water

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*Review*

# Insights into gastrointestinal virome: etiology and public exposure

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**Abstract:** Recycled wastewater is widely used owing to potential shortage of water resources for drinking purposes, recreational activities and irrigation. However, gut microbiomes of both human beings and animals negatively affects this water quality. Wastewater contamination is continuously monitored using fecal contamination indicators or microbial source tracking approaches to oppose arising enteric infections. Viral gastroenteritis is considered as a principal manifestation of waterborne pathogenic virome mediated infections mainly transmitted via the fecal-oral route. Furthermore, the acquired enteric viromes are known as the common cause of infantile acute diarrhea. Moreover, public exposure to wastewater via wastewater discharge or treated wastewater reuse has led to a significant surge of public health concerns. In this review, we discuss the etiology of waterborne enteric viromes, notably gastrointestinal virus infections in infants. Latterly, we discuss public exposure to municipal wastewater. Conclusively, infant virome is affected mostly by birth mode, dietary behavior and maternal health and could provide a signature of disease incidence. Multiphase treatment approach offered an effective means for elimination of wastewater reuse mediated public risks. The insights highlighted in this paper offer essential information for defining probable etiologies and assessing risks related to exposure to discharged or reused wastewater.

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## Enteric virome in infants

The microbiome is established as early as the gestation period, and develops into a steady state as the individual reaches early adulthood [1–5]. Infant microbiomes have been shown to be influenced by various factors involving birth mode, gestational age, antibiotic usage, geographical location, lifestyle, diet, and age [6–8]. For instance, a greater virome diversity was observed in spontaneous vaginal delivery (SVD) than in caesarean section (CS) [9]. Moreover, microbiome diversification during the early months after birth was found to be followed by a secondary expansive phase owing to diet alterations that occur after weaning [1,10,11]. Furthermore, microbiome composition of the infants' gut is linked to that of the maternal gut [12–14]. However, it was demonstrated that vertical transmission of the virome has been considerably lower than that of bacterial microbiome [12].

Interestingly, antibiotic usage acts as a stressor causing microbiome imbalances reported to cause sepsis in newborn infants by vertical transmission [15]. Antibiotic treatment reduces both the size and diversity of the bacterial community, initiates pro-phage activation and enriches phage-encoded antibiotic resistance genes that further influences the prokaryotic microbiome - associated with long-term implications [8,16–18]. Thus, such influences imposed on the microbiome via these trans-domain interactions, can be associated with probable metabolic deficiencies and inflammatory conditions [19–22]. On the

other hand, the infant virome is affected by dietary behavior and could serve as a signature of malnutrition. For instance, members of the Anelloviridae and Circoviridae were found to discriminate between both healthy twin pairs and twin pairs developing malnutrition [23].

Although the microbiome is acquired during pregnancy or even at birth, the gut virome develops post-natally, since the meconium was found to lack any virus-like particles (VLPs) [24]. However, VLP numbers begin to surge to about $10^8$ per g faeces in the first birth week along with the primary colonizers that arise from dietary and maternal sources in addition to the surrounding environment [25]. Consequently, the infantile virome expands and includes significant shifts in the phage community along with age because of the expansion and diversification of the bacterial communities [1]. The virome peak is reached at adulthood, displaying persistent viruses of about 80% that persist for >2.5 years [1,26]. Moreover, eukaryotic viruses, involving adenovirus, herpes simplex virus, cytomegalovirus (CMV), human parvovirus B19, enterovirus, respiratory syncytial virus and Epstein–Barr virus, were characterized from healthy mothers' amniotic fluid whilst neonates were healthy [27]. Furthermore, placental and vaginal transmission of viruses, comprising HIV, influenza, hepatitis, CMV, rubella and herpes zoster virus were also detected, rendering extra evidence of maternal-mediated infantile virome modification [28]. This eukaryotic virome encompasses these conventional pathogenic viruses and viruses of unidentified host interactions. Despite the presence of these classical pathogens, infected hosts can remain asymptomatic.

## Viral etiology

Viral gastroenteritis is mainly transmitted by the fecal-oral route. Currently, five common groups of viruses account for the most frequently occurring acute diarrheal cases worldwide, involving Adenovirus, Rotavirus, Norovirus, Hepatitis A virus and Astrovirus (discussed below).

### *Adenovirus*

Adenoviruses are members of the family *Adenoviridae* and the genus *Mastadenovirus*, comprising more than 80 human serotypes [29]. Human adenoviruses are non-enveloped icosahedral particles with a double-stranded linear DNA genome of ~34-36 kb [30] (Figure 2.3). They are currently grouped into 7 Human Adenovirus species (A-G) besides novel adenovirus types that are continuously emerging [31,32]. Types were identified in cross-neutralization assays as serotypes up to type 51, however a genotype designation was used for the more recent types based upon phylogenetic analyses of genes encoding the major capsid proteins [33].

Adenovirus infections can lead to a wide spectrum of clinical symptoms. Gastrointestinal infections are commonly caused by subgroup A, D and F, whilst subgroup B is the main cause of infections of lung and urinary tract. Subgroups C and E are, however, mainly related to infections of respiratory tract. Amongst subgroup F, serotypes 40 and 41 with serotype 31 of subgroup A are mainly associated with gastroenteritis [34]. Adenovirus infections are mostly self-limiting, with the exception of immunocompromised individuals. However, a strain of adenovirus 14 that emerged previously resulted in a fatal respiratory disease in healthy personnel [35].

Human adenoviruses are specific to humans even though adenoviruses infect a range of animals. In domestic sewage, human adenoviruses existed in notably high concentrations and their seasonal variability was insignificant [36–38]. As with most enteric viruses, adenovirus persists much better in the environment and even during sewage treatment processes than the currently used fecal indicator bacteria [39,40]. Moreover, adenoviruses are highly resistant to UV light and this significant resistance might be due to the host cell-mediated DNA repair mechanism [41]. In addition, adenoviruses have another mecha-

nism that ameliorates the DNA damage response is mediated by the E4 orf4 protein, involved in efficient adenovirus replication [42]. Therefore, adenoviruses were proposed as a virological index for water quality control due to their potential environmental stability [43].

*Rotavirus*

Rotavirus is a double stranded RNA virus composed of 11 segments of genome size ~18,550 bp [44]. These segments differ in size from 667 to 3,302 nucleotides (Figure 3). Viral capsid proteins (VP1, VP2, VP3, VP4, VP6 and VP7) are encoded by segments 1, 2, 3, 4, 6 and 9, respectively. The non-structural proteins (NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6) are encoded by segments 5, 8, 7, 10 and 11, respectively. All segments have methylated cap structures at 5′ end and a 3′UGACC consensus sequence instead of the poly-A tail [45,46].

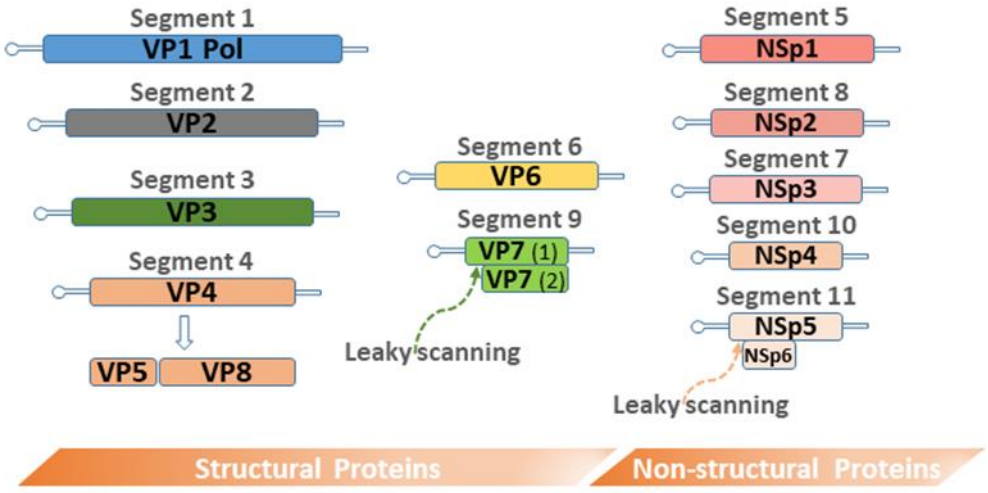


**Figure 3.** Rotavirus genome structure.

Rotaviruses are divided into seven serogroups (A-G) [47]. Rotavirus A is considered as the principal cause of severe acute gastroenteritis throughout the world and predominantly results in severe acute diarrhea in children [48,49]. The rotavirus outer capsid consists of two distinct neutralization antigens that are responsible for rotavirus attachment and entry termed as VP7 and VP4. They serve for virus classification each into a G-genotype (16 genotype) and P-genotype (27 genotype so far), respectively [49]. Despite the wide spectrum of rotavirus genotypes resulting from G/P combinations, epidemiological studies showed that the most prevalent genotypes are G1P[8], G3P[8], G4P[8], G9P[8] and G2P[4] causing up to 90% of severe RVA infections worldwide [50,51]. Furthermore, there is no clear relationship between rotavirus genotypes and the severity of disease [52].

*Norovirus*

Norovirus (NoV) is a member of the family Caliciviridae, with a polyadenylated, positive-sense, single-stranded RNA genome sized ~7.5 kb. The ≥ 40 genotypes are classified into seven genogroups (GI–GVII) [53,54]. NoV infection is considered as the most prevalent non-bacterial mediated gastroenteritis, causing ~20% of entire gastroenteritis cases worldwide [55] - particularly in 5-years old and younger children [56]. NoV causes ~685 million diarrheal episodes [57] and 200,000 deaths per year [58]. Norovirus infection symptoms occurs after an average incubation period of 24 to 48 h, typically involving vomiting, nausea diarrhea, dehydration, fever, and abdominal cramps [59].

NoV genome organization involves 3 or 4 open reading frames (ORFs). The first ORF (ORF1) encodes for 6 non-structural (NS) proteins including NS1/2 (N-Term), NS3 (NTPase), NS4 (3A-like),

NS5 (VPg), NS6 (Protease) and NS7 (RNA-dependent RNA polymerase; RdRp) which are responsible for viral replication. However, the other 2 ORFs (ORF2 and ORF3) encode 2 structural viral proteins (VP) comprising VP1 (major) and VP2 (minor capsid protein), respectively (Figure 4) [60]. These ORFs are flanked by untranslated regions (UTRs) at both ends [61]. In murine noroviruses, however, a fourth ORF is present encoding the protein VF1, that plays a role in antagonism of the innate response [62].

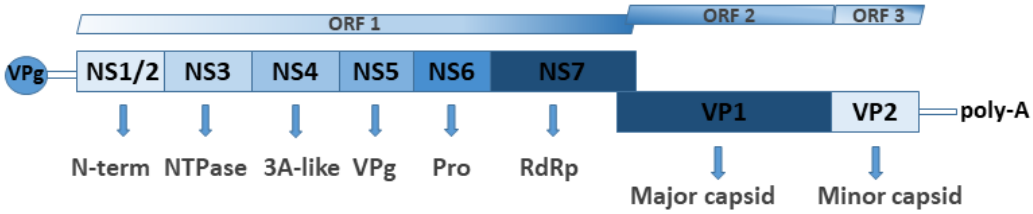


**Figure 4. Genome organization of norovirus.**

Norovirus typing was conventionally based upon sequence diversity within the capsid protein sequence. Noroviruses could be grouped into ten genogroups (GI-GX). Generally, genogroups vary by around 40–60 % of their amino acid sequence: less sequence variance (20–40%) in the case of genotypes [63]. Moreover, genotypes can be sub-divided into variants [64]. Currently, the RdRp-encoding region is used for dual genotyping of norovirus based on genotype and P type - GI.1[P1], for example [63].

Humans can be infected by more than 30 genotypes of noroviruses. Furthermore, noroviruses show significant host specificity – at variance with evidence suggesting inter-species transmission [65–67], in addition to the detection of viral RNA from human strains in different animals [67–71]. Initially, human strains of viral RNA displayed a potentially limited replication capacity in animals [72–74]. Moreover, GI and GII viruses are mainly responsible for human infections [75], GIII viruses are associated with ovine and bovine species [76,77], GV viruses are specific for murine species (mice and rats) [78,79], and GIV, GVI, and GVII viruses are associated with various carnivorous species, notably felines and canines [80–82]. Furthermore, GVIII and GIX which are novel genogroups, were detected in humans, and GX was described in bats [63]. However, there are some exceptions to the species specificity of GII and GIV genogroups. For instance, GII.11, GII.18, and GII.19 have been described in pigs [83] but not detected in humans, and GIV.1 and GIV.NA1 were associated with humans only but not found in canines or felines [61].

Analyses of outbreaks identified GII of noroviruses as the most frequently circulating strains causing gastrointestinal infections worldwide [84]. Over the past 20 years, GII.4 has become the predominant genotype resulting in 70-80% of NoV outbreaks in different countries [85]. This genotype is potentially evolving, yielding new pandemic variants including Grimsby 1995 (or US95_96), Farmington Hills 2002, Hunter 2004, Den Haag 2006b, New Orleans 2009, and Sydney 2012 [86,87]. This strain diversity arises from both genome recombinations and mutational events since significantly higher non-synonymous changes are observed in comparison with other NoVs, supporting the antigenic drift proposal - although occurring at a higher rate [88,89].

Noroviruses are mainly transmitted via the fecal-oral route, through ingestion of contaminated food or water, or, by oral contact with a contaminated fomite existing in the surrounding environment [90]. Moreover, high rates of secondary infection arise via airborne transmission, although the fomite route is more dominant [91,92]. The biological characteristics of norovirus has also been extensively studied through human feeding study volunteers [93–96]. Norovirus inocula as low as 10 viral particles were sufficient to initiate infection [95,97]. This potentially low count-mediated infection is regarded highly critical when discussing norovirus survival. Strikingly, norovirus was depicted to be of stable infectivity under freezing and thawing conditions [98], although a more recent study showed altered stability upon exposure to 3 cycles of freezing and thawing [99].

Moreover, it shows thermal resistance despite being exposure time-restricted of up to 21 min decimal reduction time (time required at a given temperature to perform a log reduction) in the temperature range of 50-72°C [100,101]. Furthermore, longer exposure time was detected at 50-60°C, despite irreversible capsid disruption at >65°C and loss of binding capacity at 72°C [102]. This high survival capacity can be demonstrated in a norovirus outbreak that occurred in a long-term care facility in which fomite-mediated survival of norovirus resulted in a continuous infection for 14 days following the initial peak of illness [103].

## Hepatitis A virus (HAV)

Hepatitis A virus belongs to the family Picornaviridae, genus Hepatovirus and is a non-enveloped positive-sense, single-stranded RNA virus of ~7.5 kb genome packaged within a 27-32 nm icosahedral capsid [104]. The HAV genome is composed of a single ORF, whose translation occurs by means of a cap-independent mechanism making the use of the internal ribosome entry site (IRES) located upstream of genome producing a polyprotein composed of ~2230 amino acids [105]. This polyprotein consists of three distinct domains (P1, P2, P3) which are further processed into 10 mature proteins by the virus-encoded proteinase, $3C^{pro}$ [104,106]. P1 encodes the four major capsid proteins VP1-VP4. The nonstructural viral proteins are comprised by the polyprotein domains P2 and P3 – also 'processed' by $3C^{pro}$ [107]. HAV displays a high degree of conservation of the antigenic determinants - notably in amino acid sequences of viral capsids – now expanded to include the recently identified HAV-like viruses [108,109]. This could have resulted from negative selection pressures imposed upon any naturally-occurring mutants, producing the observed consensus conservation [110]. Despite the high conservation of HAV, a degree of genomic sequence divergence exists defining the various HAV genotypes and the identity of sub-genogroups [110,111]. Consequently, HAV genotyping is dependent on different regions in its genome used to recognize HAV variants, including the VP1 entire region, notably the VP1 amino terminus, the 168 bp VP1–2A junction, the VP1–2B region, the VP3-2B region, the VP3 carboxy-terminus and the 5′ untranslated region [112,113]. To begin with, based on VP1–2A junction region variability (of ~15%), seven genotypes of HAV were primarily defined. However, according to the 23.7% variation denoted by the entire VP1 sequence analyses, 6 HAV genotypes (I–VI) are currently defined encompassing genotypes 1A, 1B, II, III, IV, V, and VI [114,115]. Genotypes I, II, and III infect humans and are divided into A and B subtypes, however genotypes IV to VI are called simian HAV (SHAV) since they infect non-human primates [114,116]. Amongst human HAV genotypes, subtype IA was found to be the most frequently circulating subtype worldwide [117]. Interestingly, individuals cannot be reinfected by HAV since the presence of a single HAV serotype results in neutralizing IgG production against HAV, elicited upon vaccination or even natural infection [118].

On the other hand, HAV infections can range in associated severity from asymptomatic to fulminant hepatitis mediated deaths [119,120]. However, HAV commonly causes self-limiting infections that do not lead to chronic liver disease [118,121]. Moreover, clinical manifestations can increase with age, manifested by jaundice and unusually high serum aminotransferase levels as the common symptoms - exhibited by over 70% of infected adult patients [119,122]. Furthermore, the incubation period of HAV lasts for ~15-50 days with an average of 28 days [123]. A wide range of symptoms occur upon HAV infection involving gastroenteritis, malaise, fever, nausea, anorexia, jaundice, dark urine (genitourinary symptom) and abdominal discomfort [124]. Fulminant hepatitis is considered as a rare complication associated with HAV infections that occurs in less than 1% of infected patients, with highest incidence rates in young children and the elderly with reported underlying liver illnesses [123,125]. Nucleotide substitutions at the 5′ UTR, P2 and P3 regions of the HAV genome were found to be associated with this fulminant disease [125,126].

HAV is mainly transmitted via the fecal-oral route, as well as through personal contact and exposure to contaminated water / food supplies, whereas transmission routes of the other typically hepatitis-causing viruses, in particular hepatitis B and C, involve contaminated blood or other body fluids via injection, intimate contact or perinatal period vertical transmission [127]. Remarkably, waterborne HAV outbreaks are uncommon in developed countries owing to proper sanitation procedures as well as water supply facilities [128]. On the other hand, HAV has been found to be of high stability in the surrounding environment for long periods whenever being associated with organic matter [125,129]. For instance, HAV was detected to be infectious even for more than 1 year of storage at 4°C in bottled water, with <1 log reduction owing to concentrations of the added proteins [129,130]. Moreover, HAV shows significant resistance to surprisingly low pH since it

was reported infectivity remains after treatment at pH1 for up to 5h at room temperature, for 1.5h at 38°C and at pH3 for up to 21 days at 4°C [131,132]. The environmental stability of HAV, displayed by its low pH as well as heat resistance (60 °C for 1h), could be due to the inherent molecular stability of HAV capsid, concurrent with its particular codon usage along with its unique folding pattern of the VP2 protein [108,133,134].

Ingestion of HAV-contaminated food accounts for 2–7% of all HAV-mediated outbreaks all over the world [135]. Epidemiological investigations provide a potential solution since they have succeeded previously in identifying the source of contamination. For example, a large and persistent food-borne mediated multi-state HAV outbreak occurred in Europe from 2013 to 2014, and was shown to be due to the ingestion of HAV-contaminated frozen berries. This led to over 1589 cases and 2 deaths [136,137]. Moreover, bivalve molluscan shellfish has been reported to have significant HAV levels showing various prevalence spatially and temporally [138–140]. For example, the prevalence of HAV severely declined over the years from 40% to < 8%, according to the 20 year-systematic survey carried out on bivalve molluscan shellfish from 3 estuaries in Spain [138], accompanied with a reduction in HAV cases. However, the reduction in cases could be due to increasing availability of the HA vaccine availability alongside increased surveillance that can rapidly identify contaminated food [135,141].

## Astrovirus

Human astroviruses (HAstV), are members of family Astroviridae, genus Mamastrovirus. They are non-enveloped icosahedral viruses, with a linear positive sense, single-stranded, RNA genome ranging from 6.4 -7.9 kb [142]. The genome consists of 3 ORFs comprising ORF1a, ORF1b and ORF2, flanked by 5′ and 3′ UTRs (85 and 83 nts, respectively) and a 3′ poly-A tail (Figure 5). To begin with, ORF1a and ORF1b encode 2 functional polyproteins (nsp1a and nsp1ab) encompassing a serine protease and a RNA-dependent RNA-polymerase (RdRp). ORF2 encodes the capsid proteins precursor, translated from a sub-genomic RNA (sgRNA) and comprises 2 principal domains; the highly conserved amino (N)-terminus and the hypervariable carboxy (C)-terminus [143,144]. Furthermore, between ORF1a and ORF1b, there is an overlapping region in which a unique frameshifting mechanism exists composed of a 'slippery sequence' as well as an RNA hairpin structure [145]. In addition, the viral genome encodes genomic linked protein (VPg) that plays a major role in viral infectivity (notably the TEEEY-like tract), replication of virus genome, and protein synthesis [146,147].

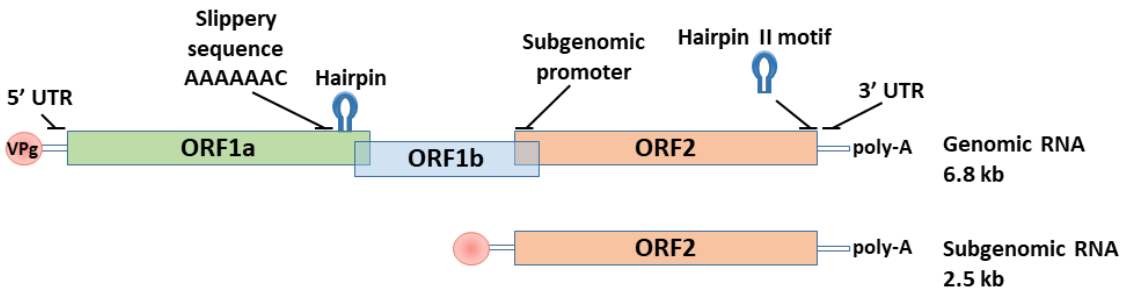


**Figure 5. Genome structure of Astrovirus.** The hepanucleotide slippery sequence and the RNA hairpin structure acts comprises the frameshifting signal that produces ORF1b translation. ORF2 is a subset of sgRNA produced during virus replication.

HAstV has been commonly associated with incidence of acute gastroenteritis in young children, immunocompromised individuals and the elderly. HAstV is responsible for sporadic non-bacterial diarrheal cases representing up to 20% and 0.5-15% of entire related outbreaks [148–150]. HAstV is considered as the second or the third major cause of infantile gastroenteritis after rota- and calciviruses [151]. Nonetheless, regional studies demonstrate significantly different relative prevalence of HAstV. For instance, in particular developing countries, 30% of all diarrheal cases were due to HAstV infection [45,152].

Gastroenteritis caused by HAstV is characterized by symptoms involving 2-3 day-watery diarrhea, vomiting, abdominal pain, malaise and headache [153]. The incubation period is somewhat longer than gastroenteritis caused by other types of virus with an average of 4.5 days [45,154]. HAstV encompasses eight genotypes, HAstV-1 to HAstV-8 [155], whilst HAstV-1 is the most common genotype identified in both wastewater and stools [156].

## Public exposure to municipal wastewater

People are exposed to wastewater by various means. The main exposure routes are frequent recreational activities and surface water drinking. Moreover, shellfish production is regarded as indirect route of exposure. Molluscs are filter feeders and consequently contaminated water pathogens are concentrated because of filtration leading to consumer infection [157,158]. Moreover, enteric viruses were detected in 50% to 60 % of the total mussel samples obtained from a bioremediation mussel farm [159]. Notably, non-enveloped viruses, such as noroviruses and hepatitis A virus, can survive in the bivalves' tissues and are highly resilient to degradation [160,161]. Moreover, virus particle size was found to define whether the particle is degradation resistant or susceptible [158]. For example, <200 nm VLPs are typically of higher degradation resistance when compared to bacteria [162]. Human infectious diseases owing to consumption of contaminated filter-feeders and recreational activities in wastewater-polluted coastal waters account for annual cost of $12 billion [163]. Moreover, wastewater-mediated irrigation, in particular sprinkler irrigation, generates aerosols that can cause infection upon direct exposure or ingestion of irrigated crops [164]. It is important to note that RNA of the pandemic SARS-CoV-2 was detected in treated wastewater that represents a critical issue for usage in irrigation [165]. Toilet flushing and groundwater production render other routes of direct exposure to wastewater [166,167].

## Discharge of wastewater

Wastewater is commonly discharged to surface water resources. In addition to the public health concerns, faecal contamination of wastewater can negatively influence water environments which are essential for fishing, drinking water and recreation. Enteric viruses are considered the main cause of waterborne illnesses associated with recreation water involving pools, spas, rivers, etc. and can reach waters via accidental release of faeces or body fluids [168]. Moreover, a surge in non-enteric diseases has been reported to arise from wastewater contaminated with significant viral contamination [169,170]. Wastewater treatment (WWT) performance guidelines have been established for reclamation and reuse. These guidelines are concerned with microorganism levels and the degree of treatment, whereas receiving waters risk management mainly depends on faecal indicator bacteria monitoring [171]. Unfortunately, these currently-used bacterial indicators cannot meet the full criteria of the ideal water quality indicators [172]. On the other hand, excreted enteric viruses can be detected in wastewater, but wastewater treatment plant (WWTP) may not completely eliminate viruses in terms of their concentration and infectivity, thus exhibiting receiving water-related health risk [173]. Furthermore, enteric virus presence in water does not necessarily link to the bacterial indicators' detection as *Escherichia coli* and coliforms [174]. Additionally, bacteriophage survival in water is more similar to human enteric viruses than the presently used bacterial indicators [171]. Thus, traceability procedures provide a mandate to determine faecal contamination sources so that the risk could be assessed to initiate a proper water management to counteract it at its source.

## Reuse of treated water

Reusing wastewater is determined by economic factors - either for recirculation of organic matter to act as natural fertilizers or because of shortage of water resources [175,176]. For instance, wastewater and greywater (households' wastewater with no fecal contamination) have been used for irrigation of agricultural products, as well as indoor activities involving toilet flushing and even for potable use [177–181]. However, intensive treatment measures are certainly required to meet the suggested wastewater reuse guidelines, in particular for greywater in which significant coliform loads may exist. However, performance of treatment procedures relying on coliform elimination may be biased and exaggerated owing to the capability of these bacteria to multiply within the greywater system.

Wastewater reuse is currently a frequent practice in many countries. For example, treated wastewater is utilised in agriculture and landscaping in many countries – i.e. Egypt, Saudi Arabia,

Italy, Cyprus, Malta, Spain or the USA [182–186]. It is, for instance, used in Egypt for irrigation of sandy soils to raise the soil content of organic matter and improving capacity of cation exchange [187]. In Saudi Arabia, 25% of treated wastewater in 2010 was used to irrigate landscapes in public parks of many cities [188]. In the Netherlands, particularly in Amsterdam, wastewater was regarded as a rich resource of organic matter that could be recovered and reused involving alginic acid, cellulose, bioplastic, and biogas besides phosphorus obtainment [189]. Moreover, wastewater reuse has converted Singapore into a universal hydrohub via implementation of novel water technologies that led to meet 30% of its water demands and is intended to increase to 55% by 2060 [190]. Wastewater reuse usually demands higher standards of treatment, since it may well contain higher pathogen content than greywater [191]. Greywater reuse is, therefore, much easier when separated from wastewater [192]. However, water contamination is possible in all pathways to an extent leading to the need for adequate safety measures prior to establishment of new systems. In this regard, Singapore has approved a potential multi-phase approach to water reuse involving primary sedimentation followed by activated sludge and microfiltration then ultrafiltration, reverse osmosis and eventually disinfection by ultraviolet radiation exposure [190]. This approach can also be highly beneficial to eliminate or even significantly reduce public risks associated with the reuse of various wastewater streams. However, the targets should be well defined and proper assessment tools should be available to ensure that it meets the recommended guidelines of safe water reuse.

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