in a suburb of Lyon. No deaths were reported. The source of infection could not be identified (Schmitt and Bitar 2005).
Outside hospitals, there have been no reports of outbreaks of recurrent cases of disease following consumption or use of drinking-water kept cool and not subjected to prolonged periods of stagnation (WHO 2002).

Ecology

**Regrowth in distribution systems/biofilms**

Aquatic biofilms provide comfortable ecological niches in which *Legionella* settle and proliferate. At temperatures between 20°C and 50°C, legionellae frequently colonize water distribution systems where biofilms may be the reservoir for recolonization of water (Walker 1993). *Legionella* spp are able to colonize all parts of internal distribution systems of hospitals, hotels and buildings (Yu 2000) (Perola 2005). According to some authors, the biofilms may support the survival and growth of legionellae outside a host cell (Rogers 1992). Other works found that amoebae were required for multiplication of the bacteria within biofilms (Murga 2001) (Wadowsky 2004) (Kuiper 2004).

Concentrations have been found to be as much as 10 times higher in biofilms from faucets than from water collected from the faucets (Ta 1995). There is some evidence that pipe material can also affect colonization of legionellae (WHO 2002).

**Occurrence**

Although usually in low numbers, *Legionella* can commonly be found in natural aquatic environments and water supplies that meet drinking water standards (Leclerc 2004). The ubiquitous nature of legionellae in water means that water supplies, regardless of their source, may contain *Legionella* sp in low quantities. It has often been recovered in surface water and also in groundwater (Brooks 2004). Costa et al. reported that some strains were persistent in groundwater for at least 12 years (Costa 2005). In Canada, *L. pneumophila* and other *Legionella* species have been recovered from drinking water with low concentration levels (Dutka 1984) (Tobin 1986).

The organisms have also been found in sewage-contaminated coastal waters of Puerto Rico (WHO 2002). With the exception of thermal waters and water in tropical regions, legionellae are found only in low concentrations in natural environments (< 1cfu/ml in groundwater).

**Reservoirs**

Aquatic biofilms and protozoa constitute important natural reservoirs of legionellae. But other natural niches may exist and have not yet been identified.

**Survival**

The ubiquitous nature of *Legionella* reflects its ability to survive under varied water conditions, including temperatures from 0 to 63°C, and pH from 5 to 8.5. Temperature is a factor that significantly influences *Legionella* survival and growth. *L. pneumophila* multiplies at temperatures between 20°C and 50°C with maximal growth between 30°C and 40°C (Leclerc 2004).

It is now clear that Legionellae are natural intracellular parasites of aquatic protist hosts, mostly amoebae such as *Acanthamoeba, Hartmanella, Valkampfia* and *Naegleria* which are “amplifiers” for legionellae in the environment. As natural reservoirs, protozoan phagocytes amplify *L pneumophila* in fresh and potable water supplies. Batch experiments with tap water showed that *L. pneumophila* did not multiply in the absence of protozoa (Wadowsky 2004).

Free-living amoebae appear to be very important in legionellosis. Intracellular growth even enhances the infectivity of *L. pneumophila* to human-derived cells (Fritsche 1998) (Leclerc 2004). Protozoa (*Acanthamoeba*) can enable Legionellae to survive drinking water treatment processes including disinfection and be carried by the mains water distribution into buildings (Kilvington 1990). This added resistance may play a large role in the survival of this bacteria and in its ecology and persistence in the distribution systems of cooling towers and hot tubs and other environments.

In low-nutrient environments, *L pneumophila* is able to enter in a viable but non culturable state, a process that is now well documented as starvation survival. This state can also result from other stresses such as temperature, antibiotics, salt and chlorine (Roszak and Colwell 1987). The addition of amoebae to the dormant bacteria appears to result in the resuscitation of *Legionella* to a culturable state (Steinert 1997).
Legionella’s ability to develop symbiotic relationships with other members of heterotrophic flora bacteria is thought to be important for their survival and proliferation in water. Only few studies have attempted to characterize the interactions between water bacteria and Legionella. Legionellae have been shown to survive all phases of wastewater treatment and they apparently survive after discharge into the ocean (Palmer 1993).

Inactivation/removal

As for other bacteria, physical removal treatments such as coagulation, flocculation, sedimentation and filtration will reduce the number of Legionella in finished water.

Published data on inactivation are summarized in annex 1. UV light systems at doses commonly used in drinking water treatment may be useful for localized disinfection, keeping in mind that there are no distal residual effects (WHO 2002). Legionella strains are killed within 3 min on exposure to hot water at 70 degrees C and exposure to ultraviolet light at 90 mW.s/cm² (Miyamoto 2000). Legionella species are susceptible to ozone. The major problem with ozone is the poor penetration to all parts of the system.

Long-term disinfection has been obtained with continuous copper-silver ionization techniques. But there are no publications indicating the effectiveness of ionization in cooling towers.

In comparison to indicator organisms such as E. coli, a higher CT value is necessary to achieve a comparable level of reduction in Legionella using chlorine and chloramine dioxide. It has been shown that dichloramine as a disinfectant in municipal and hospital systems (rather than the more traditional free chlorine) is effective in controlling Legionella. Recently chlorine dioxide, applied as a continuous treatment, was identified as the most effective for controlling L. pneumophila in a domestic water system (Thomas V 2004). But the same study showed that amoebae resisted all the treatments applied and probably acted as reservoirs for L. pneumophila, allowing quick re-colonization of the system once the treatments were interrupted.

Several studies showed the potential protection of Legionella by biofilms and amoebic hosts from the effects of disinfectants, low PH and heat (Miyamoto 2000) (Storey 2004).

Surrogates

Esherichia coli (or thermotolerant coliforms) is not a reliable index for the presence/absence of Legionella (WHO 2004).

No suitable indicators have been identified to signal increasing concentrations of Legionella spp. in building plumbing systems. There is some evidence that increasing Legionella concentrations are accompanied or preceded by an increase in other bacteria, resulting in an elevated HPC (Heterotrophic Plate Count) measurement (i.e. > 100cfu/ml) (WHO 2002). However, the correlation between HPC and Legionella is not consistent.

Environmental detection

- Culture methods are available: isolation usually requires concentration of the bacterial flora by centrifugation or membrane filtration, followed by heat (50°C for 30 mn.) or acid (pH 2.2 for 5 mn.) treatment before inoculation on to media containing antibiotics. Kusnetsov et al. (Kusnetsov 1994) provided a study of the comparison of various culture methods for the detection of Legionella spp. in water samples.

  ISO 11731 method is consistent with the original method developed by the CDC and with standard methods used in Australia and Singapore (AU/NZ, 3896). A similar method is used in France (AFNOR T90-431) (McCoy 2004).

  But all these culture methods require 10 days to yield confirmed results and cannot detect VBNC bacteria.

  Saint et al. (Saint 1998) described a convenient alternative to standard confirmation procedures for Legionella and Legionella pneumophila employing the EnviroAmp Legionella system and seroagglutination.

- Legionella spp. can also be detected by immunological methods: Yamamoto et al. (Yamamoto 1993) used a direct immunofluorescence assay for the detection of L. pneumophila (sero groups 1 to 6) in
cooling tower waters. But this approach lacks sensitivity for the detection of *Legionella* in water samples and does not distinguish between living and dead cells.

Molecular methods have been developed to detect *Legionella* spp or/and *L. pneumophila* in water samples, including fluorescence in situ hybridization (Manz 1995) (Declerck 2003) (Buchbinder 2002) or the most widely used approach based on PCR-based detection (Catalan 1994) (Maiwald 1994) (Ballard 2000) (Levi 2003). A PCR assay was reported for the identification and discrimination of *Legionella longbeachae* serogroups 1 and 2 (Saint and Ho 1999). PCR also appears to be a reliable method for cooling tower waters (Chang 1995) (Yamamoto 1993) (Koide 1993). However, cooling water often contained PCR-inhibiting substances that could result in false negative PCR results for *Legionella* (Declerck 2003). Catalan et al. (Catalan 1997) developed a nested-PCR method to detect *Legionella pneumophila* in wastewater. Recently, two real-time PCR assays were developed: one for the direct confirmation of *Legionella* primary cultures (Giglio 2005), the other for rapid detection of *Legionella pneumophila* in water (Fiume 2005).

Some PCR-diagnosis kits are available including Aqua Screen® Water Test System (Minerva Biolabs GmbH) and AGFOOD kit (Biotools). But all these PCR-based-detection methods cannot differentiate between dead or living cells and are essentially qualitative.

Few quantitative PCR approaches have been developed. One study (Wellinghausen 2001) has developed a quantitative assay using real-time LightCycler PCR for the detection of legionellae in hospital water. This approach needs to be validated and compared to the standard culture method. Recently, a quantitative real-time PCR assay was developed and validated in combination with an immuno-magnetic separation system for the quantitative determination of *Legionella pneumophila* in water samples (Yanez 2005). This promising approach should allow to quickly monitor water samples for the risk assessment of environmental contaminations.

It must be noted that a real-time PCR assay for quantification of *Acanthamoeba* (a well known natural reservoir for *Legionella*) was recently described (Riviere 2005).

**Typing methods:**

Both environmental strains and clinical isolates can be successfully subtyped by molecular techniques such as ribotyping, macrorestriction analysis by pulsed-field gel electrophoresis or RAPD (Lepeuple 2004) (Van Belkum 1996) (WHO 2002).

Today the use of PCR-based detection methods is of great use in screening samples in outbreak investigation but it does not eliminate the need for culturing samples to quantify viable *Legionella* from water systems.

No rapid quantitative PCR assay (using real-time PCR) to quantify *Legionella* / *L. pneumophila* in water systems has been described in the literature, nor any PCR-based approach which can determine viability (although such methods have been developed by some GWRC partners).

**Research needs**

For more than two decades since it was first recognized, legionellosis has represented a continuing public health challenge. There are still many gaps in our knowledge of the ecology and epidemiology of this organism.

**Ecology**

Further research should be carried out on the normal behavior of *Legionella* species and its natural environmental habitat and ecological niches.

For example, the need for protozoa for the proliferation of *Legionella pneumophila* in aquatic habitats is still not fully understood and remains controversial. Little is known about the range of this parasitic association and there may be other undiscovered protist hosts.

The interactions between *Legionella* and other bacteria in such diverse habitats as free water and biofilms remain also unknown.
Occurrence:
There is a need to acquire more data on the occurrence and level of contamination of the different species of *Legionella*, particularly in treated water and in groundwater by using molecular methods.

Epidemiology

The role of biofilms and protozoa in the epidemiology of legionellosis should be further investigated. What is the impact of the transmission mode (within biofilms, within protozoa or as free-living bacteria) on the outcome of the disease following exposure to *Legionella pneumophila*?

The host susceptibility factors for legionellosis should be investigated.

Treatment

Work is needed to determine if control of *Acanthamoeba* spp. is efficient to control the presence of *Legionella* in water supplies. More broadly, as for Mycobacteria, water treatment approaches that minimize selection for treatment resistant pathogens, the survival of protozoa, biofilm formation and production of disinfection by-products need to be investigated.
Non-tuberculosis mycobacteria (NTM)

<table>
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<td>Relative infectivity</td>
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<td>Important animal source</td>
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</table>

Adapted from table 7.1 "Waterborne pathogens and their significance in water supplies" (WHO 2004).

Microbiology

**General description**

The tuberculous or "typical" species of *Mycobacterium*, such as *M. tuberculosis*, *M. bovis*, *M. africanum* and *M. leprae*, have only human or animal reservoirs and are not transmitted by water. By contrast *Mycobacteria* spp. that have been implicated in waterborne transmission are generally known as atypical mycobacteria or mycobacteria other than tuberculosis (NTM) and are natural inhabitants of a variety of water environments. Environmental opportunistic mycobacteria include both slowly and rapidly growing mycobacteria. By far, the most important slowly growing species are *Mycobacterium avium* and *M. intracellulare*, called the *M. avium* complex (MAC) (also called *M. avium-intracellulare*) responsible for the majority of non-*M. tuberculosis* mycobacteria infections in developed countries (Horsburgh 1996) (WHO 2004).

MAC is included in the USEPA Drinking water contaminant candidate list (U.S. Environmental Protection Agency. 1998; U.S. Environmental Protection Agency. 2005).

They are aerobic, Gram positive, rod-shaped, and acid fast bacteria. They differ from other bacteria by having a complex cell wall with high lipid content, which is used in identification of the organisms using acid-fast staining.

**Classification**

*Mycobacterium* is the single genus in the family Mycobacteriaceae, order Actinomycetales. Over 70 *Mycobacterium* species have been defined, at least 30 of which cause disease in human and animals. Among environmental opportunistic mycobacteria, the main potential human pathogens species are: *M. avium*, *M. intracellulare*, *M. chelonae*, *M. kansasii*, *M. marinum*, *M. fortuitum* and *M. ulcerans*.

Human health effects

**Clinical features**

Atypical *Mycobacterium* spp. are opportunistic bacterial pathogens which can cause a range of diseases involving the skeleton, lymph nodes, skin and soft tissues, as well as the respiratory, gastrointestinal and genitourinary tracts. Manifestations include pulmonary disease, Buruli ulcer, osteomyelitis and septic arthritis in people with no known predisposing factors (WHO 2004). Pulmonary disease resembling tuberculosis may be associated with *M. kansasii*, *M. avium-intracellulare*; lymphadenitis may be associated with *M. scrofulaceum*, *M. avium* complex; skin ulcers and soft tissue wound infections may be associated with *M. fortuitum*, *M. cheloni*, *M. ulcerans* and *M. marinum*. Current research suggests a possible role for MAC organisms in the development of Crohn’s disease (Hermon-Taylor 2000), an inflammatory bowel disease similar to Johne’s disease in sheep, cattle and goats.

**Dose – response data**

The infectious dose is unknown.

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Target populations

For most immunocompetent people, exposure to MAC has no effect on health, or possibly causes localized infection. But it constitutes a major cause of disseminated infections in immunocompromised patients, commonly causing death in HIV-positive persons (WHO 2004).

Diagnosis

Diagnosis is difficult and time-consuming. It may be suggested by seeing acid-fast bacilli in sputum or during the histological examination of biopsy specimens, then it is confirmed by culture on appropriate media, which can take up to two months (Hunter 1997).

Treatment

Treatment of the infection depends upon the sensitivity of the infecting organism to specific antibiotics. As many as 4-6 drugs may be used to treat some infections and treatment may require 6 months to 2 years. Certain lymph node infections and skin lesions can be surgically removed.

Geographical distribution

Worldwide.

Epidemiology

Transmission routes

Transmission of nontuberculous mycobacteria to man occurs by various mechanisms depending on the species (Dailloux 1999). The process by which these bacteria are transmitted is not completely understood. Principal routes of infection appear to be inhalation, contact and ingestion of contaminated water as well as consumption of contaminated food. But there is no evidence for person-to-person transmission.

Waterborne outbreaks

Epidemiological studies suggest that natural or drinking water are the principal source of human contamination (von Reyn 1994). Mycobacterial infections linked to contaminated hospital water, particularly hot water systems, have been recognized for many years (Aronson 1999). Infections are generally sporadic. But in all these cases, there is only circumstantial evidence of a causal relationship between the occurrence of bacteria in drinking-water and human disease (WHO 2002). Infections have been linked to contaminated water in spas (Lumb 2004).

Ecology

Mycobacteria can be recovered from a wide variety of environmental niches (natural water, drinking-water, biofilms, aerosols, soils, foods, plant and animals).

Regrowth in distribution systems/biofilms

MAC and other mycobacteria have been shown to be present in biofilms (WHO 2004) (Carter 2003) (September 2004) where they can proliferate with densities usually between $10^3$ and $10^4$ cfu/cm² (Schulze-Robbecke 1992). Some species, such as M. kansasi, can colonize cold water distribution systems whilst M. xenopi and M. avium are commonly associated with hot water systems (WHO 2004). High numbers of atypical mycobacteria may occur in distribution systems after events that dislodge biofilms, such as flushing or flow reversals.

Resistance to ozone and chlorine-based disinfectants is undoubtedly one reason why MAC and other mycobacteria grow and persist in drinking water distribution systems. In fact disinfection of water can lead
to selection of *M. avium* and *M. intracellulare* and other mycobacteria by killing competitor organisms (Falkinham 2001).

One study recently showed that the survival of *M. avium* in a model drinking water distribution system was dependent upon a complex interaction between pipe surface, nutrient levels and disinfectants (Norton 2004). This study suggested that reducing the biodegradable organic material in drinking water, control of corrosion, maintenance of an effective disinfectant residual, and management of hot water temperatures can help limit the occurrence of *M. avium* complex in drinking water biofilms.

**Occurrence**

Data are limited because isolation of atypical mycobacteria from water is laborious and time-consuming. MAC and some other mycobacteria have been isolated from natural waters throughout the world, including lakes, rivers, ponds and streams, sea water and wastewater. They also have been frequently recovered from drinking-water systems before and after treatment, from distribution systems and from raw source waters (Falkinham 2001) (Le Dantec 2002) (LeChevallier 2001). Although the data confirm that members of the genus *Mycobacterium* are isolated in drinking water, the numbers and frequencies of recovery of *M. avium* and *M. intracellulare* are low.

Environmental mycobacteria have been isolated in bottled table waters in Greece (Papapetropoulou 1997). MAC has been recovered from pools and spas in the Netherlands (Havelaar 1985).

**Reservoirs**

Nontuberculous mycobacteria are acquired from the environment (soil, water, dust, tissues of domestic animals...), but specific reservoirs of these organisms leading to human disease have not been defined (Horsburgh 1996).

Although environmental opportunistic mycobacteria have been isolated from wild and domestic birds and animals, the zoonotic potential of MAC infections remains unclear (Falkinham 2002).

**Survival**

Not extensively documented.

The physiological characteristics of non-tuberculous mycobacteria provides an understanding of their ecological distribution and survival: 1) they can grow over a wide pH range and they grow best at low pH values (around 5); 2) they grow microaerobically; 3) *M. avium* strains can grow over a wide range of temperatures, up to 45°C or more (up to 57°C), unlike *M. intracellulare* which can grow only up to 42°C; 4) they grow equally well in water with high levels of salt and are found in large numbers in brackish swamps and estuaries; 5) they are hydrophobic and collect largely at air-water interfaces; 6) they are relatively resistant to heavy metals and may be isolated from water with high levels of metal pollution; 7) their high resistance to disinfection by chlorine undoubtedly contributes to their persistence in drinking water systems (Leclerc 2004).

One study (Archuleta 2002) suggested that the remarkable stress survival exhibited by *M. avium* further suggests persistence in a large range of environments. Recently, one study found that *Mycobacterium avium* enters a state of metabolic dormancy in response to starvation in the environment (Archuleta 2005).

*M. avium* is capable of survival and growth in phagocytic protozoa and amoebae (*Acanthamoeba polyphaga* and *A. castellanii*) and such growth can increase its virulence and provide protection from antimicrobial agents (Steinert 1998). One study reported that protozoa intracellular growth of *M. avium*, *M. intracellulare*, and *M. scrofulaceum* was 40-fold faster than that of water-grown isolates (Strahl 2001).

**Inactivation/removal**

Published data on inactivation are summarized in annex 1. MAC are relatively resistant to chlorine, chloramine, chlorine dioxide, and ozone (Taylor 2000). (Falkinham 2003) suggests that simultaneous exposure of *M. avium* and other mycobacterial cells to chlorine at high temperatures might result in increased eradication of *M. avium* from public water supplies. Other opportunistic environmental mycobacteria, including *Mycobacterium kansasii*, *Mycobacterium marinum*, *Mycobacterium fortuitum*, *Mycobacterium phlei*, and *Mycobacterium chelonae*, have been shown to be relatively resistant to chlorine at concentrations used in municipal water systems for disinfection.
M. avium were killed within 3 min when exposed to hot water at 70 degrees C and exposed to ultraviolet light at 90 mW.s/cm² (Miyamoto 2000).

**Surrogates**

*Esherichia coli* (or thermotolerant coliforms) is not a reliable index for the presence/absence of atypical mycobacteria (WHO 2004).

No suitable indicators have been identified to signal increasing concentrations of MAC organisms in water systems. There is some evidence that the presence of *M. avium* is associated with turbidity in raw water (Falkinham 2001) but further investigation on this issue is needed.

Currently the presence of mycobacteria in water is not regulated by any country or international organization.

**Environmental detection**

Culture-based methods for isolation of MAC and other slow-growing mycobacteria is difficult as these organisms grow very slowly and tend to be dominated by other, more rapidly growing organisms.

Chemical decontamination methods were developed to reduce overgrowth problems but they may destroy some mycobacterial cells. This step is not necessary for drinking water samples. Several studies have been conducted to try and determine the optimum decontamination method but there is no clear consensus as to the best method.

Alternative purification methods based on IMS have been proposed for the specific enrichment of target cells (WHO 2004).

Reference culture methods to isolate NTM from water include four main steps: concentration by filtration or centrifugation, decontamination of the sample, growth on a selective media and enumeration of samples. These culture-based methods are laborious and time-consuming (weeks or months), cannot detect VBNC bacteria and do not allow species identification.

To overcome these limitations, molecular methods have been increasingly used for several years. Detection and identification of NTM to the species level have been reported using a culture enrichment step and PCR-restriction fragment length polymorphism (PCR-RFLP) in tap water (Chang 2002) and in bottled table waters (Papapetropoulou 1997).

FISH has been developed to visualize mycobacteria in situ in culture. This technique constitutes a promising approach particularly for biofilm samples but still needs extensive development (Stender 1999) (Hongmanee 2001) before being operational on environmental water samples.

Specific PCR tests have been developed: a PCR detection of *Mycobacterium ulcerans* in the environment (Stinear 2000), a real-time PCR method for the quantification of *M. ulcerans* DNA (Rondini 2003) and a PCR detection of *Mycobacterium avium* complex (MAC) (MacGregor 1999). Recently, two detection tests specific for *Mycobacterium avium paratuberculosis* from water samples have been developed: a real-time PCR assay for quantitative detection (Rodriguez-Lazaro 2005) and an IMS-PCR assay (Whan 2005). Currently all these available alternative molecular methods are at the experimental stage and validated protocols do not yet exist.

Several commercial tests for rapid species-level identification of mycobacteria have been developed (INNO-Lipa Mycobacteria kit from Innogenetics and Accuprobe from Genprobe) but these tests lack specificity and can give false-positive results.

Fingerprinting methods such as pulsed-field gel electrophoresis (PFGE), RAPD, or Inter-insertion sequence PCR, have proven very useful in epidemiological studies (WHO 2004). A new approach for genotyping mycobacteria in clinical samples using DNA chip technology has been reported (Troesch, Nguyen 1999). Extensive research work must be carried out before this promising approach can be operational in environmental water samples.
Research needs

The following issues can constitute priorities for future research on atypical mycobacteria:

**Analytical methods**

Develop specific tests and testing strategies for atypical mycobacteria.
- **Culture-based methods:** Improvements in the methodology for isolation and enumeration of mycobacteria in water and biofilms samples are necessary. For example by developing a more selective media or isolation conditions which will enable only MAC or other mycobacteria to grow and that will not require a decontamination step.
- **Molecular methods:** Develop and validate specific PCR tests for rapid detection and quantification of pathogenic environmental mycobacteria species and strains.

**Virulence factors**

Virulence factors are poorly understood for mycobacteria. The lack of knowledge of virulence markers hinders the identification of the sources of MAC infection. There is a need to identify genetic or phenotypic markers of pathogenic strains. Genetic markers are the basis for modern molecular detection methods and their knowledge will allow the development of pathogen-specific molecular tests.

**Epidemiology**

The host susceptibility factors for MAC infection should be investigated.

**Biofilm**

Biofilm might play a role in the survival of virulent mycobacteria strains. Further studies should be carried out on this topic in order to identify approaches for inhibiting *M. avium-intracellulare* (and other mycobacteria) biofilm formation and colonization, for example, by identifying *M. avium* genes involved in biofilm formation and investigating the effect of disinfectants on the establishment of biofilm.

Additional studies are needed on the role of amoebae and protozoa in the survival, proliferation and the virulence of atypical mycobacteria in biofilms.

Studies on the transfer of resistance to antibiotics and virulence factors in drinking water biofilms should be an area of future research.

**Waterborne transmission**

Limited data exist to evaluate the risk of *M. avium* complex infection from water.
- Further epidemiological studies using molecular biology techniques such as pulsed field gel electrophoresis should be carried out in order to investigate the role of water as the principal source of contamination.
- More systematic studies of drinking water systems over long period of times and of the efficacy of water treatment steps need to be carried out.
- Additional research is needed on the occurrence, survival and reservoirs of MAC in water.

**Inactivation**

- There is a need to develop disinfection strategies, which are effective on MAC.
- Additional research on the susceptibility of atypical mycobacteria to treatment processes is needed. More broadly, as for *Legionella*, water treatment approaches that minimize selection for treatment resistant pathogens, the survival of protozoa, biofilm formation and production of disinfection by-products need to be investigated.
- Furthermore, data are limited on the effectiveness of control measures that could be applied to reduce the potential risk of these organisms. As the eradication of nontuberculous mycobacteria in water is not possible, there is a need for preventive measures, which could minimize their presence and proliferation, particularly in biofilm of drinking-water systems.

**Risk assessment**

Dose-response data, in particular for MAC, are needed to establish public health goals for potable water supplies (WHO 2004).
Viruses
Adenovirus

<table>
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<tr>
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<tr>
<td>Resistance to chlorine</td>
<td>Moderate</td>
</tr>
<tr>
<td>Relative infectivity</td>
<td>High</td>
</tr>
<tr>
<td>Important animal source</td>
<td>No</td>
</tr>
</tbody>
</table>

Adapted from table 7.1 “Waterborne pathogens and their significance in water supplies” (WHO 2004).

Microbiology

General description

Adenoviruses are medium-sized (90-100 nm), nonenveloped icosahedral viruses containing double-stranded DNA. First isolated from the adenoids of children in 1953, they are the only human enteric viruses with double stranded DNA.

They are featured on the U.S. Environmental Protection Agency drinking water contaminant candidate list (CCL) (U.S. Environmental Protection Agency. 2005) (U.S. Environmental Protection Agency. 1998).

Classification

Human adenoviruses belong to the family of Adenovirus and the genus of Mastadenovirus. They have been classified into six subgenera (A to F) and 47 immunologically distinct types (serotypes).

Human health effects

Clinical features

Adenoviruses can cause a variety of clinical syndromes depending on the infecting serotype:

- Subgenera A-E are generally associated with respiratory infections, conjunctivitis, pharyngitis, pneumonia, acute and chronic appendicitis, exanthematous disease, bronchiolitis, acute respiratory disease, febrile illness (sore throat, glands) (Baum 1995).
- Subgenus F, which includes types 40 and 41, is consistently associated with gastroenteritis. The two serotypes, 40 and 41 are referred to as enteric adenoviruses. They are considered second only to rotaviruses as the primary causes of gastroenteritis in children (Uhnoo 1984).

Adenoviral infections typically last from a couple of days to a week. Severe respiratory infections can last longer and cause persistent symptoms, like a cough. Pneumonia can last from 2 to 4 weeks. For some adenovirus serotypes, the clinical spectrum of disease associated with infection varies depending on the site of infection; for example, infection with adenovirus 7 acquired by inhalation is associated with severe lower respiratory tract disease, whereas oral transmission of the virus typically causes no or mild disease.

Dose – response data

The infectious dose in humans is unknown but presumably is 10-100 virus particles.

Target populations

Adenoviruses are opportunistic pathogens, as they tend to affect children, the elderly and the immunocompromised. The virus is also associated with crowding and stress.

Diagnosis

Antigen detection, polymerase chain reaction assay, virus isolation, and serology can be used to identify adenovirus infections (from throat, conjonctival swabs, or stools).
Adenovirus typing is usually accomplished by hemagglutination-inhibition and/or neutralization with type-specific antisera. Since adenoviruses can be excreted for prolonged periods, the presence of the virus does not necessarily mean it is associated with disease.

**Treatment**

Most infections are mild and require no therapy or only symptomatic treatment. Because there is no virus-specific therapy, serious adenovirus illness can be managed only by treating symptoms and complications of the infection.

Vaccines have been developed for adenovirus serotypes 4 and 7, but only for purposes of preventing Acute Respiratory Disease (ARD) among military recruits.

**Geographical distribution**

Worldwide. The prevalence of adenoviruses is high in both developed and developing countries.

**Epidemiology**

**Transmission routes**

All types of adenoviruses are transmitted by faecal-oral route, by direct person-to-person contact, and occasionally by indirect waterborne or foodborne transmission.

For non enteric adenoviral infections, airborne spread through coughing and sneezing is probably common.

**Waterborne outbreaks**

Although the potential health risks constituted by adenoviruses in water sources and supplies are widely recognized (U.S. Environmental Protection Agency, 1998) (American Water Works Association, 1997) (WHO, 2002), no food or waterborne outbreak associated with drinking water has ever been reported. The only waterborne outbreaks reported in the literature have been associated with inadequately chlorinated swimming pools. These outbreaks are generally associated with types 3 and 4 and result in pharyngoconjunctivitis (Papapetropoulou and Vantarakis, 1998) (Harley, 2001).

**Ecology**

**Regrowth in distribution systems/biofilms**

They are environmentally inert and only replicate in their hosts. Their persistence in biofilms has never been documented.

**Occurrence**

Human adenoviruses (Ads) are ubiquitous and can be found in the environment where contamination by human faeces and sewage has occurred.


Data are limited about the prevalence of enteric adenoviruses 40 and 41 in water sources. Levels of contamination in water are not documented. The public health significance of adenoviruses presence in water remains unknown.

**Survival**

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Not extensively documented. Adenoviruses have been found to be quite persistent in both tap and sea water. They can survive for days to weeks (Enriquez 1995). Enriquez et al. reported that adenoviruses (Ad 40 and Ad 41) were much more stable than either poliovirus type 1 or hepatitis A. Infectivity is undiminished by refrigeration (4 deg C) for 70 days (American Water Works Association 1997).

**Reservoirs**

Humans are the only known reservoir of the organism.

**Inactivation/removal**

Published data on removal and inactivation are summarized in annex 1. Adenoviruses are unusually stable to chemical or physical agents and adverse pH conditions, allowing for prolonged survival outside the body.

The most effective germicides (with a minimum 15 min. contact time) are: Phenol (5%); Sodium hypochlorite (household bleach diluted to 200 ppm or 10%).

The virus is also effectively killed by heat, at 56 degrees °C (American Water Works Association 1997). Inactivation can be achieved with chlorination levels comparable to those used in water treatment. A dose of 0.1 mg/l of free chlorine and contact times (60 to 237 min) is sufficient to inactivate Adenovirus type 40 (Thurston-Enriquez 2003) (American Water Works Association 1997).

Adenoviruses are much more resistant to ultraviolet light (UV) than any other pathogen of concern because their double-stranded DNA genome allows them to use the host cell enzymes during replication to repair UV damage in the DNA (Gerba 1996). The UV doses for a one log reduction, or 90% removal, for Adenovirus type 40 and 41 are 30 mW-s/cm² and 24 mW-s/cm² respectively (Qin and Gerba 1996).

The effectiveness of treatment processes used to remove adenoviruses requires validation.

**Surrogates**

Adenoviruses have been detected in drinking water supplies that met accepted specifications for treatment, disinfection and conventional indicator organisms. Because of the higher resistance of the viruses to disinfection, *E coli* (or, alternatively coliforms) is not a reliable index of the presence/absence of adenoviruses in drinking-water supplies (WHO 2004).

**Environmental detection**

Most serotypes grow well in standard cell culture line except fastidious adenovirus serotypes 40 and 41 for which no standard culture method is available.

The virus may be identified by electron microscopy (Thomas 1999) and ELISA (Uhnoo 1984) but this method is not sensitive enough for environmental water samples.

The basic method for concentration of adenoviruses from water is the same as for enteroviruses. A large number of molecular sensitive and specific tests have been developed to detect all types of adenoviruses in water: PCR or nested-PCR based detection (Lee and Kim 2002) (Cho, Lee et al. 2000) (Pina 1998) (Castignolles 1998) (Puig 1994) (Schvoerer 2000).

Several studies recently reported the detection of infectious adenoviruses in water by a combination of cell culture and PCR (Ko 2003) (Greening 2002) (Lee 2004). Recently a quantitative real-time PCR assay has been described to quantify human adenovirus genomes in urban rivers (Choi 2005), but samples with positive results obtained by real-time PCR gave negative results by tissue culture, suggesting that the adenoviruses from the analysed samples were non infectious.

All the molecular methods available are at the experimental stage and validated protocols do not yet exist. To date analysis for adenoviruses in water is likely to be restricted largely to research purposes.
Calicivirus

<table>
<thead>
<tr>
<th>Health significance</th>
<th>Persistence in water supplies</th>
<th>Resistance to chlorine</th>
<th>Relative infectivity</th>
<th>Important animal source</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>Long</td>
<td>Moderate</td>
<td>High</td>
<td>Potentially</td>
</tr>
</tbody>
</table>

Adapted from table 7.1 “Waterborne pathogens and their significance in water supplies” (WHO 2004).

Microbiology

**General description**

Caliciviruses are small, nonenveloped, single-stranded RNA organisms about 7.5Kb long. They are spherical and about 27-40nm in diameter and generally display a typical surface morphology resembling cup-like structures.

Caliciviruses are featured in the Contaminant Candidate List (CCL) (U.S. Environmental Protection Agency. 2005) (U.S. Environmental Protection Agency. 1998).

**Classification**

Human caliciviruses (HuCVs) have been grouped into two genera: *Norovirus* and *Sapovirus* (Berke 1997)

*Sapovirus* spp. demonstrate typical calicivirus morphology and were called classical caliciviruses in the past. Sapporo virus is the prototype strain for *Sapovirus* (Parshionikar 2003).

*Norovirus* spp. (previously termed Norwalk-like viruses or small round structured viruses) generally fail to reveal the typical morphology. They have been divided into genogroups I and II, each with a number of distinct genetic clusters. Norwalk virus is a prototype strain for genogroup I, while Snow Mountain virus is a prototype strain for genogroup II (Ando 2000).

The Norwalk virus was the first human calicivirus identified by electron microscopy during a gastroenteritis outbreak in Norwalk, Ohio, in 1972 (Kapikian 1972).

Human health effects

**Clinical features**

Human caliciviruses, especially the *Norovirus* genus, are a major cause of acute nonbacterial gastroenteritis worldwide and are believed to be one of the major causes of waterborne disease.

The illness leads to vomiting and/or diarrhea, and abdominal cramps. Fever and respiratory symptoms have been reported in a small number of cases. The disease is marked by an abrupt onset, 24-28 h after exposure, but is typically mild and self-limiting, only lasting 12-60h (Kaplan 1982). However, children and persons with compromised immunity may need hospitalization for dehydration. Since some cases involve vomiting only and no diarrhea, the condition is also known as “winter vomiting disease”. Infections by HuCVs induce a short-lived immunity (WHO 2004).

**Dose – response data**

The infectious dose is low, about 10-100 viral particles (Green 1997). A dose-response model for norovirus is currently being developed and should be published soon.

**Target populations**

*Norovirus* causes illness in people of all age groups, whereas *Sapovirus* predominantly causes illness in children.
**Diagnosis**

Detection in stools has been done by electron microscopy (EM), immune electron microscopy (IEM) but these methods are not very sensitive and require highly skilled microscopists as well as expensive equipment. In 1995, reverse transcriptase polymerase chain reaction (RT-PCR) was introduced, and has since become the laboratory standard.

**Treatment**

There is no specific antiviral treatment. Oral rehydration therapy is the recommended treatment prescribed to avoid severe dehydration.

**Geographical distribution**

Worldwide.

**Epidemiology**

Noroviruses are the viruses most commonly associated with food and waterborne outbreaks of gastroenteritis (Green 1997). 50% to 80% of gastroenteritis outbreaks in the United States, Japan and some European countries were attributed to noroviruses (Lopman 2003) (Blackburn 2004) (Dedman 1998) (Hedberg and Osterholm 1993) (Hedlund 2000).

**Transmission routes**

The virus is transmitted by faecal-oral route through contaminated food, water and by person-to-person contact. Contaminated surfaces, such as door handles, telephone handsets, and water taps, have been shown to carry caliciviruses in detectable amounts, thereby forming an important mechanism for virus transmission (Brown 1999). Transmission by aerosol produced by vomiting is probably common (Hedberg and Osterholm 1993).

**Waterborne outbreaks**

The first well-documented outbreak of *Norovirus* gastroenteritis linked to drinking water occurred in a Washington State elementary school in the USA in May, 1978 (Taylor 1981). Since that time, many waterborne outbreaks of *Norovirus* associated to drinking water have been reported worldwide (Kukkula 1999) (Huffman 2003; Nygard 2003) (Kaplan 1982) (Haffliger 2000) (Parshionikar 2003) (Hedberg and Osterholm 1993) (Kukkula 1997) (Schvoerer 1999). Waterborne outbreaks associated with swimming in contaminated recreational water have also been reported (Kappus 1982) (Baron 1982). Moreover, numerous outbreaks due to HuCV-contaminated foods, such as shellfish, salads, and deli sandwiches have been reported (Johansson 2002).

**Ecology**

*Regrowth in distribution systems/biofilms*

As with all viruses, HuCVs are environmentally inert and only replicate in their hosts (human). Their persistence in biofilms has never been documented.

**Occurrence**

The distribution of HuCVs in the environment reflects the distribution of human faecal contamination. The occurrence of caliciviruses in water has been studied only since molecular detection methods have been available. Noroviruses are frequently detected in various types of environmental water samples, such as sewage (Lodder 1999), river water (Gilgen 1997), well water (Nygard 2003) (Beller 1997), seawater (Katayama 2002), and even mineral waters (Beuret 2000) (Beuret 2002) and tap water (Kukkula 1999) (Haramoto 2004).
But RT-PCR based detection cannot determine whether or not the virus is infectious and thus as suggested by Gassilloud et al. (Gassilloud 2003), positive results must be interpreted with caution in terms of public health. Levels of contamination are not documented.

**Reservoirs**

The main reservoir of infection appears to be humans but recent data suggest that calves and pigs may be reservoir hosts of Norovirus as well (van Der Poel 2000) (van Der Poel 2003). It is believed that caliciviruses emerged from ocean reservoirs, with subsequent zoonotic and interspecies movement (Smith 1998).

**Survival**

Little is known about the survival of caliciviruses in the environment. One study (Kadoi 2001) reported the survival of a cultivable model virus (feline calicivirus strains) in marine water: infective viruses can survive at 10 °C or lower temperature during 30 days.

**Inactivation/removal**

Published data on removal and inactivation are summarized in annex 1. Knowledge of efficient inactivation methods for HuCVs is limited and based on studies with cultivable model viruses, mainly feline calicivirus (FCV) (Thurston-Enriquez 2003) (Doultree 1999) (Duizer 2004).

Caliciviruses remained infective to volunteers following: pH 2.7, 20% ether at 4°C for 18 hours, temperatures of 60°C for 30 minutes, and chlorine levels consistent with those found in drinking water systems (Keswick 1985). Other work showed that feline calicivirus was surprisingly resistant to chlorine, requiring 1000 mg/l of freshly reconstituted granular hypochlorite for complete inactivation (Doultree 1999). There is a strong need for further studies on disinfection of these persistent organisms.

Duizer et al. (Duizer 2004) reported incomplete inactivation of FeCV by 70% ethanol and 300 ppm free chlorine.

In recent studies, FCV appears to be UV resistant (Nuanualsuwan 2002) (De Roda Husman 2004).

The effectiveness of treatment processes used to remove HuCVs requires validation.

**Surrogates**

Due to higher resistance of the viruses to disinfection, *E coli* (or, alternatively, thermotolerant coliforms) is not a reliable index of the presence/absence of HuCVs in drinking-water supplies (WHO 2004).

**Environmental detection**

HuCVs cannot be propagated in available cell culture systems. Their detection depends mainly on reverse transcription-PCR. But RT-PCR fails to determine virus infectivity and does not give the level of contamination.

Conventional filter adsorption-elution methods are the most widely used for concentrating the virus from large volume of water (Sobsey 1980) (Schwab 1995) (Gilgen 1997) (Gassilloud 2003). Haramoto et al. (Haramoto 2004) reported a new concentration method using a cation-coated filter. A new procedure for concentration of enteric viruses from coastal water using a negatively charged membrane was described (Katayama 2002).

A large number of studies reported the development of RT-PCR assays to detect *Norovirus* from sewage (Lodder, Vinjé et al. 1999), environmental water samples (Myrmel 1999) (Gilgen, Germann et al. 1997) (Parshionikar 2003) (Schwab 1995) and mineral water (Beuret 2003).
Immunomagnetic separation combined with RT-PCR was developed to detect *Norovirus* (genogroup I) in water (Myrmel 2000). Haramoto developed a new method for concentration and detection of Norovirus by TaqMan-RT-PCR in tap water (Haramoto 2004). But this test is not quantitative.

A single analytical process using ultrafiltration combined with high-density oligonucleotide array has been developed to simultaneously detect waterborne pathogens including bacteria, viruses (Enterovirus, Hepatitis A, Norovirus) and parasites (Guillot 2003) (see Enterovirus section).

In the future, there is a need to develop a rapid method to quantify HuCV contamination levels.

The method currently used for typing *Norovirus* is sequencing and subsequent phylogenetic analysis of RT-PCR products (Schvoerer 1999). One study reported a simple method for genotyping noroviruses using oligonucleotide array (Vinje 2000).

At the present time, molecular tests for Noroviruses are only available in specialist laboratories and cannot be used for routine testing of water samples. Commercial test kits are not yet available. Evaluation studies and standardization of the procedures are necessary to get the new methods approved by health authorities; this is the basic requirement for routine application.
Enterovirus

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<tr>
<td>Relative infectivity</td>
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</tr>
<tr>
<td>Important animal source</td>
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</tr>
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</table>

Adapted from table 7.1 “Waterborne pathogens and their significance in water supplies” (WHO 2004).

Microbiology

**General description**

Enteroviruses (EVs) are a large group of small (20- to 30-nm in diameter) nonenveloped single-stranded RNA viruses with an icosahedral symmetry. Enteroviruses have been the most studied in the past because they are easily isolated by standard cell culture methods. EVs (Coxsackievirus and Echovirus) are included on the U.S. Environmental Protection Agency drinking water contaminant candidate list (CCL) (U.S. Environmental Protection Agency. 2005) (U.S. Environmental Protection Agency. 1998).

**Classification**

The genus *Enterovirus* is a member of the Picornaviridae family. This genus consists of 69 serotypes that infect humans: poliovirus types 1-3, coxsackievirus types A 1-A 24, coxsackievirus types B 1-B 6, echovirus types 1-33 and the numbered enterovirus types EV 68- EV 71. Members of the genus are collectively referred to as enteroviruses. Other species of the genus infect animals other than humans, for instance the bovine group of enteroviruses (WHO 2004).

**Human health effects**

**Clinical features**

Enteroviruses are one of the most common causes of human infection. However most infections are unapparent and lead to a non-specific febrile illness referred to as the “summer flu” (Dagan 1996). Some serotypes, however, may cause serious clinical syndromes such as poliomyelitis, encephalitis, meningitis, myocarditis, and pericarditis (Melnick 1990). Enterovirus 71 infections lead to blisters in the oral cavity, palms and soles, ulcers and fever (hand-foot-mouth disease). Most infections, particularly in children, are asymptomatic, but still lead to the excretion of large numbers of the viruses, which may cause clinical disease in other individuals (WHO 2004).

**Dose – response data**

The infectious dose is low: 1-10 infectious particles. Different dose – response models based on experiments on humans have been described for echovirus 12 and polyovirus 1 and 3 (Regli 1991).

**Target populations**

Everyone is at risk of EV infection. Infants, children, and adolescents are more likely to be susceptible to EV infection, due in part to a lack of prior immunity and to poor hygiene habits.

**Diagnosis**

Diagnosis can be performed by cell culture from the throat, swabs and faeces. Serological diagnosis may also be used but is of limited value due to large number of strains.
**Treatment**

No specific treatment is available for enteroviral infections. Treatment focuses on management of complications. No vaccine is available for non-polio enteroviruses.

**Geographical distribution**

Worldwide.

**Epidemiology**

Some EV serotypes have been responsible for large and serious epidemics throughout the world in recent years: Echovirus caused several large epidemics of aseptic meningitis in Japan, Europe, the Middle East and Cuba (Kirschke 2002). EV 71 has caused major outbreaks of hand-foot-and mouth disease worldwide since 1995 (Ho 1999). Rapid genetic evolution of some new strains of EVs as well as development of anti-viral drug resistance have been reported (Nel and Weyer 2004).

**Transmission routes**

EVs are transmitted via the faecal-oral route. Person to person contact and inhalation of airborne viruses or viruses in respiratory droplets are considered to be the predominant routes of transmission of enteroviruses in communities. Persons may also become infected by contact with respiratory secretions (e.g., saliva, sputum, or nasal mucus). Transmission from drinking water could also be important, but this has not yet been confirmed (WHO 2004).

**Waterborne outbreaks**

Despite the wide occurrence of enteroviruses in the water environment, few water-related outbreaks have been reported. The limited knowledge on the role of waterborne transmission could be related to a number of factors, including the wide range of clinical symptoms, frequent asymptomatic infections, the diversity of serotypes and the dominance of person to person spread (WHO 2004).

The only outbreak of poliomyelitis linked by an epidemiological study to drinking water occurred in Taiwan in 1982, affecting 1031 persons (Kim-Farley 1984). Coxsackievirus has been associated with numerous common source outbreaks, including at least two documented waterborne outbreaks (Ikeda 1993). Echovirus-30 was responsible for a large waterborne outbreak of aseptic meningitis in Belarus in summer/autumn of 1997 (Amvrosieva 2001).

**Ecology**

**Regrowth in distribution systems/biofilms**

As with all viruses, EVs are environmentally inert and only replicate in their hosts. One study reported the tendency for virus accumulation within drinking water biofilms (Quignon 1997); the role of biofilm in the survival and persistence of viruses remains unclear.

**Occurrence**

The virus is shed in the faeces of persons with both asymptomatic and symptomatic infection.

Recently, Vivier et al. (Vivier 2004) reported the monitoring of drinking water for the presence of enteroviruses by cell culture-PCR, over a period of 1 year: Enteroviruses (predominantly coxsackie B viruses) were detected in 11% and 16% of the drinking water samples (that met bacteriological standards) from two treatment plants. EVs have also been isolated from swimming pools (Keswick 1981). In addition, EVs are found in food and have been isolated from shellfish.

Levels of contamination are not extensively documented (because the molecular methods increasingly used do not readily allow quantification). Levels of 1-100 pfu/l of enteric viruses in contaminated surface water, of 1-10 pfu/100 l. in less polluted surface water, 1-10 pfu/1000 l. in treated drinking water were reported (Payment 1981) (Hurst 1991). In the future, further research should be done to determine the public health significance of EV occurrence in water.

**Reservoirs**

Humans are the only reservoir of EVs.

**Survival**

Not extensively documented. These viruses are quite resistant to environmental stresses and stable under acidic conditions (pH 3). Their persistence was shown in water supplies over 1 month at 20°C (WHO 1993). Ultraviolet radiation reduces survival, and survival in fresh waters is longer than in marine water (Hunter 1997). Skraber et al. compared the survival in river water of infectious polioviruses to somatic coliphages and poliovirus genomes considered as potential indicators. At three different temperatures (4°C, 18°C and 25°C), somatic coliphages and poliovirus genomes persisted longer than infectious polioviruses. According to these results and previously reported results (Hot 2003) the authors suggested that poliovirus genomes detected by RT-PCR may be useful for predicting the presence of waterborne viruses (Skraber 2004).

**Inactivation/removal**

Published data on removal and inactivation are summarized in annex 1. Hurst et al. (Hurst 1991) reported the removal of enteric viruses from various stages of conventional water treatment. EVs have been shown to be particularly resistant to chlorine and UV inactivation (Blackmer 2000) (Payment 1985) (Batigelli 1993).

**Surrogates**

Enteroviruses have been detected in drinking water supplies that met accepted specifications for treatment, disinfection and conventional indicator organisms. Because of the higher resistance of the viruses to disinfection, *E coli* (or, alternatively coliforms) is not a reliable index of the presence/absence of enteroviruses in drinking-water supplies (WHO 2004).

**Environmental detection**

A standard cell culture method is available for most enteroviruses (Fout 1996). But this approach is time consuming (6-15 days) and not very sensitive.

Although cell culture still remains the "gold standard" for enterovirus isolation, RT-PCR -which is more rapid and sensitive- has become widely used.

**Concentration:**

The method of choice to concentrate viruses from water is the adsorption/elution technique (APHA 1998) using positively charged filters (Fout 1996) (Sobsey 1980) with improvements for groundwater (Dahling 2006).
or electronegative filters (Beuret 2003). Recently a new concentration method using tangential flow ultrafiltration has been described (Bigliardi 2004).

**Purification:**
Schwab et al. have developed a broad-spectrum immunocapture method for concentration and purification of enteric viruses before RT-PCR detection (Schwab 1996). Four commercial kits for viral RNA extraction from water concentrates were evaluated: NucliSens and Centricon-QIAamp proved very sensitive, efficient and robust methods (Burgener 2003).

**Detection:**
Numerous RT-PCR methods used for water samples have been reported to detect polioviruses (Tansuphasiri 2000), enteroviruses (Abbaszadegan 1999) or simultaneously several viruses: adenoviruses and enteroviruses (Cho 2000), noroviruses and enteroviruses (Beuret 2003), enteroviruses and hepatitis A viruses (Li 2002) (Tsai 1994), adenoviruses, enteroviruses and hepatitis A viruses in sewage and shellfish (Formiga-Cruz 2005) and enteroviruses, hepatitis A virus, Norwalk virus, reoviruses, and rotaviruses (Fout 2003).

A single analytical process using ultrafiltration combined with high-density oligonucleotide array (GeneChip technology from Affymetrix, Santa Clara, Ca.) to simultaneously concentrate and detect waterborne pathogens including bacteria, viruses (Enterovirus, Hepatitis A, Norovirus ) and parasites in treated and distributed water samples has been described (Guillot 2003). This new process is at the experimental stage and requires significant improvements, particularly in terms of specificity and sensitivity.

However conventional RT-PCR does not determine the infectivity of the viruses and does not allow easy quantification.


Only one study reported the quantitative detection of pathogenic human enteroviruses from surface waters by TaqMan RT-PCR (Donaldson 2002).

Typing methods such as PCR-Restriction Fragment Length Polymorphism (Kuan 1997) (Vivier 2001) and PCR and sequencing (Oberste 1999) have been used.

Currently all the available molecular methods are at the experimental stage and validated protocols do not yet exist. In the future, evaluation studies and standardization of these new methods will be necessary to get them approved by the authorities.
Hepatitis A

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<td>Relative infectivity</td>
<td>High</td>
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<tr>
<td>Important animal source</td>
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</table>

Adapted from table 7.1 “Waterborne pathogens and their significance in water supplies” (WHO 2004).

Microbiology

**General description**

Hepatitis A virus (HAV) is a small (27-28nm) spherical, nonenveloped (naked) positive strand RNA virus, about 7.5Kb in length. HAV has a single antigenic serotype.

**Classification**

HAV is classified under the genus Hepatovirus within the *Picornaviridae* family.

Human health effects

**Clinical features**

Hepatitis A is an acute, usually self-limiting infection of the liver caused by hepatitis A virus (HAV). Symptoms typically include fever, malaise, anorexia, nausea and abdominal discomfort, followed by dark urine and jaundice (Koff 1992). Complications of hepatitis A include relapsing hepatitis, cholestatic hepatitis and fulminant hepatitis. Chronic infection with HAV does not occur. In children below six years of age, HAV infection is usually asymptomatic, with only 10% developing jaundice (Yang 1988).

The average incubation period is 28 days, but may vary from 15–50 days. Approximately 10–12 days after infection the virus can be detected in blood and faeces. Virus excretion may begin up to a week before the symptoms are apparent, making control difficult.

Infection with HAV induces lifelong immunity.

**Dose – response data**

The infectious dose in humans is unknown but presumably is 10-100 virus particles (WHO 2002).

**Target populations**

The severity of disease and mortality may be dependent on the general state of health (immunodeficiency and malnutrition…) and increase in older age groups.

**Diagnosis**

Hepatitis A is diagnosed by finding IgM-class anti-HAV in serum collected during the acute or early convalescent phase of disease. Commercial kits are available. Detection of the virus or viral antigens in the stools is of limited value for routine diagnosis.

**Treatment**

No specific treatment is currently available. Vaccines are available for persons 2 years of age and older.
Geographical distribution

HAV has a worldwide distribution.

Epidemiology

An estimated 1.5 million clinical cases of hepatitis A occur every year. The prevalence of clinical disease has typical geographically based characteristics. In countries where inadequate sanitary conditions prevail, the virus persists in the environment and almost 100% of the population acquires infection in childhood. In developed, industrialized countries HAV has ceased to circulate in the environment and the general population. Here, infections predominantly occur in adults travelling to endemic areas or exposed at home to thus infect individuals or members of high risk groups (e.g. children in day care centres, i.v. drug users) (Grabow 2002) (WHO 2000).

Transmission routes

Transmission occurs primarily through the faecal-oral route, and is closely associated with poor sanitary conditions. The most common modes of transmission include close personal contact with an infected person (usually among household contacts or sexual partners) and ingestion of faecally contaminated water and food. Infection can also be spread in association with injecting and non-injecting drug use. Asymptomatic and non-jaundiced HAV-infected persons, especially children, are an important source of HAV transmission (Staes 2000). Outbreaks by person-to-person spread commonly occur among families, institutions and troops (day-care centres, school and institutions for mentally ill people and food establishments) (Banker 2003).

Waterborne outbreaks

Food and waterborne outbreaks of HAV in developed countries have been recognized for over 40 years, but are infrequently reported (Fiore 2004). Epidemiological evidence to link hepatitis infection to food and water sources is sparse because of the long incubation period (Seymour and Appleton 2001). In developing countries they are uncommon because of high levels of immunity in the resident population (Fiore 2004).

Waterborne outbreaks have often been associated with water supplies that complied with generally accepted guidelines for indicators and treatment procedures (Grabow 2002). In 1972 an outbreak of HAV affected 49 children and one adult in a rural elementary school in Alabama, probably associated with contaminated drinking school water (Baer and Walker 1977). In 1989 an outbreak of hepatitis A affecting 47 people in central Italy was linked to a single brand of bottled mineral water (Stroffolini 1990). Coursag et al. reported for the first time a waterborne outbreak of acute hepatitis simultaneously due to HAV and HEV observed in 1993 in Djibouti (Coursaget 1998). In the United States, the highest incidence of waterborne infectious hepatitis occurred between the 1950s and the 1970s with 47 outbreaks reported (Craun 1996). Since the early 1990s, very few outbreaks of HAV have been reported to the CDC (De Serres 1999).

The largest ever recorded outbreak of HAV infection occurred in Shanghai in 1988 involving 250,000 people and was traced to consumption of raw clams (Halliday 1991). Outbreaks associated with recreational water contact were also reported (Mahoney 1992) (Bryan 1974).

Ecology

Regrowth in distribution systems/biofilms

As all viruses, HAV only replicates in its hosts (human or animal). The presence and persistence of HAV within biofilms have not been investigated.

Occurrence

The ecology in the environment mirrors that of the distribution of human faecal contamination (both asymptomatic and symptomatic infection).
Using molecular biology techniques, HAV has been found in environmental water (Grabow 2002), HAV has been detected in sewage and wastewater (Jothikumar 1998) (Espigares 1999), in polluted rivers and in drinking water (Schvoerer 2000) (Morace 1993) (Pina 2001) (Taylor 2001) (Jothikumar N 2000).

Further study is required to determine the public health significance of HAV occurrence in water. Levels of contamination in environmental waters are insufficiently documented (because molecular methods available to date are not quantitative).

**Survival**

Not extensively documented. The survival of infectious HAV greatly depends on the temperature. Under favorable conditions (ambient temperatures) HAV may survive in the environment for months (Mbithi, Springthorpe 1991). It could survive in wastewater and groundwater for 90 days or more at 10°C. Crance et al. (Crance 1998) reported that infectious HAV remain stable at 4°C throughout 92 days in synthetic seawater. Hepatitis A virus and poliovirus were shown to survive in excess of one year in mineral water stored at 4°C (Biziagos 1988).

**Reservoirs**

Humans are the only known reservoir of the organism. But a variety of non-human primates are susceptible to HAV under experimental laboratory conditions and transmission of the virus from chimpanzees to humans during close contact is well documented (Zuckerman and Thomas 1993). But to date there is no evidence that animals may serve as a significant reservoir for HAV. This question needs to be answered.

**Inactivation/removal**

To date HAV removal or inactivation by water treatment and disinfection processes has not been extensively documented. The published data are summarized in annex 1.

The virus is relatively stable at low pH levels (as low as 3) and moderate temperatures, but is inactivated by high temperature (at 85°C/185°F for 1mn).

Only 2% glutaraldehyde, a quaternary ammonium formulation containing 23% HCl (toilet bowl cleaner), and sodium hypochlorite (greater than 5,000 micrograms/ml of free chlorine) reduced the virus titer by more than 99.9% (Mbithi 1990).

HAV is resistant to disinfection by some chemical disinfectants (phenolics, iodine-based products, alcohols, and solutions of acetic, peracetic, citric, and phosphoric acids).

HAV appears to be relatively resistant to chlorine (Peterson 1983) (Li 2004) (Grabow 1983). In the United States a level of 5 mg chlorine/l with a contact time of 1 min is recommended for inactivation of hepatitis A virus.

**Surrogates**

Due to the higher resistance of the viruses to disinfection, *E coli* (or, alternatively, thermotolerant coliforms) is not a reliable index of the presence/absence of HAV in drinking-water supplies (WHO 2004).

**Environmental detection**

HAV is not readily detectable by routine cell procedures. It needs a prolonged incubation in cell culture requiring at least 4-5 weeks.

The method of choice to concentrate viruses from water is the adsorption/elution technique (APHA 1998) using positively or negatively charged membrane filters (Fout 1996) (Sobsey 1980), followed by organic flocculation for secondary concentration.
An amplified enzyme-linked immunosorbent assay (A-ELISA) for detecting and quantifying hepatitis A virus in estuarine water samples was reported but this approach was time consuming and not very sensitive (Nasser 1987).

Thus, the most feasible current approach to detect HAV from water samples is based on molecular techniques (mainly RT-PCR).

Numerous RT-PCR methods have been reported to detect HAV alone or in combination with other viruses in environmental waters: detection of HAV in wastewater (Espigares 1999) (Divizia 1998); simultaneous detection of rotavirus, HAV, and small round structured viruses in river water (Gilgen 1997), detection of enterovirus and HAV (Li 2002); detection of HAV and HEV in drinking water (Jothikumar N 2000), detection of HAV and rotavirus in spring water samples (Brassard 2005), detection of poliovirus, HAV and rotavirus in sewage and ocean water (Tsai, Tran et al. 1994), detection of HAV, rotavirus and enterovirus in shellfish and sediment (Le Guyader 1994); detection of enteroviruses, HAV, Norwalk virus, reoviruses, and rotaviruses (Fout 2003) and recently detection of adenoviruses, enteroviruses and HAV in sewage and shellfish (Formiga-Cruz 2005).

A combination of immunocapture followed by RT-PCR from environmental water samples has also been described (Abd El Galil 2004) (Jothikumar 1998) (Monceyron and Grinde 1994).

A single analytical process using ultrafiltration combined with high-density oligonucleotide array has been developed to simultaneously detect waterborne pathogens including bacteria, viruses (Enterovirus, Hepatitis A, Norovirus) and parasites (Guillot 2003) (see Enterovirus section).

RT-PCR is highly sensitive and specific but it does not determine the infectivity of the viruses or the level of contamination.

However, Bhattacharya et al. (Bhattacharya 2004) showed that RT-PCR could discriminate between infectious and non-infectious hepatitis A viruses following UV inactivation treatment. The procedure may be adapted to other viruses and inactivating agents.

An integrated cell culture/polymerase chain reaction (ICC/RT-PCR) technique was recently developed for the detection of infectious HAV alone or in combination with enteroviruses in environmental waters (Reynolds 2004). Though promising, this approach is still not ideal as it is time consuming (at least 48H), expensive and not readily applicable for routine diagnostic work.

Recently, Sanchez et al. (Sanchez 2006) reported the detection of HAV using commercial quantitative real-time RT-PCR tests. This approach will have to be optimized and validated on environmental water samples.

Nested PCR amplification and partial sequencing have been used in molecular epidemiology studies (Pina 2001) (Sanchez 2004).

All available molecular methods are currently at the experimental stage mainly for research purposes and validated protocols do not yet exist.
Hepatitis E

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<td>Relative infectivity</td>
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<tr>
<td>Important animal source</td>
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</tbody>
</table>

Adapted from table 7.1 “Waterborne pathogens and their significance in water supplies” (WHO 2004).

Microbiology

General description

Hepatitis E (HEV) is a new waterborne infectious viral agent, first characterized in the 1990s. HEV is a small (27-34 nm) spherical, non-enveloped positive strand RNA virus, with a single serotype, about 7.2kb in length; it was described for the first time in 1983 (Balayan 1983).

Classification

HEV was previously classified into the family Caliciviridae. However, it is now reclassified into the Hepatitis E-like viruses as an unassigned genus (Berke 2000). There are indications of antigenic variation, and possibly even differences in serotypes of the virus (WHO 2004). But to date only a single serotype has been recognized.

Human health effects

Clinical features

The disease caused by HEV is called hepatitis E, or enterically transmitted non-A non-B hepatitis. Other names include faecal-oral non-A non-B hepatitis, and A-like non-A non-B hepatitis.

Hepatitis caused by HEV is clinically indistinguishable from hepatitis A disease. The incubation period, however, is longer (6-8 weeks). Symptoms include discomfort, anorexia, abdominal pain, arthralgia, fever and vomiting. Patients generally excrete HEV for 1-2 weeks. The disease is usually self-limited and resolves in 2 weeks, leaving no sequelae.

HEV infection in children is mostly asymptomatic or causes a very mild illness without jaundice (anicteric) that goes undiagnosed (Grabow 2002).

Dose – response data

The infectious dose is unknown.

Target populations

The disease is most often seen in young to middle aged adults (15-40 years old) with a relatively low mortality rate of 0.1-1%.

Pregnant women are especially susceptible to severe disease, and excessive mortality has been reported in this group (up to 30%) (Balayan 1997) (WHO 2002).

Diagnosis

Diagnosis is performed by detecting specific IgM antibodies in the patient's serum.

Treatment

Most cases of hepatitis E only require non-specific supportive treatment consisting of rest and analgesics. No available therapy can alter the course of acute infection.
There is currently no vaccine available.

**Geographical distribution**

Hepatitis E is mostly confined to tropical and subtropical areas (China, Asia, Africa, Mexico and Central America). Hepatitis E in developed countries is generally restricted to persons who travel to an endemic area (Langer 1996) (Balayan 1993). But recent evidence indicates that HEV might also be prevalent at a very low level in Europe (Clemente-Casares 2003).

Increased travel for business and tourism to HEV endemic areas and increasing globalization of food markets by industrialized countries have the potential of introducing HEV into new geographical areas.

**Epidemiology**

The risk factors for HEV infection are related to poor sanitation in large areas of the world and HEV shedding in faeces.

**Transmission routes**

The virus is primarily transmitted through the faecal-oral route. HEV is transmitted mainly by faecally contaminated water. Foodborne transmission has been suggested but not demonstrated conclusively. Person-to-person transmission is uncommon (Aggarval and Naik 1994). There is no evidence for sexual transmission or for transmission by transfusion.

**Waterborne outbreaks**

Large outbreaks associated with sewage-contaminated drinking water have been reported in Asia (mainly India and China), Africa, the Middle East, and North America (Mexico) (Mast and Krawczynski 1996). One of the first known waterborne outbreaks was in Delhi, India in 1955-1956; approximately 29,300 people became ill (Vishwanathan 1957). In 1991 the largest waterborne outbreak of HEV occurred in Kanpur, India, with an estimated total of over 79,000 cases of disease (Nail 1992). To date no U.S. outbreak has been reported but several sporadic cases have occurred (Kwo 1997).

**Ecology**

**Regrowth in distribution systems/biofilms**

As with all viruses, HEV is environmentally inert and only replicates in hosts (human or animal). The presence and persistence of HEV within biofilms have not been investigated.

**Occurrence**

The ecology of this virus in the environment mirrors that of the distribution of animal or human faecal contamination. Due to lack of any practical detection techniques, the occurrence of HEV virus in water has not been documented to date.

**Reservoirs**

The reservoir of HEV has not yet been established, although recent findings have suggested that at least some strains of HEV may be zoonotic (Clayson and al. 1996) (Meng 2000). Some animals including domestic animals (mainly pigs), cattle, goats and even rodents may serve as reservoirs of HEV infections in humans and many human infections may originate from water sources polluted by animal waste. The zoonotic aspect of HEV needs to be more investigated and confirmed in the future.
Survival

Not documented. The only clue to the behavior of HEV in the water environment is provided by the detection of the virus in raw and treated wastewater (Pina 1998) (Jothikumar N 1993), which suggests that the virus can survive in hostile environments.

Inactivation/removal

HEV removal or inactivation by water treatment have not yet been studied. But data on waterborne outbreaks suggest that HEV may be as resistant as other enteric viruses to disinfection processes.

Surrogates

Due to the likelihood that the virus has a higher resistance to disinfection, E coli (or, alternatively, thermotolerant coliforms) is not a reliable index of the presence/absence of HEV in drinking-water supplies (WHO 2004).

Environmental detection

No method is currently available for routine analysis of water and food. HEVs are undetectable by conventional cell culture methods.

Some studies reported the development of molecular detection methods to isolate HEV from water or food (shellfish).

As for most viruses, the environmental detection of HEV requires two steps: concentration and detection. The use of a membrane filter-based adsorption and elution method (Grimm and Fout 2002) or a filtration column filled with granular activated carbon (GAC) (Jothikumar N 1995) has been proposed for concentration of HEV viruses from large volume water samples (at least 100 liters).

Some RT-PCR-based detection methods have been reported for environmental samples (Jothikumar N 1993) (Jothikumar N 1995) (Grimm and Fout 2002). Jothikumar et al. (Jothikumar N 2000) developed a duplex RT-PCR for simultaneous detection of Hepatitis A and E viruses isolated from drinking water. Two recent studies reported a quantitative real-time PCR assay for HEV: one in human faeces (Orru 2004) and the other in clinical and environmental samples (Jothikumar 2006).

Currently the few available molecular methods are in the experimental stage and restricted to research purposes. These protocols should be optimized and validated before being recognized as official methods.
### Rotavirus

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<td>Important animal source</td>
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Adapted from table 7.1 “Waterborne pathogens and their significance in water supplies” (WHO 2004).

**Microbiology**

**General description**

First detected in 1973 in Australia from children with acute diarrhoea (Bishop 1973) rotaviruses are non-enveloped double-stranded RNA viruses approximately 70 nm in diameter. Using electron microscopy, intact virus particles have the appearance of a wheel - hence the name “rota.”

**Classification**

Rotaviruses are classified with the *Reoviridae* family. Seven serological groups have been identified, three of which (groups A, B, and C) infect humans with group A being the most important human pathogen, whereas others infect a wide spectrum of animals (Cukor 1984) (Jiang 2002).

**Human health effects**

**Clinical features**

The illness starts abruptly with fever, vomiting and diarrhoea, which normally last 24-48 hours, although diarrhoea can last up to five days. The incubation period for rotavirus disease is approximately 1-2 days. Immunity after infection is incomplete, but repeated infections tend to be less severe than the original infection. For persons with healthy immune systems, rotavirus gastroenteritis is a self-limited illness, lasting for only a few days.

Rotaviruses are excreted in very large quantities in the faeces of infected subjects at a rate of $10^{11}$ virus particles per gram.

**Dose – response data**

As little as one infectious particle of rotavirus can trigger disease in animal (Graham 1987) or human models (Ward 1986). A $\beta$-Poisson dose-response model based on experimentation on humans has been described (Teunis 2000).

**Target populations**

Humans of all ages are susceptible to rotavirus infection but rotavirus most commonly affects infants and young children.

**Diagnosis**

Diagnosis may be made by rapid antigen detection of rotavirus in stool specimens. Strains may be further characterized by enzyme immunoassay (several commercial kits are available for group A rotavirus) or reverse transcriptase polymerase chain reaction (RT-PCR), but such testing is not commonly done.

**Treatment**

Treatment is non-specific and consists of oral rehydration therapy to prevent dehydration. About one in 40 children with rotavirus gastroenteritis requires hospitalization for intravenous fluids.

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In 1998, a live, attenuated rotavirus vaccine (Rotashi eld, Wyeth Laboratories, Marietta, PA) was licensed in the United States and recommended for routine immunization of U.S. infants. However, 9 months later, the use of Rotashield was suspended because some children who received the vaccine developed bowel obstruction. Efforts are ongoing to develop other rotavirus vaccines, and within the next few years, a rotavirus vaccine may be available (Parashar 2003).

Geographical distribution

Worldwide.

Epidemiology

Human rotaviruses (HRVs) are the most common cause of severe diarrhoea among children, worldwide (Cukor 1984). It is estimated that rotavirus is responsible for the deaths of approximately 500,000 children each year, over 80 percent of them in developing countries (Parashar 2003). In developed countries deaths are relatively scarce, but rotavirus gastroenteritis is the most frequent reason for admission of young children to hospital (Glass 1997). Group A rotavirus is endemic worldwide. It is the leading cause of severe diarrhoea among infants and children. In temperate areas, it occurs primarily in the winter, but in tropical areas it occurs throughout the year. Group B rotavirus is responsible predominantly for adult diarrhoeal disease, and therefore designated as adult diarrhoea rotavirus or ADVR. Group C rotavirus has been associated with rare and sporadic cases of diarrhoea in children in many countries.

Transmission routes

The primary mode of transmission is the faecal-oral route. Person-to-person transmission and the inhalation of airborne HRVs or aerosols containing the viruses would appear to play a much more important role than the ingestion of contaminated food or water (Craun 1991) (WHO 2004).

Person-to-person spread through contaminated hands is probably the most important means by which rotaviruses are transmitted in very small communities such as pediatric and geriatric wards, day care centers and family homes.

Waterborne outbreaks

Foodborne and particularly waterborne spread are probably a significant route of transmission in developing countries, but in developed countries reports are rare. Several waterborne outbreaks of rotavirus have been reported: in a school in Rio de Janeiro in May, 1980 (Sutmoller 1982), in 1981 in Colorado (Hopkins 1984), and two very large outbreaks in China affecting more than 12,000 adults (Hung 1984). More recently, a large infantile gastroenteritis outbreak occurred in Albania; it was primarily caused by rotavirus (Villena 2003) (Divizia 2004). All of the outbreaks of rotavirus have been associated with a direct faecal contamination of the water supply or improper treatment.

Ecology

Regrowth in distribution systems/biofilms

As all viruses, HRVs are environmentally inert and only replicate in their hosts (human). The presence and persistence of HRVs within biofilms have not been investigated.

Occurrence

The distribution in the environment reflects the distribution of human (both symptomatic and asymptomatic) faecal contamination.
Rotaviruses have been found in treated and tap water (Keswick 1984) (Gratacap-Cavallier 2000) (van Zyl 2004), in river water in Switzerland (Gilgen 1997) and in Spain (Abad 1998). No studies reported their presence in groundwater.
They are present in large amounts in wastewater and sewage (Hejkal 1984) (Dubois 1997) (Smith 1982) (Pusch 2005).
Levels of contamination are not documented.
Further research is needed to determine the public health significance of their occurrence in water.

Survival

Not extensively documented.
Rotaviruses can survive for weeks in potable and recreational waters and for at least 4 hours on human hands (Ansari 1991).
Temperature is an important factor, with low temperatures favoring viral survival in natural waters. Raphael et al. (Raphael 1985) found no significant drop in rotavirus titer after 64 days at 4°C in raw water, treated tap water or filtered water. However, a 99% drop in titer was observed after 10 days at 20°C.
The virus is stable to storage in ambient tropical temperatures for more than 2 months (Fischer 2002).

Reservoirs

Humans are the only reservoir for HRVs.
However, animal rotaviruses have been detected in drinking water; the role of water in the spread of animal strains to human populations and the emergence of reassortant strains have thus been suggested and require further investigation (Gratacap-Cavallier 2000).

Inactivation/removal

Published data on removal and inactivation are summarized in annex 1. Rotaviruses are inactivated below pH 3 and above pH 10. They are stable to freeze-thawing, sonication, incubation at 25°C overnight or at 37°C for 1 h and to treatment with acid, ether and chloroform (Estes 1979).
Rotaviruses are relatively resistant to commonly used hard-surface disinfectants and hygienic hand-wash agents. (Ansari 1991).
Rotaviruses have been reported to be more resistant to chloramines and ultraviolet light disinfection than enteroviruses and more resistant to inactivation by preformed chloramines and ozone than poliovirus type 1 (Crabtree 1996) (Abad 1994).

Surrogates

Due to a higher resistance of the viruses to disinfection, E coli (or, alternatively, thermotolerant coliforms) is not a reliable index of the presence/absence of HRVs in drinking-water supplies (WHO 2004).

Environmental detection

Rotaviruses known as fastidious viruses do not grow in routine cell culture.
The standard methods for the diagnosis of specific infectious rotaviruses include electron microscopy (Brandt 1983), immunological methods such as latex agglutination or enzyme immunosorbent assays (Doern 1986) but these approaches are laborious and not sensitive enough to be applied to water samples.
The basic method for concentration of rotavirus form large volume water samples includes adsorption and elution from positively charged microporous filters (Sobsey 1980) with some modifications (Mehnert 1993) (Gilgen 1997) (Guttman-Bass 1983).
Immunofluorescence assays have been successfully developed to detect rotavirus in sewage (Agbalika 1985) (Smith 1982) (Guttman-Bass 1987) (Mehnert 1993).
Water samples are mostly analyzed by RT-PCR (Jothikumar N 1995) (Tsai 1994) (Gratacap-Cavallier 2000) (Kittigul 2005) (van Zyl 2004), often combined with the detection of other viruses: detection of
hepatitis A virus and rotavirus in spring water samples (Brassard 2005), detection of enterovirus, rotavirus, hepatitis A virus, and small round structured viruses in water samples (Gilgen 1997). RT-PCR-based detection is highly sensitive and rapid (a few hours), but it cannot differentiate between infectious and non-infectious viruses.

To determine the infectivity, some approaches have been proposed combining cell culture, immunofluorescence and flow cytometry. (Abad 1998) (Bosch 2004) or cell culture and ELISA (Sellwood 1995). Though they seem promising, these approaches are still not ideal as they are time consuming (8-10 days), expensive and not readily applicable to routine diagnostic work.

Although Kurokawa et al. (Kurokawa 2004) used real-time PCR for rapid detection of three viruses (Rotavirus, Adenovirus and Norovirus) in faecal samples; no rapid quantitative method has yet been developed and validated on environmental water samples. Currently all the available molecular methods are at the experimental stage for research purposes and validated protocols do not yet exist.

Virus research needs

Much remains unknown about waterborne viruses, namely in terms of ecology and epidemiology. For lack of appropriate diagnosis technology, enteric viruses have not been frequently identified as the aetiologic agents of waterborne disease outbreaks. There is a lack of information on the environmental survival and transport, the occurrence and impact on health as well as the efficiency of water treatment processes.

Research themes of interest to water utilities include:

**Analytical methods**

A standardized approach to virus detection in water is essential for understanding the “true” environmental prevalence of enteric viruses.

First there is an urgent need to develop rapid and reliable analytical methods to detect waterborne viruses in water.

Quantitative cell culture techniques must be developed for assaying the infectivity of caliciviruses, rotaviruses and adenoviruses types 40 and 41. These methods will serve as reference for infectivity for these viruses.

Rapid, simple, quantitative methods (TaqMan RT-PCR) to quantify viral contamination levels in water (treated and untreated) need to be developed for each type of virus. These molecular methods must eventually be evaluated and validated in order to be used as alternatives to the reference culture method, if any.

RT-PCR based detection methods are useful for rapid screening of water to provide evidence of virus occurrence. But they give no information on the infectivity of the virus. Positive results would then have to be followed up with other tests, such as the integrated cell culture-PCR assay to determine the health significance of the findings.

Further research using new molecular methods is necessary in order to acquire water-related data, to compare performance with existing cell culture methods and to correlate any positive results with human health risks by carrying out epidemiological studies.

**Occurrence**

Data on the presence and distribution of waterborne viruses in water are limited.

Little information is available on the types and levels of viruses found in groundwater.

There is a lack of quantitative data on virus occurrence in surface water, groundwater and treated water.

Further research must be carried out to determine the public health significance of the occurrence of waterborne viruses in water.
Epidemiology

The role of waterborne transmission needs to be more clearly defined and documented, particularly for adenoviruses, enteroviruses and rotaviruses. Infective dose data and susceptibility of the human population remain unknown, particularly for adenoviruses, HAV and HEV. There are few data available on the occurrence of viral waterborne outbreaks, particularly in Europe. Further epidemiological studies using molecular techniques for viruses detection should be carried out.

Ecology

Reservoirs:
Investigation must be done to determine whether animal to human transmission is possible for caliciviruses, rotaviruses and HEV.

Environmental survival and transport:
There is little information on the environmental persistence of viruses. The potential incidence of environmental factors such as humidity, temperature, seasonal variation and other geographic factors should be further evaluated.
The possible role of biofilm in the survival of viruses in drinking water systems by providing protection from disinfection should also be investigated.
The transport and fate of viruses in groundwater systems should be further investigated.

Treatment

The effectiveness of water treatment processes requires validation. Studies on the performance of water treatment processes to remove viruses from drinking water should be carried out in full-scale conventional water treatment plants and not only in pilot tests.
How viruses go through physical and chemical treatment barriers and enter finished drinking water supplies needs to be understood.
The effectiveness of water treatment and disinfection processes must be evaluated for virus aggregates.

Indicators

There is a need for new, relatively simple and easy to-use-indicators, to assess the presence of infectious viruses in water. Suitable indicator organisms based on model systems using similar non-pathogenic cultivable human or mammalian viruses may be the most promising alternative.
The presence of waterborne viruses may reliably be monitored by practical indicator systems consisting of appropriate combinations of suitable indicators.
Protozoa
Cryptosporidium

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Adapted from table 7.1 Waterborne pathogens and their significance in water supplies (WHO 2004).

Microbiology

General description

Cryptosporidium is now one of the most commonly identified intestinal pathogens throughout the world (WHO 2004). It was discovered to infect humans only in 1976, and waterborne transmission was confirmed for the first time in 1984. Cryptosporidium is an obligate, intracellular, coccidian parasite with a complex life cycle including sexual and asexual replication, which infect the gastrointestinal tract of animals and humans. Thick-walled spherical oocysts with a diameter of 4-6 µm are shed in faeces. The oocyst containing four sporozoites constitutes the environmentally resistant transmission stage of the parasite. The whole life cycle of Cryptosporidium can be completed within a single host.

Classification

Cryptosporidium is an apicomplexan protozoan parasite. All species of Cryptosporidium are classified taxonomically within the family Cryptosporidiidae, suborder Eimeriorina, order Eucoccidiorida, subclass Coccidiasina and class Coccidia. Eleven species are currently recognized within the genus Cryptosporidium. C. parvum has been segregated into genotype 1 (recently named Cryptosporidium hominis) (Morgan-Ryan 2002) which is restricted to humans and genotype 2 (C. parvum) which infects humans and various mammalian hosts. While C. parvum and C. hominis are the primary species known to infect humans, recent studies suggest that C. muris, C. meleagridis, C. felis, C. canis and a cervine genotype may also cause diarrhea in humans (Fayer 2004) (Carey 2004). The taxonomy of Cryptosporidium species is unclear and is still being developed using molecular techniques. New species and genotypes of Cryptosporidium are being identified for which the infectivity for humans is not clear. A user-friendly database called CryptoDB representing a collaborative effort to locate all genome data for Cryptosporidium parvum and hominis is accessible through a free searchable website (http://CryptoDB.org).

Human health effects

Clinical features

Cryptosporidiosis is the illness caused by exposure to Cryptosporidium. The average incubation period varies widely but is usually about 7 days. In normally healthy individuals, cryptosporidiosis is usually characterized by an acute self-limiting diarrhoeal illness, commonly lasting one to two weeks, from which the patient recovers fully. Other symptoms are nausea, abdominal cramps and vomiting and mild fever. In patients who are immuno-suppressed, including those with AIDS, the disease is much more severe and persistent. Illness can last for several months or until deaths. Non-gastrointestinal illness, such as cholecystitis, hepatitis and respiratory disease may also occur in such individuals. The peak intensity of oocysts shedding, with an average concentration of 10^7/g, coincides with the peak intensity of clinical symptoms.
**Dose – response data**

The infectious dose is not clearly known. Experiments in human volunteers indicated a median infective dose of 132 oocysts and a minimum infective dose of less than 30 (DuPont 1995). Studies using animal models showed that as few as 1 to 10 oocysts may initiate an infection (Leclerc 2004). A mathematical model based on data from the Milwaukee outbreak suggested that some individuals developed cryptosporidiosis following ingestion of only one oocyst (Haas and Rose 1994). Exponential dose-response models based on experimentation on humans have been described (Haas 1996, Teunis 1999). More recently, the variability in infectivity between different *Cryptosporidium* isolates and between hosts was investigated (Teunis 2002a, Teunis 2002b).

**Target populations**

Anyone can have cryptosporidiosis but immuno-compromised persons (e.g. AIDS patients) as well as young children and the elderly are particularly at risk.

**Diagnosis**

Diagnosis is usually based on microscopic demonstration of oocysts in faeces. Oocysts are usually stained by acid-fast staining procedure. Other methods include direct or indirect immuno-fluorescence microscopy and ELISA using commercially available kits (Quintero-Betancourt 2002). None of these techniques are species-specific nor can they determine whether oocysts are infectious or not. Only molecular techniques, including PCR, can help determine the possible source and risk to human health.

**Treatment**

As yet there is no effective specific medical treatment.

**Geographical distribution**

Worldwide.

**Epidemiology**

**Transmission routes**

*Cryptosporidium* is transmitted by the faecal-oral route. The major route of infection is person-to-person contact. Other sources of infection include the consumption of contaminated food and water and direct contact with infected farm animals and possibly pets.

**Waterborne outbreaks**

Numerous reports of outbreaks of cryptosporidiosis related to drinking water have been described mainly in North America, the UK, and Japan (Fayer 2004) (Hlavsa 2005). The first occurred in 1984 in Texas, with sewage-contaminated groundwater as the source of the infection (D'Antonio 1985). The largest documented waterborne outbreak occurred in Milwaukee, USA, in 1993 when more than 400,000 people were infected by the drinking-water supply (MacKenzie 1995). The total cost of illness associated with this outbreak has been estimated at 96.2 million US dollars (WHO 2004). In European countries other than the United Kingdom, few *Cryptosporidium* outbreaks have been reported. The first outbreak occurred in 1995 in The Netherlands, but the source of transmission was not identified (Van Asperen 1996). A second outbreak due to the contamination of a water tank was reported in Italy in 1997 (Pozio 1997). In France, *Cryptosporidium* was the likely causative agent of two waterborne outbreaks that occurred in the south of the country in 1998 (Guyonnet 2000) and in eastern France in 2001 (Dalle 2003). Most outbreaks were caused by *C. parvum* genotype 1 (recently called *C. hominis*), even in areas where the majority of sporadic cases were caused by *C. parvum* genotype 2. Waterborne outbreaks of cryptosporidiosis have been attributed to contaminated drinking water, from both surface-water and ground-water sources, and to recreational water, including swimming pools. Outbreaks caused by drinking-water have been attributed to contamination of the source water by heavy rainfall or melting snow, to sewage contamination of wells, to inadequate treatment or treatment
deficiencies and to combinations of these factors. However, many outbreaks have been associated with potable water even when processing systems had been operated in accordance with conventional standards of water treatment, and while current microbiological standards were met. Swimming-pool associated outbreaks have also been widely reported (WHO 2002). In addition, foodborne transmission and traveler's diarrhea related to cryptosporidiosis have been documented.

Ecology

Regrowth in distribution systems

Oocysts are environmentally inert and cannot multiply outside their hosts. The role of biofilm in the persistence of oocysts remains insufficiently documented, although oocysts of Cryptosporidium have been shown to be able to attach to and persist in biofilms (Piriou 2003).

Occurrence

Cryptosporidium oocysts are ubiquitous in surface waters worldwide. Reported concentrations are generally in the range 0.01-100 per liter (WHO 2002). The sources of environmental contamination can be both human sewage and animals. Groundwaters that mix with surface water or other sources of contamination (e.g., surface run-off) may contain low levels of Cryptosporidium (<1/liter). They also have been detected in treated water samples in the range 0.001-0.3/liter. Surveys conducted in various regions of the United States demonstrated the presence of Cryptosporidium oocysts in 67-100% of wastewaters, 24-100% of surface waters, and 17-26.8% of drinking waters (Rose 1991). Although more and more studies have collected data on Cryptosporidium species occurrence and infectivity (Xiao 2001)(Le Chevallier 2003) over the past few years, information is still lacking about the health significance of the presence of Cryptosporidium oocysts in water, in particular whether human pathogenic species are present and whether the oocysts are viable and infectious.

Reservoirs

Many animal reservoirs, including farm livestock, pets and wildlife have been identified (Graczyk 1997). They spread oocysts to humans and the water environment, particularly through agricultural and human effluents.

Survival

The robust nature of C. parvum oocysts enables them to survive for months in surface water. (Carey 2004) summarized the effects of environmental selected factors on the viability and infectivity of C. parvum oocysts; the main data are reported below.

Oocysts are able to survive for several months in water kept at 4°C. Temperature has a large effect on oocyst survival. Warmer temperatures can accelerate oocyst degradation, although oocysts are known to remain infective up to 12 weeks when stored in water at 25°C. At extreme temperatures, oocyst viability and infectivity are adversely affected. Recently one study (King 2005) reported that oocysts incubated at 4 degrees C and 15 degrees C remained infective over the 12-week holding period, whereas a 4 log(10) reduction in infectivity was observed for both 20 and 25 degree C incubation treatments at 12 and 8 weeks, respectively, for all water types examined. The authors also showed that temperature inactivation at higher temperatures was a function of increased oocyst metabolic activity, as measured by oocyst ATP content.

Rapid freezing inactivates oocysts as compared with slow freezing, which is typically found in the natural environment.

C. parvum oocysts are susceptible to inactivation by dessication.

Oocysts were also able to survive for extended periods in seawater during 8 to 12 weeks at varying temperatures and salinities, similar to those in which Eastern oysters survive. When in contact with faeces, oocysts were considered to develop an enhanced cell impermeability which might increase the robustness of the oocysts when exposed to environmental pressures (Robertson 1992).

Inactivation/removal

Published data on removal and inactivation are summarized in annex 1.
Chemical treatment:
*Cryptosporidium parvum* oocysts are resistant to inactivation by common disinfectants such as free chlorine and monochloramine in the concentrations used in water treatment. Ozone more effectively inactivates *Cryptosporidium* oocysts: Effective oocyst inactivation was achieved (>2 log_{10} units) 2 mg of ozone/liter (for 10 min) at 20°C (Keegan 2003). But the effectiveness of ozone decreases at lower temperatures. Sequential disinfection of oocysts with different chemical agents can result in greater inactivation levels (Carey 2004).

Physical removal or inactivation:
Due to their small size, *Cryptosporidium* oocysts pass through most conventional filters, rendering physical removal difficult. Reverse-osmosis filters and 1µm absolute filters have been shown to remove oocysts. Recently, one study demonstrated that membrane filtration of the public water supply was effective in reducing the risk for sporadic human *Cryptosporidium* infection in a population in England between 1996-2002 (Goh 2004).

Freezing and thawing at more than 72°C for 1mn or 45°C for 10-20mn effectively inactivate oocyst (Carey 2004).

Investigations based on infectivity assays have shown that UV light irradiation inactivates oocysts. Effective oocyst inactivation – as measured by cell culture -TaqMan PCR - was achieved (>2 log_{10} units) with low-pressure UV lamps (20 mJ/cm²) (Keegan 2003). *G. muris* cysts and *C. parvum* oocysts exposed to medium-pressure UV doses of 60 mJ/cm² or higher did not exhibit resistance to and/or reactivation following treatment (Belosevic 2001). It was recently demonstrated that *C. hominis* oocysts (the predominant genotype in human cryptosporidiosis infections) displayed similar levels of infectivity and had the same sensitivity to UV light as *C. parvum* (Johnson 2005).

Surrogates
Owing to the exceptional resistance of the oocysts to disinfectants, *E coli* (or, alternatively, thermotolerant coliforms) cannot be relied upon as an index for the presence/absence of *Cryptosporidium* oocysts in drinking-water supplies.

Continuous turbidity or particle count monitoring can give an early warning of particle breakthrough and alert operators to an increased risk of the presence of oocysts in the treated water.

Legislation:
The UK legislation requires that oocysts must not be present in drinking water at a concentration higher than 1 per 10 litres.

Environmental detection
Concentration, purification, and detection are the three key steps in all methods that have been approved for routine monitoring of waterborne oocysts.

Methods for total enumeration (dead and live oocysts):

- Approved methods USEPA 1622 (*Cryptosporidium*) and USEPA 1623 (*Cryptosporidium* and *Giardia*): Both methods include filtration (Pall Gelman Envirotech™ HV filter or Idexx/Genera Filta-Max™ filter), Immuno-magnetic Separation (IMS) and Immuno-fluorescence Assay (IFA). Confirmation using differential interference contrast microscopy (DIC) and nucleic acid stain (DAPI) can be added (USEPA 1999a) (USEPA 1999b).
- Drinking water inspectorate DWI approved method (UK): This method is similar to the USEPA methods, using Idexx/Genera Filta-Max™ filter for concentration (DWI 1998).
- French standardization association (AFNOR) approved method NFT90-455: this method is similar to the USEPA methods.
In a study designed to evaluate the recovery efficiencies of *C. parvum* oocysts from various filtration cartridges (Lee 2004), the Filta-Max filter proved to perform the best.

- Flow cytometry has been used for selective separation of *Cryptosporidium* oocysts and *Giardia* cysts from samples as an alternative to IMS purification (Medema 1998). The development of a protocol using flow cytometry to quantitate *Giardia* cysts and *Cryptosporidium* oocysts in water samples was recently described (Gratacap-Cavallier 2000) (Hsu 2005).

- Alternative methods to concentrate water samples have been described, including cross-flow filtration, continuous-flow centrifugation (Higgins 2003) and vortex-flow filtration. Graczyk et al. (Graczyk 1997) investigated the use of the cellulose-acetate membrane filter dissolution method to concentrate and recover viable and infective oocysts.

Conventional approaches however cannot discriminate among the different species of *Cryptosporidium*. Only molecular biology techniques are able to specifically detect human pathogenic species and genotypes.

- Since 1991, many PCR-based detection methods, including IMS-PCR have been described (Laxer 1991) (Johnson 1995) (Hallier-Soulier and Guillot 2000) (Rochelle 1997). Recently Jiang et al. (Jiang 2005) developed and evaluated different direct extractions of *Cryptosporidium* DNA from water concentrates. An immunocapture-based polymerase chain reaction (PCR) assay for simultaneous detection of *Cryptosporidium parvum* oocysts and *Giardia intestinalis* cysts in surface water has been reported (Rimhanen-Finne 2002).

- Several recent studies described protocols based on PCR-RFLP to detect and discriminate human pathogenic species, *Cryptosporidium parvum* and *C. hominis* in water samples (Ochiai 2005) (Xiao 2004).

- A single analytical process using ultrafiltration combined with high-density oligonucleotide array has been developed to simultaneously detect waterborne pathogens including bacteria, viruses and parasites (*Cryptosporidium* and *Giardia*) (Guillot 2003) (see Enterovirus section).

- Several methods using real-time PCR to quantify *C parvum* oocysts in water samples have been published (Higgins 2001) (Fontaine and Guillot 2002) (Fontaine and Guillot 2003). A multiplex real-time PCR (qPCR) assay for simultaneous quantification of *G. lamblia* and *C. parvum* in water and sewage has been reported (Guy 2003).

Currently all the available molecular methods are at the experimental stage for research purposes and validated protocols do not yet exist.

**Methods for determining oocyst viability:**

- In vitro excystation: the method is not very sensitive and is difficult to apply on a routine basis.

- Incorporation of vital dyes: DAPI/PI procedure or SYTO9 staining. To date interpretation remains difficult and results are not reproducible (WHO 2002).

Several molecular approaches have been described:

- FISH (Fluorescent in situ hybridization) technique (Vesey 1998) (Smith 2004) (Lemos 2005): This technique does not appear sensitive enough for application to environmental water concentrates that contain autofluorescent algae and mineral particles.

- The combination of in vitro excystation with PCR amplification (Wagner-Wiening 1995). In vitro excystation /PCR tests show the same limitations as excystation itself.

- RT-PCR: The detection of mRNA transcripts from an induced heat shock protein 70 (*hsp70*) gene has been shown to be sensitive enough to detect small numbers of viable oocysts in environmental water concentrates (Stinear 1996) (Kaunzer 1998) (Hallier-Soulier and Guillot 2003). One study described the use of nucleic acid sequence-based amplification (NASBA) with subsequent electrochemiluminescent detection for the specific and sensitive detection of viable oocysts of *Cryptosporidium parvum* in environmental samples (Baeumner 2001). As the RT-PCR -based
methods above, this approach targets *C. parvum* heat shock protein hsp70 mRNA. Jenkins *et al.* (Jenkins 2000) described an RT-PCR assay directed at mRNA encoding the enzyme amyloglucosidase (CPAG) to differentiate viable from non-viable *C. parvum* oocysts.

As for PCR assays available, all these molecular methods which determine viability are at the experimental stage for research purposes and validated protocols do not yet exist.

**Methods for determining oocyst infectivity:**

- Animal infectivity: this assay has long been considered the method of choice for determining the infectivity of oocysts, but is impractical for use on a routine basis. It requires extensive space and equipment.

- Cell culture based methods: Foci Detection Method (FDM) combines cell culture and immunofluorescence assay (Slifko 1997). This method is time-consuming and tedious.

- An in vitro infectivity assay using cell culture combined with RT-PCR to detect the infectious *C. parvum* oocysts has been described (Rochelle 1997).

- A new approach called cell culture quantitative sequence detection (CC-QSD) which combines quantitative TaqMan PCR method with cell culture method has been recently developed (Di Giovanni 2005) (Keegan 2003). To date these promising molecular approaches are at the experimental stage and need optimization and standardization before routine use on environmental water samples. At the present time, there is an absence of adequate methods to detect the presence of viable/infectious oocysts of *Cryptosporidium* human pathogenic species.

Several typing methods are available to discriminate human and animal *Cryptosporidium* species and genotypes such as PCR-RFLP and RAPD. Straub *et al.* (Straub 2002) developed an oligonucleotide microarray to differentiate between closely related *C. parvum* isolates and *Cryptosporidium* species.

**Research needs**

There are obvious needs to be addressed from the clinical standpoint – e.g. therapeutic drugs, vaccine development, infective dose - as well as in epidemiology concerning the actual incidence of cryptosporidiosis, but we will focus on needs with respect to waterborne oocysts. There are a number of areas relating to *Cryptosporidium* that require further research including aspects of monitoring, analysis, water treatment and disinfection efficiency, environmental survival and distribution, transport and fate of oocysts in soils and groundwater systems.

**Analytical method**

Clearly the most critical requirement is for standardized, improved oocyst recovery and detection methodology. The numerous published PCR-based methods identify the lack of an optimized standard method for clinical, environmental, and public health investigations of cryptosporidiosis. The ability to distinguish viable and/or infectious oocysts would greatly facilitate interpretation of oocyst presence in waters.

There is a need to develop reliable, routine tests which specifically detect human pathogenic species (*C. parvum* and *C. hominis*) and genotypes and determine oocyst viability. RT-PCR constitutes a promising approach but requires optimization, simplification and validation.

Methods for rapid and reliable quantification of *Cryptosporidium* species are also required.

**Taxonomy**

Further investigation including international collaborations are needed to resolve the current difficulties concerning taxonomy, genotyping, phylogenetic analysis and characterization of *Cryptosporidium*. In fact research on *Cryptosporidium* has recently resulted in a considerable progress due to available molecular methods for studying the diversity within the genus. Numerous genotyping studies yielded an enormous amount of data concerning this parasite. However there is an urgent need to review and update the sequence data for *Cryptosporidium* available in public databases. Considerable work must be done for the resolution of the taxonomy and the number of valid species in the genus *Cryptosporidium*. 

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Ecology

The potential incidence of environmental factors (humidity, temperature, seasonal variation, other geographic factors) should be further determined. The role of biofilm in the survival of pathogenic Cryptosporidium remains insufficiently investigated. The impact of predation is another issue that has not been addressed in survivability experiments. Little information is available on the significance to the environmental ecology of the state in which oocysts occur in water, i.e., suspended or attached to particles. Concerning groundwater, transport and fate of Cryptosporidium in groundwater systems should be further investigated.

Occurrence and distribution

Little is known about the species distribution of Cryptosporidium parasites in environmental samples. Studies using molecular typing tools to identify Cryptosporidium isolates from human, animal or environmental samples including water and sludge need to be carried out. The objective will be to determine which Cryptosporidium species or genotypes are present in Europe, the USA and other parts of the world, how they are spatially distributed, how they circulate in ecosystems, and whether geographic origin is predictive of genotypes. In addition, the health significance of the presence of oocysts in water is not documented for lack of adequate methods for viability determination. It would be critical to determine if Cryptosporidium oocysts detected in particular in groundwater and in treated water samples are viable and potentially infectious and whether human pathogenic species are present. National databases should be established to provide comprehensive information on the occurrence of oocysts in both source and treated water.

Treatment

Additional research into improving water treatment and sewage treatment practices is needed, particularly in testing the efficiency of ozone in oocyst inactivation using Cryptosporidium human pathogenic species. Studies on the performance of treatment processes to remove oocysts from drinking water should be carried out in full-scale conventional water treatment plants and not only in pilot tests. In addition, further evaluation and development of the use of bacterial spores or other surrogates to assess treatment performance are necessary.
Giardia

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Adapted from table 7.1 Waterborne pathogens and their significance in water supplies (WHO 2004).

Microbiology

**General description**

*Giardia* is an ubiquitous parasite affecting humans and a wide range of domestic and wild mammals. *Giardia duodenalis* (syn. *Giardia intestinalis*; *Giardia lamblia*) is the most common intestinal parasite of humans worldwide (Marshall 1997).

*Giardia* has a relatively simple life cycle consisting of a flagellate trophozoite that multiplies in the gastrointestinal tract and an infective thick-walled cyst that is shed intermittently but in large numbers in faeces. The cyst is the infective form, which is responsible for the transmission of the parasite and the infection of humans and animals.

*Giardia duodenalis* cysts are elliptical, 8-12µm long and 7-10µm wide. The cyst wall is 0.3-0.5 µm thick and has a fibrillous structure. Two to four nuclei are found in each cyst, together with axonemes of the flagella of the trophozoite (WHO 2004).

**Classification**

The taxonomy of *Giardia* at the species level remains unclear because only limited morphological differences are present between different species. In addition the high degree of genetic heterogeneity found in human and animal isolates makes speciation difficult. It is still unclear how and whether this heterogeneity is related to host specificity and pathogenicity of *Giardia* (WHO 2002).

Six species in the genus are currently considered valid: *Giardia duodenalis* (synonym *G. lamblia* or *G. intestinalis*) in a wide range of mammals including humans, *Giardia agilis* in amphibians, *Giardia muris* in rodents and reptiles, *Giardia ardeae* in the grey blue heron and *Giardia psittaci* in birds, and *Giardia microti* in muskrats and voles (Sulaiman 2004).

Two major groups of *G. duodenalis* have been recognized in humans, assemblages A and B (Thompson 2000).

**Human health effects**

*Giardia* has been known as a human parasite for 200 years. Giardiasis is considered to be a re-emerging infection because of its association with outbreaks of diarrhoea in child-care centres (Thompson 2000).

**Clinical features**

The symptoms of giardiasis are acute diarrhoea, abdominal cramps, bloating and excessive flatulence. In most cases, the infection is acute and self-limiting, with a duration of 2-4 weeks. The incubation period can be anything from 1 to 75 days, but on average it is 7-10 days.

The severity of the illness can vary considerably and only about a quarter of infected people show symptoms of the illness. Malabsorption of food can lead to considerable loss of weight and in children it can be a cause of failure to thrive.

Excretion of cysts varies between $10^6$ and $10^8$ per gram of stools but a significant proportion of the stool samples do not contain detectable levels of *Giardia*.
**Dose – response data**

The infectious dose has been estimated to be as low as 10 cysts for *Giardia* (Adam 2001). Exponential dose-response models based on experimentation on humans have been described (Rose 1991, Regli 1991).

**Target populations**

Giardiasis affects all age groups, although the highest incidence is in children at the age of 1-4 years, particularly in developing countries. It is also a common cause of traveller's diarrhoea.

**Diagnosis**

Diagnosis is usually made by light microscopic examination of a faeces sample. Procedures using the enzyme immunoassay (EIA) or a direct fluorescent-antibody method have been developed (Marshall 1997). None of these techniques are species-specific nor can they determine whether cysts are infectious or not.

**Treatment**

Giardiasis is readily treated by a number of different drugs (Minenoa 2003), but if left untreated it can persist for a number of years (Hunter 1997). The two drugs that seem to be most effective are metronidazole and tinidazole.

**Geographical distribution**

Worldwide

**Epidemiology**

**Transmission routes**

Transmission is through the faecal-oral route. Consumption of contaminated drinking water or food is source of infection. However, there are many other possible ways of becoming infected with *Giardia* such as person-person contact, animal-person contact, contaminated swimming pools and other recreational waters (rivers and lakes), or foreign travel - giardiasis is also known as "travellers diarrhoea". Several authors suggest a zoonotic potential for giardiasis (van Keulen 2002) (Thompson 2000) but the infectivity of animal strains for humans has not yet been demonstrated.

**Waterborne outbreaks**

Waterborne outbreaks of giardiasis have been reported for some 30 years. They are a major public health problem in many industrialized nations, including the United Kingdom, Sweden, Australia, New Zealand, Canada and the United-States. The first outbreak was documented in 1965 at Aspen, CO (Craun 1986). The first UK waterborne outbreak occurred in the Bristol area in 1985 affecting 108 persons (Jephcott 1986). Similar to cryptosporidiosis, waterborne outbreaks of giardiasis have been attributed to contaminated drinking water, from both surface-water and ground-water sources, and to recreational water, including swimming pools.

Outbreaks caused by drinking-water have been attributed to surface water treatment failure, contamination of the source water, and leakage into the distribution system or to combinations of these factors. However many outbreaks have been associated with potable water even when processing systems had been operated in accordance with conventional standards of water treatment, and while current microbiological standards were met (WHO 2002). Swimming -pool associated outbreaks have also been widely reported (Hunter 1997).

In addition, foodborne transmission and traveller's diarrhoea related to giardiasis have been documented.
Ecology

Regrowth in distribution systems/biofilms

Cysts are environmentally inert and cannot multiply outside their hosts. Cysts of Giardia are thought to attach to and persist in biofilms. As for Cryptosporidium, the role of biofilm in the persistence of Giardia remains poorly investigated.

Occurrence

As Cryptosporidium oocysts, Giardia cysts are ubiquitous in surface waters worldwide. Reported concentrations are generally in the range 0.01-100 per liter (WHO 2002). The sources of environmental contamination can be both human sewage and animals. They are widely found in lakes and rivers especially where there is wildlife, which uses these water sources. Concentrations as high as 88,000 cysts per liter have been found in raw sewage and as high as 240 per liter in surface waters (Wallis 1996). Giardia cysts have been detected in 81% of raw water samples and 17% of filtered water samples in the United States (Marshall 1997). Groundwaters that mix with surface water or other sources of contamination (e.g., surface runoff) may contain low levels of Giardia (<1/liter).

Giardia cysts are commonly found in untreated and in treated sewage. An investigation study of four wastewater treatment plants in Italy revealed that Giardia cysts were ubiquitous, whereas Cryptosporidium oocysts were quite rare (Cacciò 2003).

It is not clear whether or not seasonality is a general feature of Giardia contamination: results available are contradictory (Cacciò 2003). However, most studies on Giardia contamination of water have been limited to estimating the prevalence and little information has been published on the contaminating species. Recently the distribution of Giardia duodenalis genotypes and subgenotypes in raw urban wastewater in Milwaukee was investigated (Sulaiman 2004).

The public health importance and contamination sources of Giardia cysts found in water are largely unclear.

Reservoirs

Giardia spp. affect a wide range of animals, including domestic pets, farm animals and wild mammals, but there is no unequivocal evidence that organisms from these sources have caused infections in humans.

Survival

Giardia cysts have been shown to survive in water for up to 2 months at temperatures as low as 8°C (Meyer 1980). G muris cysts remained viable for 1 to 3 months depending on the temperature and type of water sample (deRegnier 1989). Under natural conditions, the die-off rate for Giardia is higher and more temperature-dependent than for Cryptosporidium, varying between 0.015 log_{10} –units per day at 1°C and 0.28 log_{10} per day at 23°C (WHO 2002).

Inactivation/removal

Published data on removal and inactivation are summarized in annex 1.

Chemical treatment:

The effectiveness of standard chemical disinfectants such as chlorine and chloramine against Giardia cysts is limited. 99.99% reduction can be achieved with a CT of 180-530 mg.min/litre with chlorine, depending on the temperature and pH of the water. At CT values of 4.7-28 mg.min/litre chlorine dioxide reduces Giardia by 99% (WHO 2002).

Ozone is the most effective agent against Giardia cysts: at 20°C the CT for 99% inactivation of G. intestinalis cysts is 0.6 mg.min/liter (Finch 1993). But the effectiveness of ozone decreases at lower temperatures.

Physical removal or inactivation:

Cysts are particulate and are fairly readily removed by conventional processes used in drinking water treatment plants such as coagulation, settling, rapid filtration and slow sand filtration. A well-operated
treatment plant based on chemical coagulation, settling and filtration should achieve at least 99.9% cyst removal. Micro and ultra filtration can remove over 99.99% as long as the integrity of the system is maintained. Irradiation with UV light is currently the most promising form of disinfection or inactivation for Giardia. Work is in progress to identify the conditions in which UV radiation may be used with confidence. (Belosevic 2001) found that G. muris cysts and C. parvum oocysts exposed to medium-pressure UV doses of 60 mJ/cm² or higher did not exhibit resistance to and/or reactivation following treatment.

Surrogates

Due to the exceptional resistance of the cysts to disinfectants, E. coli (or, alternatively, thermotolerant coliforms) cannot be relied upon as an index for the presence/absence of Giardia cysts in drinking-water supplies.

Continuous turbidity or particle count monitoring can give an early warning of particle breakthrough and alert operators to an increased risk of the presence of oocysts in the treated water.

Environmental detection

Methods for the analysis of a water sample for Giardia cysts are broadly the same as for Cryptosporidium oocysts. Concentration, purification, and detection are the three key steps in all methods that have been approved for routine monitoring of waterborne parasites.

Methods for total enumeration (dead and live cysts):

- Approved method USEPA 1623 (Cryptosporidium and Giardia). This method includes filtration (Envirocheck cartridge), Immunomagnetic Separation (IMS) and Immunofluorescence Assay (IFA). Confirmation using differential interference contrast microscopy (DIC) and nucleic acid stain (DAPI) can be added (USEPA 1999).

- The French standardization association (AFNOR) approved method NF T 90-455: this method is similar to the USEPA 1623 method.

- Flow cytometry has been used for selective separation of Cryptosporidium oocysts and Giardia cysts from samples as an alternative to IMS purification (Medema 1998). Recently Hsu et al. (Hsu 2005) described a protocol using flow cytometry to quantify Giardia cysts and Cryptosporidium oocysts in water samples.

- Alternative methods to concentrate water samples have been described, including cross-flow filtration, continuous-flow centrifugation (Higgins 2003) and vortex-flow filtration. But conventional approaches cannot discriminate among the different species of Giardia and Cryptosporidium. Only molecular biology techniques are able to specifically detect human pathogenic species and genotypes.

- Since 1991, several PCR-based detection methods, including IMS-PCR, have been described (Mahbubani 1991) (Rochelle 1997) (Mahbubani 1998). A PCR- and gene probe-based detection (Mahbubani 1992) and a PCR-RFLP assay (Sulaiman 2004) have been described for differentiation of Giardia duodenalis from other Giardia spp. An IMS-PCR assay for simultaneous detection of Cryptosporidium parvum oocysts and Giardia intestinalis cysts in surface water has been reported (Rimhanen-Finne 2002).

- A single analytical process using ultrafiltration combined with high-density oligonucleotide array has been developed to simultaneously detect waterborne pathogens including bacteria, viruses and parasites (Cryptosporidium and Giardia) (Guillot 2003) (see Enterovirus section).

But PCR-based detection gives qualitative results only.
Recently a multiplex real-time PCR (qPCR) assay for simultaneous quantification of *G. lamblia* and *C. parvum* in water and sewage has been reported (Guy 2003).

All molecular methods currently available are at the experimental stage for research purposes and validated protocols do not yet exist.

**Methods for determining oocyst viability:**

- **In vitro excystation** (Hoff 1985): the method is not very sensitive and difficult to apply on a routine basis.

- Incorporation of vital dyes: according to Taghi-Kilani et al. (Taghi-Kilani 1996) SYTO-9 staining was correlated to animal infectivity. The determination of *Giardia* cyst viability in water samples has been reported by combination of immunofluorescence, fluorogenic dye staining (DAPI/PI) and differential interference contrast microscopy (Thiriat 1998). Another study described a viability assay using fluorogenic dyes, fluorescein diacetate (FDA) and labelled monoclonal antibodies (Jarmey-Swan 2000). But to date methods available cannot be used on a routine basis because microscopic interpretation remains difficult (WHO 2002).

- Electrorotation as a rapid method for determining the viability of *Giardia* has been reported (Dalton 2001).

These different approaches need optimization and standardization before routine use on environmental water samples.

**Molecular approaches based on RT-PCR have also been described:**

- **RT-PCR**: Methods based on detection of giardin mRNA (Mahbubani 1991) and of heat shock-induced mRNA (Abbaszadegan 1997) have been reported. Simultaneous detection of viable *C. parvum* oocysts and *Giardia* cysts from large-volume water samples with wound fiberglass cartridge filters and reverse transcription-PCR has also been described (Kaucner 1998).

At the present time, there is an absence of adequate reference methods to detect the presence of viable/infectious cysts of *Giardia* human pathogenic species.

Several typing methods are available to discriminate human and animal *Giardia* species and genotypes such as PCR-RFLP (Homan 1998) and RAPD (Paintlia 1999) (Pelayo 2003) and a sequence characterization of the triosephosphate isomerase (TPI) gene (Sulaiman 2004).

**Research needs**

*Giardia*-related issues are similar to those of *Cryptosporidium* and research has often focused on both parasites. This explains why, as for *Cryptosporidium*, there are a number of areas relating to *Giardia* that require further research including aspects of monitoring, analysis, water treatment and disinfection efficiency, environmental survival and distribution, the zoonotic potential transmission, transport and fate of cysts in soils and groundwater systems.

Clearly the most critical requirement is for standardized, improved cyst recovery and detection methodology. The ability to distinguish viable and/or infectious cysts would greatly facilitate interpretation of *Giardia* cyst presence in waters. There is a need to develop reliable; routine tests which specifically detect the human pathogenic species (*G. intestinalis*) and which determine the viability of the cysts. RT-PCR constitutes a
promising approach but requires optimization, simplification and validation. Molecular typing methods which can discriminate between the different strains of *G. intestinalis* from human or animal sources would be useful for epidemiological studies. Methods for rapid and reliable quantification of *Giardia* cysts species are also required.

**Taxonomy:**
Further investigation is needed to resolve the current difficulties concerning taxonomy, genotyping, and characterization of *Giardia*. Considerable work must be done for the resolution of the taxonomy and the number of valid species in the genus *Giardia*.

**Zoonotic transmission:**
Little is known about the relative infectivity and the intensity of the infection caused by the different strains of *G. intestinalis* of human or animal origin. The zoonotic transmission of giardiasis, although it is often addressed in the literature, remains to be demonstrated.

**Ecology:**
The potential incidence of environmental factors (humidity, temperature, seasonal variation, other geographic factors) should be further determined. The role of biofilm in the survival of pathogenic *Giardia* remains poorly investigated. The impact of predation is another issue that has not been addressed in survivability experiments. Little information is available on the significance to the environmental ecology of the state in which cysts occur in water, i.e. suspended or attached to particles. Concerning groundwater, transport and fate of *Giardia* in groundwater systems should be further investigated.

**Occurrence and distribution:**
Little is known about the species distribution of *Giardia* parasites in environmental samples. Studies using molecular typing tools to identify *Giardia* isolates from human, animal or environmental samples including water and sludge need to be carried out. In addition, the health significance of the presence of cysts in water is not documented for lack of adequate methods for viability determination.

**Treatment:**
Additional research to improve water treatment and sewage treatment practices is needed, particularly in testing the efficiency of ozone and UV in cyst inactivation. Studies on the performance of treatment processes to remove cysts from drinking water should be carried out in full-scale conventional water treatment plants and not only in pilot tests.
Cyclospora

<table>
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<td>Resistance to chlorine</td>
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<td>Relative infectivity</td>
<td>presumably high</td>
</tr>
<tr>
<td>Important animal source</td>
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</tr>
</tbody>
</table>

Adapted from table 7.1 Waterborne pathogens and their significance in water supplies (WHO 2004).

Microbiology

General description

Cyclospora is a newly described waterborne and foodborne pathogen. The first known human cases of illness caused by Cyclospora infection were reported in 1979 (Ashford 1979). But it is only since about 1990 that the role of the organism in human disease has been identified. Cyclospora is a single-cell, obligate, intracellular, coccidian protozoan parasite. It produces thick-walled spheroid oocysts of 8 to 10µm in diameter, and each oocyst has two ovoid sporocysts with two sporozoites each. The oocysts stain red with modified acid-fast stains and autofluoresce under UV light.

The life cycle has not yet been completely elucidated. One of the fundamental features of the biology of this organism is the fact that Cyclospora oocysts are non infectious in freshly excreted stool. The oocysts are thought to require from days to weeks outside the host, under favorable environmental conditions, to sporulate, and thus become infectious (Herwaldt 2000).

Classification

Cyclospora is a member of the subphylum Apicomplexa, class Sporozoasida, subclass Coccidiasina, family Eimeriidae. Molecular phylogenetic analysis suggests that the genus is closely related to the genus Eimeria (WHO 2002).

Of the 17 known Cyclospora species, C. cayetanensis is the only species of the genus thus far associated with human illness (Shields 2003).

In the past, it used to be called by such names as cyanobacterium-like, coccidia-like, and Cyclospora-like bodies (CLBs).

Human health effects

Clinical features

Cyclospora is a microscopic parasite that infects the small intestine of humans with a disease that is called cyclosporiasis. Sporozoites are released from the oocysts when ingested and penetrate epithelial cells in the small intestine of susceptible individuals. Clinical symptoms include water diarrhoea, abdominal cramping, weight loss, anorexia, myalgia and occasional vomiting and/or fever and last an average of 7 weeks. In AIDS patients, infections tend to last up to 4 months. Relapsing illness often occurs (Marshall 1997).

The incubation period is variable but averages 7 days with low or moderate numbers of unsporulated oocysts being excreted for up to 60 days.

Asymptomatic infection occurs and probably is most common in settings where Cyclospora is endemic.

Dose – response data

While unknown, the infectious dose is presumed to be low, as for Cryptosporidium.

Target populations

Anyone can suffer Cyclospora infection.
Diagnosis

Light microscopy, acid-fast staining, sporulation assay can be used. Molecular methods are increasingly used: a nested-PCR-RFLP protocol has been developed to specifically detect C. cayetanensis in stool (Orlandi 2000)

Treatment

Cyclospora infection is treatable with a combination of two sulfa-based antibiotics: trimethoprim and sulfamethoxazole.

Geographical distribution

In the past, Cyclospora infection was usually found in people who lived or travelled in developing countries. However, nowadays Cyclospora infection occurs worldwide but appears to be most common in tropical and subtropical areas (Herwaldt 2000). It is endemic to North, Central and South America, Southeast Asia, the Caribbean islands, India and parts of Eastern Europe (Sterling and Ortega 1999).

Epidemiology

A seasonal distribution of infection, coinciding with wet or warm months of the year has been suggested (Herwaldt 2000).

Transmission routes

It is transmitted by the faecal-oral route. The parasite can be transmitted by ingestion of contaminated water or food (fresh fruit and vegetables) with either sporulated oocysts or unsporulated oocysts that have time to sporulate before consumption. Direct person-to-person transmission by faecal exposure is unlikely, because excreted oocysts must sporulate to become infective.

Waterborne outbreaks

Several outbreaks of Cyclospora infection (or isolated cases) have been linked to waterborne transmission (Slifko 2000). The first reported cases were among the staff of a hospital in Chicago, USA, in 1990. The infections were associated with drinking tap water that had possibly been contaminated with stagnant water from a rooftop storage reservoir (Centers for Disease Control 1991). To date, the only case of Cyclospora transmission through water was reported in 1994 in Nepal, where drinking-water consisting of a mixture of river and municipal water was associated with infections of 12 to 14 soldiers. The United States and Canada experienced a number of large foodborne outbreaks in the 1990s (Herwaldt 2000). From 1990 to 2000, there were 11 foodborne outbreaks of cyclosporiasis in North America that affected at least 3600 people caused by produce imported from countries where Cyclospora is endemic (Mansfield 2004). In 1996, the largest ever reported outbreak of cyclosporiasis, affecting more than 1,400 persons in North America was associated with eating fresh raspberries from Guatemala. Most likely, faecally-polluted water used for spraying biocides on fruit was the indirect source (Leclerc 2004).

Ecology

Regrowth in distribution systems/biofilms

Does not multiply outside host. Its possible persistence in biofilm has not yet been documented.

Occurrence

Data is limited because of the lack of sensitive and specific environmental screening methods. Dowd et al. (Dowd 2003) reported the presence of Cyclospora cayetanensis in source water used for consumption around the city of Guatemala using molecular approach. In a waterborne outbreak in Nepal,
Cyclospora oocysts were found in the drinking water supply although chlorine residuals remained at acceptable levels (0.3-0.8 ppm) (Rabold 1994). C. cayetanensis oocysts were also detected in wastewater using microscopic and molecular techniques (Sturbaum 1998).

Reservoirs

The primary source of contamination is human faeces containing oocysts. Other sources may exist but have not yet been identified. Host range remains unknown including if animals infected are a source of infection for humans. Cyclospora-like oocysts have been recovered in several animals such as ducks, chickens, dogs, including non-human primates (Sterling and Ortega 1999). But their phylogenetic relationship to C. cayetanensis remained uncertain due to the limited availability of molecular techniques to differentiate and speciate these isolates.

Survival

Little is known. To maintain transmission, Cyclospora must survive in the environment long enough both to sporulate and to be ingested thereafter by a susceptible host. A moist environment is probably more conducive to survival than a dry one. Cyclospora sp. are likely to survive longer at lower temperatures when suspended in water. Preliminary studies have shown that oocysts subjected to -20°C for 24H and exposure to 60°C for one hour cannot be induced to sporulate. Oocyst storage at 4°C or 37°C for 14 days retards sporulation (Smith 1997).

Inactivation/removal

Little information is available regarding the ability of water- treatment processes to remove or inactivate Cyclospora sp. oocysts. Due to size (8-10µm in diameter), it is likely that physical removal will be similar to that achieved with Giardia and Cryptosporidium (WHO 2002). According to WHO (WHO 2002), the oocysts are resistant to disinfection and are not inactivated by chlorination practices generally applied in the production of drinking water (WHO 2002).

Surrogates

Owing to the resistance of the oocysts to disinfectants, E coli (or, alternatively, thermotolerant coliforms) cannot be relied upon as an index of the presence/absence of Cyclospora in drinking-water supplies.

Environmental detection

No reference methods have been specifically developed to detect Cyclospora in environmental samples. Cyclospora sp. oocysts being larger than C. parvum and smaller than G. intestinalis cysts, it is therefore assumed that concentration techniques developed for Cryptosporidium and Giardia will prove effective for sampling and recovering Cyclospora sp. oocysts from water concentrates (WHO 2002). Traditional microscopic identification currently used in stool samples has been applied to environmental samples. Microscopic techniques use size and morphology to identify Cyclospora oocysts and take advantage of the oocyst's autofluorescence under UV light. But they are difficult, labor-intensive and cannot distinguish Cyclospora species. Currently there is no commercially available polyclonal or monoclonal antibody against Cyclospora oocysts. Electrorotation as a rapid method for determining the viability of Cyclopora cayetanensis has been reported (Dalton 2001). There is a lack of laboratory methods for assessing the viability and infectivity of Cyclospora oocysts. There are neither in vitro culture methods nor in vivo amplification models.

Molecular techniques have first been developed for clinical samples and have recently been applied to environmental samples. But the current PCR-RFLP protocol (Orlandi 2000) used in stool samples cannot distinguish between C. cayetanensis and Cyclospora species from other primates. Orlandi et al. (Orlandi
developed primers for PCR detection to differentiate between C. cayetanensis, nonhuman primate species of *Cyclospora*, and *Eimeria* species. An extraction-free, filter-based protocol to prepare DNA templates for use in PCR to identify *C. cayetanensis* has been described (Orlandi 2000). Recently, one study reported the development of a PCR-RFLP assay for detection of *Cyclospora cayetanensis* in environmental waters without microscopic confirmation (Shields 2003). This new protocol should be of great use for environmental microbiologists and public health laboratories. To date, only one study reported the development of a quantitative real-time PCR assay for *Cyclospora cayetanensis* (Varma 2003). This promising protocol should be validated on environmental water samples.

Research needs

There is a need for a better understanding of the environmental biology and survival as well as the epidemiology of this newly described emerging pathogen.

**Environmental survival**

Little is known about the effects of various environmental conditions on the rate of sporulation and on the viability of unsporulated and sporulated oocysts. What is the impact of fluctuating environmental conditions on the rate of sporulation?

Little is known about conditions that may favor the survival of *Cyclospora*. It is unknown if *Cyclospora* is more resistant to environmental stresses than *Cryptosporidium*, which is excreted fully sporulated.

*Cyclospora* oocyst distribution and occurrence in the environment remain poorly documented.

**Epidemiology**

**Transmission to humans:**

What is the infectious dose? No animal or human exposure studies have been undertaken.

Do *Cyclospora* other than *C cayetanensis* infect humans? The number of species of *Cyclospora* that are infective to human beings is not known, as well as whether human-derived oocysts are infectious to non-human hosts.

Other potential sources of infection such as possibly insects as transport hosts should be investigated. Epidemiological studies will help clarify these transmission issues.

The genetic and antigenic diversity within *C cayetanensis* species should be further studied. Do the different variants behave differently (e.g. sporulation rate, infectivity and clinical manifestations)?

Another fundamental issue is how the environment, including water, becomes contaminated with *Cyclospora* and whether humans are the only sources of oocysts.

**Treatment**

Little is known about treatment that will effectively inactivate *Cyclospora* oocysts. The effectiveness of drinking water treatment and disinfection regarding the elimination of *Cyclospora* oocysts needs to be documented.

**Analytical method**

Robust and efficient detection, viability and typing methods are required to better assess risks and to further epidemiological understanding. Molecular approaches seem to be the most promising.
Microsporidia

<table>
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<th>Health significance</th>
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<tbody>
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<td>High</td>
</tr>
<tr>
<td>Important animal source</td>
<td>Yes</td>
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</table>

Microbiology

**General description**

Microsporidia are intracellular eukaryotic parasites, which have the potential for zoonotic and environmental, including waterborne, transmission. They are included in the Contaminant Candidate List (CCL) (U.S. Environmental Protection Agency 2005) (U.S. Environmental Protection Agency 1998). These organisms are small, single-celled, obligate intracellular parasites. They produce environmentally resistant spores with a diameter of 1.0-4.5µm and a characteristic coiled polar filament for injecting the sporoplasm into a host cell to initiate infection. Their host range is extensive and includes most invertebrates and all classes of vertebrates.

**Classification**

The term "Microsporidia" is a non-taxonomic designation commonly used to describe a group of obligate intracellular protozoa belonging to the phylum Microspora and which were recently reclassified with fungi (Weiss 2001).

Approximately 143 genera and more than 1,200 species are known. Since the first documented case in 1985, at least six genera (*Enterocytozoon*, *Encephalitozoon* (including *Septata*), *Nosema*, *Pleistophora*, *Vittaforma* and *Trachipleistophora*) including 14 species have been reported to infect humans (Franzen 1999). *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* are the most common causes of human infections.

**Human health effects**

**Clinical features**

Since the recognition of immunosuppression due to HIV infection, microsporidia have become recognized as important human pathogens. The most common symptoms are diarrhoea, weight loss, abdominal pain, vomiting and fever. Prolonged illness for up to 48 months has been reported in AIDS patients. Infections are self-limiting in immunocompetent individuals. The incubation period is still unknown. The species *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* are the most prevalent microsporidian parasites that cause gastrointestinal infection.

The pathology of microsporidia infection varies depending on the organs that are infected. Other documented infections include keratitis, conjunctivitis, hepatitis, peritonitis, myositis and central nervous system infection (Marshall 1997).

**Dose – response data**

The infectious dose is unknown, but it may be low because as few as 100 spores cause infection in mice (Didier 1994).

**Target populations**

While rare cases of acute self-limiting infections in immunocompetent individuals exist, most infections are primarily associated with immunocompromised hosts (Marshall 1997).

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**Diagnosis**

The diagnosis of microsporidiosis relies on direct visualization of spores in clinical specimens by light or fluorescent microscopy. Trichrome stain and optical brighteners are used to detect spores in faeces, urines, respiratory secretions and other aspirates. Electron microscopy remains an important method but its sensitivity is relatively poor. Molecular diagnosis techniques have been used as alternatives (da Silva 1996).

**Treatment**

The most effective drugs for treating microsporidiosis in humans currently include albendazole, which is effective against the *Encephalitozoon* species but not against *Enterocytozoon bieneusi*, and fumagillin, which has broader anti-microsporidia activity but is toxic in mammals, suggesting a need to identify better drugs (Didier 2004).

**Geographical repartition**

Worldwide. Microsporidiosis infections have been reported in persons from all continents.

**Epidemiology**

**Transmission routes**

The route of infection has not been fully characterized. Microsporidia are likely transmitted by the faecal-oral route. Ingestion or possibly inhalation of spores by a susceptible host from a contaminated environmental source as well as direct contact with infected individuals or animals have been suggested as possible transmission routes (Didier 2004).

**Waterborne outbreaks**

A single reported drinking water outbreak of microsporidiosis occurred in the summer of 1995 in France where approximately 200 cases of microsporidiosis were identified, mostly in AIDS patients (Cotte 1999). However, the source of the organism and faecal contamination of the drinking water supply were not demonstrated. Foodborne associations have been suggested but not yet demonstrated.

**Ecology**

**Occurrence**

Not extensively documented for lack of environmental detection methods. Microsporidia spores can be introduced into the environment from stools, urine or respiratory secretions of infected hosts. Avery et al. (Avery 1987) detected microsporidia from concentrated ditch water. By using molecular methods, several studies have detected these organisms in water. *E. intestinalis* spores have been found in tertiary sewage effluent, and in surface and ground water (Dowd 1998) (Dowd 2003). Spores of *E. bieneusi* have been detected in small numbers from the Seine River in France, suggesting that the risk of waterborne transmission to humans is limited (Sparfel 1997) (Fournier 2000). Fournier et al. (Fournier 2002) found a positive sample of unknown species of microsporidia in swimming pool water. Human pathogenic species of microsporidia were detected in irrigation water used for crop production (Thurston-Enriquez 2002).

**Reservoirs**

Although several animal hosts have been identified, the reservoirs of human microsporidia are still unknown.
*E. bieneusi* was isolated from farm and companion animals including cattle, pigs, dogs, cats and chickens (Reetz 2002) (Rinder 2000) and also from immunodeficient macaques (Chalifoux 2000). Recently Sulaiman et al. (Sulaiman 2003) reported the first isolation of *E. bieneusi* in wild, fur-bearing mammals. However, if *E. bieneusi* may result in zoonotic infection, no direct evidence of transmission from animals to humans has yet been documented.

**Survival**

Not extensively documented.

In distilled nonchlorinated water, some microsporidia spores can survive extreme temperatures, variations in pH, and multiple freeze-thaw cycles and remains viable for up to 10 years. Spores of *E. intestinalis* stored in water were still infective after 12 months and 3 months at 10 °C and 25°C, respectively (Li 2003).

The survival of *Encephalitozoon* species in sea water at 10 and 20° C lasts between 2 to 12 weeks depending on the species, which suggests that these organisms can potentially remain infectious long enough to become widely dispersed in estuary and coastal waters (Fayer 2004).

**Inactivation/removal**

Published data on removal and inactivation are summarized in annex 1.

Data are limited about the response of microsporidia to water treatment processes. The small size of the organisms is likely to make them difficult to remove by filtration process.

Preliminary results showed that conventional drinking water treatment consisting of coagulation, sedimentation, and mixed media filtration allowed the removal of *E. coli* (2.67 log) and *E. intestinalis* (2.47 log) (Gerba 2003).

*Encephalitozoon intestinalis* spores are more susceptible to chlorine disinfection than *Giardia* cysts, but much more resistant than enteric bacteria and viruses (Gerba 2003). According to Johnson et al. (Johnson 2003) spores of *Encephalitozoon* are sensitive to chlorination. Recently John et al. (John 2005) studied chlorine and ozone disinfection of *Encephalitozoon intestinalis* spores. They found that Chlorine CT values for 99% (2-log(10)) reduction ranged from 12.8 at pH 6 to 68.8 at pH 8 (mgmin/l). Ozone CT values were approximately an order of magnitude less at 0.59-0.84mgmin/l, depending on initial concentration of *E. intestinalis*. Further studies should be conducted on other species of microsporidia that are infectious to humans.

*E. intestinalis* seems to be very sensitive to inactivation by UV light. The ultraviolet dose required for a 3-log(10) or 99.9% reduction in the number of infective spores of *Encephalitozoon intestinalis* was determined to be 8.43 mWs/cm(2) (Huffman 2002) (John 2003). However the sensitivities of other species to UV disinfection have not been evaluated.

**Surrogates**

Due to the lack of information on sensitivity of infectious species of microsporidia to disinfection, the reliability of *E coli* (or, alternatively, thermotolerant coliforms) as an index for the presence/absence of these organisms from drinking-water supplies is unknown.

**Environmental detection**

Standard methods for the sampling, identification, and enumeration of microsporidia in water are not yet available.

Recently a method for the concentration and recovery of microsporidia from tap water was developed (Stine 2005).

Because of the small size of human pathogenic microsporidia, light microscopic methods are not appropriate for water samples and do not allow identification and species determination. Immunofluorescence assays (IFA) have been developed but the available polyclonal and monoclonal antibodies have shown cross-reactivity among species (Dowd 1999). IFA for routine screening of water did not reveal to be an acceptable approach. Recently a protocol for recovering *Encephalitozoon intestinalis* spores from waters by centrifugation and immunofluorescence microscopy was described (Li 2003).
A quantitative cell culture infectivity assay for *E. intestinalis* has been described (John 2003). Though this approach is very useful for health risk assessment studies, in vitro cultivation of microsporidia is laborious, time consuming and thus not suitable for routine use. In addition no in vitro cultivation methods for the main human pathogen species *E. bieneusi* are available.

Alternative molecular methods have been recently developed. A fluorescent in situ hybridization assay has been developed for the detection of the human-pathogenic microsporidia, *Encephalitozoon hellem* in water samples (Hester 2000), but this approach does not seem to be sensitive enough for water samples with low contamination levels.

An original approach (Moura 2003) described the use of MALDI-TOF mass spectrometry to characterize four human pathogenic species of *Microsporidia*. This may be a potential alternative to characterize microsporidian isolates.

Several PCR-based detection assays for the main pathogen species *E. bieneusi* and *E. intestinalis* have been described (Sparfel 1997) (Fournier 2000) (Dowd 1999). An IMS-PCR assay has been developed for *E bieneusi* in water samples (Sorel 2003).

The above methods currently available are at the experimental stage and validated protocols do not yet exist. In addition they give qualitative results only. A single study reported the quantitative detection of *Encephalitozoon* human-pathogenic species using real-time PCR (Hester 2002). The performance of this method must be evaluated on a large number of water samples.

To date no quantitative molecular method has yet been described for *E bieneusi* in water samples. No methods which determine the viability of pathogenic microsporidia spores are available. Typing methods such as Restriction Fragment Length Polymorphism (RFLP) analysis of amplicons can be used to determine genus, species and strain types of various microsporidia.

**Research needs**

To date, the mode of transmission and environmental occurrence of microsporidia have not been elucidated due to lack of sensitive and specific screening methods. Little is known about the occurrence of these protozoan parasites in environmental water sources. Further studies are needed to determine whether water supplies may be sources of human pathogenic microsporidia.

Although several animal hosts have been identified recently, the relevant reservoirs of human microsporidia are still unknown. Also, the routes of spreading are unknown. Is microsporidiosis a zoonotic disease that will be transmitted through close contact with infected animals or is contaminated surface water responsible for transmission and is it a relevant reservoir?

Inactivation: The effectiveness of drinking water treatment processes including both traditional and novel disinfection technologies needs to be evaluated regarding the removal of microsporidia.

In order to carry out the research work above, reliable and validated methods are needed to detect and quantify human pathogenic species of microsporidia (in particular *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis*) in water. There is also a need to develop methods that determine the viability status of microsporidia detected in drinking water.
Acanthamoeba

<table>
<thead>
<tr>
<th>Health significance</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Persistence in water supplies</td>
<td>Long</td>
</tr>
<tr>
<td>Resistance to chlorine</td>
<td>High</td>
</tr>
<tr>
<td>Relative infectivity</td>
<td>High</td>
</tr>
<tr>
<td>Important animal source</td>
<td>No</td>
</tr>
</tbody>
</table>

Adapted from table 7.1 Waterborne pathogens and their significance in water supplies (WHO 2004).

Microbiology

General description

*Acanthamoeba* spp. are microscopic free-living amoebae (10-50µm in diameter) that inhabit a variety of air, soil, and water environments. The organism is characterized by a life cycle of feeding and replicating trophozoite and dormant cyst stages; the trophozoite form, under unfavourable conditions, such as an anaerobic environment, will develop into a dormant cyst that can resist extreme temperatures (-20°C to 56°C), disinfection and desiccation (WHO 2004).

*Acanthamoeba* was included in the first Contaminant Candidate List (CCL) (U.S. Environmental Protection Agency 1998) but not in the second list (U.S. Environmental Protection Agency 2005).

Classification

*Acanthamoeba* spp. has been placed in the Family *Acanthamoebidae*. The genus *Acanthamoeba* spp. has been divided into three morphological groups (I, II, and III) based on cyst size and shape (Marciano-Cabral 2003). The genus contains some 20 species of which *A. castellanii*, *A. polyphaga* and *A. culbertsoni* are known to be human pathogens. However a revision of the taxonomy of the genus through DNA-based approaches is under way.

Human health effects

Clinical features

*Acanthamoeba* spp. are opportunistic pathogens that produce a multifocal encephalitis called granulomatous amebic encephalitis (GAE), a chronic central nervous system disease of immunocompromised hosts, which includes headaches, stiff neck, nausea, vomiting, malaise, confusion and hallucinations, loss of balance and control of movement and seizures. It is a rare but usually fatal disease. This organism also has been associated with cutaneous lesions and sinusitis in AIDS patients and other immunocompromised individuals. *Acanthamoeba* is also a leading cause of destructive keratitis, a chronic ulceration in contact lens wearers. It is a rare disease, which can occur in healthy individuals and may lead to impaired vision, permanent blindness and loss of the eye (Nel 2004). *Acanthamoeba culbertsoni* causes GAE, whereas *A. castellanii* and *A. polyphaga* are associated with acanthamoebic keratitis and acanthamoebic uveitis (WHO 2004).

The prevalence of antibodies to *Acanthamoeba* and the detection of the organism in the upper airways of healthy persons suggest that infection may be common with few apparent symptoms in the vast majority of cases. The pathogenicity of *Acanthamoeba* seems to be related to certain strains and low levels of IgA antibodies in the tears of infected individuals (Nwachuku 2004).

Dose – response data

Dose response experiments in animals suggest that at least 1000 trophozoites are necessary to cause *Acanthamoeba* eye infection. Risk from *Acanthamoeba* keratitis is complex, depending upon the virulence of the particular strain, exposure, trauma, or other stresses to the eye, and host immune response (Nwachuku 2004).
The infectious dose is unknown for GAE.

**Target populations**

GAE is an opportunistic infection that occurs mainly in immunodeficient individuals such as AIDS sufferers. Keratitis occurs mainly in otherwise healthy people with known risk factors.

**Diagnosis**

Diagnosis of infection includes direct microscopy of wet mounts of cerebrospinal fluid or stained smears of cerebrospinal fluid sediment, light or electron microscopy of tissues, in vitro cultivation of *Acanthamoeba*, and histological assessment. Immunocytochemistry, chemiluminescent dye staining, PCR, and analysis of DNA sequence variation have also been employed for laboratory diagnosis (Marciano-Cabral 2003).

**Treatment**

Eye and skin infections are generally treatable. But the resistance of *Acanthamoeba* cysts to most antimicrobial agents makes acanthamoeba keratitis one of the most difficult ocular infections to treat with a mean treatment period of more than 5 months and surgical interventions in 50% of cases. Reports of successful treatment of *Acanthamoeba* GAE infection are scarce.

A number of health agencies recommend that only sterile water should be used to prepare wash solutions for contact lenses (WHO 2004).

**Geographical distribution**

The organism is ubiquitous and is probably found worldwide. (Marshall 1997)

**Epidemiology**

**Transmission routes**

The mode of transmission of GAE has not been well established, but drinking water is not considered to be a source of infection. Inhalation of amoebae through the nasal passages and lungs or introduction through skin lesions have been suggested as possible routes of transmission (Marciano-Cabral 2003). Acanthamoebic keratitis has been associated with soft contact lenses being washed with contaminated home-made saline solutions or contamination of the contact lens containers. Although the source of the contamination is unknown, tap water is one possibility. Human-to-human transmission has not yet been reported.

**Waterborne outbreaks**

Keratitis is the only water-related syndrome caused by *Acanthamoeba* spp. Most episodes of keratitis occur during warm weather and often follow water exposure or a history of swimming in lakes and ponds while wearing contact lenses (Marshall 1997). Recently Nwachuku et al. (Nwachuku 2004) reported a relationship between seasonal distribution of keratitis and abundance of *Acanthamoeba* in surface waters.

Cases of acanthamoebic keratitis have also been associated with drinking water due to use of tap water in preparing solutions for washing contact lenses (Marshall 1997) (Kilvington 2004) (Radford 2002).

**Ecology**

**Regrowth in distribution systems/biofilms**

*Acanthamoeba* may persist and grow in biofilm in domestic water supplies where they may serve as reservoirs for the presence and transmission of other human pathogens.
Reducing the presence of biofilm organisms is likely to reduce food sources and growth of *Acanthamoeba* in distribution systems (WHO 2004).

**Occurrence**

The wide distribution of *Acanthamoeba* in the natural environment makes soil, airborne dust and water all potential sources. *Acanthamoeba* have been found in surface waters (Tsvektova 2004), occasionally in marine water and sediments, in tap water (Lorenzo-Morales 2005), in bottled water, swimming pools and spas and sewage (Nwachuku 2004). *Acanthamoeba* spp. also have been isolated from vegetation, from animals including fish, amphibia, reptiles, and mammals.

**Reservoirs**

Their natural ecological niche is unknown.

**Survival**

*Acanthamoeba* feed on bacteria in the environment by trapping them in their cytoplasm. Trophozoites can exist and replicate in water while feeding on bacteria, yeasts and other organisms. Depending on the species, *Acanthamoeba* can grow over a wide temperature range in water, with the optimum temperature for pathogenic species being 30°C (WHO 2004). Both trophozoites and cysts can retain viable bacteria and may serve as reservoirs for bacteria with human pathogenic potential including *Legionella pneumophila* (Kilvington 1990), *Burkholderia cepacia* (Landers 2000), *Mycobacterium avium* (Steinert 1998), *H. pylori* (Winiecka-Krusnell 2002) and *E. coli* serotype O157 (Barker 1999). Thus *Acanthamoeba* may serve as a host for a variety of bacteria, as well as one species of yeast, *Cryptococcus neoformans* (Marciano-Cabral 2003) but it can also protect the bacterial agents against chlorination and other disinfection measures.

**Inactivation/removal**

The published data on removal and inactivation are summarized in annex 1. Few studies have documented the efficiency of current drinking water treatment processes on *Acanthamoeba*. Compared with *Giardia* and *Cryptosporidium*, *Acanthamoeba* is relatively large and is amenable to removal from raw water by filtration. Hoffmann et al. (Hoffmann 2000) demonstrated the efficiency of complete treatment lines, and especially of the clarification steps, to reduce *Acanthamoeba* and other amoebae. *Acanthamoeba* cysts can withstand extreme levels of temperature, desiccation, and disinfection (Marciano-Cabral 2003). All of the Candidate Contaminant List (CCL) organisms for which data were available were found to be fairly sensitive to chlorine dioxide, except for *Acanthamoeba* cysts, which were found to be the most resistant. The cysts of *Acanthamoeba castellantii* were more resistant to ozone than other protozoa and viruses (Gerba 2003). These results have been confirmed by recent studies (Loret 2004, Loret 2005). *Acanthamoeba* cysts are very resistant to chlorine, bromide and iodine inactivation as well as ultraviolet light. The trophozoites (active growing form) are much more sensitive to chlorine and other disinfectants used in drinking water treatment. They are however more resistant than bacteria (Nwachuku 2004).

**Surrogates**

*E. coli* (or alternatively, thermotolerant coliforms) cannot be used as an index for the presence/absence of *Acanthamoeba* spp.

**Environmental detection**

The classical microbiological method relies on in vitro culture of amoebae. The basic procedure is the use of non-nutrient agar with *E. coli* as a food source. Once the amoebas are established in a bacterized culture, antibiotics can be added to kill off bacteria and the amoebas can be transferred to the appropriate
axenic medium (Schuster 2002). This method is time consuming (2-3 days to 2 weeks), does not allow identification at the species level and cannot distinguish pathogenic from non pathogenic strains.

The use of newer molecular methods such as PCR can provide a more rapid and specific means to identify *Acanthamoeba* in environmental samples. Lorenzo-Morales et al. (Lorenzo-Morales 2005) used PCR amplification with a genus-specific primer pair to detect *Acanthamoeba* in water sources. A recent study (Riviere 2005) described a real-time PCR assay for quantification of *Acanthamoeba* trophozoites and cysts. This promising approach needs to be compared with the conventional microbiological method and then validated.

A variety of molecular methods for genotyping and identification of *Acanthamoeba* strains have been used in clinical samples and environmental water samples such as RFLPs of complete or partial nuclear rRNA18S, variation in complete mitochondrial 16S rRNA, analysis 18S rRNA sequences (Schroeder 2001) (Tsvetkova 2004), and randomly amplified polymorphic DNA analysis of whole-cell DNA (Ortega-Rivas 2005).

**Research needs**

*Acanthamoeba* is a relatively newly described pathogen of growing importance because it is responsible for severe pathologies in immunocompetent and immunosuppressed individuals and because it may serve as a potential vector for bacterial infections from water sources. Much remains unknown about the epidemiology and ecology of this organism.

**Ecology**

*Acanthamoeba* – *bacteria* interaction:

Some species of bacteria can grow and reproduce within the cytoplasm and become symbionts. In particular, little is known about the interaction between *Acanthamoeba* and Legionellae. More information is required on the role of *Acanthamoeba* spp. as potential vectors for bacterial infections from water sources.

Bacterial endosymbionts may also play a role in the pathogenicity of *Acanthamoeba*. This topic should be investigated.

**Occurrence:**

For risk assessment purposes, more information is required on the occurrence and the types of *Acanthamoeba* in tap water and ground water. Information on the type of treatment the water received and/or the level of residual chlorine should be known. Moreover, assessment of the pathogenicity of *Acanthamoeba* strains in tap water by cell culture and molecular methods would be valuable.

**Epidemiology**

The health effects of *Acanthamoeba* and the significance of water in its transmission should be further investigated in epidemiological studies.

**Treatment**

The effectiveness of drinking water treatment and disinfection regarding the elimination of *Acanthamoeba* needs to be more documented.

In addition, further research should determine if control of *Acanthamoeba* spp. is needed to better control pathogen transmission through water supplies.

**Environmental detection method**

A rapid quantitative detection method (based on real-time PCR) should be validated on water samples to be used as an alternative to the conventional culture method.
Research areas covered by GWRC members

This section summarizes the information received from AWWARF, CRC, DWGW/TZW, KIWA, Suez, USEPA, Veolia, WERF, WRC, and WRF.

It appears from this information that the research activities conducted or supported by the GWRC members on waterborne pathogens are mainly oriented towards management strategies and have so far covered the following areas:

Source water / catchment
- Pathogens monitoring in raw water (development of methods, organisation of surveys)
- Development of source control strategies
- Identification of mechanisms / modelling of pathogens transport / survival in surface water, aquifers, soils

Drinking water production
- Colonization of filters with pathogens
- Pathogens repair after disinfection
- Treatment efficacy / optimization
- Assessment of surrogates

Drinking water distribution
- Control / monitoring of regrowth / intrusion of pathogens in distribution systems

Domestic water systems
- Control of pathogens regrowth
- Evaluation of treatment efficacy

Water and health
- Outbreak detection
- Role of water in endemic level of microbial disease
- Quantitative microbial risk assessment
- Detection of infectivity / viability / virulence
Conclusion

Over the past decade, new microbiological detection techniques have emerged in order to track specific pathogens both for routine monitoring of water and for the investigation of disease outbreaks. Molecular detection methods especially based on PCR, RT-PCR and recently real-time PCR have been increasingly developed, but still need to be enhanced in the future. There are probably very few or no groups of pathogens for which no molecular detection method has not yet been developed.

But real-time quantitative PCR methods are still lacking for some viruses (calicivirus, rotavirus) and bacteria (Yersinia, Aeromonas, Helicobacter and Shigella), and standard culture methods are still lacking for Helicobacter, adenovirus serotype 40 and 41, calicivirus, hepatitis E virus, and rotavirus.

In the future, evaluation studies, comparison of performance with existing cell culture methods, and standardization of these new methods, as well as development of their capacity to detect viability and virulence, will be necessary to receive their approval from legislative authorities. Whatever the detection method, sample preparation and recovery of micro-organisms, prior to analysis, will also have to be improved.

Knowledge on waterborne pathogens has significantly been heightened over the past 15 years namely by the development of molecular biology tools but many important questions remain unanswered. With the purpose of identifying priorities for research, one of these important questions would be the determination of the real burden of waterborne disease. Most of the available statistics are based on outbreak data, which probably reflect only a minor part of the burden. Studies aiming at evaluating endemics and their causes would be necessary.

May water be a significant route for the dissemination of Helicobacter pylori, Aeromonas, Burkholderia, non-tuberculosis mycobacteria, Cyclospora, microsporidia, as well as adenoviruses, enteroviruses or rotaviruses? The presence of these pathogens in distributed waters should be investigated, traceability studies from source to tap should be conducted and links with epidemiological data should be established.

The removal and inactivation of pathogens by drinking water production processes should be better documented, especially for Campylobacter, hepatitis E virus, and Cyclospora, for which no specific data are available.

Concerning bacteria, the emergence of antibiotic-resistant strains of enteric bacterial pathogens such as Salmonella spp., Shigella spp., E.coli O157, and Campylobacter spp. is a serious challenge and many aspects are poorly understood. May drinking water biofilms play a role in the transfer of antibiotic resistance and virulence factors? This question should be an area of future research.

Amongst emerging aquatic pathogens, Legionella pneumophila and non-tuberculous mycobacteria are unique in terms of their ability to proliferate in hot water systems, their environmental ubiquity, their ability to proliferate under certain circumstances within protozoan cells and their resistance to disinfection, but a great deal remains unknown.

Concerning protozoa, Cryptosporidium and Giardia arouse significant media attention due to the magnitude of recent outbreaks. Many research studies have essentially focused on Cryptosporidium, which constitutes a higher health risk than Giardia because no treatment is available against cryptosporidiosis which can be fatal for immunocompromised individuals. In the future, attention should be given to the emerging protozoa Cyclospora and Microsporidia for which waterborne transmission is plausible but unconfirmed.

The role of Acanthamoeba spp. and other amoebae as reservoirs and transmission routes for waterborne pathogens in drinking water systems also requires further research.

A key scientific challenge in the coming years will indisputably be waterborne viruses, about which much remains unknown. Waterborne viruses are increasingly being suspected as major causative agents of gastrointestinal infections. Their incidence is probably dramatically underestimated due in part to the difficulties inherent to both their specific clinical diagnosis and measurement in drinking water. There are many areas where knowledge is poor regarding the epidemiology of viruses as well as their occurrence and behaviour in the environment and the effectiveness of water treatment processes for their removal;
this is partly due to the fact that no straightforward and reliable environmental detection methods are available. In addition, many - as yet uncharacterized – pathogenic viruses might emerge in the future.

Most research efforts are currently cast on viruses, bacteria and protozoa in drinking water but other potentially new, little-known infectious agents such as fungi, prions and viroids are likely to prove dangerous, and their presence in water may constitute a risk to human health.

Annex 2 summarizes these conclusions in the form of a table showing the state of knowledge for each micro-organism considered in the present study.
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Appendix
### ANNEX 1: Treatment data

#### Treatment data: clarification

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Treatment</th>
<th>Filter type</th>
<th>Water Flow Rate (gpm)</th>
<th>Filter Loading Rate (gpm/ft²)</th>
<th>Raw Water Quality (Turbidity NTU, Concentration)</th>
<th>Effluent Quality (Turbidity NTU)</th>
<th>PH</th>
<th>Scale</th>
<th>Reduction (log)</th>
<th>Reference</th>
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<tr>
<td>E.coli O157 H7</td>
<td>Coagulation + Sedimentation + Filtration</td>
<td>Anth./Sand</td>
<td>1</td>
<td>2–8</td>
<td>3.5–3.9</td>
<td>6*E7 CFU/L</td>
<td>0.11–0.21</td>
<td>7.78 (average)</td>
<td>0.75 (average)</td>
<td>Harrington, Xagoraraki et al. 2003</td>
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<td>E.coli O157 H7</td>
<td>Coagulation + Sedimentation + Filtration</td>
<td>Anth./Sand/Garnet</td>
<td>1</td>
<td>4</td>
<td>7.1–10.1</td>
<td>6*E7 CFU/L</td>
<td>0.1–0.12</td>
<td>7.41 (average)</td>
<td>1.0 (average)</td>
<td>Harrington, Xagoraraki et al. 2003</td>
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<td>Anth./Sand</td>
<td>1</td>
<td>4</td>
<td>7.1–10.1</td>
<td>6*E7 CFU/L</td>
<td>0.08-0.09</td>
<td>5.7 (average)</td>
<td>2.0 (average)</td>
<td>Harrington, Xagoraraki et al. 2003</td>
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<td>E.coli O157 H7</td>
<td>Coagulation + Sedimentation + Filtration</td>
<td>Anth./Sand</td>
<td>1</td>
<td>4</td>
<td>6*E7 CFU/L</td>
<td>pilot</td>
<td>0.7 (average)</td>
<td></td>
<td>Xagoraraki, Harrington et al. 2004</td>
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<tr>
<td>E.coli ATCC 25922</td>
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<td>GAC/Sand/Gravel</td>
<td>0.4–0.47</td>
<td>5–10</td>
<td>1*E8 CFU/L</td>
<td>1</td>
<td>pilot</td>
<td>2.67 (optimal)</td>
<td>Gerba, Riley et al. 2003</td>
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<td>Anth./Sand</td>
<td>1</td>
<td>2–8</td>
<td>3.5–3.9</td>
<td>2*E7 CFU/L</td>
<td>0.11–0.21</td>
<td>7.78 (average)</td>
<td>3.2 (average)</td>
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<td>2*E7 CFU/L</td>
<td>0.1–0.12</td>
<td>7.41 (average)</td>
<td>3.5 (average)</td>
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<td>Adenovirus</td>
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<td>1</td>
<td>4</td>
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<td>&lt;0.2</td>
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<td>GAC/Sand/Gravel</td>
<td>0.4–0.47</td>
<td>5–10</td>
<td>1*E6 TCID50/L</td>
<td>1</td>
<td>pilot</td>
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<td>4</td>
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<td>1.85 (average)</td>
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<td>0.65 (average)</td>
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<td>1*E7 CFU/L</td>
<td>0.4–2.0</td>
<td>full</td>
<td>0.3 (average)</td>
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<th>Effluent Quality</th>
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## Treatment data: disinfection – chlorine

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CDF- Chlorine Demand Free; TAP-Treated Drinking Water; BDF- Buffer Demand Free; PEW-Potomac Estuarine Water; HTE-Human Tank Effluent
## Treatment data: disinfection - chloramines

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PBW-Phosphate Buffered Water
## Treatment data: disinfection – chlorine dioxide

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## Treatment data: disinfection - ozone

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**DW**-Distilled Water; **ODF**-Ozone Demand Free; **ASE**-Activated Sludge Effluent
## Treatment data: disinfection - UV

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GM-Growth Medium; SDW-Sterile Distilled Water; PBS-Phosphate Buffered Saline
Treatment data: references


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## ANNEX 2: Map of knowledge

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<tr>
<td><strong>Calicivirus</strong></td>
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<tr>
<td><strong>Enterovirus</strong></td>
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<tr>
<td><strong>Hepatitis A</strong></td>
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<tr>
<td><strong>Hepatitis E</strong></td>
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<tr>
<td><strong>Rotavirus</strong></td>
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<tr>
<td><strong>Cryptosporidium</strong></td>
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<tr>
<td><strong>Giardia</strong></td>
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<tr>
<td><strong>Cyclospora</strong></td>
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<tr>
<td><strong>Microsporidia</strong></td>
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<tr>
<td><strong>Acanthamoeba spp.</strong></td>
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</tbody>
</table>

- ■ documented
- □ unsufficiently documented
- ○ not documented

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