> Pathogenic Microorganisms and Viruses in Groundwater

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SUMMARY

Groundwater represents the quantitative most important freshwater resource on our planet. Generally well protected by overlaying soil and sediment layers, it is a valued source for drinking water. Along with demographic development and global change the quality of groundwater is increasingly challenged by anthropogenic impacts including the direct and indirect introduction of potentially harmful pathogenic microorganisms and viruses. Does this pose a new risk to human health? This review paper provides a brief summary on (1) the diversity of pathogens continuously or occasionally found in groundwater, (2) their sources and ways of entry to groundwater systems, (3) their transport and attenuation, as well as (4) their survival and potential growth in aquifers. Further sections of the paper focus on (5) drinking water contamination and disease outbreaks, (6) developments in pathogen detection and the use of indicator organism. Finally, the report (7) highlights knowledge gaps and open research questions and provides recommendations for safeguarding groundwater and drinking water resources.

1. INTRODUCTION

Groundwater is one of men's most important sources for life. Globally groundwater accounts for approximately one third of all freshwater resources and represents around 99% of available freshwater (UNESCO 1999; Danielopol *et al.* 2003). In most countries worldwide it is used as the main source of drinking water. Groundwater usage in Europe ranges from only 13% in Norway up to 100% in Denmark, Lithuania and Austria (EEA 1999; UNECE 1999; Ashbolt 2004; WHO 2006; BGR 2007). The average proportion of groundwater as drinking water in US states is about 65% and hence quite similar to the European consumption. In Germany, about 70% of the drinking water derives from aquifers. However, there is a huge variation between the federal states, with Bremen, Hamburg, Saarland and Schleswig-Holstein as the top groundwater consumer states (100%), whereas the proportion of groundwater in drinking water in Berlin and Saxony is only about 25% and 33%, respectively (BGR 2007).

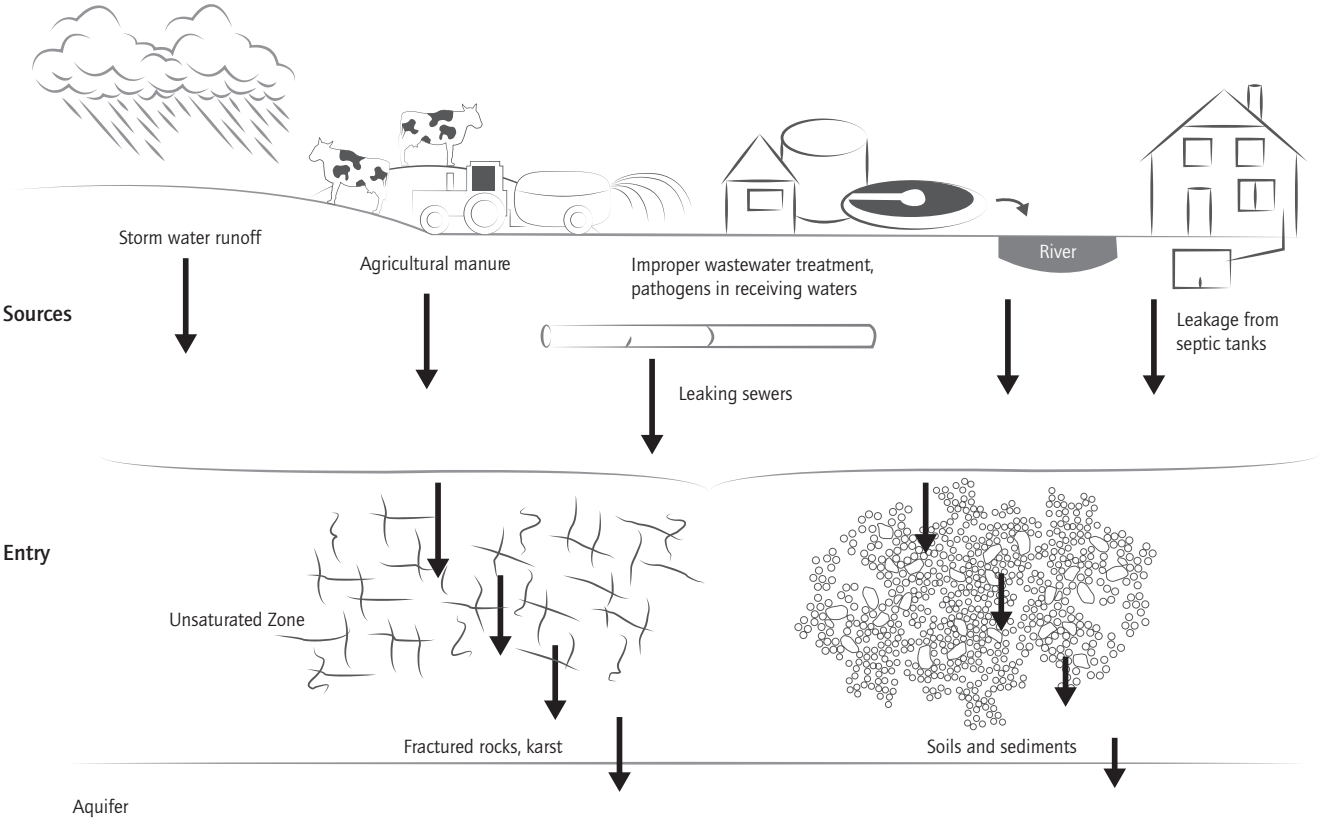
There is logic behind the utilization of groundwater as drinking water source. Naturally, groundwater ecosystems are well protected by overlaying soil and sediment layers. Water from precipitation recharging aquifers needs to pass these zones which act as effective mechanical and biological filters, hence providing a natural clean-up of newly generated groundwater. In aquifers, the biological components, mainly microorganisms, provide the valuable ecosystem services of water purification and storage at high quality for decades and centuries (Herman *et al.* 2001; Avramov *et al.* 2010). Nevertheless, today groundwater faces increasing threats from anthropogenic impacts (Sampat 2000), including contamination with pathogenic microorganisms and viruses (Pedley and Howard 1997). Thus, although drinking water in Germany is the best controlled food (Bundesregierung 2009) and therefore generally considered to be safe, there is a steady and possibly increasing risk for human health from contaminated groundwater.

Groundwater for a long time has been thought completely free of microbial contaminants and viruses, believing that vertical transport times are long enough and microbial survival too short to reach the aquifers. However, the risks of water contamination are obvious when having a look at the manifold small and huge endemic outbreaks from pathogenic microbes and viruses in the last two centuries which could be linked to contaminated groundwater and drinking water consumption (Andersson and Bohan 2001; OECD 2003; Craun *et al.* 2006; Craun *et al.* 2010). Today, there is no doubt that pathogenic microorganisms and viruses can be found everywhere in the environment. Some relevant pathogenic microorganisms even became or always have been members of natural microbial communities (e.g. species of the genus Legionella; Fliermans *et al.* 1981; Fliermans 1996; Steinert *et al.* 2002). Others may survive for years and decades or even reproduce in the environment. There are multiple sources of contamination including wastewater entering different environmental compartments and manure applied to fields in agricultural areas. Pressures on terrestrial and aquatic environments may increase in the future due to global change. It may be suggested that pathogenic microorganisms and viruses will more often enter soil, river banks and aquifers along with irregular storms, flood and drought events (Foster and Chilton 2003; Schwarzenbach *et al.* 2010). The efficiency of natural attenuation of pathogenic agents in soils, vadose zone sediments and porous, fissured or karst aquifers is highly dependent on the matrix characteristics and environmental conditions. In regions with thin soil layers, fractured rocks or in karst regions, aquifers are especially vulnerable to contamination. In contrary, extended soil layers are a good protection shield against pathogens entry. More specific, biologically active environmental compartments, such as soils, often have been observed to support fast and efficient pathogen elimination (Hurst *et al.* 1980; Hurst 1988; Nasser *et al.* 2002). Unfortunately, hardly any of the various antagonistic biological processes have been elucidated in detail. Thus, it is in time to newly interpret information available on pathogens ecology and reconsider strategies in groundwater and drinking water protection as well as current standards in water quality control and drinking water treatment.

2. SOURCES AND ENTRY

Due to growing populations and expanding land use, sources of pathogen contaminated wastes steadily increase hence also raising the potential pollution of groundwater reservoirs with infectious agents all around the world. This is mainly true for pathogens originating from human and animal faeces. Sources of faecal contamination in groundwater potentially include (i) leakage from on-site sanitation systems such as septic tanks or sewers, (ii) underground storage tanks, (iii) disposal systems, (iv) animal manure and compost, and (v) from (accidental and non-accidental) wastewater discharge or (vi) sewage sludge applied to fields in agricultural areas (Reynolds and Barrett 2003; Gerba and Smith 2005; Arnone and Walling 2007; Fig. 1). Additionally, (vii) surface waters receiving treated or untreated sewage from human sources or livestock enterprises and discharge from non-point sources like urban and agricultural runoff are a steady source and reservoir of pathogenic agents (Kirschner *et al.* 2009).

Fig. 1: Major sources of faecal pathogens and potential entry paths to groundwater.



Today's sewage treatment plants are highly efficient in removing and transforming most organic and inorganic pollutants contained in wastewater; with some exceptions. Although the number of (potentially) pathogenic microorganisms and viruses contained in raw wastewater is reduced by 1-3 orders of magnitude during classical treatment, the elimination processes are incomplete leading to a continuous release of pathogens to the recipient and surface waters (Hirn 1980; Omura *et al.* 1989; Rose *et al.* 1996; George *et al.* 2002).

Especially during periods characterized by increased bacterial and viral infections in the population (e.g. winter time in Germany), the incoming numbers of pathogens in wastewater may rise dramatically. A person that is infected with *Campylobacter* may excrete between 1 x 10^6 and 1 x 10^8 bacteria per gram faeces (Taylor *et al.* 1993). Assuming 200 g of faeces being produced by a person each day, this accounts for 2 x 10^8 to 2 x 10^{10} *Campylobacter* specimen. The numbers are even higher with viruses. The faeces of a person infected with Adenovirus may contain up to 1 x 10^{11} virus particles per gram (Wadell 1984; Albert 1986). A possible cycle between increased numbers of pathogenic agents released with faeces from infected persons to wastewater treatment plants and consequently surface waters and groundwater and the distribution of these agents with drinking water is currently discussed (e.g. Soddemann, pers. comm. 2010; see also section 6).

Sources of pathogens, which verifiable caused epidemic outbreaks of waterborne diseases are most often related to improper management of wastewater disposal being the top-of-the-list responsible cause of direct pathogens entry to aquifers (Lawson *et al.* 1991; Kukkula *et al.* 1997; Willocks *et al.* 1998). In the US leaked septic fluids from tanks or cesspools, compared to all human sources, account for the highest overall quantity of wastewater discharged directly to groundwater, and are therefore a major concern regarding the potential risks to human health (Miller 1980; Yates 1985; Bloetscher and Van Cott 1999). In comparison, other point sources, such as leachates associated with land disposal of industrial and human wastes, are of only minor concern related to groundwater contaminants (Miller 1980; Ritter *et al.* 2002).

A serious source of pathogens entering soils and groundwater is diffuse contamination (= non-point sources), for example from spreading of manure to fields and crops. Similar to human faeces, animal manure may contain high concentrations of pathogenic organisms (>10^6 per gram of faeces) including bacteria, viruses, protozoa and helminths, potentially causing zoonotic diseases in humans (Cotruvo *et al.* 2004; Gerba and Smith 2005). This poses a serious risk for human health in rural areas (Bianchi and Harter 2002; Cotruvo *et al.* 2004), arising for example from frequent contamination of private wells that are used for drinking water supply of farms. A statistical evaluation of 1,200 rural farms in the US revealed that approximately one-third of the private wells were, from a hygienic and bacteriological point of view, contaminated (Goss and Barry 1995).

Outbreaks of waterborne diseases in developing countries frequently correlate with flooding events resulting in a direct pulse response of shallow groundwater contamination to rainfall (Godfrey *et al.* 2005). Similar in Europe, strong rainfalls after the application of manure foster the contamination of shallow groundwater. In urban areas run-off from the mainly sealed surface captured in the public drainage system often exceed water holding capacities of water treatment plants. Consequently, flooding of sewage plants results in high loads of pathogens in the recipient. Additionally, public water drainage systems discharge their surplus untreated into nearby surface waters. Further down the line, floods trigger the fast import of water carrying contaminants including pathogenic agents into bank sediments and aquifers.

The ex-filtration of wastewater and thus the release of pathogens into groundwater via leaking sewers is a common source in urban areas. A significant proportion of wastewater is lost to the underground during transport from households and industry to water treatment plants. Investigations from the late 1980s documented potential sewage ex-filtration at a length of about 22% of the total sewer system in Germany. For Munich the amount of wastewater loss was estimated with 5% of the total sewer flow (Reynolds and Barrett 2003). Subsequent studies in Germany estimated the yearly leakage of wastewater from partly damaged sewage systems into soil and groundwater with several 100 million m^3 (Eiswirth and Hötzl 1997). These values underline the continuous contamination of groundwater in urban areas. There is also a small risk of cross contamination since underground drinking water distribution networks in Germany are often in close vicinity to sewage pipes, and are suggested to have a similar frequency of leaks.

Proper wastewater management, modern sanitation and groundwater protection strategies efficiently contribute to a reduction of pathogen entry into the subsurface. This is often not implemented in developing countries. Consequences of poverty and overpopulation are becoming most noticeable in problems related to hygiene and sanitation. Sanitation standards

are often outdated and insufficient and most of the sewage is discharged to the environment without any treatment (UNESCO 1999). Groundwater and/or drinking water in these regions differ fundamentally in its quality from what is generally served in developed countries (Schwarzenbach *et al.* 2010). More than half of the world's population will still not be connected to public sewerage systems in the next 20 years (UNESCO 2009).

3. DIVERSITY OF PATHOGENS IN GROUNDWATER AND RELATED DISEASES

Groundwater is an active and quantitatively very important component of the hydrological water cycle. Pathogens which appear in soil and surface waters, regularly or at least occasionally enter aquifers. It is therefore not surprising that most of the known pathogenic microorganisms and viruses have been found in groundwater.

3.1 BACTERIA

There is a great diversity of pathogenic bacterial groups and species potentially harmful to human health, of which representatives are frequently detected in groundwater and even drinking water. The majority's natural habitat is the gastrointestinal tract of humans and animals which they leave unintentionally via the excretion of faeces. Most known pathogenic bacteria belong to the family *Enterobacteriaceae*. Members of the *Enterobacteriaceae* are not necessarily pathogenic. Many representatives are widely distributed in soils and aquatic environments (Stevens *et al.* 2003). The most prominent members of the *Enterobacteriaceae* are the coliforms including a heterogeneous mix of different genera and species which vary considerably in terms of their pathogenic properties and virulence. Members of the coliform group, including the total coliform bacteria, the thermotolerant coliform bacteria, *Escherichia coli* and faecal streptococci are to date the most important indicators of faecal contamination (Gleeson 1997; Ashbolt *et al.* 2001; Leclerc *et al.* 2001). A common characteristic of this group is the growth at 37 °C. Thermotolerant coliforms, including *E. coli*, are even able to grow at up to 44 °C (WHO 2006). Among the pathogenic representatives of the *Enterobacteriaceae* are several *E. coli* strains responsible for severe infections, namely enteropathogenic *E. coli* (EPEC), enterotoxic *E. coli* (ETEC) and enterohaemorrhagic *E. coli* (EHEC) (Nataro and Kaper 1998). The *Enterococcus* group, a subgroup of the faecal streptococci is known to be, within the coliforms, most resistant to environmental conditions and changes and carry resistances to several antibiotics (McFeters *et al.* 1974; Hartke *et al.* 1998; Sapkota *et al.* 2007). One member of this group, *E. faecalis*, tolerates high temperatures (up to 45 °C), high pH values (9.6), and high salt concentrations (up to 6.5% sodium chloride) and is therefore also a valuable bacterial indicator for determining the extent of faecal contamination of water (Foulquie Moreno *et al.* 2006; Farnleitner *et al.* 2010). The coliform group and its advantages and disadvantages as indicators are discussed in more detail in section 7.

Further typical bacterial pathogens distributed by the faecal-oral route and occasionally found in groundwater include species of *Salmonella* and *Shigella*, and, emerging mainly in developing countries, species of *Vibrio* with *Vibrio cholerae* being the most prominent representative. The genus *Salmonella* and *Shigella* are also members of the *Enterobacteriaceae*, however, they are not considered coliforms.

Table 1: Pathogens found in groundwater.

PATHOGEN	MAJOR DISEASES	SOURCES
Bacteria		
Escherichia coli	Gastroenteritis, Haemolytic Uraemic Syndrome (enterotoxic *E. coli*)	Human faeces
Salmonella spp.	Enterocolitis, endocarditis, meningitis, pericarditis, reactive arthritis, pneumonia	Human and animal faeces
Shigella spp.	Gastroenteritis, dysentery, reactive arthritis	Human faeces
Campylobacter jejuni	Gastroenteritis, Guillain-Barré syndrome	Human and animal faeces
Yersinia spp.	Diarrhoea, reactive arthritis	Human and animal faeces
Vibrio cholerae	Cholera	Human faeces and freshwater zooplankton
Legionella spp.	Pneumonia (Legionnaires' disease)	Thermally enriched water
Pseudomonas aeruginosa	Pneumonia, urinary tract infections, bacteremia	Soil and water
Mycobacterium spp.	Pulmonary disease, skin and soft tissue disease	Soil and water

Viruses		
Poliovirus	Poliomyelitis	Human faeces
Coxsackievirus	Fever, pharyngitis, rash, respiratory disease, diarrhoea, haemorrhagic conjunctivitis, myocarditis, pericarditis, aseptic meningitis, encephalitis, reactive insulin-dependent diabetes	Human faeces
Echovirus	Respiratory disease, aseptic meningitis, rash, fever	Human faeces
Enteroviruses 68-71	Polio-like illness, aseptic meningitis, hand, foot and mouth (E71), epidemic conjunctivitis (E70)	Human faeces
Hepatitis A	Fever, nausea, jaundice, liver failure	Human faeces
Hepatitis E	Fever, nausea, jaundice	Human faeces
Norovirus (Norwalk virus)	Gastroenteritis	Human faeces
Calicivirus	Gastroenteritis	Human faeces
Astrovirus	Gastroenteritis	Human faeces
Sapovirus	Gastroenteritis	Human faeces
Orthoreovirus	Gastrointestinal and upper respiratory disease	Human faeces
Rotavirus A and C	Gastroenteritis	Human faeces
Coronavirus	Gastroenteritis	Human faeces
Adenovirus	Respiratory disease, gastroenteritis	Human faeces
Protozoa		
Cryptosporidium parvum	Cryptosporidiosis (gastroenteritis)	Water, human and other mammal faeces
Giardia lamblia	Giardiasis (chronic gastroenteritis)	Water and animal faeces
Entamoeba histolytica	Dysentery	Human and animal faeces
Acanthamoeba spp.	Encephalitis, Keratitis	Human faeces
Naegleria fowleri	Meningoencephalitis	Warm water
Toxoplasma gondii	(congenital) Toxoplasmosis (Encephalitis)	Human and animal faeces

Resource: Adapted from Macler and Merkle 2000; Ashbolt 2004.

As shown in Table 1 several pathogenic bacteria are causing acute gastrointestinal illness. The outbreak of disease is caused through growth inside the host (e.g. *Salmonella*) or by release of toxins (e.g. *Campylobacter*, *Shigella* and some species of *E. coli*). Newly recognized pathogens from faecal pollution include *Campylobacter* and *Yersinia* (Szewzyk *et al.* 2000). Outbreaks of *campylobacteriosis* show an increasing trend (USEPA 2006). Exclusively carried and shed by humans, contaminations with *Shigella* are less widespread than contaminations with *E. coli*.

There are numbers of other pathogens of serious health concern readily detected in the environment. For example, different species of *Staphylococcus*, a common antibiotic resistant organism, are often found biofilm-associated (Hall-Stoodley *et al.* 2004; Singh *et al.* 2009). They are frequently disease triggers of severe chronic infections in hospitals. Their occurrence in groundwater could be documented by a study on a large number of wells in Turkey. *Staphylococcus* was found in about 30% of all groundwater samples tested (Aydin 2007).

Half of the world's population is assumed to be infected with *Helicobacter pylori* and in developing countries even 70 to 90% of the population seems to carry this pathogen (Dunn *et al.* 1997). Infections are harmless in the majority of cases, however, part of the infected individuals suffer from chronic gastritis or even gastric cancer (Ernst and Gold 2000). The potential transmission of *H. pylori* via drinking water was supported by epidemiological studies in developing countries but also independent findings of

H. pylori in Europe and the USA provided evidence for infection via well water (Engstrand 2001; Park *et al.* 2001; Krumbiegel *et al.* 2004). Nevertheless, more data are needed on the occurrence of this pathogen in the environment and explicitly in groundwater.

There are a few pathogenic and potentially pathogenic bacteria that naturally occur in aquatic environments, reproducing without any specific host, e.g. members of the genus *Legionella*, *Pseudomonas aeruginosa* and several species of the genus *Mycobacterium* (Riffard *et al.* 2001; Leclerc *et al.* 2002; Brooks *et al.* 2004). These may cause infectious disease, however, mainly in compromised and only rarely in healthy individuals (Rusin *et al.* 1997; Falkinham *et al.* 2001; Sharma *et al.* 2003; Ashbolt 2004; Fok 2005). Infecting their host they take opportunity of weakened defence mechanisms and hence are called opportunistic microorganism. They typically inhabit surface waters or appear to grow in biofilms in water pipes causing regular problems in drinking water distribution systems. Representatives of *Pseudomonas* and *Mycobacterium* potentially cause pneumonia or skin diseases (Tab. 1). Growth of several species of *Legionella* is best in warm waters, in-house hot water systems and cooling towers (Park *et al.* 2003). A potentially new habitat are zones of aquifer thermal heat storage, areas in the subsurface to which warm water is pumped during summer to be used in winter for heating purposes (Brielmann *et al.* 2011). In natural groundwaters opportunistic pathogens are usually found in only small numbers and thus the risk of infection or disease outcome in healthy individuals is low.

3.2 VIRUSES

The smaller a particle, the easier it may pass filters such as soil and sediments. Hence, the penetration of pathogenic viruses to aquifers seems much more likely than for pathogenic bacteria and protozoa (Schijven and Hassanizadeh 2000). The risk of viral contamination of water is further increased because of the extremely high numbers by which enteric viruses are shed into the environment. Their numbers in infected individuals range from 10^5 up to 10^{11} per gram of stool (Fong and Lipp 2005). Moreover, viral pathogens from human and animal faeces have much longer survival times in water than most intestinal bacteria, are generally more infectious than bacteria and protozoa and are remarkable resistant to common disinfection treatments (Fong and Lipp 2005). These features make pathogenic viruses the most important candidates for faecal contamination of groundwater.

To date more than 15 different groups of enteric viruses are known including more than 140 different serotypes that can be found in the human gut (Leclerc *et al.* 2002). Therefore, the group of enteric viruses comprises the highest diversity among all waterborne pathogens (Wyn-Jones and Sellwood 2001). The enteric viruses of major concern belong to the families *Picornaviridae* (poliovirus, enterovirus, coxsackievirus, hepatitis A virus, and echovirus), *Caliciviridae* (norovirus, calicivirus, astrovirus, and sapovirus), *Reoviridae* (reovirus and rotavirus), *Adenoviridae* (adenovirus), and *Coronaviridae* (coronavirus). The hepatitis E virus, once considered a calicivirus, now belongs to an unassigned genus, the hepatits E like-viruses (Berke and Matson 2000). Most of these mammalian viruses are nonenveloped (except the enveloped coronavirus) RNA viruses. Adenoviruses exclusively bear a genome of double-stranded DNA (Fong and Lipp 2005).

Besides the taxonomic affiliation of the individual viruses, there is a functional and physiological systematic that combine some of the potential viral pathogens to the enteroviruses. Members of this group include the *polioviruses*, *coxsackieviruses* A and B, echovirus and four ungrouped enteroviruses (types 68 to 71). One main characteristic of enteroviruses is the extreme resistance to environmental stress and stability under acidic conditions (to pH 3). Common diseases caused by enteroviruses are aseptic meningitis or respiratory diseases, or poliomyelitis in the case of *poliovirus*. As obvious from Table 1 the major disease caused by most of the enteric viruses is acute gastroenteritis, one of the most common illnesses, affecting both adults and children. Members of the familiy *Caliciviridae*, including norovirus, calicivirus, astrovirus, and sapovirus, are probably the most important cause of gastroenteritis in adults and adolescents. In contrast, infections with rotaviruses are the most important viral causes of severe gastroenteritis in infants and young children (Tyring *et al.* 2006).

The species coronavirus, also a potential pathogen of viral gastroenteritis, is the only enveloped virus listed here. Infectivity of these viruses depends on an intact lipid layer envelope which is generally more susceptible to environmental changes in physical and chemical parameters. Although reduced environmental stability of enveloped viruses compared to more robust non-enveloped viruses is known, coronavirus was surprisingly shown to be quite resistant in cold water, remaining infectious for more than 100 days in pasteurized settled sewage (Casanova *et al.* 2009; Gundy *et al.* 2009). The potential high risk for human health from transmission of waterborne coronavirus was underlined by the evidence that coronaviruses from a faulty sewage system were responsible for the major outbreak of severe acute respiratory syndrome (SARS) in Hong Kong in 2003 (Peiris *et al.* 2003).

Members of the family *Adenoviridae* frequently occur in aquatic environments potentially causing respiratory and gastrointestinal diseases (Tab. 1) in both adults and children and were included in the 'Contaminant Candidate List' (CCL, the document was first published in March 1998 with the intention to inform about potential hazardous pathogens in public water systems) in 1998 as part of the Safe Drinking Water Act (SDWA) by the US Environmental Protection Agency (USEPA 1998). To date, fifty-one different serotypes of human adenoviruses have been identified and adenoviruses are considered to be the second most important viral pathogen of childhood gastroenteritis following rotavirus (Crabtree *et al.* 1997; Gu *et al.* 2003). The risk of adenoviral contamination of groundwater and drinking water is considerably high as adenoviruses are up to 60 times more resistant to water treatment by UV irradiation when compared to RNA viruses, such as enteroviruses and hepatitis A virus (Meng and Gerba 1996; Gerba *et al.* 2002; Mena and Gerba 2009).

3.3 PROTOZOA

Most common species of human pathogenic protozoa include the zoonotic *Cryptosporidium parvum*, *Giardia lamblia*, and *Toxoplasma gondii* as well as *Entamoeba histolytica* which potentially cause severe diarrhoe, encephalitis or even dysentery in the infected individuals (Smith and Smith 1990; Exner and Gornik 2004). Generally infections with the four mentioned protozoan pathogens are self-limited in healthy individuals. However, they can cause life threatening diseases in elderly, immunocompromised hosts or unborn children. Within parasites *E. histolytica* infections are third in terms of lethality, right behind malaria-causing plasmodia and schistosomes (Marshall *et al.* 1997). While giardiasis and amoebiasis can effectively be cured with drugs, at present no efficient drug treatment exists for cryptosporidiosis (Smith and Smith 1990; Chakrabarti and Chakrabarti 2009).

Free-living amoebae are common members of aquatic microbial communities. Some representatives, e.g. *Acanthamoeba spp.* and *Naegleria fowleri*, have been documented to cause disease in humans (Schuster and Visvesvara 2004). *Naegleria fowleri* occur in warm water bodies and may cause primary amoebic meningoencephalitis (Blair *et al.* 2008; Laseke *et al.* 2010).

At present, several symbiotic and pathogenic interactions between bacteria and free-living protozoa or even higher organisms (e.g. copepods) are described (Bichai *et al.* 2008; Thomas *et al.* 2009). For example, increased numbers of the protozoan parasites Naegleria spp. generally go along with a contamination of the water with coliforms. Some species of protozoa were shown to interact with *Legionella* spp., and might play a role in its distribution (Visvesvara and Stehr-Green 1990). In many cases the human pathogenic species *L. pneumophila* was identified as the bacterial endosymbiont (Rowbotham 1980; Barker and Brown 1994; Park *et al.* 2003). This aspect adds a new perspective to protozoan pathogenicity and should be considered for the determination of waterborne protozoan diversity. However, high numbers of bacterial infected amoebae were, so far, found only in cooling towers, but not in natural environments (Berk *et al.* 2006).

3.4 FUNGI AND HELMINTHS

There is little information regarding groundwater and fungi in general (Schlosser 2011) and to date pathogenic fungi have not been implicated in waterborne outbreaks. Hence water works appear to have only little expertise with waterborne fungi (Paterson *et al.* 2009). Current control strategies and water treatment against fungi are poorly understood, and the few studies conducted are inconsistent (Niemi *et al.* 1982; Nagy and Olson 1985; Frankova and Horecka 1995; Kelley *et al.* 2003). Chemical coagulation with iron or sand filtration are currently discussed as the most promising removal strategies of fungi during water treatment while chemical disinfection of fungal spores was found to be most effective with chlorine dioxide and ozone (Niemi *et al.* 1982; Kelley *et al.* 2003). Also the group of helminths include waterborne pathogens that potentially cause human disease. However, parasitic helminths, e.g. *Ascaris lumbricoides*, are typical pathogens in surface water of developing countries. Their transmission into groundwater is unlikely, due to the size of the organism and their eggs, and if so, they are easily removed from raw water during drinking water production by filtration (Ashbolt 2004; Levantesi *et al.* 2010).

4. TRANSPORT AND ATTENUATION

Potential entry of pathogenic microorganisms and viruses into groundwater strongly depends on the vulnerability of the aquifer which is assigned by several parameters regarding thickness, structure and activity of the soil and vadose sediment layers covering the aquifer, the hydrological regime and the type of contamination source (Harter and Walker 2001; Chilton 2006). Preferential flow and transport pathways such as fractures, fissures, and heterogeneities in soil and porous sediments, as well as abandoned boreholes substantially increase the risk of pathogens entry into an aquifer (Cronin *et al.* 2003).

4.1 SOILS AND THE UNSATURATED SEDIMENTS

Soils and unsaturated sediments are effective natural barriers constituting mechanical and biological filters. A pathogen that enters unsaturated soil and sediments is challenged with a multitude of complex processes (Ginn *et al.* 2002). In the uppermost layers filtration and adsorption constitute important attenuation mechanisms (Pekdeger and Matthess 1983; Yavuz Corapcioglu and Haridas 1984). The effect of filtration is mainly related to the sediment grain size distribution (Jin *et al.* 2000; Chu *et al.* 2001). Fine sediments with lots of dead end pores support effective straining of microorganisms and viruses. Maybe the most important process is adsorption to soil particles and sediment surfaces. It is mainly determined by the hydrophobicity of the sediment, soil and sediment moisture, the pH, the ionic strength and composition of the soil water (Dizer *et al.* 1984). Moreover, cell wall properties of pathogens are a crucial factor. Adsorption may be irreversible or reversible (Gerba 1984). Recharge of high amounts of rainwater may lead to a lower ionic strength of the pore water due to dilution potentially causing the release of formerly attached pathogens (Carlson *et al.* 1968; Duboise *et al.* 1976; Landry *et al.* 1979; Bales *et al.* 1993).

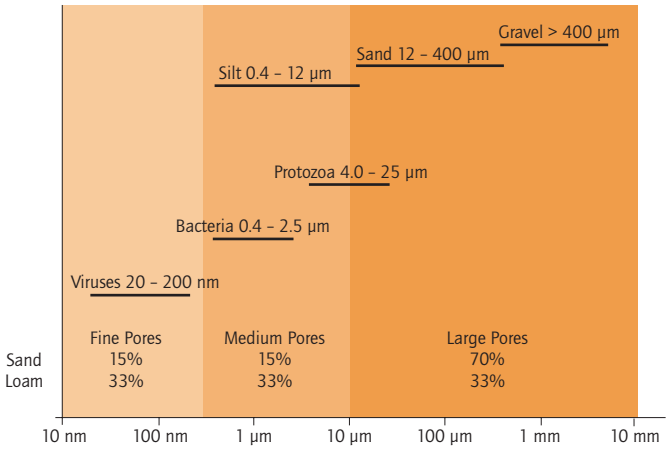
Not surprisingly, the size of pathogens matters for its transport and attenuation (Tab. 2, Fig. 2). As shown for bacteriophages, larger particles exhibited a better retardation (Aronino *et al.* 2009).

Table 2: Average size ranges of selected microorganisms and viruses.

CLASS	MICROORGANISM	SIZE
Virus	Bacteriophage	0.02-0.2 µm diameter
	Poliovirus	0.03 µm diameter
Bacteria	Bacterial spores (*Bacillus*, *Clostridia*)	1 µm
	E. coli	0.5 µm x 1.0 µm x 2.0 µm
	Salmonella typhi	0.6 µm x 0.7 µm x 2.5 µm
	Shigella spp.	0.4 µm x 0.6 µm x 2.5 µm
Protozoa	*Cryptosporidium oocysts*	4.0-6.0 µm diameter
	Giardia	7.0-14.0 µm diameter
	Enteroamoeba histolitica	20-25 µm diameter

Resource: Adapted from Pedley *et al.* 2006.

Fig. 2: Size range of pathogens compared to aquifer matrix characteristics (at the top pore size ranges of silt, sand and gravel are shown).



Resource: Adapted from Matthess and Pekdeger 1981.

Table 3: Factors affecting transport and attenuation of microorganisms and viruses in groundwater.

CHARACTERISTICS OF THE PATHOGEN	AQUIFER PROPERTIES
Size	Groundwater flow velocity
Shape	Dispersion
Organism Type	Pore size (intergranular or fracture)
Cell motility	Kinematic/effective porosity
Density	Organic carbon content (solid)
Growth phase	Temperature
Surface charge	Chemical properties of groundwater (ionic strength, pH, etc.)
Inactivation rate (die-off)	Mineral composition of aquifer/soil material
(Ir)reversible adsorption	Predatory microflora (bacteria, protozoa, fungi, algae, etc.)
Physical filtration	Moisture content Pressure

Resource: Adapted from West *et al.* 1998.

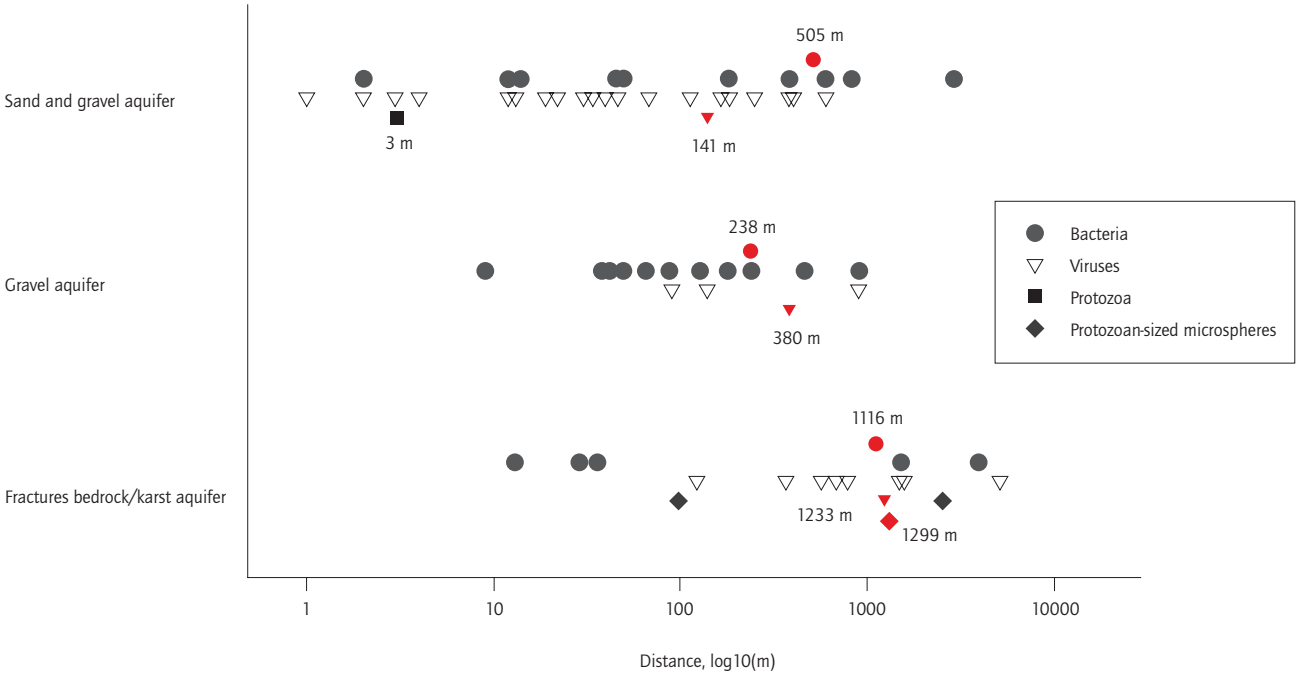
Further factors taking influence on the transport and attenuation of pathogens through the unsaturated zone include the sediment mineral composition, the organic matter content and the biological activity (Tab. 3). Global change, including new climatic dynamics (irregular heavy storm and rain events, long-term drought periods) as well as a change in land use may locally or even regionally lead to a deterioration of natural attenuation capacity of soils (Foster and Chilton 2003; Morris *et al.* 2003).

In most studies the soil zones and the unsaturated sediments turned out to carry a higher attenuation capacity when compared to the saturated subsurface (Lance and Gerba 1984; Powelson *et al.* 1990; Powelson and Gerba 1994; Chu *et al.* 2001; Chu *et al.* 2003). To give one example, infectious viruses were reduced by 8 orders of magnitude in dune sand within 30 m transport distance (equalled 25 d of travelling) from the source at the surface. The same degree of reduction took a 40 d travel distance when injected to a deeper saturated subsurface at the same site (Schijven 2001).

4.2 THE SATURATED ZONE

Arrived in the saturated zone, pathogens are distributed by advection, dispersion and diffusion (Harvey 1993; Sen 2010). Aquifer porosity and water flow velocity contribute two important variables determining the attenuation and transport of pathogens in aquifers (Taylor *et al.* 2004; Pang 2009). In this context, the type of aquifer, porous, fissured or karst, which is strongly related to groundwater flow velocities, significantly affects the travelling distance of the pathogenic agents (Tab. 3, Fig. 3).

Fig. 3: Transport distances of pathogens in different aquifer matrices. The average travel distances are represented by red symbols. Data collected from the literature.



Best attenuation of pathogens is achieved in porous sediments (Logan *et al.* 2001; Stevik *et al.* 2004). Clay, silt and fine sand deposits are characterized by flow velocities of only centimetres to decimetres per day. Middle sand, coarse sand and gravel-cobble aquifers typically exhibit high groundwater flow velocities in the range of meter to tens of meters per day (Matthess and Pekdeger 1985; Chilton and Seiler 2006). Water flow in fissured aquifers exhibits a strong variation with less than a meter per day to hundreds of meters per day. Highest flow velocities and longest travel distances for all pathogens are found in the big channels of karst aquifers often with thousands of meters a day (Matthess and Pekdeger 1985; Fig. 3). Therefore, karst aquifers are extremely susceptible to contamination due to their low filtration capacity (Leibundgut 1998; Kačaroğlu 1999; Goeppert and Goldscheider 2010). Consequently, waterborne disease outbreaks are often related to non-porous media aquifers, such as fractured bedrock (metamorphic rock) or karst (limestone) (Fong *et al.* 2007). However, also in porous aquifers microbes and viruses may travel long distances of several hundred meters (Fig. 3). This can, at least partly be explained by the pronounced structural heterogeneity of aquifers. Surprisingly, in porous sandy aquifers the average travel distance of bacterial cells is almost 4-times higher compared to the smaller viral particles (Fig. 3). Reduced retardation of larger particles can be explained by the phenomenon of pore-size exclusion in porous sediments which obviously not applies to coarse gravel aquifers as smaller viruses travel furthest under these conditions (Fig. 3; Sinton *et al.*, 2000).

Retention of pathogens in porous aquifer systems depends on mainly the same physical, chemical and biological parameters already discussed for the unsaturated zone. The extent of retardation of pathogens is highly dependent on water chemistry and surface charge of both the aquifer matrix and the cells of the pathogens (e.g. Thurston *et al.* 2001; McCarthy and McKay 2004; Branford *et al.* 2005). Additionally, further cell- and particle-specific factors such as the physiological state (e.g. starvation) or growth conditions can affect bacterial transport behaviour (Yang *et al.* 2006; Haznedaroglu *et al.* 2008; Haznedaroglu *et al.* 2009).

Direct comparison of the transport of *E. coli* and *Campylobacter jejuni* in saturated porous media revealed a very species-specific transport behavior, mainly due to differences in cell properties (Bolster *et al.*, 2006). Transport characteristics of both organisms were analyzed taking into account their cell geometry, hydrophobicity and electrophoretic mobility. *C. jejuni* exhibited a significant greater negative surface charge than *E. coli*. Thus, *C. jejuni* was removed more efficiently by metal-oxide-coated sands (Ø 250-350 µm). In contrast, attenuation of *E. coli* cells was more pronounced in all setups with uncoated quartz sand, resulting in greater transport distances of *C. jejuni*. This was the case, although cells of *C. jejuni* were slightly longer, narrower, and less spherical than *E. coli* cells. This contradicts what was repeatedly found in other studies, that a spherical cell shape favors transport in porous media (Weiss *et al.* 1995; Dong *et al.* 2002; Salerno *et al.* 2006; Bolster *et al.* 2009). These exemplary results are of great significance as they demonstrate that pathogenic *C. jejuni* might be present in zones of aquifers not reached by the indicator organism *E. coli*. Even within different strains of mammal *E. coli* isolates a large diversity in transport behaviour was observed (Bolster *et al.*, 2009). Again, this was related to a strain-specific surface charge and hydrophobicity. Foppen *et al.* (2010) analyzed the transport behaviour of 54 different *E. coli* strains in terms of their outer membrane lipopolysaccharide (LPS) composition under identical flow conditions in saturated quartz sand. The attachment efficiencies varied by two orders of magnitude within the strains tested. No single factor out of all cell characteristics tested (cell shape, motility, surface charge, cell aggregation, LPS composition, variation in gene expression) was of statistical significance to solely explain variations in attachment and transport. Unfortunately, most studies on bacterial transport have been conducted in columns packed with quartz sand. Recent studies including *E. coli* O157:H7, as well as other pathogenic bacteria, *i.e. Yersinia enterocolitica* and *E. faecalis*, revealed that attachment efficiencies calculated for *E. coli* and *E. faecalis* were lower in columns packed with loamy agricultural sand when compared to a setup with quartz sand (Schinner *et al.* 2010). Hence, it is questionable to what extent experiences from laboratory studies can be used to predict attenuation and transport behaviour of pathogens in different environmental compartments (Unc and Goss 2004).

The attenuation of viral particles in the saturated zone is also strongly affected by virus particle properties, e.g. the varying content of hydrophobic substances in their capsids and varying isoelectric points (Powelson and Gerba 1994). Filtration efficiency, as was shown for non-infectious recombinant noroviruses, is highly dependent on the nature and magnitude of electrostatic interactions that develop between the virus particle and the matrix (Redman *et al.* 1997). High amounts of dissolved humic substances and organic matter in general compete with viruses for hydrophobic sites at sediment surfaces (Dizer *et al.* 1984; Lance and Gerba 1984). Consequently, the risk of potential entry into aquifers and long distance transport of pathogenic viruses, as shown for *poliovirus*, are increased at high organic matter conditions (Gerba *et al.* 1975; Farrah *et al.* 1978; Powelson *et al.*

1991). Comparative adsorption studies on human enteroviruses, simian rotavirus and five bacteriophages underlined, similar to pathogenic bacteria, that adsorption behaviour is highly strain specific (Goyal and Gerba 1979).

Although adsorption in sediments tends to be very effective in retarding the major mass of virus particles within only decimeters to meters of unsaturated soil and saturated aquifer sediments, numerous exceptions of successful viral entry into groundwater systems have been documented. Finally this is a matter of the source virus concentration. As demonstrated for the model virus PRD-1, a strong decrease in abundance was observed within 1 to 5 meters in uncontaminated and contaminated sediments, respectively. However, a small part of the virus populations continued to migrate with groundwater flow without further decrease in concentration (Blanford *et al.* 2005).

Studies on *C. parvum* transport in saturated porous media showed that retardation of protozoan oocyst is a complex process that is very sensitive to water ionic strength and flow velocity. Again, the sediment grain size was found a critical factor (Kim *et al.* 2010). These results underline that retardation of protozoan oocysts generally is very efficient in fine-grained sediments (Fig. 3). Several studies demonstrated that protozoan oocysts from *C. parvum* are efficiently removed from water and wastewater by sand filtration (Chapman and Rush 1990; Timms *et al.* 1995; Logan *et al.* 2001). A significant amount of initially deposited oocysts, however, might be remobilized due to time-dependent detachment (Harter *et al.* 1999).

Transport of *Giardia cysts* through aquifer sands also seems to be mainly controlled by straining. A hundred percent retention of *Giardia cysts* was observed in sediment columns packed with fine sand (Ø 150 µm). A recovery of only 1.8% was found with coarse sand (700 µm). The amendment of manure suspensions, surprisingly, resulted in a strong increase in oocyst concentration in the effluent varying from 75 up to 172%. These results highlight that many pathogen transport studies neglecting the simultaneous presence of manure (as the source) are in risk to underestimate real oocyst transport distances in manure-contaminated sediments (Bradford *et al.* 2006).

5. SURVIVAL AND GROWTH

Terrestrial and aquatic environments are generally hostile habitats for most human pathogenic microorganisms and viruses, and fortunately an efficient decay based on physical, chemical and biological retardation, inactivation and destruction of pathogens takes place. Environmental factors, such as exposure to light (at the surface), water characteristics including temperature, pH and ionic strength as well as the adsorption of pathogenic agents to soil and sediment particles are currently considered main factors affecting pathogens survival (Keswick and Gerba 1980; Fattal *et al.* 1983; Gerba 1983; Yates *et al.* 1985; Burkhardt *et al.* 2000). Since the decay of pathogenic agents at the generally low temperatures in the subsurface is suggested to be slow, physical and chemical attenuation processes are of major importance (Riemer 1983; Matthess and Pekdeger 1985; Filip *et al.* 1986). The factors that lead to the destruction of pathogenic agents in aquifers are hardly known, but may be mainly based on biological activities, *i.e.* microbial antagonism (Tab. 4; Keswick and Gerba 1980; Yates *et al.* 1987; Hurst 2000).

In surface waters the majority of pathogenic microorganisms and viruses are readily inactivated when exposed to solar radiation (visible light and UV) (Brookes *et al.* 2004). Two other factors which effectively contribute to pathogen decay at the surface are temperature and desiccation (Tab. 4). By contrast, after infiltration into the subsurface transport, attenuation and survival of pathogens are mainly influenced by attachment and sorption to soil and sediment particles (Buchan and Flury 2008). However, adsorption to soil particles does not necessarily result in inactivation of the pathogen.

Table 4: Factors that influence the survival of pathogens in the subsurface.

FACTOR	INFLUENCE
Temperature	Long survival at low temperatures, rapid die-off at high temperatures. For some faecally-derived bacteria high temperatures might give rise to growth.
Moisture content	Desiccation is detrimental to most microorganisms (spores excepted). An increased rate of reduction will occur in drying soils. This is of most relevance in the unsaturated zone.
Sunlight	More rapid die-off at the soil surface due to UV irradiation.
pH	Bacteria die-off more rapidly in acid soils (pH 3-5) than in alkaline soils. The pH influences the adsorption of microorganisms and viruses to the soil matrix and indirectly influences survival.
Microflora	Soil bacteria and fungi may produce exo-enzymes that damage the structure of faecal microorganisms, while amoebae and other protozoa may feed on them. Bacterial survival is shorter in natural soils than in sterilised soils, but for viruses no clear trend is observed.
Organic carbon content	The presence of organic carbon increases survival and may give rise to the regrowth of bacteria.
Cations	Certain cations have a thermal stabilising effect on viruses and increase virus survival. Cations also enhance virus adsorption to soil and this indirectly increases survival, as viruses appear to survive better in the adsorbed state.

Resource: Adapted from Gerba 1984.

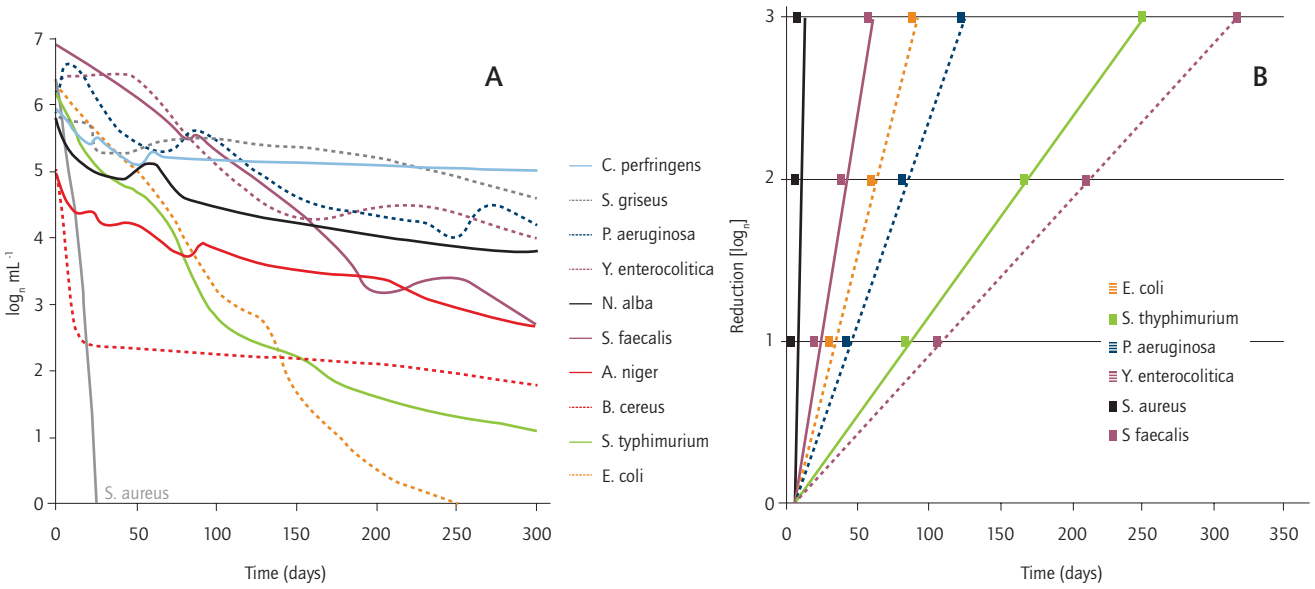
Pathogen survival in groundwater has been studied in various experimental setups including laboratory batch and microcosm experiments and incubation of pathogens in groundwater using membrane chambers or dialysis tubes. However, although most of these studies mimic the situation in aquifers in various ways, the data obtained for pathogen survival need to be critically interpreted in terms of survival rates in the environment.

5.1 SURVIVAL OF BACTERIAL PATHOGENS

There is a pile of literature on the survival of pathogenic bacteria in the aquatic environment. Survival periods reported are very group-, species- and strain-specific and last, in exceptional cases, for up to several years (e.g. spores). Moreover, there are pathogenic bacteria which have been reported to reproduce in soils and aquatic environments as well as in water distribution systems and house water installations. In the following, the pathogen-specific variations and factors related to survival and growth are described by selected examples.

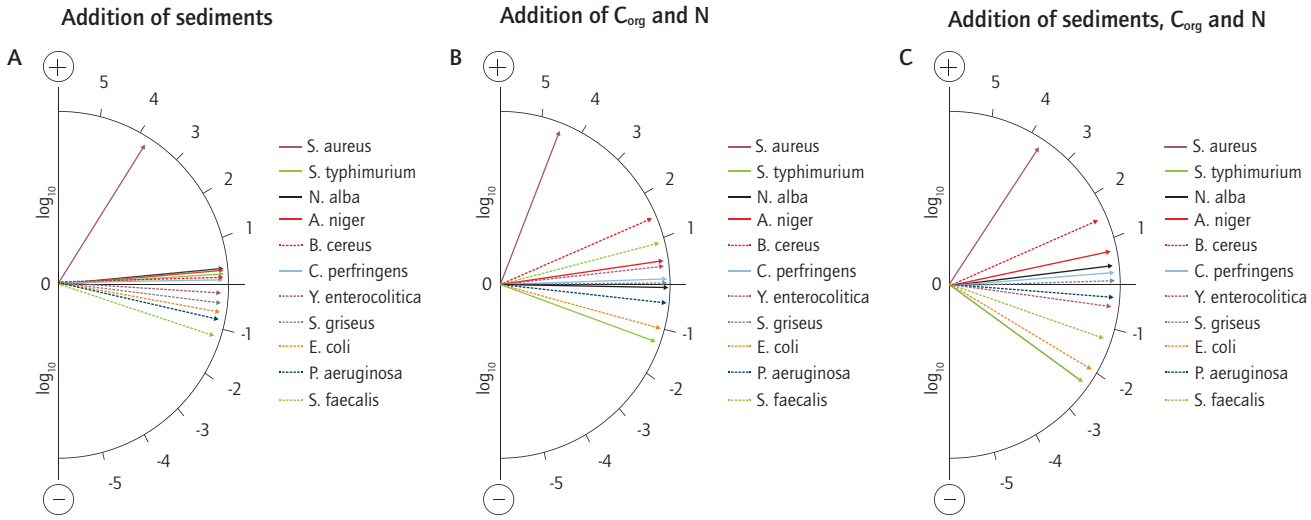
In the framework of an interdisciplinary research project funded by the German Federal Environment Agency (UBA), several working groups evaluated the effectiveness of the 50 days protection zone, as it is applied in Germany to safeguard drinking water production (see section 6). In a series of batch and column experiments numerous model microorganisms (bacteria and fungi) and viruses were tested for their persistence in sterile and non-sterile groundwater (Filip *et al.* 1986).

Fig. 4: Survival (detectability) of various model microorganisms in groundwater (pH = 7.3, O_2 = ≥ 0.8 mgL-1, DOC = 3.4 mgL-1, PO_4 = 0.25 mgL-1) batch experiments at 10±1 °C lasting for 300 days. *Escherichia coli*, *Salmonella thyphimurium*, *Pseudomonas aeruginosa*, *Yersinia* enterocolitica, *Staphylococcus aureus*, *Streptococcus faecalis*, *Bacillus cereus*, *Clostridium perfringens*, *Streptomycos griseus*, *Nocardia alba*, *Aspergillus niger*.



Resource: Data compiled from Filip *et al.* 1986.

Fig. 5: Effect of sediment and nutrient (organic carbon [C_{org}; DOC = 1.1 gL-1] and nitrogen [NO_3 = 50 mgL-1] onto the survival (detectability) of various model microorganisms in groundwater batch experiments at 10±1 °C lasting for 100 days. The final concentration of microbes in unamended batch bottles containing only groundwater served as reference. The experiment lasted for 100 days. For the effects observed with the originally three different sediment treatments (addition of middle sand fraction, coarse sand fraction and natural sand) we depicted a mean value. In case of *Staphylococcus aureus*, 25 d data and 30 d data have been extracted for (A) and (B, C), respectively. Also in case positive as well as negative effects have been found with different sand fractions, a mean trend is given. For persistence and disappearence of the various strains tested in unamended groundwater see Fig. 4. Arrows pointing in the positive (+) direction indicate growth, those directed in the negative (–) direction indicate cell inactivation and decay. *Escherichia coli*, *Salmonella thyphimurium*, *Pseudomonas aeruginosa*, *Yersinia* enterocolitica, *Staphylococcus aureus*, *Streptococcus faecalis*, *Bacillus cereus*, *Clostridium perfringens*, *Streptomycos griseus*, *Nocardia alba*, *Aspergillus niger*.



Resource: Data compiled from Filip *et al.* 1986.

As can be seen in Fig. 4A, when starting batch incubations with cell densities of 1 x 10^5 to 1 x 10^7, almost all strains tested could still be detected after 300 days at 10°C in groundwater. Only *Staphylococcus aureus* and *Bacillus megaterium* (data not shown) readily disappeared decreasing in numbers by more than six orders of magnitude within 15 to 25 days. *E. coli* fell below detection after 250 days (Fig. 4A). For *Bacillus cereus* an initial decrease was observed which then labelled off for the residual time of the experiment. A similar pattern was observed for *Clostridium perfringens*, which was most probably caused by spore formation in both strains (Filip *et al.* 1986). The decay rates for selected strains are shown in Fig. 4B. The results of Filip *et al.* (1986) are in agreement with findings from others. Weber *et al.* (1982) reported the persistence of *E. coli*, Y. enterocolitica, P. aeruginosa and other enterobacteria of more than 300 days in cold drinking water (4°C).

Filip *et al.* (1986) further tested the effect of adding (1) sediment of varying grain size, (2) organic carbon (1.1 gL-1) and nitrate (50 mgL-1), or (3) a combination of both, sediment and substrate, to the batch experiments. After an incubation time of 100 days it was found that the presence of sediments resulted either in no or only a slight reduction of suspended microorganisms when compared to the numbers incubated in groundwater only (Fig. 5). With a few strains a slight increase in suspended microbes were observed. The combination of both treatments revealed growth for some strains, while others decreased faster in numbers in the presence of C_{org}, N, and sediments (Fig. 5C).

The set of treatments allows drawing some conclusion on factors influencing the survival, decay or growth of pathogenic microbes, which is discussed exemplarily for selected strains. *E. coli*, for example, showed a faster disappearance from the water phase with both treatments which 'added up' when combined (Fig. 5C). This is contradicting other studies, which postulated longer survival times for *E. coli* when adsorbed to particles. In most cases, extended survival times were detected in sediments when compared with overlying waters (Merkli 1974; Gerba and McLeod 1976; Filip *et al.* 1983). No 'additive' effect could be observed in the experiments of Filip *et al.* (1986) for *Streptococcus faecalis*, *i.e.* the addition of C_{org} and N allowed longer survival, while the presence of sediment significantly reduced the cell numbers in the water phase. When combining both treatments,

the sediment effect was found determining (Fig. 5C). Although the experiments of Filip and co-workers revealed very interesting findings with respect to the survival of pathogenic bacteria and fungi in groundwater, the mechanisms behind the decrease or increase of cell numbers are not clearly understood.

It was believed for a long time that enteric bacterial pathogens are not able to grow outside its human host (Burgess 1998), and growth and replication of pathogens in the environment is the exception but not the rule (Camper *et al.* 1991; Pedley *et al.* 2006). However, there is growing evidence that individual pathogenic bacteria are able to reproduce in soil and aquatic systems. This has been shown, for example, for *Vibrio cholerae* (strain O1 Ogawa Eltor) und *E. coli* (strain O157) which grew in sterile oligotrophic river and pond water (Vital *et al.* 2007; Vital *et al.* 2008). Growth of both strains was positively correlated with temperature. *Vibrio cholerae* even showed a positive growth rate in non-sterile waters (Vital *et al.* 2007), which allows the conclusion that growth of specific pathogens in groundwater can not be ruled out.

5.2 PERSISTENCE AND PROLIFERATION OF PATHOGENIC VIRUSES

Viruses are particles without own metabolism. In that way they may be compared to resting stages of bacteria and protozoa, such as spores and cysts, respectively. This may also explain the comparably long persistence of pathogenic viruses in the environment (Fig. 6). At the surface, irradiation is an important factor promoting viral inactivation and decay (Fattal *et al.* 1983; Sinton *et al.* 2002; Brookes *et al.* 2004). Another important parameter is temperature, while low temperatures support a long persistence (Yates *et al.* 1985). Consequently, at typical groundwater temperatures of ≤ 15°C viruses may survive and stay infectious for several hundred days (Fig. 6). In groundwaters, examined all over the US, only temperature, out of several physical-chemical parameters measured, significantly correlated with the survival or inactivation rate of viruses. Similar observations were made for viral decay in soils (Yeager and O'Brien 1979).

Among enteric viruses, members of the familiy *Adenoviridae* are known to be very stable in natural aquatic environments. The same seems to be true for *poliovirus* as early studies described enormous stability of infectious particles in cold groundwater for up to 550 days (Althaus 1983; Fig. 6). However, in comparative tests, adenovirus serotypes 40 and 41 were found much more stable in 4°C tap water than hepatitis A and *poliovirus* 1. Prolonged incubation at 4°C revealed a total persistence time of 304 days for serotype 41 (Enriquez *et al.* 1995). These results demonstrate common discrepancies regarding persistence data most likely due to experimental variations. Recent studies on adenovirus serotype 2 (AdV2) repeatedly assessed viral survival in groundwater via simultaneous analyses of each sample by both a cell culture assay (infective particles) and real-time reverse transcription (RT)-PCR (total particles) (Charles *et al.* 2009; Ogorzaly *et al.* 2010). AdV2 survived at least 364 days in groundwater at 12°C and viral DNA was detectable for 672 days of the 728 day monitoring period (Charles *et al.* 2009).

By contrast to adenovirus, to date no adequate cell culture assay exists to determine the number of infectious particles of human pathogenic noroviruses (Duizer *et al.* 2004a). The detection of viral nucleic acid in groundwater samples by quantitative RT-PCR is helpful but does not reveal information on infectivity (Duizer *et al.* 2004b). Thus, during the last years several culturable viral surrogates (e.g. murine norovirus, feline calicivirus, *poliovirus* or phage MS2) were tested with respect to inactivation rates and persistence in water. In these studies murine norovirus turned out to be the most promising candidate as a human norovirus surrogate, as both viruses revealed comparable nucleic acid stability in surface water (25°C) and groundwater (4°C) (Kadoi and Kadoi 2001; Allwood *et al.* 2003; Wobus *et al.* 2006; Bae and Schwab 2008). However, up to now data on survival of human noroviruses in groundwater and drinking water are sparse and partially contradictory (Beuret *et al.* 2002; Lamothe *et al.* 2003; Sanchez *et al.* 2005; Ngazoa *et al.* 2008; Charles *et al.* 2009).

Survival of enteroviruses, including coxsackie- and *polioviruses*, at low temperatures was shown for weeks to months in shallow groundwater (Wellings *et al.* 1975) and also exposed to deep groundwater (Keswick *et al.* 1982; Bitton *et al.* 1983). Filip *et al.* (1986) tested survival of selected model viruses, *i.e. coxsackievirus* A9, echovirus 7 and *poliovirus* 1 in groundwater in laboratory batch experiments at 10°C. It was observed that the virus titer dropped by only 2 to 3 orders of magnitude within 260 days. All three of the tested viruses showed at the end of the experiment higher numbers in the controls (autoclaved groundwater), pointing at antagonistic effects of the natural groundwater microbial community.

Further factors discussed to influence the survival and decay of pathogenic viruses in aquatic environments, include the concentration of dissolved oxygen and redox conditions. A removal study with bacteriophages ΦX174 and MS2, two surrogates for hu-

man pathogenic viruses, revealed that removal during transport through an anoxic aquifer was considerably lower compared to that in oxic aquifers due to lower inactivation and adsorption rates. One explanation for higher inactivation rates in aerobic sediments might be the presence of ferric oxihydroxides resulting in higher adsorption rates of pathogenic agents (Abudalo *et al.* 2005). Consequently the authors suggest to differentiate between oxic and anoxic aquifers regarding to groundwater protection and to extend the microbial protection zones around anoxic aquifers (Van der Wielen *et al.* 2008).

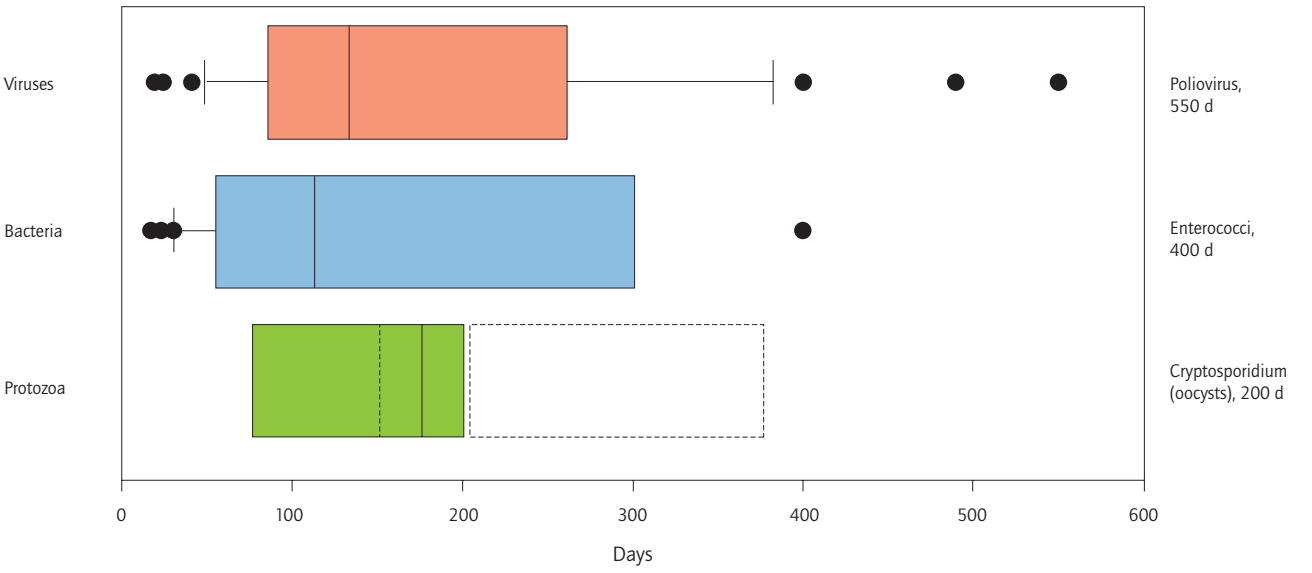
Studies on different enteric viruses and bacteriophages also underlined the role of adsorption as controlling factor in viral survival (Hurst *et al.* 1980). Noteably, most of the studies published took into account only the virus fractions suspended in the water phase. What really happens to virus particles adsorbed to sediment is generally unknown.

5.3 SURVIVAL OF PATHOGENIC PROTOZOA

Protozoa are able to outlast long periods of time as oocysts which are insensitive to environmental stress and many treatments procedures applied during drinking water production. However, data on protozoan survival in groundwater and subsequent infectivity in humans are sparse as only in a few studies infectious oocysts could be detected in aquifers due to the influx of contaminated surface water (Hancock *et al.* 1998). At the land surface survival of protozoa and their cysts are heavily influenced by temperature, desiccation and freezing, respectively. Drying of *C. parvum* cysts on microscope slides for more than 2 h at room temperature resulted in 100% death (Robertson *et al.* 1992). In soil and sediments protozoan oocysts are efficiently retarded by filtration and adsorption. Inactivation rates of *Cryptosporidium* in surface water matrices and sterile river water were found to be very low for a temperature range of 5 to 20°C (Robertson *et al.* 1992; Medema *et al.* 1997; Chauret *et al.* 1998). *Cryptosporidium* oocysts that were held in sterile water at 15°C remained infectious in mice and cell cultures for 7 months (Fayer 2004). For groundwater at 5°C, survival times of protozoan cysts were calculated to range between 176 days and more than 200 days (Fig. 6; Robertson *et al.* 1992; Ives *et al.* 2007).

The results obtained on survival and persistence of pathogenic bacteria, viruses, and protozoa in natural groundwater need to be viewed and interpreted critically, before transferred to the situation *in situ*. First, the various experiments conducted in the lab and in the field strongly differed according to their experimental setup, model pathogens used, sampling and detection protocols applied. Jansons *et al.* (1989) nicely demonstrated the significant difference of seeded vaccine strains or natural viral isolates in terms of sorption and inactivation. Second, in most studies, the focus was only on the suspended fraction of pathogens. Rarely

Fig. 6: Survival times of pathogens in groundwater and drinking water. Only survival times in the temperature range between 4 and 15°C were summarized from the literature. Data of protozoan survival in groundwater are scarce (green box) and do not reflect general survival times of oocysts in the environment. To better illustrate the high environmental persistence of oocysts, also published experimental data from different water types have been introduced (dashed box).



the sediment associated (adsorbed, attached) fraction was further evaluated for its long-term fate.

5.4 MICROBIAL ANTAGONISMS

It was repeatedly suggested that microbial activity and competition with autochthonous microorganisms, summarized here as microbial antagonism, supports a faster decay of pathogens in the environment (Keswick and Gerba 1980; Althaus *et al.* 1982; Filip *et al.* 1986; Yates *et al.* 1987; Hurst 2000). Processes may include grazing of bacteria and viruses by protozoa, bacterial endoparasitisms, bacterial production of toxins and lysogenic enzymes. Althaus *et al.* (1982) provided a comprehensive summary of early literature on microbial antagonism. However, the question if such processes are of relevance in groundwater could so far not be satisfactorily answered.

An early study of Bagdasaryan (1964) showed that virus inactivation was greater in intact soil compared to sterile soil incubations while others, 16 years later, found no significant differences between active and inactive, as well as aerobic and anaerobic set-ups (Hurst *et al.* 1980). Very often, the fact that survival of viruses and bacteria is longer in sterile waters or waters of low microbial activity was quoted as direct evidence for antagonistic interactions (McCoy and Hagedorn 1979; Matthess and Pekdeger 1985; Althaus 1983). Similarly, faecal pathogens, such as *poliovirus* 1 and *E. coli*, were found more stable in groundwater than in sea and river water, which was attributed to the higher microbial activity in surface waters (Bitton *et al.* 1983). Microbial activity is directly influenced by the concentration of organic matter and nutrients, temperature, oxygen and light. These factors also have been shown to have a direct influence on the decay of individual pathogens. For example, correlations between increased virus inactivation rates, higher activity of indigenous microorganisms in groundwater, and the water oxygen concentration have been observed (Yates *et al.* 1988; Jansons *et al.* 1989; Gordon and Toze 2003). Hurst (1988) impressively demonstrated that the presence of aerobic microorganisms significantly influenced virus survival, resulting in a two- to threefold increase of viral inactivation.

Hirsch and Rades-Rohkohl (1983) tested 217 aerobic bacterial isolates from groundwater against *E. coli* K12. More than 20% of the strains showed inhibitory effects to *E. coli*, about 8% showed aggregation, and about 7% stimulated the model pathogen. Cliver and Herrmann (1972) demonstrated the inactivation of Cocksackie virus type A-9 by bacterial (*Pseudomonas aeruginosa*) proteolytic enzymes and the uptake of ^{14}C-label from the virus coat protein by the bacterial cells. Also Lipson and Stotzky (1985) indicated that bacteria may utilize viruses as growth substrates. Nasser *et al.* (2002) reported the effect of different microbial enzyme activities on virus reduction in saturated soil. Low concentrations of protease pronase led also to an inactivation of Cocksackie virus type A-9. *Pseudomonas aeruginosa* extracellular enzymes additionally reduced hepatitis A virus, while *poliovirus* type 1 and MS2 bacteriophages seemed insensitive to these treatments. Virus decay in the environment may be further caused by biological degradation of the viral genome by free nucleases and destruction of viral capsids by proteases (Gerba 1987).

It may further be expected that pathogenic viruses are also reduced by grazing protozoa, similar to natural prokaryotic bacteriophages (Gonzalez *et al.* 1992; Suttle and Chen 1992). Gonzalez and Suttle (1993) estimated that flagellates feeding on viral particles can thus gain up to 9% of the carbon they obtain from ingested bacteria. The authors also indicate that it may need a critical number of bacteriophages to serve as a sufficient carbon source for protozoa. Without doubt, protozoa may also ingest viruses and large viruses might be taken up preferentially (Murray 1995), thus part of the pathogenic viruses can be suggested to be reduced by grazing, what needs to be addressed in future investigations.

5.5 MICROBIAL STRATEGIES FOR A LONG-TERM SURVIVAL

Several behavioural and physiological strategies of bacteria, including encapsulation, attachment and participation in microbial biofilms or a shift to low-activity or dormant stages, successfully contribute to a longer survival in the environment (LeChevallier *et al.* 1988). The embedment of pathogens in microbial biofilms efficiently provides protection against external factors such as water treatment. For unencapsulated *Klebsiella pneumoniae* attachment to glass slides was shown to increase its resistance to chlorination 150-fold (LeChevallier *et al.* 1988). Biofilms in aquatic environments and especially in drinking water distribution systems are places for the long-term persistence of potential pathogens such as *E. coli*, *Legionella*, *Klebsiella* and/or *Pseudomonas* (Banning *et al.* 2003; Taylor *et al.* 2009; Declerck 2010). However, because real biofilms hardly occur in oligotrophic, energy-limited aquifers, numbers of *Legionella* spp. detected in groundwater were always lower than those detected in man-made environments (Costa *et al.* 2005; Diederen *et al.*

2007). *Legionella* species might survive also protected in protozoan amoeba. The human pathogenic *L. pneumophila* proliferates in various amoebal hosts (Rowbotham 1980). Fortunately, so far high numbers of infected amoeba have been found only in cooling towers but not in natural environments. Therefore, this association between bacteria and amoeba might be favoured only under special environmental conditions (Berk *et al.* 2006).

Together with a multitude of natural non-pathogenic bacteria some faecal pathogens developed a unique strategy to survive in oligotrophic, energy-limited conditions. These bacteria, including the species *E. coli*, *E. faecalis*, *V. cholerae*, *Shigella spp.*, and *Campylobacter* spp. may change their physiological stage and enter a low-active or dormant, viable but not culturable state. This state, characterized by a very low content of nucleic acids and significantly smaller cell size, allow the organisms to resist unfavourable conditions (Colwell *et al.* 1985; Rollins and Colwell 1986; Byrd *et al.* 1991; Ravel *et al.* 1995; Rahman *et al.* 1996; Arana *et al.* 1997). Although still alive, partially still infectious or at least able to return to an infectious stage they are no longer detectable by traditional enrichment assays (Oliver 2005).

One of the most persistent bacterial states of dormancy is the survival in spores. The production of spores, as known for species of the genus *Bacillus* and *Clostridium*, allows surviving hostile conditions such as the physical and chemical treatments used in drinking water production. Fortunately, efficient adsorption of *Cl. perfringens* spores to sand particles was shown, however the attachment was reversible for most of the spores dependent on their total concentration (Schijven *et al.* 2003).

6. CONTAMINATION OF DRINKING WATER AND OUTBREAK OF WATERBORNE DISEASES

The top candidates of harmful pathogens in aquatic environments changed during the last centuries due to the improvement of water hygiene. In the 19th century life-threatening bacterial outbreaks including cholera and typhoid fever dominated waterborne diseases (Exner and Koch 2011). Today, and especially in Europe and the USA, human populations are mainly challenged by waterborne gastrointestinal illnesses that are self-limited or curable in most cases of infections. The responsible agents are high infectious and environmental resistant pathogenic viruses including norovirus, rotavirus, adenovirus or members of the enteroviruses, besides individual bacterial pathogens such as *Campylobacter* species or *E. coli* (Domingo and Ashbolt 2010). It is worth mentioning that the risk of infection when consuming contaminated drinking water is 10- to 10,000-fold greater with viruses than with pathogenic bacteria. However, some pathogens of great concern in the past are still serious causative agents for waterborne epidemics. The bacterial pathogen *Vibrio cholerae* was indicated as the causative agent in the first documented waterborne outbreak in 1892 and it seems to be still a serious risk for human health in other regions of the planet as is demonstrated by the current cholera epidemic in Haiti with nearly 210,000 infected individuals and more than 4,000 deaths (CDC 2010).

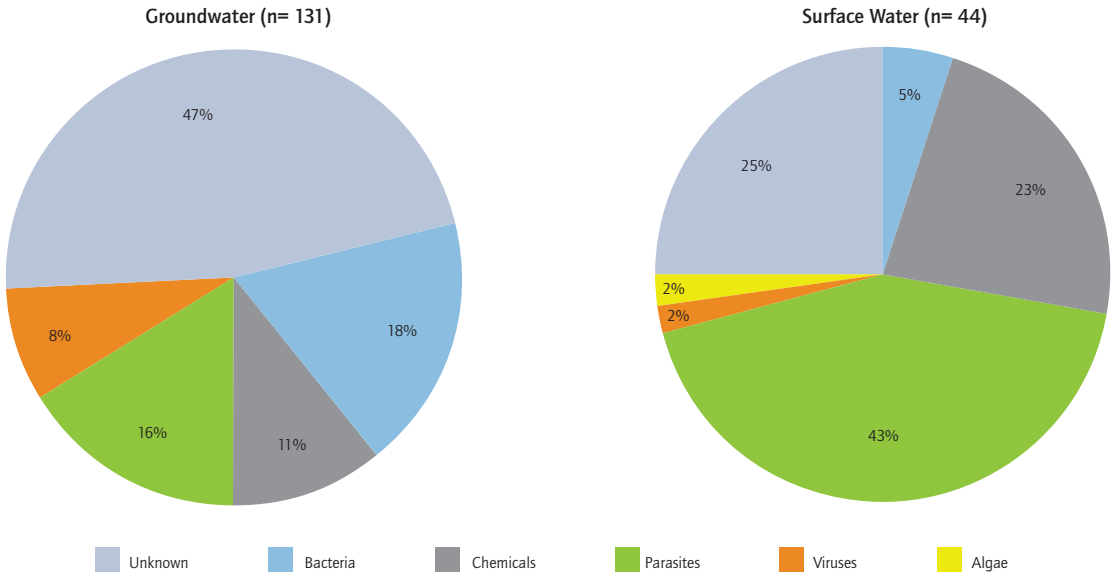
6.1 WATERBORNE DISEASE OUTBREAKS

There is a long history of severe outbreaks of waterborne disease. Historical outbreaks in Germany are documented for typhoid fever (*Salmonella typhi*) in Berlin in 1889, Gelsenkirchen in 1901, Pfortzheim in 1919, Hannover in 1926, and in Neuötting twice in 1946/47 and 1948. The last severe outbreak of cholera in Germany (*V. cholerae*) dates back to 1892 in Hamburg (Althaus *et al.* 1982). A very recent outbreak of dysentery (*Shigella spp.*) took place 1978 in Ismaning (Bavaria) (Althaus 1983). In all cases, the outbreaks were connected to contaminated groundwater and/or drinking water.

Also nowadays pathogens are frequently detected in groundwater and drinking water, and still regular small and even larger disease outbreaks occur in Germany, Europe and all over the world, which can directly or indirectly be related to groundwater and drinking water consumption.

A comparison of etiologic agents and their impact in outbreaks via groundwater or surface water in the United States

Fig. 7: Etiologic agents associated with drinking water outbreaks in the US between 1989-2000 distinguished into the type of source water, *i.e.* groundwater or surface water. Unknown = acute gastrointestinal illness of unknown cause (etiology). n = number of outbreaks traceable to either a groundwater or surface water source. An outbreak is generally defined as two or more people experiencing similar illness traceable to a common drinking water source. The three primary categories of pathogens, bacteria, viruses, and parasites (including protozoa), are highlighted in terms of colour.



Resource: Adapted from the Centers for Disease Control and Prevention, Gerba 2004.

(US) is shown in Fig. 7. The significance of viral transmission via groundwater becomes obvious as the viral contribution in waterborne outbreaks due to groundwater is four-times higher than surface water related outbreaks. Similarly, bacteria are preferentially transmitted via groundwater, while the risk of parasitic (including protozoa) outbreaks is highest with surface water (Fig. 7). It is important to mention that a large portion (47%) of etiologic agents in groundwater is still unknown.

Contaminated groundwater is the most commonly reported source of waterborne disease in the US, associated with 64% of the drinking water outbreaks between 1989 and 2002 (Fong *et al.* 2007). An even increasing tendency of groundwater associated pathogen outbreaks was observed between 2003 and 2006. In these years the National Center for Infectious Disease (CDC) documented 11 pathogen associated drinking water outbreaks in the US and in 82% of all cases contaminated groundwater was found to be the major source (Liang *et al.* 2006; Yoder *et al.* 2008). However, waterborne outbreak documentation is difficult as it is not possible to backtrack all waterborne outbreaks to the relevant water source and outbreaks are often underreported because those infected do not always seek medical care (Craun *et al.* 2006; USEPA 2006).

Some of the recent severe waterborne outbreaks due to pathogenic bacteria, viruses and protozoa in contaminated groundwater are listed in Table 5. Pathogenic pollution of groundwater is often attributed to the failing of septic systems and uncontrolled release of sewage. Moreover, water supply wells in regions with fractured rocks or karst are highly vulnerable to faecal pollution as impressively demonstrated by three major disease outbreaks in Walkerton, Texas and Ohio (Tab. 5; Barwick *et al.* 2000; Worthington *et al.* 2002; Fong *et al.* 2007). A minor norovirus outbreak in Wisconsin in June 2007 also occurred due to highly vulnerable hydrogeological settings as an 85 m deep well in a fractured dolomite aquifer was contaminated by faecal pathogens (Borchardt *et al.* 2010). Settings like this are especially at high risk of contamination during times of snowmelt or irregular heavy precipitation events and flooding as it was also demonstrated by several studies on waterborne outbreaks in Canada (Charron *et al.* 2004; Schuster *et al.* 2005; Thomas *et al.* 2006). Unfortunately, such extreme hydrological events accumulate in the future due to climate change.

Table 5: Recent major groundwater-borne outbreaks.

OUTBREAK/IMPLICATIONS	PATHOGEN	REASON	REFERENCE
Salmonellosis, May/June 1965, Riverside, California, USA; 16,000 infected individuals.	Salm*onella typhimurium*	Unknown source	Boring *et al.* 1971; Report 1971
Gastroenteritis and Hepatitis A, June 1980, Georgetown, Texas, USA; ~ 8,000 (79%) infected individuals.	Enterovirus, Rotavirus, *Coxsackievirus*, Hepatitis A	Leaky sewage line	Hejkal *et al.* 1982
Viral gastroenteritis, March 1981, Eagle-Vail, Colorado, USA; 56 (44%) infected individuals	Rotavirus	Improper working sewage treatment plant	Hopkins *et al.* 1984; Hopkins *et al.* 1985
Bloody diarrhoe, Dec 1989/Jan 1990, Missouri, USA; 243 infected individuals, 4 died.	*E. coli* O157:H7	Unchlorinated water supply	Swerdlow *et al.* 1992
Viral gastroenteritis, April 1994, Finland; up to 3,000 (50%) infected individuals.	Norwalk Virus (Norovirus), Adenovirus, Rotavirus	Spring flood, contamination by polluted river water	Kukkula *et al.* 1997
Diarrhoe, August 1995, Idaho, USA; 82 (35%) infected individuals.	*Shigella sonnei*	Heavy rainfall, improperly drained sewage	CDC 1996
Cryptosporidiosis, spring 1997, North Thames, UK; 345 infected individuals.	*Cryptosporidium*	Contaminated water from treatment works	Willocks *et al.* 1998

Cryptosporidiosis, 1998, Brush Creek, Texas, USA; 1,300-1,500 infected individuals.	*Cryptosporidium*	Spill of raw sewage (karst aquifer)	Bergmire-Sweat *et al.* 1999
Gastroenteritis/Campylo-bacteriosis, May 2000, Walkerton, Ontario, USA; ~ 2,300 infected individuals, 7 died.	*E. coli* O157:H7, *Campylobacter*	Heavy rainfall, cattle manure contamination	Hrudey *et al.* 2003
Gastroenteritis, August 2000, Gourdon, France; ~ 2,600 (37%) infected individuals.	*Campylobacter* coli, Rotavirus, Norovirus	Contamination by agricultural run-off, failure in the chlorination system	Gallay *et al.* 2006
Viral gastroenteritis, summer 2004, South Bass Island, Ohio, USA; ~ 1,450 infected individuals	*Campylobacter* spp., Norovirus, *Giardia* spp., and *Salmonella typhimurium*	Contamination with untreated or partially treated sewage (karst region)	USEPA 2006; Fong *et al.* 2007; O'Reilly *et al.* 2007

In less developed countries with lower sanitation standards there is a clear correlation of climate and groundwater contamination in rural and urban areas. The burden of improper installed on-site sanitation or inadequately protected wells and boreholes is increased by a direct pulse-response between heavy rainfalls and pathogenic contamination (Lewis *et al.* 1982; Lewis and Chilton 1984; Godfrey *et al.* 2005).

Also in the USA a correlation between enteric waterborne outbreaks and precipitation becomes obvious as more than half of the 548 reported outbreaks between 1948 and 1994 were related to heavy rainfalls (Curriero *et al.* 2001).

In industrialized countries, virus infections took over as the most common cause of waterborne disease, mainly gastroenteritis. Meanwhile, norovirus infections are considered to be the most common cause of gastroenteritis in the western world (Besner *et al.* 2011).

Approximately half of all waterborne diseases outbreaks in the US from groundwater consumption are presumed to be of viral origin (Borchardt *et al.* 2007). In 2003, Abbaszadegan *et al.* performed a survey of 448 utility wells in 35 states of the USA. In this study approximately one third of groundwater supplies sampled were found to be positive for infective enteric viruses (including enterovirus, rotavirus, and hepatitis A) or viral indicators as detected by molecular methods (RT-PCR). As the detection limit for the molecular assays is rather high, *i.e.* a minimum of 9.3 virus particles in 1 mL as shown for enteroviruses (Donaldson *et al.* 2002), the real number of contaminated wells can be suggested much higher.

The main causative agents of bacterial gastroenteritis that are characterized by relatively high infectivity are *C. jejuni* and *E. coli* O157:H7, with *C. jejuni* found to be one of the leading causes for acute gastroenteritis worldwide (Swerdlow *et al.* 1992; Allos 2001; Hrudey *et al.* 2003; Besner *et al.* 2011). The infectivity of *E. coli* O157:H7, also known as enterohaemorragic *E. coli* (EHEC) is one of the highest known for bacterial pathogens. As few as 10-100 EHEC cells compared to 500-1000 *C. jejuni* cells and 10^5 *Salmonella* cells can cause infections (Pachepsky *et al.* 2006; Besner *et al.* 2011).

An additional risk may arise in the future as more and more bacterial strains are detected carrying antibiotic resistance (McKeon *et al.* 1995). The analyses of E.coli strains in sewage revealed a broad resistance against several antibiotics, especially in hospital sewage. These strains might cause severe disease when released into the environment (Reinthaler *et al.* 2003). Another potential hazard of antibiotic resistance are antibiotic-resistant enterococci that have been detected in swine manure. With the application of manure to agricultural land, these may enter soils and aquifers and are potentially distributed with groundwater (Sapkota *et al.* 2007). The high risk of water pollution by agricultural land use is highlighted by a comparison of agricultural lands with non cultivated natural areas in Finland. While faecal indicators such as *E. coli* and streptococci where detected in only half of the samples in non cultivated land (up to 100 bacteria per 100 mL) probably originating from wild animals, coliform concentrations in agricultural areas were frequently more than tenfold higher and hence vastly exceeded that of treated wastewater (Niemi and Niemi 1991).

Environmental transmission, mostly due to farm animals, is also relevant for zoonotic protozoan pathogens such as Cryptospiridia and *Giardia*. These pathogens are frequently involved in waterborne outbreaks due to their relatively high infectivity; ingestion of less than 10 oocysts can lead to infection. However, most of these outbreaks were related to contaminated surface waters or groundwater directly influenced by polluted surface water (Hancock *et al.* 1998; Semenza and Nichols 2007; Valderrama *et al.* 2009). Due to the bigger size of protozoan pathogens (oocysts of *Cryptosporidium*, 4-6 µm) compared to bacteria (≈ 1-2 µm) and

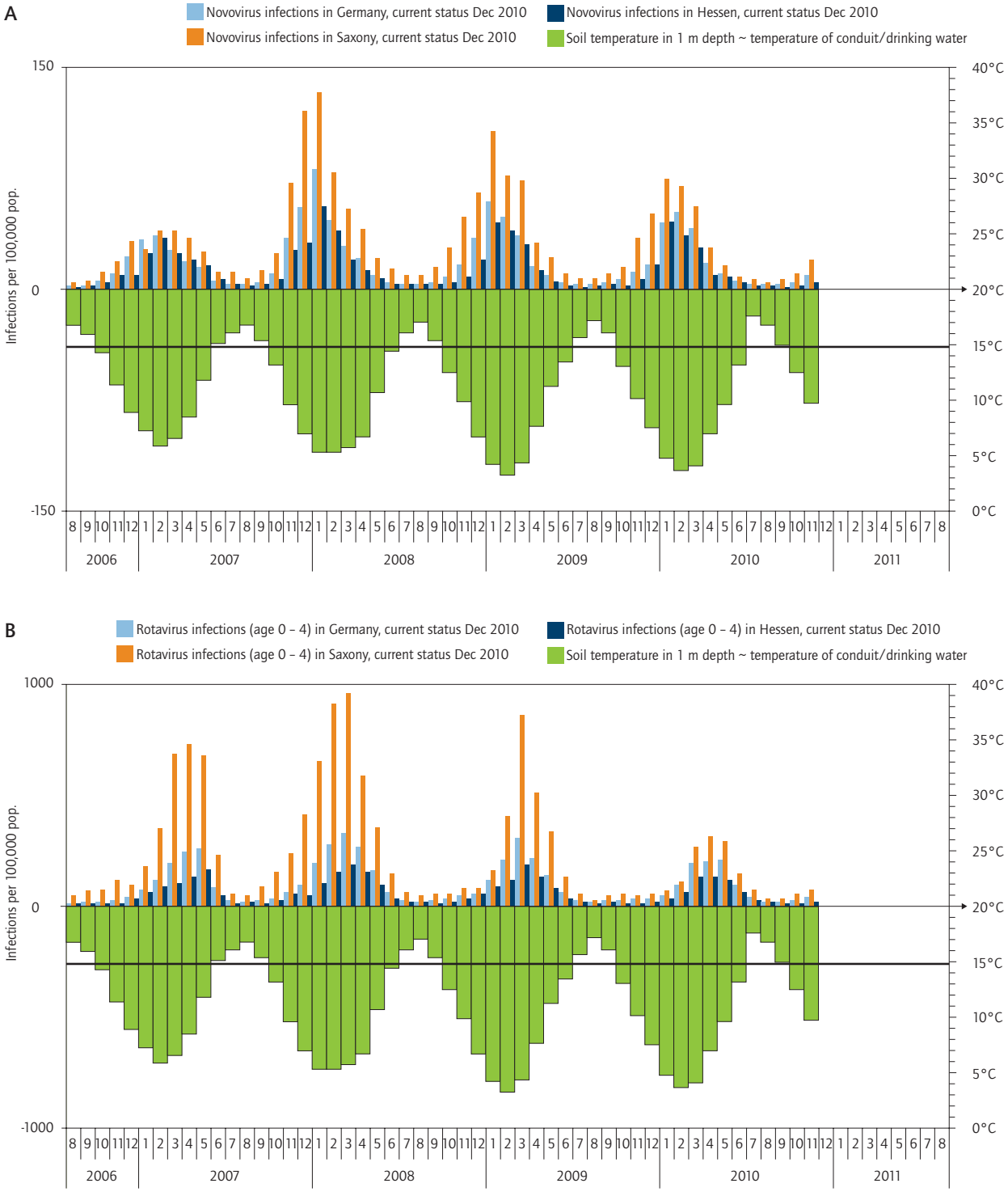
viruses (20-200 nm) well protected groundwater aquifers are generally at low risk with respect to protozoan contamination. However, contaminated or improper treated groundwater contributed 18% of all outbreaks caused by *Giardia* and *Cryptosporidium* (Howard *et al.* 2006; USEPA 2006). In the case surface water is used for drinking water supply and routinely applied water treatment processes as coagulation and filtration fail, cryptosporidiosis may cause severe epidemics as impressively demonstrated by one of the largest waterborne outbreaks in the US history in 1993 in Milwaukee with more than 400,000 infected individuals (Mac Kenzie *et al.* 1994). Highest concentrations of protozoa, e.g. *Cryptosporidium*, are found in urbanised or agricultural waters compared to relatively low concentrations in pristine waters (LeChevallier *et al.* 1991; Rose 1997; Medema *et al.* 2006). To reduce the risk of groundwater contamination in rural areas constructed wetlands have emerged as a promising technology for sanitation of wastewater effluents with low operational maintenance cost (Redder *et al.* 2010).

6.2 SEASONALITY OF PATHOGEN OCCURRENCE

There is some seasonality observed with the occurrence of pathogens in aquatic systems. This seasonality is mainly correlated to water temperature. While the warmer temperatures in late summer go along with *Salmonella*, *Campylobacter* or *E. coli* infections (Kovats *et al.* 2004; Kovats *et al.* 2005; Fleury *et al.* 2006), the cold season is characterized by the occurrence of noro- and rotaviruses, which can persist over months to years at low temperatures (Cook *et al.* 1990; Levy *et al.* 2009; Keller *et al.* 2010). A wintertime seasonality of norovirus, for example, causing the so called 'winter vomiting disease' was observed between 2005 and 2008 when 253 outbreaks could be related to norovirus in Toronto, Canada (Greer *et al.* 2009). In accordance to published data, the current monitoring of noro- and rotavirus infections in drinking water in Germany also indicates a seasonality of these viral pathogens (Soddemann, pers. comm. 2010).

The number of infections by noroviruses seems to go parallel with cold temperatures in the subsurface (Fig. 8A). Similar results are available for rotavirus infections in Germany. However, the maxima of rotavirus infections are slightly shifted and occur a few months later (Fig. 8B). These results provoked some speculation on distribution of noroviruses and rotaviruses with drinking water during winter time (Soddemann 2006).

Fig. 8: Norovirus (A) and rotavirus (B) infections in the federal states of Saxony and Hessen compared to the average overall infections in Germany displayed along with soil temperature.



Resource: By courtesy of W. Soddemann, 2010; data from Robert Koch Institut (RKI).

Another fact, which supports this argument is, that in federal states of Germany that produce their drinking water almost exclusively from groundwater (e.g. Hessen) the numbers of infections are significantly lower than in federal states where predominantly surface water is used (e.g. Saxony; Fig. 8; Soddemann, pers. comm. 2010).

It is known that a significant number of viruses enter together with treated wastewater rivers and streams. In times of epidemics, this load of viruses released from infected patients results in increased concentrations in wastewater and finally in much higher concentrations in the water which leaves the sewage treatment plant. In surface waters, at the low winter temperatures, decay of the pathogenic viruses is slow. Additionally, irradiation, as an important factor linked to viral reduction, exhibits a reduced intensity in winter. Similarly, microbial activity in surface waters and sediments is low at that time. Taking these circumstances into account, there is an improved persistence of viruses and where surface water is used for drinking water production, a high risk for the entry of viruses into drinking water.

Irrespective of sporadic infection events no waterborne outbreaks of enteric viruses via drinking water due to contaminated groundwater and water treatment issues are known for Germany. This is, as repeatedly argued because of the high standards in groundwater protection and the German drinking water ordinance (Trinkwasserverordnung). Single drinking water related outbreaks in Switzerland and Germany only occurred due to obvious failures in water treatment or break-in of sewage (Maurer and Sturchler 2000; Szewzyk *et al.* 2006).

The newly proposed regular spreading of pathogenic agents with drinking water (Soddemann, pers. comm. 2010) still awaits confirmation by drinking water analysis. This indeed is not trivial, as no simple plate and cell culture assays exist for norovirus and the detection limit with molecular methods is not sufficient at all for the monitoring of low concentration of viruses (e.g. 1 virus particle per litre or several litres of water), which still may constitute an infective dose and thus a high risk to human health. New techniques for the concentration of virus particles from large quantities of water are urgently needed (see section 8).

Currently seasonal endemics of human influenza viruses are also discussed to potentially occur through transmission via groundwater. The fact that endemics mostly occur local regarding virus subtypes and areas and that they are generally restricted to colder regions might be explained by their increased stability in cold drinking water (Soddemann 2005; Mauckner and Soddemann 2007). Human influenza viruses were also detected in faeces of several mammals, including swine and cattle (Graves and Oppenheimer 1975; Zhou *et al.* 1996; Webster 1998; Brown 2000). Therefore the transmission from the environment to natural waters and groundwater can not be excluded at the moment.

6.3 ECONOMIC IMPACT OF WATERBORNE OUTBREAKS

Waterborne outbreaks do, besides the risk of human infection, have a severe impact on our economic and social system and places a major burden on the health system. Payment (1993) estimated the total costs of infectious gastrointestinal illness, the major disease of waterborne outbreaks, in the US to be more than $20 billion per year. This is a major part of the estimated annual costs of $6.5-$34.9 billion for total microbial foodborne illness, demonstrating the great significance of waterborne outbreak (Buzby and Roberts 1997). A more detailed study on the costs of loss of productivity because of sick leave during a waterborne gastroenteritis outbreak affecting 1,600 people in Denmark were amounted to 1,600,000 Danish kroner (about $280,000 or €215,000, respectively) (Laursen *et al.* 1994). The costs for enteric viral infections per individual were calculated in the US based on 1997-1999 data. Direct and indirect costs ranged from $88/case for a norovirus infection to $1,193/case for enterovirus aseptic meningitis for non-hospitalized cases. Direct costs of hospitalization were even higher and ran from $887/case for norovirus to $86,899/case for hepatitis A (Nwachuku and Gerba 2006). These data impressively demonstrate the importance of proper water management and control of waterborne disease spread, not only to prevent life threatening illnesses but also to keep the economic damage of waterborne infections as low as possible.

6.4 GROUNDWATER PROTECTION REGULATIONS

Risk assessment is one of the major tasks for the prevention of contamination of groundwater that is intended for drinking water supply. As shown in Table 6 most countries in the world apply a similar system of protection zones, comprising of three individual zones to protect drinking water production wells.

The dimensions of the zones are very similar. Zone I is dedicated to protect the direct neighbourhood of the production well. Size of zone II is derived from tracer tests or hydrogeological modelling and accounts for individual groundwater travel times. Zone

Table 6: Comparison of protection zones in different countries.

COUNTRY	INNER PROTECTION ZONE (WELLHEAD)	MIDDLE PROTECTION ZONE	OUTER PROTECTION ZONE
	Travel time and/or radius of zone		
Australia	50 m	10 years	Whole catchment
Austria	< 10 m	60 days	Whole catchment
Denmark	10 m	60 days or 300 m	10-20 years
Germany	10-30 m	50 days	Whole catchment
Ghana	10-20 m	50 days	Whole catchment
Indonesia	10-15 m	50 days	Whole catchment
Ireland	100 days or 300 m	–	Whole catchment or 1000 m
Oman	365 days	10 years	Whole catchment
Switzerland	10 m	Individually defined	Double size of middle zone
United Kingdom	50 days and 50 m minimum	400 days	Whole catchment

Resource: Adopted from Chave et al. 2006.

III in most cases encompasses the whole catchment. Obvious differences in the wellhead protection zone are found for very arid countries as indicated for Oman (Tab. 6). In Arab regions were water resources are very limited, protection zones are used primarily to ensure adequate control over abstraction rates (Chave *et al.* 2006).

The groundwater well risk assessment in the US, which is laid down in the 'Guidelines for the Delineation of Wellhead Protection Areas' (USEPA 1987) is different to most other countries as no certain zoning system is proposed. Hence, the implementing authority can decide and plan the appropriate procedure in each individual case which can reduce costs. Additionally, the preparation of a contingency plan and the plan for the siting of new wells (vulnerability maps) is required. The lack of a zoning system might be due to the large variations of conditions from state to state. However, the guidelines leave much room for arbitrary delineations of protection zones and decisions might be difficult to defend legally (ACSAD-BGR 2003). By contrast, no vulnerability maps that might be essential to delineate protection zones in karst aquifers are included in the German guidelines.

Further, no contingency or emergency response plan is included in the law and regulations so that most water supply companies prepare their own plans. However, a comparison of the inexpensive well-established German wellhead protection area (WHPA) delineation models to the more cost-intensive USEPA-recommended WHPA delineations for agricultural settings revealed that several German delineations fitted well with an advanced numerical EPA module. Hence German WHPA methods might be a cost-saving alternative for farm operators in the US (Strobl and Robillard 2006). To further improve groundwater protection and to reduce the risk of infection via water a new European 'multi barrier system' was appointed in 1980 and especially the German drinking water suppliers have agreed on this advanced protection concept. The approach includes three major principles, (1) the protection of drinking water resources, (2) high technical standards regarding to the production, treatment, storage and distribution of drinking water, and (3) a proper operating domestic installation (Castell-Exner 2001; Szewzyk *et al.* 2006). In summary, the German regulations have proven to be effective in groundwater protection in a global point of view as to date no waterborne outbreaks of enteric viruses via drinking water due to improper water treatment are documented (Szewzyk *et al.* 2006).

6.5 WATER TREATMENT

The production of safe drinking water generally requires specific processing steps including physical (filtration, UV light), biological (aerobic microbial purification) and chemical treatments (free chlorine, chlorine compounds, ozone). In the past artificial groundwater recharge or bank filtration was proved to be highly effective in natural attenuation of contaminants including pathogens and emerged nowadays as a way of drinking water supply of growing importance (Balke and Zhu 2008). Physical filtration is known to efficiently remove the majority of bacterial and protozoan pathogens, however, viruses have been shown to pass

Table 7: CT values (disinfectant concentration and contact time, mg x min/L) for 99% inactivation at 5°C.

PATHOGEN	FREE CHLORINE (PH 6-7)	PREFORMED CHLORAMINES (PH 8-9)	CHLORINE DIOXIDE (PH 6-7)	OZONE (PH 6-7)
E. coli	0.034 – 0.05	95 – 180	0.4 – 0.75	0.02
Poliovirus 1	1.1 – 2.5	768 – 3740	0.2 – 6.7	0.1 – 0.2
Rotavirus	0.01 – 0.05	3806 – 6476	0.2 – 2.1	0.006 – 0.06
Phage f2	0.08 – 0.18	–	–	–
G. lamblia cysts	47 – 150	–	–	0.5 – 0.6
G. muris cysts	30 – 630	1400	7.2 – 18.5	1.8 – 2.0[a]
C. parvum	7200[b]	7200[c]	78[b]	5 – 10[c]

a Values for 99.9% inactivation at pH 6-9.
b 99% inactivation at pH 7 and 25°C.
c 90% inactivation at pH 7 and 25°C.

Resource: Adapted from Clark *et al.* 1993.

many filter systems (Szewzyk *et al.* 2006). Additionally, viruses (especially adenoviruses) are known to be very resistant against UV treatment, a habit which they share with bacterial spores and the protozoan *Acanthamoeba*. By contrast, most vegetative bacterial cells and oocysts of Cryptospridium and *Giardia* are highly susceptible to UV inactivation; between 19 and 28-times more sensitive compared to viruses (Hijnen *et al.* 2006).

A similar picture can be drawn according to traditional chemical treatment of water by chlorination or ozonation. The significantly increased viral persistence is best illustrated by the need for much higher doses and intensities of disinfection agents and treatments, respectively (Tab. 7). The wide range given is mainly related to water pH and exposure time, as well as water temperature. The efficiency of chlorination or ozonation, for example, is decreasing along with water temperature. Moreover, elimination of viruses by chlorination, ozone treatment or UV irradiation is insufficient with aggregated microorganisms or in biofilms (Lechevallier and Au 2004).

Protozoan oocysts are most resistant to chlorination (Tab. 7, Korich *et al.* 1990; Betancourt and Rose 2004). Viruses, on the other hand, are more resistant to inactivation than bacterial cells but are still effectively inactivated by free chlorine. Most pathogens seem to be quite resistant to chloramines. By contrast, ozone is a very powerful oxidant and a much better biocide than the other antimicrobial compounds (Tab. 7). It is therefore very useful as primary disinfectant.

It is worth to mention that disinfection by-products of chlorination, ozonation or UV treatment have been linked to cancer and reproductive defects (Amy *et al.* 2000). Better knowledge about the fate of pathogenic microorganisms and viruses in soil and groundwater allows optimizing disinfection needs and the kind of disinfection. Therefore, current research also focuses on potential alternative non-chemical water treatments. It has been shown that viruses (ΦX174 and MS2) could be efficiently removed by nanoscale zero-valent iron (nZVI) by inactivation and/or adsorption (You *et al.* 2005). However, application needs to be further evaluated since nZVI may also interact with other organisms. If unspecific reactions are comparable to free chlorine which is much more active against bacteria compared to viruses, nZVI may potentially also kill autochthonous bacteria in groundwater (Keane 2009).

7. USE OF INDICATOR ORGANISMS AND METHODOLOGICAL LIMITATIONS

Table 8: Coliform group and faecal streptococci.

GROUP	CHARACTERISTICS	
	Growth	Cells per g faeces
Total coliforms	37±1°C	10^7 – 10^8
Faecal coliforms	44±1°C	
E. coli	44±1°C	
Faecal Streptococci	45±1°C	10^6
Enterococci	10 – 45°C, pH 9.6, NaCl 6.5%	

7.1 INDICATOR ORGANISMS

Routine monitoring of hygienic water quality is essential to rule out waterborne disease outbreaks. This has led to guidelines for the assessment of water quality in various types of water bodies, including bathing waters and most important drinking water (Barrell *et al.* 2000). For the purpose of routine monitoring an indicator system is of great use as it is impossible to screen the high number of water samples for each specific known pathogen. Early, the group of coliform bacteria (total coliforms) was identified as indicator that is (1) universally present in human and warm-blooded mammal faeces in large numbers, (2) easy to detect, (3) not reproducing in natural aquatic environments or water distribution systems, and (4) behaving similar regarding persistence and water treatment as the waterborne pathogens of concern (WHO 1996). Coliforms themselves are, with some exceptions, non-pathogenic. All members of the total coliform group belong to the *Enterobacteriaceae*, sharing common characteristics including being gram-negative, non spore forming, oxidase negative, and rod-shaped facultative anaerobes that are defined by their ability to ferment lactose (using the enzyme galactosidase) with the production of acid and gas (Gilpin and Devane 2008). Because, the total coliforms also include organisms of environmental origin, there value as indicator for faecal contamination is frequently discussed. In Germany, the total coliforms lost importance as formerly mandatory indicator with a set quantitative threshold (0/100 mL) in the new drinking water ordinance (Trinkwasserverordnung), and stay as a qualitative indicator only (Exner and Koch 2011). The group of total coliforms is sometimes further distinguished into the subgroup of faecal coliforms. Members of the faecal coliforms are all thermotolerant (Tab. 8).

Escherichia coli is the most prominent coliform among the intestinal microflora and its presence is therefore a good indication for faecal contamination. Furthermore, faecal streptococci, especially enterococci are used as indicators. They are characterized by longer survival times than *E. coli* and are thus very stable in the environment (section 5, Fig. 6). Hence, their presence might be a hint to previous faecal contaminations (McFeters *et al.* 1974; Geldreich 1976; Edberg *et al.* 1997). However, some *E. coli* species can also be found in natural waters with no known sources of faecal contamination and environmental species also known within the enterococci (Carrillo *et al.* 1985; Brtkova *et al.* 2010).

Most laboratories responsible for water quality monitoring routinely test for the presence of total coliforms, faecal coliforms, *E. coli*, or enterococci (Leclerc *et al.* 2001). The guideline values for the number of individual indicator allowed to be present in safe drinking water are internationally accepted with only minor variations (Tab. 9).

Table 9: International guidelines for drinking water.

	TOTAL COLIFORM	*E. COLI*	MONITORING REQUIREMENTS	
			Population	Samples/months
United States (USEPA 1990)	0/100 mL (95%) A consecutive sample from the same site must be coliform-free	0/100 mL (100%)	–	ca. 1/1000 inhabitants
Canada (Ministère de la santé 1996)	10/100 mL (90%) None should contain more than 10 CFU/100 mL a consecutive sample from the same site must be coliform-free	0/100 mL (100%)	< 5000 5000 – 9000 > 9000	4 samples/month 1/1000 inhabitants 90+ (1/10000 inhabitants)
WHO (1994)	0/100 mL (95%)	0/100 mL (100%)	–	–
Germany (Trinkwasser-verordnung, 2001)	0/100 mL	0/100 mL (100%)	–	–

Resource: Adapted from Rompré *et al.* 2002.

Since coliform bacteria, faecal coliforms and *E. coli* are used as indicators for faecal contamination, their appropriateness and reliability is continuously discussed. Major points of discussion include (i) their natural occurrence in the environment, (ii) their possible growth outside their hosts, (iii) huge variation in survival between individual species and strains. Without doubt, their common use as surrogates of bacterial enteric pathogens requires knowledge on their behaviour in natural environments.

There is a pile of studies, which proved that coliforms and especially *E. coli* do not reproduce under natural conditions. For example, more than 40 field and laboratory survival experiments of Mancini in 1978 reported no coliform growth in natural waters. Similar results were obtained exclusively for *E. coli* revealing survival times in a range of 4 to 12 weeks (Edberg *et al.* 2000). However, there is also a number of contradicting studies. Several hints of reproducing *E. coli* are available from raw wastewater studies, were growth is enabled by nutrient-rich conditions (Niemi *et al.* 1987; Ashbolt *et al.* 1997). The growth of selected pathogenic and non-pathogenic enteric bacteria was observed in river water at 30°C, which was, however, not sufficient for a survival at 5°C (Hendricks 1972). Similarily, growth of *E. coli* O157 was shown under low carbon conditions in sterile freshwater at temperatures between 20 and 30°C. At temperatures below, no growth could be observed or was negligible (Vital *et al.* 2008). These data are promising. The common characteristics of oligotrophic and 'cold' groundwater represent hostile conditions for *E. coli*. The fact that up to date there is no strong evidence for growth of *E. coli* in groundwater or drinking water systems favours its application as common indicator organism of faecal groundwater contamination (Leclerc *et al.* 2001). However, does the absence of *E. coli* also guarantee the absence of other pathogenic bacteria?

The use of faecal coliforms and E.coli as universal indicators of groundwater contamination needs to be considered carefully. There are many enteric pathogens that behave differently to *E. coli*, particularly with respect to transport behaviour, survival, and disinfection resistance (Stevens *et al.* 2003). Several studies on *E. coli* and other enteric pathogens showed that the different cell properties of the individual strains tested significantly changed their transport behaviour (Bolster *et al.* 2006; Bolster *et al.* 2009). Even within *E. coli* isolated from different host species (Dombek *et al.* 2000; Carson *et al.* 2001) or even from the same host species (Kudva *et al.* 1997; Galland *et al.* 2001; Vali *et al.* 2004) a high genetic variability was observed. These genetic differences went along with differences in cell surface characteristics that again affected transport and/or cell motility in the environment (Yang *et al.* 2004; Morrow *et al.* 2005).

A critical point in safeguarding drinking water quality is that common tests for faecal contamination are generally done with freshly produced drinking water and not with source water prior to its treatment. As some pathogenic bacteria show a higher resistance to chlorination and UV-radiation than *E. coli* or total coliforms, the absence of *E. coli* does not exclude the presence of others. An example is the Salmonellosis outbreak due to *Salmonella typhimurim* in Riverside, California in 1965. Routine bacteriological test revealed no significant contamination (Report 1971). Also pathogenic viruses and protozoa have been shown to be much more resistant against common chlorination (Tab. 7; Engelbrecht *et al.* 1980; Korich *et al.* 1990; Li *et al.* 2002) and have been detected in water free of *E. coli* (< 1 colony in 100 mL) and other indicator bacteria (Payment *et al.* 1994; Lees 2000; Szewzyk *et al.* 2006).

The situation of using bacterial indicators as surrogates for faecal contamination potentially including pathogenic viruses and protozoa, besides pathogenic bacteria, is unsatisfactory. As already mentioned, in Germany drinking water quality is typically diagnosed for the absence of *E. coli* and faecal enterococci in 100 mL after treatment (Szewzyk *et al.* 2006). Because of their long-term persistence in the environment and their much higher resistance against some of the common water treatment procedures, the absence of *E. coli* does clearly not prove the absence of enteric viruses. Contrariwise, the presence of E.coli makes it very likely that enteric viruses are also present. And indeed, studies on drinking water wells demonstrated the inadequacy of bacterial indicators for the assessment of virus contamination. Although most of the wells considered (> 80%) were tested negative for coliforms almost 90% were tested positive for enteric viruses (Wellings *et al.* 1975).

Currently, intensive research is directed to find adequate indicators that are able to reliably indicate viral contamination in water samples (Ashbolt 2004; current research project – see discussion below). Studies concentrate on the main subgroups of bacteriophages, the F-RNA and the somatic bacteriophages (Bitton *et al.* 1983; Snowdon *et al.* 1989; Palmateer *et al.* 1991; Ijzerman *et al.* 1994; Woody and Cliver 1994; Leclerc *et al.* 2000; Grabow 2001; Espinosa *et al.* 2009). To date, a number of frequently used model bacteriophages, *i.e.* MS2, PRD1 and ΦX174, provide first estimations on removal rates of pathogenic viruses during sediment passage in column studies and during artificial groundwater recharge (Schijven and Hassanizadeh 2000; Schijven *et al.* 2003; Harvey and Ryan 2004). A comparable removal was observed for the model virus MS2 and the enteric virus *coxsackievirus* B4, but not with *poliovirus* 1 (Schijven *et al.* 2003). MS2 is very common in surface waters and/or treated wastewater that is used for groundwater recharge. Moreover, MS2 is very stable in the environment poorly adsorbing to surfaces (Schijven and Hassanizadeh 2000). Therefore, these authors suggested that the F-specific RNA bacteriophage MS2 may well fulfill the function of a worst-case viral indicator. It needs to be considered, however, that bacteriophages may replicate in nature and the concentration of autochthonous coliphages in unpolluted aquatic environments is high (Moriñigo *et al.* 1992; Gleeson 1997; Leclerc *et al.* 2000; Tallon *et al.* 2005).

But also model viruses of human and non-human origin within the enteric viruses itself, e.g. the very environmentally resistant adenoviruses, are currently tested for their applicability as universal viral faecal indicators (Ley *et al.* 2002; Choi and Jiang 2005; Griffin *et al.* 2008; Ahmed *et al.* 2010).

Besides bacteriophages several microorganisms were intensively tested for their adequacy as surrogates in the indication of pathogens where *E. coli* might fail (Tallon *et al.* 2005). The list includes *Cl. perfringens* and *Clostridium* spores as surrogates for contamination with protozoan oocysts (Schijven *et al.* 2003; Tyagi *et al.* 2006). However, limitations are given to the fact that *Clostridium* is an anaerobic organism and present in faeces in relatively low numbers.

7.2 METHODS USED IN PATHOGENS DETECTION

The sensitive and specific detection of pathogens in groundwater is one of the biggest challenges as microbial and viral agents are generally present in very low numbers (Girones *et al.* 2010). In case of faecal pollution, fast and reliable detection methods are required to ensure the rapid identification of pathogen type and its origin, which constitute the most important initial steps to prevent epidemic waterborne disease outbreaks.

To date, detection of pathogens in drinking water and groundwater depend mainly on cultivation techniques and increasingly on steadily improving molecular methods. The most traditional approaches which have been developed for the detection of faecal indicator organisms (total coliforms and subgroups) are the multiple-tube fermentation (MTF) technique, the aerobic plate count and the membrane filter (MF) technique using different specific media and incubation conditions (Tortorello 2003). Although more rapid enzymatic and molecular based methods (e.g. PCR) are available today, these assays are not yet fully developed and have serious limitations in specificity and resolution

(high detection limit). For these reasons, the relatively simple to perform and inexpensive MF technique is still the approach most commonly used for routine enumeration of coliforms in drinking water (Rompré *et al.* 2002). Unfortunately, for many well known and emerging pathogens no appropriate standardized cell culture system exists (Dong *et al.* 2008). Also biofilm associated bacteria or bacterial pathogens that have entered a viable but not culturable (VBNC) state cannot be detected by cell culture assays (Cenciarini-Borde *et al.* 2009). Consequently, molecular methods such as the real-time or quantitative PCR (qPCR) technique are used with an increasing frequency for qualitative and quantitative assessment. Despite the many advantages of qPCR including speed, high sensitivity and specificity, detection limit is often reached due to very low pathogen concentration in environmental samples. Thus, initial enrichment steps (see below) are needed (Noble and Weisberg 2005).

Approaches for the detection of viable microorganisms in aquatic environments include fluorescent microscopy or the highly specific fluorescent *in situ* hybridization (FISH) technique (Bottari *et al.* 2006). FISH has been successfully applied in studying bacterial pathogens in water, sewage and sludge, and biofilms (Gilbride *et al.* 2006). In contrast to FISH which is mainly used for community analyses, flow cytometry (FCM) can be used for microbial analysis at both the community and single-cell levels. FCM combined with immunological staining (immunofluorescent assays) have been successfully used for the detection of aquatic pathogenic protozoa. A recently developed FCM protocol for the detection of *C. parvum* using a specific monoclonal antibody-conjugate revealed a detection limit of 2 x 10^3 oocysts mL-1 (Barbosa *et al.* 2008). Another FCM-based protocol was used for the fast detection of *L. pneumophila*e in water with a detection limit of 500 cells L-1. Füchslin *et al.* (2010) described a 'speed' protocol based on immunomagnetic separation and FCM, which allows sample analysis within 3 h. Using specific antibodies, this approach could be applied also for a great variety of other pathogenic microorganisms (Wang *et al.* 2010). The application of FCM could also be an additional or alternative method to the molecular PCR-based detection of pathogens. Although PCR-based techniques are highly sensitive in the nucleic acid detection of potential pathogens, it is not possible to distinguish between dead, inactivated or still infectious cells or particles. Microfluidics-FCM is proposed to be a new rapid and inexpensive approach for the detection of pathogenic bacteria (Sakamoto *et al.* 2007).

As faecal groundwater pollution can be of both human and animal origin, microbial source tracking methods (MST) emerged in the recent past (Meays *et al.* 2004; Cimenti *et al.* 2007; Field and Samadpour 2007). These methods include several approaches such as culturing, phenotypic and genetic analyses, and chemical analyses. The main focus within MST methods today lies on genetic fingerprinting of faecal microorganisms.

Keeping in mind that only one single viral particle might be sufficient for human infection a low detection limit is of great significance regarding to human health risks. To date more than 100 types of enteric viruses of faecal origin are known but only a few can be detected by established cell culture systems. PCR-based methods are, in this respect, the most promising tool for pathogenic virus detection in the future (Rodriguez *et al.* 2009). However, due to the high detection limit, analysis of pathogens (mainly viruses) in water requires a two-stage process, consisting of (1) pathogen enrichment either by ultracentrifugation or adsorption to charged microfiltration membranes, followed by (2) the application of infectivity tests or specific molecular analysis. Commonly used filtration techniques rely on combined adsorption and elution procedures, e.g. filtration of water through electropositive filters or glass wool columns or ultrafiltration (Donaldson *et al.* 2002; Haramoto *et al.* 2005; Rajal *et al.* 2007; Lambertini *et al.* 2008; Hill *et al.* 2010). However, for testing the putative presence of enteric viruses in drinking water, large sampling volumes (10-30 m^3) need to be screened. The problem of low numbers of target microorganisms and viruses is accelerated by difficulties in a quantitative extraction and amplification of nucleic acids from environmental samples. This is mainly due to co-extraction of inhibitory substances such as humic compounds (Lebuhn *et al.* 2003). Most virus detection assays are restricted to the amplification of viral nucleic acids by molecular techniques including several modified protocols of the PCR method such as reverse transcriptase (RT)-PCR, nested PCR, integrated cell culture PCR (ICC-PCR), or qPCR. Real-time qPCR allows the quantitative determination of nucleic acid copies and is currently the preferentially used molecular approach for the analysis of viral genomes in clinical and environmental samples (Espy *et al.* 2006).

8. RÉSUMÉ - KNOWLEDGE GAPS AND RECOMMENDATIONS

8.1 STATUS QUO

The use of naturally, well protected groundwater is generally the safest way of drinking water supply for human consumption. In Germany about 70% of all drinking water is produced from groundwater and spring water (Statistisches Bundesamt 2009). However, the continuous growth of the world´s population with all its consequences compromises our natural groundwater resources in many ways and not in all areas groundwater quantity and quality is sufficient. Alternatively, drinking water is produced from surface water via bank filtration and artificial groundwater recharge. On average drinking water produced by bank filtration or infiltration (artificial groundwater recharge) in Germany is about 8% (Statistisches Bundesamt 2009). In Berlin, as much as 75% of the drinking water originates from river bank filtration (Schmidt *et al.* 2003). In consequence, such areas have a partly closed water cycle. Treated wastewater which leaves the sewage treatment plants into rivers is to some extent 'reused' for drinking water production. Water recycling and reuse increases the risk of contamination by persistent pathogens, such as viruses.

8.2 CHALLENGES

Groundwater in Germany is a well protected resource. In contrast to most other countries of the world a comprehensive area-wide groundwater protection is legally defined (SRU 1998). Based on the epidemiological data available, the risk of waterborne outbreaks from drinking water which was produced from groundwater seems negligible (Botzenhart and Fleischer 2009; Preuß *et al.* 2011). However, there are still a number of risks which need to be considered and even today a waterborne disease outbreak caused by contaminated groundwater and drinking water is possible. The risks are mainly associated with (1) a malfunction of wastewater storage (leaks in septic tanks), treatment (flooding of sewage treatment plants and/or collapse of microbial communities in there) and transport (leakages in sewers and pipes), (2) insufficient attenuation of pathogens in river bed sediments and soils (along and after heavy rain falls and floods), (3) excessive application of manure, (4) contaminated surface water, and (5) deficient protective layers (thin soil zones on top of karst systems). In such cases, a severe contamination and risk to human health is given. Finally, there is some vital discussion on a possible regular distribution of pathogenic viruses during the cold season with drinking water produced from surface water (Soddemann 2005, 2006). These speculations await urgent evaluation.

In this final section of the paper it was tried to identify and highlight the lack of knowledge with respect to the distribution, survival, ecology, and detection of pathogenic microorganisms and viruses in groundwater and drinking water in general. Based on the information condensed in this review and the identified gaps of knowledge, recommendations for future scientific research and water management actions are expressed. Especially the recommendations for an improved safeguarding of water quality are emphasized with a focus on drinking water independent of its source. Some of the topics listed have already been discussed before in the framework of an expert forum organized by the German Federal Environment Agency (UBA) in 2005 (Szewzyk *et al.* 2006). Our résumé is primarily from the point of view of microbial ecologists and unlikely complete.

8.3 RECOMMENDATIONS

8.3.1 Next generation enrichment techniques

While there are strict thresholds for the microbial contamination of drinking water, there are so far only recommendations for pathogenic viruses (Botzenhart and Fleischer 2009). With regard to the USEPA recommendations for drinking water quality, the probability of an infection by pathogens via consumption of drinking water should not exceed one infection per 10,000 persons per year ('tolerable infection risk'; Rose and Gerba 1991). The WHO developed the concept of 'Disability Affected Life Years' (DALY), which considers the concentration of viral particles, their infection potential, and the outcome of an illness (WHO 2004). The concept allows calculating the maximum acceptable virus concentration in drinking water. Since for pathogenic viruses – and the same is true for protozoa (e.g. *Cryptosporidium*) - already one particle is an infective unit, this concept runs into a severe problem of detection limits. For rotavirus, as an example, a target value of < 1 virus particle in 32 m^3 of drinking water is recommended, which equals less than 1 DALY per 100,000 consumers (WHO 2006). To take these recommendations serious, large sample volumes need to be screened in the future. To date there is no routinely applicable technique, which allows the quantitative enrichment and recovery of viral particles from large water volumes (> 10 m^3) in a reasonable time (< 1 day).

Recommendation → Development of new enrichment techniques.

Directly connected to the problem of enrichment is the problem of sensitivity of our cultivation-dependent and cultivation-independent (molecular) detection methods. While plate assays (CFU and PFU) are able to detect 1 single cell or virus particle per subsample (generally 1 ml), the same copy number is needed in 50 µl (= 20 cells/particles ml-1) for PCR, or even more if some inefficiency during nucleic acid extraction is assumed. The difference between the USEPA/WHO recommendations and the current detection limits is in the range of 7-8 orders of magnitude.

Recommendation → Sensitivity improvement of molecular assays.

8.3.2 Methodological developments and challenges

> 8.3.2.1 Reliable indicators of viral and protozoan contamination

To date the global water monitoring concepts and surveillance regulations regarding hygienic (pathogenic) contamination of water are based on the coliform indicator principle, *i.e.* on bacterial indicators exclusively. The WHO (2006) states that the absence of *E. coli* is not an appropriate indicator which guarantees the absence of pathogenic viruses. However, to date the surveillance for pathogenic enteric viruses is not mandatorily defined in any regulations with respect to groundwater and drinking water protection. Without doubt due to their high infectious potential, their small size (regularly pass standard filter systems) and their robustness (withstand some of the common water treatment procedures) separate viral indicators are urgently needed. Within the enteric viruses, coliphages and adenovirus have been considered useful indicator viruses (Pina *et al.* 1998; Jiang 2006; Szewzyk *et al.* 2006). The same story is valid for protozoa. To date, the assessment of parasitic pathogens is not mandatory. A modern integrative water quality concept should consider all groups of pathogens, *i.e.* bacteria, viruses and protozoa.

Recommendation → Development of an integrative indicator concept.

> 8.3.2.2 Improvement and development of molecular tools

Pathogen-specific detection using molecular methods require the quantitative extraction and amplification of nucleic acids from environmental samples. To date, extraction often is inefficient and amplification is inhibited by the presence of natural constituents in water and sediments, such as humic substances and iron species. These inhibitory compounds are enriched together with the organisms of interest (*i.e.* pathogens). Consequently, inefficient extraction adds on top to the already 'high' detection limit.

Recommendation → Development and testing of appropriate sample extraction and clean-up protocols.

> 8.3.2.3 Infectivity tests for all prominent pathogens

The moment, the quantification of all pathogens of interest is possible, the question of their infectivity in a given water sample remains. Thus, we need to have in hands tests for infectivity for all frequently occurring pathogenic microorganisms and viruses. To give one example, although today norovirus is the most prominent causative reason for gastroenteritis epidemics, detection of infectious norovirus particles is still not possible due to the lack of an appropriate in vitro cell culture system.

Recommendation → Development of cell culture assays for relevant pathogens.

> 8.3.2.4 Consideration of genotypes/serotypes in case of contaminations and outbreaks

In case of contaminations and along with epidemiological studies, the different genotypes (e.g. in case of norovirus) and/or serotypes (e.g. in case of adenovirus) shall be considered. This is suggested to speed up the identification of the contamination source and therefore might help to prevent more widespread epidemic waterborne outbreaks through improved surveillance of water sources (Nygard *et al.* 2003; Maunula and Von Bonsdorff 2005; Koh *et al.* 2008). Moreover, it has been shown that virus serotypes exhibit a significantly different behaviour in the environment (Fong *et al.* 2010; H.C. Selinka, pers. comm. 2010).

Recommendation → Consideration of genotypes and serotypes in forensic research.

> 8.3.2.5 A simple modular set for the estimation of pathogen reduction

There is a typical reduction in concentration of pathogens associated with different environmental compartments. The load of pathogenic viruses in wastewater is generally reduced by 1-3 orders of magnitude (1-3 log levels) during treatment in a conventional sewage plant. A comparable reduction of pathogens is achieved in a vital top soil zone. This leads to the idea of a

simple modular set for the estimation of pathogen reduction useful in an initial risk assessment. First, the system of interest is distinguished into its main environmental compartments which themselves are characterized by typical reduction potentials. The individual reduction potentials (including attenuation and elimination) are summed up for the compartments from the source of the water (e.g. wastewater) to the raw water (e.g. groundwater) in case of drinking water production via bank filtration.

8.3.3 Improved monitoring of drinking water

> 8.3.3.1 Drinking water produced from surface water

Surface waters, especially close to effluents of sewage treatment plants, carry a higher load of pathogens compared to the naturally well protected groundwater. In consequence, wherever surface water is used for drinking water production (e.g. via bank filtration or artificial groundwater recharge) a frequent end monitoring of the raw water is proposed including irregular events of heavy rainfall and snow melt.

Recommendation → Systematic evaluation of possible viral contamination of drinking water from bank filtration during times of increased viral infections.

> 8.3.3.2 Retain samples

Maunula *et al.* (2005) suggested the implementation of an easy to handle monitoring strategy for water works. The regular (daily or weekly) storage of a representative subsample of the freshly produced drinking water allows the back tracking of the contaminant source in case of waterborne disease outbreaks. Retain samples are stored for 50 days. This is suggested a cost-saving improvement of risk management. We further propose also to store in parallel representative volumes of raw water (1-5 L), where the number of pathogens in case of contamination is likely much higher and thus easier to detect.

Recommendation → Retain samples from raw water and drinking water.

> 8.3.3.3 Epidemiological monitoring

In Germany, single and multiple noro- and rotavirus infections seem to occur throughout the whole year, but with increasing numbers of infected persons during winter time (RKI 2011). For some bacterial pathogens (e.g. *Salmonella*) the peak of documented infections is in summer. The path of transmission is not always clear. To date individual infections are epidemiologically not documented, which in many cases prevents the correlation between disease and source.

Recommendation → Further developed epidemiological documentation of waterborne disease outbreaks.

8.3.4 Improved water management

To better assess the risk of viral infection through drinking water the WHO proposed a 'water safety plan (WSP)'. This concept suggests the combined assessment of raw water quality together with the efficiency of the applied water treatment methods. If the virus concentration in raw water and the efficiency of the respective treatment are known it is possible to estimate the risk of virus contamination of drinking water and therefore the risk to human health (WHO 2004). The 'multi barrier system' applied in Germany is comparable to the WSP of the WHO and is composed of a systematic resource protection as the first step followed by adequate treatment techniques. Finally, the drinking water is tested for the absence of faecal indicators. Not in all cases, the resource protection and the treatment techniques are sufficient (Szewzyk *et al.* 2006). It is thus recommended that viral risk calculations, as recommended by the WHO, are applied additionally to improve the safeguarding of drinking water. This includes, as already mentioned before, a regular control of the raw water taking into consideration also extreme climatic and hydraulic events such as stormwater run-off, flooding or snowmelt (Szewzyk *et al.* 2006).

Recommendation → Improved water quality management strategies. Concepts of groundwater and drinking water protection should be expanded by taking into account the concept of 'aquifer vulnerability' (Harter and Walker 2001; Chilton 2006). In areas with a high aquifer vulnerability protection zones need to be delineated more carefully.

8.3.5 Legal framework - groundwater protection zones

The data summarized in section 5 impressively show, that pathogens may survive or persist at low temperatures in the subsurface for months to several years. Moreover, some pathogens may even reproduce in the environment. This is not in accordance with the 50 days dwelling distance of groundwater protection zone II as applied in Germany and other countries (Tab. 6). Indeed, the transport distance of pathogens in aquifers (and river banks) is much less than just the product of survival time and

flow velocity (Althaus 1983). Physical and chemical processes as well as biological activities contribute to a generally efficient attenuation of pathogens. This was also the conclusion of a large multidisciplinary project funded by the German Federal Environment Agency (UBA) 25 years ago (Filip *et al.* 1986). However, contamination with faecal indicators is regularly detected in karst systems and fissured aquifers. We also see a potential risk for contamination of groundwater in highly heterogeneous porous aquifers.

Recommendation → We thus recommend, from a microbiological point of view, to delineate protection zones based on extensive tracer experiments with non-pathogenic representatives of prominent pathogens or appropriate surrogates taking into account also extreme climatic and hydraulic situations.

8.3.6 Basic research

> 8.3.6.1 Viral surrogates

Several model viruses, e.g. the F-specific bacteriophage MS2, somatic phages (PRD-1, ΦX174) or bacteriophages infecting *Bacteroides fragilis* have been proposed surrogates of pathogenic viruses, which show behaviour of transport and attenuation in the environment similar to some pathogens (Tartera and Jofre 1987; Leclerc *et al.* 2000; Grabow 2001; Van der Wielen *et al.* 2008). Model viruses are needed for many purposes, for example tracer experiments in the field. During the last years the murine norovirus, which has been successfully propagated in cell culture and can reproducibly be detected by RT-PCR, emerged to be a promising surrogate for the human-pathogenic counterpart (Bae and Schwab 2008).

Recommendation → Identification and testing of appropriate viral surrogates.

> 8.3.6.2 Attenuation and survival of pathogens in *situ* – The unseen fraction

First of all, most information available with respect to the transport and attenuation of pathogens originate from lab studies and/or the use of surrogate pathogens. To derive a more reliable picture for the situation *in situ* more field investigations are needed. Second, most studies concentrated exclusively on the recovery of pathogens from water passing through soil and sediments. Rarely, the fraction of strained and adsorbed or actively attached pathogens have been tackled in more detail. There is evidence that this fraction may stay alive and infectious for long periods of time, thus forming a reservoir. This aspect is, to our opinion, of relevance especially in situations where environmental compartments (soil and sediments) are continuously infiltrated with water carrying pathogens which then leads to an accumulation of pathogens over time, *i.e.* where bank filtration is used for drinking water production. At a certain time point sorption places at the sediment particle surfaces may be occupied and the natural attenuation capacity decreases. In case of changing hydrochemical (e.g. ionic strength) or hydraulic conditions (e.g. flow velocity) initially adsorbed pathogens may be released and transported further downgradient.

Recommendation → Studies on pathogen transport and survival in sediments (soils, hyporheic zone of streams, river bank sediments and aquifers) are needed considering the temporally immobilized sediment-bound fraction of pathogens.

> 8.3.6.3 Elimination processes and microbial antagonism

Human pathogens when released into the environment are usually affected by many abiotic and biotic processes which, in concert, determine their stability and survival. It has been repeatedly shown that disappearance (retardation and/or elimination) of pathogens is faster in biologically active systems (Hurst *et al.* 1980; Hurst 1988; Nasser *et al.* 2002). Moreover, direct antagonistic effects onto *E. coli* have been shown for autochthonous groundwater bacterial isolates (Hirsch and Rades-Rohkohl 1983). However, so far there is only sparse information on antagonistic activities of microorganisms causing pathogens elimination. A better understanding of natural elimination processes (natural barrier functions) will contribute to improve water protection and management and might safe costs of common treatment procedures.

Recommendation → Elucidation of biotic antagonistic processes and their significance in the elimination of pathogens.

> 8.3.6.4 Ecology of pathogens – Strategies for long-term persistence

Microorganisms (and viruses) developed a multitude of strategies to survive harsh, hostile conditions. To be released from human or animal intestine into the environment are without doubt harsh conditions for pathogens. It has been shown that pathogenic bacteria and protozoa partly cope with this situation shifting into a viable but not culturable (VBNC) state or forming spores and cysts (Roszak and Colwell 1987; Oliver 2005; Karanis *et al.* 2007). This inconsequence potentially leads to the

situation that pathogens are present in low-activity (potentially still infectious) or dormant state, but they are no longer detectable with traditional cultivation-based approaches (Oliver 2005).

Recommendation → A better understanding of pathogens ecology and improved cultivation independent detection methods are needed.

> 8.3.6.5 Pathogens with antibiotic resistance

The increasing numbers of drug-resistant bacterial strains in the environment and the possibility of horizontal gene transfer between autochthonous and allochthonous species and specimen are of great scientific interest as well as socioeconomic concern. Along with the applications of various antibiotics in modern animal livestock production, an increasing antibiotic resistance among gastrointestinal bacteria is observed (Sapkota *et al.* 2007). Moreover, the potential risk of environmental contamination by antibiotic-resistant bacteria derived from hospital sewage was demonstrated (Reinthaler *et al.* 2003). The fate of these genetically modified organisms in the environment and the probability of horizontal gene transfer in soil and aquatic environments await detailed investigation.

Recommendation → Scientific research on the fate of drug-resistant microorganisms in the environment.

> 8.3.6.6 General framework – standards for lab tests

To our opinion, it would be helpful to establish standards for performing bench-scale studies on attenuation and survival or persistence of pathogens as there is still a tremendous variability within experimental findings observed. It is suggested to set up routine protocols for the propagation and preparation of seeded test organisms. Moreover, the inclusion of standardized internal controls, e.g. the bacteriophage MS2 or *E. coli*, will contribute to minimize the variability of survival data that are reported for pathogenic microorganisms and viruses in groundwater (John and Rose 2005).

Recommendation → Agreement on standard protocols and experimental design.

> 8.3.6.7 Current research - joint project on detection, transport and elimination of pathogenic viruses in water

Currently, a national multidisciplinary research project funded by the German Research Foundation (DFG) tackles several of the 'open' topics mentioned above. Two working groups (M. Seidel & R. Niessner, Institute of Hydrochemistry, Technical University Munich (TUM); M. Exner & C. Koch, Institute for Hygiene and Public Health, University of Bonn), focus on the development of high-throughput filtration techniques (ultra- and nanofiltration). The colleagues from the TUM furthermore test selective methods for subsequent enrichment based on immunoseparation and automated analytical microarrays. The working group from the German Federal Environment Agency (H. C. Selinka & I. Chorus, UBA, Berlin) investigates the fate of pathogenic viruses (noroviruses and adenoviruses) in surface water and try to optimize protocols for nucleic acid extraction and qPCR assays; the later topic in collaboration with colleagues from the University in Bonn (Ch. Drosten, Institute of Virology, University of Bonn Medical Centre). Identification and testing of promising indicator viruses is an additional topic of these two working groups. Factors determining the efficiency of pathogenic virus reduction during wastewater treatment and the improvement of mathematical models with predictive power on virus elimination are tackled by colleagues from Hannover (K.-H. Rosenwinkel & S. Wolter, Institute for Sanitary Environmental Engineering and Waste Engineering, Leibniz University of Hannover). Last but not least, the authors of this review currently conduct lab and field experiments to evaluate the contribution of microbial processes (grazing and bacterial lyses) to the elimination of pathogenic viruses in aquatic systems. In summary, the scientific program of this joint research project on pathogenic viruses in water addresses major aspects in relation to an improved and sustainable drinking water quality management.

9. EMPFEHLUNGEN

(deutsche Version des Originaltextes unter 8.3)

9.1 TECHNISCH-METHODISCHE WEITERENTWICKLUNG MODERNER FILTERSYSTEME ZUR ANREICHERUNG PATHOGENER VIREN

Obwohl aktuell strenge Richtlinien in Bezug auf mikrobielle Kontaminationen von Trinkwasser gelten, gibt es bezüglich pathogener Viren derzeit nur Empfehlungen (Botzenhart und Fleischer 2009). Nach Vorgabe der USEPA sollte die Wahrscheinlichkeit einer Infektion je 10.000 Personen und Jahr durch Pathogene im Trinkwasser nicht übersteigen („tolerable infection risk"; Rose und Gerba 1991). Auch die WHO hat ein Konzept entwickelt („Disability Affected Life Years", DALY), welches die Konzentration von Viruspartikeln, deren Infektionspotential und das Ausmaß der jeweiligen Erkrankung berücksichtigt (WHO 2004). Dieses Konzept erlaubt es, die maximale tolerierbare Viruskonzentration im Trinkwasser, zumindest theoretisch, zu ermitteln. Wenn man berücksichtigt, dass bei pathogenen Viren (und teilweise auch bei pathogenen Protozoen, wie z. B. *Cryptosporidium*) bereits ein Partikel als infektiöse Einheit gilt, stößt man bei Viren schnell an methodische Grenzen. Für Rotaviren wird beispielsweise ein Richtwert von < 1 Viruspartikel in 32 m^3 Trinkwasser empfohlen, der weniger als 1 DALY pro 100.000 Verbrauchern entspricht (WHO 2006). Reicht es für *E. coli* 100 ml Wasser zu filtrieren, müssen in Anbetracht dieser Richtwerte und Empfehlungen für Viren künftig enorme Probenvolumen analysiert werden. Derzeit gibt es jedoch noch keine standardisierten Anreicherungsmethoden, die es ermöglichen, infektiöse Viruspartikel aus solch großen Wasservolumina (> 10 m^3) in angemessener Zeit (< 1 Tag) aufzukonzentrieren. Die Entwicklung von Filtrationssystemen zur Anreicherung von pathogenen Viren ist daher dringend notwendig, um zukünftig eine sichere Trinkwasserüberwachung zu gewährleisten.

Ein weiteres Problem stellt die Sensitivität bestehender kultivierungs-abhängiger und kultivierungs-unabhängiger (molekularer) Nachweismethoden dar. Ein Plattennachweis (CFU bzw. PFU) erlaubt theoretisch die Detektierung einer einzelnen Zelle bzw. eines infektiösen Partikels in einem Milliliter Flüssigkeit. Aus statistischen Gründen sind aber mehr als 10 Kolonien bzw. Plaques notwendig für eine reproduzierbare Auswertung. Für einen PCR-Ansatz benötigt es bereits mehrere Partikel in nur 10-50 µl Probe, was einer Anzahl von mehr als 20 Zellen bzw. Partikeln pro ml entspricht. Bei Umweltproben treten zusätzlich Verluste bei der Extraktion und Aufreinigung von Nukleinsäuren auf, die den sensitiven Nachweis erschweren. Vergleicht man die Empfehlungen von WHO und USEPA mit den heutigen technischen Möglichkeiten, so liegt die Herausforderung in der Anreicherung von Virenpartikeln um 7 bis 8 Größenordnungen.

9.2 INTEGRATIVES INDIKATORKONZEPT

Die Sicherstellung von hygienisch unbedenklichem Trinkwasser basiert heutzutage international auf einem bakterien-spezifischem Überwachungsprinzip. Trinkwasser wird routinemäßig auf bakterielle Indikatoren (zwingend *E. coli*, nicht zwingend Coliforme) getestet. Weitere Pathogene (Viren und Protozoen), die unter Umständen weitaus schwerwiegendere Erkrankungen verursachen können, werden dabei nicht erfasst. So weist die WHO (2006) ausdrücklich darauf hin, dass trotz Abwesenheit von *E. coli* die potentielle Gefahr einer Trinkwasserkontamination mit pathogenen Viren besteht. Dennoch gibt es bisher keine verpflichtenden Regelungen zur routinemäßigen Überwachung von Grundwasser bzw. Trinkwasser auf pathogene enterische Viren. Das hohe virale Infektionspotential, ihre geringe Größe (Viren passieren häufig Standard-Filtersysteme) und ihre hohe Widerstandsfähigkeit (besonders auch gegen die physikalische und chemische Wasseraufbereitung) unterstreichen die Notwendigkeit eines erweiterten Indikatorkonzepts. Aktuell werden Coliphagen und Adenoviren als virale Indikatoren diskutiert (Pina *et al.* 1998; Jiang 2006; Szewzyk *et al.*, 2006). Auch der Nachweis auf parasitäre Protozoen, die ebenfall regelmäßig in noch infektiösem Zustand die Trinkwasseraufbereitung überwinden, ist bei der Trinkwasseranalyse bis heute noch nicht gesetzlich vorgeschrieben. Aufgrund der zunehmenden Kontaminationsquellen, dem steigenden Grund- und Trinkwasserbedarf und der daraus resultierenden Zunahme der Trinkwasseraufbereitung empfehlen wir ein integratives Indikatorkonzept, welches alle relevanten Pathogenen mit einbezieht.

9.3 WEITERENTWICKLUNG VON DNA/RNA-EXTRAKTIONSMETHODEN

Wie bereits zuvor erwähnt, setzt der erregerspezifische molekulare Nachweis von Pathogenen aus Umweltproben eine quantitative Extraktion der Nukleinsäuren sowie deren effiziente Amplifikation voraus. Durch natürlich im Wasser und Sediment vorkommende hemmende Substanzen, wir z.B. Huminstoffe und Eisenverbindungen, ist die Extraktion bei aktuell verwendeten Methoden selten vollständig und der qualitative bzw. quantitative Nachweis erschwert. In der Regel kommt es bei einer Anreicherung von Pathogenen auch zu einer parallelen Anreicherung der inhibitorischen Stoffe. Die Weiterentwicklung und Erprobung verbesserter Extraktions- und Aufreinigungsprotokolle muss vorangetrieben werden.

9.4 BIOLOGISCHE TESTVERFAHREN/INFEKTIONSTESTS FÜR ALLE RELEVANTEN KRANKHEITSERREGER

Da der alleinige (molekularbiologische) Nachweis von Krankheitserregern in Wasserproben noch keinerlei Rückschlüsse auf deren Infektiosität zulässt sind dringend verlässliche Infektiositätstests für alle relevanten pathogenen Mikroorganismen und Viren erforderlich. Dies wird besonders am Beispiel der Noroviren deutlich. Obwohl Noroviren heutzutage nachweislich die häufigste Ursache für den Ausbruch viraler Gastroenteritis-Epidemien sind, konnte bisher kein geeignetes in vitro Zellkultursystem für den Nachweis von infektiösen Noroviren entwickelt werden.

9.5 BERÜCKSICHTIGUNG VON GENO- UND SEROTYPEN IN DER URSACHENFORSCHUNG

Im Falle einer Trinkwasserkontamination und im Hinblick auf epidemiologische Studien könnte die Bestimmung der jeweiligen Genotypen (z.B. im Falle der Noroviren) oder der Serotypen (z. B. im Falle der Adenoviren) entscheidend zu einer verbesserten Trinkwasserüberwachung beitragen. Die exakte Identifizierung des jeweiligen Krankheitserregers erlaubt die Suche nach möglichen Kontaminationsquellen und hilft somit entscheidend bei der Eindämmung von wasserbürtigen Ausbrüchen (Nygard *et al.* 2003; Maunula und Von Bonsdorff 2005; Koh *et al.* 2008). Da mittlerweile bekannt ist, dass verschiedene Virus-Serotypen auch ein unterschiedliches Verhalten in der Umwelt zeigen können, sollte die Typisierung zukünftig für eine verbesserte Ursachenforschung in der Epidemiologie herangezogen werden (Fong *et al.* 2010; H.C. Selinka, pers. Mitt. 2010).

9.6 ENTWICKLUNG EINES EINFACHEN MODULAREN SETS VON REDUKTIONSPOTENTIALEN ZUR RISIKOBEWERTUNG

In Abhängigkeit von den Umweltbedingungen und Wegstrecken, kommt es in verschiedenen Umweltkompartimenten (Luft, Boden, Fluss, Aquifer) zu einer unterschiedlich starken Abreicherung von Pathogenen. Die Abwasseraufbereitung in einer konventionellen Kläranlage, beispielsweise, gewährleistet eine Veringerung der Anzahl an pathogenen Viren um etwa 2 log-Stufen. Dies entspricht dem Reduktionspotential einer biologisch aktiven Bodenschicht. Reduktionspotentiale typisch für verschiedene Umweltkompartimente können in ein einfaches modulares Set zur theoretischen Abschätzung der Konzentrationsabnahme von Pathogenen entlang eines Eintragspfades einfließen und erlauben eine erste Risikoabschätzung. Im Falle der Trinkwassergewinnung durch Uferfiltration könnten so z.B. die einzelnen Reduktionspotentiale vom Ausgangsprodukt (z. B. Abwasser) bis hin zum Rohwasser (z. B. Grundwasser) aufsummiert werden und somit bereits Rückschlüsse auf das Risiko einer Verunreinigung des Trinkwassers gezogen werden.

9.7 MODERNE KONZEPTE ZUR ROHWASSERÜBERWACHUNG VOR ALLEM BEI DER PRODUKTION VON TRINKWASSER AUS OBERFLÄCHENWASSER

Im Gegensatz zum viel besser geschützten Grundwasser enthalten Oberflächenwässer, in die geklärtes Abwasser eingespeist wird eine erhöhte Anzahl an pathogenen Mikroorganismen und Viren. Daher wird speziell bei der Trinkwassergewinnung aus Oberflächenwasser, z. B. durch Uferfiltration oder künstliche Grundwasseranreicherung, empfohlen, eine regelmäßige Endproduktkontrolle am Rohwassers durchzuführen und hierbei besonders auch unregelmäßige klimatische bzw. hydrologische Ereignisse wie Starkregen oder Schneeschmelze zu berücksichtigen. Hierzu zählt auch die systematische Bewertung einer möglichen viralen Kontamination von Trinkwasser in Zeiten erhöhter Infektionsaufkommen.

9.8 RÜCKSTELLPROBEN VON ROH- UND TRINKWASSER ZUR VERBESSERTEN DOKUMENTATION VON WASSERBÜRTIGEN ERKRANKUNGEN

Erst kürzlich wurde für Wasserwerke die Einführung einer einfach zu praktizierenden Methode zur besseren Trinkwasserüberwachung empfohlen (Maunula *et al.* 2005). Nämlich, die regelmäßige (täglich oder wöchentlich) Lagerung einer repräsentativen Stichprobe des frisch produzierten Trinkwassers und des Rohwassers soll im Falle eines wasserbürtigen Krankheitsausbruches einen schnellstmöglichen Rückschluss auf die Kontaminationsquelle ermöglichen. Diese Rückstellproben können problemlos über einen Zeitraum von 50 Tagen gelagert werden und wären somit eine kostengünstige Ausweitung des Risikomanagements.

9.9 EPIDEMIOLOGISCHES DOKUMENTATIONSNETZ FÜR WASSERBÜRTIGE ERKRANKUNGEN

In Deutschland kann eine gewisse Saisonalität von Infektionskrankheiten beobachtet werden. Virale Infektionen, z. B. durch Noro- oder Rotaviren verursacht, treten gehäuft in den kalten Wintermonaten auf (RKI 2011). Erkrankungen mit bakteriellen Krankheitserregern wie z.B. Salmonellen überwiegen dagegen im Sommer. In beiden Fällen sind die Übertragungswege nicht immer eindeutig geklärt. Da einzelne Infektionen heutzutage epidemiologisch noch nicht dokumentiert werden, kann oftmals der wichtige Bezug zwischen Erkrankung und Ursache/ Kontaminationsquelle nicht hergestellt werden. Ein verbessertes epidemiologisches Dokumentationsnetz könnte bei der Suche nach möglichen Kontaminationsquellen helfen und somit dazu beitragen, die rasche Ausbreitung wasserbürtiger Ausbrüche zu verhindern und die Qualitätssicherung von Trinkwasser nachhaltig zu entwickeln.

9.10 WEITERENTWICKLUNG VON WASSERMANAGEMENTSTRATEGIEN (ÜBER MULTI-BARRIEREN-KONZEPT HINAUS, ANALOG ZUM WATER SAFETY PLAN)

Die WHO hat zur besseren Risikoabschätzung von viralen Infektionen durch kontaminiertes Trinkwasser einen so genannten „Water Safety Plan (WSP)" erstellt. Zur besseren Beurteilung der Trinkwasserqualität berücksichtigt dieses Konzept sowohl die Qualität des Rohwassers als auch die Effizienz der angewendeten Wasseraufbereitungsmethode. Bei bekannter Viruskonzentration im Rohwasser und der kalkulierbaren Effizienz der jeweiligen Wasseraufbereitung, ermöglicht dieses Konzept eine Risikoabschätzung (WHO 2004). In Deutschland kommt als vergleichbares Konzept ein so genanntes dreistufiges Multibarrierensystem zur Anwendung, welches primär den systematischen Ressourcenschutz, eine angemessene Aufbereitung und eine ordnungsgemäße Verteilung des Trinkwassers bis hin zur Hausinstallation beinhaltet. Da jedoch diese Schutzmaßnahmen nicht immer ausreichend sind und das Trinkwasser final nur auf die Abwesenheit bakterieller Fäkalindikatoren gestestet wird (Szewzyk *et al.*, 2006), wird nahe gelegt, das deutsche System um von der WHO empfohlene Schritte zu erweitern. Wie bereits zuvor erwähnt sollten für eine verlässliche regelmäßige Kontrolle des Rohwassers gerade auch extreme klimatische und hydraulische Bedingungen, wie z.B. Starkregenereignisse, Überschwemmungen oder auch Schneeschmelze, miteinbezogen werden (Szewzyk *et al.*, 2006). Moderne Wassermanagementkonzepte sollten zudem die Vulnerabilität von Einzugsgebieten und Grundwasserleitern berücksichtigen (Harter und Walker 2001; Chilton 2006). Hierdurch könnten gerade in Gebieten mit erhöhter Gefahr einer Kontamination des Grundwasserleiters die Schutzzonen besser ausgewiesen werden.

9.11 AUSWEISUNG VON GRUNDWASSERSCHUTZGEBIETEN UNTER BERÜCKSICHTIGUNG VON EXTREMEN KLIMATISCHEN UND HYDROLOGISCHEN BEDINGUNGEN

Wissenschaftliche Studien belegen, dass Pathogene im Untergrund bei niedrigen Temperaturen über Monate bis zu mehrere Jahre hin überdauern können. Des Weiteren gibt es Hinweise, dass sich einige pathogene Bakterien auch außerhalb ihres Wirtes vermehren können. Es stellt sich daher die Frage, ob die in Deutschland und vielen anderen Ländern gültige 50-Tage-Linie der Grundwasser-Schutzzone II ausreichend Schutz vor einer Kontamination mit pathogenen Mikroorganismen und Viren gewährleistet. Gerade in Karst und Kluftleitern werden pathogene Verunreinigungen regelmäßig detektiert. Ein besseres Verständnis der komplexen physikalischen und biologischen Prozesse die zu einem Rückhalt bzw. dauerhaft zu einer Elimination von Pathogenen beitragen ist notwendig. Wir empfehlen, in Risikogebieten Schutzzonen auf Basis von weitläufigen Tracerexperimenten unter Verwendung von nicht-pathogenen Vertretern bekannter Krankheitserreger zu überprüfen und dabei auch extreme klimatische und hydraulische Situationen zu berücksichtigen.

9.12 MODELL- UND REFERENZVIREN FÜR FORSCHUNGSZWECKE

In der Vergangenheit wurden zahlreiche Modellviren (z.B. der F-spezifische Bakteriophage MS2, die somatischen Phagen PRD-1 oder Φ174, oder auch Bakteriophagen die spezifisch *Bacteroides fragilis* infizieren) die sich bezüglich Transport und Rückhalt in der Natur ähnlich wie ihre pathogenen Verwandten verhalten als mögliche nicht-pathogene Vertreter (surrogates) getestet (Tartera und Jofre 1987; Leclerc *et al.* 2000; Grabow 2001; Van der Wielen *et al.* 2008). Gerade im Hinblick auf Tracerstudien im Gelände kommt diesen Modellviren eine große Bedeutung zu. Am Beispiel des nicht-kultivierbaren hochinfektiösen Norovirus wird die Notwendigkeit der Identifizierung und Testung geeigneter Modellviren deutlich. In den letzten Jahren ist es gelungen ein murines Norovirus erfolgreich in Zellkultur zu vermehren und

reproduzierbar durch RT-PCR nachzuweisen (Bae und Schwab 2008). Dieses, für den Menschen ungefährliche Virus, könnte in zukünftigen Studien als Ersatzvirus für das menschliche Norovirus fungieren und somit zu einer verlässlicheren Trinkwasserüberwachung beitragen.

9.13 FELDVERSUCHE ZU TRANSPORT UND ÜBERLEBEN VON PATHOGENEN IN SEDIMENT

Die Erkenntnisse, die bisher zu Transport und Rückhalt von Pathogenen in der Umwelt gesammelt wurden, stammen überwiegend aus Laborversuchen und Experimenten mit nicht-pathogenen Modellorganismen und -viren. Daher fordern wir für eine besseren Abschätzung der in *situ* Verhältnisse vermehrt Versuche im Feld. Bisher konzentrierten sich die meisten Studien zudem beinahe ausschließlich auf die Analyse und Wiedergewinnung von Pathogenen aus der wässrigen Phase (Sickerwasser, Porenwasser, Grundwasser). Viruspartikel haften, zum Beispiel, sehr rasch an Sedimentoberflächen an. Diese können über einen langen Zeitraum infektiös bleiben und somit als ein Reservoir an Krankheitserregern verstanden werden. Dieser Aspekt der möglichen Aufkonzentration von unerwünschten Keimen und Viren an der Festphase sollte besonders im Hinblick auf die stetige Zunahme von Wasserrecycling und Trinkwassergewinnung (z. B. Uferfiltration) berücksichtigt werden. Durch die zunehmende Belegung der Adsorptionsstellen kann sich die natürliche Rückhaltekapazität des Untergrunds über die Zeit erschöpfen. Starke Änderungen in der Wasserzusammensetzung, vor allem die Verringerung der Ionenstärke, kann zu einer erneuten Freisetzung ursprünglich adsorbierter pathogener Partikel führen. Es wird daher empfohlen besonders auch den Transport und die Persistenz von Pathogenen im Sediment (Böden, hyporheische Zone von Flüssen, Grundwasserleiter) im Hinblick auf die reversibel immobilisierte Fraktion an Pathogenen zu untersuchen.

9.14 BEITRAG BIOTISCHER ANTAGONISTISCHER PROZESSE ZUR ELIMINIERUNG VON PATHOGENEN

Es gilt mittlerweile als erwiesen, dass das Verschwinden von Pathogenen in Böden und Sedimenten (durch Rückhalt oder Elimination) mit der biologischen Aktivität korreliert (Hurst *et al.*, 1980; Hurst 1988; Nasser *et al.*, 2002). Gegenüber *E. coli* konnten sogar direkte antagonistische Effekte durch autochthone Grundwasserbakterien nachgewiesen werden (Hirsch und Rades-Rohkohl 1983). Insgesamt betrachtet ist bisher jedoch nur sehr wenig über antagonistische mikrobielle Effekte die aktiv zur Elimination von Pathogenen in der Umwelt beitragen und die dahinter stehenden Prozesse bekannt. Ein besseres Verständnis dieser natürlichen Eliminationsprozesse könnte entscheidend dazu beitragen den Trinkwasserschutz und das -management weiter zu verbessern und Kosten für die physikalisch-chemische Wasseraufbereitung einzusparen.

9.15 MODERNE METHODEN ZUM NACHWEIS VON INFEKTIÖSEN DAUERSTADIEN

Mikroorganismen (und Viren) haben zahlreiche Strategien entwickelt um ungünstige Lebensbedingungen zu überstehen. Von pathogenen Bakterien und Protozoen kennt man heute so genannte „Viable But Not Culturable (VBNC)" Stadien, sowie die Ausbildung von Sporen oder Zysten (Roszak und Colwell 1987; Oliver 2005; Karanis *et al.*, 2007). Diese morphologisch und physiologisch veränderten Zustände zeichnen sich durch eine niedrige zelluläre Aktivität aus und ermöglichen es diesen Organismen außerhalb ihres Wirtes lange Zeit zu überdauern. Pathogene mit einer niedrigen Zellaktivität oder in einem Überdauerungsstadium sind oftmals nicht oder nur sehr schwer mit herkömmlichen kultivierungs-abhängigen Nachweismethoden zu erfassen (Oliver 2005). Da Krankheitserreger in diesen Dauerstadien jedoch weiterhin als potentiell infektiös gelten, birgt dies zusätzliche Risiken für eine sichere Trinkwasserüberwachung. Ein besseres Verständnis der Ökologie von Pathogenen und die Weiterentwicklung möglicher kultivierungs-unabhängiger Nachweisverfahren können entscheidend zur Prävention wasserbürtiger Ausbrüche beitragen.

9.16 ÖKOLOGIE VON ANTIBIOTIKA-RESISTENTEN PATHOGENEN MIKROORGANISMEN IN DER UMWELT

Die Zunahme an multiresistenten Bakterienstämmen in der Umwelt und der potentielle horizontale Gentransfer zwischen autochthonen und allochthonen Arten sind nicht nur von großem wissenschaftlichem Interesse sondern betreffen auch sozioökonomische Belange. Aufgrund der heutzutage oftmals unbedachten Anwendung zahlreicher Antibiotika in der modernen Tierzucht kann eine deutliche Zunahme an Antibiotika-Resistenzen innerhalb von gastrointestinalen Bakterien beobachtet werden (Sapkota et al 2007). Ein weiteres Risiko einer Grundwasserbelastung mit multiresistenten Bakterien bergen zudem immer häufiger die Abwässer von Krankenhäusern (Reinthaler *et al.*,

2003). Das Verhalten dieser genetisch veränderten Organismen in der Umwelt ist bisher weitestgehend unbekannt und sollte wissenschaftlich genauer untersucht werden.

9.17 EINFÜHRUNG INTERNATIONALER STANDARDS FÜR LABOR- UND FELDVERSUCHE

Im Anbetracht der großen Unterschiede innerhalb der zahlreichen experimentellen Beobachtungen die bisher hinsichtlich Verhalten (Rückhalt, Adsorption) und Überleben bzw. Persistenz von Pathogenen gefunden wurden, sollte angedacht werden, für die Durchführung solcher Studien im Labormaßstab zukünftig Standardrahmenbedingungen einzuführen. Sowohl die Verwendung einheitlicher Protokolle für die Vermehrung und Präparation von eingesetzten Testorganismen als auch die standardisierte Einführung interner Kontrollen, wie z. B. der Bakteriophage MS2 oder *E. coli*, könnten helfen die große Streuung der dokumentierten Überlebensdaten zu einzelnen pathogenen Mikroorganismen und Viren im Grundwasser zu minimieren (John und Rose 2005).

9.18 AKTUELLE FORSCHUNG – VERBUNDPROJEKT ZU NACHWEIS, TRANSPORT UND ELIMINATION VON PATHOGENEN VIREN IM WASSER

Ein multidisziplinäres Forschungsprojekt, welches von der Deutschen Forschungsgemeinschaft (DFG) finanziert wird, bearbeitet derzeit einige der angesprochenen Fragestellungen. Zwei Arbeitsgruppen (M. Seidel & R. Niessner, Institut für Hydrochemie, Technische Universität München, TUM; M. Exner & C. Koch, Institut für Hygiene und Öffentliche Gesundheit, Universität Bonn) arbeiten beispielsweise an der Entwicklung von Hochdurchsatz-Filtrationstechniken (Ultra- und Nanofiltration) zur Anreicherung von pathogenen Viren. Zwei Arbeitsgruppen am Deutschen Umweltbundesamtes (UBA, Berlin) erforschen unter der Leitung von H. C. Selinka & I. Chorus das Verhalten von pathogenen Viren (Noroviren und Adenoviren) in Oberflächenwasser und bei der Sedimentpassage. Es wird zudem versucht Protokolle zur Nukleinsäure-Extraktion zu optimieren (Zusammenarbeit mit Ch. Drosten, Institut für Virologie, Universität Bonn). Ein weiteres Ziel ist zudem die Identifizierung neuer Indikatorviren. Die Effizienz der Viruselimination während der Abwasseraufbereitung und mögliche beteiligte Faktoren werden von Kollegen aus Hannover (K.-H. Rosenwinkel & S. Wolters, Institut für Siedlungswasserwirtschaft und Abfalltechnik, Leibniz Universität Hannover) untersucht. Im Mittelpunkt steht hierbei die Entwicklung und Verbesserung von mathematischen Modellen zur Vorhersage der Effizienz der Viruselimination während der Abwasseraufbereitung. Die Autoren dieses Gutachtens sind selbst an der Erforschung von mikrobiellen Prozessen und deren Beitrag zur Elimination von pathogenen Viren in aquatischen Systemen interessiert. Im Mittelpunkt stehen Versuche zum Grazing von Viren durch Protozoen und Experimente zur Lyse von Viren durch bakterielle Exoenzyme.

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