Global Distribution of Human-Associated Fecal Genetic Markers in Reference Samples from Six Continents


1Research Group Environmental Microbiology and Molecular Diagnostics 166-5-3, Institute of Chemical, Environmental and Bioscience Engineering, TU Wien, 1060 Vienna, Austria
2Interuniversity Cooperation Centre Water & Health, Vienna, Austria
3Molecular Diagnostics Group, IFA-Tulln, Institute of Chemical, Environmental and Bioscience Engineering, TU Wien, 3430 Tulln, Austria
4Institute of Hydraulic Engineering and Water Resources Management, TU Wien, 1040 Vienna, Austria
5Environment & Public Health Research and Enterprise Group, School of Environment and Technology, University of Brighton, BN2 4GJ Brighton, U.K.
6Institute for Water Quality and Resource Management, TU Wien, 1040 Vienna, Austria
7CSIRO Land and Water, Ecosciences Precinct, 41 Boggo Road, Qld 4102, Australia
8Department of Genetics, Microbiology and Statistics, University of Barcelona, 08028 Barcelona, Spain
9Department of Biochemistry, Makerere University, P.O. Box 27755 Kampala, Uganda
10Affordable Water Limited, 1011 Auckland, New Zealand
11Department of Biosciences, Sokoine University of Agriculture, PO BOX 3038, Morogoro, Tanzania
12Laboratorio de Aguas y Suelos, Instituto de Investigaciones para la Industria Química (INIQUI), Consejo Nacional de Investigaciones Científicas y Técnicas y Universidad Nacional de Salta, CP 4405 Salta, Argentina
13Environment and Climate Change Canada, Canada Centre for Inland Waters, Burlington, L7S 1A1, Ontario, Canada
14Bavarian Environment Agency, 86179 Augsburg, Germany
15Department of Epidemiology and Environmental Health Sciences, Georgia Southern University, Statesboro, 30460 Georgia, United States
16Department of Fisheries and Wildlife, Michigan State University East Lansing, 48824 Michigan, United States
17Institute for Hygiene and Applied Immunology, Water Hygiene, Medical University of Vienna, 1090 Vienna, Austria
18New Industry Creation Hatchery Center, Tohoku University, 980-8579 Sendai, Japan
19Departamento de Análises Ambientais, CETESB - Cia. Ambiental do Estado de São Paulo, 05459-900 São Paulo, Brasil
20Singapore Centre for Environmental Life Sciences Engineering and School of Civil and Environmental Engineering, Nanyang Technological University, 637551 Singapore
21U.S. Environmental Protection Agency, Office of Research and Development, 45268 Cincinnati, Ohio, United States
22VRVis Research Center, 1220 Vienna, Austria
23Research Division Biochemical Technology, Institute of Chemical, Environmental and Bioscience Engineering, TU Wien, 1060 Vienna, Austria
24Division Water Quality and Health, Department Pharmacology, Physiology and Microbiology, Karl Landsteiner University of Health Sciences, 3500 Krems an der Donau, Austria

Supporting Information

ABSTRACT: Numerous bacterial genetic markers are available for the molecular detection of human sources of fecal pollution in environmental waters. However, widespread application is hindered by a lack of knowledge regarding geographical stability,
1. INTRODUCTION

The disease burden from poor water quality, sanitation, and hygiene is estimated to be responsible for up to 4% of all deaths worldwide.¹,² Limited access to safe drinking water has led the World Health Organization to develop strategies for managing water quality with the goal to protect and promote human health.³ To provide information about microbiological water quality, cultivation of standard fecal indicator bacteria, such as E. coli and enterococci, are typically used according to certified standard procedures.⁴,⁵ However, standard fecal indicator bacteria measurements do not provide information about the origin of fecal pollution, because these organisms are present in the feces of most warm blooded animals.⁶ The field of microbial source tracking (MST) seeks to develop methods allowing for the discrimination between different animal sources of fecal pollution to improve water quality management.⁷

A useful MST assay should have high source-specificity (low number of false positives) and excellent source-sensitivity (low number of false negatives).⁸ MST assay specificity and sensitivity are typically evaluated based on repeated testing of reference fecal and wastewater samples often collected in close proximity to the research laboratory.⁹–¹¹ For example, Boehm et al. (2013) evaluated specificity and sensitivity of 41 MST methods with more than 100 reference samples collected from the California area.¹² To date, the performance of many MST assays described in the literature have not been tested for source-specificity and -sensitivity beyond the regional level.⁸,¹³–¹⁵ For this reason, it is often difficult to identify the most appropriate methods when planning a MST application in a new geographical area. To improve this situation Reischer et al. (2013)¹⁶ compared five quantitative real-time PCR (qPCR) methods targeting human and ruminant pollution sources by testing a large collection of reference fecal samples from 16 countries demonstrating that tested genetic markers were broadly distributed regardless of the location from which the fecal samples originated. The study also emphasized the investigation of the quantitative distribution of genetic marker concentrations in the target and non-target fecal samples in order to fully assess performance.

This present study seeks to build upon previous research by providing insights into the occurrence and concentration of human-associated bacterial genetic markers in raw and biologically treated municipal wastewater from multiple geographical locations around the globe. Three widely applied MST bacterial qPCR assays (BacH, BacHum-UCD and HF183/BFDrev), and two recently modified or developed qPCR assays (HF183/BacR287 and Lachno2, respectively) were challenged using wastewater samples collected from 29 facilities spanning 13 countries across six continents. Wastewater samples included both urban and rural plants serving a wide range of population sizes. Considerable effort was made to ensure standardization in sampling collection, handling, and processing. Marker concentration data found in wastewater were used in an exemplary modeling application using the QMRACatch tool. In addition, the new human-associated marker HF183/BFDrev, HF183/BacR287 and Lachno2 were challenged against a previously established collection of reference fecal samples from six continents¹⁶ to compare their ability to correctly differentiate fecal sources.

2. EXPERIMENTAL METHODS

2.1. Sample Collection and Wastewater Selection Criteria. The requirements and guidelines for cooperation partners in this MST evaluation project were defined in 2013. In brief, detailed standard operating procedures for sampling and filtration were distributed to all cooperating partners including a demonstrational video showing important filtration and filter packing steps (cf. Supporting Video File) to ensure that sample processing was standardized. Partners were also required to use an online sampling protocol to collect metadata. To improve comparability, polycarbonate membrane filters (0.2 μm Millipore, Isopore Membrane Filter – GTTP, Cork, Ireland), preprinted labels, and vials for sample processing were provided to all partners by the lead laboratory (TU Wien). Partners were requested to select one urban municipal wastewater treatment plant (WWTP) with a pollution load greater than 500 000 population equivalents (PE) and one rural municipal WWTP with less than 50 000 PE (Table 1). An effort was made to select WWTPs receiving minimal levels of industrial waste. All WWTP facilities consisted of mechanical treatment followed by either activated sludge or fixed film treatment. Data on treatment capacity (PE), sewage system (separate, combined), and details of possible inputs from industry or livestock, were provided by the respective WWTP operators.

2.2. Sampling, Shipment, and Quality Control. Five hundred milliliter grab samples were taken at all WWTP sites during the morning hours (before 09:00 local time) under dry weather conditions (no rain in the preceding 36 h). Raw wastewater samples were collected at the post mechanical screening stage, while the biologically treated wastewater samples were taken postsecondary sedimentation/clarification prior to any advanced (tertiary) treatment (e.g., ultraviolet irradiation, chlorination,
2.4. DNA Extraction. DNA from the filters was recovered at the TU Wien laboratory by phenol/chloroform extraction as previously described. Cells were lysed with CTAB buffer solution, glass beads and a FastPrep-24 Instrument (MP Biomedicals Inc., Irvine, CA) at a speed setting of 6 m s−1 for 30 s. Polycarbonate membrane filters were completely dissolved at this step and the DNA was purified with follow-up washing procedures. The extracted DNA was dissolved in 10 mM TRIS HCl, pH 8, and stored at −80 °C for no longer than 21 days prior to qPCR analysis. Every extraction event was accompanied by a blank extraction control. The concentration of extracted DNA was measured with Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, UK) on an Anthos Zenyth fluorometric plate reader (Beckman Coulter, Wien, Austria) to check for loss of DNA during extraction. For one sample the DNA concentration was below the detection limit. This sample was removed from further analyses.

2.5. qPCR Measurement and Quality Control. The following host-associated fecal genetic 16S-rRNA-gene markers were quantified by qPCR: HF183/BFDrew,11 HF183/BacR287,8 BacH,16 and BacHum-UCD,9 all of which target human-associated Bacteroidetes, and Lachno2 targeting a human-associated Firmicutes clade. In addition, the general Bacteroidetes marker, AllBac was used as a quality control to assess the ability to amplify DNA extracted from wastewater and rule out the presence of PCR inhibition in the sample extract dilutions.21 Samples with two matching concentrations (i.e., the ratio [concentration 1:100]/[concentration 1:10]) was between 0.5 and 2) in the 1:10 and 1:100 dilutions were judged free of PCR inhibitor in the 1:10 dilution. qPCR measurements were performed on a Rotorgene Q Cycler (Qiagen, Hilden, Germany). A QAGility liquid handling robot (Qiagen) was used to prepare qPCR reactions in a total volume of 15 μL, with 2.5 μL of sample DNA, 7.5 μL of Rotor-Gene Multiplex PCR Kit (Qiagen) and 400 pg L−1 bovine serum albumin (Roche Diagnostics, Mannheim, Germany). For the AllBac qPCR assay 600 nmol L−1 primer AllBac296f, 600 nmol L−1 primer AllBac412r, and 25 nmol L−1 TaqMan MGB probe AllBac375Bhq were used.21 Additionally, as an internal amplification control (IAC) 500 nmol L−1 primer IPC-ntb2-fw, 500 nmol L−1 primer IPC-ntb2-re, 200 nmol L−1 ROX probe IPC-ntb2-probe and 10 copies of IAC Template IPC-ntb2 plasmid DNA were added to each AllBac qPCR reaction. For the BacHum-UCD assay 400 nmol L−1 primer BacHum-160f, 400 nmol L−1 primer BacHum-241r and 80 nmol L−1 TaqMan MGB probe BacHum-193p were used. For the HF183/BFDrew assay 1000 nmol L−1 primer HF183, 1000 nmol L−1 primer BFDREV and 80 nmol L−1 TaqMan MGB probe BFDFAM were used. For the HF183/BacR287 assay 1000 nmol L−1 primer HF183, 1000 nmol L−1 primer BacR287 and 80 nmol L−1 TaqMan MGB probe BacP234MGB were used. For the BacH assay 200 nmol L−1 primer BacH_f, 200 nmol L−1 primer BacH_r, 100 nmol L−1 each of TaqMan MGB probes BacH_pC and BacH_pT were used. For the Lachno2 assay 1000 nmol L−1 primer Lachno2F and 80 nmol L−1 TaqMan MGB probe Lachno2P were used.

Quantification was based on plasmid standard dilutions. The respective plasmid stock for each assay was diluted in an unspecified background of 500 μg L−1 poly(dI-dC) (Roche Diagnostics, Mannheim, Germany) to avoid adsorption of plasmid DNA from the respective plasmid media and high concentrations of salt or coagulation). Tertiary-treated wastewater was not included, because tertiary treatment methods may vary by WWTP and tertiary-treated wastewater was not included, or coagulation). Tertiary-treated wastewater was not included, because tertiary treatment methods may vary by WWTP and a large proportion of the investigated plants did not have any tertiary treatment steps. Sampling points were chosen in turbulent zones to promote good mixing and samples were collected about 20 cm below the water surface. Samples were stored in 500 mL sterile glass bottles in the dark at <4 °C and immediately transported to the respective collaboration partner laboratory for filtration (holding time <6 h), prior to shipping to TU Wien. For each sampling event, four 50 mL replicate subsamples were filtered and filters were immediately frozen at −20 °C (two filters were shipped to TU Wien; the other two were kept by the local cooperation partner as backups). On each sampling occasion an unused filter was put directly into a 2 mL extraction vial as a blank filter control. Shipment services were carried out in accordance with international law by qualified logistics companies and under controlled frozen conditions on dry ice. Sample filters were only used for DNA extraction if dry ice was still present upon arrival at TU Wien in Austria.

2.3. Additional Animal and Human DNA Sample Collection. The fecal DNA samples used to compare false-positive and false-negative rates in the different human-associated markers were collected and extracted during a previous study. In brief, reference sample collection was collected during the period 2007 to 2008 and consisted of 280 fecal samples from six continents including 61 human and 219 nonhuman fecal samples from various sources such as agricultural and wild animals (for details see Supporting Information (SI)).

Table 1. Characteristics of Investigated Disposal Systems and Wastewater Treatment Plants (WWTP)24

<table>
<thead>
<tr>
<th>country (site location)</th>
<th>sewage system</th>
<th>influence</th>
<th>population equivalent (PE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argentina (rural)</td>
<td>separated</td>
<td>slight</td>
<td>350 000</td>
</tr>
<tr>
<td>Argentina (urban)</td>
<td>combined</td>
<td>strong</td>
<td>600 000</td>
</tr>
<tr>
<td>Australia (rural)</td>
<td>separated</td>
<td>no</td>
<td>50 000</td>
</tr>
<tr>
<td>Australia (urban)</td>
<td>separated</td>
<td>slight</td>
<td>500 000</td>
</tr>
<tr>
<td>Brazil (rural)</td>
<td>separated</td>
<td>no</td>
<td>19 100</td>
</tr>
<tr>
<td>Brazil (urban)</td>
<td>separated</td>
<td>slight</td>
<td>4 400 000</td>
</tr>
<tr>
<td>Canada (rural)</td>
<td>separated</td>
<td>slight</td>
<td>20 000</td>
</tr>
<tr>
<td>Canada (urban)</td>
<td>combined</td>
<td>no</td>
<td>500 000</td>
</tr>
<tr>
<td>Germany (rural)</td>
<td>combined</td>
<td>slight</td>
<td>16 800</td>
</tr>
<tr>
<td>Germany (urban)</td>
<td>combined</td>
<td>moderate</td>
<td>1 000 000</td>
</tr>
<tr>
<td>Japan (rural)</td>
<td>separated</td>
<td>no</td>
<td>10 200</td>
</tr>
<tr>
<td>Japan (urban)</td>
<td>separated</td>
<td>slight</td>
<td>300 000</td>
</tr>
<tr>
<td>N. Zealand (rural)</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>N. Zealand (urban)</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Singapore (urban)</td>
<td>separated</td>
<td>moderate</td>
<td>1 700 000</td>
</tr>
<tr>
<td>Spain (rural)</td>
<td>separated</td>
<td>no</td>
<td>45 100</td>
</tr>
<tr>
<td>Spain (urban)</td>
<td>separated</td>
<td>slight</td>
<td>384 000</td>
</tr>
<tr>
<td>Tanzania (rural)</td>
<td>combined</td>
<td>no</td>
<td>3 000</td>
</tr>
<tr>
<td>Tanzania (urban)</td>
<td>combined</td>
<td>no</td>
<td>10 000</td>
</tr>
<tr>
<td>Uganda (rural)</td>
<td>separated</td>
<td>no</td>
<td>750</td>
</tr>
<tr>
<td>Uganda (urban)</td>
<td>separated</td>
<td>no</td>
<td>320 000</td>
</tr>
<tr>
<td>UK (rural)</td>
<td>combined</td>
<td>no</td>
<td>14 600</td>
</tr>
<tr>
<td>UK (urban)</td>
<td>combined</td>
<td>strong</td>
<td>3 500 000</td>
</tr>
<tr>
<td>USA (rural)</td>
<td>separated</td>
<td>no</td>
<td>3 500</td>
</tr>
<tr>
<td>USA (urban)</td>
<td>combined</td>
<td>no</td>
<td>16 000</td>
</tr>
<tr>
<td>USA (rural)</td>
<td>combined</td>
<td>moderate</td>
<td>29 800</td>
</tr>
<tr>
<td>USA (urban)</td>
<td>combined</td>
<td>strong</td>
<td>142 000</td>
</tr>
<tr>
<td>USA (urban)</td>
<td>combined</td>
<td>moderate</td>
<td>3 000 000</td>
</tr>
<tr>
<td>USA (urban)</td>
<td>combined</td>
<td>moderate</td>
<td>480 000</td>
</tr>
</tbody>
</table>

Abbreviations: na: not available; influence of industrial and agricultural pollution sources was assessed based on expert knowledge by local partners after consultation with plant operators.
DNA to reaction vials at low plasmid concentrations (cf. SI). A total of at least eight 10-fold serial dilutions of plasmid standard \((10^9-10^7\) gene copies) were performed in each qPCR run. Every run also included several no-template and DNA extraction controls.

Each wastewater DNA sample was analyzed in two dilution steps of the original extract (10- and 100-fold dilution) and each dilution in duplicate reactions, in order to check for a possible qPCR inhibition. Additionally, an IAC was run in duplex with the AllBac assay to monitor for qPCR amplification inhibition. Inhibition was assumed to be present if the threshold cycle \((Ct)\) value of the IAC was shifted to higher \(Ct\) values by more than one cycle. All qPCR runs in this study revealed a calculated PCR efficiency between 90% and 105% and no-template and extraction controls were consistently negative (i.e., fluorescence never exceeded threshold). The qPCR standard dilutions ranging from \(10^9\) to \(10^7\) targets per reaction were used in a linear regression model for calculation of the qPCR calibration curve. Results for wastewater investigations were reported as marker equivalents per filtered wastewater volume (ME vol\(^{-1}\)) as previously described. Samples with replicate standard deviations of the ct-value >1 in the 10-fold DNA extract dilutions were considered to be not quantifiable and were not considered for further analysis.

Results for the fecal DNA setup were measured in the 1:4 dilution of the fecal DNA extracts. They are reported directly as genetic marker copies per qPCR reaction in the same manner as previously published data on the same samples. DNA extracts were reanalyzed using the AllBac assay and the results indicated that no DNA degradation had occurred during storage (data not shown).

**2.6. Data and Statistical Analysis.** All qPCR data were expressed as \(\log_{10}(x + 1)\), where \(x\) is the calculated concentration before applying the logarithm to it. To estimate \(\log_{10}\) reductions of the MST markers during wastewater treatment Monte Carlo simulations of the marker concentrations [i.e., \(\log_{10}(\text{influent}) - \log_{10}(\text{effluent})\)] were performed. As stop criterion a maximum of 100 000 simulated cases or a confidence level of 95% with a threshold of 1% for the mean was set. Visual and statistical data analyses were done with Visplore 2.0 (VRVis GmbH, Austria, Vienna) and Sigma Plot 13.0 (SPSS Inc., Chicago, IL). For multiple comparison of groups One-way ANOVA was used and if significant differences between two or more groups were detected a Tukey Post-Hoc test was performed. To account for multiple statistical testing, statistical significance levels were corrected according to Bonferroni.

To support correct comparisons of the variability of the log-normally distributed data, the multiplicative standard deviation \((s^*)\) was calculated for the measured results. \(s^*\) is a measure for the variation of \(\log_{10}\) normal distributed data and describes the shape of the distribution. When the geometric mean is multiplied with or divided by \(s^*\), the resulting values are the higher and lower limits of an interval which covers 68.3% of the midrange of the distribution.

In an exemplary model application the collected human-associated HF183/BacR287 concentrations measured in this study were used for recalibration of the quantitative microbial risk assessment tool QMRACatch. QMRACatch is a catchment-based, easy-to-use, interactive computational tool to simulate concentrations of fecal indicators and intestinal pathogens at a point of interest (e.g., recreational water uses or drinking water production) and to assess associated microbial infection risks. Host-associated genetic fecal markers are used to calibrate the model for the specific situation of fecal emissions at the considered habitat. QMRACatch (free download at www.waterandhealth.at) consists of the following model components: (i) a hydrological process model including fate and transport of health-related microbes/viruses in rivers and river/floodplain systems, and (ii) QMRA for drinking water safety management or during recreation/bathing activities for the investigated environment.

The necessary input data consist of measured MST-marker concentrations and measured or assumed pathogen data in the fecal pollution sources (raw and treated wastewater). The model output consists of simulated concentrations of health-related microbes/viruses in the wastewater and the receiving water, and the treatment requirements (log-reductions) for health-related water safety management.

For the exemplary QMRACatch model application the case study of Drex et al. (2016) at the Danube River in Austria was used. The collected HF183/BacR287 MST marker concentrations in raw and treated wastewater from rural areas (data set from this paper, \(n = 18\)) were used as input dataset for the five selected wastewater treatment plants emitting into the Danube River (details on the methods are provided in the SI) in order to evaluate the general applicability of the recovered data set from around the globe as a surrogate for raw and treated waste emission concentrations of human-associated genetic markers.

### 3. RESULTS

#### 3.1. Occurrence of Human-Associated Genetic Markers in Wastewater.**

Selected human-associated genetic fecal markers were measured in raw and biologically treated wastewater from 29 municipal and rural wastewater treatment plants (Table 1). Genetic markers were detected in all raw and treated wastewater samples (100%). IAC testing and measurement at different sample dilutions confirmed the absence of qPCR amplification inhibition.

#### 3.2. Concentrations of Human-Associated Genetic Markers in Wastewater.**

The concentration of all human-associated genetic markers in raw and treated wastewater samples is shown in Figure 1. HF183/BFDFREV showed the lowest concentration of all markers in raw wastewater with a median of \(\log_{10} 7.2\) ME 100 mL\(^{-1}\), whereas, HF183/BacR287 and BacH genetic marker concentrations were slightly higher with medians of \(\log_{10} 7.8\) and \(\log_{10} 7.8\) ME 100 mL\(^{-1}\), respectively. BacHum-UCD was detected with a median of \(\log_{10} 7.5\) ME 100 mL\(^{-1}\) and Lachno2 with a median of \(\log_{10} 8.0\) ME 100 mL\(^{-1}\). Only HF183/BDF REV was significantly different from other markers (Lachno2 and BacH) in raw wastewater (Tukey test, Bonferroni corrected significance \(p < 0.001\), see SI Table S4).

HF183/BFDFREV also showed the lowest concentrations of all assays in treated wastewater with a median of \(\log_{10} 4.6\) ME 100 mL\(^{-1}\), while the BacHum-UCD, HF183/BacR287 and BacH genetic markers were higher with medians of \(\log_{10} 5.2\), \(\log_{10} 5.3\) and \(\log_{10} 5.3\) ME 100 mL\(^{-1}\), respectively. Lachno2 had a median concentration of \(\log_{10} 6.0\) ME 100 mL\(^{-1}\) in treated wastewater. In treated wastewater only Lachno2 results were significantly different from all other markers except for BacH (Tukey test, Bonferroni corrected significance \(p < 0.001\), see SI Table S5).

No statistical differences were observed between wastewater collected in rural and urban areas (Mann-Whitney Rank Sum Test, \(p < 0.05\), Bonferroni corrected, anonymized raw data is presented in SI Tables S2 and S3). Hence, data from rural and urban locations were pooled for all subsequent...
analyses. We also refrained from comparing the data set on a WWTP to WWTP or country-to-country basis, because the sample numbers in each separate country were too low to allow meaningful conclusions to be drawn. Correlation analysis of the pooled data set revealed a statistically significant association between all five genetic markers ($p < 0.001$) with corresponding Spearman rank coefficients ranging from 0.83 to 0.91 in raw sewage and from 0.86 to 0.93 in treated wastewater (SI Table S6).

To investigate variability between data sets, multiplicative standard deviation $s^*$ analysis was used. In raw wastewater, the $s^*$ for BacHum-UCD ($s^* = 4.9$), HF183/BFDrev ($s^* = 5.0$), and BacH ($s^* = 4.2$) were very similar, except HF183/BacR287 with somewhat higher variability ($s^* = 6.4$). In contrast, variability in biologically treated wastewater was much higher with $s^*$ values increasing by an average factor of 1.5 (range 1.2−1.8) during treatment (Figure 1).

3.3. Reductions in Marker Concentrations during Wastewater Treatment. Monte Carlo simulation was used to estimate genetic marker reduction during wastewater treatment. The median $\log_{10}$ genetic marker reductions achieved by secondary wastewater treatment (without disinfection) were 2.1 for BacHum-UCD, 2.2 for HF183/BFDrev, 2.3 for HF183/BacR287 and 2.2 for BacH. Lachno2 showed a lower reduction compared to Bacteroidetes genetic markers with a median of $\log_{10} 1.7$ (Figure 2 and SI Figure S1−S5).

3.4. Performance Trends Based on Reference Fecal Sample Testing. Relative distributions of false-negative and false positive results were estimated for HF183/BFDrev, HF183/BacR287 and Lachno2 using a previously reported reference fecal sample collection.16 The detection frequency of genetic markers in reference human samples (target source) was 83% for Lachno2, 58% for HF183/BFDrev, and 62% for HF183/BacR287 compared to previously published values of 77% for BacH and 87% for BacHum-UCD. Among the tested assays, Lachno2 showed the highest incidence of “false positives”, with a median $\log_{10} 1.0$ copies per reaction in nonhuman reference samples. In contrast, the newly reported HF183/BacR287 and HF183/BFDrev did not reveal any detectable signals in most nontarget samples (Figure 3). Marker concentrations were also related to DNA concentrations in the DNA extracts. The distributions of marker concentrations $g^{-1}$ fecal DNA are presented in SI Figure S6 displaying the same relative distributions as Figure 3. Furthermore, correlation analysis of concentrations of all the markers in the fecal samples was performed to investigate differences between the human-associated markers. The corresponding Spearman’s Rank coefficients ranged from 0.25 to 0.76 (see SI Table S7 and Figure S7).
An example of such an application is the recently developed “QMRAcatch” tool, which integrates QMRA with catchment-based hydrological process modeling to predict fecal pollution levels as well as the associated infection risk for bathing or drinking water. It employs MST markers for source-specific calibration and verification of the hydrological water quality model and uses reference pathogens to simulate pollution and infection risk scenarios using QMRA.28 QMRAcatch has been used to simulate human-associated fecal pollution in a complex river/floodplain area and for estimating the required reductions of microorganisms and viruses to ensure safe water supply.29

To assess their usefulness for modeling purposes, HF183/BacR287 concentrations found in raw and treated wastewater at rural WWTPs in this current study were used to recalibrate the QMRAcatch model applied in the previous study on human-associated fecal pollution29 (details on the method are provided in the SI). The new data could successfully replace the original calibration data from Austrian rural WWTP26 as model input. SI Figure S8 and Table S8 show that using the novel global data set to simulate values for marker concentrations in wastewater sources and receiving waters in the study area resulted in an equally tight fit of the simulated with observed concentrations at the sampling sites in the catchment. Thus, the results were highly compatible with the original outcomes based solely on Austrian data.29 Beyond being a demonstration for the applicability of the MST marker data in modeling approaches, this result also indicates that the data collected in this study might serve as a best available approximation of marker levels in areas where no data on human-associated marker concentrations in wastewater currently exists. Other applications of MST data include source-specific fecal contaminant transport modeling37 and epidemiological investigations.38

In general, the measured marker concentration levels correspond to those found in a recent study26 which investigated the occurrence of MST genetic markers in Austrian and German WWTPs ranging from small, household-sized plants, to facilities serving large populations over the course of a 12-month period. The results of both the recent and the current studies have important implications for wastewater treatment efficacy testing. Most human-associated genetic markers exhibited a 2 orders of magnitude reduction after wastewater treatment with the exception of Lachnospiraceae. Lachnospiraceae are Gram-positive bacteria and may be more resilient to treatment processes or even capable of growing in specific niches within sewerage systems and treatment plants.39,40 It should be emphasized that wastewater investigated in this study went through primary and secondary (biological) treatment, but no advanced tertiary treatment such as ultraviolet radiation disinfection or chlorination. The tertiary treatment stage was omitted from this investigation because methods are very diverse and many participating countries have not implemented any wastewater disinfection technologies. Nevertheless, reduction values found in this study were similar to other studies investigating reduction values of bacterial and viral genetic markers in wastewater treatment, both with and without disinfection.26,41,42 However, future studies investigating the influence of disinfection on human-associated genetic markers are warranted.

Municipal wastewater plays an important role in the pathway of human fecal pollution and associated pathogenic agents entering the environment and ultimately affecting public health.7 The high concentration of genetic markers found in wastewater samples during this study provides further evidence to demonstrate that these MST approaches serve as useful
4.3. Comparison of Human-Associated Genetic Marker Trends. Careful examination of human-associated genetic marker occurrence in wastewater and fecal samples suggests that some markers may be more suitable than others for water quality management. While correlations between human-associated genetic marker concentrations were strong among wastewater samples, a different trend was observed with human fecal DNA tests. This is likely due to the composition of wastewater representing fecal waste from a group of individuals resulting in a homogenized mixture. In contrast, individual fecal samples contain genetic markers from a single gut system potentially reducing the sensitivity of a human-associated MST method. It should also be noted that the fecal DNA reference samples used in this study contained relatively low DNA concentration due to extensive DNA purification. This led to higher limits of detection for these samples and correspondingly to a generally higher false-negative rate and a lower false positive rate than might have been observed in more concentrated samples. The HF183/BFDrev assay in particular has been suspected to be unable to detect very low marker copy numbers. Also the fecal DNA extracts had been stored at −80 °C for several years between the two studies which might also affect marker concentrations. Another important factor to consider is the MST genetic marker itself. Results indicate strong correlations in genetic marker concentrations between HF183/BFDrev, HF183/BacR287, and BacHum-UCD, while correlations to BacH were much weaker. In fact, HF183 genetic markers share the same forward primer while the BacHum-UCD forward primer has a 16-base overlap with the HF183 forward primer. Therefore, these three genetic markers likely detect the same human-associated Bacteroidetes clade. However, there are differences in the performance of these Bacteroidetes genetic markers, with slightly higher concentrations and correspondingly lower false-negative rate for BacHum-UCD, contrasted by lower false-positive rate for HF183/BFDrev and HF183/BacR287. This “trade-off” between source-sensitivity and -specificity is often encountered in MST approaches. In contrast to the Bacteroidetes genetic markers, Lachno2 targets Firmicutes contributing to a different performance pattern with slightly higher concentrations in wastewater and human feces, but high concentrations in animal fecal samples. This difference in performance could have important ramifications for future water quality applications.

4.4. Implications for Water Quality Management. Our findings demonstrate that human-associated genetic markers tested in this study are highly sensitive tools for the detection and quantification of sewage contamination across six continents. However, the observed lower sensitivity with individual human fecal samples suggests that these genetic markers may not be as useful in scenarios where few individuals are contributing to the human fecal pollution load. In addition, no genetic marker achieved 100% specificity indicating that a single MST method may not be suitable across all geographic locations and the importance of verifying sensitivity and specificity with local reference samples prior to initiating a MST water quality study. Other strategies such as source profiling, the use of conditional probabilities, or machine learning approaches could also help to evaluate the utility of a particular MST genetic marker or group of markers to correctly identify human fecal contamination. Limitations in source-specificity might also be compensated by combining bacterial MST genetic markers with promising viral methods or human mitochondrial DNA approaches. In addition, study findings may have important implications for calibrating future microbial fecal pollution and QMRA models using novel genetic marker occurrence information from reference samples.

**REFERENCES**


