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Emerging Pathogens in Wastewaters

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Abstract This chapter contains a review of the most important emerging problems related to infectious diseases that have or may have some relationship to wastewater. It includes pathogenic viruses, bacteria and protozoa, as well as prions, and genes such as those implicated in resistance to antibiotics or virulence factors (which are transferred between different bacteria, and can influence the emergence of more noxious infectious microorganisms). After a short revision of the causes found in the emergence and re-emergence of infectious diseases, there is a detailed review of the main emergent infectious agents transmitted by the water route, and whose presence in sewage might have public health implications.

Keywords Wastewater · Emerging · Bacteria · Viruses · Protozoa · Pathogens

1 Introduction

The early history of infectious diseases was characterised by sudden, unpredictable outbreaks, frequently of epidemic proportion. Scientific advances in the late 19th and early 20th century resulted in the prevention and control of many infectious diseases, particularly in the industrialised nations. In developing countries infectious disease is still the first cause of mortality, because poverty does not allow implementation of the measures applied in the industrialised countries. Despite a century of progress, infectious diseases still cause enormous human suffering, deplete scarce resources, impede social and economic development and contribute to global instability. The potential for even greater dissemination looms as a continuous threat for humanity [1].

Recent worldwide outbreaks indicate the potential for the sudden appearance of infections in currently unaffected populations. Cryptosporidiosis both in industrialised and developing countries [2] and cholera in the Americas [3] are good examples. Additionally, new infectious diseases, often with unknown long-term public health impact (Ebola virus, *Legionella*, Hantaan virus, *Camplylobacter*, *E. coli* O157:H7, human immunodeficiency virus (HIV), hepatitis C, West Nile virus or severe acute respiratory syndrome (SARS) to mention some [4–9]) or with known public health impact but not considered as infectious (e.g. *Helicobacter pylori* [10])) continue to be identified. New agents are regularly added to the list, particularly with the availability of nucleic acid amplification and recognition techniques for detecting and identifying otherwise non-cultivable microorganisms.

All these observations have lead to an increasing interest in emerging microbial threats to health and multiple initiatives have been taken for an effective surveillance and control of these emerging infectious diseases. As always, when a new terminological concept or term appears, multiple definitions appear in parallel. Following two of them in our opinion covers all possible meanings of the term.

Thus in 1992, the US Institute of Medicine (IOM) [11] defined an emerging infection as any new, re-emerging or drug-resistant infection whose incidence

in humans has increased within the past two decades or whose incidence threatens to increase in the near future.

In one of the first articles published in the *Journal of Emerging Infectious Diseases*, which is published by the National Center for Infectious Diseases (USA Center for Disease Control and Prevention), it was written that emerging infectious diseases can be defined as infections that have newly appeared in a population or have existed but are rapidly increasing in incidence or geographic range [12].

However, it is difficult to decide whether there is a true increase in the incidence of a given infection, or whether our ability to detect the pathogen more frequently now than previously is being perceived as an emergence. Indeed in the past 20 years the ability of microbiologists to detect, enumerate and characterise microorganisms has increased enormously. Firstly, the number of microorganisms cultivable by the traditional microbiological procedures has increased. Secondly, molecular techniques, mainly those based on nucleic acid amplification, have increased the range of detectable microorganisms. The most important of these techniques being the polymerase chain reaction (PCR), nucleic acid sequence based amplification (NASBA) for amplifying DNA and reverse transcriptase-polymerase chain reaction (RT-PCR), nucleic acid recognition (hybridisation in its multiple methodological varieties) and characterisation (restriction patterns and sequencing) . The paramount examples of an improvement in our abilities to detect them are, among many others, *Helicobacter pylori* and *Cryptosporidium parvum*, and examples of truly emerging pathogens are enterotoxigenic *Escherichia coli* O157:H7 and *Vibrio cholerae* O139.

In the present chapter we will review the emerging diseases considered in the broader sense.

2
Factors in Infectious Disease Emergence

The reasons underlying the emergence of water-borne diseases can, at least, only be discussed in terms of possibilities. In many aspects they differ substantially from the causes found in the emergence of chemical contaminants. However, factors causing infectious disease emergence can be identified in virtually all cases. Among them we find those described below.

Ecological changes, including those due to economic development and land use, are found in the origin of the expansion of some infectious diseases. Thus the construction of dams that introduces changes in water ecosystems, deforestation/reforestation; floods and flood/drought cycles, the effects of extensive cattle rising in wildlife are all factors found in the origin of the expansion of some infections diseases. Argentine, Bolivian and Korean hemorrhagic fever, hantaviruses infections, Lassa fever and lyme disease are due to ecological changes that favour human contact with alternative hosts, mainly rodents, and vectors.

Climatic change produces adjustments in the geographical distribution of some vectors that transmit the infectious agents, thus increasing the geographical areas endemic for some infections. Expansion of Dengue in some areas of Central-South America seems to be due to the global warming that allows the vectors to reach new geographic areas. Also, areas where there are the conditions for emerging pathogenic biotypes of *Vibrio cholerae* seem to be increasing as a consequence of global warming.

Human demographic factors such as population growth, changes in age distribution and migration from rural areas to the cities and from some geographical areas to others are certainly important in the emergence and re-emergence of some infectious diseases. Examples of this group are acquired immunodeficiency syndrome (AIDS) and a re-emergence of malaria in certain areas.

Human behaviour is also responsible for a number of emerging infectious problems. Thus sexual behaviour, intravenous drug abuse or use of high density facilities contributes to the spread of a number of infections of different characteristics. Hepatitis B and C, AIDS, and the fast spreading of some respiratory diseases are among this group of emerging infections.

International travel and commerce as well as the rapid movement of goods and people favour the rapid dissemination of emerging pathogens. Ebola, Marburg, West Nile encephalitis and monkey pox in the United States, malaria and the fast spreading of newly re-assorted influenza virus or of new biotypes of cholera are examples of this group.

Technology and industry are also found in the origin of some problems. Globalisation of food supplies, changes in food processing and packaging, rendering processes, organ or tissue transplantation, drugs causing immunosuppression, air conditioning and widespread use of antibiotics are, among other causes, at the root of some problems. Examples of this group are legionellosis, haemolytic uremic syndrome (*Escherichia coli* O157:H7), hepatitis B and C, AIDS and bovine spongiform encephalopathy in cattle.

Mutation and genetic interchange produces many changes in the microorganisms, rendering them able to infect new hosts, to be more virulent (pathogenic) or to be resistant to one or more antibiotics. This aspect has probably always occurred, but becomes more important as a consequence of several of the previous factors. Influenza virus, Rift Valley viruses, cholera and haemolytic uremic syndrome (*Escherichia coli* O157:H7) are all infectious diseases in which genetic changes or new genetic re-assortments are identified as factors involved in their emergence or re-emergence.

Last but not least, there is a substantial breakdown in public health measures in many areas of the world that influences the emergence or re-emergence of some infectious diseases. Limitation or reduction in prevention programs, inadequate sanitation, and absent or inadequate vector control measures have indeed increased the propagation of old and new pathogens. Examples are the resurgence of tuberculosis in the United States and some countries of Western Europe, cholera in refugee camps in Africa and diphtheria in the former Soviet Union.

3 Emerging Pathogens in Sewage

Many pathogens are expected to be found in sewage under certain circumstances, since many of them are found either in faeces or in urine, although their numbers will vary according to the epidemiological status of the population. As an example, nucleic acids homologous to human immunodeficiency virus (HIV), the causal agent of AIDS, has been reported in wastewater [13], though these results are controversial and though the role of the faecal-oral route in HIV transmission is null. Also, the coronavirus causing severe acute respiratory syndrome (SARS) is expected to be found in sewage in areas with infected individuals since it causes diarrhoea in about 25% of infected individuals [14] and has been found in faeces of some of the infected macaques [15], though the role of the faecal-oral route in transmission of the SARS coronavirus is not yet known. But, we only have to worry about those pathogens in sewage that are transmitted through water, mostly those transmitted by the faecal-oral route, and consequently we will limit this article to this group.

Table 1 summarises the emerging water-borne pathogens. These are the pathogens that we will comment on below. Additionally, we will comment on some other emerging problems such as prions, bacterial genes encoding for resistance to antibiotics and genes encoding for virulence factors.

The presence of most of them in sewage is important because their transmission is by the faecal-oral route. Consequently, contamination of drinking water, recreational waters and food with sewage containing these pathogens

Table 1 Major emerging infectious diseases transmitted through water

Agent	Category	Main disease
Cryptosporidium parvum	Protozoa	Acute enterocolitis
Legionella pneumophila	Bacteria	Legionellosis
Campylobacter jejunii	Bacteria	Gastroenteritis
Escherichia coli O157:H7	Bacteria	Haemorrhagic colitis and haemolytic uremic syndrome
Helicobacter pylori	Bacteria	Gastric ulcers
Vibrio cholerae O139	Bacteria	Cholera
Hepatitis E virus	Virus	Hepatitis
Cyclospora	Protozoa	Acute enterocolitis
Toxoplasma gondii	Protozoa	Toxoplasmosis
Mycobacterium (atypical)	Bacteria	–
Norovirus and Sapovirus	Virus	Acute gastroenteritis
Yersinia enterocolitica	Bacteria	Acute diarrhoea

means a significant health hazard. The presence in sewage of bacteria such as *Legionella* or *Mycobacterium* is not considered to be an additional health hazard since they are constituents of the normal microbiota of fresh waters; they grow in the biofilms of the pipelines of drinking water supply networks, and the contribution of sewage in their transmission is considered to be negligible.

4 Pathogens and Indicators in Sewage

In sewage contaminated by faecal material, either human or animal, a great number and variety of microorganisms are found. Although some of the microorganisms excreted by humans and animals are pathogens, the great majority are not. Neither the pathogens nor the non-pathogens that originate in the gut replicate once outside the gastrointestinal tract, although it cannot be excluded that some of them replicate under some exceptional circumstances. However, they can survive quite successfully outside the gastrointestinal tract. Some survive much more successfully than others. Generally speaking, virus and some forms of protozoa (oocysts) persist more than bacteria, and among bacteria, those that make spores survive better than those that do not. Many of the microorganisms excreted through faeces are found in similar numbers in sewage of both human and animal origin and in sewage from different geographical areas. The microorganisms contributed by humans or animals are similar, though studies are being made in order to be able to track the sources of faecal contamination for a better management of water resources. Some of these microorganisms in all sorts of faecal samples are used as surrogate indicators for the assessment of water quality. Among these surrogate indicators there are groups of species like total coliforms, faecal coliforms, faecal streptococci; sulfite reducing clostridia, coliphages, etc., on which definitions are based in a given detection and enumeration method or methods. They have been used for many years. At present they are being progressively substituted by species or genera (*Escherichia coli*, *Enterococcus* and *Clostridium perfringens*) when techniques are available.

In contrast, the presence of pathogens in sewage depends on the epidemiological status of the population, and accordingly their densities may vary from zero to millions per millilitre. Consequently, there are important differences in their densities between different periods of time in the same geographical location and between geographical areas or countries. Usually the densities and diversity of pathogens in sewage of developing countries is significantly higher than in industrialised countries, although this may not be applicable for emerging pathogens. For example, the numbers of antibiotic-resistant microorganisms are higher in industrialised countries than in the developing ones. When available, data on occurrence and densities of emerging pathogens in sewage belong to industrialised countries, and consequently it is difficult to compare between the occurrence of emerging pathogens in industrialised and developing countries.

5
Pathogens Detection in Sewage

Detection of pathogens in water samples and consequently in sewage present serious difficulties, which arise from the difficulty itself of cultivating some pathogens and their small densities as compared to those of the background flora. Problems in detecting and counting pathogens differ for viruses, bacteria and protozoa. Traditional methods for viruses and bacteria rely on the capability to reproduce them, whereas those for protozoa rely on direct observation.

5.1
Traditional Methods

Viruses only replicate when susceptible cells are available. In this case, they can be counted either by direct enumeration of plaques of lysis (each plaque is associated to an infectious unit and receives the name of plaque-forming units, PFU) on a monolayer of cells covered by a layer of semisolid culture media, or by quantal methods based on the determination of the presence/absence of viruses in a given volume of sample assayed on a monolayer of susceptible cells covered by liquid culture media. A combination of a significant number of subsamples, tested at different volumes, allows statistical estimations (most probable number, MPN, or 50% tissue culture infectious dose, $TCID_{50}$) of the number of infectious viruses. But, a significant proportion of the emerging viruses do not grow in any of the available cell lines. This is the case of the emerging viruses, hepatitis E viruses, noroviruses and sapovirus, whose transmission is associated with water.

Bacteria are usually grown on mixtures of suitable nutrients, which can be extremely variable depending on the bacteria. Also, bacteria have different requirements regarding oxygen concentration in the culture media. Providing that suitable media are available, bacteria, as in the case of viruses, can be counted either by direct enumeration of colonies (each colony is associated to a cultivable bacterium and receives the name of colony forming units, CFU) on a layer of solid culture media, or by quantal methods based on the determination of the presence/absence of bacteria in a given volume of sample assayed on liquid culture media. A combination of a significant number of sub-samples tested at different volumes allows a statistical estimation (MPN) of the number of cultivable bacteria. Currently used culture methods for pathogenic bacteria are time-consuming and laborious, requiring prolonged incubation, selective enrichment to reduce the growth of the background flora, and biochemical identification. Additionally, bacterial pathogens may also enter a viable but non-cultivable state due to starvation and physical stress and selective media do not efficiently recover stressed microorganisms, and indeed most pathogens are stressed in sewage.

Traditional methods for parasitic protozoa are based on microscopic observation, which is time-consuming, extremely tedious and requires trained

personnel. Direct observation can be improved by immunostaining with specific antibodies. Techniques such as flow cytometry or laser scanning immunostained protozoa are useful tools for detecting and enumerating parasitic protozoa. These techniques still have some drawbacks such as the presence of interfering autofluorescent particles (e.g. unicellular green algae that do not distinguish between live and dead oocysts). A number of chemical stains (DAPI, PI, SYTO, etc.) have been used in order to determine the viability of the protozoa observed by microscopy, though no one seems to provide comparable results to the ones provided by infection of susceptible cells or hosts.

5.2 Molecular Methods

Over the last decade molecular techniques, mostly PCR-based systems including RT-PCR, PCR, quantitative PCR or detection of amplified DNA by hybridisation in DNA microarrays, have been applied to develop improved detection methods for pathogens. Due to its high sensitivity, specificity and rapid results, PCR is presented as an alternative to conventional methods. However, environmental application of PCR presents several problems.

Firstly, environmental samples may contain inhibitory substances with a significant effect on the activity of enzymes (reverse transcriptase and *Taq* polymerase) used in amplifying nucleic acids. In this case purification steps may be necessary. Secondly, the low proportion of pathogens compared to the background flora causes amplification problems. A pre-enrichment step can overcome this problem. However, this pre-enrichment approach again causes problems in quantification. Thirdly, these techniques do not allow distinction of whether the amplified nucleic acids correspond to microorganisms that conserve their infectious potential, or belong to microorganisms that have not yet lost their integrity and in which nucleic acids have not yet been degraded, but which have lost their infectious potential. This third aspect can be overcome in bacteria and protozoa by amplifying the messenger-RNA of those genes that are the first ones to be stimulated when replication is stimulated by external signals.

As a consequence of all these methodological constraints, data on pathogens in sewage are scarce and incomplete. In many cases only presence/absence data are available, and when quantitative data are available, very likely they represent an underestimation of the actual values.

6
Pathogenic Viruses

6.1
Hepatitis E Virus

Among the various viruses causing hepatitis (A, B, C, delta and E), only hepatitis A virus (HAV) and hepatitis E viruses (HEV) are transmitted by the faecal-oral route, and only HEV is considered as emerging. Hepatitis E virus is a major cause of acute hepatitis in many areas of Africa, Asia and America (Mexico), where HEV is considered endemic [16]. In some of these areas it is associated with more than 50% of sporadic cases of acute hepatitis. It was responsible for one of the broadest water-borne hepatitis outbreaks to have occurred in the last 50 years, which affected more than 500,000 individuals in India. It affects mainly young adults and the disease is generally self-limited. However, sometimes hepatitis E has severe complications and a high case fatality rate, particularly in pregnant women [17]. Industrialised countries have traditionally been considered as non-endemic for HEV; most cases in these countries have been considered imported either by travellers or immigrants. Nevertheless, nucleic acid characterisation of some HEV strains isolated from humans in North America and Europe show genetic divergence with strains from HEV-endemic countries [18]. Evidence that some animals, mostly swine, can be reservoirs of HEV are accumulating; swine and human strains isolated in a given geographical area show an important degree of genetic similarity [19]. Transmission of HEV infection during outbreaks primarily occurs through contaminated water [20].

By using a seminested RT-PCR amplification of the viral fraction purified from sewage, genomes of HEV viruses have been detected in raw sewage in industrialised countries like France, Spain and the USA [21, 22]. One of these sewage samples containing genomes of HEV produced infections after being injected to monkeys [21]. This indicates that the viruses occurring in sewage were infectious. Quantitative data about their occurrence in sewage are not yet available, but hepatitis E viruses has been detected by RT-PCR in sewage volumes as small as 1 mL [21]. Certainly, densities of hepatitis E viruses in sewage from endemic areas will be much higher than the densities in sewage from industrialised countries.

6.2
Norovirus and Sapovirus

Though this viral infection may not be considered as truly emerging, since it was first reported in 1972 [23] the availability of molecular detection methods has allowed us to recognise these viruses as the most commonly identified cause of infectious gastrointestinal disease in Western European communities [24] and the USA [25]. They have been included in the list for this reason. These

groups contain many viruses. Norwalk, Snow Mountain and Sapporo viruses are the best known and most frequently reported as causal agents of outbreaks. Collectively they were previously known as Norwalk-like virus, or small round structured viruses [26] and were included in the genus calicivirus. Now they are classified as Norovirus (Norwalk and Snow Mountain among others) and Sapovirus (Sapporo virus among others). Acute gastroenteritis, with vomiting and diarrhoea, caused by the noroviruses is mild and self-limiting in the absence of other factors. It presents a short incubation (24–48 h) and duration of the disease, but the patient excretes huge amounts of viruses, up to 10^{11} g^{-1} faeces, for a couple of days. A large human reservoir of infection, a very low infectious dose and the ability to be transmitted by a variety of oral-faecal routes [24, 25] contribute to the prevalence of infection by noroviruses. Numerous water-borne outbreaks are well documented [27, 28]. Noroviruses also affect animals, and recent data suggest that calves and pigs may be reservoir hosts of these viruses [29].

Noroviruses have been described in domestic sewage [30] by nucleic acid amplification techniques. Values of 10^3–10^5 genomes per 100 mL of raw sewage are found under normal circumstances, but the numbers may increase to more than 10^7 when there is an outbreak in a given community [30]. Recently, a quantitative RT-PCR has been developed for the detection of noroviruses in sewage, and they have been found in 96% of raw sewage samples tested in Southern England with values as high as 10^4 mL^{-1}, and with a wide variety of strains [31]. These data indicate that noroviruses are widespread in the population or Western Europe, even in non-epidemic situations.

7
Pathogenic Bacteria

7.1
Helicobacter pylori

Helicobacter pylori, a Gram-negative, microaerophilic bacterium, has been implicated in the aetiology of most chronic gastritis, peptic ulcer disease and is believed to play a major role in gastric cancer [32]. Water supplies contaminated with faecal material may be the source of *H. pylori* transmission [33]. This is particularly relevant in developing countries where the municipal water supplies are not adequately treated and water is obtained from rivers and other untreated sources.

H. pylori is difficult to cultivate and detection in sewage by culture requires a previous enrichment of the sample by immunoseparation. This impedes quantification, but allows detection. Thus cultivable *H. pylori* have been detected in sewage from industrialised countries [34, 35, 37] and developing countries [36].

7.2 *Campylobacter*

Campylobacter are characterised as fastidious gram negative, non-spore-forming, motile, microaerophilic, spiral shaped microorganisms. *Campylobacter* species occur in the reproductive and intestinal tracts of man and animals. Some species are pathogenic. *Campylobacter jejunii* and *Campylobacter coli* are those more frequently isolated from humans.

C. jejunii is now the leading cause of bacterial gastroenteritis in industrialised countries [38, 39]. Human campylobacteriosis is a severe disease, often leading to serious sequels and sometimes resulting in death. Severe sequels include Guillain Barré Syndrome and reactive arthritis. It is recognised as a zoonotic infection. It seems to be particularly adapted to the avian intestinal tract, thus causing poultry to be the primary vehicle of transmission to humans, though *Campylobacter* is also detected in faeces of other animals. *Campylobacter* cells may enter the environment, including drinking water, through the faeces of animals or infected humans. It is usually associated with food-borne transmission, but recently it has been associated with water-borne outbreaks [38–42] related to contamination of drinking source water by sewage, farm slurries, land run off and contamination with avian wildlife faeces, followed by inadequate disinfection.

There are several problems concerning detection of *Campylobacter* cells in water, including the small numbers and slow growth rates of the organisms. The traditional methods currently used are time-consuming and laborious, requiring prolonged incubation, selective enrichment to reduce the growth of the background flora, and biochemical identification. *Campylobacter* cells may also enter a viable but non-cultivable state due to starvation and physical stress. Recently, methods based on the PCR assay, after enrichment of the samples in non-selective media, seem to provide the best method for *Campylobacter* detection.

Virtually all surface waters contain *Campylobacter* and consequently so does sewage. Concentrations ranging from 10^2 to 10^5 CFU per 100 mL of raw sewage have been reported [43, 44].

7.3 *Escherichia coli* O157:H7

Escherichia coli is a Gram negative, facultative anaerobic bacterium, with curved rod cells. It is a major component of the microbiota of human and animal gut. It is non-pathogenic for humans and animals, and is without any doubt the best known living being. Molecular biology has known most of its breakthroughs in strain K12 of *E. coli*. However, some strains are pathogenic. Among those, *Escherichia coli* O157:H7 is the better known, though not the only one. Other *E. coli* serogroups (O1, O5, O18, O26, O103 and O11, among others) have been described as causing the same diseases as O157 [45, 46]. First

described in 1982, this biotype produces haemorrhagic colitis, with serious complications. Haemolytic uremic syndrome, in around 5% of infected people, was followed by death or permanent renal failure in 3–5% of cases [47]. Its pathogenicity has been attributed in part to the production of enterotoxins known as Shiga-toxins. Numbers of outbreaks and sporadic cases have been increasing since then. It is particularly abundant in cattle faeces. It is transmitted by the faecal-oral route. Food contamination with animal faeces is the main route of transmission, but water-borne outbreaks have been reported [48, 49]. Although generally considered to be a problem in the industrialised countries, it has recently been isolated in developing countries. Joint infections with *Shigella* have been described as having devastating effects in Africa. It is excreted by infected humans and animals and consequently it is expected to be found in sewage.

Methods for direct detection on routine analysis are not available and consequently data on its occurrence and numbers in sewage are very scarce and apparently contradictory, probably as a consequence of methodological problems. They have been isolated in highly polluted river waters at cell densities of 10^2–10^4 mL^{-1} [50] and in urban sewage and calves wastewater at 1–10 mL^{-1} (C. Garcia and X. Bonjoch, personal communication).

7.4 *Vibrio cholerae* O139

Vibrio cholerae is a Gram negative, facultative anaerobic bacterium, with straight or curved rod cells. It is known as the causative agent of cholera. *V. cholerae* is now recognised as an autochthonous member of the microbiota in many aquatic environments such as riverine and estuarine waters as well as in the gut of zooplankton [51]. Among 193 recognised serogroups only two, O1 and O139, have been associated with cholera in humans; some of the other serogroups (O10, O12) can cause sporadic cases of diarrhoea [52]. The difference lies in the fact that O1 and O139 produce the cholera toxin and a colonisation factor that facilitates colonisation of the human gut. Both are coded by genes introduced into the host strain by bacteriophages, and this horizontal transmission of genes seems to be the cause of the emergence of pathogenic strains [53].

From 1817 to the present, seven distinct pandemics (worldwide epidemics) of cholera have occurred. The first six were cause by *V. cholerae* O1, but the last that appeared at the end of 1992 was caused by *V. cholerae* O139 [54, 55]. In Europe, several countries were affected by cholera in the 1990s [56, 57]. At the moment both persist and coexist in different areas of the world, mostly in developing countries, where outbreaks occur in a regular seasonal pattern and are associated with poverty and poor sanitation. A clone of serogroups O37 showed epidemic potential in the 1960s [58], though it did not produced a pandemic situation. The close evolutionary relationship among O1, O139 and O37 and the implication of lysogenic conversion (horizontal transfer of genes mediated by

bacteriophages) of the two virulence genes indicates that new clones with epidemic potential will likely emerge in the future [54, 55, 58].

V. cholerae transmission is water-borne. It is excreted in great amounts by infected persons and consequently it is found in sewage during outbreaks. Numbers as high as 10^5 mL^{-1} of sewage-contaminated areas and irrigation water have been described [51]. Moreover, *V. cholerae* may find the conditions to replicate in some oxidising ponds used to treat wastewaters [59]. As said before, *V. cholerae* persists in different areas of the world, mostly developing countries, where outbreaks occur in a regular seasonal pattern. Where and how it persists in the periods in which there is no disease remains to be elucidated.

7.5
Yersinia enterocolitica

Yersinia enterocolitica is a Gram negative, facultative anaerobic bacterium, with straight rod cells. It is an important food- and water-borne bacterium that is known to cause a variety of gastrointestinal problems. Most commonly, it causes acute diarrhoea. Post-infectious sequelae are manifested in the form of reactive arthritis. World-wide surveillance data on *Y. enterocolitica* show great changes in distribution over the past two decades, for example the colonisation of America by European strains, and bring forth its emerging nature [60]. However, at present, the importance of *Y. enterocolitica* as an emerging water-borne pathogen needs to be elucidated.

Usually isolated from terrestrial and fresh water ecosystems, data available on the presence of *Yersinia* in sewage are very scarce. Using an enrichment procedure *Yersinia* spp were isolated in 90.6% of sewage samples in Germany [61] and in 90% of samples in Argentina [62], of which approximately 50% were *Y. enterocolitica*.

7.6
Legionella pneumophila

It is a Gram negative, aerobic bacterium with straight rod cells. *Legionella pneumophila* serogroup 1 is most frequently associated with the human disease. *Legionella* is a common inhabitant, usually in low numbers, of aquatic environments and of water supply networks [63]. Temperature and other factors influence *Legionella* survival and growth [64]. Hot water tanks, cooling systems and towers create the conditions for bacterial growth, and consequently act as amplifiers of *Legionella* [65]. Evidence also indicates that amoebae may be a natural host for *Legionella*, playing an important role in the transmission of infection [63]. *Legionella* infection can lead to the two forms of the disease, legionellosis, a purulent pneumonia, and Pontiac fever, a self limiting non-pneumonic disease consisting of fever and mild constitutional symptoms. Infection is the consequence of inhalation of contaminated aerosols.

Legionella has been detected in sewage. Values for *Legionella* cells of over 10^3 mL^{-1} have been detected in primary and secondary sewage effluents, and their numbers do not decline through the treatment process [66, 67]. However, it is thought that sewage does not play a role in its transmission.

7.7
Non-tuberculous Mycobacteria

The non-tuberculous mycobacteria include the former "atypical mycobacteria", which are free living saprophytes that are widely distributed in the environment including water [68], but that can also live and grow in animal tissues. They are not contaminants picked up from another source but residents able to survive and grow in water, and it is thought that water may play a role in their transmission. Their resistance to many disinfectants contributes to their persistence in drinking water, which can even be colonised by these bacteria [69]. There is increasing evidence that these non-tuberculous mycobacteria can cause disease [70]. Increases in the immunodeficient population and the prevalence of non-tuberculous mycobacteria in water systems contribute to an emerging problem of water-borne mycobacterial infections. Recently, environmental opportunistic mycobacteria, including *Mycobacterium avium*, *M. terra*, and the new species *M. immunogenum*, have been implicated in outbreaks of hypersensitivity pneumonitis or respiratory problems in a wide variety of settings by exposure to aerosols [71]. However, at present, the importance of non-tuberculous mycobacteria as an emerging water-borne pathogen needs to be elucidated. They have been detected in sewage samples [72], but sewage does not seem to play a role in transmission.

8
Pathogenic Protozoa

8.1
Cryptosporidium parvum

Cryptosporidium, a coccidian parasite, has only recently been recognised as a cause of water outbreaks of cryptosporidiosis. It affects man and other animals. The disease in man is typically mild and self limiting; it may involve diarrhoea, abdominal cramps, low grade fever and headache. In severely immunocompromised hosts, particularly patients suffering with AIDS, it is a life threatening disease: usually there is persistent watery diarrhoea with other affected body sites (e.g. respiratory, biliary) [73]. No chemotherapy is available. The causal agent, *Cryptosporidium* is a homoxenoeus protozoon that develops in the intestinal microvilli. Two types of oocysts are formed; thin-walled oocysts, which cause reinfection of the host, and acid-fast thick-walled oocysts

which are voided by faeces. The taxonomy of the genus is still unsettled. *Cryptosporidium parvum* is the species that infects man.

The outbreak in Milwaukee, with just over 400,000 cases, has been one of the largest documented water-borne incidents in the last decades [72]. Many outbreaks have been associated with potable water.

Transmission by the water route is through acid-fast thick-walled oocysts, which have been shown to be very resistant to the disinfectant doses applied to drinking water. *Cryptosporidium parvum* has been regarded as a problem in industrialised countries. However, the organism has also been isolated from stools of diarrhoeic patients in developing countries [74]. A broad variety of animal reservoirs including farm livestock, pets and wildlife spread oocysts to the water environments. The infectious dose is extremely low. Recent molecular studies indicate the existence of two genotypes, one infecting only humans and the other infecting both humans and animals.

The most frequently used detection methods are direct microscopic observation, indirect (e.g. flow cytometry and laser scanning) detection of immunostained oocysts or by PCR amplification of some specific gene. However, these methods do not distinguishing infectious from non-infections oocysts. Using different hystochemical stains, amplification of the mRNA of some genes related to activation of cell replication and replication in cell cultures has been used to determine the degree of infectiousness of the oocysts, but only infection of hosts (e.g. mice) give an unequivocal response to the question. These technical difficulties limit knowledge of the occurrence and levels of *Cryptosporidium* in the water environment.

Since *Cryptosporidium* is excreted with faeces it is found in sewage. Densities of immunostained oocysts in raw sewage in industrialised countries range from 10^2 to 10^3 L^{-1} under normal circumstances [75–77]. Higher numbers indicate an epidemic situation.

8.2 *Cyclospora cayetanensis*

Cyclospora is emerging as an opportunistic pathogen and may have water-borne routes of transmission. As with *Cryptosporidium*, it has high infection rates in severely immunocompromised hosts, particularly AIDS patients [78]. Before 1995, these parasite protozoa were primarily described in gastroenteritis among children living in poor sanitary conditions in developing areas. Recently, a few outbreaks have been linked to water-borne transmission [79]. In spite of the existence of methodological limitations in its detection, *Cyclospora* has been detected in a limited number of water samples. The importance of *Cyclospora cayetanensis* as an emerging water-borne pathogen needs to be elucidated.

Since *Cyclospora* is excreted by faeces it should be expected in sewage, though data on its occurrence and densities are scarce. *Cyclospora* ssp. genomes were detected by nested PCR in 50% of 1 L sewage samples tested in Peru where cyclosporiasis is endemic [80].

8.3
Toxoplasma gondii

Toxoplasma gondii is an intracellular parasitic protozoa and the causal agent of toxoplasmosis, which is an acute or chronic disease that affects humans and other animals throughout the world. Infection may be asymptomatic, or a simple lymphadenopaty or the disease can be generalised with e.g. hepatitis, pneumonia, myalgia, meningoencephalitis, etc. Latent infection may persist for years. Vertical transmission of toxoplasmosis from an acutely infected pregnant woman can cause serious disease in the foetus. In humans, infection may occur by ingestion of sporulated oocysts (10–15 μm), among other routes of transmission. Since they are found in faeces of infected animals and persons the oral-faecal transmission occurs. Water has been identified as a source of *T. gondii* infection in outbreaks both in developing and industrialised countries [81, 82]. Recent epidemiological studies performed in Brazil indicate the potential importance of oocyst transmission by the water route in this region [83].

At the moment there are no data available about the presence of *Toxoplasma* oocysts in sewage or drinking water. Methods for testing *Toxoplasma* have been dependent on animal inoculation, which is not suitable for the isolation and identification of the parasite in water samples. However, if they are in the faeces of infected humans and animals such as cats, goats, pigs and sheep, they may be expected to be present in sewage.

9
Prions

9.1
Bovine Spongiform Encephalopathy

Bovine spongiform encephalopathy (BSE), the causal agent of mad cow disease, is transmitted by the ingestion of proteinaceous agents called prions, which accumulate in the brain and spinal cord of infected bovines. There is evidence that a new variant of Creutzfeld Jacob Disease in humans is similar to the BSE agent [84]. At present, however, there is no epidemiological evidence to clearly identify the route(s) of transmission of BSE to humans. The most likely source of exposure has been through consumption of beef products that included infected offal (brain spinal cord) before it was banned from human food in late 1998. However, some concern has been expressed about the risks of transmission of BSE to humans through BSE prions discharged to the aquatic environment through wastewaters from rendering plants and abattoirs and through leaching of landfills. However, there is no epidemiological or experimental evidence to identify water as a vehicle of transmission of BSE to humans.

The concern originates from the fact that all known prions are extremely resistant to the thermal and chemical treatments that are commonly used to in-

activate agents of infectious diseases and to the fact that prion infectivity decays rather slowly in the environment. Thus, the extraordinary stability of the prions to physical and chemical inactivation is today considered to be the major cause of the BSE epidemic resulting from the feeding of inefficiently inactivated meat-and-bone meal to cattle. The lack of epidemiological evidences on the route(s) of transmission makes the quantitative risk assessment methods the only instruments for estimating the risk of exposure to BSE though various potential routes, including those from environmental disposal of BSE-infected residues. The most realistic approaches indicate that many individuals will be infected with very low numbers of prion proteins by contaminated water consumption, well below (many orders of magnitude) the infectious dose for man. The low numbers are mainly because of dilution and will never reach the necessary number even after cumulative life consumption. In contrast, a much lower number of individuals will reach the sufficient infectious dose by consumption of highly contaminated beef products. Risk assessment studies indicate that even considering the worst possible scenario, which excludes the host barrier, the probability of humans being infected by consumption of water contaminated with the bovine spongiform encephalopathy prions is extremely remote [85]. To our knowledge there are no data on occurrence of infectious BSE-prions in sewage.

10
Genes

It is well known that some genes can be transmitted by horizontal (or lateral) transference from one bacterium to another and that genes that may affect pathogenic bacteria are also present in non-pathogenic bacteria that can act as donors of those genes. Some of these genes, as virulence genes or genes coding for resistance to antibiotics, are important because they can make those bacteria that incorporate them more dangerous. This horizontal transfer may affect some of the pathogens transmitted from one bacterium to another in water environments. At present, one question is what is the role of the water environment, particularly of sewage, in the spread of these genes between the bacteria present in sewage? Secondly, what is the actual role of some of the genes considered above in the emergence or re-emergence of infectious diseases? Due to the high microbial biomass and the abundance of nutrients, wastewater represents a suitable habitat for horizontal gene transfer.

10.1
Virulence Factors Genes

Many bacterial pathogens have a number of genes that are not found in all strains of the species. Many of these genes encode for characteristics related

to the pathogenicity of a given microorganisms, and are known as virulence factors. Examples are genes encoding for enterotoxins, haemolysins, adhesins, intimines, enterocyte effacing factor, necrotising factors, etc. Many of these genes are susceptible to being transferred from one bacterium to another through the existing mechanisms of horizontal transfer, mainly through phage-mediated transduction and plasmid-mediated conjugation.

Let us consider *Escherichia coli* O157:H7, which has been included among the emerging pathogens. The genome of one strain of serotype O157:H7 has been sequenced [86] and compared to the genome of strain K12 [87], which is the strain used for most of the experiments on the molecular genetics of *E. coli*. The differences between the two genomes are remarkable. Considering the open reading frames, it has been estimated that the chromosome of O157:H7 contains 5,416 genes; 1,387 of these genes are absent in K12, which has only 4,405 genes, of which 528 are absent in O157:H7. Making the comparison in base pairs, 4.64 Mb for K12 versus 5.98 Mb for O157:H7, the difference is about 22.4%. It is thought that the majority of additional genes or sequences would have been acquired by horizontal transfer. Among the differences due to horizontal transfer we find phages that encode for enterotoxins. Additionally, strains of O157:H7 contain transferable plasmids that encode for some virulence factors. Among them is the pO157 plasmid that carries the gene of an enterohaemolysin and the outer membrane protein intimin [88].

Some of the enterotoxins produced by the most virulent strains of *Escherichia coli* O157:H7 are very similar to the enterotoxin produced by *Shigella* spp. and for this reason they were first called "Shiga-like toxins" and later on "Shiga toxins" (Stx) [89]. There are two Stx, Stx1 and Stx2. The toxicity of these toxins can be evidenced on Vero cells and for this reason they are also known as verotoxins. The presence of the verotoxins, mainly Stx2, is very frequently associated with a greater virulence of the strains that produce it and it is thought to be implicated in the progression of the infection from enterocolitis to the haemolytic-uremic syndrome [88]. Up to seven variants of Stx2 have been described [89, 90]. These variants, besides differing in the genetic sequence, might differ in the mechanism of action. In the middle 1980s, O'Brien et al [91], first described that the genes coding for Stx1 and Stx2 in strain 933 of *Escherichia coli* O157:H7 were included in the genome of two prophages, which are the genomes of bacteriophages (bacterial viruses) integrated in the host chromosome. After induction with UV light these phages performed specialised transduction converting the transduced strains into verotoxigenic. Thus, genes encoding Stx1 and Stx2 were transmitted horizontally between *E. coli* strains. Latter on it has been shown that this is frequent in many verotoxigenic strains, belonging to serotypes other than O157:H7 [90, 92] and also in non-*E. coli* enterobacteria such as *Citrobacter* and *Enterobacter* [93, 94].

Nowadays it is known that there is a great diversity of bacteriophages that carry in their genome the genes coding for Stx1 and Stx2 and that there is a certain diversity of bacteria that can be infected and converted to verotoxigenic by

them [90–92, 95]. It is also known that some antibiotics and animal growth promoters (e.g. quinolones, trimetroprim, carbadox and furazolidona) extensively used nowadays induce replication of the converting phages and consequently might augment the horizontal transfer of genes encoding for enterotoxins [96, 97]. Also, horizontal transfer has been shown to occur in the gut of mice [98].

But, it remains to be elucidated whether slurries and sewage are potential environments for the horizontal transmission of those genes. The question is, does transduction occurs in slurries and sewage? At the moment, this question cannot yet be answered but there are some data that indicate that it is not only possible, but probable. Firstly, the number of bacteria carrying the *stx2* genes have been reported to be about 1:1,000 with respect to *E.coli* in urban sewage [99], and there are more non-O157:H7 strains that carry the *stx2* gene than O157:H7. Secondly, bacteriophages carrying the *stx2* gene are found in sewage in significant amounts [100] and persist in water longer than the bacterial host does [101].

10.2 Antibiotic Resistance Genes

Sewage and wastewater treatment plants are potential hot spots for horizontal transfer of antimicrobial resistance genes among bacteria.

Firstly, this is because different kinds of bacteria (pathogenic or not) resistant to a great variety of antibiotic have been reported in sewage [102–106]. Frequently the genes coding for resistance to antibiotics are transferable from one bacterium to another by horizontal transfer. Moreover, transfer of antimicrobial resistance has been demonstrated by in situ experiments into municipal sewage treatment plants [107]. Accordingly, these genes can be transferred from non-pathogenic to pathogenic bacteria or to indigenous bacteria (e.g. *Acinetobacter* [108]) that may maintain the genes in the aquatic environment.

Secondly, this is because recent studies have shown the occurrence of various antibiotics in wastewater [109, 110]. Generally much higher concentrations of antibiotics than those found in wastewaters are necessary to inhibit the growth of resistant bacteria, but the concentration of antimicrobial agents in municipal wastewaters can affect susceptible bacteria [111] and consequently have the potential to select in favour of resistant bacteria. Some reports indicate a greater proportion of bacteria resistant to antibiotics in treated sewage than in sewage [102, 103], but there are other reports that indicate the contrary [104]. This is an aspect that needs further investigation.

Taking into consideration the increasing isolation of antibiotic-resistant bacteria, the huge amounts of antibiotics used and the substantial amounts of antibiotics found in wastewaters, it has become urgent to study the actual role of the aquatic environment in the spread of antibiotic-resistance genes.

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