GLOBAL WATER PATHOGEN PROJECT

**PART THREE. SPECIFIC EXCRETED PATHOGENS: ENVIRONMENTAL AND
EPIDEMIOLOGY ASPECTS**

PATHOGENIC MEMBERS OF ESCHERICHIA COLI & SHIGELLA SPP. SHIGELLOSIS

Cristina Garcia-Aljaro

University of Barcelona
Barcelona, Spain

Maggy Momba

Tshwane University of Technology South Africa
Pretoria, South Africa

Maite Muniesa

University of Barcelona
Barcelona, Spain

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Citation:

Garcia-Aljaro, C., Momba, M. and Muniesa, M. (2017). Pathogenic members of *Escherichia coli* & *Shigella* spp. Shigellosis. In: J.B. Rose and B. Jiménez-Cisneros, (eds) Water and Sanitation for the 21st Century: Health and Microbiological Aspects of Excreta and Wastewater Management (Global Water Pathogen Project). (A. Pruden, N. Ashbolt and J. Miller (eds), Part 3: Specific Excreted Pathogens: Environmental and Epidemiology Aspects - Section 2: Bacteria), Michigan State University, E. Lansing, MI, UNESCO. <https://doi.org/10.14321/waterpathogens.24>

Acknowledgements: K.R.L. Young, Project Design editor; Website Design: Agroknow (<http://www.agroknow.com>)

Last published: October 30, 2017

Summary

Shigella spp. and pathogenic *Escherichia coli* are Gram-negative, facultative anaerobe bacteria belonging to the genus *Shigella*, within the family *Enterobacteriaceae*. *Shigella* spp. cause a spectrum of diseases including bacillary dysentery (shigellosis), a disease characterized by the destruction of the colonic mucosa that is induced upon bacterial invasion. Although *Shigella* are divided into four species (*S. dysenteriae*, *S. flexneri*, *S. boydii*, *S. sonnei*) its separation from pathogenic *E. coli* is based on historical precedence. Clinically and diagnostically, *Shigella* spp. are similar to enteroinvasive *E. coli* (EIEC), sharing many of the same virulence factors and biochemical characteristics. *Shigella dysenteriae* is considered the most virulent, and can produce the potent cytotoxin Shigatoxin, closely related to Stx1 in pathogenic *E. coli*.

Pathogenic *E. coli* consist of a group of serotypes linked with severe human intestinal and extra-intestinal illnesses. They combine a set of virulence factors used to separate them into six groups (enteropathogenic: EPEC, enterotoxigenic: ETEC, enteroaggregative: EAaggEC, enteroinvasive: EIEC, enterohaemorrhagic: EHEC and diffusely adherent: DAEC). One of their most dangerous group is EHEC due to their virulence factors that produce Shiga toxins (Stx), which can result in hemolytic uremic syndrome. The majority of the genes coding for virulence factors in *E. coli* are encoded in mobile genetic elements, and the difference between non-pathogenic and pathogenic strains is largely a result of the incorporation or loss of these elements.

Shigella spp. have humans as the most common reservoir although infections have also been observed in other primates. Many pathogenic *E. coli* are zoonotic pathogens, while others have humans as the only known reservoir. Both groups are geographically ubiquitous and infections are reported wherever humans reside.

Both *Shigella* spp. and pathogenic *E. coli* are spread through the fecal-oral route, and transmission is typically through: ingestion of contaminated foods (washed with fecally contaminated water, or handled with poor hygiene), drinking contaminated water (or via recreational waters) or by person-to-person contact. Both may contaminate waters through feces from humans and for *E. coli* also from domestic animals and wild birds. These pathogens enter water bodies through various ways, including sewage overflows, sewage systems that are not working properly, animal manure runoff, and polluted urban storm water runoff. Wells may be more vulnerable to such contamination after flooding, particularly if the wells are shallow, have been dug or bored, or have been submerged by floodwater for long periods of time. Occurrence of *E. coli* O157 and other serotypes carrying *stx2* gene in raw municipal sewage and animal wastewater from several origins has been described. In addition, since land application is a routine procedure for the disposal of both animal (manure) and human waste of fecal origin (direct deposition or sludge), the presence of *Shigella* and pathogenic *E. coli* has also been described there, and shows

surprising long-term survival in these substrates.

E. coli's role as an indicator organism of fecal pollution is described in another chapter, but as such it is always present in relatively high amounts whenever feces is present. *E. coli* is therefore considered a useful surrogate of pathogenic *E. coli* and *Shigella*, however, as most pathogenic *E. coli* are lactase-negative, they are not detected in standard water quality media used to enumerate *E. coli*. Hence, molecular methods targeting virulence factors are used to distinguish pathogenic variants from commensal, non-pathogenic ubiquitous *E. coli* strains. The problem is that the mosaic genetic structure of these strains, containing most virulence genes encoded in mobile genetic elements that might be present or not, can make it hard to resolve commensal *E. coli*. Moreover, the detection of the mobile genetic elements free in water bodies, as happens with Stx phages, add a further level of complexity in identifying infectious, pathogenic *E. coli*.

It is generally assumed, although with limited actual data, that fate and transport of fecal indicator *E. coli* is indicative of the intracellular, *Shigella* spp. and pathogenic *E. coli*'s environmental behavior. Most data have been collected on *Shigella* spp. and EHEC, and unless otherwise noted in this Chapter, commensal *E. coli* results can probably be extrapolated to provide rates of inactivation of the pathogenic members.

Shigella and pathogenic *Escherichia coli*

1.0 Epidemiology of the Disease and Pathogen(s)

1.1 Global Burden of Disease

1.1.1 Global distribution

Shigella spp. and pathogenic *E. coli* are ubiquitous Gram-negative rod shaped bacilli largely associated with mammalian or avian hosts, classified in the genus *Shigella*, within the family *Enterobacteriaceae* (The et al., 2016). Both pathogens are transmitted through the fecal-oral route. *Shigella* spp. can induce a symptomatic infection via an exceptionally low infectious dose (<10 bacteria), as opposed to the various diarrheagenic *E. coli* pathovars, which have infectious doses of at least four orders of magnitude greater (Kothary and Babu, 2001).

1.1.2.1 *Shigella* spp.

Shigella is typically an inhabitant of the gastrointestinal tract of humans and other primates (Germani and Sansonetti, 2003; Strockbine and Maurelli, 2005; WHO, 2008). The first report on the isolation and characterization of bacteria causing bacillary dysentery, later named *Shigella*, was published by Japanese microbiologist Kiyoshi Shiga at the end of the 19th century (Schroeder and Hilbi, 2008). By means of the fecal route of transmission, *Shigella* can be also found in fecally contaminated material and

water but shows a low survival rate without the optimal acidic environment in the intestinal tract. Asymptomatic carriers of *Shigella* can exacerbate the maintenance and spread of this pathogen, particularly where sanitation is poor or non-existent.

Although *Shigella* spp. have been awarded their own genus which is divided into four species (*Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii*, *Shigella sonnei*), its separation from *E. coli* nowadays is only historic (Karaolis et al., 1994; Pupo et al., 2000; The et al. 2016). These bacterial pathogens are largely responsible for shigellosis or bacillary dysentery.

Globally, *Shigella* species are not evenly distributed. *S. dysenteriae* is mainly found in densely populated areas of South America, Africa and Asia. *S. flexneri* usually predominates in areas where endemic shigellosis occurs, while *S. boydii* occurs sporadically, with the exception in India where it was first identified. *S. sonnei* is mostly reported from developed regions of Europe and North America (Germani and Sansonetti, 2003; Emch et al., 2008). *S. dysenteriae* type 1 affects all age groups, most frequently in developing regions. The median percentages of isolates of *S. flexneri*, *S. sonnei*, *S. boydii*, and *S. dysenteriae* were, respectively, 60%, 15%, 6%, and 6% (30% of *S. dysenteriae* cases were type 1) in developing regions; and 16%, 77%, 2%, and 1% in industrialized areas (Kotloff et al. 1999). Shigellosis can also be endemic in a variety of institutional settings (prisons, mental hospitals, nursing homes) with poor hygienic conditions.

1.1.1.2 *Escherichia coli*

The primary habitat of *E. coli* has long been thought of as the vertebrate gut since first described as *Bacterium coli commune* by a German pediatrician, Dr. Theodor Escherich, which he isolated from the feces of an infant patient (Escherich, 1885). This bacterium is a highly adaptive bacterial species that comprises numerous commensal and pathogenic variants adapted to different hosts, but which also survives in extraintestinal environments and particularly in fecally-polluted water. Healthy humans typically carry more than a billion commensal *E. coli* cells in their intestine. In the environment outside the body, *E. coli* is commonly found in fecally contaminated areas (Savageau, 1983). However, there are non-pathogenic *E. coli* strains that are thought to be largely environmental, and not of enteric origin (Ashbolt et al., 1997; Luo et al., 2011).

Pathogenic *E. coli* strains associated with intestinal diseases have been classified into six different main groups based on epidemiological evidence, phenotypic traits, clinical feature of the disease and specific virulence factors: enteropathogenic: EPEC, enterotoxigenic: ETEC, enteroaggregative: EAaggEC, enteroinvasive: EIEC, enterohaemorrhagic: EHEC and diffusely adherent: DAEC. Several *E. coli* strains cause diverse intestinal and extraintestinal diseases by means of virulence factors that affect a wide range of cellular processes. Their reservoir and distribution can vary depending on the group and are described in the following sections. While some groups are

frequently associated to developing regions (ETEC, EPEC, EIEC), other groups are predominant in developed regions and often zoonotic (EHEC).

1.1.2 Symptomatology

1.1.2.1 *Shigella* spp.

Shigella is a pathogen with a low infectious dose, capable of causing disease in otherwise healthy individuals. Infection with *Shigella* spp. causes a spectrum of diseases ranging from a mild watery diarrhea to severe dysentery (shigellosis). The dysentery stage of disease correlates with extensive bacterial colonization of the colonic mucosa, and the destruction of the colonic mucosa that is induced upon bacterial invasion (Schroeder and Hilbi, 2008). *Shigella* spp. are common etiological agents of diarrhea among travelers to less developed regions of the world, and tend to produce a more disabling illness than enterotoxigenic *E. coli* (Kotloff et al., 1999), the leading cause of travelers' diarrhea syndrome.

Worldwide burden of shigellosis has been estimated to be between 150 and 164.7 million cases, including 163.2 million cases in developing countries, of which 1.1 million result in deaths (Germani and Sansonetti 2003; Parsot 2005; Emch et al. 2008). Since the late 1960s, pandemic waves of Shiga dysentery (*S. dysenteriae* type 1) have appeared in Central America, south and south-east Asia and sub-Saharan Africa, often affecting communities in areas of political upheaval and natural disaster. Shigellosis can also be endemic in a variety of institutional settings (prisons, mental hospitals, nursing homes) with poor hygienic conditions. When pandemic *S. dysenteriae* type 1 strains invade these vulnerable groups, the attack rates are high and dysentery often becomes a leading cause of death (Kotloff et al. 1999). *Shigella dysenteriae* type 1 affects all age groups, but most frequently in developing regions. The epidemics of shigellosis in these countries largely affect children under 5 years and account for 61% of all deaths attributable to shigellosis in this age group (Germani and Sansonetti 2003; Emch et al. 2008).

Shigella infections also occur in industrialized countries, largely due to *S. sonnei*, which is thought to have evolved from other *Shigella* some 400 years ago in Europe (The et al. 2016). Important epidemics reported in Western countries in the last decades (Central America in 1970:11200 cases, 13000 deaths, Texas/USA in 1985:5000 cases, Paris in 1996: 53 cases) were mostly linked to ingestion of contaminated lettuce (Kapperud et al. 1995). Despite the severity of the disease, shigellosis is self-limiting. If left untreated, shigellosis persists for 1 to 2 weeks and patients recover.

The estimated Disability Adjusted Life Years (DALY) was 5.4 million in 2010 (Kirk et al., 2015), whereas the DALY per 100,000 persons was 43 in Africa, 38 in the Eastern Mediterranean countries and 1 in America.

1.1.2.2 Pathogenic *Escherichia coli*

The pathogenic variants of *E. coli* have potential to

cause a wide spectrum of intestinal and extra-intestinal diseases such as urinary tract infection, septicemia, meningitis, and pneumonia in humans and animals (Smith et al. 2007). Symptoms of disease include abdominal cramps and diarrhea, which may be bloody. Fever and vomiting may also occur. Most patients recover within 10 days, although in a few cases the disease may become life-threatening, particularly for infants or aged patients (Tarr et al., 2005).

With a large range of pathologies, pathogenic *E. coli* is an important cause of human morbidity and mortality worldwide. While surveillance of pathogenic *E. coli* infection is well established in many developed countries, which may be apparent in geographical differences in terms of infection incidences, many infections may go unrecognized due to lack of routine testing. This has created substantial gaps in knowledge about the mortality and case-fatality ratios. However, mortality from diarrhea is overwhelmingly a problem of infants and young children in developing countries where each year *E. coli* contributes to this burden by being one of the important causes of death due to diarrhea (Bern, 2004).

E. coli strains, particularly serotypes such as O148, O157 and O124 are implicated in acute diarrhea and gastroenteritis transmitted through consumption of

contaminated water (Kaper et al. 2004; Abong'o and Momba 2008; Cabral 2010). With lack of adequate clean water and sanitation in many developing countries, ETEC serotypes are an exceedingly important cause of diarrhea, especially to children under five years old. These strains are responsible for several 100 million cases of diarrhea and several thousand deaths on a yearly base. ETEC serotypes are the most common cause of travelers' diarrhea followed by EAggEC, affecting individuals from developed regions travelling to developing areas (Bettelheim 2003; Scheutz and Strockbine 2005; WHO 2010).

The estimated DALY for EPEC is 9.7 million per year (data from 2010). The DALY per 100,000 persons varies between the different regions from 140 in Africa to 5 in America and 57 in the Eastern Mediterranean countries (Kirk et al., 2015). In the case of ETEC, they are responsible for a DALY of 5.9 million yearly, and the DALY per 100,000 persons also varies considerably between the different regions (Africa, 109, America, 5 and Eastern Mediterranean countries, 35). STEC have an estimated DALY of 26,900 per year, representing DALY per 100,000 persons of 0.05 in Africa to 0.3 in America.

Table 1 illustrates the incidences of pathogenic *E. coli* and *Shigella* infections.

Table 1. Reported number of cases associated with pathogenic *Shigella* and *E. coli* in different countries

Area	Period of Study	Microorganism	Total number of cases	Reference
Afghanistan	2011	<i>S. dysenteriae</i>	756	Martin et al., 2012
Angola	2012 to 2013	<i>E. coli</i> (EPEC ^b)	344	Gasparinho et al., 2016
Argentina	2003	<i>E. coli</i> (VTEC ^c or STEC ^d)	15	Gomez et al., 2004
Austria	2007	<i>E. coli</i> O157	45	Much et al., 2009
Austria	2015	<i>S. sonnei</i>	13	Lederer et al., 2015
Austria	2001; 2005 to 2007	<i>E. coli</i> (EHEC ^e)	17	Much et al., 2009
Belgium	2007	<i>E. coli</i> (VTEC or STEC)	12	De Schrijver et al., 2008; Buvens et al., 2011
Brazil	2011	<i>E. coli</i> O26:H11	3,910	Assis et al., 2014
Cambodia	2005	<i>E. coli</i> (EAggEC ^f)	24	Nakajima et al., 2005
Canada	2000	<i>E. coli</i> O157	5,000	Hrudey et al., 2003
Canary Islands	2005	<i>S. sonnei</i>	14	Alcoba-Flórez et al., 2005
Denmark	2006	<i>E. coli</i> (ETEC ^g)	217	Jensen et al., 2006
Denmark	2010	<i>E. coli</i> (EHEC)	260	Ethelberg et al., 2007

Area	Period of Study	Microorganism	Total number of cases	Reference
Denmark	2003 to 2004	<i>E. coli</i> O157:H-	25	Jensen et al., 2006
DR Congo	2003	<i>E. coli</i> (EPEC ^h)	463	Koyange et al., 2004
Egypt	2005	<i>S. sonnei</i>	71	McKeown et al., 2005
Egypt	2014	<i>S. dysenteriae</i>	100	McKeown et al., 2005
Finland	2012	<i>E. coli</i> (EHEC)	2	Jalava et al., 2014
Finland	2007 to 2008; 2012	<i>E. coli</i> (VTEC or STEC)	1,000	Lienemann et al., 2011
France	2011	<i>E. coli</i> O104:H4	8	Delannoy et al., 2015
Germany	2011	<i>E. coli</i> O104:H4	3,078	Bielaszewska et al., 2011; Muniesa et al., 2012
Japan	1996	<i>E. coli</i> O157	9,578	Ikeda et al., 2000
Korea	2004	<i>E. coli</i> (VTEC or STEC)	103	Kato et al., 2005
Korea	2013	<i>E. coli</i> (EAggEC)	54	Shin et al., 2015
Malaysia and Singapore	2005	<i>S. sonnei</i>	6	Kimura et al., 2006
Mexico	2000 to 2013	<i>E. coli</i> (ETEC)	1,230	Cortés-Ortiz et al., 2002
Netherlands	2005	<i>E. coli</i> O157	32	Doorduyn et al., 2006
Netherlands	2007	<i>E. coli</i> O157	36	Friesema et al., 2007
Netherlands	2008 to 2009	<i>E. coli</i> O157: H-	20	Greenland et al., 2009
New Zealand	2001	<i>S. boydii</i>	30	Hill et al., 2002
Peru	2011	<i>E. coli</i> (EHEC)	10	Gonzaga et al., 2011
Russia	2006	<i>S. sonnei</i>	23	Schimmer et al., 2007
Slovakia	2004	<i>E. coli</i> O157	9	Liptakova et al., 2004
Spain	2000	<i>E. coli</i> O157	200	Muniesa et al., 2003
Takjikistan	2010	<i>E. coli</i> (ETEC)	342	Wei et al., 2014
Turkey	2011	<i>E. coli</i> O104:H4	8	Jourdan-da Silva et al., 2012
Turks and Caicos Islands	2002	<i>S. sonnei</i>	78	Gaynor et al., 2009
UK	1994	<i>E. coli</i> O157	100	Upton and Coia, 1994
UK	2005	<i>E. coli</i> O157	118	Pennington, 2000
UK	2009	<i>E. coli</i> O157	36	Pennington, 2014

Area	Period of Study	Microorganism	Total number of cases	Reference
USA	2003 to 2012	<i>E. coli</i> O157	4,928	Heiman et al., 2015
USA	2010	<i>Shigella flexneri</i>	3	CDC, 2010
USA	2000 to 2010	<i>E. coli</i> (EPEC)	6,778	CDC, 2010; Painter et al., 2013
Vietnam	2014	<i>S. sonnei</i>	15	Kim et al., 2015

^aReported cases are either associated with outbreaks or with sporadic clusters of disease. Reported cases show a great variability depending on the country and the period of study and should not be accounted for different geographical incidences; ^bEPEC: Enteropathogenic *E. coli*; ^cVTEC: Verotoxigenic *E. coli*; ^dSTEC: Shigatoxigenic *E. coli*; ^eEHEC: Enterohemorrhagic *E. coli*; ^fEAggEC: Enteroaggregative *E. coli*; ^gEPEC: Enteropathogenic *E. coli*.

1.2 Taxonomic Classification of the Agents

As members of the genus *Shigella* and *Escherichia*, *Shigella* spp. and pathogenic *E. coli* share 80 to 90% genomic similarity (Brenner et al. 1972). Moreover, shigellosis produces inflammatory reactions and ulceration on the intestinal epithelium followed by bloody or mucoid diarrhea, which is also caused by EIEC. Yet in spite of their similarity, it is possible to genetically identify and separate pathogenic *E. coli* from other *Shigella* using molecular techniques (Ud-Din and Wahid 2014). It has been also pointed out that *Shigella* spp. are intracellular pathogens, whereas intestinal pathogenic *E. coli* (IPEC) strains are facultative pathogens with a broad host range and diverse mechanisms of infection (Croxen and Finlay 2010).

1.2.1 Physical description of the agent

1.2.1.1 *Shigella* spp.

Shigella is divided into four species and at least 54 serotypes based on their biochemical and/or the structure of the O-antigen component of the LPS present on the cell wall outer membrane: *S. dysenteriae* (subgroup A with 16 serotypes), *S. flexneri* (subgroup B with 17 serotypes and sub-serotypes), *S. boydii* (subgroup C with 20 serotypes) and *S. sonnei* (subgroup D with 1 serotypes) (Simmons and Romanowska 1987; Talukder and Asmi 2012).

Shigella spp. have a very low infection dose, causing bacillary dysentery, a clinical pathology consisting of cramps, painful straining to pass stools (tenesmus), and frequent, small-volume bloody, mucoid diarrhea. The disease follows an incubation period of 1-4 days with fever, malaise, anorexia, and sometimes myalgia. Stool analysis reveals watery diarrhea with the presence of a large numbers of leukocytes. When the diarrhea turns bloody, then dysentery is declared. In healthy adults, symptoms subside in 2-5 days. In children and the elderly, loss of water and electrolytes may lead to dehydration and even death. Few cases show neurological symptoms and HUS because some *Shigella* produce Stx, which is not essential

for invasion, but which can increase the severity of the disease (Brooks et al., 2007).

The fundamental event in the pathogenesis of bacillary dysentery is invasion of the epithelial cells of the colon. The bacteria reach the lamina propria and trigger an acute inflammatory response with mucosal ulceration and abscess formation. In well-nourished individuals, the infection does not extend beyond the lamina propria. Otherwise, epithelial cell penetration can occur, causing intracellular multiplication, lateral movement to the adjacent cells and cell-to-cell invasion. *Shigella*, however, does not penetrate further to survive into deeper tissues (Carayol and Tran Van Nhieu, 2013).

The mechanism of *Shigella* pathogenesis is very similar to that from EIEC, but differs in that EIEC has a higher infective dose, there is less person-to person spread and outbreaks of EIEC are usually foodborne or waterborne. In addition, kidney failure is not observed in EIEC infection that usually does not possess Stx (Parsot, 2005).

1.2.1.2 Pathogenic *Escherichia coli*

Pathogenic *E. coli* isolates have been divided into subgroups in the following ways:

Serology: *E. coli* is serologically divided in serogroups and serotypes on the basis of specific antigens produced by structural components of the bacterium. The O-type antigens are somatic (cell surface) lipopolysaccharides, whereas the H-type antigens are components of the bacterial flagella. Many strains express a third class of antigens, the capsular or K antigens, which are occasionally used in serotyping (Stenutz et al. 2006). F-type antigens are additional surface fimbrial antigens whose identification provides important information for more detailed characterization of strains. Serotyping is usually used in pathogen detection. Some isolates were classified as serogroup ONT, which refers to a provisional designation for as-yet-unclassified or non-standardized O-antigen groups (Gyles 2007).

Pathogenicity: Human pathogenic strains usually colonize other animal species asymptotically. Based on their ability to cause disease in humans, *E. coli* strains are subdivided into three categories: i) non-pathogenic or commensal *E. coli*, ii) intestinal pathogenic *E. coli* (IPEC) and iii) extraintestinal pathogenic *E. coli* (ExPEC). ExPEC strains are comprised from two pathovars: uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC) (Kaper et al. 2004; Smith et al. 2007). However, since ExPEC are less or not related to waterborne infections, these will not be further discussed in this chapter.

Waterborne transmission involves the intestinal pathogens that are also known as diarrheagenic *E. coli*. Based on their virulence factors, phenotype and pathology diarrheagenic, *E. coli* strains (Kaper et al. 2004) are classified as:

Enteropathogenic *E. coli* (EPEC): The main virulence properties of EPEC are attaching and effacing lesions (A/E) associated with pathogenicity island known as locus of enterocyte effacement (LEE).

Enterotoxigenic *E. coli* (ETEC): ETEC adhere to small bowel enterocytes and induced watery diarrhea. The main virulence factors are production of heat-stable enterotoxin (STs) or the heat-labile enterotoxin (LT) or a combination of these.

Enteroadgregative *E. coli* (EAggEC): The characteristic of this group is aggregative adhesion mediated by the genes located on a family of virulence plasmids, known as pAA plasmids (Harrington et al. 2006).

Enterohaemorrhagic *E. coli* (EHEC): EHEC isolates often share numerous virulence factors with other pathogenic *E. coli* strains, but are distinguished by their production of Shiga toxins (Stx), which can lead to potentially life threatening diseases. Stx genes are encoded in the genome of temperate bacteriophages (O'Brien et al. 1984). Serotype O157:H7 is one of the most cited to cause outbreaks.

Enteroadgregative hemorrhagic *E. coli* (EAHEC): This is a new pathogenic group of *E. coli* characterized by the presence of a Stx2-phage integrated in the genomic backbone of Enteroadgregative *E. coli* (EAggEC). The most notorious example is the O104:H4 strain that caused the outbreak in Germany in May 2011, the largest outbreak ever reported caused by a pathogenic *E. coli* (Bielaszewska et al., 2011; Muniesa et al., 2012).

Enteroinvasive *E. coli* (EIEC): These strains are most closely related to *Shigella* spp. and it is generally considered that they should form a single group. This group differs from others diarrheagenic *E. coli* because it includes obligate intracellular bacteria. EIEC can cause an invasive inflammatory colitis, and occasionally dysentery, but mostly manifests as watery diarrhea.

Diffusely adherent *E. coli* (DAEC): A heterogeneous group that induces a cytopathic effect characterized by the development of the long cellular extensions that wrap around adherent bacteria. DAEC have been implicated in

cases of diarrhea in children less than five years old as well as in recurring urinary tract infections in adults (Servin 2014).

Other divisions of *E. coli* are based on phylogenetic properties or for their source of isolation, such as environmental isolates or avian pathogenic *E. coli* or simply for the possession of specific virulence marker (i.e. Shiga toxin).

Shiga toxin-producing *E. coli* (STEC): Konowalchuk et al. (Konowalchuk et al., 1977) reported the feature of a group of *E. coli* isolates that produced a cytopathic effect on Vero cells in culture. This resulted in a term "verotoxigenic *E. coli*" or "Vero cytotoxin-producing *E. coli*" (VTEC). In 1983 the toxin from one of the Konowalchuk isolates was purified (O'Brien and LaVeck 1983) and it was demonstrated that it possessed many of the properties of Shiga toxin produced by *Shigella dysenteriae* type 1. Shiga toxin-producing *E. coli* (STEC) and Verocytotoxin or Verotoxin-producing *E. coli* (VTEC) have been used in the literature since this time. They are equivalent terms, and refer to *E. coli* strains that possess one or more of the stx genes family (Kaper et al. 2004) through acquisition of one or more Stx-encoding temperate bacteriophage(s).

Even though STEC were first implicated in disease in the early 1980s (Riley et al. 1983), they have rapidly become important emergent pathogens in developed countries. Until today, more than 400 different serotypes of *E. coli* have been reported to encode Stx and large varieties of them have been implicated in human disease (García-Aljaro et al. 2004; Mora et al. 2005; Mathusa et al. 2010). Nevertheless, some STEC serotypes found in cattle or in foods have never, or only very rarely, been associated with human disease (Karmali et al. 2003).

To underline the differences in virulence between groups of STEC serotypes, these strains are classified into five seropathotypes (A-E), based on their incidence and association with severe diseases and outbreaks. The most notorious serotypes of STEC that have demonstrated to cause severe diseases in humans are denoted as EHEC. One of the most dangerous groups of pathogenic *E. coli* is EHEC and its main virulence factors are the Shiga toxins (Stx). The lack of effective treatment and the potential for large-scale outbreaks of diarrhea with life threatening complication have placed EHEC on a list of worldwide threat to public health (Karmali et al. 2003).

Besides *E. coli* and *Shigella dysenteriae* type I (Bartlett et al. 1986), stx gene sequences, although rarely, have been detected in other members of *Shigella* spp. (O'Brien et al. 1977; Bartlett et al. 1986; Beutin et al. 1999), in *Enterobacter cloacae* (Paton and Paton 1996), *Citrobacter freundii* (Schmidt et al. 1993) and, outside of *Enterobacteriaceae* family, in *Campylobacter* spp. (Moore et al. 1988).

1.3 Transmission

1.3.1 Routes of transmission

Shigella and pathogenic *E. coli* are excreted in feces and can be transmitted by ingestion of contaminated water or food, or through contact with animals or infected people (<http://www.cdc.gov/ecoli/general/>). These are present in the stools of infected persons while they have diarrhea and for a period of time after the disease.

Pathogenic *E. coli* are usually transmitted through consumption of contaminated water or food, such as undercooked meat products and raw milk. They have been reported universally, most of which have caused water and food borne disease outbreaks (Cunin et al., 1999; Jackson et al., 2000; Abong'o and Momba, 2008). An epidemiological study done in US estimated that O157 and non-O157 STEC transmission occurs in 85% due to consumption of contaminated food (Mead et al., 1999). A large number of foods have been implicated in STEC transmission. The ruminants are the main carriers of EHEC, and therefore, foods of animal origin have been commonly identified as an important transmission vehicle. It has been estimated that in USA, ground beef accounts for 41% of food-borne outbreaks involved in *E. coli* O157:H7 infection (Rangel et al., 2005). Many outbreaks have been linked to undercooked meat, and during the 1982 outbreaks, the organism was cultured from a suspected batch of hamburger patties. Meat sources other than undercooked hamburger meat involved in transmission of *E. coli* O157:H7 include roast beef, pork, poultry, lamb and turkey (Su and Brandt, 1995; Welinder-Olsson and Kaijser, 2005). Other mammals (pigs, horses, rabbits, dogs, cats) and birds (chickens, turkeys) have been occasionally found infected. A study conducted in the Eastern Cape Province of South Africa has pointed out that drinking water, meat and meat products (biltong, cold meat, mincemeat, and polony) and vegetables (cabbages, carrots, cucumbers, onions and spinach) are potential sources of *E. coli* O157:H7 that are potentially capable of causing diarrhea in HIV/AIDS individuals (Abong'o and Momba, 2008).

Dairy products are also an important route for STEC transmission. Consumption of unpasteurized milk and cheese have been reported as a cause of infection (Gyles, 2007) as well. *E. coli* O157:H7 was isolated from the feces of healthy cows who had supplied raw milk consumed by the patients affected in the outbreak (CDC, 2000).

Besides food of animal origin, a large number of vegetables as sprouts, lettuce, coleslaw, and salad have also been implicated in STEC infections and outbreaks. Most of them caused by transmission of the pathogen through manure applied as fertilizer or the water used for irrigation as discussed below.

In addition, some serotypes as *E. coli* O157:H7 can survive at low pH and transmission has also been associated with low pH products, like fermented salami, mayonnaise, yogurt and unpasteurized apple cider (Erickson and Doyle, 2007; Gyles, 2007).

The spread of STEC can be facilitated environmentally: via manure applied to fields, contaminated water in

irrigation or food processing, poor hygiene and poor equipment sanitation. In addition, *E. coli* can survive for substantial periods of time on stainless steel and plastic. Therefore, these surfaces can serve as intermediate sources of contamination during food processing operations or food preparations (Erickson and Doyle, 2007; Söderström et al., 2008; Karmali et al., 2010; Dechet et al., 2014).

Waterborne transmission was first reported in two outbreaks (Dev et al., 1991; Swerdlow et al. 1992) and is now clearly an important transmission pathway. Other routes of infection are swimming in contaminated water (Samadpour et al., 2002), which appears to be an important issue, as well as the contaminated or unchlorinated drinking water (Hunter, 2003; Lascowski et al., 2013). The use of irrigation water containing the pathogen appears as an important source of outbreaks, particularly with certain vegetable products as sprouts (Buchholz et al., 2011; Dechet et al., 2014).

Person to person transmission of STEC also has been described in both day care and hospital settings, and from animals to human transmission in zoos and farm. In general, it is less common in community-wide outbreaks. Nosocomial *E. coli* O157:H7 infections have also been reported (Su and Brandt, 1995; Rangel et al., 2005).

In contrast, the best way of transmission of *Shigella* is person to person spread and the primary transmission is by fecal-oral route by people with contaminated hands. Hands can be contaminated through a variety of activities, such as touching surfaces (e.g., toys, bathroom fixtures, changing tables, diaper pails) that have been contaminated by the stool from an infected person.

1.3.2 Reservoirs

Different reservoirs can be found within the heterogeneous group of *Shigella* and *E. coli* (Croxen et al., 2013):

Unlike other pathogens, humans and primates are the principal reservoirs of *Shigella*, and it is within their intestinal tract where the major reservoir is found. From infected carriers, *Shigella* are spread by several routes, including food, finger, feces and flies. In addition, flies and living amoebae have been reported to play a role in *Shigella* transmission, where this pathogen can survive, but it is not known if they can serve as reservoirs (Hale and Keusch, 1996). It has been shown experimentally that free-living amoebae *Acanthamoeba* have the ability to uptake virulent and non-virulent *S. sonnei*. The bacteria can grow inside their cysts in experimental settings, providing a microhabitat that protects *Shigella* from the outside environment. However, free-living amoebae harvesting *Shigella sonnei* have not been found (Saeed et al., 2009).

EPEC: As with other diarrheagenic *E. coli*, EPEC is transmitted from host to host via the fecal-oral route through contaminated surfaces, weaning fluids, and human carriers. Although rare, outbreaks among adults seem to

occur through ingestion of contaminated food and water; however, no specific environmental reservoir has been identified as the most likely source of infection (Nataro and Kaper, 1998).

ETEC: Exposure to ETEC is usually from contaminated food and drinking water and therefore an environmental reservoir is suspected. Some examples of high-risk foods contaminated with etiological agents for traveler's diarrhea include food that is left at room temperature, table-top sauces, certain fruits, and food from street vendors.

EHEC and extensively STEC: These can be found in the gut of numerous animal species, but ruminants have been identified as a major reservoir. Usually domestic animals are asymptomatic carriers of STEC (Caprioli et al., 2005). The STEC isolated from cattle and other animals belong to a variety of serotypes and an individual animal can carry more than one serotype, including both O157 and non-O157 serotypes. Some of the STEC isolated from animals are of the same serotype as human isolates and many of these have been associated with severe disease in humans (Mora et al., 2005; Gyles, 2007). Cattle prevalence was associated with the presence of Stx-producing bacteria in water and in the environment of cattle (Renter et al., 2005).

EAggEC: While atypical EAggEC has also been identified in calves, piglets, and horses, animals are not an important reservoir of human-pathogenic typical EAggEC. However, since EAggEC isolates are so heterogeneous, it is premature to rule out animal reservoirs for under-characterized lineages of EAggEC (Uber et al., 2006).

EIEC: Conventional host-to-host transmission of EIEC is mediated via the fecal-oral route mainly through contaminated water and food or direct person-to-person spread. Because many EIEC outbreaks are usually foodborne or waterborne, in addition to its human reservoir, an environmental reservoir is suspected (Crohen et al., 2013).

DAEC: It is detected in ill or in healthy age-matched controls who did not present diarrhea at rates similar to those for DAEC detection in ill patients. However, it is unknown how DAEC is transmitted or its reservoir (Crohen et al., 2013).

STEC: when considering the presence of Stx, the reservoirs would depend on the background of the strain, as for example EAggEC carrying Stx phage would have a human reservoir while an EHEC carrying Stx phage would be found in ruminants. Nevertheless, there are environmental reservoirs of STEC and, in contrast to the clinical isolates, they display intermediate stages of occurrence of virulence factors (García-Aljaro et al., 2004; Martínez-Castillo et al., 2012), suggesting an environmental pool of circulating *E. coli* with diverse virulent potential and degrees of persistence to environmental stressors.

1.3.3 Incubation period

Incubation period of *Shigella* and pathogenic *E. coli*

depends on the serotype. It varies from twelve hours to seven days but usually shows a median of three to four days (World Health Organization, WHO) (<http://www.who.int/>).

1.3.4 Period of communicability

Shigella and *E. coli* are communicable during the acute phase and while the infectious agent is present in feces. These pathogens can be shed some days after the disease when symptoms are not present anymore. For O157:H7, the duration of excretion of the pathogen is usually a week or less in adults and up to 3 weeks in children (Locking et al., 2011), although excretion can extend up to 14 weeks. Based on date of onset of diarrhea and date of last positive stool sample, the median duration of excretion among primary cases from the RNA analysis can be of 19 days (range 2 to 52 days). For children aged <5 years, the median duration of excretion can be 29 days (range 5 to 37 days) (O'Donnell et al., 2002). *Shigella* can be found in feces usually no longer than four weeks. Asymptomatic carriage and excretion of the pathogen may persist for months.

1.3.5 Population susceptibility

Shigella affects travelers in developing areas, can also cause disease to the autochthonous population. Severe and life-threatening complications can occur, especially in areas of the developing world where the disease is epidemic or endemic. In these areas, mainly young children (often malnourished and strongly immunosuppressed) are infected, and lack access to adequate treatment (Kotloff et al., 1999).

In turn, *E. coli* is a commensal pathogen, but some groups of populations present different susceptibilities to the different strains. While most STEC are strict pathogens, ETEC or EIEC are known to be the main cause of the traveler's diarrhea, affecting visitors from other areas while the local population shows a low incidence of symptomatic infections.

Children and the elderly men who have sex with men, those with immune deficiency disorders, by attendance at child care or contact with a child in child care, and in international travelers who do not take adequate food and water safety are more susceptible to these infections than healthy adults that, in some cases, can be asymptomatic carriers of the pathogen. A study by Momba et al. (2008) predicted a possible link between *E. coli* O157:H7 from drinking water and diarrheic conditions of both confirmed and non-confirmed HIV/AIDS patients visiting Frere Hospital for treatment in the Eastern Cape province of South Africa (Momba et al., 2008).

One of the most significant public health concern stemming from EPEC, ETEC or EAggEC infections is malnourishment in children in developing countries, as persistent infections lead to chronic inflammation, which damages the intestinal epithelium and reduces its ability to absorb nutrients (Nataro and Kaper, 1998; Crohen et al., 2013).

Some studies show immunological susceptibility, such as the blood type, to certain infections by ETEC (Croxen et al., 2013). HUS infection caused by Shiga toxin-producing strains show higher incidence in children than in adults (Tarr et al., 2005). Exceptions can be found in the O104:H4 strain causative of the German outbreak, that because of its genetic background as EAggEC, and attributable to its mechanism of colonization, showed higher incidence of HUS in adults (Bielaszewska et al., 2011; Muniesa et al., 2012).

1.4 Population and Individual Control Measures

1.4.1 Vaccines

Management of diarrheal diseases in the face of an exploding population can best be accomplished through multiple interventions. Many treatment and prevention tools are currently available, but the full potential of adding vaccines to the control process is yet to be realized. Among the many causes of diarrheal disease, *Shigella* and enterotoxigenic ETEC are the two most important bacterial pathogens for which there are no currently licensed vaccines (Camacho et al., 2014; Walker, 2015).

Efforts have been devoted to the development of vaccines and there are several vaccine candidates: attenuated, vectored or inactivated targeting the bacterial cells, targeting one or various virulence factors (toxins, fimbriae, adhesins, intimins etc). Some approaches include the use of conjugates of heterologous O-specific polysaccharide purified from *Shigella* LPS covalently coupled to protein carriers in order to induce stronger and longer-lasting T cell-dependent immune responses (Walker, 2015). The development of outer membrane vesicles (OMVs) displaying membrane antigens as vaccine candidates has also been conducted.

Since Shiga toxin, either in *E. coli* or *Shigella* is the main virulence factor leading to the most undesirable consequences of *E. coli* infections, Stx has been proposed as a target for vaccination. Antigenicity is conferred by the B-subunit of the toxin and efforts have been devoted towards it (Marcato et al., 2005; Gupta et al., 2011).

Other approaches include vaccines against some of the most virulent serotypes. In this sense, vaccines against *E. coli* O157:H7 or non-O157 serotypes have been explored (Andrade et al., 2014; Garcia-Angulo et al., 2014).

1.4.2 Hygiene measures

The prevention of infection requires control measures at all stages of the food chain, from agricultural production on the farm to processing, manufacturing and preparation of foods in both commercial establishments and household kitchens (Maranhão et al., 2008; Bentancor et al., 2012). To prevent water-borne transmission, prevention requires a proper water treatment. Strategies to prevent transmission and spread of these pathogens include:

- proper hand washing

- improvements in sanitary conditions and freshwater supplies
- correct treatment of recreational waters
- consumption of bottled water or municipal water that has been treated with chlorine or other effective disinfectants
- consumption of only pasteurized milk, juice, or cider
- consumption of well-cooked meat from a known source. Beef should be thoroughly cooked. Minced beef should be cooked until all pink color is gone
- properly washing of fruits and vegetables, especially those that will not be cooked
- visits to farms, particularly of children, should include strict hand washing and avoiding direct contact with animals
- minimize person to person transmission by instructing cases and contacts on the importance of washing hands with soap and water for at least 15 seconds and drying thoroughly, prior to handling food and after using the toilet
- ensure adequate hygiene in childcare centers, especially frequent supervised hand washing with soap and water and disinfection of toys and surfaces

2.0 Environmental Occurrence and Persistence

2.1 Detection Methods

Detection of pathogens in water and other environmental samples is hampered by the presence of the intrinsic non-pathogenic populations naturally occurring in such environments, which are normally found at higher concentrations than the pathogens themselves. This fact, together with the low infection dose reported for *Shigella* and some pathogenic *E. coli* such as STEC have prompted the need for the development of high specific and sensitive methods to overcome these limitations.

2.1.1 Culture based methods

Culture based methods have been used for water examination for over a century (Pyle et al., 1995). These methods have the main limitation that many bacteria present in these environments are non-culturable or have lost the culturability due to different environmental stresses (changes in osmolarity, lowered pH, UV irradiation and nutrient starvation) but are still viable (VBNC) and are able to escape detection by standard methods (Bogosian and Bourneuf, 2001). In this respect, different molecular methods are currently available which are capable to circumvent these limitations. However, culture methods are still the preferred choice in many areas of the world due to their higher simplicity and cost for routine analysis, particularly in developing countries.

2.1.1.1 *Shigella*

Detection of blood and mucus in the stool is a highly predictive sign of the presence of *Shigella* although it is not sufficient to distinguish them from other invasive bacteria such as *Campylobacter jejuni*, EIEC, *Salmonella* or *Entamoeba histolytica*. Its detection strongly depends on

the adequate collection and transportation of fecal samples (preferably stool samples) to the laboratory. Fresh stool samples should be processed preferably within 2 hours but can be kept in Cary-Blair medium or buffered glycerol-saline transport medium at 4°C for no more than 48 hours (Sur et al., 2004; WHO, 2005b).

Differentiation of *Shigella* from *E. coli* is difficult due to their close genetic relationship although they have particular biochemical characteristics such as the inability to ferment lactose (*S. sonnei* can ferment lactose after prolonged incubation) and do not produce gas from carbohydrates. They are negative for urease, oxidase, do not decarboxylate lysine and give variable results for indole (with the exception of *S. sonnei* that is always indole negative) and they are positive for catalase with the exception of *S. dysenteriae* Type 1. Commercial selective isolation media include McConkey agar, deoxycholate citrate agar (DCA) and a moderate selective medium such as xylose-lysine deoxycholate (XLD). After incubation for 18 to 24 hours, *Shigella* species form colorless colonies on McConkey agar and pink red colonies with no black center on XLD agar, with some strains with a pink or yellow periphery. Colonies on DCA agar are colorless although *S. sonnei* may form pale pink colonies because of late lactose fermentation. Suspected colonies are inoculated onto Triple sugar iron (TSI) or Kligler iron agar. In these media *Shigella* displays a non-motile phenotype and do not produce hydrogen sulfide or other gas, do not ferment lactose and therefore the tubes will have a yellow butt and a red slant (Sur et al., 2004). Finally, confirmation can be made by biochemical testing such as the biochemical miniaturized galleries and serology testing using agglutination tests is used to type the isolate into the four groups (A, B, C, D) corresponding to the 4 *Shigella* species (*S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei*), which in turn can be subtyped.

2.1.1.2 *E. coli*

EPEC: It is performed by culturing the samples onto McConkey agar, followed by testing lactose-fermenting colonies with specific antisera. Cell culture is also used to demonstrate localized or diffuse adherence to Hep-2 or HeLa cells.

EAggEC: EAggEC are recognized by a characteristic adherence in HEp-2 cells “stacked brick-like” pattern, and the production of a heat-stable toxin.

ETEC: Cell culture techniques are used to demonstrate the production of a heat-labile enterotoxin (LT) or the suckling mouse assay to detect the production of a heat-stable enterotoxin (ST). The rabbit ileal loop assay is also used to detect the production of toxins

EHEC: Detection of *E. coli* O157:H7 and non-motile isolates (H) is based on two biochemical characteristics that differ from the majority of *E. coli* strains due to its clonal relationship (Whittam et al., 1993): the absence of β -D-glucuronidase activity and the inability to ferment sorbitol within 24 hours. Commercial agars such as Sorbitol McConkey Agar (SMAC), which contains sorbitol as carbohydrate source can be used alone or supplemented with cefixime and tellurite, to make it more restrictive (CT-SMAC), is routinely used for the detection of this serotype in food and clinical samples (Zadik et al., 1993). It supports growth of O157 and inhibits the growth of most *E. coli* strains.

Other agars include the Chromagar and Rainbow Agar media, which rely on the detection of β -glucuronidase activity (Bettelheim, 1998). However their use in environmental samples is hampered by the high number of interfering bacteria which mimic the growth of the *E. coli* O157 cells and they do not differentiate between toxigenic and non-toxigenic strains (Sata et al., 1999; LeJeune et al., 2001, 2006; Garcia-Aljaro et al., 2005a). Its effectiveness can be improved by including an immunomagnetic separation (IMS) step using magnetic beads coated with anti-O157 antibodies to capture O157 cells prior to the seeding of the samples onto CT-SMAC (Wright et al., 1994; Chapman et al., 2001). Variations of this method include analysis of IMS-enriched samples by the most-probable-number technique (Fegan et al., 2004), the use of immunomagnetic-electrochemiluminescent detection (Yu and Bruno, 1996) or the addition of a colony immunoblot detection step after CT-SMAC growth of the colonies (Garcia-Aljaro et al., 2005a) to clean up interfering bacteria.

The O157:H7ID agar (now chromID™ O157:H7), contains chromogenic substrates for detecting β -D-galactosidase, an enzyme present in virtually all *E. coli* strains together with β -D-glucuronidase, which is present mainly in *E. coli* not belonging to STEC O157:H7/H serotypes (Figure 1), and sodium deoxycholate to increase selection for *Enterobacteriaceae* (Bettelheim, 2005), generating green-blue colonies easy to detect among other groups.

Figure 1. Lack of β -glucuronidase activity in *E. coli* O157:H7 strain EDL933 (left) and a non-pathogenic *E. coli* K-12 (right) observed in Chromocult agar medium

There are unusual strains of serotype O157:H7/H⁻ able to ferment sorbitol and with positive β -D-glucuronidase activity, that have also been involved in some outbreaks (Ammon et al., 1999). Some agars are able to detect by a different color those colonies produced by atypical strains (RAPID'E.coli O157:H7 Agar).

In any case, the suspected O157 colonies isolated from the above-mentioned agars should be confirmed by testing with *E. coli* O157 antiserum or latex reagents (Chapman, 1989) although biochemical confirmation of the species is also required because of the cross-reactions of the O157 antibodies with other species.

2.1.2 Molecular methods

Immunological methods for the detection of *Shigella* have been investigated. Among others detection of the pathogen by fluorescent antibody staining (Albert et al., 1992) or by colony blot immunoassays (Szakal et al., 2003) have been proposed although they require a laboratory infrastructure. Immunochromatographic dipsticks are available for the rapid detection of *S. flexneri* (Nato et al., 2007), *S. dysenteriae* Type 1 (Taneja et al., 2011) and more recently *S. sonnei* (Duran et al., 2013). The latter relies on the clonality of majority of *S. sonnei* virulent strains that share a somatic antigen whose antigenic properties depend on the presence of disaccharide repeating subunits containing two unusual amino sugars (2-amino-2-deoxy-L-altruronic acid and 2-acetamido-4-amino-2,4,5-trideoxy-D-galactose) in the O-side chains. The dipstick can be stored at room temperature for two years without refrigeration in a humidity-proof plastic bag, facilitating its use in developing countries.

A variety of immunological methods for the detection and enumeration of pathogenic *E. coli* have been developed. For instance, detection of *E. coli* O157:H7 or STEC by a colony immunoblot assays and enzyme-linked-immunosorbent-assays (ELISA) has been reported (Law et al., 1992; Kehl et al., 1997). These methods rely on the use

of monoclonal or polyclonal anti-O157 antibodies to capture O157 cells or anti-Stxs antibodies. In the case of O157 immunodetection, cross-reactivity in the immunoassays with other microorganisms different from *E. coli* O157 strains has been reported. Anti-O157 antibodies may cross-react with *Escherichia hermannii*, *Salmonella* O group N, *Hafnia alvei*, *Yersinia enterocolitica* or *Citrobacter freundii* (Borczyk et al., 1987; Nataro and Kaper, 1998) and therefore the colonies must be confirmed biochemically.

Commercial kits for the detection of STEC have been developed. The ProSpecT Shiga toxin *E. coli* Microplate assay (Alexon-Trend, Ramsay, Minn.), an enzyme immunoassay for detection of Shiga toxins directly from stools (Gavin et al., 2004), and the immunochromatographic method Duopath Verotoxin Kit (Oxoid, Basingstoke, Engl.) which is intended for detecting the toxins from the isolated strains (Park et al., 2003) are designed for Stx detection, among others. On the other hand, immunochromatographic strips for detection of specific serotypes such as *E. coli* O157 have also been reported (Jung et al., 2005).

As it happens with other pathogens, PCR-based detection protocols targeting genes coding for the most important virulence factors of the different pathogenic groups have been developed.

PCR-based methods have been explored as rapid tool to detect *Shigella* showing comparable sensitivity to conventional culture detection methods (Frankel et al., 1990; Hsung et al., 1997; Islam et al., 1998; Dutta et al., 2001; Thiem et al., 2004; Cho et al., 2012) although they are not yet standardized and are not routinely used, these methods are the preferred method for the detection of this pathogen in the environment due to the difficulty of analyzing large number of non-lactose fermenting colonies that are present in the environment (Faruque et al., 2002; Hsu et al., 2007). One of the target genes most frequently used is the invasion plasmid antigen (*ipaH*) which is suitable for the simultaneous detection of all four *Shigella* species and EIEC (Sethabutr et al., 1994; Dutta et al., 2001; Faruque et al., 2002; Thiem et al., 2004). A two-step

method based on a first immunocapture using specific monoclonal antibodies against *Shigella* followed by 16S rRNA amplification by PCR using universal primers and sequencing (Peng et al., 2002).

In spite of their similarity, it is possible to genetically identify and separate EIEC from *Shigella* using marker genes such as *uidA* gene and *lacY* gene (Ud-Din and Wahid, 2014). Other methods are based on the combination of culture-based and molecular methods in order to distinguish EIEC from *Shigella* (Hsu et al., 2010). The method consisted in a first isolation of presumptive colonies on xylose lysine deoxycholate (XLD) agar, followed by detection and sequencing of the invasion plasmid antigen H (*ipaH*) by PCR, and a final biochemical test and serological assay.

For EPEC, the most commonly virulence factors detected are the intimin (*eaeA*), responsible of adhesion of bacteria to the cell surface (Figure 1), the bundle-forming pili (*bfpA*) or the EPEC adherence factor (EAF) (Aranda et al., 2004). In the case of EHEC, *stx*, *eae* and *hlyA*, have been successfully used to detect STEC in food, animal and environmental samples (Gannon et al., 1993; Paton and Paton, 1998; Chapman et al., 2001; Ibekwe et al., 2002; Omisakin et al., 2003; Spano et al., 2005). Similarly, the use of the most-probable-number in combination with a nested-PCR allowed the quantification of *stx*₂-carrying

bacteria in wastewaters (García-Aljaro et al., 2004). O-serotyping by traditional PCR assays for the most common STEC serotypes, O26, O55, O91, O103, O104, O111, O121, O113 and O157, have also been developed (Paton and Paton, 1999). Real-time PCR using SYBR green or TaqMan probes are applied reducing the time required for analysis (Li and Drake, 2001; Ibekwe et al., 2002; Yoshitomi et al., 2006). The major drawback of these methods is the presence of PCR inhibitors in different complex matrices present in the environment that may render false negative results. In any case, although PCR is a sensitive assay for detection of STEC bacteria, it only detects the presence of bacteria, but does not isolate them, which in the final result is required to determine the etiological agent responsible for an outbreak (Paton and Paton, 1998). These handicaps can be overcome by the use of other molecular methods, which are based on colony hybridization with selective media and then detection of the *stx* genes with DNA probes (Newland and Neill, 1988; Blanch et al., 2003). A colony blot for the *stx*₂ gene, using Chromocult coliform agar, detects 1 STEC colony in 1,000 to 10,000 coliform colonies agar (García-Aljaro et al., 2004) (Figure 3). Combinations of culture base methods and PCR have also been reported (Heijnen and Medema, 2006).

Figure 2. *E. coli* O157:H7 cells adhering to HeLa cells after 24 hours of contact. Staining Giemsa

Figure 3. *Stx* -positive *E. coli* strains detected in swine wastewater by colony blot hybridization. A. Colonies grown in Chromocult agar and transferred to a nylon membrane. B. autoradiograph of the colonies in (A) hybridized with a *stx*_{2A}-DIG labeled probe. Arrows point at the *stx* - positive colonies

2.2 Data on Occurrence

In the case of *Shigella*, epidemiologic studies have reported seasonal fluctuations in the number of shigellosis outbreaks worldwide (Engels et al., 1995). *Shigella* as well as *E. coli* outbreaks have been linked to different water sources (recreational spray fountains, lakes, swimming pools and ground water) and food consumption as well as person-to-person contact (see (Warren et al., 2006)). Although public health reports indicate that food-borne *Shigella* infections are more common than waterborne infections in the US and other industrialized countries (Smith, 1987), the isolation of the pathogen from the environment is probably underestimated due to the low infectious dose as well as the possibility of this bacterium to enter into the VBNC state (Colwell et al., 1985; DuPont et al., 1989; Islam et al., 1993).

The literature available on the occurrence of pathogenic *E. coli* and *Shigella* in the environment is scarce due to the difficulty for the detection and isolation of these pathogens from environmental samples. However, microbiological, epidemiological and environmental studies of cases have associated different uses of water with human outbreaks: recreational, drinking, irrigation, wastewater. As discussed

above, the natural reservoirs of enteropathogenic strains are humans (EPEC, ETEC, EIEC and EAggEC) or domestic animals (ETEC, EHEC), whereas humans is the unique recognized reservoir of *Shigella spp.* Therefore, the occurrence of these pathogens in the environment is mainly linked to transmission through the fecal-oral route from these reservoirs.

2.2.1 Excreta in the environment (Fecal Waste, Night Soil, Dry Latrines)

In the case of *Shigella*, it is estimated that infected individuals excrete 10^6 to 10^8 *Shigella* per g of stool (Gaurav et al., 2013). The prevalence of *Shigella* in stools varies depending on the study, ranging from 1.4 to 12% in human feces (Table 2) Kallergi and co-workers investigated the presence of different enteropathogens including *Shigella* and EPEC in children feces in Greece (Kallergi et al., 1986). *Shigella* was detected in 4.46% of stool samples with *S. sonnei* being the most prevalent species (60%), followed by *S. flexneri* (39.17%) and *S. boydii* (0.83%). Similarly, EPEC were detected in 4.38% of samples with serotypes O26:K60, O119:K69 and O127:K63 being the most frequently isolated serogroups.

Table 2. Occurrence of pathogenic *E. coli* and *Shigella* in human and animal excreta

Area	Period of Study	Microorganism	Matrix	Detection Method	Sample Volume, gm	Percent Positives (# of Samples)	Reference
France	2002	STEC ^a	Pig feces	PCR ^b detection	25	5.7% (5/88)	Vernozy-Rozand et al., 2002
France	2002	<i>stx</i> genes	Cattle manure	PCR detection	25	8.0 % (4/45)	Vernozy-Rozand et al., 2002
France	2002	<i>stx</i> genes	Pig manure	PCR detection	25	0.0% (0/10)	Vernozy-Rozand et al., 2002
France	2002	VTEC ^c and STEC	Cattle feces	PCR detection	25	8.0% (4/48)	Vernozy-Rozand et al., 2002
Greece	1984 to 1985	EPEC ^d	Human feces	Isolation	NR ^e	4.4% ^f (118/2,693)	Kallergi et al., 1986
Greece	1984 to 1985	<i>Shigella spp</i>	Human feces	NR	NR	4.5% ^f (121/2,693)	Kallergi et al., 1986
India	2012	<i>Shigella spp</i>	Human feces	Isolation	NR	2.6% (8/311)	Gaurav et al., 2013
Jordan	1987 to 1988	<i>Shigella spp</i>	Human feces	Isolation	NR	1.4% ^g (4/283)	al-Lahham et al., 1990
Nigeria	2000	<i>S. flexneri</i>	Human feces	Isolation	NR	12.0% (71/593)	Udo and Eja, 2004
Nigeria	2000	<i>S. sonnei</i>	Human feces	Isolation	NR	8.8 % ^f (52/593)	Udo and Eja, 2004
USA	1995	<i>E. coli</i> O157	Cattle feces	Isolation by plating or enrichment	10	1.5% ^h (6/399)	Zhao et al., 1995
USA	2002	<i>E. coli</i> O157	Cattle feces	Direct plating and enrichment	25	7.5% (44/589)	Omisakin et al., 2003

^aSTEC: Shigatoxigenic *E. coli*; ^bPCR: Polymerase chain reaction; ^cVTEC: Verotoxigenic *E. coli*; ^dEPEC: Enteropathogenic *E. coli*; ^eNR: Not reported; ^fIn children; ^gIn food handlers; ^hControl herds

The average numbers of non-pathogenic *E. coli* excreted by humans and warm-blooded animals range from 10^4 to 10^5 CFU/g in cattle to 10^7 to 10^8 CFU/g in humans, although most animals excrete around 10^7 CFU/g of feces (Drasar and Barrow, 1985). In the case of *E. coli* O157:H7, it is excreted by both symptomatic and asymptomatic infected humans as well as animals including cattle, pigs, sheep, wild birds and others (Kudva et al., 1996; Wallace et al., 1997; Verweyen et al., 1999; Elder et al., 2000; Johnsen et al., 2001; Muniesa et al., 2003; 2004). In fact, cattle are considered as the main reservoir of EHEC O157 (Elder et al., 2000). Though the fecal excretion of *E. coli* O157 by cattle is only transient, typically lasting 3 or 4 weeks, *E. coli* O157 can be repeatedly isolated from environmental sources on farms for periods lasting several years (Hancock et al., 1997; Mechie et al., 1997). The concentration of the pathogen in cattle feces is estimated to be between 10^2 to 10^5 CFU/g (Zhao et al., 1995; Omisakin et al., 2003). In addition, there are cattle that excrete more *E. coli* O157 than others, known as super-shedders (Chase-Topping et al., 2008), that can contribute to a wider environmental spread.

2.2.2 Sewage

The study of the prevalence of *Shigella* and pathogenic *E. coli* in municipal sewage or animal wastewater is important to provide reference values since these wastewaters are one of the main contributions of fecal contamination to different waterbodies. However, their real prevalence is difficult to be estimated due to the difficulty of isolating these species in the environment. For reference, *E. coli* O157 and other STEC are commonly present in human and animal wastewaters at estimated values of 10 to 10^2 CFU/100 ml for municipal sewage and 10^2 to 10^3 CFU/100 ml for animal wastewaters from slaughterhouses (García-Aljaro et al., 2005b). A recent study by Teklehaimanot et al. (2014) demonstrated the non-compliance of the overall quality of South African municipal wastewater effluents with the South African special standard of no risk (zero *E. coli*/ 100 ml) and the stringent WHO standard (≤ 200 *E. coli*/ 100 ml) stipulated for the purpose of unrestricted irrigation. Fecal pollution loads in treated effluent impacted the quality of the receiving-water bodies pessimistically. Microbial counts of the receiving water bodies by far exceeded the South African standard limits recommended for domestic purpose (zero *E. coli*/ 100 ml) and aquaculture use (<10 *E. coli*/ 100 ml). Furthermore, the receiving water bodies' quality showed non-compliance with WHO standards stipulated for intermediate contact recreational use (< 1 *E. coli*/ 100 ml) (Teklehaimanot et al., 2014).

The prevalence of different *Shigella* species varies depending on the geographic location. In South Africa, Teklehaimanot et al., (2014) occurrence rates of *S. dysenteriae* at up to 40 to 60% of wastewater effluents samples and their respective receiving waterbodies

(Teklehaimanot et al., 2014; 2015). For the health risk assessment, the daily combined risk of the infection for this bacterial species was above the lowest acceptable risk limit of 10^{-4} as estimated by WHO for drinking water. These studies showed that poor quality of the inadequately treated wastewater effluents and their receiving waterbodies could pose potential health risks to the surrounding communities in peri-urban areas.

In China, Peng and co-workers (2002) studied the prevalence of *Shigella* in sewage samples collected from hospital and residential areas. 65.8% and 14.3% of samples were positive for the presence of *Shigella* by specific immunocapture-PCR and conventional bacterial culture detection, respectively. In this study the serotype most frequently detected was *S. flexneri* Type 2 (42.9%), followed by *S. flexneri* Type 3 (8.6%) and *S. sonnei* (8.6%), and *S. dysenteriae* Type 1 (5.7%).

Different STEC serotypes have been isolated from sewage carrying different virulence factors. For example, in the study of García-Aljaro and co-workers, one-hundred and forty-four STEC strains belonging to 34 different serotypes (0.7% belonging to serotype *E. coli* O157:H7) were isolated from urban sewage and animal wastewaters in Spain (García-Aljaro et al., 2005b). Other studies in the same field have shown that the prevalence of one major virulence factor from STEC, the *stx₂* gene, has a mean value of 10^2 *stx₂* genes/ml in municipal sewage (García-Aljaro et al., 2004). A proportion of 1 gene-carrying colony per 1,000 fecal coliform colonies in municipal sewage and around 1 *stx₂* gene-carrying colony per 100 fecal coliform colonies in animal wastewaters were observed being in agreement with other studies (Ibekwe et al., 2002; Chern et al., 2004).

2.2.3 Manure

As stated above *E. coli* O157:H7 is shed in manure by cattle transiently. According to Samadpour et al. (2002) the prevalence of *stx₁* and *stx₂* genes in cattle feces was around 18%. Therefore, soils fertilized with contaminated poultry or bovine manure compost as well as the use of contaminated irrigation water in agriculture has been reported as transmission routes for *E. coli* O157:H7 to the vegetables grown in these fields (Valcour et al., 2002). The consumption of contaminated raw vegetables such as lettuce or other leaf crops can transmit the pathogen to humans. One of the first cases of infection with *E. coli* O157:H7 was linked to the use of manure from cow as a fertilizer (Santamaria and Toranzos, 2003).

2.2.4 Surface waters

In spite of the limitations to study the prevalence of *Shigella* environmental samples, the presence of *Shigella* in surface waters has been investigated by several authors. Faruque et al demonstrated for the first time the existence of environmental *Shigella* from river and lake waters in Bangladesh (Faruque et al., 2002) (Table 3). Their results

suggested that the presence of these strains in these samples was not probably due to a recent fecal contamination event, but have persisted in the environment representing a potential source of virulence genes that may contribute to the emergence of virulent strains in the environment. Similarly, other authors have reported the isolation of *S. flexneri* 2b isolates from a freshwater lake in Bangladesh which differed from standard clinical strains. The isolates showed atypical API 20E profiles with only glucose and mannose positive tests that only provided a 69.3% identity score, and they lacked the virulence genes

with the exception of *ipaH* and a megaplasmid (Rahman et al., 2011). The isolates with apparent clonal origin were recovered after one-year interval indicating a strong environmental selection pressure for persistence in the environment. Wose Kinge and Mbewe, (2010) investigated the occurrence of *Shigella* in surface waters from 5 river catchments in South Africa. In The prevalence in these river catchments varied from 10 to 88% depending on the river catchment studied. *Shigella* was also detected in lakes and rivers from Bangladesh, United States, or Taiwan (Table 3).

Table 3. Occurrence of pathogenic *E. coli* and *Shigella* in wastewater, other surface water and soils

Area	Period of Study	Microorganism	Matrix	Detection Method	Sample Volume	Percent Positive (# of Samples)	Concentration Average CFU ^a /L, MPN ^b /L, or CFU/g	Reference
1. Wastewater and sludge								
France	2002	STEC	Clarifiers from waste storage lagoons and wastewater treatment plants	PCR ^c detection	25 g	53 to 80%	NR ^d	Vernozy-Rozand et al., 2002
Germany	2003 to 2004	STEC	Human wastewaters	PCR detection	NR	25%	NR	Burckhardt et al., 2005
South Africa	2011	<i>S. dysenteriae</i>	Wastewater effluent and receiving waterbody	Isolation	100 ml	40%	1.55E+03 to 1.41E+07 CFU	Teklehaimanot et al., 2014, 2015
Spain	2012	<i>E. coli</i>	Raw sludge	MPN	NR	NR	3.16E+06 MPN/g dry matter	Astals et al., 2012
Spain	2001 to 2004	<i>E. coli</i> O157, STEC	Animal wastewaters	PCR detection	250 µL	NR	1E+03 to 1E+04 CFU	Garcia-Aljaro et al., 2005b
Spain	2001 to 2004	<i>E. coli</i> O157, STEC	Human wastewaters	PCR detection	250 µL	NR	1E+02 to 1E+03	Garcia-Aljaro et al., 2005b
USA	NR	stx genes	Human wastewaters	NR	NR	51%	NR	Higgins et al., 2005
2. Other surface waters								
USA	1974	<i>Shigella</i> spp.	Recreational water	Isolation	100 ml	29%	NR	Rosenberg et al., 1976
USA	1999	<i>E. coli</i> O157:NM	Recreational lake	Isolation	NR	43%	NR	Feldman et al., 2002
USA	2010	<i>E. coli</i> O157	Water samples from farms	MPN	100 ml	21.4%	2.5E+04 MPN	Benjamin et al., 2013
3. Soil and compost								
USA	2010	<i>E. coli</i> O157	Soil surrounding farmyards	Plate count	10 g	1.7%	1.1E+05 CFU/100 g	Benjamin et al., 2013
USA	2008	<i>E. coli</i> O157:H7	Compost	MPN	30 g	6%	NR	Brinton Jr et al., 2009

^aCFU: Colony forming units; ^bMPN: Most probable number; ^cPCR: polymerase chain reaction; ^dNR: Not reported

An important percentage of outbreaks associated with the use of contaminated recreational waters in the United States during 1971 to 2000 were related to pathogenic *E. coli* or *Shigella* (Table 3 and 4). In these cases the source of contamination of recreational water is frequently related to the bathers themselves, but also to the poor microbiological conditions of the bathing waters or unchlorinated water in swimming pools and pad pools that become contaminated

with sewage and wild or domestic animal feces (McCarthy et al., 2001; Feldman et al., 2002; Lee et al., 2002; Samadpour et al., 2002; Craun et al., 2005). Structural problems and inadequate filtration or disinfection of recirculating water at children's spray parks have also been related to *E. coli* O157:H7 and *S. sonnei* outbreaks (Gilbert and Blake, 1998; CDC, 2006).

Table 4. Occurrence of pathogenic *E. coli* and *Shigella* in natural waters

Area	Period of Study	Microorganism	Matrix	Detection Method	Sample Volume, ml	Percent Positives	Concentration Average CFU ^a , MPN ^b or GC ^c /L	Reference
Bangladesh	1993	<i>Shigella</i> spp	Lakes and rivers	Isolation	10	12.0%	NR ^d	Islam et al., 1993
Canada	1999	<i>E. coli</i> O157:H7	River	Isolation	90	0.9%	NR	Johnson et al., 2003
Greece	1996	<i>S. sonnei</i>	Groundwater	Isolation	300	41.4%	NR	Alamanos et al., 2000
Japan	1993	<i>E. coli</i> O157:H7	Well	Isolation	NR	80 to 100%	1.9E+03 (CFU)	Akashi et al., 1994
Japan	1999	<i>stx2+</i> strains <i>E. coli</i> O157:H7	River	PCR ^e detection	40	NR	1E+05 to 1E+08 (GC) 1E+05 to 1E+07 (GC)	Kurokawa et al., 1999
Japan	2001	<i>ipaBCD</i> , <i>ipaH</i> , <i>stx1 E. coli</i> genes	River	PCR detection	NR	10.9%	NR	Faruque et al., 2002
South Africa	2007	<i>Shigella</i> spp	Lake	Isolation	1	10 to 88%	NR	Wose Kinge and Mbewe, 2010
South Africa	2008	<i>E. coli</i> <i>S. dysenteriae</i>	Groundwater	Plate count	100	77%	120 to 580 (CFU)	Mpenyana-Monyatsi and Momba, 2012
Taiwan	2002	<i>Shigella</i> spp	Streams	Plate count	100	3.3%	730 (CFU)	Hsu et al., 2008
USA	1974	Fecal coliforms	Estuary water	Plate count	100	NR	2 to 1.7E+05 (CFU)	Gerba and McLeod, 1976
USA	2007	<i>E. coli</i> O157:H7 <i>eaeA</i> gene	Stream	MPN	100	58% 95.5%	1E+05 (MPN)	Cooley et al., 2007
USA	2009	<i>stx2</i> gene <i>stx1</i> gene <i>rfbO157</i> gene	River	PCR detection	NR	53.7% 11.9% 13.4%	NR	Duris et al., 2009
USA	2002 to 2004	<i>stx</i> genes	River	PCR detection	1	12.9 to 29%	NR	Duris et al., 2009

^aCFU: Colony forming units; ^bMPN: Most probable number; ^cGC: Gene copies ; ^dNR: Not reported; ^ePCR: Polymerase chain reaction

The presence of virulence markers of STEC in river waters from Indiana and Michigan in the US was analyzed by PCR (Duris et al., 2009) (Table 4). The *eaeA* gene was found almost ubiquitously (95.5% of samples), followed by the *stx₂* gene (53.7%) and *stx1* (11.9%). The higher prevalence of the *eaeA* gene could be related to the presence of this gene in other non-O157:H7 strains, such as

EPEC were it has also been reported (Ogata et al., 2002; Nguyen et al., 2006).

2.2.5 Ground waters

Waterborne diseases caused by contaminated ground water have increased in the past decades (Santamaria and

Toranzos, 2003) (Table 4). For example, *E. coli* O157:H7 and *Campylobacter* contaminating the water supply from a well in a nursery school in Japan in 1990 caused a severe outbreak affecting 174 children (Akashi et al., 1994). There were unfortunate circumstances that facilitated the water contamination and outbreak: heavy rains accompanied by flooding, *E. coli* O157:H7 and *Campylobacter* spp. present in cattle manure from a sear farm, a well subject to surface water contamination and a water-treatment system that may have been overwhelmed by increased turbidity. *S. sonnei* caused also a large outbreak in Greece in 2000 linked to groundwater contamination (Alamanos et al., 2000). Mpenyana-Monyatsi and Momba assessed the quality of 100 boreholes that supply drinking water to rural areas of the North West Province in South Africa (Mpenyana-Monyatsi and Momba, 2012)(Table 3). The results of molecular study revealed among other

pathogenic bacteria, the presence of *E. coli* and *Shigella dysenteriae*. These finding showed convincing evidence that groundwater supplies in rural areas of this province pose a serious health risk to consumers.

2.2.6 Drinking waters

Shigella as well as *E. coli* have been detected in drinking water, although their presence is normally linked to the occurrence of an outbreak due to inappropriate control measures especially in developing countries. Wastewater or municipal sewage discharges can also contaminate water supplies (Table 5). A discharge of treated sewage into stream water contaminated a drinking water supply with *E. coli* O157 and *Campylobacter* causing an outbreak (Jones and Roworth, 1996). *Campylobacter jejuni* and *Shigella* spp were also found in a water pond that was used for drinking in Ohio (CDC, 2010).

Table 5. Occurrence of pathogenic *E. coli* and *Shigella* in drinking waters, food and beverages

Area	Period of Study	Microorganism	Source Environment	Detection Method	Percent Positive (# of cases)	Reference
1. Drinking waters						
Swaziland	1992	<i>E. coli</i> O157:NM	Drinking water	Isolation	42% (327/778)	Effler et al., 2001
UK	1966	<i>Shigella</i> spp	Drinking water	Isolation	49% (98/201)	Green et al., 1968
USA	1989 to 1990	<i>E. coli</i> O157:H7	Unchlorinated water supply	Isolation	80% (194/243)	Swerdlow et al., 1992
2. Food and beverages						
Germany	2011	O104:H4	Sprouts	Isolation	NR ^a	Buchholz et al., 2011; Muniesa et al., 2012
USA	2003	<i>E. coli</i> O157:H7	County fair beverages	Isolation	91.7% ^b (117/128)	Bopp et al., 2003

^aNR: Not reported; ^bSample volume: 100, 250 or 500 ml

As stated above, *Shigella* has also been linked with drinking water outbreaks worldwide (Table 5).

On the other hand, extreme meteorological and climatological conditions are also considered a significant risk factor for water contamination and have been linked to different outbreaks. For example, heavy rainfall occurred several days before the occurrence of one of the largest outbreaks involving *E. coli* O157:H7 and *Campylobacter* in Walkerton in Canada (Auld et al., 2004). Another large outbreak of *E. coli* O157 in 1992 in Southern Africa was also related to extreme climatological and meteorological situations (Effler et al., 2001).

The use of unchlorinated water supplies has also been linked to waterborne outbreaks such as the *E. coli* O157:H7 outbreak that affected 243 people in Missouri at the end of 1989 (Swerdlow et al., 1992). In the Highlands of Scotland, the distribution of an untreated and unprotected private

water source in a rural area where animals grazed freely seemed to be the cause of an outbreak in 1999 (Licence et al., 2001). A study by Momba and co-workers (2008) pointed out that the poor microbiological quality of drinking water and especially the presence of pathogenic *E. coli* O157:H7 was linked to the endemic outbreak of diarrheal diseases in the Eastern Cape communities of South Africa (Momba et al., 2008).

Some infections have also occurred at agricultural fairs where beverages made with fairground water were consumed. In fact, several studies have suggested that the attendance at summer agricultural fairs in the United States contributes to the seasonal peak in incidence of outbreaks (CDC, 1999). In this respect, the largest reported waterborne outbreak of *E. coli* O157:H7 in the United States was a co-infection outbreak with *Campylobacter jejuni* affecting 775 persons in New York state following a county fair in 1999 (Bopp et al., 2003).

2.2.7 Seawaters

Shigella as well as *E. coli* have been shown to enter into the VBNC in seawater (Xu et al., 1982; Islam et al., 1993) and therefore it is difficult to assess the real prevalence of these species in these environments. Concentration of fecal coliforms ranging from 2 to $1.7 \times 10^4/100$ ml were reported by Gerba and MacLeod at various sites with various degrees of fecal contamination (Gerba and McLeod, 1976).

2.2.8 Sludge

The presence of *E. coli* in sludge has been investigated by different authors. For example, Astals et al reported values for *E. coli* of up to $6.5 \log_{10}$ units/g dry matter in raw sludge (Astals et al., 2012). Although sludge digestion has shown reduction of pathogens, generic *E. coli* were still detected in all sludge samples whereas *Shigella* was isolated only from 1 out of 3 sludge samples. This is not surprising since temperature is one of the main factors determining the survival of *Shigella* (Dudley et al., 1980) (Table 4).

2.2.9 Soil

The presence of pathogenic bacteria in soil is a risk for spreading them to different water sources or even crops after rainfall events or even to crops through irrigation. The presence of *E. coli* O157 in sediments from a produce farm and surrounding area in Central California was investigated by Benjamin and co-workers. The pathogen was detected in 1.7% of the overall soil samples (0.6% from farms), with no seasonal variations, and was not correlated with the presence of generic *E. coli* (Benjamin et al., 2013). Brinton and co-workers reported an average value of 10^5 CFU/g of generic *E. coli* in composts from 50 different plants, with 36 out of the 50 below 10^2 CFU g⁻¹ (Brinton Jr. et al., 2009).

Beach sand and sediment has also been suggested as reservoir for fecal bacteria in recreational waters. In fact, sand may contain 2 to 100 times more fecal bacteria than water and therefore is likely to be a major non-point source for beach contamination (Bogosian and Bourneuf, 2001; Wheeler et al., 2003).

2.2.10 Irrigation water and on crops

Different outbreaks have been associated with the consumption of contaminated vegetables worldwide. For example, bagged baby spinach was the cause of an *E. coli* O57:H7 multi-state outbreak in the US, orromaine lettuce, that was responsible for an outbreak of *E. coli* O145 in Arizona (Table 5). The most recent and important outbreak taking into consideration the number of cases took place in Germany in 2011 and was linked to the presence in sprouts of the EAggEC O104:H4 serotype, that acquired *stx* genes through transduction by an Stx phage (Buchholz et al., 2011; Muniesa et al., 2012). Contaminated irrigation water, runoff from livestock facilities, application of improperly composted manure, and wildlife fecal depositions are thought to be the main contamination sources (Steele and Odumeru, 2004; Doyle and Erickson, 2008; Heaton and

Jones, 2008), although secondary, indirect contamination can occur at many places along the production chain, during post harvest interventions, during processing, or in storage (Wachtel and Charkowski, 2002; Delaquis et al., 2007). The internalization ability of enteric pathogens into the plants has been demonstrated by several authors. There is a possibility to enter through the leaves or roots but also through damaged tissue (Takeuchi et al., 2001) although leaves seem the most probable route under field conditions (Hirneisen et al., 2012), and therefore the microbiological quality is of high importance to prevent the risk of foodborne disease specially for vegetables that are eaten with minimal processing.

The prevalence of pathogenic bacteria in water samples from farms in Central California were investigated (Table 4). The recurrence of outbreaks related to produce suggests that contamination with the pathogen may occur through irrigation water or manure applied to the crops. Conditions that favor regrowth may increase the risk of spread of this microorganism (Ravva and Korn, 2007).

The U.S. Food and Drug Administration investigated the presence of foodborne pathogens, including *E. coli* O157:H7 and *Shigella* among others, on imported (broccoli, cantaloupe, celery, parsley, scallions, loose-leaf lettuce, and tomatoes) and domestic produce (cantaloupe, celery, scallions, parsley, and tomatoes) (FDA, 2001; 2003). *E. coli* O157:H7 was not detected in any of the samples. However, *Shigella* contamination was found in 9 out of 671 imported samples including 3 out of 151 cantaloupe samples, 2 out of 84 celery samples, 1 out of 116 lettuce samples, 1 out of 84 parsley samples, and 2 out of 180 scallion samples (FDA, 2001). *Shigella* contamination was found in 5 out of 665 total domestically grown samples: 1 out of 164 cantaloupe samples, 3 out of 93 scallion samples, and 1 out of 90 parsley samples (FDA, 2003).

2.2.11 Fish and shellfish

Shigella and *E. coli* have been isolated from fish and shellfish from sea and freshwater (Dartevelle and Desmet, 1975; Singh and Kulshrestha, 1993; Singh and Kulshreshtha, 1994; Balière et al., 2015a; Balière et al., 2015b). In Singapore, *Shigella* was detected in about 10% of samples of shellfish and prawns (Lam and Hwee, 1978). In India, *E. coli* was detected in 17% of fish and shellfish samples, mainly in freshwater fishes (13 out of 17 isolates), including serotypes O2, O3, O20, O87 and O128. *Shigella* was detected in 2.9% of fish from freshwater (Singh and Kulshrestha, 1993; Singh and Kulshreshtha, 1994). In this latter study, a total of 7 isolates of *S. dysenteriae* were isolated from 185 samples of seafoods including 4 freshwater fish out of 96, 2 marine fish out of 37, 1 prawn out of 14 and 0 out of 26 freshwater molluscs.

2.2.12 Air

Houseflies have been linked to the transmission of *E. coli* (Sola-Gines et al., 2015) as well as *Shigella* (Chompook, 2011). However, airborne transmission is not expectable for enteric pathogens.

2.3 Persistence

The survival of pathogens in natural environments is of great concern not only for their persistence but also for the possibility of these microorganisms to regrow, which increases the chances to find a suitable host. Bacteria are excreted from their natural hosts and once they reach the environment, they face a number of stressing factors (nutrients scarcity, sunlight, temperature, pH, and moisture variations, without forgetting predation). The survival rate of the different microorganisms depends therefore on the particular characteristics of their surrounding environment. *Shigella* and *E. coli* as many other enteric bacteria have been suggested to be able to enter into a dormancy state (the VBNC state) in certain environmental conditions. In this state cells show very low levels of metabolic activity but are not able to grow on routine bacteriological media where they would normally grow (Oliver, 2005). Therefore, their detection and isolation from environmental samples is difficult, which limit the study of their real persistence in the environment. Consequently, the majority of persistence studies have been performed in micro or mesocosm with spiked bacteria previously grown in the laboratory, providing limiting information. According to Islam et al., (1993) *S. dysenteriae* entered into the VBNC state 2 to 3

weeks after inoculation in a laboratory microcosm and remained detectable by fluorescence microscopy up to 6 weeks (Islam et al., 1993). The importance of these VBNC in pathogenicity has been demonstrated by several investigators, which showed that VBNC *E. coli* cells caused fluid accumulation into ligated rabbit ileal loops, as well as the possibility to recover culturability (Grimes and Colwell, 1986). The existence of these cells was supported by Makino and co-workers (Makino et al., 2000), who found that around 0.75 to 1.5 culturable cells had been responsible for an outbreak of *E. coli* O157:H7 in Japan in 1998 that was considered too low for causing infection. In the case of *S. dysenteriae* type 1, the cells maintained the capability of active uptake of methionine and its incorporation into protein, as well as cytotoxicity for cultured mammalian cell lines, produced Shiga toxin and the capability to adhere to intestinal epithelial cells (Rahman et al., 1996).

Available data about *E. coli* persistence in different environments is mostly related to commensal non-pathogenic *E. coli* used as indicator. Data about the persistence of *Shigella* is scarcer. In Tables 6 and 7, persistence expressed as log₁₀ reductions in a given time (days) is presented.

Table 6. Reported persistence of *E. coli* and *Shigella* in wastewaters, sludge and soils

Area	Microorganism	Matrix	Temperature°C	Log ₁₀ Reduction	Survival Days	Log ₁₀ Reduction/Day	Reference
1. Wastewaters, wastes and sludge							
Tunisia	<i>Shigella</i>	Domestic treatment plant effluent	4 or RT ^a	2 to 3	30	0.067 to 0.10	Ellafi et al., 2010
			4 or RT	5		0.167	
USA and others	<i>E. coli</i>	Domestic sewage	8	1 to 2	91	0.011 to 0.022	Wang and Doyle, 1998
USA and others	<i>E. coli</i>	Domestic untreated sewage	25	≥3	49 to 84	0.036 to >0.061	Wang and Doyle, 1998
		Untreated sewage	10	2	1 to 29	0.069 to 2	
		Sewage sludge	10	2	0 to 56	0.036 to >2	
UK	<i>E. coli</i>	Abattoir wastes	10	2	26 ±6	0.063 to 0.1	Avery et al., 2005
		Cattle slurry	10	2	12 ±4	0.125 to 0.25	
		Creamery waste	10	0.5 to 1	64	0.008 to 0.016	
Spain	<i>E. coli</i>	Sludge	10	>3	60	>0.05	Pascual-Benito et al., 2015
			22	<1	60b	<0.027	
			37	>7	20 ^b	>0.35	
USA	<i>E. coli</i>	Manure slurry	4	1	21.5	0.046	Himathongkham et al., 1999
			20	1	4.75	0.211	
			37	1	3.18	0.314	

Area	Microorganism	Matrix	Temperature ^a C	Log ₁₀ Reduction	Survival Days	Log ₁₀ Reduction/Day	Reference
USA	<i>E. coli</i>	Dairy wastewater	NR ^c	1	0.5 to 9.4	0.106 to 2	Ravva et al., 2006
2. Soils							
USA	<i>E. coli</i>	Non sterile soil	4	7	100	0.07	Garzio-Hadzick et al., 2010
USA	<i>E. coli</i>	Soil and sediment	4	≤1	50	0.02	Bogosian et al., 1996
USA	<i>E. coli</i>	Soil and sediment	14	~2	50	0.04	Garzio-Hadzick et al., 2010
USA	<i>E. coli</i>	Non sterile soil	20	7	60	0.117	Bogosian et al., 1996
USA	<i>E. coli</i>	Soil and sediment	24	>2	50	0.04	Garzio-Hadzick et al., 2010
USA	<i>E. coli</i>	Non sterile soil	37	7	12	0.583	Bogosian et al., 1996
USA	<i>E. coli</i>	Sterile soil	4, 20 or 37	No decline	100	0	Bogosian et al., 1996

^aRT: Room temperature (25°C); ^bhours; ^cNot reported

Table 7. Reported persistence of *E. coli* in fresh and marine waters

Area	Matrix	Temperature, °C	Log ₁₀ Reduction	Survival Days	Log ₁₀ Reduction/Day	Reference
Japan	Seawater	27	3 to 4	15	0.2 to 0.27	Miyagi et al., 2001
Spain	River water (in situ)	NR ^a	2.9 ^b	7	≥0.286	Muniesa et al., 1999
Spain	River water	10	2	6	0.33	Avery et al., 2008
Tunisia	Seawater	NR	3	1	3	Ellafi et al., 2009
	Seawater	NR	8	30	0.27	Ellafi et al., 2009
USA	River water	4	7 ^c	12	>0.58	Bogosian et al., 1996
USA	Sterile artificial seawater	4	No decline	50	NR	Bogosian et al., 1996
USA	Freshwater lake: without sand	4	<1	20	<0.05	Sampson et al., 2006
	Freshwater lake: with sand	4	<1	20	<0.05	
USA	Freshwater lake	10	2	12.9	0.15	Avery et al., 2008
USA	River water	20	7 ^c	8	0.87	Bogosian et al., 1996

Area	Matrix	Temperature, °C	Log ₁₀ Reduction	Survival Days	Log ₁₀ Reduction/Day	Reference
USA	Sterile artificial seawater	20	4	NR	NR	Bogosian et al., 1996
USA	Freshwater lake: without sand	25	3 ^c	20	>0.15	Sampson et al., 2006
	Freshwater lake: with sand	25	2	20	0.10	
USA	River water	37	7 ^b	6	>1.17	Bogosian et al., 1996
USA	Sterile artificial seawater	37	5 ^b	60	>0.08	Bogosian et al., 1996

^aNR: Not reported; ^b*E. coli* O157:H7; ^cFell below detection limit

All species of *Shigella*, once excreted, are very sensitive to environmental conditions and die rapidly in few days, especially when dried or exposed to direct sunlight (WHO, 2005a). In a microcosm study survival of *S. dysenteriae* and *S. flexneri* survived for up to 6 and 16 days, respectively, and the survival showed a positive correlation with the initial bacterial counts, as well as low temperature (Ghosh and Sehgal, 2001). Rahman showed a survival of *Shigella* for up to 4 days in river water (Rahman et al., 2011), although prolonged persistence of *Shigella* in the cytoplasm of free-living amoebae has also been demonstrated, suggesting that this protozoon could be a possible environmental host of *Shigella* such as in the case of other genera like *Legionella*, *Mycobacterium*, *Campylobacter* and *Listeria* (Greub and Raoult, 2004; Jeong et al., 2007; Schmitz-Esser et al., 2008). Although amoeba are commonly found in natural aquatic systems and in soil (Rodríguez-Zaragoza, 1994; Zaman et al., 1999) the increase in sludge disposal activities, which may contain high numbers of protozoans like *Acanthamoebae* is a growing concern for the survival of this pathogen (Jeong et al., 2007). *S. sonnei* and *S. dysenteriae* survived for more than 3 weeks when incubated in the presence of *Acanthamoeba castellanii* and increased between 10 fold and 100 fold, respectively, after 3 days of co-cultivation (Saeed et al., 2008). *Shigella* has also shown a higher resistance to chlorine when incubated together (King et al., 1988).

E. coli can survive in surface or groundwater for days to months depending on the environmental conditions. Concerning the effect of temperature on the survival, most studies performed with *E. coli* K12 indicate that cool waters increase the ability of *E. coli* and *E. coli* O57:H7 to survive in a variety of conditions (Brettar and Hofle, 1992; Smith et al., 1994; Bogosian et al., 1996; Wang and Doyle, 1998), with a clear decline in fresh water at 37°C and in seawater at 15°C (Flint, 1987; Gauthier and Le Rudulier, 1990), although other studies performed with other *E. coli* strains

showed that there is a decline at lower temperatures (5°C) but not at warmer temperatures (González et al., 1992; Fish and Pettibone, 1995). For example, the persistence and survival of *E. coli* K12 in recreational water and soil was studied by (Bogosian et al., 1996)(Table 6 and 7). The decline of *E. coli* was higher in nonsterile water than in nonsterile soil. In nonsterile water *E. coli* counts dropped by more than 7 log₁₀ units in 12 days at 4°C, 8 days at 20°C and only 6 days at 37°C. In contrast, in nonsterile soil, the persistence was higher with 100 days at 4°C to give a reduction of 7 log₁₀ units, around 60 days at 20°C and 12 days at 37°C. In sterile soil no decline after 100 days at the different assessed temperatures. In sterile artificial seawater at 4°C or sterile river water at 4 or 20 °C the *E. coli* counts remained constant for over 50 days. However at 37°C there was a decline of 4 log₁₀ units in 60 days in sterile river water, similarly to *E. coli* decline in artificial seawater at 20°C. At 37°C the inactivation rate was higher (more than 5 log₁₀ units in 60 days). The persistence of an environmental *E. coli* in a lake microcosm showed similar results (Sampson et al., 2006) (Table 6).

The presence of soil and sediment has been shown to protect and stabilize *E. coli* cells (Davies et al., 1995; Fish and Pettibone, 1995; Sampson et al., 2006; Rehmann and Soupier, 2009; Garzio-Hadzick et al., 2010) (Table 7) which may provide bacteria a protective niche with available soluble organic matter and nutrients but also a shield against UV sunlight as well as predation by protozoa (Jamieson et al., 2005; Koirala et al., 2008). In the study of Williams and co-workers (Williams et al., 2013) a rapid decay in the number of cells was observed over the first 7 days after inoculation of *E. coli* O157 in an agricultural soil when stored at 4 °C or 15 °C for up to 120 days, although the pathogen can persist at a low metabolic state for prolonged periods (Jones, 1999). According to Garzio-Hadzick et al. (2010) a higher content of fine particles in the sediments increased the survival of manure borne *E. coli*, similarly to other reports (Burton et al., 1987),

although this is in controversy with the work of Cinotto (Cinotto, 2005) in which a higher survival of *E. coli* was shown in sediments with larger particles (between 125 to 500 μ m). On the other hand, the presence of organic carbon has also been shown to increase *E. coli* survival (Garzio-Hadzick et al., 2010). Noteworthy, Seo and co-workers demonstrated that *E. coli* O157:H7 previously incubated with manure or soil showed an increase in the survival rate in a laboratory microcosms compared to LB-grown *E. coli* O157:H7 (Seo and Matthews, 2014).

In finished sludge, a different reduction rate for *E. coli* has been reported depending on the previous digestion process applied to the sludge (Pascual-Benito et al., 2015). A reduction of more than 3 log₁₀ units in mesophilic sludge stored at 22 °C for 60 days and more than 7 log₁₀ units when stored at 37 °C for 20 days. In the case of thermophilic digested sludge, storage at 22 °C for 60 days produced less than 1 log₁₀ reduction, whereas more than 1 log₁₀ units in thermophilic sludge stored for 5 days at 37 °C was observed (Table 7).

The survival of different pathogenic groups such as EHEC has also been studied in manure (Table 7) and manure slurry (Kudva et al., 1998), sediments of cattle drinking reservoirs contaminated with feces (Faith et al., 1996; Hancock et al., 1998; LeJeune et al., 2001), domestic sewage (Muniesa et al., 1999) and seawater (Gagliardi and Karns, 2000; Maule, 2000; Miyagi et al., 2001; Avery et al., 2005). In some of these matrixes inoculated *E. coli* O157:H7 has been recovered after long periods of time (Islam et al., 2004; Semenov and Franz, 2008), even after 21 months in a manure pile from inoculated sheep. In contrast, the pathogen declined rapidly in manure, manure slurries, and wastewater from dairy lagoons (Himathongkham et al., 1999; Avery et al., 2005; Ravva et al., 2006). Studies by Islam and co-workers showed that *E. coli* O157:H7 in compost and in irrigation water could persist for 154 to 217 days in soils receiving the compost or irrigation water, with *E. coli* O157:H7 spiked strains detected in lettuce and parsley for up to 77 and 177 days (Islam et al., 2004). In another study, *E. coli* O157 as well as O157:H32 declined rapidly in 4 to 5 days below the limit of detection and more rapidly from filter-sterilized wastewater, which was attributed to the removal of organic matter by the filters. In steam-sterilized wastewater two strains were able to grow during the first 2 days but were undetectable after 15 days of incubation (Ravva and Korn, 2007) with a starting concentration of 10⁵ CFU/ml. Spiked EHEC O157 persists in river water similarly to *E. coli* naturally occurring in domestic sewage (Muniesa et al., 1999). Similarly, the presence of algae in the water ecosystems such as the green filamentous algae *Cladophora* spp. commonly found in nearshore beach freshwater lakes has shown to increase its survival (Table 7). The presence of the algae was shown to protect against natural UV radiation in a microcosm setting simulating a lake environment (Beckinghausen et al., 2014). Besides, algae blooms have been shown to provide an ideal organic matrix that facilitates bacterial attachment and growth of *E. coli* among other bacterial pathogens, which can reach levels of 1 x 10⁵ CFU/g of algae, and therefore could be

regarded as an environmental reservoir (Byappanahalli et al., 2003; Whitman et al., 2003; Badgley et al., 2012) and persist for more than 48 days attached to *Cladophora*. Similarly, STEC and *Shigella* have also been associated with algae (Ishii et al., 2006) although no differences in the persistence was observed in the case of *Shigella* which was detected for up to 2 days attached to *Cladophora* at room temperature (Englebert et al., 2008). Another factor with great influence on the survival of *E. coli* is solar radiation (Whitman et al., 2004; Wilkes et al., 2009).

Shigella as well as *E. coli* have a great capability to adapt to nutrient starvation and harsh environments by regulating gene expression. Ellafi and co-workers demonstrated that *Shigella* were able to adapt and survive in domestic treatment plant effluent by adapting cell metabolism and physiology; the strains underwent changes in the biochemical and enzymatic characteristics of the isolates and increased their adherence to KB cells (Ellafi et al., 2009, 2010) (Table 7). The authors observed also modifications of the resistance to antibiotics probably due to structural modifications of the cellular envelope. The population decreased around 5 log₁₀ units after 1 month (2 to 3 log₁₀ units after 24 hours) showing a more rapid decline at room temperature than 4°C (Ellafi et al., 2009). Similar results were obtained for *E. coli* O157:H7 and *E. coli* O157:H32 by (Ravva and Korn, 2007) and other authors, who reported changes in protein expression enzyme activities in different *E. coli* strains in the NVBC cells (reviewed in Dinu et al., 2009).

3.0 Reductions by Sanitation Management

There is an abundant corpus of information regarding reductions of *E. coli* in sanitation management. Nevertheless, many studies are focused on *E. coli* as indicator microorganism, hence not directly evaluating pathogenic species. Although otherwise indicated, results of indicator *E. coli* could be correlated with its pathogenic counterpart and other *Enterobacteriaceae*, as *Shigella*. The following section refers to reductions either in pathogenic *E. coli*, when available, and if not to indicator *E. coli* or fecal coliforms.

3.1 Excreta and Wastewater Treatment

Waterless sanitation system (WSS) is defined as the disposal of human waste without the use of water as a carrier; it is also used for waterless toilets. Often the end product is used as a fertilizer. In developed countries, dry sanitation toilets were initially designed for use in remote areas for practical and environmental reasons. However, increasing environmental awareness has led to some people using them as an alternative to conventional systems. In developing countries they can be a low cost, environmentally acceptable, hygienic option. They have found useful in situations where no suitable water supply or sewer system and sewage treatment plant is available to capture the nutrients in human excreta. Reduction due to the different treatments in different matrices is summarized in Table 8-13.

3.1.1 Dry Sanitation with inactivation by storage

In general, the storage of residues is hygienically harmless if thermophilic composting occurs at temperatures of 55°C for at least two weeks or at 60°C for one week. For safety reasons however the WHO (http://www.who.int/water_sanitation_health/wastewater/gsuww/en/) recommends a composting at 55°C to 60°C for one month with a further maturation period of 2 – 4 months in order to ensure a satisfactory bacterial reduction.

When storage is the only treatment at ambient temperature (2 to 20°C), long term storage up to 1.5 to 2 years will eliminate most bacteria, bacterial pathogens

(including *Shigella* and *E. coli*), substantially reduce viruses, protozoa and parasites. Only some soil ova may persist. The regrowth of *E. coli* would not be considered. If the temperature is higher (> 35°C), storage will reduce bacteria in > 1 year. However, storage alone is unlikely to be a reliable pathogen destruction mechanism although fecal coliforms (including *E. coli* and *Shigella*) may diminish (Hill and Baldwin, 2012) (Table 8). Therefore, combinations of storage plus alkaline treatment (pH>9) will allow absolute elimination of *E. coli* in > 6 months. Even with combined treatment, at the temperature lower than 35°C, the presence of moisture at a temperature of more than 25°C or a pH below 9, will prolong the time for absolute elimination (Schoenning and Stenstroem, 2004).

Table 8. Reported *E. coli* (generic indicator) reductions in fecal waste under different onsite wastewater treatment methods

Area	Treatment	Log ₁₀ Initial Concentration	Log ₁₀ Final Concentration	Log ₁₀ Reduction	Reference
Nepal	Mixed latrine microbial composting toilets (MLMCs)	7.1 CFU ^a /cm ^{3b}	4.9 CFU/cm ³	2.2	Sherpa et al., 2009
Tunisia	Storage & Composting	7.0 cells/g	3.0 cells/g	4.0	Hassen et al., 2001
USA	Source separating vermicomposting toilets (SSVCs)	3.9 CFU/g	2.3 CFU/g	1.6	Hill and Baldwin, 2012
UK	Vermifilter	9.0 CFU /100 mL	6.1 CFU /100 mL	2.9	Furlong et al., 2015

^aCFU colony forming unit quantified by plate count methods.; ^bWet weight measured as a volume

3.1.1.2 Pit Latrines, vault toilets, dry toilets

3.1.1.2.1 Urine separation

It is important to know that urine in the bladder of a healthy person is sterile (meaning it contains no pathogens) or very little contaminated in comparison with feces. Pathogenic *E. coli* and *Shigella* would be less prevalent in urine and therefore be a minor problem.

Systems that collect urine in the vault together with feces produce a larger volume of leachate. This leachate has to be handled carefully in order to avoid the spreading of pathogens. The handling, discharge and treatment of excess leachate has to be considered in the planning phase of a composting toilet. For the treatment of urine from urine diverting toilets please refer to the technology review (von Münch, Winker, 2011).

Source-separated vermi-composting (SSVC), in which urine and fecal matter added at the toilet hole are separated from each other, showed a significant reduction of *E. coli*, and proved to be more efficient in pathogen

removal than other composting toilets (Table 8).

3.1.1.2.2 Variations with ash, lime, soil, urea additions

Using these systems, covering material should be added to the feces vault after each defecation. Covering material can be ash, sand, soil, lime, leaves or compost and should be as dry as possible. The purpose of adding covering material is to reduce odor, assist in drying of the feces (to soak up excess moisture, to prevent access for flies to feces and to improve aesthetics of the feces pile (for next user) and, to increase pH value (achieved when lime or ash is used).

Some studies indicated that the process of lime stabilization reduced pathogens in sludge, enabling lime treated sludge to be safely disposed-off in landfills or applied to land (Wong et al., 2001; Kocaer et al., 2003). In some cases, alkaline byproducts such as fly ash, cement kiln dust and carbide lime have also been used as a stabilizing agent. The high CaO content of alkaline fly ash makes it potentially useful as an additive in stabilization

processes for sludge and increase the removal of indicator bacteria, including *E. coli* (Kocaer et al., 2003).

Related advanced treatments using amorphous silica and hydrated lime as used to treat swine wastewater and observed removal rates of coliform bacteria and *E. coli* exceeding 3 log₁₀ with >0.1 w/v%.

3.1.1.3 Composting

Composting studies have been performed using *E. coli* as surrogator of pathogenic *E. coli* and other enterobacteria (Hassen et al., 2001) (Table 8). Sterilization induced by relatively high temperatures (60 to 55 °C) produced caused a significant change in bacterial communities.

3.1.2 Onsite sanitation

There are different on-site sanitation systems that can be used on site that need water for operating. The pour flush toilet combined by a single or twin pit is best suited for places where there is the possibility to construct new pits or there is a possibility to empty the constructed pits. Greywater helps degradation of the fecal material but excessive shorten the life of the pit (Tilley et al., 2014). Other systems include collection of blackwater in a septic tank, or in an anaerobic baffled reactor or anaerobic filter can be used to reduce the organic and pathogen load. In a well-constructed septic tank a 1 log₁₀ *E. coli* removal can be achieved. The storage effluent can be disposed through a

soak pit or leach field. A sewerage system with urine diversion can also be used although the cost of this technology is high. Urine is separated from the fecal material and can be applied to the land but brownwater has to be treated to reduce the number of pathogens (Tilley et al., 2014).

Alternate on-site sanitation systems have also been investigated. For example, the work by Furlong and co-workers developed the "Tiger toilet" which was based on the use of a worm-filter (vermifilter). This system is based in the ability of worms to efficiently reduce the number of thermotolerant coliforms in feces by 3 log₁₀ units in 210 days (Furlong et al., 2015) (Table 8).

3.1.3 Coupled Engineered and Environmental-based Systems

3.1.3.1 By waste stabilization ponds

Water stabilization ponds are used to treat wastewaters worldwide, with particular importance in tropical and warm temperate countries. The microbiological effluent quality depends strongly on the weather conditions and therefore, variation has been reported in its quality (Hickey et al., 1989; Davies-Colley et al., 1999). For example, in the study of Hickey et al., fecal coliforms ranged between 8.0 E+02 and 1.64 E+05 per 100 ml from different ponds analyzed in different seasons. Approximately 1.5 log₁₀ of *E. coli* is reduced during a period of 15 to 21 days (Cody and Tischer, 1965) (Table 9).

Table 9. Reported *E. coli* and *Shigella* reductions in wastewater by different treatments

Area	Microorganism	Treatment	Initial Concentration, Log ₁₀ MPN ^a or CFU ^b /L	Final Concentration, Log ₁₀ MPN or CFU/L	Time, Days	Log ₁₀ Reduction/day	Reference
Canada	Fecal coliforms	Aerated lagoons	7.7 (CFU)	3 (CFU)	16 to 19	0.25 to 0.29	Locas et al., 2010
Greece	<i>E. coli</i> (indicator)	Photoelectrocatalytic oxidation on TiO ₂ (2)/Ti films + solar radiation	10 (CFU)	5 (CFU)	0.063	Not applicable	Venieri et al., 2012
India	Fecal coliforms	Waste stabilization ponds	7.7 (MPN)	7.4 (MPN)	11	0.18	Tyagi et al., 2011
Spain	Fecal coliforms	Waste stabilization ponds	NR ^c	NR	24	0.08	Garcia and Becares, 1997
USA	<i>E. coli</i> (indicator)	Waste stabilization ponds	NR	NR	15 to 21	0.07 to 0.1	Cody and Tischer, 1965
USA	Fecal coliforms	Wetlands	9.0 (CFU)	6.7 (CFU)	6 to 8	0.38 to 0.5	Hench et al., 2003
	<i>Shigella</i>	Wetlands	6.8 (CFU)	5.1 (CFU)	6 to 8	0.29 to 0.38	
Reductions with no time indicated							
Belgium	Fecal coliforms	Enhanced primary (sedimentation + coagulation)	5.5 (CFU)	3.1 (CFU)	NR	2.4	Kuai et al., 1999
China	Fecal coliforms	Oxidation ditch	5.8 (CFU)	2.5 (CFU)	NR	3.3	Fu et al., 2010
China	Fecal coliforms	Primary /preliminary treatment	5.8 (CFU)	5.7 (CFU)	NR	0.1	Fu et al., 2010

Area	Microorganism	Treatment	Initial Concentration, Log ₁₀ MPN ^a or CFU ^b /L	Final Concentration, Log ₁₀ MPN or CFU/L	Time, Days	Log ₁₀ Reduction/day	Reference
Switzerland	<i>E. coli</i> (indicator)	Septic tank	NR	NR	NR	1.0 ^d	Tilley et al., 2014
USA	<i>E. coli</i>	Secondary treatment (membrane bioreactors)	5.6 (CFU)	0.4 (CFU)	NR	5.2	Francy et al., 2012
USA	Fecal coliforms	Secondary treatment (Anaerobic/anoxic digestion and biogas)	6.9 (MPN) ^e	2.4 (MPN)	NR	4.5	Mohd et al., 2015
Various countries	Fecal coliforms			NR	NR	≥2.0	Garcia-Armisen and Servais, 2007; Wéry et al., 2008; Fu et al., 2010;
		Secondary treatment (activated sludge)	Various concentrations				Francy et al., 2012
	<i>E. coli</i>			NR	NR	2.0 to 4.0	

^aMPN: most probable number; ^bCFU: colony forming unit; ^cNR: Not Reported; ^dTotal log removal observed; ^eStandard method

Sunlight exposure has been reported as the main factor that affects the disinfection process (Mayo, 1995). Depth of the pond, algal concentration, and mixing are the main factors that affect penetration of solar radiation into the pond water and therefore, disinfection efficiency. The physico-chemical conditions in the surface of the water fluctuate according to the sunlight. Photo-oxidation was the inactivation mechanism attributed to the die-off of fecal coliforms (Curtis et al., 1992). Davies-Colley and co-workers demonstrated that different mechanisms were responsible for the inactivation of *E. coli*. At high pH (pH>8.5) inactivation dependent mainly on the dissolved oxygen as well as the presence of water pond constituents, while at lower pH the damage is mainly by UVB, causing a photo-oxidation damage (Davies-Colley et al., 1999).

In a pilot waste water stabilization pond consisting in a rectangular pond with an area of 15 m² and 75 cm depth and a retention of 24 days, García and Becares (Garcia and Becares, 1997) reported an efficiency in reduction of fecal coliforms of 1.8 log₁₀. In a full scale system, with a retention time of 11 days, a reduction of around 2 log₁₀ units was observed for fecal coliforms (Tyagi et al., 2011).

3.1.3.2 Wetlands

The construction of wetlands to be used as alternative wastewater treatment has increase the interest since it represents an economical option for water sanitation in developing countries. There are numerous factors affecting the removal of microorganisms in these ecosystems, which, together with the fact that it is very difficult to construct an identical unit, make the development of predictive models for the microorganism removal efficiency very difficult (Werker et al., 2002). The different factors have been addressed separately mainly in mesocosm studies

(reviewed in (Wu et al., 2016)). In the study of Hensch and co-workers (Hensch et al., 2003) a reduction of around 3 log₁₀ units was observed for fecal coliforms, and slightly lower for *Shigella* (2.3 log₁₀ units), showing a higher inactivation in a planted mesocosm with a retention time of 6 to 8 days.

3.1.3.3 Aerated lagoons

Aerated lagoons offer a simple and affordable technology for wastewater treatment in small and rural areas. Removal of pathogens as in the case of the previously described wastewater treatments depends on many abiotic and biotic factors. The weather influence on the removal of thermotolerant coliform was studied by Locas and co-workers (Locas et al., 2010) in two aerated lagoons located at different sites in Quebec, composed by 3 and 4 cells, with a retention time of 19 and 16 days, respectively. The thermotolerant coliforms decreased by around 5 log₁₀ units and 3 log₁₀ units, under warm and cold weather conditions at both sites (Table 9).

3.1.3.4 Oxidation ditch

Oxidation ditch treatment produced a reduction of around 2.5 log₁₀ units for fecal coliforms (Fu et al., 2010).

3.1.4 Wastewater Treatment and Resource Recovery Facilities

3.1.4.1 Combined sewer overflows

Combined sewers carry wastewater from urban areas as well as storm water runoff in the same pipe. The final concentration of pathogens in the effluent depends on many factors including rainfall, and the resuspension of in-sewer

deposits, among others. Variations around 1 log₁₀ units for *E. coli* can be observed under dry weather conditions (Kim et al., 2009). Normally, during dry weather, wastewater is collected in the sewer and conveyed to a wastewater treatment plant or treated by other means. However, during rainfall events when the total volume of water cannot be handled, part of the rain and wastewater is diverted and discharged into a water body reducing the number of pathogens by a dilution effect.

3.1.4.2 Primary /preliminary treatment

The primary treatment performed in wastewater treatment plants does not produce a significant reduction in pathogens. A reduction of 0.06 log₁₀ units for fecal coliforms was observed after a primary sedimentation treatment in a full-scale waste water treatment plant (Fu et al., 2010) (Table 9).

An enhanced primary treatment based on sedimentation combined with a coagulation step using poly-electrolyte has shown to increase the removal of fecal coliforms up to 1.5 log₁₀ units (Kuai et al., 1999) (Table 9).

3.1.4.3 Secondary treatment

3.1.4.3.1 Trickling filters

A trickling filter purifies wastewater through a physical filtration/adsorption process and biological degradation. The main principle of elimination by a biological process is believed to be based on sorption, die-off and the predation of micro-organisms by macro-organisms. In the case of the trickling filter, the high variety of macro-organisms might be the major contributor to the good effect of disinfection, although disinfection appears simultaneously with nitrification (Kuai et al., 1999). The trickling filter process typically includes an influent pump station, trickling filter containing a biofilm carrier, trickling filter recirculation pump station, and a liquid-solids separation unit (Daigger and Boltz, 2011). Depending on the design, the removal of fecal coliforms by trickling filters can vary. A reduction between 2 to 4 log₁₀ units was achieved for fecal coliforms (Kuai et al., 1999), and 2 log₁₀ units for *E. coli* (Elmitwalli et al., 2003) in domestic wastewater. A lower removal efficiency (by 1 to 2 log₁₀ units) was reported in the treatment of swine wastewaters (Zacarias Sylvestre et al., 2014).

3.1.4.3.2 Activated sludge

Secondary wastewater treatment based on activated sludge has been reported to achieve a reduction in fecal coliforms by 2 log₁₀ units (Garcia-Armisen and Servais, 2007) or higher (Wéry et al., 2008; Fu et al., 2010) and between 2 and 4 log₁₀ units for *E. coli* (Francy et al., 2012). The reduction is attributed both to different abiotic and biotic factors, such as adsorption to flocs and sedimentation, the natural microorganisms die-off as well as predation.

3.1.4.3.3 Membrane bioreactors

Membrane bioreactor technology has emerged in the last decades as an alternating wastewater treatment in which membranes are submerged in the activated-sludge tank to perform solid-liquid separation process increasing the quality in the effluent compared to classical activated sludge process, although at higher cost (Sun et al., 2015). Using this technology the efficiency for *E. coli* removal can be higher than 5 log₁₀ units (Francy et al., 2012).

3.1.4.3.4 Anaerobic/ anoxic digestion and biogas systems

Anaerobic digestion is one of the most used methods for the stabilization of wastewater. Besides ensuring a safe disposal for the waste, this method has great potential for producing energy-rich biogas and nutrient-rich biosolids. The method involves four main steps; namely hydrolysis, acidogenesis, acetogenesis and methanogenesis, and are typically conducted at mesophilic (30 to 40 °C) or thermophilic temperatures (55 to 60 °C), the mesophilic requiring longer retention time. Operation at an intermediate temperature of 45 °C a 3 log₁₀ reduction for fecal coliforms was achieved (Mohd et al., 2015).

3.1.5 Biosolids/sewage sludge treatment

Sludge mesophilic and thermophilic digestion processes are widely used to reduce the number of pathogens for sludge disposal. In the work by Astals and co-workers *E. coli* was reduced by 2.2 log₁₀ units in 20 days using a mesophilic digestion process, whereas in a thermophilic digestion process produced a reduction of 4.2 in 15 days was observed (Astals et al., 2012). In an attempt to save energy and stabilize the sludge, a method combining a mesophilic anaerobic digestion for 3 days followed by a thermophilic aerobic process was developed (Cheng et al., 2015). A reduction of more than 4 log₁₀ units after a 10-day digestion was attained using this method.

Table 10. Reported *E. coli* and fecal coliform reduction from sludge by different treatments

Area	Microorganism	Treatment	Initial ConcentrationLog ₁₀ MPN ^a /g or log ₁₀ CFU ^b /g	Final ConcentrationLog ₁₀ MPN ^a /g or log ₁₀ CFU ^b /g	Time, Days	Log ₁₀ Reduction/day	Reference
China	<i>E. coli</i> (indicator)	Sludge mesophilic anaerobic digestion	6.7 (MPN) wet weight	3.5 (MPN) wet weight	25	0.13	Chen et al., 2012
China	Fecal coliforms	Mesophilic aerobic digestion + thermophilic anaerobic digestion	7.8E+06 (MPN) wet weight	4.8E+02 (MPN) wet weight	10	0.42	Cheng et al., 2015
Egypt	Fecal coliforms	Windrow composting	7.0 (MPN) dry weight	NR	7	0.7 to 1	Khalil et al., 2011
Iran	Fecal coliforms	Composting of anaerobically digested sewage sludge	6 (MPN) dry weight	BDL ^c	NR ^d	NR	Nafez et al., 2015
Spain	<i>E. coli</i>	Mesophilic aerobic digestion	6.4 (CFU) dry weight	4.2 (CFU) dry weight	20	0.11	Astals et al., 2012
		Thermophilic aerobic digestion	6.4 (CFU) dry weight	2.2 (CFU) dry weight	15	0.28	Astals et al., 2012
USA	<i>E. coli</i>	Desiccation in marine beach sand	6.0 ^e dry weight	2.0 ^e dry weight	7	0.57	Mika et al., 2009

^aMPN: Most probable number; ^bCFU: Colony forming units; quantification; ^cBDL: Below detection level; ^dNR: Not reported; ^elog₁₀ MPN/L

3.1.6 Tertiary treatment post-secondary

3.1.6.1 Lagooning

Lagoons are systems based on the treatment of wastewater in sealed ponds with micro-organisms or aquatic plants. The low cost and simplicity of their construction, operation, and maintenance has caused them to be considered one of the most important wastewater treatment technologies, especially for small cities and

towns, and in particular when the effluent is land-applied. Studies conducted with 186 ponds around the world (Von Sperling, 2005) showed median values of coliform removal efficiencies of 1.8 log₁₀ units (98% removal) for primary facultative ponds, 1.0 log₁₀ units for secondary facultative ponds (90% removal) and 1.2 log₁₀ units (94% removal) for each maturation pond in the series. However, efficiency will depend on retention times of several days, with short retention times, lagooning will only reach discrete reductions 31 % of coliforms in 10 hours in South Africa (Grabow et al., 1978) (Table 11).

Table 11. Reported *E. coli* and *Shigella* reductions from wastewater effluent by different tertiary treatments

Area	Microorganism	Treatment	Initial Concentration Log ₁₀ MPN ^a or Log ₁₀ CFU ^b /L	Final Concentration, Log ₁₀ MPN or Log ₁₀ CFU/g	Log ₁₀ Reduction	Reference
Costa Rica	Fecal coliforms	Subsurface flow Reedbeds	5.0 to 7.5 (CFU)	2.0 to 3.0 (CFU)	3.0 to 5.0	Dallas and Ho, 2005
Germany	<i>E. coli</i> (indicator)	Filtration sand filter	4.0 (CFU) (average)	2.0 (CFU) (average)	>1.6 to 2.2	Pfannes et al., 2015
Germany	<i>E. coli</i> (indicator)	Retention soil filter	7.0 (CFU)	4.0 (CFU)	2.7	Scheurer et al., 2015
Japan	Fecal coliforms and <i>E. coli</i>	Lime (amorphous silica and hydrated lime)	5.2 (CFU)	4.6 (CFU)	>3.0	Tanaka et al., 2014
South Africa	Fecal coliforms	Lagooning	NR	NR	0.2 in 10 h	Grabow et al., 1978
South Africa	Fecal coliforms	Coagulation+Lime	NR	NR	3.7	Grabow et al., 1978
South Africa	Fecal coliforms	Filtration sand filter	NR	NR	2	Grabow et al., 1978
UK	<i>E. coli</i>	Coagulation	Various concentrations ^c	Various concentrations	1.0 to 2.0	LeChevallier and Kwok-Keung, 2004
USA	<i>E. coli</i> O157	Dual and tri-medium filters ^d	NR	NR	1.0 to 2.0	LeChevallier and Kwok-Keung, 2004
USA	Fecal coliforms	Dual and tri-medium filters	>4.4 (MPN)	1.3 (MPN)	Up to 3.0	De Leon et al., 1986
USA	Pathogenic <i>E. coli</i>	Filtration membranes (ultrafiltration)	Various concentrations ^d	Various concentrations ^d	3.6 to 6.9 ^e	CDC
Various countries	Fecal coliforms	Lagooning	Various concentrations ^d	Various concentrations ^d	1.0 to 1.8	von Sperling, 2005

^aMPN: Most probable number; ^bCFU: Colony forming units; ^cinoculated in the sample; ^dDifferent studies; ^e: From raw water

^fpH 7 and 5.7;

The decay of coliforms (thermotolerant coliforms, or more specifically *E. coli*) in ponds is, from a practical point of view, accepted as being able to represent satisfactorily well the removal of pathogenic bacteria and, under many circumstances, viruses (Von Sperling, 2005). Modelling of the decay of coliforms in ponds is therefore important as a means of predicting the suitability of the effluent for reuse (agriculture or aquaculture) or discharge into water sources. The removal of coliforms is usually the controlling factor in the assessment of the quality of the pond effluent and its potential for further use or discharge, because require long detention times for the removal of coliforms to comply with WHO guidelines (Akin et al., 1989) for unrestricted irrigation (≤ 1000 MPN/100 ml).

A 90% decrease in cells of an outbreak strain of *E. coli* O157 within half a day in wastewater from dairy lagoons has been observed and attributed to the protozoa grazing (Ravva et al., 2010), since an increase in protozoa was observed when wastewater was reinoculated with *E. coli* cells.

3.1.6.2 Coagulation

Coagulation or flocculation and removal of the solid flocs eliminate bacteria bounded up within (Table 11). This method accelerates removal similar to the one occurring in activated sludge. Coagulation is commonly achieved by the addition of alum [$\text{Al}_2(\text{SO}_4)_3$], iron salts (FeCl_3), or polyelectrolytes. If additional treatments, such as lime are performed together, high removal rates can be achieved.

For example, a lime treatment that raises the pH at 11.2, followed by sedimentation with FeCl₃, can achieve a removal of 99.98% of coliforms (Grabow et al., 1978) (Table 11).

When properly performed, coagulation, flocculation and sedimentation can result in 1 to 2 log₁₀ removals of bacteria. However, performance of full-scale, conventional processes may be highly variable, depending on the degree of optimization. For example, the performance of treatment plants from various countries, average microbial removals for coagulation and sedimentation ranged from 0.13 to 0.58 log₁₀ for viruses, 0.17 to 0.88 log₁₀ for bacteria (total coliforms or fecal streptococci (LeChevallier and Kwok-Keung, 2004) (Table 11).

3.1.6.3 Filtration

Filtration, a physical process that removes microorganisms by means of a membrane or a sand filter. Filtration will remove fecal bacteria, including *E. coli* or *Shigella* with a good efficiency (Table 11).

A well-designed sand filter, is a cost-effective method that, with a sufficiently deep and a sufficiently low filtration rate, will remove a considerable proportion of fecal indicator bacteria. For instance, a slow sand filter, receiving 2 to 5 m³ per square meter per day of effluent removes around 3 log₁₀ of enteric bacteria (Grabow et al., 1978; Lalander et al., 2013). Removal increases at warm temperatures. *E. coli* is removed from 1.6 to 2.2 log₁₀ units using slow sand filters or different sand grain sizes, being those with smaller size the ones achieving higher reductions (Pfannes et al., 2015).

Similarly to sand filters, retention soil filters (RSF) remove 2 log₁₀ units of facultative pathogenic and antibiotic resistant *E. coli* from combined sewer systems that collect surface runoff as well as wastewater of industrial and domestic origin (Scheurer et al., 2015).

Construction of greywater filters using natural, locally available materials can lower the production and maintenance costs. A number of studies evaluating the use of natural and refuse materials in greywater filters. The removal efficiency of *E. coli* O157:H7 of pine, bark and activated charcoal filters at three organic loading rates showed to decrease drastically when the organic loading rate increased fivefold in the charcoal and sand filters, but increased by 2 log₁₀ in the bark filters (Lalander et al., 2013). Bark appeared as the most promising filtration approach in terms of pathogen removal.

3.1.6.3.1 Micro filtration, ultra filtration and reverse osmosis

Micro, ultra and reverse osmosis- thile microfiltration only shows moderate effectiveness in reducing Eacteria as *E. coli*, ultrafiltration, with a öore size of öore size of aööroximately 0.01 micron, has a very high effectiveness in removing acteria Efor examöle, *Campylobacter*, *Salmonella*, *Shigella*, *E. coli*) (CDC - Centers for Disease

Control); In general, devices based on filtration are able to remove bacterial contaminants regardless of their pathogenicity since physical removal is based on the size of the pathogen. Different filtration-based portable devices were able to reduce bacteria by 3.6 to 6.9 log₁₀ units from raw water. Those devices were based only on filtration through 0.2 to 0.4 mm diameter pore size filters or on reverse osmosis, being the last the most efficient for viral removal (Hörman et al., 2004).

Reverse osmosis system use a process that reverses the flow of water in a natural process of osmosis so that water passes from a more concentrated solution to a more dilute solution through a semi-permeable membrane. Pre- and post-filters are often incorporated along with the reverse osmosis membrane itself to improve bacterial removal (CDC - Centers for Disease Control).

3.1.6.3.2 Mono, dual and tri media

The terms "multilayer," "in-depth," or "mixed media" apply to a type of filter bed which is graded by size and density. Coarse, less dense particles are at the top of the filter bed, and fine, denser particles are at the bottom. Downflow filtration allows deep, uniform penetration by particulate matter and permits high filtration rates and long service runs. Because small particles at the bottom are also more dense (less space between particles), they remain at the bottom. Even after high-rate backwashing, the layers remain in their proper location in the mixed media filter bed. Water filtration through mono-media filters displaying a single layer of sand or anthracite, dual-medium (anthracite on top of sand) and tri-medium (anthracite, sand and garnet or magnetite). Previous studies showed that both dual and tri-media filter systems effectively reduced fecal coliforms numbers and even that the dual media filter outperformed the tri-media filter simply in terms of the total number of coliforms passing the filter barrier (De Leon et al., 1986). Dual or tri-medium filters achieved better *E. coli* O157 removal than just sand filtration, but no apparent differences in due to filter media configuration (dual or tri-medium filters), with differences more related to turbidity of the sample or filtration rate (Harrington, 2001).

3.1.6.4 Land treatment

The treatment of a primary or secondary effluent by application to land, with subsequent flow through the soil to underdrains or to groundwater, can be an effective method of removing bacteria (Desmarais et al., 2002). Moreover, land treatment and vegetation filter strips are among the best management practices commonly used to decrease the pollutant loads from agricultural fields and pastures, where animal manures have been applied.

Land treatment could be applied by three different methods, i) slow rate (SR), ii) rapid infiltration (RI) or iii) overland Flow (OF).

SR is the use of effluent for irrigation of crops, (land treatment) and is included in the US Environmental

Protection Agency's Process Design Manual for Land Treatment of Municipal Wastewaters (USEPA, 1977). *RI* or 'infiltration percolation' of effluent is similar to a soil-aquifer treatment. The EPA manual deals with *OF* as a wastewater treatment method in which the effluent is distributed over gently sloping grassland on fairly impermeable soils. Ideally, the wastewater moves evenly down the slope to collecting ditches at the bottom edge of the area and water-tolerant grasses are an essential component of the system. *OF* has been widely adopted in Australia, New Zealand and the UK for tertiary upgrading of secondary effluents, it has been used for the treatment of primary effluent in Werribee, Australia and is being considered for the treatment of raw sewage in Karachi, Pakistan (USEPA, 1977).

Suspended and colloidal organic materials in the wastewater are removed by sedimentation and filtration through surface grass and organic layers. Removal of total nitrogen and ammonia is inversely related to application rate, slope length and soil temperature. Overland flow systems also remove pathogens from sewage effluent at levels comparable with conventional secondary treatment systems, without chlorination. Passage through 2.5 m of sand at a rate of 0.2 m³/m²/day at 24°C reduced 5 log₁₀ units the values of fecal coliforms (Desmarais et al., 2002).

The survival of *E. coli* in general, and pathogenic strains in particular, would again vary depending on the temperature and pH, moisture, the type of soil as well as

the nutrients. Moreover, it will depend on the ability of the pathogen itself to compete with naturally occurring strains, that seems to be quite limited (Jamieson et al., 2002).

3.1.6.5 Other processes

A system similar to lagooning, but with some variations can be observed in the use of reedbeds using plastic (PET) bottle segments as an alternative low-cost media for the treatment of domestic greywater showed significant reductions either of BOD and fecal coliform (Dallas and Ho, 2005).

3.2 Disinfection as a tertiary (or post primary) treatment

3.2.1 Chlorine

As with water chlorination, the level of bacterial kill achieved in effluent chlorination increases with the dose, temperature, and contact time and as pH decreases. The additional factor of critical importance found that the chlorination of three secondary effluents is the chemical quality of the effluent being chlorinated, because free chlorine added rapidly combines with ammonia and organic compounds and disappears almost immediately. This chlorine has lower bactericidal potential than free chlorine. Chlorinating secondary effluents to use them for irrigation is a very common practice around the globe (Table 12).

Table 12. Reported *E. coli* and *Shigella* reductions by disinfection as a tertiary treatment

Area	Microorganism	Matrix	Treatment	Initial Concentration, Log ₁₀ CFU ^a /L	Log ₁₀ Reduction	Reference
Austria	Pathogenic <i>E. coli</i>	Water	Ultraviolet up to 30 mJ/cm ²	9.0	>6.0	Sommer et al., 2000
Singapore	<i>E. coli</i> O26	Wastewater	Ultraviolet at 13 mJ/cm ²	9.0	4.0	Tosa and Hirata, 1999
Spain	<i>E. coli</i> O157:H7	Diluted wastewater	Ultraviolet with a germicidal lamp	8.8	>5.8	Allué-Guardia et al., 2014
Spain	<i>E. coli</i> O157:H7	Wastewater	Chlorination in 3 minutes	7.9	>4.9	Allué-Guardia et al., 2014
Spain	Naturally occurring <i>E. coli</i>	Wastewater	Chlorination	8.0 ^b	3.6 to 4.0	Muniesa et al., 1999
USA	<i>E. coli</i> (indicator or O157)	Wastewater	Chlorination	8.8	>5.0	Rice et al., 1999; Durán et al., 2003
USA	<i>E. coli</i> O157:H7	Wastewater	Chlorination	7.0 ^c	>4.0	Chauret et al., 2008

^aCFU: Colony forming units using plate count as the quantification method;

^bonly study to have a final concentration reported 4.0 log₁₀; ^cMPN most probably number

Considering the naturally occurring microorganisms, the results of chlorinating a sewage effluent are very similar to those observed for drinking water, though it has to be considered that, in the sewage effluent, the chlorine combines very fast with ammonium and organic matter to give chloramines. Therefore, inactivation may be due to the combined effect of chlorine and monochloramine.

Chlorine inactivation have been widely studied, and many groups included *E. coli* O157:H7 and concluded that there is no difference in resistance to chlorine between pathogenic and non-pathogenic *E. coli* and concluded that the normal conditions used for water disinfection should be sufficient to inactivate pathogenic *E. coli* (Rice et al., 1999).

Therefore, a proper chlorination drastically reduces the bacterial content in water. For instance, 4 log₁₀ units of *E. coli* O157:H7 are removed by chlorine produced at a CT of 0.13 mg.min/l (Chauret et al., 2008). Chlorine at 10 ppm reduced *E. coli* O157:H7 >4.89 only after 3 minutes of contact (Allué-Guardia et al., 2014) in water and reduced from 3.5 to 4 log₁₀ units after 20 minutes when *E. coli* O157:H7 is spiked in sewage (Muniesa et al., 1999), similar to results of naturally occurring *E. coli* (3.6 to 4.4 log₁₀ units of reduction) in the same samples (Muniesa et al., 1999). Similarly, 1 minute of contact with free chlorine for 2 minutes is enough to reduce almost 5 log₁₀ units of *E. coli*, either O157:H7 or wild type *E. coli* (Rice et al., 1999). These inactivation rates are very similar to those shown with non-pathogenic *E. coli* (reduction of >5.6 log₁₀ units of *E. coli* spiked in mineral water after 3 minutes of treatment) and a reduction of 5.2 log₁₀ units of FC naturally occurring in a secondary effluent (Durán et al., 2003).

Chlorination of water supplies decreased rapidly the number of new cases after residents were ordered to boil water and after chlorination of the water supply in the waterborne outbreak of *E. coli* O157:H7 occurred in Missouri (Swerdlow et al., 1992). The failure of the chlorine disinfection equipment used for the Walkerton drinking water supply was identified as one of the major causes of these tragic outbreak occurred there (Holme, 2003). Similarly, Wang and Doyle (Wang and Doyle, 1998) showed that *E. coli* O157:H7 could survive for several weeks in autoclaved filtered municipal water, reservoir water and lake water. As expected, survival was greatest in cold water (8°C) than in warm water (25°C). Their results also indicate that this bacterium may enter a VBNC state in water.

3.2.2 Ultraviolet

The UV inactivation of microorganisms is based on the damage of their nucleic acids by UV ray. Some organisms have, however, mechanisms to repair the damage produced (Sommer et al., 2000) and one of these being photoreactivation. For drinking water, however, this is not a main concern since it use to be transported under dark conditions. However, this should be considered for treated sewage and irrigation water.

While high UV doses (e.g. 400 J/m²) show good inactivation of different strains regardless their photoreactivation abilities, (Sommer et al., 2000) pointed out that at lower UV doses there is a wide variation on the sensitivity of pathogenic *E. coli* compared with the non-pathogenic strains. These divergences seem to be related to their ability to repair the damage rather than to the serotype or other features (Sommer et al., 2000). Experimental UV treatment of a germicidal lamp reduced *E. coli* O157:H7 >4.89 log₁₀ units only after 1 minute of irradiation (Allué-Guardia et al., 2014). Whereas 6 log₁₀ units of inactivation was achieved for *E. coli* O157 at a irradiation of up to 30 J/m² (Sommer et al., 2000). Variation between strains is also shown in a study by Tosa and Hirata (Tosa and Hirata, 1999) where *E. coli* O157 showed a reduction of 6 log₁₀ units at a 33 J/m² while other strains *E. coli* O26, able to display photoreactivation, needed 130 J/m² to show a reduction of 4 log₁₀ units.

3.2.3 Natural processes

3.2.3.1 Natural sunlight

Natural sunlight inactivation has been evaluated in “*in situ*” inactivation experiments. Experiments with sewage and/or waste stabilization pond effluent mixed with river or sea water in a proportion of 10 % placed in a 560 mm depth (300 liters) open-top chambers (Sinton et al., 2002). There were remarkable differences in *E. coli* inactivation between winter (approx. 12 °C) and summer (approx. 16°C) time season. Nevertheless, natural sunlight cannot be evaluated alone, but in synergy with other factors, such as sunlight and temperature and salinity. Similarly, pathogenic *E. coli* O157:H7 was evaluated in mesocosm experiments in summer and winter seasons (Allué-Guardia et al., 2014) showing a decay of 5 and 7 log₁₀ units respectively in seven days. Salinity was not an influencing factor here and the differences were attributable to temperature and insolation.

Application of sunlight for reduction of pathogens is the rational for the SODIS technology to improve the microbiological quality of drinking water. SODIS uses solar radiation to inactivate pathogenic microorganisms, as described by professor Aftim Acra in the earlier 1980's (McGuigan et al., 2012). This technology cannot change the chemical quality of water, is not effective against chemical pollution, does not change the taste of water, is applicable at household level and is replicable with low investment costs. The SODIS technology is used by filling contaminated water into transparent plastic bottles and then exposing them to the sunlight. This method might be appropriate in rural communities with sunny climates when other disinfection methods are too expensive or not appropriate (McGuigan et al., 2012). Reduction of pathogenic *E. coli* O157 has been shown variable values, being some > of 5 log₁₀ units in 1.5 hours and a T90 of of 33.4 ± 3.7 mins (Boyle et al., 2008).

Reports on SODIS showed that the inactivation of *E. coli* during solar disinfection results from synergistic relations between the optical and thermal inactivation mechanisms when the water temperature exceeds 45°C (McGuigan et al., 1998). Furthermore, a complete inactivation of high populations of *E. coli* can be produced

in drinking water, even if high turbidity (200NTU), by exposing 1.5 l in plastic soft drink containers to strong-medium solar irradiances for periods of at least 7h. Bacteria in samples that achieve intermediate water temperatures can still be permanently inactivated if the water is of low turbidity and is exposed to irradiances of 70 mW/cm² for period of up to 7h (McGuigan et al., 1998).

Table 13. Persistent of *E. coli* and *Shigella* under natural processes

Area	Microorganism	Matrix	Treatment	Temperature, °C	Initial Concentration, Log ₁₀ CFU ^a /L	T90, Days	Reference
New-Zealand	<i>E. coli</i>	Wastewater	Natural inactivation in waste stabilization ponds	12 16	NR ^b	Summer: 0.38 Winter: 0.85	Sinton et al., 2002
New-Zealand	<i>E. coli</i>	Wastewater	Natural inactivation in raw sewage	12 16	NR	Summer: 0.14 Winter: 0.29	Sinton et al., 2002
Spain	<i>E. coli</i> O157	Untreated groundwater	Natural sunlight (SODIS) ^d	28 to 39	9	0.023 ± 0.003 ^c	Boyle et al., 2008
Spain	<i>E. coli</i> O157:H7	Water	Natural inactivation in a mesocosm	19.5 to 29 3.5 to 14.5	11	Summer: 0.33 Winter: 0.58	Allué-Guardia et al., 2014
Switzerland	<i>E. coli</i> K-12	Water	Natural sunlight (SODIS)	37	10	0.13	Berney et al., 2006
Switzerland	<i>S. flexneri</i>	Water	Natural sunlight (SODIS)	37	10	0.10	Berney et al., 2006

^aCFU: Colony forming units and quantification was plate count; ^bNR: Not reported; ^c> 5.0 log₁₀ reductions in 1.5 h; ^dSODIS: Solar disinfection under conditions of strong natural sunlight for 48 h

3.2.3.2 Solar beds (solarization)

There are several methods for reducing pathogen levels in sludge prior to land application, including sludge drying processes and lime stabilization. The technique of open air drying is used mainly on small wastewater treatment plants whenever sufficient inexpensive land is available and the local climate is favorable. This method reduces density of pathogenic bacteria by approximately 2 log₁₀ units when the ambient average daily temperature is above 0°C during 2 of the 3 months drying period (USEPA, 1999).

3.2.3.3 Dessication

Sustained moisture greatly affected *E. coli* survival in microcosm experiments performed in marine beach sand (Mika et al., 2009). Additional moisture decreased the decay rates of *E. coli* to less than one-half of the non-moistened decay rates.

Levels of *E. coli* in soil have been shown to decrease dramatically with distance from water (and decreasing water content) (Desmarais et al., 2002). Microcosm and field studies at a river showed that while desiccation would initially decrease *E. coli* levels, the subsequent addition of moisture would result in a regrowth of *E. coli* (Solo-Gabriele et al., 2000). These results indicate that although *E. coli* can be inactivated through desiccation, some cells can recover and regrow upon the addition of new moisture.

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