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Review

An Overview of Microbial Source Tracking Using Host-Specific Genetic Markers to Identify Origins of Fecal Contamination in Different Water Environments

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Abstract: Fecal contamination of water constitutes a serious health risk to humans and environmental ecosystems. This is mainly due to the fact that fecal material carries a variety of enteropathogens, which can enter and circulate in water bodies through fecal pollution. In this respect, the prompt identification of the polluting source(s) is pivotal to guiding appropriate target-specific remediation actions. Notably, microbial source tracking (MST) is widely applied to determine the host origin(s) contributing to fecal water pollution through the identification of zoogenic and/or anthropogenic sources of fecal environmental DNA (eDNA). A wide array of host-associated molecular markers have been developed and exploited for polluting source attribution in various aquatic ecosystems. This review is intended to provide the most up-to-date overview of genetic marker-based MST studies carried out in different water types, such as freshwaters (including surface and groundwaters) and seawaters (from coasts, beaches, lagoons, and estuaries), as well as drinking water systems. Focusing on the latest scientific progress/achievements, this work aims to gain updated knowledge on the applicability and robustness of using MST for water quality surveillance. Moreover, it also provides a future perspective on advancing MST applications for environmental research.

Keywords: aquatic environments; environmental DNA (eDNA); fecal contamination; host-specific marker genes; microbial source tracking (MST)

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1. Introduction

Fecal water contamination poses an adverse impact on the environment and constitutes a critical threat to public health [1,2]. This is primarily due to the fact that various enteric pathogens residing in the intestines of both humans and animals can enter water environments directly through fecal matter or indirectly through, e.g., sewage irrigation or manuring [3]. Consequently, at least 10% of the world's population is exposed to indirect fecal contamination by consuming food irrigated with wastewater (WW), while over 800,000 human deaths each year are caused by poor sanitation, hygiene, and water quality [2]. The resulting huge annual global economic loss reaches USD 12 billion [4]. Enteropathogen contaminations are frequently reported in various aquatic ecosystems of freshwater [5,6] and seawater [7,8], as well as in drinking water (DW) sources [9,10]. In this respect, the rapid and reliable identification of the pollution source(s)/origin(s) is of vital importance in terms of water quality management, remediation strategies, and informed health risk assessment.

Traditionally, fecal water contamination has been monitored by the detection/enumeration of fecal indicator bacteria (FIB), predominately *Escherichia coli* (*E. coli*) and enterococci [11,12], which are both classified as the primary microbiological parameters to assess the quality of water intended for human consumption [13]. However, FIB examination cannot provide information on potential source(s) of contamination [14,15], largely due to the low host specificity caused by genetic signatures shared ubiquitously across wide-ranging hosts.

Since the early 21st century, microbial source tracking (MST) has emerged [16,17] and has been widely applied for fecal source identification in various aquatic and terrestrial environments. It became a functional measure in environmental DNA (eDNA) studies since fecal matter is considered one of the predominant origins of eDNA [18]. In this regard, MST defines host-specific signatures of fecal eDNA (anthropo-zoogenic origins and sources), which are essential for the assessment of the environmental impacts of microbial pollution and the health risks of enteric pathogens [19–22].

MST encompasses a suite of tools capable of linking certain enteric microbes to a particular host. Generally, there are both culture-dependent and -independent methods, where the latter is more widely favored in terms of time and labor concerns [23]. The culture-independent methods refer basically to the molecular approaches consisting of library-dependent and -independent methods. For instance, Dela Peña et al. [24] established a fecal source library with rep-PCR fingerprints of a variety of hosts to study fecal contamination sources in Laguna Lake (Philippines). Similarly, an *E. coli*-based fecal library was created for source tracking on the French Atlantic coast [25]. However, the authors indicated that the uneven genotypic composition of the library with a high proportion of nondiscriminatory genotypes hampered its performance in MST analysis. Compared to the considerable efforts required to construct a library for the library-dependent approach, the microbial genetic marker-based library-independent methods enable rapid, reliable, and labor- and time-effective approaches [17].

The inventory of host-specific/associated genetic markers is constantly expanded and is increasingly exploited for qualitative and quantitative MST analyses in different water environments across the world [3,5,9,14,21,23–28]. The majority of genetic markers are developed to target the 16S rRNA gene of *Bacteroides* spp. of diverse host species, e.g., humans, ruminants, cattle, swine, horses, and birds [29–33]. Other bacterial species have also been considered for MST marker development. A *Helicobacter* spp.-associated GFD marker was applied to detect avian water pollution [34,35]. *Bifidobacterium* spp. 16S rRNA genes, *Enterococcus esp* gene, and *Lachnospiraceae* Lachno3 marker have been used as indicators for human fecal contamination [36–39]. There are also MST markers derived from human enteric viruses, such as adenovirus (AdV), polyomavirus (PyV), norovirus (NoV), and pepper mild mottle virus (PMMoV), which have been used to link pollution with human and/or domestic WW [40,41]. Recently, a novel human bacteriophage marker, crAssphage, has shown great potential for pinpointing anthropogenic/sewage-associated pollution [42–44]. It is noteworthy that human-associated MST gene markers have been found to be highly anthropogenic specific and thus are suggested as population-related reference biomarkers in the wastewater-based epidemiology (WBE) surveillance of SARS-CoV-2 [45].

Although there are a number of published review studies on MST applications in aquatic ecosystems, where most of these works have primarily addressed the diverse types/categories of genetic markers and their assay performances, fewer studies have focused on the prominent feasibility and applicability of the method. In view of this thematic shortage, it is essential to collectively evidence the practical significance of applying MST in different water environments, as revealed in the latest research progress/achievements. In this context, this review was conducted on a number of studies, findings, and reports of the most up-to-date published information on the implementation of MST applications in various waters across a wide range of spatial and geographic scales. All these aspects highlight the academic and technical significance of this research to the environmental science and research fields and thus demonstrate the originality and main scope of this review.

2. Methods

A retrospective literature study was conducted to identify research articles published between 1 January 2011 and 1 March 2022. The literature search was performed using renowned bibliographic databases, such as PubMed, ScienceDirect, Google Scholar, and Scopus.

A broad spectrum of keywords was applied to identify the most relevant publications for the defined scope of this review, such as microbial source tracking; fecal source tracking; fecal pollution; host-specific marker; gene; DNA; RNA; environment; freshwater; drinking water; groundwater; seawater; water contamination. Booleans, such as "AND" and "OR", were used for keywords combination.

After the rigorous screening, 146 articles were obtained, out of which 86 were identified as suitable for this review study. The applied selection criteria were: (a) original research articles; (b) peer-reviewed; (c) written in the English language; (d) non-duplicating.

3. Freshwaters

In all waters on the Earth, merely 2.5% constitutes freshwaters. In this small portion, approximately 1.7% is fixed in glaciers and ice sheets, while only approximately 0.2% forms surface waters composing lotic and lentic systems [46,47]. This small decimal proportion represents all available surface freshwaters that are estimated to cover around half of the world's DW needs [48].

3.1. Surface Waters

Amongst the Earth's aquatic ecosystems, surface freshwaters are the most exposed to multiple natural/environmental/zoogenic and anthropogenic factors, implicated by spatio-temporal dynamics driven by climate seasonal variabilities and extensive changes, water demands and quality crises, and increases in pollution varieties and ranges. Under the huge, direct, and immediate impacts of these multi-factors, surface waters are highly vulnerable to a larger extent than other water systems [49].

3.1.1. Lotic Ecosystems

There is a general perception that zoogenic fecal water contamination is predominantly derived from non-point/diffuse pollution sources (mainly environmental sites and rural catchments), while the contamination of anthropogenic fecal origin is predominately associated with point source pollution (mostly settlements and urbanized areas). However, urban diffuse pollution/runoff can also convey fecal contamination, whereas rural settlements also contribute to fecal point source pollution [14,50,51]. It is, therefore, essential to determine the origin(s) of fecal water contamination, zoogenic and/or anthropogenic, through proven measures (such as MST analyses). Urban watercourses are prone to contamination by human wastes, principally through, e.g., sewage leakage and the discharge of untreated/less efficiently treated WW. Human-associated MST markers, e.g., BacHum, HF183, Hum2, and Hum163, are used to examine the extent and origin of the contamination at numerous studied sites worldwide. For instance, the Pearl River, the third largest river in southern China, was subject to anthropogenic and sewage-associated pollution, as revealed by the detection of a high prevalence of Hum2, Hum163, and crAssphage human markers [52]. The developed *Lachnospiraceae* genome-based Lachno3 and Lachno12 markers exhibited high specificity in the detection of human fecal pollution in urban rivers in Wisconsin, USA [53]. Liao et al. [54] studied storm load impact on an inland urban stream in Virginia (USA) and revealed that HF183 was greatly associated with the peak flow reached in such events. In Norway, research on a culverted urban stream conducted for over 2 years discovered a high proportion of anthropogenic fecal contamination and a significant correlation between BacHum marker and contaminants of emerging concern (including pharmaceuticals and endocrine-disrupting chemicals), thus exposing potential water-related health risks for humans and the environment [14]. A novel marker, crAsspahge, was evaluated and applied to fecal source tracking in Chile's Mapocho River, and it indicated that human wastes from chlorinated WW effluent were the major pollution source, posing a public health risk [43].

Although urban lotic waters are frequently subjected to substantial human/anthropogenic pollution pressures, diverse environmental impacts on water quality changes cannot be neglected. Studies on cross-tracking sources of pollution (microbial/fecal, nutrients, phar-

maceuticals, and personal care products) revealed that fecal contamination in urban catchments had distinct footprints of animal/zoogenic origins [14]. In addition, the identified fecal origins were found to be significantly associated with eutrophication-causing nutrients. Thus, the key role of MST analyses was highlighted as a potential early warning measure of aquatic ecosystem deterioration. Furthermore, quantitative MST (QMST) applications disclosed that under distinct climatic conditions (e.g., cold/warm periods), the dominancy of fecal pollution from anthropogenic and zoogenic sources altered significantly in urban lotic waters [3,14]. In this context, more marker types, i.e., human- and animal-associated markers, have been used in combination to track diverse fecal sources in rivers, streams, and creeks across urban and rural regions. For instance, the Danube River, the world's most international river, was monitored for anthropogenic and zoogenic fecal contamination [55,56] using human (BacHum, HF183II), ruminant (BacR), and pig (Pig2Bac) markers. The studies found that humans were the primary source of pollution, while animals played a role in some areas [55,56]. Universal (Allbac), human (BacHum), ruminant (BacR), and horse (Hor-Bac) markers were used to identify fecal origin(s) in different Norwegian lotic waters and accurately pinpointed the dominant anthropogenic and zoogenic sources in urban and rural catchments, respectively [1,14,57]. In New Zealand, a marker panel including universal (GenBac3), human (HumM3, HumBac, *Bifidobacterium adolescence*), wildfowl, and canine markers were applied in an MST study of the Avon River [38]. The investigation discovered that the intrusion of untreated WW from an earthquake-damaged sewage system accounted primarily for the pollution in the river. The study also indicated that human MST markers could act as effective predictors of protozoan pathogens in water. Similarly, another multi-marker MST survey was conducted in the Tiaoxi River in China [35]. The marker set consisted of universal (BacUni and GenBac), human (HF183, BacHum and Hum2), swine (Pig2Bac), cattle (BacCow), and avian (AV4143 and GFD) markers. The survey assigned the pollution to human and avian sources; moreover, it detected a high prevalence of waterborne pathogens, such as *Campylobacter jejuni* (*C. jejuni*) and *Shigella* spp., in many locations of the river, indicating serious health risk potential.

3.1.2. Lentic Ecosystems

MST is broadly used for studies of human and environmental impacts in lakes (i.e., the most targeted lentic ecosystems) of varying scales and geographic conditions. The routine monitoring and examinations of standard FIB (*E. coli* and enterococci) are coupled with MST assays using genetic markers of both anthropogenic origin and various zoogenic sources. Apart from the extensively applied *Bacteroidales* DNA markers, other bacteria species and human mitochondrial DNA (mtDNA) are also targeted for MST analyses. In a large-scale survey of water quality in the Great Lakes recreational area of the USA [58], diverse fecal pollution sources were identified, including humans (e.g., sewer overflow and defective septic systems), pets (e.g., dogs), shore birds, and ruminants (e.g., deer and upstream farming), using respective host-specific markers, i.e., human (HF183/BacR287 and HumM2), canine (DG3), avian (GFD), and ruminant (Rum2Bac). This study emphasized the importance of applying MST for the routine and rapid resolution of variable fecal pollution sources across a vast spatial range, enabling the prioritized remediation of disparate sources. Urban and suburban lakes (e.g., Lake Parramatta in Sydney, Australia, and Lake Carroll in Florida, USA) were found to be vulnerable to human fecal pollution, as revealed by investigations using human- and sewage-associated MST markers, e.g., HF183, crAssphage, *Enterococcus faecium esp* gene, and human PyV and PMMoV [40,42,59]. Moreover, these recreational lakes were found to be directly fecally polluted by both anthropogenic and zoogenic sources, humans (e.g., swimmers and beach visitors) and animals (e.g., dogs and gulls), respectively. Using *Catellicoccus marimammalium* and human *Bacteroidales* markers, black-headed seagulls and humans were identified as polluting origins in an urban scenery lake in southern China [60]. Through the MST analysis of three inland lakes in Ohio, USA, multi-fecal sources (i.e., humans, cattle, dogs, and gulls) were characterized by targeting general *Bacteroidetes* Allbac, human HF183, bovine BoBac, canine BacCan, and seagull Gull2

markers [61]. Similarly, Shrestha et al. [62] linked the fecal contamination of beaches in Lake Michigan (USA) to human, dog, and avian sources, using human (HF183/BacR287, HumM2), dog (DG3 and DG37), and avian GFD MST markers. However, in the beach water of Lake Pontchartrain, Louisiana (USA), swimmers were found to be the primary source responsible for fecal pollution [63].

Amongst MST surveys of human and environmental impacts on the quality of aquatic lentic ecosystems, there are also findings indicating that fecal water pollution could be greatly impacted by land use patterns. For instance, agricultural and human sources were identified as the main contributors to water quality exacerbation in Laguna Lake in the Philippines [24,64]. These results were acquired through the examinations of the *E. coli* heat-labile toxin IIA (*LTIIa*) gene and heat-stable II (*STII*) gene specific to cattle and swine, human mtDNA, and genus-specific *Cryptosporidium* markers [24,64]. Swedish Lake Rådasjön, serving as a drinking water source, was fecally contaminated by sewer overflow, stormwater, and cattle (from an adjacent grazing area), which was revealed by MST of human BacH and ruminant BacR markers [65]. Similar findings were reported from freshwater lake studies in the USA, where cattle from adjacent grazing areas were the major fecal contributor to water contamination [66]. These studies indicated that in undeveloped lakes, e.g., in remote and wilderness areas, animals played a larger role than humans (less frequent visiting) in water fecal pollution. This was verified through the recent investigations of Pendergraph et al. [67] in a number of remote alpine lakes within the Absaroka Beartooth Wilderness (Montana, USA). The investigation involved MST studies using the droplet digital PCR (ddPCR) of general marker Allbac (vastly detected) and human BacH (barely detected), which attributed the fecal contamination mostly to animals. In addition, the obtained results suggested a relatively low prevalence of waterborne pathogens (e.g., human-derived *Bacteroides*) associated with minimal pollution by human fecal microorganisms [67]. However, pathogen contamination from zoogenic sources cannot be ignored, as discovered by Ahmed et al. [68] through the examination of a number of water samples collected from the Wivenhoe Reservoir in Brisbane, Australia. The reservoir was extensively used for camping and outdoor recreational activities (swimming, boating and fishing); however, the suspected sources of fecal pollution were predominantly the intensive grazing of cattle with direct access to the reservoir. This was confirmed by the MST tests applying bovine-associated *Bacteroidales* markers (i.e., BacCow-UCD, cowM3), bovine AdV, and bovine-related zoonotic pathogens (i.e., *Campylobacter* spp., *Salmonella* spp., and *E. coli* O157), which revealed bovine fecal contamination associated with the presence of zoonotic pathogens [68].

3.2. Groundwaters

Groundwater (GW) constitutes the largest freshwater reservoir (97%) available for human use in the world [69]. It is a vital drinking water source in many countries, especially in low-income regions [70]. Globally, approximately 2.2 billion people are dependent on GW for their daily consumption [71]. However, GW is prone to the contamination of enteric pathogens. An increased risk of human infectious diseases in association with polluted GW has been noticed. Therefore, the routine and close quality monitoring of GW, e.g., involving MST analyses, is pivotal to mitigating potential health risks.

GW wells, although beneath the Earth's surface, are still under various pressures from land-related pollution sites; hence, they are exposed to a similar variety of fecal contamination sources as surface waters. In practice, a like range of anthropogenic- and zoogenic-associated genetic markers is applied in GW microbial quality studies. It is noteworthy that anthropogenic fecal pollution is mostly attributed to underground impacts, e.g., defects in sewer infrastructure, leakages from sewage networks, and the infiltration of untreated WW, for instance, leaching from malfunctioning septic systems contaminating the adjacent wells, which was confirmed by MST assays using human HF183 marker [72,73]. The zoogenic fecal pollution of GW originates predominately from animals' surface activities (e.g., pasturing, farming, and manuring). In response to these various contamination sources, different

human-/animal-associated genetic markers were established and adopted for field surveys. For instance, a panel of host-specific markers, i.e., human (BacHum, HF183), ruminant (BacCow, BacR), swine (Pig2Bac, PF163), and canine (BacCan), were used for tracking fecal pollution in a number of drinking water wells of various types and at different depths in Kathmandu Valley, Nepal [74]. The results explicitly pointed out that human markers were detected in higher ratios in built-up areas, while ruminant markers were prevalent in agricultural regions. Comparatively, variable pollution sources across different regions of Ontario, Canada, were found in numerous private wells that were examined over multiple years by Krolik et al. [75]. Some were linked to human wastes from faulty septic systems, as revealed by the human-specific marker BacHum, while others were ascribed to cattle, as confirmed by the bovine-specific marker BacBovine. Similarly, animal feces were highly suspected to be the leading cause of drinking water well contamination in remote rural areas of Pueblo Nuevo, Nicaragua, indicated by the detection of human- and bovine-specific markers [76]. Zoogenic sources of GW fecal contamination were also identified in Japan, as *Bacteroidales* markers for ruminants (BacR) and swine (Pig2Bac) were found in both public and private wells [41]. In addition, PMMoV, norovirus genogroup II (NoV GII), and rotavirus A (RoV A) were also detected in the examined wells, suggesting the anthropogenic impact on GW quality generating a health concern. The identified pollutions were most likely associated with frequent flooding episodes, causing the infiltration of overloaded surface runoff carrying livestock wastes and untreated WW [41]. Health threats were also identified in the studies of private wells in Kewaunee County, Wisconsin, USA [77]. Contamination caused by human and zoonotic enteric pathogens resulted from the application of cattle manure and defects in private septic systems. In these studies, human- and bovine-specific *Bacteroidales* markers together with associated viral and protozoan enteric pathogens, were used to assign the polluting sources and provided the data input for quantitative microbial risk assessment (QMRA). The analysis suggested that the contamination of private wells could account for 301 cases of acute gastrointestinal illness (AGI) per year, among which most (230 cases) were associated with cattle fecal sources [77].

Agricultural areas can accumulate great anthropo-zoogenic pollution impacts (from settlements, livestock, and farming); thus, MST approaches were also devised for the quality evaluation of GW from agricultural wells, i.e., those supplying water primarily for food and livestock production (e.g., breeding, culturing, farming, gardening, and irrigation). Recent studies by Carrey et al. [78] discovered mixed sources of nitrate, including humans, pigs, ruminants, and poultry, in agricultural wells in Catalonia (Spain). These sources were determined by *Bifidobacterium* genetic markers specific for human (HMBif), ruminant (CWBif), poultry (PLBif), and *Bacteroidales* swine (Pig2Bac) markers, in conjunction with multi-isotopic analysis. Alsalah et al. [79] investigated wells in agricultural fields in Saudi Arabia using human- and chicken-associated *Bacteroides* markers and found GW to be heavily contaminated by sewage. Furthermore, a high abundance of antibiotic-resistant bacteria (ARB) was detected in the harvested fruits irrigated with the affected GW, which raised another critical health concern [79].

MST forensics is also applied to assess the GW qualities of springs and wells in karst regions, which tend to be susceptible to the pollutants from the surface and underground due to their specific geological structures. This, for instance, was investigated over a large area in the USA (Illinois, Wisconsin, Kentucky, and Missouri) by Zhang et al. [80] using human-, swine-, and bovine *Bacteroidales*-specific markers, along with environmental measures. These results indicated that human and animal wastes have largely contaminated the local karst GW systems, where the contamination was worse in spring water in comparison to well water. Similar findings were also revealed in the study of Diston et al. [81], where the spring water in karst St Imier Valley (Switzerland) was found to be more degraded than the well water, as impaired by human and ruminant fecal wastes. Ohad et al. [82] implemented qPCR-based MST to assess human/animal fecal impacts on adjacent karst springs under dry/wet seasonal conditions in northern Israel. They found signatures of human, bovine, and swine markers fluctuating over the seasons; however, human contamination

occurred even during the dry season, suggesting a continuous and direct exposure of the springs to septic systems. Stange and Tiehm [83] reported that following heavy rain, an increased amount of FIB was observed, along with high levels of antibiotic resistance genes (ARGs) and human-specific MST markers, in a karst spring in Germany, which was largely attributed to the sewer overflow.

3.3. Drinking Water Systems

The provision of safe potable water at the point-of-use is of paramount importance globally. According to the UN's MDG report [84], 1.9 billion people have gained access to piped drinking water (DW) since 1990. However, there are still 663 million people using unimproved DW sources, among which half of them live in sub-Saharan Africa, and one-fifth live in South Asia. Microbial pathogen contamination poses an adverse impact on DW quality, which is greatly associated with public health. The contaminated DW can cause severe gastrointestinal illnesses, to which young children are particularly susceptible, and induce epidemic outbreaks affecting large communities. Among all the possible causes, raw source water contamination, treatment deficiency, and distribution network failure are the most common ones [10]. MST genetic markers are used as indicators to discriminate fecal polluting sources between humans and non-human species in each linked part of the DW system, i.e., from raw water to the point-of-use along the DW supply chain.

Fresh surface waters and GW are the greatest DW sources on Earth. MST based on host-specific DNA signatures (markers) is extensively applied to assess the cause(s) of fecal contamination in these waters, which have been already addressed in the above sections of this review. However, it is worthwhile to gather extensive information regarding the impacts on DW systems. For instance, multi-factors such as land use patterns related to variable agricultural activities, road management, and surface water intrusion were identified as the main causes of the declined quality of potable water supplied from small systems of shallow GW in rural areas of Finland [85]. These impacts were assessed based on the *Bacteroidales* general marker GenBac3 and human HF183 used for MST assay, together with amplicon sequencing-based bacterial community mapping and coupled physicochemical parameters. In a survey of community water sources (e.g., tube wells and ponds) and home-stored water in a rural area of Odisha in India, the universal GenBac3, human HF183, and cattle BacCow markers were detected in 74%, 36%, and 82% of the studied households, respectively [86]. These results indicated that both animal and human wastes contaminated the well water sources, while cattle were assumed as the dominant source. Similar detections in source and stored water were reported from sub-Saharan African countries. In Kenya, a number of rural households used fecally contaminated DW, as confirmed by human (HF183), avian (GFD), and ruminant (BacR) markers [87]. In addition, ruminant signatures were detected more frequently in stored waters as compared to source waters. In Cameroon, source water and home-stored water in rural (the Far North region) and urban (four neighborhoods in Maroua) regions were examined through two separate studies [88,89], using human (HF183 and gyrB), ruminant (Rum2Bac), and bird (GFD) MST markers. Their outcomes unveiled that mixed and varied anthropo-zoogenic polluting sources were observed in urban areas, most likely caused by varying population densities and ethno-economic characteristics, while persistent and ubiquitous ruminant fecal contamination was prevalent throughout the studied rural areas and enrolled households. In addition, zoonotic pathogens (e.g., *Campylobacter*, *Salmonella* and *Cryptosporidium*) were molecularly identified in the fecally contaminated DW sources (wells and boreholes) and household water containers in the studied rural and urban sites, which were associated with registered local gastrointestinal illnesses [88,89]. Notably, MST toolkits have greatly supported the timely identification of DW fecal contamination, causing the largest waterborne *Campylobacter* outbreak (2019) in Norway [21]. At that time, zoogenic sources (with primarily horse fecal contribution assessed through a fecal source apportionment profile (FSAP)) were discovered to contaminate an old caved DW holding pool from which water was distributed to approximately 15,000 people [21]. Similarly, non-human sources

were characterized as problematic in water quality studies comparing the conditions before and after the construction of a DW treatment plant in the Galapagos Islands [90]. It was indicated that, despite a significant decrease in microbial contamination after the intervention, improvements in treatment and distribution infrastructures were still needed since fecal contamination derived from non-human sources remained in some post-distribution sites. Failures in the DW distribution systems led to two DW outbreaks (2016 and 2018) in Finland, during which MST examinations using GenBac3 and HF183 contributed largely to confirming contamination and attributing it to the intrusion of municipal WW into the DW distribution network through pipe breakage [9].

4. Sea/Salt Waters

Over 97% of all water on the Earth is salt water, whereas the majority (96.5%) covers the Earth's surface (seas and oceans), some (around 1%) represent saline GW [47]. In this regard, it is of critical importance to apply fecal source tracking to assess the quality of seawaters, where pollution can result in huge negative impacts on diverse marine ecosystems. Related problems have already become prominent along coastlines, which are composed of many recreational beaches and bathing waters. In these areas, fecal pollution can arise from diverse sources, e.g., sewer overflows, drainage of stormwater, urban and rural runoff, leakage of septic or sewage systems, and human activities [28,91]. The examination of FIB (i.e., *E. coli* and enterococci) in these waters has been widely implemented for routine monitoring programs. However, the need to discriminate polluting sources among a large variety of possible hosts warrants the extensive application of MST tools in different marine and coastal regions across the world.

A considerable number of studies have focused on recreational beach waters, which are greatly associated with human activities and public health. Humans/sewage inputs have been frequently identified as the major pollution sources on public urban beaches [92], urban runoff-impacted beaches [93], marine beaches [94], coastal beaches [95], and surfing beaches [96], often using HF183 and HumM2 MST markers. Bathers and beach users/visitors were considered to contribute directly and significantly to the resulting fecal pollution, which is exacerbated by stormwater discharge. In addition, indirect human inputs are reported, especially in the discharge zones of inefficiently treated sewage. For instance, marine waters along the coastlines of the Southwest United States and Mexico were found to be under constant human fecal contamination as a result of receiving effluents from the nearby WW treatment plant [97,98]. This was verified through multiple microbial source tracking tools; i.e., digital droplet PCR (ddPCR) was adopted for MST assays, and the findings (WW-impacted pollution) were in agreement with the data analyzed by the 16S rRNA gene sequencing-based SourceTracker algorithm [98].

Apart from direct/indirect anthropogenic contamination, zoogenic fecal sources are also often detected. Dog and seabird/gull feces are most frequently found to contaminate sand and waters, e.g., Poche Beach, USA [99]; bathing waters in Wells, USA [100]; strands in Dublin Bay, Ireland [44]; and the Goleta surf zone, USA [101], using various human-, gull-, and canine-associated MST markers. Notably, MST findings enable the launch of timely and effective measures and actions, not only restrictions for humans (and accompanying pets) but also a best management practice (BMP) to inhibit environmental pollution sources. For instance, a gull abatement BMP was successfully executed in California (USA), leading to substantial quality improvements in the affected beach water, which was validated through MST analyses with gull (GullMST), canine (DogMST), and human (HumMST) source tracking markers [99].

In addition to marine waters, brackish waters (e.g., lagoons and estuaries) are also greatly impacted by fecal contamination deriving from various environmental and anthropogenic sources. In a recent sanitary survey, a shallow coastal lagoon in Manly Beach, Sydney (Australia), was found to be contaminated mainly by sewage overflows and dog feces, as disclosed by MST using human HF183/Bac242 and dog BacCan-UCDmodif markers [102]. Similar findings were reported in urban estuarine waters where sewer

overflows originated from combined sources, such as human wastes from peri-urban settlements, and animal feces (e.g., avian and dogs) transported by stormwater runoff were detected using respective human (e.g., HF183, crAssphage CPQ_056, Lachno3), avian GFD, and dog BacCan-UCD host-specific markers [103,104]. Additionally, coastal waters in estuarine regions used for shellfish harvesting and aquafarming have been quality examined. Fecal contaminations in these waters are closely related to land-use patterns; thus, impacts from various polluting sources can be expected. For instance, harvesting waters for oysters in Elorn estuary (France) were investigated based on sources of fecal contamination suspected from nearby livestock farms and a WW treatment plant [105]. Human-, ruminant-, and pig-associated *Bacteroidales* MST markers, Hum-1-Bac, Rum-2-Bac, and Pig-2-Bac, were applied, respectively. The MST method proved efficient and useful in pinpointing the main pollution origin, where humans were identified as the predominant fecal contamination source in oysters and water in the harvesting areas of the estuary, while animal MST markers were rarely detected [105]. A similar application of different host-specific *Bacteroides* markers (human—HF183; pig—Pig-2-Bac; poultry—qCD; and ruminant—BacR) was implemented to track fecal contamination origins and sources in two major areas for aquaculture (fisheries and shellfish farms) in South Korea [106]. The assays revealed clear variation in concentrations related to the characteristic of areas near water, i.e., the prevalence of anthropogenic fecal contamination associated with residential areas and the dominance of zoogenic (mainly poultry) fecal pollution caused by livestock breeding [106]. Apart from the land-use patterns, the impact of seasonal/climatic variations on water quality and fecal pollution in estuaries have also been observed. As indicated in the pioneering MST study conducted in the tropical region of Central America, shellfish harvesting waters did not reveal obvious anthropogenically derived pollution, deduced from the absence of human/domestic WW-associated markers, such as human HF183, and viral PyV and PMMoV markers [34]. However, FIB concentrations in those waters varied significantly over periods, with the greatest counts during the rainy season, suggesting fecal pollution through runoff and sediment resuspensions [34].

The MST studies in salt waters along with the other aquatic environments worldwide, are summarized in Table 1. The presented findings retrieved from the most updated and relevant publications demonstrate the robustness and great applicability of using genetic marker-based MST for rapid and reliable fecal source tracking. With this defined focus in mind, the review is not projected to address the technical/methodological aspects of each individual marker being used in each respective study in great detail. These technical details can be easily retrieved from the original publication with available access.

Table 1. MST studies using various host-associated genetic markers for source(s) determination of fecal contamination in distinct water environments.

Water Types	Study Sites	Countries	MST Markers Used	Fecal Pollution Sources	References
Lotic ecosystems	The whole Danube River and the main tributaries	Germany, Austria, Slovakia, Hungary, Croatia, Serbia, Romania, Bulgaria, and Ukraine	Allbac, BacHum, HF183II, BacR, and Pig2Bac	Humans (major), animals (minor)	[56]
	Danube River in Vienna	Austria	BacHum, HF183II, BacR, and Pig2Bac	Humans, sewage, animals	[55]
	Mpocho River	Chile	Viral human faecal marker-crAssphage CPQ_064, HF183, and norovirus GII	Wastewater	[43]
	Pearl River	China	Hum2, Hum163, and crAssphage	Humans, sewage	[52]
	Tiaoxi River	China	BacUni, GenBac, HF183, BacHum, Hum2, Pig2Bac, BacCow, Av4143, and GFD	Humans, birds	[35]

Table 1. *Cont.*

Water Types	Study Sites	Countries	MST Markers Used	Fecal Pollution Sources	References
	Avon River	New Zealand	GenBac3, HumM3, HumBac, wildfowl-, and canine-associated markers	Wastewater intrusion	[38]
	Urban rivers	USA	Lachno3 and Lachno12	Sewage	[53]
	Inland urban stream	USA	HF183 and general marker	Humans	[54]
	Culverted urban stream	Norway	Allbac, BacH, and BacR	Humans (major), animals	[3,14]
	Rural creaks	Norway	Allbac, BacH, BacR, and Hor-Bac	Humans, wastewater, agricultural runoff, animals	[14,23]
Lentic ecosystems	Great Lake Basin	USA	HF183, BacR287, HumM2, Rum2Bac, DG3, and GFD	Dogs, birds	[58]
	Lake Parramatta	Australia	HF183, crAssphage DPQ_056, PMMoV, BacCan-UCD, cowM2, and GFD	Humans, sewage	[40]
	Lake Carroll	USA	Sewage-associated *E. faecium* esp gene, HF183, and human PyVs	Humans	[59]
	Laguna Lake	Philippines	Host-associated *E.coli* marker, heat-labile toxin (*LTIIA*), heat-stable II (*STII*), cattle and swine marker, human mtDNA, and genus-specific *Cryptosporidium* markers	Humans, cattle, ducks	[24,64]
	Lake Michigan	USA	HF183/BacR287, HumM2, DG3, DG37, and GFD	Humans, dogs, birds	[62]
	Lake Rådasjön	Sweden	BacH and BacR	Sewer overflow, cattle	[65]
	Absaroka Beartooth Wilderness	USA	Allbac and BacH	Animals (major), humans (minor)	[67]
	Wivenhoe Reservoir	Australia	BacCow-UCD, cowM3, and viral B-AVs (bovine adenovirus) marker	Bovines	[68]
	Lake Pontchartrain	USA	HF183 and cowM3	Humans (swimmers)	[63]
	Freshwater lakes	USA	Markers of *Methanobrevibacter smithii*, human PyVs, ruminant, human (HF183), and general *Bacteroidales*	Cattle	[66]
	Urban scenery lake	China	*Catellicoccus marimamalium* marker, human-associated *Bacteroidales*, thermophilic *Campylobacter* and *Helicobacter*	Black-headed seagull, humans (limited)	[60]
	Inland recreational lakes	USA	Allbac, HF183, BoBac, BacCan, and Gull2	Ruminants, dogs, gulls, humans	[61]
Groundwaters	Public and private water wells	USA	HF183/BacR287, human adenovirus group A, HumM2, HF183, *Cryptosporidium hominis*, group A rotavirus G1 P, and bovine CowM2, CowM3, bovine enterovirus, bovine polyomavirus, and group A rotavirus G10 P	Malfunctioning septic system/septic leaching, lawn care runoff, and land-applied cattle manure	[72,73,77]
	Drinking water wells	Nepal	BacHum, HF183, BacCow, BacR, Pig2Bac, PF163, and BacCan	Humans, ruminants, pigs	[74]
	Private drinking water well	Canada	BacGeneral, BacHuman, and BacBovine,	Humans, septic system, cattle	[75]
	Rural drinking water wells	Nicaragua	Human and bovine specific markers, *Bacteroides thetaiotaomicron*, and CowM2	Animals	[76]
	Public and private drinking water wells	Japan	BacR and Pig2Bac	Ruminants and pigs	[41]

Table 1. *Cont.*

Water Types	Study Sites	Countries	MST Markers Used	Fecal Pollution Sources	References
	Agricultural wells	Spain	*Bifidobacterium* species, HMBif, CWBif, PLBif, and Pig2Bac	Humans, pigs, ruminants, and poultry	[78]
	Wells near agricultural fields	Saudi Arabia	Human-associated *Bacteroides* spp. (*B. vulgatus*, *B. fragilis*, and *B. uniformis*), and chicken-specific metabolism inorganic ion of *B. fragilis*	Sporadic occurrence of anthropogenic contamination	[79]
	Karst springs and wells	USA	Host-specific markers for human, swine, and bovine	Humans and livestock	[80]
	Well and spring karst sites	Switzerland	Allbac, GenBac, HF183, HuBac, BacR, and Rum2Bac	Humans and ruminants	[81]
	Karst springs	Israel	GenBac, BacR, CowM2, CowM3, Pig2Bac, BacH (I/II), and HumM3	Septic systems, pigs, and ruminants	[82]
	Karst spring	Germany	Host-specific markers for human, horse, chicken, and cow	Humans/sewer overflow	[83]
Drinking water systems	Potable water from shallow wells in rural areas	Finland	GenBac3 and HF183	Agricultural activities, road and surface water intrusion	[85]
	Household-stored water	Kenya	HF183, BacHum, humM2, BacCow, Rum2Bac, BacR, and GFD	Ruminants and humans	[87]
	Source water and household-stored water	Cameron	HF183, gyrB (gyrase subunit B from human-specific *Bacteroides fragilis*), Rum2Bac, *Bacteroidales* 16S rRNA gene, and GFD	Humans, ruminants, and birds	[88,89]
	Water treatment systems	Ecuador	HF183	Animal wastes, humans (insignificant)	[90]
	Water distribution network	Finland	HF183 and GenBac3	Untreated municipal wastewater intrusion	[9]
	Water holding pool	Norway	Allbac, BacH, BacR, and Hor-Bac	Animals, horse (dominant)	[21]
Seawaters	Poche Beach	USA	Human (HumMST), gull (GullMST), canine (DogMST), and GenBac	Gulls	[99]
	Goleta Beach	USA	HF183, HumM2, DogBact, Gull2, and HoF597	Gulls (major), dogs, humans	[101]
	Surfing beaches	USA	HF183, avian, and canine markers	Humans, sewers, birds, and canines	[96]
	Recreational beaches	USA	HF183, BsteriF1, BuniF2, and HumM2	Humans, sewage	[93,94]
	Coastal recreational waters	USA	Allbac, HF183, GFD Bac32, CF128, DF475, and Gull2	Humans, gulls, and dogs	[100]
	Coastal beaches of Rio de Janeiro	Brazil	*Methanobrevibacter smithii nifH* gene marker	Humans	[95]
	Prophète Beach	France	Human-, dog-, horse-, and gull/seagull markers	Humans, dogs, and gulls	[92]
	Urban marine bathing waters (Sandymount Strand and Merrion Strand)	Ireland	crAssphage crAss_2 marker, HF183, canine, and gull genetic markers	Humans and seabirds	[44]
	Baja California	Mexico	HF183	Wastewater treatment plant discharge	[97]
	Manly Lagoon	Australia	HF183/Bac242, and BacCan-UCDmodif	Humans, sewerage overflow, and dogs	[102]
	Urban estuaries	Australia	HF183, PMMoV, crAssphage CPQ_056, *Lachnospiraceae* Lachno3, GFD, BacCan-UCD, cowM2, and HoF597	Sewage, birds, and dogs	[104]

Table 1. *Cont.*

Water Types	Study Sites	Countries	MST Markers Used	Fecal Pollution Sources	References
	Urban estuary (Golden Horn)	Turkey	*B. thetaiotaomicron* *α-1,6-mannanase* (BT-α) marker	Sewer overflow	[103]
	Elorn estuary	France	Human-, ruminant-, and pig-associated *Bacteroidales* markers	Humans	[105]
	Gulf of Nicoya	Costa Rica	HF183, human PyV, and PMMoV markers	Non-human	[34]
	Aphae Island and Goseong Bay	South Korea	Host-specific *Bacteroides* markers for human, poultry, pig, and ruminant	Poultry and humans	[106]

5. Concluding Remarks and Future Perspectives

This review scrutinizes and compiles the most relevant and up-to-date research achievements in MST forensics using host-associated genetic markers in various and distinct fecally contaminated aquatic environments. The literature survey highlights the broad feasibility and robustness of using such MST approaches for water quality assessments, particularly among different types of water environments, which have not been extensively addressed in previous MST review studies.

Host-specific markers, which are derived primarily from diverse bacterial, viral, and bacteriophage species, are under constant development. These markers have been successfully used as proxies to discern the direct or indirect fecal contamination originating from human vs. non-human sources in freshwaters (i.e., surface and groundwaters) and marine/salt waters (e.g., estuary, lagoon, and beach waters). The established and ever-growing MST toolkits, providing rapid, easily operating, and reliable analyses, are progressively implemented in routine water quality surveillance to facilitate informed management decisions. Evidently, the suite of tools has greatly served, for instance, the administrating DW infrastructures (e.g., raw freshwater sources, water treatment facilities, distribution, and supply systems), WW system surveillance (e.g., combined sewer networks, centralized treatment plants, on-site purification systems and effluent discharges) and the operation of recreational areas (e.g., controlling bathing water quality and decision making on beach closure/re-opening).

In view of the ever-expanding inventory of genetic markers, i.e., many new markers are continuously developed and introduced for use, it is vital to give sufficient consideration to their geographic variability, which may compromise the markers' sensitivity and specificity. Thus, adequate examinations using local/regional fecal reference materials are essential to verify the performance of the newly introduced marker prior to its full application for water assessments. It also must be noted that the persistence of each individual marker in divergent aquatic environments can be variable; thus, the marker decay rate under different environmental conditions (e.g., ambient temperature and length of sunlight) must be considered upon data analysis and result interpretation. Recently, MST analysis data have been utilized and integrated into the QMRA modelling system, striving to enable the prediction of enteropathogen-induced waterborne disease morbidity. Although the prognostic system has demonstrated considerable disease-predicting potential, more efforts must focus on further optimization in terms of the reliability and reproducibility of the system, especially under variable environmental and climatic scenarios.

Notably, other remaining issues must also be better addressed for the further advancement of MST technology in environmental research. For instance, currently, more than one genetic marker for identifying a single host species can be found; concerning this practical issue, it has been proposed to include more markers for species assignment in MST assays. In this way, the findings obtained from the examination of different markers can be compared and used for verification. Additionally, the implementation of other non-marker-based source tracking methods, such as chemical analyses, can also be considered to support the validation of MST findings. For instance, caffeine detection can be used to

confirm/verify the detection of the human pollution source. Nowadays, with the extensive engagement of next-generation sequencing (NGS) technology in water quality research, MST analysis can be remarkably reinforced by leveraging NGS for MST result verification, linking MST results with pathogen identification/screening and genomic mining for new MST marker development. To this end, multiple methods used in combination will synergistically enhance the MST assay's overall precision and reliability.

Regarding the final interpretation of the results, presently, most studies are, in principle, determining the fecal contamination source(s) based on the detection frequency, namely, the positive detection rate during an examining period. However, this evaluation approach is unable to provide the real-time dominant source(s) attribution, which should be determined for the study site at the actual sampling timepoint, i.e., independent of a series of data collected over time. In this regard, a better quantitative apportionment algorithm, which is able to depict the fecal contribution profile, thus allowing to pinpoint the dominant source(s), is more desirable. Such advanced analysis platform applying the FSAP has emerged, been tested, and practically implemented, e.g., during the largest waterborne *Campylobacter* outbreak in Norway (2019). Apparently, upon any emergent waterborne outbreak of variable scale, a timely determination of fecal pollution origin(s) is pivotal for the early and prompt control of the situation to mitigate the potential water-related health risks of enteric pathogens for humans and the environment. Finally, there is also an urgent need for the standardization of MST operating procedures, e.g., sample collection and preservation, microbial DNA extraction methods, and qPCR protocols for marker detection. This is of critical importance for cross-laboratory and -country data comparison, technology improvements, and further advancement of MST applications.

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Abbreviations and Acronyms: AdV—adenovirus, AGI—acute gastrointestinal illness, Allbac, BacUni, GenBac, and GenBac3—general/universal *Bacteroidales* markers, ARB—antibiotic resistant bacteria, ARGs—antibiotic resistance genes, AV4143 and GFD—avian-associated genetic markers, BacBovine, BacCow, BacCow-UCD, BoBac, CF128, cowM2, and cowM3—bovine-/cattle-/cow-associated genetic markers, BacCan, BacCan-UCDmodif, DF475, DG3, DG37, DogBact, and DogMST—canine-associated genetic markers, BacH, BacHum, BsteriF1, BuniF2, crAssphage CPQ_056, HF183, HF183II, HF183/BacR287, HF183/Bac242, HumBac, HumM2, HumM3, HumMST, Hum-1-Bac, Hum2, Hum163, and gyrB—human-associated genetic markers, BacR and Rum2Bac—ruminant-associated genetic markers, B-AVs—bovine adenovirus, BMP—best management practice, *C. jejuni*—*Campylobacter jejuni*, crAssphage—cross-assembly phage, CWBif—ruminant-specific *Bifidobacterium* marker, ddPCR—droplet digital polymerase chain reaction, DW—drinking water, *E. coli*—*Escherichia coli*, *E. coli* O157—*Escherichia coli* O157 somatic antigen, eDNA—environmental deoxyribonucleic acid, FIB—fecal indicator bacteria, FSAP—fecal source apportionment profile, Gull2 and GullMST—gull-associated genetic markers, GW—groundwater, HMBif—human-specific *Bifidobacterium* marker, HoF597 and Hor-Bac—horse-associated genetic markers, Lachno3 and Lachno12—human-associated *Lachnospiraceae* genetic markers, *LTIIa*—heat labile enterotoxin type IIa, MST—microbial source tracking, mtDNA—mitochondrial deoxyribonucleic acid, NGS—next-generation sequencing, NoV—norovirus, NoV GII—norovirus genogroup II, PCR—polymerase chain reaction, PF163 and Pig2Bac—pig-specific genetic markers, PLBif—poultry-specific *Bifidobacterium* marker, PMMoV—pepper mild

mottle virus, PyV—polyomavirus, qCD—poultry-specific *Bacteroides* marker, QMRA—quantitative microbial risk assessment, QMST—quantitative microbial source tracking, qPCR—quantitative polymerase chain reaction, RoV A—rotavirus A, SARS-CoV-2—severe acute respiratory syndrome coronavirus 2, Spp.—species, *STII*—heat-stable enterotoxin II, WBE—wastewater-based epidemiology, WW—wastewater.

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