GLOBAL WATER PATHOGEN PROJECT
PART THREE. SPECIFIC EXCRETED PATHOGENS: ENVIRONMENTAL AND EPIDEMIOLOGY ASPECTS

POLIOVIRUSES AND OTHER ENTEROVIRUSES

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Summary

Enteroviruses (EVs), including poliovirus and nonpolio enteroviruses (i.e., coxsackieviruses, echoviruses, enteroviruses) are among the most common genera of the *Picornaviridae* family that infect humans and therefore are known to circulate widely in human populations throughout the world. New genera of the *Picornaviridae* family (i.e., Saffold cardiovirus [SAFV]) *Cosavirus* [common stool-associated picornavirus] *Salivirus* [stool Aichi-like virus]) have been identified in fecal specimens and wastewaters using conventional and highly-sensitive genomic sequencing technologies. In addition, members of the *Echovirus* genus originally known as echovirus 22 and 23 have been reclassified within the genus *Parechovirus. Likewise, previous* rhinovirus species have been reclassified within the *Enterovirus* genus. Enterovirus (EV) infections are a significant cause of morbidity and mortality throughout the world, primarily in infants and young children. Nevertheless, reliable worldwide estimates of EV-related mortality are not currently available.

Enteroviruses like most enteric viruses have evolved stability to adverse environmental conditions, including thermal stability, acid stability, resistant to radiation as well as to oxidants and proteolytic enzymes, which allow survival of these viruses in the environment and facilitate their transmission through multiple environmental routes (e.g., water, food, aerosols, and virus-contaminated inanimate objects or fomites). Human wastes, such as sewage and poorly treated effluents, urban stormwater and combined sewers overflows (CSO) are the primary source of enteroviruses released into aquatic and land environments that subsequently contaminate raw source waters for potable supply, bathing waters, shellfish waters, and waters used for irrigation of crops. Groundwater sources are also vulnerable to contamination with enteroviruses through different routes including direct injection of wastes through wells, percolation of sewage sprayed over the land, leaking or broken sewer lines, seepage from waste lagoons, infiltration of sewage-polluted surface streams, and septic tank effluents. The biophysical properties of enteroviruses (i.e., small-size, genome type and the non-enveloped capsid structure of the virion) play an important role on virus survival in the environment and on the physical removal and inactivation of virus particles through conventional wastewater treatment processes. Membrane bioreactor systems (MBRs) are becoming increasingly applied in developed regions as advanced wastewater treatment technologies to produce treated effluents of very high quality applicable to wastewater discharge and recycling solutions, including non-potable or indirect potable reuse. The distribution and persistence of enteroviruses in sewage-polluted waters may vary geographically depending on the epidemiological status of the population, population density, and the extent of sanitation coverage (i.e., wastewater treatment and wastewater disposal) which influence the viral load released into the environment. Recent estimates by the World Health Organization (WHO)/United Nations Children's Fund (UNICEF) Joint Monitoring Programme for Water Supply and Sanitation indicate that despite significant progress on sanitation, in 2012, more than one third of the global population - some 2.5 billion people – do not use an improved sanitation facility, and of these 1 billion people still practice open defecation. Sewage represents a useful matrix to derive information on circulating enteroviruses in given populations and to describe the enterovirus epidemiology associated with human disease, also known as environmental surveillance.

Environmental poliovirus surveillance is a major goal of the WHO Global Poliovirus Eradication Initiative (GPEI) used to monitor pathways of poliovirus transmission of wild poliovirus and circulating vaccine-derived polioviruses (cVDPVs). A major effort to develop new, improved, and safer live polio vaccine is being promoted by the WHO, Rotary and the Bill & Melinda Gates Foundation. Human enteroviruses have been recovered worldwide from surface waters including coastal waters, rivers streams, and lakes, from ground waters, wastewaters and finished drinking water. Numerous methodological approaches have been recently developed for concentration of human enteric viruses from water and their isolation from the environment. Selection of an appropriate filtration method for the primary concentration of viruses is crucial to successful virus detection. However, an efficient method to recover all viruses has not been developed yet. Integrated cell culture and PCR (ICC-PCR) developed in the late 1980s is still applicable for detection and identification of a suite of infective viral pathogens recovered from environmental samples. The detection of enteroviruses in sources of drinking water and recreational water bodies has been broadly accepted as a marker of a possible failure of the sanitation systems and as an indicator of the potential role of water in disease outbreaks. Moreover, since the natural host for all human enteroviruses is humans, the detection and quantification of amplifiable enterovirus genetic material in environmental waters has been proposed and successfully used as a water quality assessment tool for tracking sources of human fecal pollution.

Enteroviruses and new genera within the family Picornaviridae

1.0 Epidemiology of the Disease and Pathogen

Human EVs, like all viruses, are obligate intracellular parasites that are dependent upon living host cells for the essential elements of replication (Lees, 2000). EVs including poliovirus (PV) and non-polio enteroviruses (NPEVs) (i.e., coxsackieviruses, echoviruses, EVs) are among the most common genera of the *Picornaviridae* family that infect humans and therefore are known to circulate widely in human populations throughout the world (Pallansch et al., 2013). *Picornaviridae* is considered one of the oldest and most diversified virus families, presenting a non-enveloped, single, positive-stranded RNA (ssRNA) genome packed in a ~30 nm icosahedral capsid (Racaniello, 2013). This family includes many important enteric human and animal pathogens, such as poliovirus, hepatitis A virus,

and foot-and-mouth-disease virus (Pallansch et al., 2013; Racaniello, 2013; Tapparel et al., 2013). Most EVs and other genera of enteric picornaviruses (Hepatovirus, Parechovirus, Cardiovirus, Kobuvirus, Salivirus, and Cosavirus) replicate in the epithelial cells of the gastrointestinal tract and their progeny are in fecal matter excreted into the environment.

1.1 Global Burden of Disease

1.1.1 Global distribution

Although enterovirus infections are a significant cause of morbidity and mortality throughout the world, in particular for infants and young children, reliable worldwide estimates of EV-related mortality are not currently available. Since enterovirus infections are not nationally notifiable and health authorities in different countries admit that these infections are never treated or tested, the number of enterovirus detections is likely a considerable underestimate of the true burden of disease. In the developed world, enterovirus infections result in hundreds of thousands of hospitalizations per year, with aseptic meningitis accounting for the vast majority of these cases. Enteroviral infections are more common in most developing countries, so it is reasonable to assume that significant morbidity can be attributed to these viruses globally (Pallansch et al., 2013). The disease burden in the developing world of the tropics is poorly estimated, with

the exception of poliomyelitis. Indeed, the focus for the worldwide EV surveillance network is primarily on neurological presentation such as acute flaccid paralysis (AFP), to support poliovirus eradication programs. In general, national laboratories throughout the world report enterovirus typing results from clinical specimens to exclude poliovirus and establish the epidemiology of NPEVs. The World Health Organization (WHO) and the U.S Centers for Disease Control and Prevention (CDC) have guidelines for epidemiologic, clinical, and laboratory investigations of AFP to rule out poliovirus infection (WHO, 2004).

In the United States of America, the National Enterovirus Surveillance System (NESS) is a voluntary, passive surveillance system that has monitored trends in circulating EVs since 1961. Participating laboratories (state public health laboratories and local diagnostic laboratories willing to participate) report to CDC monthly summaries of enterovirus detections by virus type (i.e., serotype), with specimen type, collection date and demographic information. CDC summarizes the data and disseminates the results as shown in Table 1. Serotype-based EV surveillance provides a mechanism for determining patterns of enterovirus circulation and for identifying predominant serotypes for a given season. NPEVs are quite common, causing an estimated 10 – 15 million or more symptomatic infections per year in the US alone (Pallansch et al., 2013).

Table 1. Reports of selected enteroviruses infections and isolation, by sex and age of source patient, specimen type and collection month, and outcome (Data from the National Enterovirus Surveillance System, United States, 1993-2005)

| | Specimen Collection Month | | | | Sex | | Age Groups (yrs) | | | | | Specimen Type | | |
| | | | Outcome[a] | | | | % | | | | | Most Common Specimen | | |
Selected Serotypes[b]	June-Oct %	Number with Known Specimen Collection Month	%Fatal	Number with Known Outcome	% Male	Number with Known Sex	<1	1 to 4	5 to 19	≥20	Number with Known Age	Type	%	No. with Known Specimen Type
Coxsackievirus A16	68.3	252	1.1	92	54.4	259	28.3	41.3	16.1	14.3	223	Other	41.2	199
Coxsackievirus A24	97.9	48	NA[c]	0	56	25	3.6	3.6	39.3	53.6	28	Other	98	49
Coxsackievirus A9	80.9	1,176	1.7	346	56.8	1,257	39.4	16.6	26.5	17.5	1,126	CSF[d]	54.4	1,048
Coxsackievirus B1	81.1	673	0	104	52.5	676	67.9	11.2	10.1	10.8	651	Resp[e]	39.9	576
Coxsackievirus B2	79.1	1,188	3.8	263	59.1	1,293	63.4	14.2	11.4	11	1,157	CSF	39.2	1,089
Coxsackievirus B3	78.3	787	5.4	130	56.6	830	63.7	14.9	12.3	9.1	766	Resp	38.6	664
Coxsackievirus B4	81.7	861	9.8	123	56.2	937	62.5	15.5	9.6	12.4	865	CSF	41.1	755
Coxsackievirus B5	83.4	1,790	1.5	325	55.3	2,041	49.2	14.9	16.8	19.1	1,844	CSF	47.1	1,634
Echovirus 11	75.1	2,437	4.6	435	57.8	2,541	54.8	15.6	13.2	16.3	2,327	CSF	42.4	2,136
Echovirus 13	62.4	93	NA	1	59.2	397	44.9	11	31.5	12.6	390	CSF	54.1	392
Echovirus 18	79.7	661	1.8	57	57.2	934	41.4	12.6	27.6	18.3	927	CSF	54	752
Echovirus 3	79.7	182	NA	15	61.5	174	54.7	23.6	13.7	8.1	161	Resp	38.8	129
Echovirus 30	78.6	3,287	3.2	310	56.3	3,265	22.3	10.9	42.6	24.2	3,068	CSF	73.8	3,010
Echovirus 4	80	636	2.8	72	54.9	687	27.6	12.9	36.1	23.4	595	CSF	65	535
Echovirus 5	74.6	453	3.8	80	57.8	457	46.4	10.9	22.6	20.1	412	CSF	44.9	352
Echovirus 6	80.5	1,379	5.9	185	57.1	1,520	41.9	14.6	24.9	18.5	1,414	CSF	52	1,284
Echovirus 7	76.5	1,214	2.5	203	57.2	1,139	57	17	14.1	11.9	1,055	CSF	44.2	952

Echovirus 9	75.5	2,810	0.2	405	57.9	2,937	29.9	13.5	36.9	19.7	2,653	CSF	67.5	2,627
Enterovirus 68	50	24	NA	1	59.1	22	29.4	35.3	11.8	23.5	17	Resp	95.7	23
Enterovirus 71	70.5	237	NA	15	58.7	223	42.1	30.7	11.9	15.3	202	Resp	40.2	174
Human Parechovirus 1	62.9	474	10.9	92	58.6	495	73	22.6	2	2.4	456	Resp	45.2	321
Human Parechovirus 2	54.5	33	NA	8	55.9	34	67.6	20.6	0	11.8	34	Resp	50	18

[a]Outcome information was available for 1983 to 1998; [b]Most common serotypes shown in bold; [c]Not available <50 observations or outcome information not available; [d]CSF: cerebrospinal fluid; Resp: respiratory specimens; [e]Resp: respiratory specimens.

Adapted from (Khetsuriani et al., 2006)

The WHO maintains an integrated global alert and response system for epidemics and other public health emergencies based on strong national public health systems and capacity and an effective international system for coordinated response (www.who.int). Although enterovirus-associated fatalities involving poliomyelitis have been more consistently investigated, WHO continues to track the evolving infectious disease situation for other enterovirus diseases including acute flaccid paralysis or "poliomyelitis-like" infections associated with NPEVs. Among these newer enterovirus infections are those associated with enterovirus-71 (EV-71) and enterovirus D68 (EV-D68). EV-71 infection induces mild symptoms (e.g., hand-foot-mouth disease [HFMD] herpangina, or fever) in young children that can also lead to severe neurological and systemic complications, including meningitis, encephalitis, and central nervous system (CNS) involvement with cardiopulmonary failure. EV-71 is emerging as the most important virulent neurotropic EV during the poliomyelitis eradication period and countries of the Asian Pacific Rim in particular have been affected by large outbreaks of EV-71-associated HFMD with severe neurologic complications or systemic disease as described in Tables 2 and 3 (Cardosa et al., 2003; Huang et al., 2014; Pallansch et al., 2013; Ryu et al., 2010). The transmission of EV-71 infections in other geographical areas is poorly understood.

Table 2. Demographic factors and clinical outcomes of Enterovirus (EV)71 – infected patients (from metropolitan areas of Taipei, Taichun, and Kaohsiun in Taiwan between May 2011 and November 2012)

Demography/Outcome	EV[a]71 Severity				p[c]
	Uncomplicated (n = 153)	Meningitis/Myoclonic Jerk (n = 193)	Encephalitis (n = 35)	CNS[b] Involvement with PE (n = 18)	
Mean age ±SD[d] (years)	3.29 ±2.64	3.32 ±2.62	2.23 ±3.87	2.49 ±0.77	0.25
Sex					
Male	93 (60%)	114 (59%)	21 (60%)	12 (67%)	0.94
Female	61 (40%)	79 (41%)	14 (40%)	6 (33%)	
Age					
› 2 y	107 (70%)	107 (55%)	21 (60%)	10 (56%)	0.05
≤2 y	46 (30%)	86 (45%)	14 (40%)	8 (44%)	
Prerequisite illness					
Preterm birth	8 (5%)	19 (10%)	4 (11%)	2 (11%)	0.37
Developmental delay	3 (2%)	6 (3%)	0 (0%)	3 (17%)	0.004
Previous admission due to EV	15 (10%)	29 (15%)	2 (6%)	0 (0%)	0.10
Interval between onset and hospital visit					
0 to 2 days	69 (45%)	102 (53%)	4 (11%)	1 (6%)	<0.001
3 to 8 days	84 (55%)	91 (47%)	31 (89%)	17 (94%)	
Clinical outcome					
Full recovery	53 (100%)	193 (100%)	34 (97%)	12 (67%)	<0.001
Morbidity	0 (0%)	0 (0%)	1 (3%)	4 (22%)	
Fatality	0 (0%)	0 (0%)	0 (0%)	2 (11%)	

[a]EV: Enterovirus; [b]CNS: Central nervous system; [c]*p* value denoting statistical significance from the analysis of the clinical outcomes of all participants and the associations among disease severity and the possible predisposing factor; [d]SD: Standard deviation.

Adapted from: (Huang et al., 2014)

Table 3. Summary of Enterovirus 71 outbreaks in Asia (1998 – 2009)

Outbreak Location, Year	HFMD[a]		Complicated HFMD[a]		
	Number of Patients	Number of Patients with EV71	Number of Patients	Number of Patients with EV71	Genotypes Detected
Brunei, Southeast Asia, 2006	≥100	34	NR[b]	NR	B4, B5
Fuyang, PRC[c] 2008	151	59	112	42	C4a
Shandong, PRC, 2007	105	55	11	6	C4a
Sarawak, Malaysia, 2000 to 2004	773	277	102	56	B4, C1, B5
South Korea, 2009	519	168	112	92	C4a, C1, C5
Taiwan, 1998	NR	NR	405	78	C2

[a]HFMD: Hand-foot-and-mouth-disease; [b]NR: Not reported; [c]PRC: People's Republic of China.

Countries of the Asia Pacific Rim affected by large outbreaks of EV71-associated hand-foot-and-mouth disease (HFMD). Best documented outbreaks with prevalence of neurologic complications of EV71 infection among outbreaks assumed to have been driven by differences of genotypes and co-infection with other viruses, such as a newly characterized adenovirus.

Adapted from (Ryu et al., 2010)

EV-D68 infection has been associated with acute respiratory illness of differing severities ranging from mild upper respiratory tract infections to severe pneumonia including fatal cases in pediatric and adult patients (Imamura and Oshitani, 2015; Oberste et al., 2004). EV-D68 has been recognized as a reemerging pathogen due to its high frequency of detection in different parts of the world since 2008 – 2009 (Table 4). In the United States, the National Enterovirus Surveillance System received 79 EV-D68 reports during 2009 – 2013 (Midgley et al., 2014).

Cases of respiratory syndrome with neurological complications, including acute flaccid myelitis, have been reported in the USA, Canada, Norway, and France (Khan, 2015; Lang et al., 2014; Milhano et al., 2015). However recent investigations indicate that EV-D68 infection is most commonly associated with respiratory illness and no definitive connection between EV-D68 and acute flaccid myelitis has been established (http://www.cdc.gov/ncird/investigation/viral/2014-15/investigation.html).

Table 4. Human enterovirus D68 cases, by cluster location and age group in Asia, Europe, and United States (2008 – 2010)

Area	Number of Cases Overall	Age Groups (Years)			
		0 to 4	5 to 9	10 to 19	≥20
Japan	11	10	1	0	NR[a]
Netherlands	24	11	1	0	12
Philippines	21	17	2	2	NR
USA (Arizona)	5	1	2	2	NR
USA (Georgia)	6	0	0	0	6
USA (Pennsylvania)	28	15	5	8	NR
Total	95	54	11	12	18

In temperate regions of the world, the majority of EV infections (70 to 80%) occur in summer and fall, whereas in the tropics, EV infections occur year-round or with increased incidence during the rainy season. Unexplained cycles of infection (i.e., 2, 3, 4 or 5 years) of some human EVs have been identified (Pallansch et al., 2013; Stellrecht et al., 2010).

1.1.2 Symptomology

Table 5 shows Picornavirus genera and enterovirus species associated with disease in humans. While most enterovirus infections are asymptomatic and mild febrile illness (e.g., respiratory illness and febrile rash illness) has been recognized as the most common symptomatic manifestation of infection, EVs are also associated with severe neurological illness, such as aseptic meningitis and encephalitis. Aseptic meningitis seems to be the most common severe clinical manifestation associated with enterovirus infection in children and adults, especially in developed countries (Pallansch et al., 2013; Stellrecht et al., 2010; Tapparel et al., 2013). PVs are perhaps the most studied and characterized of the EVs, especially given the clinical consequences of infection which can infect motor neurons of the anterior horn of the spinal cord and lead to paralytic poliomyelitis in humans (Rhoades et al., 2011). Infants infected with Coxsackievirus (CV) have been shown to be extremely susceptible to myocarditis, meningitis and encephalitis with a subsequent mortality rate as high as 10% (Rhoades et al., 2011). EV-71 is a major public health issue across Asia and of increasing concern globally, causing HFMD with potential neurological complications as previously discussed (Rhoades et al., 2011). Echoviruses are highly infectious and preferentially target infants and young children. These viruses can cause a mild, nonspecific illness similar to that of CVs. Other clinical syndromes associated with NPEV infections include acute hemorrhagic conjunctivitis caused by multiple EV serotypes such as coxsackievirus type A24 variant (CVA24), enterovirus type 70 (EV-70), and E-71; coxsackieviral myocarditis (i.e., inflammation of the myocardium) caused by coxsackievirus group B (CVB); enteroviral respiratory infections caused by Coxsackievirus group A (CVA) and CVB serotypes as well as echovirus types 6, 9, 11, 16, 17, 22, and 25; acute gastroenteritis caused by echovirus types 1, 7, 11, 13, 14, 30 and 33. Consequently, no enteroviral disease is uniquely associated with any specific serotype, and no serotype is uniquely associated with any one disease (Pallansch et al., 2013; Tapparel et al., 2013).

Table 5. Picornavirus genera and enterovirus species associated with diseases in humans (Over 111 enterovirus genotypes discovered and most reported in sewage)

Genus	Species	Genotypes	Associated Disease	Strains
Enterovirus	*Human enterovirus A* (EV-A)	*Coxsackievirus* (CV) *Enterovirus* (EV)	Encephalitis	CV-A2, CV-A3, CV-A4, CV-A5, CV-A6, CV-A7, CV-A8, CV-A16, EV-A71
			Paralysis or myelitis	CV-A2, CV-A4, CV-A5, CV-A7, CV-A8, CV-A16, EV-A71, EV-A76, EV-A89, EV-A90, EV-A91
			Meningitis	CV-A2, CV-A3, CV-A4, CV-A5, CV-A6, CV-A7, CV-A8, CV-A10, CV-A16, EV-A71
			Hand-foot-and-mouth disease	CV-A4, CV-A5, CV-A6, CV-A7, CV-A10, CV-A12, CV-A16, EV-A71
			Upper and lower respiratory disease	CV-A10, CV-A16, EV-A71
			Pleurodynia	CV-A2, CV-A4, CV-A6, CV-A10, CV-A16
			Herpangina	CV-A2, CV-A3, CV-A4, CV-A5, CV-A6, CV-A7, CV-A8, CV-A10, CV-A16, EV-A71
			Gastroenteritis	EV-A76, EV-A71, EV-A89, EV-A90
			Myopericarditis	CV-A16, EV-A71
			Pancreatitis	EV-A71
			Type I diabetes	EV-A71
			Encephalitis	CV-A9, CV-B1, CV-B2, CV-B3, CV-B4, CV-B5, E-3, E-4, E-5, E-6, E-7, E-9, E-11, E-13, E-14, E-16, E-17, E-18, E-19, E-24 E- 25, E-27, E-30, E-33
			Paralysis or myelitis	CV-A9, CV-B1, CV-B2, CV-B3, CV-B4, CV-B5, CV-B6, E-1, E-2, E-3, E-4, E-5, E-6, E-7, E-9, E-11, E-12, E-13, E-14, E-16, E-18, E-20, E-21, E-25, E-27, E-29, E-30, E-33, EV-B75, EV-B77, EV-B81, EV-B85, EV-B86, EV-B87, EV-B88, EV-B93, EV- B97, EV-B100

Genus	Species	Genotypes	Associated Disease	Strains
	Human enterovirus B (EV-B)	*Coxsackievirus Echovirus* (E) *Enterovirus*	Meningitis	CV-A9, CV-B1, CV-B2, CV-B3, CV-B4, CV-B5, CV-B6, E-1, E-2, E-3, E-4, E-5, E-6, E-7, E-9, E-11, E-12, E-13, E-14, E-15, E- 16, E-17, E-18, E-19, E-20, E-21, E-24, E-25, E-27, E-29, E-30, E-31, E-32, E-33
			Hand-foot-and-moth disease	CV-A9, CV-B1, CV-B2, CV-B3, CV-B5, E-4, E-9, E-11, E-19, EV-B84
			Upper and lower respiratory diseases	CV-A9, CV-B1, CV-B2, CV-B3, CV-B4, CV-B5, CV-B6, E-1, E-2, E-3, E-4, E-5, E-6, E-7, E-9, E-11, E-12, E-13, E-14, E-15, E- 16, E-17, E-18, E-19, E-20, E-21, E-25, E-29, E-30
			Pleurodynia	CV-A9, CV-B1, CV-B2, CV-B3, CV-B4, CV-B5, CV-B6, E-1, E-2, E-3, E-6, E-7, E-9, E-11, E-12, E-14, E-16, E-17, E-18, E-19, E-24, E-25, E-30
			Herpangina	CV-A9, CV-B1, CV-B2, CV-B3, CV-B4, CV-B5, E-3, E-6, E-9, E-11, E-16, E-17, E-25
			Conjunctivitis	E-7
			Gastroenteritis	CV-A9, CV-B2, CV-B3, CV-B4, CV-B6, E-1, E-6, E-7, E-11, E-13; E-14, E-17, E-18, E-19, E-21, E-24, E-25, E-30, E-32, EV- B84
			Myopericarditis	CV-A9, CV-B1, CV-B2, CV-B3, CV-B4, CV-B5, CV-B6, E-1, E-2, E-3, E-6, E-7, E-9, E-11, E-13, E-16
			Pancreatitis	CV-B1, CV-B2, CV-B3, CV-B4, CV-B5, E-6, E-11, E-30
			Hepatitis	CV-A9, CV-B1, CV-B2, CV-B3, CV-B4, CV-B5, E-3, E-6, E-7, E-9, E-11, E-14, E-17, E-19, E-20, E-25, E-30
			Type I diabetes	CV-A9, CV-B1, CV-B2, CV-B3, CV-B4, CV-B5, CV-B6, E-3, E-4, E-5, E-6, E-9, E-11, E-16, E-18, E-24, E-25, E-30
	Human enterovirus C (EV-C)	*Poliovirus* (PV) *Coxsackievirus Enterovirus*	Encephalitis	CV-A11, CV-A13, PV-1, PV-2, PV-3
			Paralysis or myelitis	CV-A1, CV-A11, CV-A13, CV-A17, CV-A20, CV-A21, CV-A24, EV-C96, EV-C109, PV-1, PV-2, PV-3
			Meningitis	CV-A11, CV-A13, CV-A17, CV-A19, CV-A22, CV-A24, PV-1, PV-2, PV-3
			Upper and lower respiratory diseases	CV-A21, CV-A24, EV-C104, EV-C109, EV-C117, EC-C118, PV-3
			Pleurodynia	CV-A1
			Herpangina	CV-A1, CV-A19, CV-A22
			Conjunctivitis	CV-A24
			Gastroenteritis	CV-A1, CV-A13, CV-A17, CV-A19, CV-A21, CV-A22
			Myopericarditis	PV-1, PV-3
			Hepatitis	CV-A20
			Type I diabetes	CV-A21, CV-A24, PV-1, PV-2, PV-3
	Human enterovirus D (EV-D)		Encephalitis	EV-D68, EV-D70
			Paralysis or myelitis	EV-D68, EV-D70, EV-D94
			Meningitis	EV-D68, EV-D70
			Upper and lower respiratory disease	EV-D68
			Conjunctivitis	EV-D70
			Type I diabetes	EV-D70
Cardiovirus (Saffold virus, SAFV)	*Cardiovirus*	*Cardiovirus A Cardiovirus B Cardiovirus C*	Potential association with gastrointestinal and respiratory tract infections, and neurological disorders	
Hepatovirus	*Hepatovirus A*	Hepatitis A virus	Enteric hepatitis	
Kobuvirus	*Aichi virus*	*Aichi virus A Aichi virus B Aichi virus C*	Gastroenteritis	
Parechovirus	*Parechovirus A Parechovirus B*	16 HPeV types	Potential association with gastrointestinal, respiratory and central nervous system (CNS)-related manifestations (i.e, encephalitis)	
Cosavirus	*Cosavirus A*	*Cosavirus A*	Potential association with non-polio acute flaccid paralysis and acute diarrhea	
Salivirus	*Salivirus A*	*Salivirus A*	Potential association with gastroenteritis	

Modified from Pallansch et al.(Pallansch et al., 2013) and Tapparel et al. (Tapparel et al., 2013), with the classification of Cardiovirus, Salivirus, Kobuvirus based on Virus Taxonomy: 2014 Release (International Committee on Taxonomy of Viruses) http://www.ictvonline.org/taxonomyReleases.asp Last accessed Feb. 2016.

1.2 Taxonomic Classification

1.2.1 Physical description of the agent

EVs, like other members of the family *Picornaviridae*, are non-enveloped icosahedral particles (i.e., virions) ~ 30 nm in diameter. Virions consist of a protein shell (capsid) surrounding the naked single-stranded positive (message)-sense RNA genome (Racaniello, 2013; Stellrecht et al., 2010). EVs are distinguished from other picornaviruses on the basis of physical properties, including buoyant density in cesium chloride and acid stability (Pallansch et al., 2013). EVs and cardioviruses have a buoyant density of 1.34 g/mL, while parechoviruses (HPeVs) and rhinoviruses (HRVs) have an intermediate value of 1.36 g/mL and 1.40 g/mL, respectively and such differences lie in the permeability of the viral capsid to cesium (Racaniello, 2013). EVs (except HRVs), hepatoviruses, and HPeVs are acid stable and retain infectivity at pH values of 3.0 and lower. In contrast, HRVs are labile at pH values of less than 6.0. Differences in pH stability influence the sites of replication of the virus, for instance, HRVs replicate in the respiratory tract and need not be acid stable while cardioviruses, EVs, hepatoviruses, and HPeVs pass through the stomach to gain access to the intestine and, therefore, must be resistant to low pH (Racaniello, 2013).

EVs are relatively resistant to many common laboratory disinfectants, including 70% ethanol, isopropanol, dilute Lysol, and quaternary ammonium compounds. The virus is insensitive to lipid solvents, including ether and chloroform, and it is stable in many nonionic detergents at ambient temperature. Formaldehyde, glutaraldehyde, strong acid, sodium hypochlorite, and free residual chlorine inactivate EVs (Pallansch et al., 2013; Stellrecht et al., 2010)). Chlorine disinfection is a critical step for inactivation of viruses in water and wastewater treatment processes Ideal conditions for virus inactivation are high chlorine residual, long contact time, high water temperature, low pH, low turbidity, and an absence of interfering substances (DEP, 2011)(see Section III).

In the environment, EV virions are relatively thermostable, but less so than hepatitis A virus that is distinguished from other picornaviruses by its high thermal stability (Anderson and Counihan, 2011; Pallansch et al., 2013). EVs, like other picornaviruses, have evolved thermal stability (stable at 42°C or up to 50°C in the presence of sulfhydryl reducing agents and magnesium cations) and pH stability (pH 3-9) in the extracellular environment, which is the result of the assembly of virus particles of all picornaviruses (Gerba et al., 2007; Hurst and Adcock, 2000; Hurst and Murphy, 1996; National Research Council, 2004; Racaniello, 2013). Indeed, the biophysical properties of picornaviruses such as their small-size (30 nm), genome type (ssRNA), and non-enveloped protein capsid structure of the virion are known to play an important role on the mechanisms of virus survival and transport in the environment (Bosch et al., 2006; Fong and Lipp, 2005;

Gerba et al., 2013; National Research Council, 2004; Sobsey and Meschke, 2003; Wigginton and Kohn, 2012; Xagoraraki et al., 2014). In particular, the electrostatic and hydrophobic interactions involving functional groups of the viral capsid (i.e., charged amino acids residues) are essential for capsid assembly, capsid stability, and virus particle adsorption to solid surfaces (e.g., suspended solids, sediments and environmental surfaces or fomites) (Mateu, 2011; Michen and Graule, 2010). Studies have suggested that picornaviral capsids are also stabilized by interactions with the RNA genome (Pallansch et al., 2013). Additional studies conducted with PV strains (serotype 1 virulent Mahoney and serotype 1 attenuated Sabin) have indicated that viral binding to bacteria or bacterial polysaccharides offers a selective advantage by enhancing environmental stability of the virions (Robinson et al., 2014).

The capsids of EVs and other picornaviruses are composed of four viral structural proteins (i.e., VP1, VP2, VP3, and VP4) arranged in 60 repeating protomeric units (protomers) that confer an icosahedral shape to the virion. The shell is formed by VP1 to VP3, and VP4 lies on its inner surface. VP1, VP2, and VP3 comprise the surface of the virion with a similar topology forming an eight-stranded, antiparallel β-barrel core structure. Packing of the β-barrel domains is strengthened by a network of protein-protein contacts on the interior of the capsid, particularly around the fivefold axes of symmetry. This network, which is formed by the N-terminal extensions of VP1 to VP3 and VP4, is essential for the stability of the virion (Racaniello, 2013). Similarly, a network formed by the N-termini on the interior of the capsid contributes significantly to the stability of the virions. Furthermore, studies have suggested that picornaviral capsids are stabilized by interactions with the ssRNA genome. The positive strand ssRNA genome of EVs and all picornaviruses serves as a template for both viral protein translation and RNA replication. In fact, the viral ssRNA is infectious because it is translated on entry into the cell to produce all the viral proteins required for viral replication (Pallansch et al., 2013; Stellrecht et al., 2010).

1.2.2 Taxonomy and diversity in the *Picornaviridae*

Enterovirus corresponds to the genus within the *Picornaviridae* family. This family belongs to the order *Picornavirales* and comprises 12 genera, which all contain viruses that infect vertebrates (i.e., Apthovirus, Avihepatovirus, Cardiovirus, Enterovirus, Erbovirus, Hepatovirus, Kobuvirus, Parechovirus, Sapelovirus, Senecavirus, Tremovirus, Teschovirus) (Racaniello, 2013). Picornavirus genera and enterovirus serotypes infecting humans are presented in Table 5.

Members of the Echovirus genus originally known as echovirus 22 and 23 have been reclassified within the genus Parechovirus based on distinctive biological and molecular properties. To date, 16 genotypes of human parechoviruses (HPeVs) have been identified (Harvala and

Simmonds, 2009). Likewise, the former Rhinovirus genus has been reclassified within the Enterovirus genus based on its genome organization and particle structure (Tapparel et al., 2013).

With respect to EVs, the current taxonomic classification scheme (Virus Taxonomy: 2014 Release (International Committee on Taxonomy of Viruses) http://www.ictvonline.org/taxonomyReleases.asp last accessed Feb. 2016) based on genome organization and biological properties of viruses plus high-throughput EV genome sequencing and bioinformatics analysis, divides human EVs into four species: Enterovirus A, B, C, and D. The classification scheme keeps traditional names for individual serotypes (i.e., coxsackievirus, echovirus, enterovirus, poliovirus). The following species designation criteria based on molecular and biological features have been established: HEV species share amino acid percent identities from 60% to greater than 70% within specific genomic coding regions, share a limited range of host cell receptors and a limited natural host range; have a G + C genome base composition which varies by no more than 2.5%; and share a significant degree of compatibility in proteolytic processing, replication, encapsidation, and genetic recombination.

Approximately 107 serotypes/genotypes of NPEVs have been identified based on a threshold of 25% nucleotide divergence in the VP1 coding region (Pallansch et al., 2013). The serotype of an unknown isolate is inferred by comparison of the VP1 sequence with a database containing VP1 sequences for the prototype and variant strains of all human EV serotypes according to the following suggested guidelines:

  i. A partial or complete VP1 nucleotide sequence identity of 75% (>85% amino acid) identity between a clinical EV isolate and serotype prototype strain is used to establish the serotype of the isolate, on the provision that the second highest score is <70%
  ii. A best-match nucleotide sequence identity of <70% may indicate that the isolate represents an unknown serotype,
  iii. A sequence identity between 70% and 75% indicates that further characterization is required before the isolate can be firmly identified.

VP1 nucleotide sequence comparison can be used to rapidly determine whether viruses isolated during an outbreak are epidemiologically related (Manor et al., 2007; Pallansch et al., 2013; Shulman et al., 2012; Shulman et al., 2015).

New genera of the Picornaviridae family (i.e., Cardiovirus also referred to as Saffold cardiovirus or SAFV, Cosavirus , a common stool-associated picornavirus, and Salivirus, a stool Aichi-like virus) have been identified in fecal specimens and in sewage using conventional and highly-sensitive genome sequencing technologies (Greninger et al., 2009; Jones et al., 2007; Kapoor et al., 2008; Tapparel et al., 2013). Although these viruses have been associated with disease in humans, they do not appear to be a major cause of human illness; therefore, their clinical significance remains to be fully elucidated.

It is known that EV activity in populations can be either sporadic or epidemic, and that certain EV types may be associated with both sporadic and epidemic disease occurrences. The patterns of virus shedding and routes of transmission for EVs are consistent with few exceptions. The largest amount and duration of virus shedding occurs upon primary infection with a given serotype and most primary infections occur during childhood. Consequently, young children are possibly the most important transmitters of EV within households (Pallansch et al., 2013).

1.3 Transmission

1.3.1 Routes of transmission

Transmission of human EVs to susceptible individuals occurs via fecal – oral, oral – oral and nasopharyngeal routes (respiratory droplets), and via fomites. Most EVs enter primarily by the fecal – oral route and replicate in the gastrointestinal tract, including the oropharyngeal and intestinal mucosa. Following replication, EVs may cause both localized and systemic infections, affecting many organ systems in patients of all ages. Some EVs have a restricted respiratory tropism (e.g. HRVs) while others can invade the CNS and induce neurological disease (Pallansch et al., 2013; Rhoades et al., 2011; Tapparel et al., 2013). It is well documented that the fecal – oral pathway of EV transmission predominates in areas with poor sanitary conditions, while respiratory transmission predominates in more developed areas. Consequently, the relative importance of the different modes of transmission probably varies with the particular EV and environmental settings. HRVs are traditionally associated with upper respiratory tract infection, otitis media, and sinusitis, and therefore are predominantly transmitted from person to person via direct contact or through a fomite or small or large particle aerosol (Jacobs et al., 2013).

Evidence of more specific modes of enterovirus transmission by the fecal – oral route via environmental contamination (e.g., contaminated food [shellfish] and water sources) are described in detail in specific sections of this chapter. Since EVs are enteric viruses, water-related transmission remains of primary importance for indirect human enterovirus transmission.

1.3.2 Reservoirs and fecal shedding

Contaminated water and some food sources may serve as reservoirs of the environmentally-stable EV virions, but humans are thought to make up the only important natural reservoir for replication of human EVs (Gerba et al., 2013; Pallansch et al., 2013). Most EVs, excluding Enterovirus 70 (EV70) coxsackievirus A24 (CVA24) are abundant in stool specimens of infected individuals and levels of excretion of PVs are known to reach maximal amounts of 10^6 infectious virons per gram of feces (Dowdle et al., 2002; Hovi et al., 2007). Less information is available for levels of excretion of other enterovirus genotypes; however, the consensus from previous and current references indicates that

humans excrete between 10^6 and 10^7 EVs per gram of feces (Feachem et al., 1983b; Gerba, 2000; Melnick and Rennick, 1980). The duration of intestinal virus shedding and thus the spreading (i.e., transmission) of a particular enterovirus may vary among the different genotypes. For example, longer periods of excretion have been documented for PVs and CVs (Pallansch et al., 2013). Additional studies have indicated that EV excretion may persist for up to 11 weeks after an EV infection (Chung et al., 2001). Furthermore, long-term PV excretion ranging from 10 months to 28 years has been documented for individuals with immunodeficiencies (Dunn et al., 2015; Kew et al., 1995).

1.4 Population and Individual Control Measures

1.4.1 Poliomyelitis vaccination and eradication update

Following the eradication of smallpox, paralytic poliomyelitis (i.e., AFP) caused by wild polioviruses (WPV, serotypes 1, 2 and 3) became a major focus of public health activity. Most childhood EV and PV infections are asymptomatic. Surprisingly, on average only about 1 in 200 to 1000 poliovirus infections in fully susceptible individuals results in paralytic disease and drops to 1 in a few millions for vaccinated individuals. The most common symptomatic disease caused by PVs is a mild febrile illness with or without gastrointestinal signs (i.e., abortive poliomyelitis) that occurs in 4% to 8% of individuals. Less frequently infection may lead to non-paralytic illness characterized by the typical features of viral meningitis. (Pallansch et al., 2013). Person-to-person WPV transmission can occur in the absence of the relatively rare symptomatic infections especially in highly vaccinated populations (Shulman et al., 2014).

In 1988 the World Health Assembly adopted the Global Polio Eradication Initiative (GPEI) to eradicate all three serotypes of poliovirus. This effort has involved both health professionals and more than ten million volunteers in all countries (WHO, 2014). Success of any eradication program depends on an effective intervention strategy and sensitive and easily performed surveillance systems able to detect the pathogen. For polio, intervention was based on the availability of an oral polio vaccine (OPV) consisting of live attenuated poliovirus strains and an inactivated polio vaccine (IPV) containing inactivated neurovirulent vaccine strains. Each vaccine type has advantage and disadvantages (see reviews by (Plotkin and Vidor, 2008; Shulman, 2012; Sutter et al., 2008). Both OPV and IPV were trivalent to raise antibodies against the three non-cross-reacting poliovirus serotypes. The gold standard for PV surveillance is based on investigation of the cause of all cases of AFP in children ≤15 years of old to determine whether or not the AFP was caused by poliovirus (Pallansch et al., 2013). Poliovirus is considered to be absent when the number of AFP cases investigated is 1 per 100,000 children ≤15 of age (the rate for all non-PV causes of AFP) and none were due to PV. A supplementary or alternative approach is to conduct environmental surveillance (ENVS) of sewage and other wastewaters, to test whether PVs are present in samples collected from these sources. The advantage of ENVS is that it can detect PV circulation in the absence of clinical cases since virus is excreted into sewage during

asymptomatic infections as well as symptomatic infections. Moreover, results can be semi quantitative as well as qualitative (Hovi et al., 2012; Hovi et al., 2001; Lodder et al., 2012; Manor et al., 2014).

A review of publications between 1935 and 1995 on excretion of polioviruses by Alexander and associates (Alexander et al., 1997) indicated that in most infections of naïve children, wild polioviruses were excreted for 3 – 4 weeks with a mean rate of 45% at 28 days, and 25% of the cases were still excreting during the sixth week. In contrast, fewer than 20% excreted vaccine strains after 5 weeks. Excretion of polio ranged from a few days to several months (Minor, 1992). The highest probability of detecting poliovirus positive stool samples was reported to be at 14 days after the onset of paralysis (Alexander et al., 1997; Dowdle and Birmingham, 1997) and is the basis of stool sample collection for diagnosis of polio by AFP surveillance (WHO, 2004). The Global Polio Laboratory Network of laboratories accredited by the WHO provides the surveillance arm necessary for successful eradication. Standard laboratory procedures were adopted for AFP) surveillance (WHO, 2004).

The GPEI has made significant advances towards eradication of poliomyelitis. To date, nearly 80% of the world's population lives in areas certified as polio-free. The last reported infection with WPV2 was reported in 1999 (MMWR, 2001) and type two WPV was declared eradicated by the WHO on September 20, 2015 http://www.polioeradication.org/mediaroom/newsstories/Global-eradication-of-wild-poliovirus-type-2-declared/tabid/526/news/1289/Default.aspx. This declaration prepared the way for the global withdrawal of type 2 PV from trivalent OPV by April 2016, and the subsequent destruction of all materials that contain type 2 OPV by July 2016 (WHO, 2015b) in all but a few specially designated laboratories. No circulating WPV3 has been reported since November 2012 (Kew et al., 2014). Countries where transmission of wild poliovirus has not yet been interrupted for greater than three years are Afghanistan and Pakistan. Significantly, no polio cases have been reported for more than one year in Nigeria. The international spread of WPV1 was declared a public health emergency of international concern (PHEIC) in May 2014 during the 4th IHR Emergency Committee meeting regarding the international spread of wild poliovirus http://www.who.int/mediacentre/news/statements/2015/polio-27-february-2015/en/ since continued circulation of WPV1 in endemic countries risked reintroduction of PV1 into polio-free areas of the world, for example by travelers, refugees from Africa and the Middle East.

Evidence of global interruption of transmission of WPV has been based primarily on AFP surveillance. ENVS and AFP surveillance will be critically important during the end stage of eradication and post eradication for documenting eradication and ensuring very early detection of any reemergence. ENVS will be especially important for populations with high IPV vaccine coverage, since the ratio of AFP cases to asymptomatic infections would be orders of magnitude lower than for under-vaccinated populations (Hovi et al., 2012). (The latest information on global

eradication can be viewed at http://www.polioeradication.org/). Improving methodologies and expanded use of environmental surveillance have become one of the goals of the WHO, the Rotary International and the Bill & Melinda Gates Foundation.

Live attenuated polio vaccine can be transmitted from person-to-person, a trait considered advantageous when WPV infections were endemic, but which is now problematic as the eradication endgame approaches (Dowdle et al., 2003). Specifically, like wild poliovirus, the OPV genome evolves during circulation. Some of the OPV progeny may revert to neurovirulence. AFP from reverted vaccine strains (e.g., vaccine associated paralytic poliomyelitis (VAPP)) occurs very rarely (1 per 500,000 to 1,000,000 primary vaccinations of naive individuals) and in rarely, these neurovirulent vaccine-derived strains (VDPVs) have circulated and caused outbreaks of poliomyelitis in poorly vaccinated populations (Kew et al., 2005). As a consequence there is a major effort to develop new, improved, and safer live polio vaccine stocks promoted by the WHO, Rotary and the Bill and Melinda Gates association. Three different approaches have been funded to reduce the risk for vaccine-derived poliovirus causing outbreaks of poliomyelitis and are in advanced stages of completion prior to clinical testing. These approaches include genetically engineering PV to de-optimize codon usage in the capsid region; increasing poliovirus polymerase fidelity and reducing recombination capacity; and relocating the cis-acting replication element (cre) to attenuate or minimize recombination. Alternative approaches include use of noninfectious DNA, immunogenic peptides and antiviral drugs. All will need more testing against PV.

Development of a general anti-EV vaccine appears to be very difficult due to significant EV genetic variability. However efforts have been initiated to develop a vaccine against specific EVs most notably EV-71 (Liang and Wang, 2014; Lu, 2014; Zhu et al., 2014).

1.4.2 Hygiene measures

Wastewater treatment (WWT) includes biological treatment (e.g., conventional activated sludge, waste stabilization ponds, trickling filters), physicochemical treatment (e.g., sedimentation, media or membrane filtration), conventional oxidation (e.g., chlorine and ozone), and advanced treatment technologies (e.g., membrane bioreactors, microfiltration, ultrafiltration, constructed wetlands, chemical coagulation, and disinfection with ultraviolet irradiation and ozone. WWT can also include steps that remove other hazardous contaminants of urban wastes, e.g., toxic chemicals, heavy metals, pharmaceuticals. A by-product of domestic wastewater or sewage treatment is sewage sludge, a semi-solid slushy mass, deposit, or sediment produced by sedimentation during water and sewage treatment processes. This sludge contains enteric pathogens and needs to be properly treated for recycling or disposal (discussed below in Section III). Multiple levels of wastewater treatment (preliminary, primary, secondary, and tertiary or advanced) and combination of their corresponding treatment

technologies are required to consistently reduce, remove and/or inactivate the vast numbers of viruses present in all the wastes sent into the sewer from drains and toilets (Costan-Longares et al., 2008; Francy et al., 2012; Haramoto and Otagiri, 2013; Harwood et al., 2005; Hegazy et al., 2013; Katayama et al., 2008; Kitajima et al., 2014a; Kitajima et al., 2014b; Kitajima et al., 2015; La Rosa et al., 2010; Locas et al., 2010; Ng et al., 2012; Ottoson et al., 2006a; Rock et al., 2015; Simmons et al., 2011; Simmons and Xagoraraki, 2011a; Symonds et al., 2014). The greater the exposure of the public to these contaminated sources, the greater the need for treatment to reduce all viral pathogens and hazardous contaminants present in these wastes (Gerba et al., 2013; Rock et al., 2015; Xagoraraki et al., 2014).

Person-to-person EV transmission can also be interrupted by physical hygienic means. These include directly stripping EVs from the surfaces of foods, hands, and other surfaces by adequate washing, disinfecting materials and surfaces used for food production, heating the food to inactivate virus, and using uncontaminated or treated water. Unfortunately, facilities that hygienically separate human excreta from human contact are unavailable for approximately 2.4 billion people or one third of the current global population (Graham and Polizzotto, 2013; WHO and UNICEF, 2015).

2.0 Environmental Occurrence and Persistence

Numerous methods have been used to evaluate the occurrence and persistence of EVs and other human picornaviruses in environmental waters. 2.1 of this Section details the extensive research that has been accumulated over the past few years. The current state of knowledge about the occurrence and persistence of EVs and other human picornaviruses in different environmental sources is documented in 2.2 and 2.3 of this Section. See Reviews by Faechem et al. (Feachem et al., 1980; Feachem et al., 1983a) for a review of literature before 1983.

2.1 Detection Methods

The selection of an appropriate filtration method for the primary concentration of viruses is crucial for successful virus detection (Rajal et al., 2007). Interestingly, the efficiency of virus recovery by these methods depends largely on the viral target and less on water types or method used (Cashdollar and Wymer, 2013). To date, no single efficient method to recover multiple viral pathogens from environmental waters has been developed. Adsorption of viral particles to charged microporous filters (electronegative and electropositive membranes and cartridges) followed by elution or desorption of the virus by a pH adjusted solution is perhaps the most common approach used in water analysis applications (Fong and Lipp, 2005; Fout et al., 1996; Haramoto et al., 2005; Katayama et al., 2002; Wyn-Jones and Sellwood, 1998; Wyn-Jones, 2007).

Ultrafiltration, i.e. tangential flow filtration (TFF) and hollow-fiber ultrafiltration, based on size exclusion or

entrapment of viral particles to a filter matrix, has emerged as the most promising methods for virus concentration from large volumes of water (Gibson and Schwab, 2011; Hill et al., 2005; Olszewski et al., 2005; Polaczyk et al., 2008; Rhodes et al., 2011). Furthermore, these methods have been optimized for the simultaneous concentration of multiple microorganisms (e.g., enteric bacteria, protozoa, and viruses) from diverse water matrixes, including source and finished drinking water, tap water, surface water, and reclaimed water (Gibson and Schwab, 2011; Hill et al., 2007; Hill et al., 2005; Liu et al., 2012; Morales-Morales et al., 2003; Polaczyk et al., 2008). In addition, a new type of filtration media composed of highly electropositive nanoalumina (AlOOH) fibers (~2 nm in diameter by ~250 nm in length) and microglass is currently available for concentrating EVs and other enteric viruses from large volumes of water (Gibbons et al., 2010; Ikner et al., 2011; Karim et al., 2009; Li et al., 2010). NanoCeram cartridge filters combine the benefits of standard pleated filters and nanotechnology (Karim et al., 2009; Li et al., 2010).

Cell culture (i.e., primary cell cultures, cell strains, and continuous cell lines) has been widely used for cultivation and isolation of viruses from environmental samples, especially viruses that are amenable to culture (e.g., EVs, adenoviruses, reoviruses, and rotaviruses) (Fout et al., 1996; Gerba et al., 2013). In fact, virus isolation in cell culture has long served as the "gold standard" for virus detection (Leland and Ginocchio, 2007; WHO, 2004, 2015a). However, EVs behave differently on different types of cultured cells and therefore the use of multiple appropriate cell lines is essential to increase yield and to improve the detection assay (Chonmaitree et al., 1988; Prim et al., 2013). Table 6 describes the susceptibilities of different cell lines commonly used for isolation of EV and HPeV. Cytopathic effect (i.e., morphological changes in individual cells or groups of cells induced by virus infection and easily recognizable under a light microscope) is the simplest and most widely used criterion for enterovirus infectivity; however not all EVs, cause a cytopathic effect, and in these cases other methods must suffice (Condit, 2013; Gerba et al., 2013). Quantitative measures of infectious enterovirus in environmental samples have been performed by the plaque assay or various endpoint methods that measure virus infectivity but not the number of virus particles in a preparation, many of which may be non-infectious (Condit, 2013).

Table 6. The susceptibilities of different cell lines commonly used for isolation of Enteroviruses and Human Parechoviruses

Cell Line	Susceptibility to Viruses[a]						
	Poliovirus	Coxsackievirus		Echovirus	HPeV		
		Type A[b]	Type B		1	2	3
Monkey kidney							
Rhesus	+++	+	+++	+++	+	+	unk[c]
Cynomolgus	++++	+	+++	+++	+	+	unk
Buffalo green (BGMK)	+++	+	++++	++	+	+	unk
African green (Vero)	+++	+	+++	++	+	+	unk
LLCMK2	+++	+	+++	+++	++	++	+++[d]
Human							
HeLa	+++	+	+++	+	+	+	unk
Kidney (293)	+++	+	++	+++	++	++	-
WI-38	++	++	+	+++	+	+	unk
Embryonic lung (HELF)	+++	++	+	+++	+	+	unk
MRC-5	+++	+	+	+++	+	+	unk
Rhabdomyosarcoma (RD)	+++	+++[e]	+	+++	+	+	-
HEp-2	+++	+	+++	+	++	++	-
A549	+++	+	+++	+++	+	+	-
HuT-292	+++	+++	+++	+++	unk	unk	unk
HT-29	+++	+	+++	+++	+++	+++	-
Super E-mix (combination of BGMK-hDAF[f] and Caco[g])	++	unk	++++	++++	++	++	unk

[a] Relative susceptibilities: +, minimally susceptible; ++++, maximally susceptible; -, nonsusceptible; [b] Coxsackievirus A1, A19, and A20 are not readily isolated; [c]unk: unknown (no published reports). Some viruses are difficult to isolate even on minimally susceptible cell lines; [d] Improved yields after passage; [e]Many types of CV type A only grow in RD cells; [f]BGMK-hDAF, BGMK cells expressing human decay-accelerating factor; [g]Caco, human colon adenocarcinoma cells.

No one cell line is capable of efficiently identifying the vast array of enteroviruses in sewage, thus underestimation is recognized for many studies.

Source: (Stellrecht et al., 2010)

Numerous molecular biology-based detection methods for EVs in environmental concentrates have been developed during the last decades as an alternative to cell-culture method. Molecular biological detection of enteroviral genomic RNA by conventional reverse transcription – polymerase chain reaction (RT-PCR) and by reverse transcription quantitative real-time PCR (qRT-PCR) has been widely used for direct detection and identification of human EVs in environmental water concentrates. Genus-specific primers against the highly conserved region of the 5' untranslated region (5'UTR) from all enterovirus genomes are broadly used for generic detection of a few copies of enteroviral RNA. Furthermore, the enterovirus 5' UTR PCR assay, also called pan-EV PCR, allows the detection of enterovirus serotypes that do not readily grow in cell culture standardly used in most laboratories and additional fastidious serotypes that that have been unculturable in all cells tested to date.

Highly specific identification of enterovirus serotypes can be achieved by RT-PCR and direct nucleotide sequencing of all or a portion of the genomic region that encodes capsid proteins especially VP1 (Oberste et al., 1999; Oberste et al., 2003; Kroneman et al., 2011; Nix et al., 2006; Oberste et al., 2006; Pallansch et al., 2013). These sequences can be submitted to an online tool at http://www.rivm.nl/mpf/enterovirus/typingtool/introduction that returns the genotype by comparison with a regularly updated sequence reference set of human Picornaviridae genera and enterovirus species and (sero)types (Kroneman et al., 2011).

Integrated cell culture and PCR (i.e., ICC-PCR) developed in the late 1980s still provides a reliable method for practical analysis and direct monitoring of environmental samples for EVs and other viral pathogens recovered from environmental samples (Amdiouni et al., 2012; Chapron et al., 2000; Choo and Kim, 2006; Lee et al., 2005; Reynolds, 2004; Reynolds et al., 1996). More recently, these techniques have been used in combination with viral metagenomics approaches for cell culture evaluation of the virologically quality of wastewater (Aw et al., 2014).

2.2 Data on the Occurrence in the Environment

Human EVs have been recovered worldwide from surface waters including coastal waters, rivers, streams, and lakes, from wastewaters, ground waters and finished drinking water (Boehm et al., 2003; Borchardt et al., 2007; Borchardt et al., 2012; Chapron et al., 2000; Donaldson et al., 2002; Fong and Lipp, 2005; Fout et al., 2003; Fuhrman et al., 2005; Gantzer et al., 1997; Griffin et al., 2003; Hamza et al., 2009; Hot et al., 2003; Jung et al., 2011; Pusch et al., 2005; Springthorpe and Sattar, 2007; Ye et al., 2012). EVs along with other culturable viruses (adenovirus, rotavirus, and reovirus) have been extensively monitored for regulatory purposes in the US in order to derive baseline data on the occurrence of viral agents in surface and ground waters (Fout et al., 1996). Due to their ubiquitous excretion in feces and frequent detection in untreated domestic wastewaters EVs are the best known viruses and commonly associated with water quality (Grabow, 2007; Rodriguez et al., 2012). The detection of EVs in sources of drinking water and recreational water bodies has been broadly accepted as a marker of a possible failure of the sanitation systems and as an indicator of the potential role of water in disease outbreaks (Hovi et al., 2007). Moreover, since the natural host for all human EVs is humans, the detection and quantification of amplifiable enterovirus genetic material in environmental waters has been proposed and used as a water quality assessment tool for tracking sources of human fecal pollution (Boehm et al., 2003; Fong et al., 2005; Wong et al., 2012).

2.2.1 Occurrence in sewage

The concentration and types of EVs and other picornaviruses in municipal waste waters (i.e., sewage) in different geographic locations, including highly polluted surface waters in areas where wastewater treatment is either inadequate or nonexistent, are shown in Table 7.

Table 7. Occurrence of enteroviruses and other human picornaviruses in untreated sewage worldwide

Area	Period of Study	Information on Collection	Percentage Positive (# of Samples)	Concentration Average (Range), (GC/L, PFU/L, MPN/L, IU/L)[a]	Reference
Bolivia	2012	None	100% 10/10	4.2 E+02 to 6.2 E+02 IU[b]/L	Symonds et al., 2014
China (Guangzhou)	2009 to 2012	Liede	90% 216/240	NR[c]	Zheng et al, 2013
Côte d'Ivoire (Abijan)	2008 to 2009	Yopougon, Abijan	44.1% (30/68) 51.5% (35/68)	NR	Momou et al., 2013
Côte d'Ivoire (Abijan)	2008 to 2009	Yopougon, Abijan	39% (27/68)	4.28 E+02 PFU/L	Momou et al., 2013
Germany (Leipzig)	2002 to 2003	Leipzig, Rosental	89% (8/9)	NR	Pusch et al., 2005
Iran (Sistan-Balouchestan, Tehran, Fars Province)	2006 to 2007	Several villages in Sistan and Balouchestan Province (Zabol, Zahedan, Chabahar), city of Tehran, and Fars Province	100% (197/197)	NR	Kargar et al., 2009
Israel	Jan to Aug 2013	None	40.6% (80/192)	from 0 to 2.08E+07 PFU/L	Shulman et al., 2014
Israel	1989 to 1997	Composite samples plus a few grab samples and gauze pad collections	>80% (1779/2224)	0 to 250 PFU/L	Manor et al., 1999
Italy (Milan)	2006 to 2012	Central-eastern Milan and in the Milanese hinterland (Peschiera Borromeo, Nosedo, Nosedo Est)	96.8% (311/321)	NR	Battistone et al., 2014
Japan (Osaka, Kyoto, Saitama, Shiga, Tokyo, Ibaraki)	2003 to 2004	Six WWTPs[d]	100% (72/72)	~3E+02 GC[e]/L	Katayama et al., 2008
Japan (Yamanashi Prefecture)	2011 to 2012	None	71% (10/14)	4.47E+04 to 2.8E +06 GC/L	Haramoto and Otagiri, 2013
New Zealand	2003 to 2004	From across New Zealand	60% (18/30) 100% (30/30)	5.0 to 8.3 E+03 PFU/L 6.91E+02 to 4.67 E+06 GC/L	Hewitt et al., 2011
Singapore	2007	Three water reclamation plants	94% (17/18)	NR	Aw and Gin, 2010
Slovak Republic (Banska Bystrica, Presov, Zilina, Nitra, Kosice, Trencin, Trnava, Bratislava)	2001 to 2011	Sewers from self-governing Western, Central, Eastern region	100% (3744/3744)	NR	Klement et al., 2013
South Africa (Pretoria and Johannesburg)	2001 to 2003	Daspoort Sewage Treatment Plant, Pretoria and East Rand Water Care Company, Johannesburg	32% (112/351) Enteroviruses 25% (88/351) NPEVs 7.0% (25/351) PV	NR	Pavlov, 2006; Pavlov et al., 2005
South Africa	2000 to 2002	Selected Areas	42.5% (43/100)	NR	Ehlers, 2005
Spain (Barcelona and Girona)	2001 to 2006	Northern Spain	84% (62/74)	5.4E+03 to 3.7E+04 PFU/L	Contan-Longares et al., 2008
Taiwan	2004 to 2005	Hospital sewage	31% (4/13) 46% (6/13)	NR	Chen et al., 2008

Area	Period of Study	Information on Collection	Percentage Positive (# of Samples)	Concentration Average (Range), (GC/L, PFU/L, MPN/L, IU/L)[a]	Reference
The Netherlands (Apeldoorn)	1998 to 1999	City of Apeldoorn	100% (72/72)	1.4E+02 to 8.33E+02 PFU/L	Lodder and de Roda Husman, 2005
Tunisia	2009 to 2010	Rural regions of Monastir and Madhia governments, East center part of Tunisia	30.2% (52/172)	NR	Ibrahim et al., 2014
United Kingdom (Edingburgh)	2009 to 2010	None	92.5% (37/40)	NR	Harvala et al., 2014
USA Milwaukee, Wisconsin	1994 to 2003	None	88% (94/107)	2.37E+02 to 3.35E+03 MPN/L	Sedmaket al., 2005
USA (Michigan)	2008 to 2009	Five WWTPs	100% (10/10)	3.0E+02 to 1.2E+06 GC/L 8.0 to 5.0E+02 MPN/L	Simmons and Xagoraraki, 2011b
USA (Ohio)	2008 to 2010	Composited grab samples	79% (15/19)	2.4E+02 to 2.9E+05 GC/L	Francy et al., 2012
USA (Southern Arizona)	2011 to 2012		100% (12/12)	EV: 1E+03 to 1E+06 GC/L AiV: 9.7E+04 to 2.7E+06 GC/L	Kitajima et al., 2014b
USA (Southern Arizona)	2011 to 2012		25% (6/24)	Max 3.24E+05 GC/L	Kitajima et al., 2015
USA (Southern Arizona)	2011 to 2012	Two WWTPs	46% (11/24)	2.32E+03 to 2.28E+05 GC/L	Kitajima et al., 2014a
Venezuela (Caracas)	2007 to 2008	Domestic sewers	75% (9/12)	NR	Rodriguez-Diaz et al., 2009

[a]Methods used in the different studies. Bolivia: Adsorption/elution electronegative membranes/Cell culture, results reported in infection units per liter. China: Adsorption/elution electronegative membranes; cell culture-RT-PCR. Côte d'Ivoire: Two-phase separation procedure (PEG-6000 and Dextran T40) RT-PCR and cell culture-RT-PCR. Results reported as plaque forming units per liter. Côte d'Ivoire: Two-phase separation procedure followed by cell culture and typing by seroneutralization assay (Lim Benyesh-Melnick neutralizing antibody pools A through H) and RT-PCR. PVs identified by ELISA and Intratypic differentiation testing by PCR. Germany: Ultracentrifugation and nested RT-PCR. Iran: Two-phase separation procedure [cell culture - seroneutralization assay (Lim Benyesh-Melnick neutralizing antibody pools A through H). Israel: Two-phase separation procedure for composite samples and cell culture. Italy: Two-phase separation procedure followed by cell culture - RT-PCR. Enterovirus typing by seroneutralization assay using Lim Benyesh-Melnick neutralizing antibody pools A through H. RT-PCR. PVs: ELISA and ITD testing by PCR. Japan: Adsorption/elution electronegative membranes [RT-qPCR]. New Zealand: Centrifugation/flocculation followed by hydroextraction with Polyethelene glycol – NaCl, cell culture, RT-qPCR. Singapore: Hydroextraction-Polyethelene glycol [RT-PCR]. Slovak Republic: Two-phase separation procedure (PEG-6000 and Dextran T40)[cell culture - seroneutralization assay (Lim Benyesh-Melnick neutralizing antibody pools A through H)]. South Africa: Hydroextraction: Polyethelene glycol 6000 and sodium chloride [cell culture -RT-multiplex PCR]. South Africa : Glass wool adsorption–elution technique [cell culture - RT-PCR]. Spain: Direct filtration through 0.22μm-pore-size low protein binding polyether sulfone membrane filter [cell culture]. Taiwan: Adsorption/elution electronegative membranes [RT-PCR] [nested RT-PCR]. The Netherlands: Adsorption-elution electronegative cartridge (Nominal 1.2 μm pore-sized filter) [cell culture]. Tunisia: Prefiltration/flocculation followed by hydroextraction with Polyethelene glycol [RT-PCR]. United Kingdom: Centrifugation/Filtration [RT-PCR]. United States of America: Organic flocculation (US EPA ICR virus protocol) [cell culture- Lim Benyesh-Melnick neutralizing antibody pools A through H]. USA: Adsorption-elution electropositive cartridge (Zeta Plus Virosorb 1 MDS) [RT-qPCR]. USA: Glass wool adsorption–elution technique, RT-qPCR. USA: Adsorption/elution electronegative membranes [RT-qPCR]. USA: Adsorption/elution electronegative membranes [RT-qPCR]. USA: Adsorption/elution electronegative membranes[RT-qPCR]. Venezuela: Ultracentrifugation [RT-PCR].

[b]IU: Infectious units; [c]NR: Not reported; [d]WWTP: Wastewater treatment plant; [e]GC: Gene copy.

Members of the genus Enterovirus are universally distributed among human populations and therefore are always found in sewage waters worldwide (Gerba et al., 2013; Pallansch et al., 2013).

Quantitative estimation of human EVs and other picornaviruses (i.e., viral load) in domestic wastewaters (i.e., primary source of human EVs and enteric picornaviruses to the environment) from different regions may vary due to differences and limitations of the methods

used for recovery and detection of viruses in water, differences in the viral load that may exist in human populations for each enteric virus, and daily plus seasonal fluctuations of viruses in human sewage. Nevertheless, the assessment of the total and infectious viral load in untreated and treated domestic wastewaters as well as the identification of virus types are critical to understand the fate of viral pollutants in the environment and to estimate the potential health risks from waterborne viral pathogens from discharges to waters used for recreation, shellfish farming or as sources of drinking water (de Roda Husman and Bartram, 2007; Gerba et al., 2013; Gibson, 2014; Rao et al., 1986; Symonds et al., 2009). Quantitative information of the virus concentration in various types of water, including quantitative data of the inactivation and removal of pathogenic viruses by water and wastewater treatment processes, are essential parameters for the quantitative microbial risk assessment (QMRA) process, the new concept for evaluating microbial safety of drinking water and wastewater (Haas et al., 1993; Haas et al., 1999; Hijnen et al., 2006; Mena, 2007; Soller, 2006).

Screening of waste waters for human viruses in multiple geographical areas has revealed the presence of newly described genera of the Picornaviridae family, i.e., Cosavirus, Cardiovirus, Salivirus, Kobuvirus (Alcala et al., 2010; Blinkova et al., 2009; Haramoto and Otagiri, 2013; Kitajima et al., 2014b; Kitajima et al., 2015). More recent, deep-sequencing analysis of viral metagenomes from sewage and sewage sludge (also known as sewage viromes) has indicated that these novel human picornaviruses can be found in most cases at relatively higher abundance and occurrence than members of the Enterovirus genus (Aw et al., 2014; Bibby and Peccia, 2013; Cantalupo et al., 2011; Ng et al., 2012). Taken together, these studies have demonstrated the importance of targeted molecular assays and unbiased high-throughput sequencing (i.e., metagenomics viral pathogen detection technologies) to the study of virus diversity in waste waters and to monitor shedding of known and newly characterized viral human pathogens on a population level scale.

2.2.2 Occurrence in sludge

Numerous studies conducted during the last two decades have evaluated the occurrence of EVs in biosolids and the efficiency of sludge treatment on the reduction of these and other enteric viruses (Goddard et al., 1981; Goyal et al., 1984; Guzman et al., 2007; Monpoeho et al., 2001; Pourcher et al., 2005; Schwartzbrod and Mathieu, 1986; Sidhu and Toze, 2009; Soares et al., 1994; Straub et al., 1994; USEPA, 1988). Despite the extreme heterogeneity of sludge samples and variations in sampling processing methods, these studies demonstrated the occurrence of infectious EVs and other enteric viruses in treated biosolids.

Soares et al. (Soares et al., 1994) evaluated the occurrence of EVs in undigested and anaerobically digested sludge. For undigested sludge the concentration of EVs varied between 4.36 MPN/g (most probable number per gram dry weight) to $7.00x10^2$ MPN/g with geometric average of $3.29x10^1$ MPN/g $\pm$ 0.0044. For anaerobically

digested sludge the concentration varied from <0.0062 MPN/g to $2.52x10^2$ MPN/g with a geometric average of 1.61 MPN/g $\pm$ 0.02. Removal of EVs during sludge treatment ranged from 0.5 log_{10} to >3 log_{10} with a geometric mean 1 log_{10}. The study revealed that despite sludge treatment the concentration of EVs in anaerobically digested sludge could exceed 10^2 MPN/g and therefore additional treatment was required in order to reduce the concentration of EVs to levels established for land application in the study area.

Monpoeho et al. (2001) evaluated several viral extraction techniques for simultaneous detection of EV genomes (number of copies per 10 g of dry matter) and infectious particles (most-probable-number cytopathogenic units [MPNCU]/10 g of dry matter) in different types of sludge samples. The concentration of EVs detected varied considerably from one sample to another. For primary sludge the concentrations varied from <0.3 to $2.24x10^2$ MPNCU/g and from <40 to $3.84x10^4$ genome copies/g (dry weight). The mean concentrations detected in primary sludge were 45.7 MPNCU/g and $1.37 x10^4$ genome copies/g. The equivalent values were 2.9 MPNCU/g and $9.36x10^2$ copies/g in activated sludge, 0.9 MPNCU/g and $1.06x10^3$ copies/g in thickened sludge, and 0.7 MPNCU/g and $4.8x10^3$ copies/g in digested sludge. According to these results, from primary sludge to activated sludge the concentrations of virus decreased by a factor of 16 in terms of infectivity and by a factor of 15 in terms of genomes. From activated sludge to thickened sludge viruses decreased by a factor of 3 in terms of infectivity but remained unchanged in terms of genomes. Finally, from thickened sludge to digested sludge they decreased slightly, by a factor of 1.3, in terms of infectivity and increased by a factor of 4.5 in terms of genomes. The study provided a rapid sludge testing protocol capable of determining concentrations of viral genomes and proportion of infectious virus particles in sludge samples.

Wong et al. (2010) investigated the occurrence and the quantitative levels of human EVs and other enteric viruses (human adenovirus, NoVGGI, NovGGII ,and hepatitis A virus) in class B mesophilic anaerobically digested (MAD) biosolid samples using molecular (qPCR) and cell culture methods. In this study, the levels of human adenoviruses were significantly higher than the levels of other enteric viruses and they were also detected more frequently than other enteric viruses. The average levels of EVs and adenoviruses in the MAD biosolids were $1.9x10^4$ copies/g and $5.0x10^5$ copies/g, respectively. The average level of human NoVs corresponded to $5x10^4$ copies/g (NV GGI) and $1.5x10^5$ copies/g (NV GGII). The ICC-PCR assays demonstrated the occurrence of infectious adenoviruses and EVs in the biosolid samples thereby indicating the limited inactivation effectiveness of MAD treatment as revealed in previous studies (Monpoeho et al., 2004; Soares et al., 1994). This study also highlighted the importance of monitoring for additional human pathogenic viruses (adenoviruses, NoVs) considering their frequent occurrence and levels reported in biosolids. Indeed, due to the large potential pathogen diversity in sewage sludge and biosolids, continuous surveillance of biosolids for the culturable and infective content of a broad diversity of

pathogens may be required to understand potential risks (Viau et al., 2011).

Reduction/inactivation of EVs in biosolids is discussed in Part 3.1.8 of Section 3.0 Monitoring the occurrence of infectious EVs in biosolids is required to assess pathogen disinfection efficacy and compliance with pathogen land-application standards. Furthermore, quantitative data from pathogen monitoring can be used to assess the infection risk associated with biosolids pathogen exposure. Class-B-treatment processes must yield virus reduction levels (i.e., log reduction value) established for each biosolids disinfection process (i.e., 0.5 – 2 logs) (USEPA, 1999). The European limit value for virus concentration in biosolids is 3 MPNCU (for most probable cytopathic units) per 10 g of dry matter. More recently, QMRA of biosolids exposure based on new pathogen data has demonstrated that the tradition of monitoring pathogen quality by Salmonella spp. and concentration of EVs underestimates the infection risk of pathogens contained in the biosolids (to the public), and that a rigorous biosolids pathogen treatment process is the most efficient method of reducing pathogen exposure and infection risk (Viau et al., 2011). Indeed, the use of improved risk assessment methods has been recommended to supplement technological approaches to establishing regulatory criteria for pathogens in biosolids (National Research Council, 2002). In the United Kingdom, the use of sewage sludge as a soil enhancer and fertilizer on agricultural land remains the environmentally favored option, with around 80% being applied to agricultural land. In Australia and New Zealand strict state and national guidelines specify the way in which specific biosolids can be used.

2.2.3 Occurrence in drinking water

EVs were detected in treated drinking water from supplies that had been treated and disinfected according to international specifications for the production of safe drinking water in South Africa (Ehlers et al., 2005; Vivier et al., 2004). The frequency of detection ranged from 11 to 18.7% and CV-B was the EV predominantly found in drinking water samples. In Egypt, two conventional drinking water treatment plants differing in treatment capacity were investigated for the occurrence of EVs and other enteric viruses. EVs were found in finished water samples and the infective EV count ranged between 1.6 and 33.3 plaque forming unit (PFU)/L (Ali et al., 2004). These viruses were also detected in the distribution system as a result of inadequate water pipe connections. Similarly, EVs were detected in finished water samples collected from seven conventional water treatment plants in the Montreal, Quebec, area that ranged in capacity from 2.4 to 26.4 millions of gallons per day and produced finished water essentially free of indicator bacteria. The frequency of detection of cytopathogenic viruses expressed as MPNCU/L was 7% (11 of 155) and the average density was 0.0006 MPNCU/L with the highest virus density measured corresponding to 0.02 MPNCU/L (Payment et al., 1985b). All viruses isolated were EVs (types 1, 2, and 3 PVs, CVB3, CVB4, CVB5, Echovirus 7, and untyped picornaviruses). EVs were also detected in 47.8% of tap water samples collected from different locations in three urban areas in Seoul, Korea (Lee and Kim, 2002). The level of infectious EVs was always below 10 MPN per 1000 Lbut the frequency of detection was considerably high compared to previous studies.

2.2.4. Occurrence in coastal waters

Human EVs have been found in varying concentrations in marine coastal waters as demonstrated by numerous research studies conducted from early 2000 around the world (Table 8). Previous studies from 1970s – 1980s on viruses in coastal waters focused on EVs due to the ability to detect these viruses using standard-cell culture monolayer methods. The level of EVs reported per liter of sample analyzed ranged from 0.007 to 2.6 PFU; 0.05 to 16 $TCID_{50}$; 0.05 to 6.5 MPNCU as summarized by Bosch et al. (2005). Recent investigations have introduced PCR detection and quantification of enterovirus genomes by qPCR followed by molecular characterization of the EV types including identification of other human picornaviruses by amplicon sequencing. Types 1, 2 and 3 PVs; coxsackievirus, echovirus, EVs and other human picornaviruses such as HPeVs have been isolated from marine coastal waters. The concentrations reported in the different studies included in Table 8 vary as a result of the level of sewage treatment provided and the methods used to concentrate and detect the viruses.

Table 8. Occurrence of enteroviruses in coastal waters

Area	Sample Description	Enterovirus Type Identified	Concentration Technique and Detection Assay [Cell Culture or RT-PCR/RT-qPCR	Percent Positive (# of Samples)	Concentration Average (Range), (GC/L[a], PFU/L[b], MPN/L[c], IU/L[d])	Reference
China (Tianging)	Winter seawater along Tianging coast of Bojai Bay	Poliovirys type 2	Centricon plus-70 centrifugal filter device (100-kDa molecular weight cutoff membrane) [RT-qPCR]	100.0% (10/10)	1.7E+06 to 6.3E+07 GC/L	Zhang et al., 2010

Area	Sample Description	Enterovirus Type Identified	Concentration Technique and Detection Assay [Cell Culture or RT-PCR/RT-qPCR]	Percent Positive (# of Samples)	Concentration Average (Range), (GC/L[a], PFU/L[b], MPN/L[c], IU/L[d])	Reference
Italy (Venice)	Surfzone at Lido Beach	NR[e]	Adsorption/elution electronegative membranes [RT-qPCR]	88.2% (15/17)	2.0 to 1.61E+03 GC/L	Rose et al., 2006
Japan (Fujisawa City and Tokyo)	Summer sampling: Enoshima Beach (Kanagawa Prefecture) Winter sampling: Tokyo Bay (Daiba Kaihin Park and Kasai Kaihin Park)	NR	Adsorption/elution electronegative membranes [cell culture-RT-PCR]	66.6% (4/6)[f]	NR	Katayama et al., 2002
Japan (Kanagawa Prefecture)	Tokyo Bay	NR	Adsorption/elution electronegative membranes [RT-PCR and cell culture PCR]	24.4% (11/45)[g] 40.6% (26/64)[h]	NR	Katayama et al., 2004
Spain (Barcelona)	Marine beaches in the metropolitan area	NR	Adsorption/elution glass powder VIRADEN method cell culture [RT-PCR]	50% (10/10)[i] 20.0% (4/20)[j]	1 to 1.58 E+02 PFU/10L	Moce-Llivina et al., 2005
Spain	Seawater samples collected during bathing season (June through September)	CV: 100% (CV-B1 85.7%; CV-B4 14.3%)/E	VIRADEN method (filtration and quantification of viruses adsorbed onto 3 μm-pore-size cellulose nitrate membrane filters) [cell culture]	63.3% (13/21)	Maximum 1.3 PFU/L[k]	Costan-Longares et al., 2008
USA (Sarasota, Florida)	Coastal waters off west Florida	Echovirus 20 and 24; poliovirus	Adsorption/elution electronegative cartridges (Filterite)	25% (11/24)	0.17 to 0.59 IU/100 L	Lipp et al., 2001a
USA	Nearshore waters Florida Keys	NR	Adsorption/elution electronegative cartridges (Filterite) [RT-PCR]	93.3% (14/15)	NR	Lipp et al., 2001b
USA (Charlotte Harbor estuary, Florida)	NR	NR	Adsorption/elution electronegative cartridges (Filterite) [cell culture]	21.8% (7/32)	1.0E+02 to 2.0E+02 MPN/L	Lipp et al., 2001b
USA	Key West and Dry Tortugas (Florida Keys)	Coxsackievirus A9, Coxsackievirus A16, and Poliovirus Sabin type 1	Vortex flow filtration (100kD filter) Membrex Benchmark System (Membrex Inc., Garfield, NJ, USA). [RT-PCR]	60.0% (9/15)	9.3E+02 GC/L	Donaldson et al., 2002
USA (Avalon Bay, Catalina Island, California)	Offshore, bay, shoreline, and subsurface samples	NR	Adsorption/elution glass fiber filters [RT-qPCR]	13.6% (3/22)	NR	Boehm et al., 2003
USA (Key West, Florida)	Upper, Middle, and Lower Keys	NR	Adsorption/elution electronegative cartridges (Filterite) [cell culture]	33.3% (2/6)	1.13E-01 to 1.26E-01 PFU/L	Wetz et al., 2004

Area	Sample Description	Enterovirus Type Identified	Concentration Technique and Detection Assay [Cell Culture or RT-PCR/RT-qPCR	Percent Positive (# of Samples)	Concentration Average (Range), (GC/L[a], PFU/L[b], MPN/L[c], IU/L[d])	Reference
USA (San Diego, California)	Surfzone adjacent to the Tijuana river mouth, Imperial Beach pier	Echovirus 6, 11, and 30; Coxsackievirus A5, Enterovirus B, Poliovirus SL-1, SL2, and SL-3	Adsorption/elution electronegative membranes [RT-qPCR]	100.0% (7/7)[l] 85.7% (6/7)[m]	3.0E+02 to 4.42E+04 GC/L[l] 7.0E+01 to 3.75E+03 GC/L[m]	Gersberg et al., 2006
USA (Santa Catalina Island, California)	Nearshore waters Avalon Beach (72-hour sampling)	NR	Adsorption/elution electronegative membranes [1 L] [RT-PCR]	100.0% (20/20)	NR	Boehm et al., 2009
USA (Honolulu, Hawaii)	Coastal waters, Island of Oahu	NR	Adsorption/elution electronegative membranes [RT-PCR]	50.0% (11/22)	NR	Connell et al., 2012
Venezuela (Cumana)	Nearshore coastal waters northeast Caribbean coast	Echovirus 6, Poliovirus SL-1, Parechovirus	Adsorption/elution electronegative membranes[RT-qPCR] [ICC-RT-qPCR]	46.6% (14/30)[n] 33.3% (10/30)[o]	5.3E+03 to 3.9E+04 GC/L	Betancourt et al., 2015

[a]GC /L: Genome copies per liter; [b]PFU/L: Plaque forming units per liter; [c]MPN/L : Most probable number per liter; [d]IU/L: Infectious units per liter; [e]NR: Not reported; [f]Enoshima Beach (summer), none detected in Tokyo Bay (winter); [g]Samples positive by RT-PCR; [h]Samples positive by cell culture – PCR; [i]Samples positive by cell culture; [j]Samples positive by RT-PCR; [k]Geometric mean 3.6E-01 PFU/L; [l]Wet weather surf zone; [m]Wet weather at Imperial Beach pier; [n]Samples positive by RT-qPCR; [o]Samples positive by cell culture RT-qPCR.

Solid-associated virions in marine waters are protected from inactivation by environmental stressors (e.g., sunlight irradiation, increasing temperatures, and microbial enzymatic degradation), can be transported long distances after discharge in surficial sediment pore water, and particle-bound virions in sediments can be easily resuspended by mild turbulence and water movements including tidal currents, patterns of water circulation, prevailing winds, storm action, dredging, boating and in coastal and shelf environments (Bosch et al., 2006; Dupuy et al., 2014; Gerba, 2007; Hurst and Murphy, 1996; Pianetti et al., 2007; Rao et al., 1986).

2.2.4.1 Regulatory considerations for coastal waters

Marine coastal waters have received a great deal of attention most likely due to the public health concerns associated with the contamination of shellfish growing beds and the potential exposure pathway to enteric virus infection risk from coastal recreation, e.g. swimming, water skiing, scuba diving, and surfing (Bosch et al., 2006; Dyble et al., 2008; Gerba, 2006; Griffin et al., 2003; Stewart et al., 2008).

- The European Union regulations concerning the quality of bathing waters, European Directive 76/160/ECC, stipulates monitoring for viruses, with a standard of 0 EVs per 10 liters.

Recent studies are addressing the fate and distribution of human EVs and other human picornaviruses in tropical coastal marine environments in Latin America where research studies had been limited and disposal of untreated wastewater into the coastal ocean is a common practice (Betancourt et al., 2015). Overall, these studies have highlighted several relevant findings related to the occurrence and public health significance of human EVs in the coastal environment: (i) EVs are suitable and valuable indicators of human fecal pollution in coastal waters, although they are not ideal viral indicators for all fecally excreted viruses in all situations and geographic locations; (ii) EV detection by RT-PCR/RT-qPCR is a highly sensitive and easy-to-use tool for rapid assessment of marine water quality and fecal contamination provided that adequate sample process controls are included - from concentration to genome amplification – to accurately detect and quantify EV genomes; (iii) the inclusion of ICC-RT-PCR in studies of human EVs in coastal waters serves as a further processing step for enhancing the sensitivity of the EV detection assay with multiple advantages including the capacity to reveal the circulation of specific EV serotypes and other human picornaviruses (e.g. Aichi virus and Parechovirus) in sewage-polluted coastal waters, i.e. ENVS; (iv) molecular typing methods based on RT-PCR and nucleotide sequencing of portions of the VP1 gene (i.e. direct genotyping identification) can be used to obtain location-

specific data on enterovirus serotypes in coastal waters, which is of practical value in epidemiological surveillance and in outbreak investigation as previously mentioned; and (v) most frequently EV concentrations in marine waters are not correlated with concentrations of bacterial indicators of sewage pollution as previously documented, although the simultaneous occurrence of EVs and fecal indicator bacteria in water quality monitoring can be used in most geographical settings as a reasonable indication of recent fecal contamination and therefore of chronic pollution problems in coastal waters (Betancourt et al., 2015; Boehm et al., 2003; Connell et al., 2012; Donaldson et al., 2002; Fuhrman et al., 2005; Gersberg et al., 2006; Gregory et al., 2006; Kitajima and Gerba, 2015; Lipp et al., 2001b; Lipp et al., 2001c; Moce-Llivina et al., 2005; Wetz et al., 2004; Wyer et al., 1995).

2.2.4.2 Epidemiological evidence for marine recreational disease

Epidemiological evidence of EV-associated infections transmitted by swimming in contaminated seawater has been limited, most likely due to variability and inefficiency of analytical techniques and lack of epidemiological studies that establish a link between EV illnesses and marine water exposure. Begier et al. (Begier et al., 2008) reported the first outbreak of primarily EV illnesses among travelers to Mexico that likely occurred as a result of swimming in sewage-contaminated seawater. All sea swimming exposures were associated with increased risk of EV infection, and only the time spent on swimming was significantly associated with increased risk of EV infection. QMRAs can be used to evaluate the likelihood that adverse health effects will occur following exposure to coastal recreational waters in which human viruses are present. For these purposes, effective investigations and documentation of EV outbreaks, quantitative data of EVs and thorough consideration of the sources of their infections are necessary, otherwise such assessments will remain difficult to conduct or, when conducted, will be quite uncertain in their outcomes (Parkin et al., 2003). Most commonly, fecal indicator bacteria have been used as predictors of acute gastroenteritis or gastrointestinal illness – the main health outcome – among swimmers at wastewater-impacted recreational beaches and in exploratory quantitative microbial risk assessments (Arnold et al., 2013; Wade et al., 2010). Acute gastroenteritis is a generic term used to refer to gastrointestinal illness involving diarrhea, nausea, or vomiting, which may or may not also be combined with abdominal pain, abdominal cramps, or systemic symptoms, such as fever (Parashar and Glass, 2012; Roy et al., 2006). It is also the common definition given to waterborne diseases (Annon, 1999). However, EVs cause a wide spectrum of acute disease as previously mentioned. Therefore, the use of QMRAs when investigating recreational exposure assessments to enteric viruses must be based on quantitative detection of specific viruses and not on fecal indicator bacteria, as fecal indicator bacteria-based QMRAs frequently underestimate virus occurrence and associated health risks (Symonds and Breibart, 2015).

2.2.5 Occurrence in shellfish

EV contamination has been reported in areas routinely impacted by sewage with a frequency of detection of 19% that can increase up to 45% in highly contaminated sites (Le Guyader et al., 2000). Another study reported EV detection in shellfish tissue from shellfish collected from six of nine beach (66%) sites tested. In this case study, the frequency of detection of EV was higher in shellfish than in water samples from corresponding locations (Connell et al., 2012). EVs have been detected in high quality harvesting areas in Portugal with 35% of the batches analyzed showing signs of enteroviral contamination, 33% showing hepatitis A virus contamination, and 37% showing signs of NoV contamination (Mesquita et al., 2011). Due to bioaccumulation of human enteropathogenic viruses (e.g., EVs and NoVs) by indigenous filter-feeding bivalve mollusks these organisms have been suggested as potential bio-sentinels of human waste in marine coastal waters (Asahina et al., 2009). No PVs were found in shellfish during ENVS for PV in shellfish in the Netherlands downstream from an accidental discharge of high amounts of virulent poliovirus from a vaccine production facility in Brussels (Duizer, 2015; ECDC, 2014).

Although several studies have demonstrated EV contamination in shellfish harvested from polluted and even relatively clean sites, these viruses have not been directly associated with shellfish or seafood vectored infection (Lees, 2000).

2.3 Persistence

The persistence and survival of human enteric viruses, including EVs and other picornaviruses, in the extracellular environment (e.g., water, soil, food, aerosols, and environmental surfaces or fomites), the combination of factors that influence survival (i.e., temperature and seasonal variation, pH, salinity, relative humidity, sunlight irradiation and UV light penetration, wet weather flows, viral aggregation, viral adsorption to suspended solids or surfaces, microbial biome and microbial predation), the risks for transmission of EV infections through environmental exposures (water, foods, aerosols, and fomites), and prevention strategies to reduce this risk have been extensively documented by peer-reviewed scientific research and reviewed in detail by several authors (Bertrand et al., 2012; Bosch et al., 2006; de Roda Husman et al., 2009; Gerba, 2006, 2007; Gerba et al., 2013; John and Rose, 2005; Rzezutka and Cook, 2004; Vasickova and Kovarcik, 2013; Vasickova et al., 2010; Xagoraraki et al., 2014; Yates and Yates, 1988) EV virions can survive outside the body for months under favorable environmental conditions (Bosch et al., 2006; Dowdle and Birmingham, 1997; Mattle et al., 2011; National Research Council, 2004; Pallansch et al., 2013; Templeton et al., 2004; Templeton et al., 2008; Young and Sharp, 1977). These conditions include neutral pH, moisture, low temperature: 4 – 10°C), and association with particles in untreated or partly treated waste waters that protects against inactivation by natural or artificial processes, including enzymatic (i.e., proteolytic bacterial enzymes) or UV degradation and chemical disinfection (discussed further in Section 3.2). The

biophysical properties of picornaviruses such as their small-size (30 nm), genome type (ssRNA), and non-enveloped capsid structure of the virions are known to play an important role on the mechanisms of virus survival and transport in the environment (Bosch et al., 2006; Fong and Lipp, 2005; Gerba et al., 2013; National Research Council, 2004; Sobsey and Meschke, 2003; Wigginton and Kohn, 2012; Xagoraraki et al., 2014). Processing conditions for picornavirus capsid assembly enable thermal stability (stable at 42°C or up to 50°C in presence of sulfhydryl reducing agents and magnesium cations) and pH stability (pH 3 – 9) in the extracellular environment (Gerba, 2007; Hurst and Adcock, 2000; Hurst and Murphy, 1996; National Research Council, 2004; Racaniello, 2013). Capsid protein interaction with genomic RNA genome adds stabilization (Pallansch et al., 2013). Electrostatic and hydrophobic interactions involving functional groups of the viral capsid (i.e., charged amino acids residues) are essential for capsid assembly, capsid stability, and virus particle adsorption to solid surfaces (e.g., suspended solids, sediments and environmental surfaces or fomites) (Mateu, 2011; Michen and Graule, 2010). Binding of serotype 1 polio vaccine strains (Mahoney and Sabin 1) to bacteria or bacterial polysaccharides offers a selective advantage by enhancing capsid stability in the environment (Robinson et al., 2014).

2.3.1 Persistence in aquatic environments.

Persistence of EVs in aquatic environments can be inferred from initial concentrations and T90 and T99 inactivation times, the times required to reduce the number of virus by $1\log_{10}$ and $2\log_{10}$, respectively. T90 and T99 parameters have been derived from different studies under different experimental conditions (light intensity, type of water, turbidity, and pH) and different methods to determine EV inactivation rates (cell culture versus molecular methods). As a result, wide variations in inactivation times for the same enterovirus or other members within the *Picornaviridae* family have been reported in different studies. Several examples will be provided.

T90 and T99 times for *in situ* natural inactivation of laboratory stocks of Echovirus-6 and CV-B5 were 1.5 and 3.7 days respectively, at 24 – 25°C, Salinity 36 – 37%, pH 7.7 – 8.1, turbidity 0.8 – 1.8 nephelometric turbidity units (Moce-Llivina et al., 2005). In seawater, the time required for a reduction of 2 $\log_{10}$ titer of poliovirus type 1 ranged between 3 days at 25°C and 6 days at 5°C (Callahan et al., 1995; Chung and Sobsey, 1993).

The T90 and T99 decay rates for infectivity and viral RNA genome detection of poliovirus strain Lsc-1 was 2 and 4 days, respectively, in unfiltered natural seawater at 30°C, while it was 2.2 and 4.4 days, respectively at 22°C in unfiltered natural seawater (Wetz et al., 2004). Longer time periods (lower rates of virus inactivation) were documented for filtered seawater and distilled water indicating that much of the virus decay was caused by factors biological in nature (i.e., microbial activity). Little difference was noted in the decay rates of the virus due to temperatures. In a different experiment with the same poliovirus strain

comparing sterile and non-sterile marine waters under different environmental conditions (e.g., Temperature 22 – 27°C; Salinity 8 – 35%; Turbidity <1 – 4 nephelometric turbidity units, polluted versus non-polluted water, sunlight vs darkness), the T90 values in marine waters kept in the dark ranged between 1 and 1.1 days whereas only 0.4 days were required for a $1\log_{10}$ reduction of the virus in marine waters and the inactivation rate in the polluted canal water was 3x faster than in non-polluted marine waters with sunlight (Johnson et al., 1997).

PV remained infectious for 90 days at 10°C in wastewater and groundwater and persisted for more than a year in mineral water stored at 4°C (Biziagos et al., 1988).

Differences in T90 inactivation rates for EVs in fresh water depends on the water temperature: 1 to 1.5 days at 12 - 20°C (O'Brien and Newman 1977 as cited in (Carter, 2005)) while inactivation by 6.5 to 7 $\log_{10}$ units for CV-B3, Echovirus-7 and PV1 required 8 weeks at 22°C, and a 4 to 5 $\log_{10}$ inactivation of the same viruses at 4°C required 12 weeks (Hurst, 1988a).

2.3.2 Persistence in groundwater

Persistence of EVs in groundwater can be inferred from temperature-based inactivation studies (John and Rose, 2005; Schijven and Hassanizadeh, 2000). As above, variations in inactivation times for the same enterovirus or other members within the *Picornaviridae* family have been reported: The mean inactivation rate of PV was to 0.02 $\log_{10}$ per day (range 0.005 – 0.05 $\log_{10}$ per day) between 0 and 10°C and 0.4 $\log_{10}$ per day (range 0.006 – 1.4 $\log_{10}$ per day) at 26– 30°C; the mean inactivation rate for echovirus was 0.1 $\log_{10}$ per day (range 0.05 – 0.2 $\log_{10}$ per day) at temperatures between 11 and 15°C, and 0.2 $\log_{10}$ per day-1 (range 0.06 – 0.6 $\log_{10}$ per day) at temperatures between 21 and 25°C; and the mean inactivation rate for coxsackieviruses was 0.06 $\log_{10}$ per day-1 (range 0.002 – 0.2 $\log_{10}$ per day) at temperatures between 0 and 20°C and 0.1 $\log_{10}$ per day at temperatures between 25 and 30°C.

2.3.3 Persistence In biofilms

Incorporation of enteroviruses into biofilms of drinking water distribution systems and into natural waste water biofilms protects the virus genome from degradation and to a certain extent, prevents inactivation (Skraber et al., 2009) and Vanden Bosschose (cited in (Skraber et al., 2005)). Persistent EVs may be present in biofilm sloughs in water distribution systems, and persistence of EVs in wastewater biofilms may extend the temporal dispersal of viral pollution in water environments after periods of prevalence in wastewaters.

2.3.4 Persistence In soil

Table 9 provides the data on inactivation rates of enteroviruses in soils. Poliovirus can survive for at least 12 days in soils at 37°C and for months at temperatures at or below 4°C when moisture content is above 2.9%, but are

rapidly eliminated within a few days when moisture content is ≤1.2% (Dowdle et al., 2006). Sensitivities may vary among different isolates of the same virus serotype (for example see Dowdle et al. (2006) for differences between oral poliovirus strains and wild polioviruses).

Table 9. Reported enterovirus inactivation rates in soils

Virus	Inactivation Rate Log_{10}/d	Temperature (°C)	Medium	Sand/Silt/Clay %	pH
Echovirus 1	0.057	25	Cecil clay	46/18.9/35.1	4.6
Echovirus 1	0.057	25	Ponzer muck	NR[a]	3.6
Echovirus 1	0.095	25	Cecil clay	46/18.9/35.1	4.6
Echovirus 1	0.048	25	Corolla sand	98.1/0.8/1.1	8.3
Echovirus 1	0.026	25	Kaolinite	0.5/0.5/99	NR
Echovirus 1	0.029 to 0.057	25	Bentonite	0.5/0.5/99	NR
Echovirus 1	0.032 to 0.048	25	Flushing meadows	89/8/3	NR
Echovirus 1	0.036	25	Corolla sand	98.1/0.8/1.1	8.3
Echovirus 1	0.036	25	Kaolinite	0.5/0.5/99	NR
Echovirus 1	0.048	25	Ponzer muck	NR	3.6
Echovirus 1 V239	0.007	5	Rubicon sand	92/4/4	7.1
Echovirus 1 V239	0.019 to 0.022	25	Pomello sand	89/8/3	7.1
Echovirus 1 V239	0.026 to 0.027	25	Flushing meadow sand	89/8/3	7.8
Poliovirus 1	0.019	25	Pomello sand	89/8/3	7.1
Poliovirus 1	0.007	5	Rubicon sand	92/4/4	7.1
Poliovirus 1	0.02 to 0.027	25	Flushing meadows sand	89/8/3	7.8
Poliovirus 1	0.016	25	Pomello sand	89/8/3	7.1
Poliovirus 1	0.009	5	Rubicon sand	92/4/4	7.1
Poliovirus 1	0.071	25	Ponzer muck	NR	3.6
Poliovirus 1	0.041	25	Corolla sand	98.1/0.8/1.1	8.3
Poliovirus 1	0.032	25	Flushing meadows	89/8/3	NR
Poliovirus 1	0.026	25	Bentonite	0.5/0.5/99	NR
Poliovirus 1	0.095	25	Ponzer muck	NR	3.6
Poliovirus 1	0.071	25	Flushing meadows	89/8/3	NR
Poliovirus 1	0.032 to 0.036	25	Cecil clay	46/18.9/35.1	4.6
Poliovirus 1	0.041	25	Kaolinite	0.5/0.5/99	NR
Poliovirus 1	0.095	25	Coroll sand	98.1/0.8/1.1	8.3
Poliovirus 1	0.048	25	Bentonite	0.5/0.5/99	NR
Poliovirus 1	0.029	25	Kaolinite	0.5/0.5/99	NR

[a]No: Not reporte°

Studies have shown that the extent of virus adsorption differ among the soil types and among the viruses. The rates of inactivation of poliovirus and echovirus vary due to differences between the two viruses in adsorption to soils. Poliovirus adsorbs most extensively by clay soils (cecil clay, bentonite, kaolinite) than echovirus and therefore shows longer persistence in clay soils than echovirus. In general, echovirus reductions in sandy soil columns were lower than those of poliovirus. Moreover, the extent of echovirus reduction in columns was not strongly influenced by soil texture but was greater at the higher temperature of 25°C. At 5°C both viruses show lower inactivation rates than at higher temperatures. The data reported in this table show ranges of inactivation for poliovirus and echovirus in soils when multiple assays were conducted.

Source: (Yates, 2002)

Studies conducted during the early 1980s found that EV persistence in soils was influenced by temperature, soil moisture content, degree of virus adsorption to soil, soil levels of resin-extractable phosphorus, exchangeable aluminum and the pH (Hurst et al., 1980). Table 10 shows the data on sorption behavior of enteroviruses with various types of sorbents. Variations in experimental conditions in the various studies make it difficult to draw general conclusions. Variations in experimental conditions in the various studies make it difficult to draw general conclusions. However, in general, the mechanisms of virus adsorption to solid surfaces depend on the chemical composition of the liquid phase and the nature of the solid surface. Key variables influencing sorption are pH and ionic strength of the solution, presence of compounds competing for sorption sites, functional groups, and isoelectric points of virus and sorbent. EVs can persist in soils for prolonged periods of time, especially at lower temperatures (4 – 8°C) than at higher temperatures (20 – 37°C) (Rzezutka and Carducci, 2013; Vasickova and Kovarcik, 2013) and under in anaerobic conditions (Yates, 2002). Depending on the type of virus and sorbent, sorption is dominated by electrostatic interactions, van der Waals forces, hydrophobic effects, and covalent binding to surfaces (Yates, 2002). EV adsorption to clays protect the virions from inactivation by increasing stability of the viral capsid, by adsorption of enzymes and other inactivating substances, and prevention of aggregate formation (Gerba, 2007).

Table 10. Enterovirus adsorption to soils

Virus	Adsorption Coefficient (ml/g)	Soil Texture			pH	Surface Area (m²/g)	CEC (meq/100g)	Organic Matter (%)	Remarks
		Sand (%)	Silt (%)	Clay (%)					
Coxsackievirus B3	32.33	59	13	28	8	52	23	1.4	buffer
Coxsackievirus B3	32.33	40	24	36	8	105	30	0.78	buffer
Coxsackievirus B3	2.7	92	4	4	5.5	18	5.6	0.4	buffer
Coxsackievirus B3	0.54	77	10	13	8.2	38	4.2	0.27	buffer
Coxsackievirus B3	4.88	13	16	53	4.9	15	53	1.4	buffer
Coxsackievirus B3	11.5	26	20	54	7.1	203	71	4.2	buffer
Coxsackievirus B3	249	48	13	39	4.5	84	32	0.3	buffer
Echovirus	0.0309	98.1	0.8	1.1	8.3	NR[a]	NR	NR	Secondary effluent pH 7
Echovirus	0.042	98.1	0.8	1.1	8.3	NR	NR	NR	Primary effluent pH 7
Echovirus 1	0.12	59	13	28	8	52	23	1.4	buffer
Echovirus 1	0.27	26	20	54	7.1	203	71	4.2	buffer
Echovirus 1	3.55	92	4	4	5.5	18	5.6	0.4	buffer
Echovirus 1	0.15	77	10	13	8.2	38	4.2	0.27	buffer
Echovirus 1	332.33	48	13	39	4.5	84	32	0.3	buffer
Echovirus 1	9	13	16	53	4.9	15	53	1.4	buffer
Echovirus 1	99	46	19	35	4.6	NR	NR	NR	Ground water pH 7
Poliovirus 1	99	46	19	35	4.6	NR	NR	NR	Ground water pH 7
Poliovirus 1	0.724	96.1	0.8	1.1	8.3	NR	NR	NR	Primary effluent pH 7
Poliovirus 1	99	46	19	35	4.6	NR	NR	NR	Primary effluent pH 7
Poliovirus 1	0.515	98.1	0.8	1.1	8.3	NR	NR	NR	Secondary effluent pH 7
Poliovirus 1 LSc	19	26	20	54	7.1	203	71	4.2	buffer
Poliovirus 1 LSc	4.58	77	10	13	8.2	38	4.2	0.27	buffer
Poliovirus 1 LSc	1.27	92	4	4	5.5	18	5.6	0.4	buffer
Poliovirus 1 LSc	15.67	13	16	53	4.9	15	53	1.4	buffer
Poliovirus 1 LSc	99	59	13	28	8	52	23	1.4	buffer

Virus	Adsorption Coefficient (ml/g)	Soil Texture			pH	Surface Area (m²/g)	CEC (meq/100g)	Organic Matter (%)	Remarks
Poliovirus 1 LSc	999	48	13	39	4.5	84	32	0.3	buffer

[a]NR: Not reported

The adsorption of viruses to soils and other surfaces may prolong or reduce their survival, depending on the properties of the sorbent. However, the mechanisms whereby adsorption to a solid surface prolongs or reduces virus survival have not been elucidated. Several possibilities have been suggested, including interference with the action of virucides, increased stability of the viral protein capsid, prevention of aggregate formation, and adsorption of enzymes and other inactivating substances. 25°C. Viruses, like poliovirus, are adsorbed relatively efficiently by soils with high clay content and relatively poorly by soils with a high sand content. The latter is also applicable to enteroviruses such as echoviruses and coxsackieviruses. The data presented in this table also indicate that under most test conditions, differences in water quality did not influence virus adsorption efficiency.

Source: (Yates, 2002)

3.0 Reductions by Sanitation Management

Factors affecting removal of viruses from wastewater are discussed below. Removal of virus from wastewater is often incomplete resulting in viral pollution of the environment at levels that are still sufficient for viral transmission (Okoh et al., 2010; Rodriguez-Diaz et al., 2009; Theron and Cloete, 2002). In fact, wastewater effluents are often the major source of enteric virus contamination of the environments (Simmons et al., 2011). The review by Stenstrom et al (Stenström et al., 2011) has provided a detailed description of risk of exposure and transmission of enteric pathogens related to various sanitation technologies. Applying reduction values determined for a facility measured in the past to the same type of facility today is not straightforward. On one hand, Improvements in technology and monitoring techniques for measuring inactivation/removal data over time mean extending the lower limits of detection, while on the other hand, inactivation/removal efficiencies may be overestimated for older facilities as equipment deteriorates with age time (Chong et al., 2012).

3.1 Wastewater Treatment

The following discussion is mainly limited to reviewing reports that included data on enterovirus inactivation/removal. The majority of the reports also contained data on inactivation/removal of other enteric pathogens such as non-enterovirus enteric viruses, bacteria and parasites. This comparative data, highly relevant when considering the use of indicator organisms to represent behavior of all of the enteric pathogens has not been included.

3.1.1 Onsite waterless sanitation

3.1.1.1 Passive inactivation by storage of human excreta

A general consensus is that most, but not all, enteric viruses will be removed from solid excreta by storage of 1.5 – 2 years at 20°C (WHO, 2006). Alkaline treatment at temperatures >35°C, sufficient moisture and pH >9 can reduce this time to 6 months. Elevated temperature, rising pH and ammonium concentrations are the main inactivating agents for urine stored in closed containers, resulting in a $1\log_{10}$ decrease in rotaviruses and index bacteriophages at 20°C within 35 and 71 days, respectively, but almost none at 5°C (Stenström et al., 2011). Survival times differ in different natural settings (see Table 12). Inactivation times for fecal bacteria (enteric viruses were not tested) were slower on beach sand adjacent to contaminated seawater (Zhang et al., 2015), while survival timeswere longer for particle-associated poliovirus in seawater than for free poliovirus (Rao et al., 1986).

3.1.1.2 Dry sanitation for small systems or individual households

Dry sanitation includes pit latrines, vault toilets, dry toilets, urine separating toilets, and composting toilets and the information about the survival of human pathogenic viruses during storage, treatment and utilization of composted feces is scarce and scattered over the literature (Guardabassi et al., 2003).

The data suggest that in pit latrines all pathogens are likely to be destroyed after storage of > two years (Stenström et al., 2011). Yet the requirements depending on the temperature only mandate 1 hour up to 2 weeks. Consistent with pit latrines as a source of this viral contamination is the observation that most of the ground water contamination that was associated with pit latrines was reported within 50 meters downstream of the latrines (Tillett, 2013; Verheyen et al., 2009) especially in freshly dug latrines where establishment of biofilm and maximum soil-filtering may take months to establish (Graham and Polizzotto, 2013). Viral contamination of ground water has often been associated with proximity to pit latrines where transport of the virus may occur through ground water flow in the upper part of the soil and/or by surface water runoff (Abbaszadegan et al., 2003; Borchardt et al., 2003; Borchardt et al., 2004; Fout et al., 2003; Graham and Polizzotto, 2013; Keswick et al., 1982; Sagik et al., 1980; Tillett, 2013; Verheyen et al., 2009) (see also Section 2.0). Seasonal contamination of the environment especially surface waters can occur during flooding for pit latrines

located in flood-prone areas (Stenström et al., 2011). The amount of contamination detected depends on hydrological and soil condition, rainfall including the height of the groundwater table and soil condition, the methods chosen for detection, and the viral target (Graham and Polizzotto, 2013; Rao et al., 1986; Stenström et al., 2011; Tillett, 2013). Enteroviruses were recovered from the walls of the latrine, surface soil, soil at a depth of 10 cm, food preparation area and inside the home, and on toys and dishes (Pickering et al., 2012). Vault toilets are "improved pit latrines" where excreta are sealed off from the environment by containment in closed tanks. The contents of these tanks are periodically removed and transported to wastewater treatment facilities for decontamination. Enteric viruses may undergo degradation and inactivation to varying extents during storage, while binding to organic particles may act to prolong viability. Solids must be removed for treatment to inactivate/remove viruses at waste treatment facilities (see Section 3.1.8) or for composting (this Section below).

Dry toilets do not use flush water to dilute and remove excreta. They conserve water and reduce the cost of preparation of gray water for reuse compared with flush toilets that combine wet and solid waste with the greywater (Magri et al., 2013). Virus inactivation is dependent on the retention time and the pH and ammonia content during desiccation as demonstrated for bacteriophages MS2 and ΦX (Magri et al., 2013). Six months storage after last use may be sufficient to reduce pathogens in the solids for all climates, but secondary treatment such as thermophilic composting at 50°C, longer storage, chemical treatment with urea, and heat is recommended (Rieck et al., 2012). Under low moisture conditions, a 6 to 12 month storage

time is required to reduce viruses by up to 4 $\log_{10}$ units as opposed to bacteria and some parasites which may be reduced by up to 6$\log_{10}$ (Stenström et al., 2011). While bacteria will be eliminated within 1.5 to 2 years at 4 to 20°C, some virus may still persist (Stenström et al., 2011). In contrast, Dutch legislation implies that controlled composting (exposure to 55°C for two weeks) or controlled sanitation (exposure to 70°C for one hour) ensures complete inactivation of all human pathogenic viruses occurring In human feces (Guardabassi et al., 2003).

The urine contains useful nutrients (such as phosphorus, nitrogen, potassium, sulphur and micronutrients). Urine is generally considered to have a much lower pathogen load than solid excreta, but may also contain bacteria or viruses from certain viral infections (Rieck et al., 2012) and/or be contamination by incomplete separation from solid excreta (Stenström et al., 2011). Urine can be infiltrated in soil or be treated by storage in closed containers to eliminate potential pathogens from cross contamination with solid excreta or from certain viral infections (Rieck et al., 2012; Stenström et al., 2011).

Table 11 demonstrates the relationship between type of waterless sanitation mechanism and log reduction of viruses. Elevated temperature, rising pH and ammonium concentrations were the main inactivating agents for stored urine. As for solid waste, storage time, temperature, pH and ammonia concentration influenced the inactivation/removal of the pathogens. Rieck et al (Rieck et al., 2012) reported that storage for one month was sufficient for crops that were cooked, whereas six months was required for edible crops that were eaten uncooked while others recommend different inactivation times (Stenström et al., 2011).

Table 11. Recommended inactivation conditions for reducing enteric viruses and virus surrogates in pit latrines and dry toilets to below health risk levels

Matrices Analyzed	Virus	$\mathrm{Log_{10}}$ Reductions	Time Days	Temperature °C	Other Environmental Conditions	References
Double Pit latrine	Enteric viruses	Partial to complete	>730	4- 20	NR	Stenström et al., 2011
Double Pit latrine	Bacteriophage MS2 and phiX	7	83 to 125	NR[a]	Dessication	Magri et al., 2013
Double Pit latrine	Viruses	4	180 to 275	NR	Low moisture	Stenström et al., 2011
Composting toilets[b]	NR	~Complete	365 to 730	Mesophilic	30:1 carbon:nitrogen	Berger, 2011
Composting toilets[b]	NR	~Complete	14	55	30:1 carbon:nitrogen	Berger, 2011
Composting toilets[b]	NR	~Complete	7	60	30:1 carbon:nitrogen	Berger, 2011
Composting toilets[b]	NR	~Complete	7 to 28	50 to 60	30:1 carbon:nitrogen	WHO, 2006

[a]NR: Not Reported; [b]pH 6.5 to 7.5.

Table 12 shows log reductions of enteric viruses by storage in feces, manure and urine. Factors that affect composting most are (1) the mixture of bacteria, fungi and worms; (2) temperature (including that generated from microbial degradation) and time for composting; (3) 40-70% moisture, levels most appropriate for the microorganisms and worms; and (4) probably most important, sufficient aeration (McCaustland et al., 1982; Stenström et al., 2011; USEPA, 1999b).

Table 12. Inactivation of human enteric viruses by storage in feces, manure and urine

Virus	Log$_{10}$ Reductions	Time (Days)	Temperature °C	Other Environmental Conditions	Reference
Poliovirus	1	18.7	20.8	Feces[a] Mixed waste (human/animal)	Deng and Cliver, 1992
Poliovirus	1	6.8	25	Feces[a] Mixed waste (human/animal)	Deng and Cliver, 1992
Poliovirus	1	1.3	37	Feces[a] Mixed waste (human/animal)	Deng and Cliver, 1992
Poliovirus	2	5.5	Ambient	Storage in river water	Dowdle et al., 2006
Poliovirus	2	2.5	Ambient	Storage in sea water	Dowdle et al., 2006
Poliovirus	3 4.5 4.5	14	4 18 25	Storage in river water[b]	Skraber et al., 2004
Poliovirus	1	26	NR[c]	Storage in open sewage canals	Dowdle et al., 2006
Poliovirus	4	83 to 84	25	Passive storage composting Feces	Guardabassi et al., 2003
Poliovirus	4	20 to 21	40	Passive storage composting Feces	Guardabassi et al., 2003
Hepatitis A Virus	1 to 2	100	Low ambient	Passive storage composting contaminated manure	Guardabassi et al., 2003
Hepatitis A Virus	1 to 2	625 to 1250	Low ambient	Feces Passive storage composting	Guardabassi et al., 2003
Hepatitis A virus	4	139	25	Feces Passive storage composting	Guardabassi et al., 2003
Hepatitis A Virus	4	29	40	Feces Passive storage composting	Guardabassi et al., 2003
Rotavirus	1	35	20	Urine	Stenström et al., 2011
Rotavirus	1	71	20	Urine	Stenström et al., 2011

[a]pH 6.9; [b]pH 7.7 to 8.1; [c]NR: Not reported.

Composting toilets are dry toilets where solid and liquid excreta are broken down over time under aerobic conditions by microorganisms (bacteria and fungi) and/or redworms or manure worms into a humus-like end product that can be used to recycle nutrients. Virus survival in composted human feces depends not only in the conditions used for composting, but also in the storage conditions and synergistic interactions between them (Dumontet et al., 1999; Guardabassi et al., 2003).

As diagnostic tests evolve and limits for detection drop, inactivation/removal of pathogens to levels below detection becomes more difficult and may require prolonged composting times, elevated temperatures and even some form of secondary treatment at an external site (Berger, 2011; WHO, 2006).

- Conservative estimates for removal of enteric pathogens under mesophilic batch composting conditions may require composting for 1 to 2 years, while only two weeks or one week are required when composting under thermophilic composting

temperatures of 55°C or 60°C, respectively (Berger, 2011) while the WHO recommends even longer composting times of one month at 55°C to 60°C with a further 2 – 4 month maturation period (WHO, 2006).

- A much less conservative estimate is that the pathogens can be destroyed by composting for one month, one week, one day, or one hour when the composting is performed at 43°C, 46°C, 50°C, and 62°C, respectively.
- Enteric viruses may need shorter times for inactivation compared to enteric pathogens such as helminth eggs.

When deciding which inactivation conditions should be used, the minimum requirements of inactivation should be sufficient to inactivate enteric viral pathogens equivalent to HAV which were shown to remain viable in dried feces stored at 25°C and 42% relative humidity for 30 days (McCaustland et al., 1982). Proper maintenance of composting conditions is important to reduce or eliminate the potential for pathogen transmission to personnel involve in crying out the maintenance which consists mainly of adding bulking agents (biodegradable, carbon rich wood chips, grass clippings, sawdust, chopped straw, etc.) and removing and handling of the finished compost (McCaustland et al., 1982; USEPA, 1999b). Appropriate personal protective equipment (nitrile gloves under rubber gloves, work boots, goggles, disposable polyethylene suits or their equivalent, and face masks more for protection against flies than respiratory protection) should be worn when crying out these tasks (Burton and Dowell, 2011). Risk for infection be EVs and other enteric pathogens that have not yet been inactivated is highest when there is a very short time interval between the last addition of excreta and removal of compost as can occur when there is only a single collection chamber rather than multiple containers whose use can be rotated (Berger, 2011).

3.1.2 Water-based sanitation – onsite and small systems

Septic tanks are onsite, relatively small sewage treatment facilities consisting of a holding tank where solids settle and together with non-settleable materials within the scum layer undergo anaerobic degradation and an outlet for the wastewater to flow into draining fields where clarified effluent percolates into the soil. Johkasou are septic tanks with an obligate aerobic treatment process with some versions including urine separation and membrane filtration (Gaulke, 2006). Settlement of solids and hydraulic retention time are the major processes influencing pathogen removal from wastewater in septic tanks (Canter and Knox, 1985). Most viruses are removed in the soil drainage field depending on death rate, flow rate, filtration, adsorption on soil particles, microbial competition and antagonism, moisture, pH, sunlight, and temperature, while $<2 \log_{10}$ are removed during sedimentation (Hagedorn, 1994; Lusk et al., 2014). In both systems, the solids are periodically removed (see Section 3.1.8 on treatment of biosolid waste) and viruses will accumulate in these solids.

Enteric viruses are typically reduced by in the effluent less than $0.3 \log_{10}$ (Stenström et al., 2011; WHO, 2006). Storage and treatment conditions are important resulting in a 17 to 80 day variation in the number of days for $1\log_{10}$ reduction of PV (Snowdon et al., 1989) (Table 13). Depending on regulations, solid septic waste may be disposed in the environment or preferable receive further treatment in centralized facilities. As in Sections 3.1.1 and 2.0. one of the main health hazards is the ability of viruses to penetrate into groundwater and reach potable water wells (Abbaszadegan et al., 2003; Borchardt et al., 2003; Borchardt et al., 2004; Fout et al., 2003; Keswick et al., 1982; Sagik et al., 1980). Contamination of groundwater with EV and other enteric pathogens from septic tanks depends on: (i) soil properties and the depth of the vadose zone (a subsurface zone of soil or rock containing fluid under pressure that is less than that of the atmosphere) more than on system design; (ii) Soil type and temperature - sandy soils with seasonally high water tables can cause groundwater contamination with enteric pathogens under suboptimal conditions; (iii) soil texture - risks are highest in coarse textured (sand) soils when water tables are shallow (e.g., small vadose zone or saturated soil conditions) and in winter when temperatures are low; and (iv) clay content - large reductions in enteric pathogens are possible when the clay content of the drainage field is at least 15%, the vadose zone is at least 1 m deep, and when the drainage field distribution lines do not become submerged in the groundwater (Butler and Smith, 2002). To achieve a level of safety according to risk assessment and geostatistical investigation by the USEPA, septic tank fields should be located 80 – 325 m away from abstraction wells, depending on aquifer and source characteristics to allow for natural reduction of up to $11 \log_{10}$ between source and ground water (Butler and Smith, 2002).

Table 13. Poliovirus reductions by septic tanks as water-based sanitation

Virus	Log$_{10}$ Reductions	Time Days	Temperature °C	Other Environmental Conditions	References
Poliovirus Type 1	1	80	5	Lab model - septic tank effluent alone	Snowdon et al., 1989
Poliovirus Type 1	1	19	5[a]	Lab model - septic tank effluent mixed with dairy manure slurry	Snowdon et al., 1989
Poliovirus Type 1	1	72	15	Lab model - septic tank effluent alone	Snowdon et al., 1989

Virus	Log$_{10}$ Reductions	Time Days	Temperature °C	Other Environmental Conditions	References
Poliovirus Type 1	1	20	15	Lab model - septic tank effluent mixed with dairy manure slurry	Snowdon et al., 1989
Poliovirus Type 1	1	19	25	Lab model - septic tank effluent alone	Snowdon et al., 1989
Poliovirus Type 1	1	17	25	Lab model - septic tank effluent mixed with dairy manure slurry	Snowdon et al., 1989

[a]Significantly shorter inactivation times when manure was added were attributed to progressive strong adsorption of virus to manure solids such that the virus was no longer extractable and at least in part, to bacteria and perhaps to the organic acids in the slurry (Snowdon et al., 1989).

3.1.3 Waste stabilization ponds

Waste stabilization ponds are used extensively worldwide for wastewater treatment including use as systems supplying reclaimed wastewater for agricultural purposes (see review by Verbyla and Mihelcic (Verbyla and Mihelcic, 2015)). Verbyla and Mihelcic analyzed reports describing 71 different ponds (anaerobic, facultative, maturation, surface aerated ponds, and any of these used in conjunction with other wastewater treatments). They calculated an average 1log$_{10}$ reduction in enteric virus for every 14.5 to 20.9 days of retention (with up to 54 days for two standard deviations of reported values). The wide variations in physical, chemical, and biological characteristics of the ponds make it difficult to relate inactivation/reduction of viruses directly to fluid retention times. Ponds are frequently integrated with primary and secondary wastewater treatment (see Section 3.1.5). Inactivation/removal of viruses, particles, and particle-associated viruses during these other steps will influence the viral loads reaching and subsequently being processed in the ponds (Symonds et al., 2014).

Climate, temperature, pH and ammonium concentration, sunlight, interaction of virus with particles and microorganisms, and pond geometry, hydraulic conditions, and retention time are important factors in determining whether, and to what extent, the maximum reported reduction of enteric viruses by 4log$_{10}$ in waste stabilization ponds (WHO, 2006) can be approached (Stenström et al., 2011; Verbyla and Mihelcic, 2015; WHO, 2015a). For example, Oragui et al (Oragui et al., 1987) reported 3 log$_{10}$ reduction for enteroviruses in a series of anaerobic, facultative and retention ponds with an overall retention time of 21 days at a temperature of 25°C. The relative contribution of each of the above factors towards inactivation/removal depends on the type of virus and to some extent on the micro flora and fauna communities residing in the ponds (Verbyla and Mihelcic, 2015). Some factors, such as sunlight, may work directly on the virus and/or indirectly by forming chemically reactive intermediates or radicals. Estimated average inactivation times for poliovirus in sludge formed in waste stabilization pools varied from 180 days at 2°C to 26 days at 23°C (WHO, 2015a). Some ponds also include further UV, ozone, peracetic acid, and/or chlorination steps to remove residual enteric pathogens (See Sections 3.2).

3.1.4 Wetlands

Dissolved biodegradable material is removed from wastewater in wetlands by microorganisms (bacteria, fungi, and actinomycetes) in suspension, growing on soils, and growing on aquatic plants. The plants and algae provide oxygen for aerobic degradation, while anaerobic degradation can occur in settled material. Effluents compared to influents have lower BOD and generally lower pH due to biological activities. Plants also play an active role in taking up nitrogen, phosphorus, and other compounds from the wastewater (Robert A. Gearheart in (USEPA, 1993)). Variation in EV reduction may be high due to wide changes in physical conditions and biological compositions of plants and microorganisms in different wetlands. A combination of elevated water temperature and biological activities in natural wetland water reduced PV1 by 1 to 4 log$_{10}$ (Rachmadi et al., 2016) and EV removal ranged from 2 to 3 log$_{10}$ (Williams et al., 1995). Additional data on removal of EVs in wetlands for wastewater treatment are given in Table 14. Virus persistence (see Section 2.3) is also relevant when considering the minimal time required for removal/inactivation of EVs in wetlands. A viral inactivation step to reduce the load of enteric viral pollutants prior to influx reduces the risk for transmission. However, once EVs enter wetlands, there was significantly more inactivation/removal in the presence of aquatic plants (Gersberg et al., 1987; Karim et al., 2008; Schaub et al., 1980) and aerobic organisms in sandy loams (Hurst, 1988b).

Table 14. Enterovirus reduction by environment-based systems: lagoons and ponds

Matrices Analyzed	Virus	Log$_{10}$ Reductions	Time Days	Temperature °C	Other Environmental Conditions	References
Anaerobic, facultative and retention ponds	Enterovirus	3	21	25	NR[a]	Oragui et al., 1987
Lagoons and ponds	Enteric viruses and coliphage	1	14.5 to 20.9	Variable	Review of literature on 71 ponds	Verbyla and Mihelcic, 2015
Oxidation pond	Poliovirus	3	70	18 to 23	NR	Kott et al., 1978
Wetlands	Enterovirus	2 to 3	0.25	22 to 27	Gravel bed hydroponics constructed wetlands	Williams et al., 1995
Gravel-based constructed wetlands[b]	Poliovirus	0.8 to 1.0	NR	NR	Presence of plants significantly increased removal	Karim et al., 2008
Land treatment - managed aquifer recharge	Enterovirus	1 to 3.7	NR	NR	Recharge of ground water by filtration through soil	Betancourt et al., 2014

[a]NR:Not Reported; [b]pH 6.9 to 7.9.

3.1.5 Aerated lagoons

Aerated lagoons are large holding basins where wastewater is aerated to facilitate and accelerate biological degradation of BOD. There are two types, one where energy is supplied to keep the suspension from settling and the other where particles in the suspension settle and then are degraded anaerobically. The retention times are usually short ranging from 6 to 18 days. BOD removal varies from 75 to 90 percent depending on dwell times. Efficiency depends on physical factors (type of soil, surface area, depth, wind action, sunlight, temperature, season), chemical factors (organic material, pH, solids, concentration and nature of waste), and biological factors (type of bacteria, type and quantity of algae, activity of organisms, nutrient deficiencies, toxic concentrations) (Annon, 2015a; USEPA, 2000a).

In early studies of serial ponds, Bausum (Bausum et al., 1983) reported between 2 and 3 log$_{10}$ reductions of enteric viruses in different seasons and higher reduction (3 log$_{10}$) in the first pond in each series, presumably to precipitation of particle bound viruses. In another study 1 log$_{10}$ removal/inactivation of enteric viruses only occurred under warm ambient conditions and to a lesser extent than coliphages that ranged between 2.8 and 3.4 log$_{10}$. (Locas et al., 2010). Removal/inactivation of coliphages in sunlight in summer (3 log$_{10}$) was more effective than in winter (1 log$_{10}$) (Fujioka and Yoneyama, 2002).

3.1.6 Oxidation ditches

Microorganisms from activated sludge (see Section 3.1.7.4) are added to digest the biosolids from sewage in oxidation ditches. Stirring aids digestion by increasing the amount of dissolved oxygen available to the microorganisms. In an oxidation ditch, only about 15% of the original BOD ends up as sludge. At the end of each digestion cycle, part of the activated sludge is recycled into the next batch, while the remaining sludge is removed and processed as described in Section 3.1.8.

Viable virus may be concentrated in the sludge by-product, while less than 1 log$_{10}$ of virus may be removed from the effluent of oxidation ditches (Okoh et al., 2010; Simmons and Xagoraraki, 2011b). Viruses may remain viable for prolonged periods of time, There was no decrease in EV in sludge kept at 5°C for 38 and 17 days obtained from an extended aerated ditch or an oxidative ditch, respectively (Berg et al., 1988).

3.1.7 Wastewater treatment plants (WWTPs)

Many reports describe log$_{10}$ reductions between influents and effluents rather than virus concentrations at each stage of treatment. Data representative of both types of reports will be presented in the text and in Table 15.

In treatment plants wastewater undergoes a series of treatment steps before effluents are returned to the environment. The treatment process can be divided into three major sequential stages (Annon, 2015c). In primary treatment, up to 50% of the suspended particles and up to 20% of the BOD are removed by mechanical means. This is followed by secondary treatment where microorganisms that decompose organics remaining after primary treatment using aerobic and/or anaerobic metabolic processes remove between 70 – 90% of the biological and at least 70% of the chemical oxygen demand that was

carried over. Advanced WWTPs can also incorporate a tertiary treatment step to remove more organic material or filtration to remove turbidity and other impurities and can incorporate disinfection steps.

There are many variations available for each of these steps resulting in a plethora of possible combinations and a real difficulty in determining the contribution of each individual component toward reduction/inactivation of enteric viral pathogens. There are also wide variations in populations within catchment areas of WWTPs and in the mix of waters being processed (domestic, domestic mixed with varying amounts of industrial and or agricultural wastewater).

A report by Hewitt et al (Hewitt et al., 2011) comparing ten different WWTP configurations (moving bed biofilm, trickle filter activated sludge with stabilization pond, activated sludge, waste stabilization pond, and waste stabilization pond with wetland), serving different sized catchment areas (population range: <1100 to >1,000,000) with mixed source of wastewater (domestic, domestic plus industrial and/or industrial waste) is illustrative of the difficulty in making simple comparisons. Despite these large differences, they have provided some methods that enabled comparisons independent of the differences. They found that enterovirus profiles and variations of concentrations were generally independent of population size in influents however higher variability was seen for mid and especially small sized populations. They also reported as expected that viable viruses were likely to be isolated from effluents collected before disinfection. This in part reflects the situation where there are a large number of infected individuals in populations, e.g., the populations have a similar epidemiological distribution of viruses, and differs from the situation where there are one or at most a few infected individuals in a population at the initial stage of an emerging viral infection (see discussion in Section 1.4.1 on Eradication and ENVS for PVs.).

Wastewater treatment facilities may be subject to overloads (influx of fluid volumes greater than the maximum volume the WWTP was designed to handle) resulting in larger loading of pathogens into soil, potable, recreational and agricultural surface waters and even groundwater from various stages during processing. An example is excessive rainfall that overwhelms the physical capacity of the WWTPs of older systems where rainwater is not collected separately from sewage, pit latrines (Section 3.1.1), etc. Unusually high pulses of EVs, for example during accidental release of wild neurovirulent poliovirus from a virus production facility (discussed in Section 1.4.1), may result in release of large amounts of virus even if all stages of wastewater treatment function at maximum efficiency. Finally, WWTPs may release viruses when components systems suffer mechanical failure.

3.1.7.1 Primary /preliminary treatment

The primary treatment step is not designed to actively remove/inactivate enteric viral pathogens. Inactivation/removal is passive and dependent on ambient physical and environmental conditions, WWTP component design, flow rates, the amounts of large and micro solids to which enteric pathogens can bind, and retention times (discussed in more detail in other parts of Section 3.0). Enteric viruses content may be reduced in the effluent from primary treatment by up to $1\log_{10}$) after flocculation (Section 3.1.7.5) but they accumulate in the sediment (Section 3.8) where they must be inactivated before sediment is disposed or used.

3.1.7.2 Secondary treatment

Data on enteric virus removal after secondary treatment is also presented in Table 15. The secondary treatment step is also not designed to actively remove/inactivate enteric viral pathogens. Enteric viruses are passively removed from the effluent with the smaller particles to which they are adsorbed. By "removed" we mean that they are accumulated in the sludge which itself must be periodically removed and processed to reduce biological pollutants (see Section 3.1.8). The 1972 US Federal Water Pollution Control Act that mandated secondary treatment of municipal sewage in the USA has ensured that there is plenty of sludge that must be processed (Rao et al., 1986). In steps involving recirculation of activated sludge to seed the microorganisms for biodegradation, viral concentrations may paradoxically increase in various compartments (Rao et al., 1986) (see Sections 2.2.1, 3.1.7.4, and 3.1.7.5). Pathogenic viruses are present in effluents from secondary treatment (Rao et al., 1986) and in the sludge byproduct of the process.

Table 15. Poliovirus reduction by wastewater treatment plants (WWTPs)

Treatment	Virus	Log$_{10}$ Reductions	Temperature	Other Environmental Conditions	Reference
Secondary treatment	Enterovirus	0.7 to 2.1	NR[a]	Primary settlement/ activated sludge	Lodder and de Roda Husman, 2005
Secondary treatment	Poliovirus	1 to 2	Ambient	Removal fine particles	Dowdle et al., 2006
Secondary treatment	F specific RNA bacteriophage	2.1	Ambient	Screening and grit removal / activated sludge	Flannery et al., 2012
Sedimentation	Viruses base line removal[b]	0	NR	Simple[c]	WHO, 2011
Filtration (Granular media)	Viruses base line removal	0.5 to 1	NR	Rapid (RG) slow sand (SS)	WHO, 2011
Secondary and tertiary treatment	Enterovirus	≥3.2	NR	Activated sludge/Filtration in sand filter/UV/Chlorine disinfection	Costan-Longares et al. 2008
Secondary and tertiary treatment	Enterovirus	≥1.9	NR	Activated sludge/settling pond/oxidation pond/ constructed wetland	Costan-Longares et al. 2008
Secondary and tertiary treatment	Enterovirus	≥4.0	NR	Activated sludge/coagulation-flocculation/Filtration in a multilayer filter/UV/Chlorine disinfection 0.3 ppm	Costan-Longares et al. 2008
Secondary and tertiary treatment	Enterovirus	≥4.2	NR	Activated sludge/Coagulation-flocculation/Decantation/ Filtration in sand filter/UV/ Chlorine disinfection	Costan-Longares et al. 2008
Membrane bioreactors	Enterovirus	3.6	NR	Primary settling/Membrane bioreactors	Simmons et al., 2011

[a]NR: Not reported; [b]Baseline Removals are those typically expected in actual field practice when done by relatively unskilled persons who apply the treatment to raw waters of average and varying quality and where there are minimum facilities or supporting instruments to optimize treatment conditions and practices. This is different from Maximum Removals which are those possible when treatment is optimized by skilled operators who are supported with instrumentation and other tools to maintain the highest level of performance in waters of predictable and unchanging quality (e.g. a test water seeded with known concentrations of specific microbes)" (WHO, 2011); [c]Simple Sedimentation refers to the physical settling of large particles and large sedimentable microbes to which virus may have been bound in the absence of any additional factors or treatments that enhance this physical settling process. Sedimentation varies with storage time and the concentration of particulates in the water.

Secondary treatments that incorporate a filtration step (i.e., tertiary treatment) remove more fine particles that in turn reduces effluent virus content by 1 to 2.4log$_{10}$ (Dowdle et al., 2006). A cautionary note: low levels of enteroviruses detected in influent in many studies may result in an overestimation of the efficiency of secondary treatment (Qiu et al., 2015). Secondary treatment may provide substantial but not complete removal of enteroviruses. A protective stabilizing effect has been observed when poliovirus adsorbs to the high amounts of silts and suspended solids associated with wastewaters (Smith et al., 1978).

3.1.7.3 Filtration and trickling filters

Table 16 shows the effect of combined wastewater treatment processes that include tricking filters for removal of enteroviruses from wastewaters. Log removal for each treatment step are given in this table.

Table 16. Enterovirus reductions by wastewater treatment

Area	Virus Type (Unit of Measurement)	Average or Range Log$_{10}$ Reductions	Number of Samples (Facilities)	Description of Treatment Train	Reference
Canada (Alberta)	Enteroviruses (GC qPCR)	2.55±1.28	16 (1)	Primary sedimentation plus activated sludge	Qiu et al., 2015
		0.58±0.96	16 (1)	Activated sludge followed by UV disinfection	
		2.06±1.06	16 (1)	Activated sludge plus ultrafiltration	
		2.81±1.06	16 (1)	Primary sedimentation/Activated sludge/UV disinfection	
		4.61±1.12	16 (1)	Primary sedimentation/activated sludge/ultrafiltration	
Italy	Enterovirus (GC[a] qPCR)	0.18 (NR[b] to 1.5)	250 (5)	Grid separation, primary sedimentation, biological treatment by activated sludge and final disinfection with chlorine.	La Rosa et al., 2010
New Zealand	Culturable enteric viruses (MPN cell culture)	1.37 to 2.82	30 (10)	Moving bed biofilm reactor or activated sludge before UV disinfection	Hewitt et al., 2011
	Enterovirus (GC qPCR)	1.39 to 5.13			
Spain (Mallorca)		0.7	13 (1)	Lagooning	Gomila et al., 2008
		0.5	13 (1)	UV disinfection	
USA	Culturable enteric viruses (MPN cell culture)	1.89±1.09 (0.86 to 3.23)	5 (1)	Grit removal followed by activated sludge. Filter type: Cloth. Filter media Fabric. Chlorine disinfection	Rose et al., 2004
		3.04±0.57 (>3.43 to >4)	5 (1)	Pre-chlorination, Grit removal followed by activated sludge. Filter type: traveling bridge. Filter media: sand. Chlorine disinfection	
		4.27±0.55 (3.6 to 4.81)	5 (1)	Grit removal (primary clarifier) followed by flocculation with cationic polyelectrolyte and activated sludge. Filter type: monomedium. Filter media: anthracite (1.2 m). Chlorine disinfection	
		3.25±0.37 (2.86 to 3.59)	5 (1)	Grit removal followed by activated sludge. Filter type: dual media. Filter media: anthracite (0.8 m) sand (0.25 m). Chlorine disinfection	
		2.66±0.47 (>2.26 to >3.41)	5 (1)	Grit removal; equalization basin followed by nitrification. Filter type: continuously backwashed upflow filter. Filter media: sand (1.2 m) UV disinfection (medium pressure)	
		4.04±0.49 (3.53 to 4.54)	5 (1)	Grit removal followed by biological nutrient removal. Alum added to secondary clarifier. Filter type: dual media. Filter media: anthracite (0.6 m) sand (1.2 m). Chlorine disinfection	

Area	Virus Type (Unit of Measurement)	Average or Range Log$_{10}$ Reductions	Number of Samples (Facilities)	Description of Treatment Train	Reference
USA	Culturable enteric viruses (MPN cell culture)	2.41±0.56 (1.2 to 3.2)	11 (11)	Preliminary, primary, and secondary (activated-sludge) treatment, as well as phosphorus removal, and disinfection using chlorine	Sedmak et al., 2005
USA	Enterovirus (GC qPCR)	3.6 (2.9 to 4.3)	8 (1)	Primary and secondary treatment membrane bioreactor	Simmons et al., 2011
		2.9 (2.0 to 3.7)		Primary and secondary treatment activated sludge	
	Culturable enteric viruses (MPN cell culture)	4.4		Primary and secondary treatment and UV disinfection	
		2.4 (1.9 to 5.0)		Primary and secondary treatment and chlorine disinfection	

[a]GC: Gene copies; [b]NR: Not reported; [c]PFU: Plaque forming units.

3.1.7.4. Activated sludge

Conventional activated sludge (CAS) secondary processing usually involves biological degradation after seeding pre-settled sewage from primary processing with microorganisms present in processed sludge. While viruses are removed by wastewater treatment, the concentration may increase in the activated sludge. Harwood et al (Harwood et al., 2005) reported an average 3 log$_{10}$ reduction of EVs after CAS treatment among six WWTPs in the US. Viable EVs were isolated from 73% of the secondary treatment samples, and 31% of the tertiary disinfected effluent and 100% of the influent samples.

Sedmack et al (Sedmak et al., 2005) reported isolation of EVs in only 62% of the effluent samples and 99.9% of the influent samples from a conventional WWTP in Milwaukee, WI, USA, with minimal and maximal concentration reductions of 1.2 and 3.2 log$_{10}$ MPN/L of EVs during a nine-year study. The Milwaukee WWTP consisted of preliminary, primary, and activated-sludge secondary treatment, as well as phosphorus removal, and disinfection using chlorine. In Canada, a 2.55 ±1.28 log$_{10}$ of EV was removed by activated sludge secondary treatment, (Qiu et al., 2015).

La Rosa et al. (2010) reported low rates (0.19 log$_{10}$) for removal of enterovirus nucleic acids in the effluent from five Italian conventional activated sludge wastewater treatment plants that included grid separation, primary sedimentation, secondary biological treatment and final disinfection with chlorine. 96% of the intake samples and 86% of the effluents samples were enterovirus RNA-positive and many of the enterovirus nucleic acid-positive effluents were shown to contain low levels of culturable EVs. Hewitt et al. (2011) reported slight reductions of culturable EVs in WWTPs of different size compared with a 2 log$_{10}$ reduction of viral nucleic acids by molecular assay for moving bed biofilm reactors (MBBR) and conventional activated sludge (CAS) plants serving large populations and conventional plants that served small populations. Flannery et al.

(Flannery et al., 2012) reported a 2.1 log$_{10}$ reduction in F-specific RNA bacteriophage in a conventional coupled primary (screening and grit removal) and secondary (activated sludge including primary settlement, aeration, and final settlement) WWTP.

3.1.7.5 Membrane bioreactors

Membrane bioreactors (MBRs) are a wastewater treatment technology in which membranes are submerged in the activated sludge tank to separate out solids. In theory viruses should not penetrate the membranes, but as the size of membranes increase, so do the chances for imperfections that would allow virus to penetrate the membrane barrier.

MBR processing was more efficient in microbial removal/inactivation than CAS reactors (Zhang and Farahbakhsh, 2007). A much higher 4.1 – 6.8 log$_{10}$ (average 5.1] decrease in EVs between influent and effluent waters was reported for a full scale MBR system when virus was measured by quantitative RT-PCR (Simmons et al., 2011) compared to CAS. This study also illustrated the importance of viral attachment to biosolids. When particles in the influent to the membrane were allowed to settle, the settled solids attached EVs removing 2.5 log$_{10}$ of the starting amount.

MBRs using UV for tertiary disinfection removed enterovirus more efficiently than CAS reactors using chlorine for disinfection, while chlorine treatment had little additional inactivation effect on enterovirus concentrations (Table 17) (Francy et al., 2012). Tertiary treatment in CAS reactors added a 0.5log10 removal of EVs to the total reduction from secondary treatment (Ottoson et al., 2006b). The reduction in culturable EVs was slight compared to a 2log10 reduction by molecular assay for moving bed biofilm reactors and CAS plants serving large populations and CAS plants serving small populations (Hewitt et al., 2011). As discussed above, factors including isoelectric points, hydrophobicity, temperature, pH and hydraulic retention

times account for wide variations in virus removal. Ottoson et al. (2006a) compared CAS, MBR and upflow anaerobic sludge blankets (UASB) reactors run in parallel at the same pilot plant in Sweden and reported a 1.7, 1.8 and 0.5

log_{10} reduction in EVs, respectively. Of significance was their finding that 36% of the effluent samples from the CAS reactor contained enteroviral genomes, a similar number for the MBR line and even higher for the USAB line.

Table 17. Enterovirus reduction by tertiary treatment

Treatment	Virus Type	Log$_{10}$ Reductions	Other Environmental Conditions	Reference
Membrane bioreactors (MBR)[a]	Enterovirus	2.2 to 4.7 1.1 to 3.7	MBR (full scale) secondary conventional MBR (full scale) + UV secondary convent. + UV	Francy et al., 2012
Membrane bioreactors	Enterovirus	5.1	MBR (full scale)	Simmons et al., 2011
Membrane bioreactors[a]	Enterovirus live Enterovirus genomes	<1 ~2	MBR (full scale) medium and large	Hewitt et al., 2011
Membrane filtration	Viruses base line removal[b]	0 3 3 3	Micro (MF), Ultra (UF), Nano (NF), Reverse osmosis (RO)	WHO, 2011
Membrane filtration	Viruses base line removal	0	Fiber and fabric	WHO, 2011
Membrane bioreactor	Enterovirus	1.8	MBR: 30 mm drum filter MBR with a submerged microfilter, membrane module (0.4 mm)	Ottoson et al., 2006a
Porous membranes	Viruses base line removal	NR[c]	Porous ceramic and carbon block filtration	WHO, 2011
Tertiary treatment post secondary	Enterovirus	2		Dowdle et al., 2006
Enhanced[d] coagulation with FeCl$_3$	Coxsackie virus B6, Echovirus 11, Poliovirus	1.8 to 3	Coagulant dose of 40 mg/L FeCl$_3$ and a pH below 6.5	Mayer et al., 2008

[a]Run at ambient temperatures; [b]Baseline Removals are those typically expected in actual field practice when done by relatively unskilled persons who apply the treatment to raw waters of average and varying quality and where there are minimum facilities or supporting instruments to optimize treatment conditions and practices. This is different from Maximum Removals which are those possible when treatment is optimized by skilled operators who are supported with instrumentation and other tools to maintain the highest level of performance in waters of predictable and unchanging quality (e.g. a test water seeded with known concentrations of specific microbes)" (WHO, 2011); [c]NR:Not Reported; [d]pH of 6.5, 20°C at 51 minutes contact time.

The biological and biochemical interactions that occur during membrane bioreactor processing and the physical and hydrodynamic processes of MBRs are very complex (see the review by Zuthi et al. (2012) on modeling these processes in order to design and maintain efficient MBRs). While many of the bioprocesses resemble those that occur in conventional activated sludge (CAS) plants during steady state processing, they are influenced by physical and hydrodynamic parameters specifically associated with MBRs. Most notably are problems associated very long sludge retention times compared with conventional activated sludge reactors (Section 3.1.7.4). Longer retention times allow greater buildup of soluble microbial products, extracellular polymeric substances and higher

concentrations of suspended solids that increase viscosity, alter dynamic microbiological equilibriums, alter the oxygen supply available for biological degradation processes, reduce membrane permeability, increase membrane fouling, affect trans-membrane pressure and flux, and may contribute to a greater tendency for foaming and higher energy costs.

The complexity produces an apparent paradox relating to removal/inactivation of EVs. On one hand, there is a significant decrease in the amount of enterovirus between the influent the effluent of MBR WWTPs. On the other hand, during eight months of observation of the secondary

processing stage, the concentrations of enterovirus virus influent to the membrane, e.g., samples taken after the point of addition of returned sludge, were 1.6 $\log_{10}$ higher (p-value < 0.05) compared with the concentration in primary influent (5.2 $\log_{10}$) and effluent concentrations (Simmons et al., 2011). This increases the difficulties in processing sludge for reuse or disposal (see Section A.8.). Research pertaining to membranes and improvements in membrane performance for MBRs is reviewed annually in Water Environment Research (for example, see Pellegrin (Pellegrin et al., 2011).

Williams (2003) reported that the effectiveness of micro and ultra filtration membrane reverse osmosis for exclusion of biological contaminants varied widely (from 0 – 3 $\log_{10}$) depended on the characteristics of the organic contaminant (polarity, size, charge, etc.), physiochemical properties of the membrane, and the reverse osmosis operating conditions (feed pH, operating pressure, etc.). William's review did not refer to use of reverse osmosis to exclude biological contaminants. Howeverin theory, pathogens that are larger in size than the pore size of the membrane should be prevented from passing through the membrane (exclusion based on size). In practice, there is a pore size distribution throughout the filter membrane in addition to imperfections in the membrane or the support system for the membrane that allow a proportion of virus to pass through (Antony et al., 2012).

3.1.7.6 Anaerobic /anoxic digestion and biogas

Poliovirus inactivation of 3 $\log_{10}$ within 2 hours under thermophilic anaerobic sludge digestion was reported at temperatures ranging between 51°C to 56°C (Popat et al., 2010).

There are many different treatment configurations for anaerobic/anoxic digestion and biogas formation from biosolids produced during treatment of wastewater and new technologies chemical cell destruction, micro-sludge, increased digestion of biomass and biogas production by using NaOH and high-pressure disruptors to break up bacterial cells are emerging (USEPA, 2006). Ultrasonic cell destruction using acoustic waves instead of NaOH, broke up cellular material in the biosolids reducing anaerobic oxidation times, and increased collectible biogas availability for energy production. Electrocoagulation using an electric current to introduce aluminum ions to suspended biosolids, increased particle aggregation until aggregates precipitated, while gas bubbles floated the precipitated aggregates to the surface where they were removed along with >1 $\log_{10}$ coliform. Yet most of these techniques have not been evaluated for EV reductions.

Mass reduction by thermal hydrolysis reduces biomass by dewatering by 0.07 – 0.9 $\log_{10,}$ and when followed by oxidation at 160°C and 100 psi of pressure and then anaerobic digestion, destroyed pathogens and broke cells reaching high levels of volatile solid destruction and biogas production. Three phase anaerobic digestion (fatty acid digestion; anaerobic thermophilic gas digestion) followed by digestion at temperatures >35°C improved pathogen destruction (Willis and Schafer, 2006), increased biogas production and produced Class A biosolids (defined in Section 3.1.8). Laboratory scale anaerobic digestion with low levels of ozone (0.06 g of ozone per gm of dissolved solids) increased average digestion of biosolids by 0.5 $\log_{10}$ and increased biogas production by 0.2 $\log_{10}$ over conventional anaerobic digesters.

Critical factors affecting subsequent process operation performance included conditions and inoculum during start-up of anaerobic digesters and subsequent temperature fluctuations in reactors running between 46 to 53°C (a range too high for mesophilic microorganisms and below an optimal 55°C for hemophilic organisms (De la Rubia et al., 2013). In upflow anaerobic sludge blanket (UASB) reactors startup may take 2 to 8 months (Chong et al., 2012). Energy recovered from the biogas produced by anaerobic processing is a useful byproduct but is not sufficient by itself to justify anaerobic processing for energy recovery (Kumar, 2011). Combining a mesophilic anaerobic and a thermophilic aerobic processor increased methane production by 24-fold when the feed sludge and re-circulated sludge received thermal alkaline pretreated together (Cho et al., 2013).

3.1.8 Biosolids

One of the byproducts of wastewater treatment is the accumulation of sludge or biosolids precipitated during primary and secondary treatment that must be disposed. Table 18 shows reductions of enterovirus in processed activated sludge.

Table 18. Enterovirus reduction by biosolid treatments

Treatment	Virus Type	Log$_{10}$ Reductions	Time Days	Temperature ºC	Other Environmental Conditions	Reference
Activated sludge	Poliovirus in soil	1	Few days <1hr 12 180 1.5 20	NR[a] Ambient 37 4 Summer Winter	Moisture <2.9% Dry air stream Moisture >2.9% Moisture >2.9% Moist Moist	Dowdle et al., 2006
Biosolids treatment (review of literature)	Enteroviruses	3.6 3.5 1.3	NR	NR	COM[b] TPAD[c] MAD[d]	Viau et al., 2011
Biosolids treatment	Enteroviruses	2	NR	NR	In 3 WWTPs where sludge treated by MAD or dewatering	Simmons and Xagoraraki, 2011b
Biosolids treatment (33% activated sludge + 67% raw sludge)	Enteroviruses	1 to 3	20 days	~35 ~49	Mesophillic Thermophilic	Berg and Berman, 1980
Conventional Activated sludge	Enteroviruses	1.7	NR	NR	CAS[e]: Pre-sedimentation, activated sludge with biological nitrogen removal, pre-denitrification and chemical precipitation	Ottoson et al., 2006
Conventional Activated sludge	Poliovirus	2.5	NR	NR	Labscale simulation	Wen et al., 2009
Upflow anaerobic sludge blanket	Enteroviruses	0.5	NR	NR	UASB: Presedimentation, two UASB reactors in series, chemical precipitation and a final 20 mm drum filter	Ottoson et al., 2006

[a]NR:Not Reported; [b]COM: Compost; [c]TPAD: Temperature-phased anaerobic digestion; [d]MAD: Mesophilic anaerobic digestion; [e]CAS: Conventional activated sludge.

Simmons et al. (2011) reported on accumulation of EVs in the sludge itself for three WWTPs with the following primary, secondary and tertiary configurations (1) (MBR) / Mesophilic anaerobic digestion (MAD) /UV; (2) activated sludge /dewatering/chlorination; and (3) rotating biological contactors /MAD/chlorination. The initial influent virus concentration, 4.1 (MPN)/100L (equivalent to 100kg), was concentrated 2x10^5-fold to yield an average of 1.1 to 3.2 (MPN)/gm in the biosolids.

There was an average 4.2 log$_{10}$ (range 1.9 to 5.0 log$_{10}$) reduction in infectious virus in sludge after treatment in a study of five WWTPs (Simmons and Xagoraraki, 2011b). Wong et al. (2010) and Monpoeho et al. (2001) reported an average of 4 log$_{10}$ MPN/gm in class B sludge after dewatering (four WWTPs) or MAD (thirteen WWTPs). Differences in recovery levels after dewatering and some of the variation in the number of enterovirus positive samples between the two reports may have been due to differences in plant location and virological tests used. Song et al reported better results in terms of reduction of volatile solids and equivalent methane production when anaerobic digestion was separated into two stages involving thermophilic then mesophilic digestion than either process alone, although removal of coliform bacteria was similar for both configurations (Song et al., 2004). An additional number of researchers reported

<1 log$_{10}$ reductions for EVs between raw and processed biosolids (reviewed in Sidhu and Toze, 2009).

Joo et al. (2015) reviewed over 500 recent publications with the aim of extracting information on sustainable approaches for minimizing biosolids production and maximizing reuse options. They reviewed thermal, chemical, electromagnetic, ultrasonic, microwave, mechanical as well as integrated combinations of these pre-treatment methods (composting, vermicomposting, aeration, recovery of nutrients especially phosphorus and nitrogen by use of struvite, or calcium phosphate for precipitation, mixed thickening and high elutriation of sludge), electrodialytic process, cross-flow microfiltration, and a hybrid process of low pressure wet oxidation and nanofiltration) for reducing biosolids production by making organic material in biosolids more available for biodegradation, for reduction of hazardous substances, and for extracting nutrients to enhance and expand reuse options. A combined alkaline and ultrasonic pretreatment for cell disintegration was most effective balancing chemical pretreatment with energy consumption and in reducing biosolids volume by producing 20% more solubilization than either process alone. Three main systems for composting included aerated static piles,

enclosed systems and turned windrow systems (Kumar, 2011). Composting with a mixture of bacteria and fungi can be performed on un-separated waste or on waste from which indigestible materials have first been removed with compost quality dependent on the characteristics of the waste and degradation achieved (Kumar, 2011). An alternative, vermicomposting, is land surface intensive and takes longer (Kumar, 2011). Conditions including moisture, pH, temperature C:N ratios and microorganism composition (described above for composting toilets, Section 3.1.1) are important for composting of sludge. The USEPA 2006 report (USEPA, 2006) updated in 2013 (USEPA, 2013) reviewed the pros and cons of emerging technologies for different processing stages for treating biosolids including viruses in the USA. Research remains to be done to determine actual viral reduction/inactivation efficiencies. Stages included biosolids thickening, conditioning, stabilization, dewatering, thermal conversion and drying and the processes were categorized as embryonic (bench scale, full scale outside of US) or innovative (tested at full scale in <25 locations in USA, implemented since 2001, and/or available overseas with some use in USA). Established technologies (used in more than 25 sites in the USA) were excluded from the report.

There are two classes of sludge depending on its intended land application and whether biosolids are pathogen-free (Class A) or only significantly reduced (Class B) (USEPA, 1994). Land application " is the spreading, spraying, injection, or incorporation of sewage sludge, including a material derived from sewage sludge (e.g., compost and pelletized sewage sludge), onto or below the surface of the land to take advantage of the soil enhancing qualities of the sewage sludge". There are many possible WWTP system component configurations that can be assembled to degrade sludge using mesophilic and/or thermophilic, aerobic or anaerobic degradation (for examples see Speece et al (Speece et al., 2006)). Use of anaerobic degradation has lagged behind aerobic degradation (Speece et al., 2006). Important factors leading to increased efficiency of anaerobic processes were close microbial proximity to the sludge at mesophilic or thermophilic ranges especially at the start of degradation; availability of inorganic nutrients to reduce volatile fatty acids, and the reactor configuration (Speece et al., 2006). Virus survival in composted human feces depends not only in the conditions used for composting, but also in the storage conditions (Dumontet et al., 1999). For example, viruses can survive the composting process when certain zones of the heap are not exposed or exposed for too short times to the temperatures required for viral inactivation. Average concentrations and reductions of a variety of enteric pathogens in biosolids by composting (COM) and temperature-phased anaerobic digestion (TPAD) for Class A and mesophilic anaerobic digestion (MAD) for Class B biosolids as well as the annual log probability of viral infections associated with land applications and aerosols were examined in detailed reviews by Viau et al. (Viau et al., 2011; Viau and Peccia, 2009). Values for viability and molecular-based determinations were compared. The mean $\log_{10} \pm$ SD/dried gram in USA and European Union of enterovirus and fecal coliform concentrations in stabilized dried biosolids (number of samples used for average in

USA, and EU)] were 0.4±0.69, 1.7±1.11, (8, 3) after COM; 0.2±0.25, none analyzed, (2, 0) after TPAD; and 0.9±0.69, 4.15+0.5, (7, 5) after MAD processing. The $\log_{10}$ reductions for EVs compared with fecal coliform reduction for COM, TPAD and MAD processing were approximately 3.6±1.1 vs 5.6±0.8; 2.8 vs 3.5±1.0; and 1.3±0.4 vs 1.6±0.5, respectively. Berg and Berman (Berg and Berman, 1980) reported recovery of more EVs from mixtures of one-third activated sludge and two-thirds raw primary sludge digested under mesophilic than hemophilic conditions with an average dwell time of 20 days in digesters fed intermittently on a daily basis (min-max influx 380 to 11,600 plaque forming units (PFU)/100ml; after mesophilic digestion 30 to 410 PFU/100ml, approximately a 1 $\log_{10}$ reduction; while after hemophilic digestion <4 to 5 PFU/100ml a reduction of 2 to 3 $\log_{10}$; compare to 6 $\log_{10}$ for fecal coliform bacteria).

Mesophilic anaerobic degradation is the most common method for generating Class B biosolids in the US (Wong et al., 2010). Land and agricultural use of class B sludge has been regulated by the US EPA to reduce pathogen transmission "by allowing time for natural processes to further reduce pathogen levels". Our awareness of the potential health risks from new and emerging enteric pathogens, our ability to test for them, and the increasingly lower limits of detection and quantitation since the time this legislation was formulated has increased exponentially and many of the consequences and lessons from this expanded knowledge have been highlighted in this and other chapters of this book. The major health risk associated with land use of processed activated sludge still appears to be airborne exposure although accidental ingestion also poses a risk, however the list of potential pathogens and conditions necessary for their removal/inactivation have led many state governments in the US to adapt more stringent rules for land use (Viau et al., 2011). More information on pathogen and indicator content in biosolids and risks of infection from ingestion and inhalation have been can be found in the review by Viau et al. (2011).

Viau et al. (2011) also report a growing trend to convert Class B to Class A sludge to reduce the amount of pathogens returned to the environment by adding composting, dewatering, or adding both hemophilic and mesophilic treatment steps. However, even US EPA standard Class A sludge with $< 10^3$ colony forming units (CFU) bacteria/ dry gram were not enteric pathogen-free and presented a health risk to humans in increasing order through contaminated food ingestion, contaminated groundwater ingestion, aerosol inhalation and accidental direct ingestion...and that is only for the limited number of enteric pathogens that were tested and reported in the literature.

Sludge containing enteric viruses accumulates in lagoons of WWTPs using this process (USEPA, 2002a, 2002b). Virus accumulation is higher in cold climates because of reduced microbial degradation ,but can also be higher during summer because of a shortage of dissolved oxygen (USEPA, 2002a).

3.1.9 Land treatment

Land treatment of municipal wastewater involves a downwards flow along a grade to remove organics and suspended solids by biological oxidation, sedimentation and filtration through the soil (USEPA, 1981; USEPA, 1991). There are three main options: slow flow rate, rapid infiltration and overland flow, with an upper range for remaining fecal coliform of <10, <200, and <2,000 per 100 ml, respectfully. Vegetation and microorganisms participate in pathogen removal in slow flow treatment, water percolates through permeable soil to ground water in rapid infiltration, while in overland flow, the slope is covered by grass growing in relatively impermeable soil or sub-soil. Enteric virus removal, primarily through adsorption, is best in slow flow rate systems (USEPA, 1981). In rapid flow systems removal efficiency depends on initial concentration, hydraulic loading rate, type of soil, and distance traveled through the soil. When influx viral loads are high, virus can flow off into surface water or can enter the ground water especially if soil permeability is high (USEPA, 1981). (See Section 2.3 on enteric virus survival in soil and surface waters.) When water is applied using sprinklers, virus may also be present on plant surfaces, so crops that are eaten raw should not be used and there should be a sufficient time delay between last application and grazing (usually measured in weeks). The water quality of overland flow is equivalent to that after secondary treatment without disinfection (removal of <1.5 $\log_{10}$). Aerosols may spread viable enteric viruses up to 100 meters, especially when sprinklers are used. Rainwater facilitates sub-soil transport (Duboise et al., 1976). Managed aquifer recharge (MAR) systems improve water quality using natural filtration of surface water, storm water, or reclaimed water through soil to recharge ground water.

In MAR systems, virus concentrations have been shown to drop below detection when groundwater residence times exceeded 14 days (equivalent to a 1 to 5 $\log_{10}$ reduction depending on virus type) and there was a 1.8 to >3.7 $\log_{10}$ reduction in enterovirus genomes (Betancourt et al., 2014). In another study (Sprenger et al., 2014) of an MAR virus was no longer detectable after 50 meters of infiltration and 119 days of underground passage. Microorganisms present in the ground water enhance inactivation/removal of virus (Gordon and Toze, 2003).

3.2 Disinfection

Final reductions/inactivation of residual biologically viable pathogens often requires an additional tertiary inactivation step using disinfectants such as chlorine, ozone, paracetic acid or UV radiation (Jacangelo and Trussell, 2002; Mezzanotte et al., 2007; Nocker and Gerba, 2010; Okoh et al., 2010; Rossi et al., 2007).

Enterovirus reduction/inactivation studies measure the amount of viable virus or physical components of the virus in effluent compared with that in influent wastewater at WWTPs using viruses "naturally" present in sewage or after spiking the influent with known amounts of virus. It is important to keep in mind that inactivation of spiked virus may not be equivalent to that for the same virus naturally present in sewage (Payment et al., 1985a; Tree et al., 2003) due to differences in the way the virus is presented during the different disinfection processes. Moreover, inactivation and the contact time required for inactivation vary for different disinfectants and different pathogens (Jacangelo and Trussell, 2002) making it difficult to generalize or compare studies since even different EVs have different inherent resistance to disinfectants (Payment et al., 1985a). Stability of the disinfectant may also be inversely related to its effectiveness, although this may be compensated for by prolonged persistence (Akin et al., 1982). Finally, the effect of multiple inactivation steps such as UV with chlorination may be synergistic (indicator bacteria (Wang et al., 2012); poliovirus with chlorine and copper or silver ions (Yahya et al., 1992).

Disinfectants, mechanisms of action, and factors influencing the efficiency of inactivation are reviewed in depth in Morato et al. (Morato et al., 2003) and Nocker and Gerba (Nocker and Gerba, 2010). Chlorine, chlorine compounds and UV may be less effective against enteric viruses than against bacteria used as indicator organisms (Blatchley et al., 2007; Theron and Cloete, 2002). Inactivation or disinfection refers to the rendering the pathogen nonviable while removal refers to the physical separation or exclusion of the pathogen (see discussion on membranes and ultrafiltration in Sections 1.4.2, 3.1.7 and 3.2.4).

Initial inactivation is often considered to be a first order chemical reaction dependent on the type of disinfection and the ratios of the disinfectant and the microorganism (Akin et al., 1982). Suspended solids to which viruses adsorb usually negatively affect the efficiency of disinfection and digestion (Fong and Lipp, 2005; Schijven and Hassanizadeh, 2010; Templeton et al., 2008) as may virus aggregation (Schijven and Hassanizadeh, 2010), although under some conditions binding to suspended particles may enhances disinfection (see review by Templeton et al., 2008). All chemical disinfection processes produce disinfection byproducts (DBPs) (Annon, 2015c; Blatchley et al., 2007; Doederer et al., 2014; Jacangelo and Trussell, 2002; Verma et al., 2014; WHO, 2011), however the risk of illness and death from pathogens outweighs the risk from exposure to DBPs (WHO, 2011).

3.2.1 Chlorine

Chlorine disinfection in sewage suggest that particle-bound viruses are extremely important see review by Tempelton (Templeton et al., 2008). Inactivation of poliovirus present in sewage was much less efficient than when the same amount of unbound virus was added as a spike to the sewage (Tree et al., 2003). Cl_2 concentrations of 4 – 10 mg/L for 2 hours were able to inactivate 4 $\log_{10}$ of seeded virus but released virus could be detected in receiving waters even after chlorination (Blatchley et al., 2007). In a comparison of six WWTPs, Rose et al. (2004) reported approximately a 2 $\log_{10}$ removal of enteric viruses by biological treatments and another 0.3 to 1 $\log_{10}$ by filtration with pre-chlorinated shallow sand filters

correlating with the highest levels of enteric virus removal most likely due to inactivation by the chlorine. Enteric viruses were not detected in effluents from two of the WWTPs that used either pre-chlorinated sand filters or UV after reduction of nutrients (minimal ammonia available to form chloramines).

Concentration, pH, and contact time are the factors most influential in disinfection by chlorination using chlorine gas, sodium hypochlorite solution, calcium hypochlorite, chloramine dioxide and chloramines (Akin et al., 1982; Annon, 2003; Engelbrecht et al., 1980). Inactivation times varied 44 fold among six different poliovirus and non-poliovirus serotypes at the same pH and between 5-fold to 192-fold when comparing inactivation at pH 6.0 and at pH 10.0 (Akin et al., 1982; Engelbrecht et al., 1980).

Higher concentrations of chlorine (>1000 mg per min per liter) are needed for wastewater that is reused than for wastewater that is simply disposed (40 to 80 mg per min per liter) (Blatchley et al., 2007). Inactivation is less effective when organic particles interact with free and combine chlorine species (Okoh et al., 2010). Excessive use of chlorine is now avoided to reduce the risk for formation of DBPs (Verma et al., 2014). Chlorine and chlorine compounds are less effective against enteric viruses than against bacteria (Rose et al., 2004; Theron and Cloete, 2002) (see discussion of appropriate indicator organisms). However amounts of chlorine must be used that are sufficient for disinfection of enteric viruses despite DBP formation, since the risk of illness and death from pathogens outweighs the risk from exposure to DBPs (WHO, 2011).

Little data are found on other chlorine based oxidants and their use in wastewater. Jin et al. (2013) has reported that the virucidal concentration of chlorine dioxide on Hand Foot and Mouth Disease Enterovirus 71, (Section 1.0, an emerging enterovirus pathogen) was higher at pH 8.2 than at 5.6 and 7.2 and as expected higher at 36°C than at 4°C.

3.2.2 Ultraviolet and microwave inactivation

Virus inactivation by UV is dependent on interaction between high-energy photons and nucleic acids within viral particles that form pyrimidine dimers, other photoproducts, tertiary structural changes, and at high doses (>1,000 mW • s/cm^2) may also affect the capsid protein and generate RNA-protein linkages (Cutler and Zimmerman, 2011; Hijnen et al., 2006; Kurosaki et al., 2003; Nuanualsuwan and Cliver, 2003b; Okoh et al., 2010). UV inactivation appears to be effective against all pathogenic organisms although susceptibility of different EVs varied (Hijnen et al., 2006)). Specifically, 3 log$_{10}$ reductions of echovirus 1, echovirus 11, CV-B3, CV-B5, poliovirus 1, and human adenovirus type 2 were achieved by doses of 25, 20.5, 24.5, 27, 23, and 119 mW/cm^2, respectively. Inactivation occurs with first order kinetics except at very low (little or no inactivation) or high energy levels (tailing off effect) (Hijnen et al., 2006; Nuanualsuwan and Cliver, 2003b). Suspended particles and dissolved UV adsorbing organic

and inorganic chemicals decrease the efficiency of inactivation of free virus (Hijnen et al., 2006; Templeton et al., 2004; Templeton et al., 2007). The affect on UV inactivation of particle-bound viruses has been reviewed by Templeton (Templeton et al., 2008).

In contrast to chlorination, large amounts of DBPs are not formed (Ngwenya et al., 2013) and overdose is not possible (Cutler and Zimmerman, 2011). Inactivation of enteric viruses with UV appears more efficient than with chlorine compounds (Blatchley et al., 2007; Hijnen et al., 2006). A study comparing five different treatment configurations (Simmons and Xagoraraki, 2011b) found that those using UV removed/inactivated 2 log$_{10}$ more enterovirus on average than those with chlorine although the decontamination step itself with chlorine inactivated 0.6log$_{10}$ more enterovirus than the UV step.

See Mudhoo and Shrma (Mudhoo and Sharma, 2011) for a review on the use of microwave technology for disinfection, which may be related to heat generation.

3.2.3 Ozone

Ozone (O$_3$), one of the strongest oxidants and disinfectant can be generated on-site at water treatment facilities using high voltage treatment of dry oxygen or air. Ozone inactivates by direct oxidation/destruction, of structural components (cell walls, viral capsids), chemical interaction by-products of ozone decomposition, and direct damage to nucleic acids, with dose, mixing, and contact time determining the effectiveness of the inactivation (Roy et al., 1981; USEPA, 1999a).

In sewage, the degree of virus inactivation (>5 log$_{10}$) was rapid and biphasic with inactivation dependent on ozone concentration and the amount of organic matter in the effluent (Katzenelson and Biedermann, 1976; Katzenelson et al., 1979). pH during inactivation did not appear to play a significant role even though EVs tend to clump when pH is below 6.0 (Katzenelson et al., 1979).

3.2.4 Nanotechnology

Nanoparticles (1-100 nm) are an emerging method for decontamination of wastewater (see reviews by Theron et al., 2008, Qu et al., 2013, and Ngwenya et al., 2013). Nanoparticles have large surface areas per unit mas, short inter-particle diffusion distances, size and shape dependent catalytic properties, pore size and secondary structures that can be manipulated, and they can be coupled to different chemical groups to change or enhance targeting and inactivation of enteroviruses in pretreatment, in slurry reactors or absorbers, and for tertiary and post-tertiary processing (Qu et al., 2013; Theron et al., 2008).

Among the most promising nanomaterials with antimicrobial properties are metallic and metal-oxide nanoparticles, as well as TiO$_2$ photocatalysts (Theron et al., 2008). Photocatalitic nanoparticles can be used in combination with UV and may be an alternative to chlorine-based disinfection (Theron et al., 2008). Recyclability,

fouling, reduction of fouling by antimicrobial nanomaterials such as nano-Ag and carbon nanotubes, and safety are areas of research that will determine our ability to incorporate nanotechnology into sustainable wastewater treatment.

Appendix A. Environmental Surveillance in Support of Poliovirus Eradication

Testing of sewage samples and other waste waters for human pathogenic viruses (also known as sewage or environmental surveillance (ENVS)) may reveal significant information about the epidemiological characteristics of multiple enteric viral infections in the population (e.g. natural circulation and changes in circulation of viruses, potential global spread, transmission pathways of circulating virus types) which is valuable for planning and implementing public health responses and interventions (see discussion on ENVS in polio eradication vaccine section) . Sewage surveillance is especially useful in urban settings with limited clinical surveillance. This strategy has been applied around the world to assess the prevalence of EVs (poliovirus and nonpolio EVs) and other picornaviruses circulating in the communities, with important implications toward an understanding of temporal, seasonal, and geographical patterns of disease occurrence, i.e., sporadic, endemic, or epidemic viral circulation, including emergence and reemergence of viral infections in the population (Betancourt et al., 2010; Hovi et al., 2007; Hovi et al., 2012; Khetsuriani et al., 2010; Lodder and de Roda Husman, 2005; Palacios and Oberste, 2005; Rodriguez-Diaz et al., 2009; Sedmak et al., 2003; Shulman et al., 2000; Shulman et al., 2012; Apostol et al., 2012; Harvala et al., 2014; Ibrahim et al., 2014; Li et al., 2014; Richter et al., 2011; Tao et al., 2012; Wang et al., 2014; Yang et al., 2012). The monitoring of sewage waters can also be used as an early warning system for the release, introduction or re-introduction of pathogenic viruses (e.g. poliovirus) from an intentional, natural, or accidental biological contamination (ECDC, 2014; Shulman et al., 2012; Sinclair et al., 2008).

ENVS for poliovirus, which involves testing of sewage and other waste waters for polioviruses, has been widely used to assess the elimination of wild poliovirus (WPV) circulation in a given human population and to demonstrate the successful interruption of PV transmission as part of the WHO Global Poliovirus Eradication Initiative, GPEI (Deshpande et al., 2003; El Bassioni et al., 2003; Hovi et al., 2007; Hovi et al., 2012; Hovi et al., 2001; Matsuura et al., 2000; Shulman et al., 2014b; Avan der Avoort et al., 1995; WHO, 2013a). This strategy is also useful for monitoring the circulation of genetically divergent vaccine-derived polioviruses (VDPVs) and for assessing population immunity of populations vaccinated with inactivated polioviruses (IPVs) (Battistone et al., 2014; Dedepsidis et al., 2007; Hovi et al., 2013; Hovi et al., 2012; Nakamura et al., 2015). The advantage is that environmental surveillance can detect poliovirus circulation in populations in the absence of clinical cases of paralysis since the virus is excreted into sewage during asymptomatic infections as well as symptomatic infections. Standard procedures identifying viruses and especially poliovirus in wastewater have not yet been adopted. However, the US Environmental Protection Agency (USEPA) Manual of Methods for Virology (Berg et al., 1984) and WHO Laboratory Manuals for Environmental Surveillance (WHO, 2015a) have been updated to contain state-of-the-art procedures that have undergone extensive testing. One of the main goals has been and continues to be optimization of sample collection and processing. Moreover while results from environmental surveillance are usually qualitative, they can be semi quantitative as well (Hovi et al., 2001; Lodder et al., 2012; Manor et al., 2014).

Environmental surveillance is sometimes considered as supplementary to human surveillance by health organizations. In contrast to AFP surveillance, standardized methods for environmental surveillance are developed by WHO (WHO, 2015a).

OPV strains are present in sewage in countries where OPV is part of the vaccination program making it much more difficult to screen sewage for the presence of vaccine-derived viruses and wild viruses in mixtures containing much higher amounts of vaccine strains. Additional difficulties in detecting polio by environmental surveillance arise from the fact that shedding may depend in part on immune status and competence of the infected human host and shedding may be intermittent (Dowdle et al., 2006). Molecular assays offer one of the best ways to distinguish and even easily quantitate the amount of non-polio vaccine viruses in these mixtures (Hindiyeh et al., 2014; Kilpatrick et al., 2011; Kilpatrick et al., 1998; Kilpatrick et al., 2009).

The following 6 steps are essential for implementing a successful poliovirus environmental surveillance program (Hovi et al., 2012; Shulman et al., 2012): (i) Identify the target population for the surveillance The optimal size of the population in the catchment area should be between 100,000 and 300,000, and sites should be selected for ease of access and lack of contaminants that can inhibit laboratory molecular and viability studies.; (ii) Ensure the necessary resources and expertise are in place for collection transportation analysis, storage and decontamination.; (iii) Design a practical sampling plan that includes the type of site (sewage or other), the type of sample (grab (semi-quantitative) vs trap (not quantitative), the logistics needed for sampling (all lab and non lab needs including storage and transportation of the sample to the diagnostic lab), the duration and frequency of sampling (repeated sampling over extended time improves sensitivity and makes interpretation of negative findings more reliable), and clear lines for communication); (iv) WHO certified sampling processing facilities with appropriately trained personnel, standard sampling protocols, quality controls, data management systems, established turn-around-times for virological testing , results and reporting and appropriate safety standards. SOPs should include rules for storage of the sample and downstream byproducts, for safe disposal after they are no longer needed and contingency plans in case of spills and accidental release; (v) Detection, interpretation of results, and reporting (SOPs for identification of viruses, establish turn-around times, and actual reporting of results); and (vi) a clear contingency plan for reacting when non-vaccine polioviruses are detected.

In almost all cases, the amount of poliovirus in sewage is low, and detection requires a concentration step (WHO, 2015a). Concentration procedures include (i) a two phase separation after addition of Dextran T40 and PEG 6000 to the sewage (can easily be implemented in all laboratories of the GPLN); (ii) PEG600/NaCl precipitation (requires heavy centrifuge and rotors not available in all labs); and (iii) Tangential Flow Ultrafiltration (currently being evaluated). Of the 3, the latter may provide the best solution since early trials indicate much higher viral recovery and it offers the possibility of on-site use to significantly reduce one of the current bottlenecks of environmental surveillance, e.g., the need to store and transport large volumes of contaminated sewage under cold chain conditions).

The WHO standard detection protocol (WHO, 2015a) requires viral isolation by tissue culture. The cell lines used, the number of replicate cultures used for viral challenge, and the inoculation volumes an even the number of days post-inoculation have not yet been standardized and these all influence results especially when virus is in low concentration.

Three examples will be briefly presented to show the usefulness of environmental surveillance in the GPEI; environmental surveillance in Egypt (Blomqvist et al., 2012; El Bassioni et al., 2003; Hovi et al., 2005; WHO, 2013b), in Israel (Hindiyeh et al., 2014; Manor et al., 2007 ; Manor et al., 1999; Manor et al., 2014; Moran-Gilad et al., 2015; Shulman et al., 2014a; Shulman et al., 2006; Shulman et al., 2012; Shulman et al., 2014b;; WHO, 2013c) and in Finland/Slovakia/Estonia (Blomqvist et al., 2004; Hovi et al., 2013; Hovi et al., 2012; Roivainen et al., 2010).

In Egypt, environmental surveillance was initiated in September 2000 while wild poliovirus was still endemic but in the last stages of elimination. Results in 2001-2002 revealed widespread silent circulation of wild type 1 poliovirus, helped to identify gaps in routine AFP surveillance, and was helpful in targeting surveillance and immunization so that by the end of 2006 Egypt was successful in eliminating endemic circulation of wild polio. In 2005 and 2010 type 2 vaccine derived polioviruses excreted by anonymous individuals (aVDPVs) were isolated and wild type 1 polio originating from Pakistan was isolated from sewage in Cairo in December 2012.

Countrywide routine monthly environmental surveillance was initiated in Israel in 1989 in response to the last outbreak of poliomyelitis from wild poliovirus. Environmental surveillance identified the introduction, local silent circulation of wild polio, and documented the successful efforts to control the circulation with tOPV in the Gaza District between 1995-96 (Manor et al., 1999; Shulman et al., 2012). Advanced molecular analysis techniques were developed to distinguish between multiple introductions of wild poliovirus and local endemic circulation (Manor et al., 2007). In 2005 Israel switched to exclusive use of inactivated polio vaccine for routine polio vaccination and OPV strains disappeared from sewage within a few months. Between 1998 and 2014, neurovirulent, vaccine derived polioviruses (aVDPVs) were periodically isolated from sewage from central Israel, once

in Jerusalem, and twice from Haifa. Molecular analysis suggested that the aVDPVs were excreted by at least three different persistently infected immune-deficient individuals (Shulman et al., 2006). After 2005 when sewage was OPV-free, all polio that was isolated was of potential interest. Plaque assay was used to indicate the amount of virus recovered from each sewage sample. During this time, the highly sensitive environmental surveillance used in Israel enabled tracking of the location of the individuals who were excreting the aVDPVs as they moved from city-to-city and even within a single city (Shulman et al., 2012). Finally, in 2013, environmental surveillance detected a sharp rise in the number of poliovirus plaques that analysis confirmed as wild type 1 (WPV1) (Manor et al., 2014). Sequence and phylogenetic analysis revealed that the imported wild type 1 poliovirus originated from Pakistan and was closely related to the wild type 1 poliovirus isolated in December 2012 in Cairo from sewage (Shulman et al., 2014a). Poliovirus isolated from AFP cases in Syria in 2014 were also related to the viruses in Israel and Egypt (WHO, 2013c). Specific RT-PCR primers were designed to detect this wild type virus by environmental surveillance (Hindiyeh et al., 2014). More importantly these primers and probe were validated for use in semi-quantitative analysis of RNA extracted directly from concentrated sewage allowing an initial turn-around time of a week after sewage collection (Hindiyeh et al., 2014; Manor et al., 2014; Shulman et al., 2014b). Results were confirmed by standard tissue culture two weeks later. The results from qRT-PCR were correlated with the number of plaques so that when the environment became flooded with OPV strains after supplementary immunization activity (SIA) with bivalent OPV (OPV without a serotype 2 component) in August 2013, plaque-equivalent wild poliovirus loads could continue to be provided to the Israel Polio Oversight Committee for outbreak control. The environmental surveillance helped successfully pinpoint the epicenter of silent circulation and target the cohort with the highest probability of detecting silent circulation by stool survey of identified individuals (Moran-Gilad et al., 2015; Shulman et al., 2014b). The Polio Regional Certification Committee (RCC) required that the same sensitive methods that had been used to identify the silent circulation of the WPV, be used to document its disappearance during the 12 months after the last positive environmental surveillance sample was collected on April 3rd 2014 before Israel could be declared WPV1-free. As a consequence of very high population immunity to polio (>95%,), there were no polio-AFP cases throughout the entire episode (Feb 2013- April 2015).

The Polio Laboratory in Finland recovered highly diverged aVDPVs from sewage in Finland, Slovakia and Estonia over extended periods of time (Blomqvist et al., 2004; Hovi et al., 2013; Hovi et al., 2012; Roivainen et al., 2010). Molecular analysis strongly suggested that immune-deficient individuals were shedding these VDPVs. These findings may indicate that persistent excretors may be less rare than believed on the basis of identification of known persistent excretors and failed efforts to identify new ones by screening immune deficient individuals in multinational studies. Environmental surveillance also documented the cessation of an extended shedding of a type 2 VDPV without intervention or identification of the shedder (Hovi et al., 2013).

There are a number of challenges for ENVS that need to be addressed. In the field these include interpretation of data from open sewers where water flow, depth and contamination and the number of persons in the catchment area are poorly documented, unknown effects of pooling samples from different locations to reduce workload, and the high cost and difficulty of transporting large volumes of sewage within and between countries in time frames that results will be are relevant for intervention. In the lab they include space personnel and interpretation of data especially negative results. On-site processing would help to alleviate some of these difficulties. In the lab they involve the process of virus concentration, elution from particles and filters, and reduction of co-extraction of inhibitors.

In conclusion ENVS will be a critical element of surveillance during polio eradication and in the post eradication era. It will play an important role in polio-endemic countries, countries where poliovirus has been reintroduced and in polio-free countries. It is especially helpful in countries where there is sub-optimal AFP surveillance and high risk for transmission and for countries with very high vaccine coverage (the example given here is Israel) where there is a significantly reduced likelihood for development of poliomyelitis after infection with neurovirulent poliovirus strains and risk of much higher numbers of silent infections by non-vaccine polioviruses before any cases occur. The World Health Organization has recommended a global cessation of the use of live serotype two in OPV vaccines by April 2016 and withdrawal of the use all live vaccine strains within two years of the last case of poliomyelitis from wild poliovirus (WHO, 2015b). Environmental surveillance is especially important for populations with high IPV vaccine coverage, since the ratio of AFP cases to asymptomatic infections would be orders of magnitude lower than for under-vaccinated populations. The objective of ENVS for eradication is to measure disappearance of endemic strains, imported strains, finally live vaccine strains and to provide early warning of importation or re-emergence. Sewage samples from the polio ENVS can be and have been used to identify circulation of other enteric viruses.

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