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Doctoral Thesis

Detection Strategies for Fecal Pollution along the Urban Waste Water Path

supervised by

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Abstract

Protecting and monitoring of water quality is essential because inadequate management of urban, industrial or agricultural pollution sources often leads to high levels of biological and chemical contaminants. In this context the WHO considers unsafe water and inappropriate sanitation as one of the leading global risk factors for disease, after malnutrition, hypertension, unsafe sex and alcohol.

Fecal pollution is a serious threat since it can contain high concentrations of intestinal pathogens. As a result, gathering information on the source(s) of fecal pollution has become increasingly important. The discipline of microbial source tracking (MST) is considered a valuable approach to investigating the origin of fecal pollution. Host-associated genetic *Bacteroidetes* fecal markers have been increasingly used over the last years as very promising tools for MST, especially in order to detect municipal and domestic wastewater input into water resources. However, information on the occurrence, persistence, treatment resistance, and methodical performance characteristics (e.g. specificity or sensitivity) of human-associated genetic fecal *Bacteroidetes* markers along the communal or domestic wastewater path is still very limiting.

The aim of this thesis was to investigate four aspects of the use of human-associated genetic *Bacteroidetes* markers for MST:

- the stability of genetic *Bacteroidetes* markers, *E. coli* and enterococci in raw and treated wastewater for the potential use in automated sampling procedures at waste water treatment plants (WWTP).
- the dynamics and occurrence of human-associated genetic *Bacteroidetes* markers in raw and treated wastewater differing in catchment size and type at selected and well described Austrian WWTP locations.
- the wider geographic distribution of human-associated genetic *Bacteroidetes* markers to determine the markers' potential global applicability.
- a comparison of genetic *Bacteroidetes* markers to standard fecal bacteria (i.e. *E. coli* and enterococci) and to other alternative fecal pollution indicators (i.e. human specific Adenovirus and JC Polyomavirus markers as well as bacteriophages infecting *Bacteroides thetaiotaomicron*) in raw and treated wastewater.

The results presented in this thesis demonstrate the suitability of the investigated microbiological parameters for automated sampling procedures in municipal WWTP. It was shown that automated sampling provides a reliable technique to obtain representative samples for microbiological analysis.

Irrespectively of catchment size and season, human-associated genetic *Bacteroidetes* fecal markers could be detected consistently in raw and biologically treated wastewater of the investigated 14 Austrian WWTPs.
The obtained results covering wastewater samples from 29 sites in 13 countries on 6 continents confirm that human-associated genetic *Bacteroidetes* fecal markers are ubiquitous and occur worldwide in high concentrations. They are highly sensitive for the detection of communal and domestic wastewater pollution around the globe, while their source-specificity indicates need for improvement. It thus remains advisable to evaluate the markers under the local circumstances in order to choose the best assays for the corresponding study. Finally it can be concluded that these markers are very promising candidates to complement water quality monitoring, if additional information on human-associated fecal pollution is needed.

Kurzfassung

Die Nutzung von Wasserressourcen angemessener Qualität setzt seit jeher sowohl Schutzmaßnahmen als auch Kontrolle voraus. Diese angestrebte Qualität kann durch landwirtschaftliche Aktivitäten, unzureichende kommunale Abwasserentsorgung, als auch durch industrielle Abwässer gefährdet werden.

Die Hauptquelle für mikrobiologische Wasserkontaminationen sind menschliche sowie tierische Fäkalien, welche potentiell sehr hohe Konzentrationen an intestinalen Krankheitserregern aufweisen können. Die Herkunftsbestimmung fäkaler Verunreinigungen wird in diesem Zusammenhang immer wichtiger, um zielgerichtete Schutzmaßnahmen im Einzugsgebiet vorzunehmen. Dafür wurden in der Vergangenheit mit zunehmender Häufigkeit quantitative Nachweisverfahren für wirtsassoziierte genetische Fäkalmarker auf Basis intestinaler bakterieller *Bacteroidetes*-Populationen herangezogen. Diese Verfahren fanden und finden insbesondere in der Detektion von häuslichen und städtischen Abwässern Anwendung. Betreffend der Abundanz und Persistenz im Abwasser sowie deren Verhalten während der Abwasserbehandlung existiert jedoch noch eine Vielzahl unbeantworteter Fragestellungen.

Diese Dissertation hatte zum Ziel, vier wesentliche offene wissenschaftliche Aspekte betreffend der Anwendbarkeit genetischer Fäkalmarker entlang des kommunalen und häuslichen Abwasserpfades auf Basis von *Bacteroidetes* Populationen zu untersuchen. Diese beinhalten, i) die Fragestellungen der Persistenz von *Bacteroidetes* Markern in Roh- und behandeltem Abwasser sowie deren Vergleich mit *E.coli* und Enterokokken, ii) ihre Eignung für automatische Probenahmen in Kläranlagen, iii) deren Vorkommen und Variabilität in Abwässern aus österreichischen Kläranlagen mit unterschiedlicher Größe und Charakteristika und Vergleich mit Standard und alternativen viralen genetischen Fäkalmarkern sowie, iv) die Untersuchung der weltweiten Anwendbarkeit zur Detektion kommunaler und häuslicher Abwässer.

Die in dieser Dissertation dargestellten Resultate belegen die hinreichende Persistenz und somit Eignung von genetischen Fäkalmarkern auf Basis von *Bacteroidetes* Population, *E. coli* und Enterokokken für automatisch durchgeführte Probenahmen in Kläranlagen. Weiters konnte das ubiquitäre Auftreten der genetischen Fäkalmarker in österreichischen, sowie in weiterer Folge, weltweit untersuchten Roh- und biologisch behandelten Abwässern demonstriert werden. Untersuchungen der Spezifität der angewandten genetischen Fäkalmarker zeigten jedoch ein nicht zu vernachlässigendes Verbesserungspotential auf. Um genetische Fäkalmarker, basierend auf *Bacteroidetes* Population, derzeit anwenden zu können, sollten diese im Vorfeld im Untersuchungsgebiet getestet und gegebenenfalls durch alternative virale Marker ergänzt werden, um eine ausreichende Aussagekraft zu erlangen. Nichtsdestotrotz stellen genetische Fäkalmarker, basierend auf

Bacteroidetes Populationen, eine signifikante Erweiterung der Methodik dar und werden zukünftig bei vielen Anwendungen zur Überwachung der Gewässer- und Wasserqualität an Bedeutung gewinnen.

Contents

1 Introduction

Water quality impacts human health at all stages of life. Inadequate management of urban, industrial or agricultural wastewater can lead to dangerous levels of biological and chemical contaminants in the water resources, such as drinking water, of millions of people. Contaminated water may transmit diseases such as cholera, dysentery or typhoid fever. In fact, unsafe water resources and inappropriate sanitation is still one of the leading global risk factors for disease, after malnutrition, hypertension, unsafe sex and alcohol (Stevens et al. 2009, WHO 2013). Bain et. al estimated that 1.8 billion people use contaminated water for drinking purposes and that more often water was contaminated in rural (41%) than in urban areas (12%) (Bain et al. 2014).

At the end of the 19th century Robert Koch established the technical skills and tools mankind still relies on it to grow, isolate, and enumerate specific microbial populations in water. Based on these techniques fecal indicators such as *Escherichia coli (E. coli*) and enterococci can be detected with great sensitivity (Tallon et al. 2005). *E. coli* and enterococci have been part of the standard fecal indicator bacteria (SFIB) for the detection of general microbial fecal pollution for more than 100 years (Tallon et al. 2005, Yates 2007). As such they are routinely determined for water quality monitoring purposes by membrane filtration or most probable number method in trained laboratories (ISO 2000, 2001a, Stalder et al. 2011). Furthermore, the WHO references SFIB and their selective cultivation as a regulatory and methodological basis for fecal pollution analysis (WHO 2004).

In 1966 Bonde postulated that an ideal fecal indicator

- should always be present in human and warm blooded animal feces,
- must not be able to multiply in aquatic environments or soil,
- must occur in greater numbers than pathogens,
- should not be a pathogen itself,
- have a defined persistence and resistance in the environment where they occur,
- must be unambiguously identifiable by simple characteristics and reliable tests (Bonde 1966).

During the last decades it became apparent that SFIB cannot meet all these criteria. However, occurrence in high abundance in fecal excrements and wastewater and showing a defined degradation or die-off rate, out of the intestinal system still

remains a basic requirement (Byappanahalli et al. 2012, Byappanahalli et al. 2006, Ishii and Sadowsky 2008).

During the last years, the information on the source of fecal pollution is gaining increasing importance (Farnleitner et al. 2014, Wuertz et al. 2011). Knowing the pollution source allows target oriented measurements and supports risk assessment. For example, the combination of general fecal pollution monitoring, microbial source tracking, and microbial risk assessment was successfully demonstrated for alpine karst springs in Austria for sustainable drinking water resources management (Farnleitner et al. 2014, Stalder et al. 2011). SFIB, as indicators of general fecal pollution, do not provide information on the source of fecal pollution, since they occur in human as well as in most animal fecal sources (Harwood et al. 2014).

The field of microbial sources tracking (MST) was developed in the 1970s as an attempt to determine the dominant sources of fecal contamination in environmental waters. At present the list of potential source identifiers and parameters has become extremely complex. However, MST is still a young science and far from offering standardized procedures. Host-associated genetic fecal markers, which target specific bacterial, viral or mitochondrial populations, are often used to determine the source of fecal pollution. Wastewater specific chemicals are also investigated for their ability to trace contaminations in water bodies (Hagedorn et al. 2011).

For bacterial fecal source tracking, different polymerase chain reaction (PCR) based assays were developed during the last 15 years, which target, human- or animal-associated feces and sewage (Farnleitner et al. 2014, Harwood et al. 2014, Kildare et al. 2007, Reischer et al. 2006). To provide useful information the genetic markers have to possess a number of important performance criteria. Firstly, a potential genetic fecal marker has to show host-specificity, in other words it should be specifically associated with the suspected source of fecal pollution, namely the feces of the targeted host group. Secondly, a potential genetic marker has to show host-sensitivity, that means, it should occur in all members of the targeted host group in high numbers (Ahmed et al. 2013, Farnleitner et al. 2014). In this regard, a genetic fecal marker should also exhibit temporal and geographical stability. Finally, genetic fecal markers should show defined and characterised decay rates to support straightforward selection in order to fulfil the specific monitoring purpose (Ahmed et al. 2013, Hagedorn et al. 2011, Harwood et al. 2014).

Human-associated *Bacteroidetes* markers are especially promising, since they are potentially valuable tools to sensitively highlight municipal and domestic wastewater input into water resources. However, up to date there is very limited information on the applicability of these markers available. Although several studies on fecal sensitivity and specificity performance characteristics were performed, investigations were limited to local fecal sample setups (Ahmed et al. 2013, Green

et al. 2014, Layton et al. 2013). Evaluation on a more global level is still very scarce (Reischer et al. 2013). Knowledge on the potential seasonal and daily dynamics in wastewater is also not available. Furthermore no data or sufficient information on the persistence in raw and treated wastewater and the extent of removal during treatment exist. Investigations on the removal of SFIB during wastewater treatment were based on grab sampling, neglecting daily wastewater quality dynamics.

The aim of this thesis was to improve the basic understanding on the occurrence and dynamics of human-associated genetic fecal *Bacteroidetes* markers along the human water path. Four aspects were covered in detail in order to better understand the applicability of human-associated genetic fecal *Bacteroidetes* markers for water quality monitoring. Firstly, the stability of genetic bacterial markers in raw and treated wastewater was investigated regarding its potential use at automated sampling of raw and treated wastewater at WWTPs. Furthermore, the occurrence of human-associated *Bacteroidetes* genetic markers in wastewater of 14 well characterized municipal and domestic WWTPs in Austria was investigated in order to determine their suitability for the detection of sewage pollution regarding catchment size and season. In addition the occurrence of human-associated *Bacteroidetes* genetic markers was determined in grab samples of raw and treated wastewater from 29 rural and urban sites in 13 countries on six continents with a focus on geographic distribution. Standard fecal indicator bacteria and alternative genetic viral markers were also compared to the genetic fecal Bacteroidetes marker.

2 Stability of fecal indicators and human-associated genetic fecal markers in municipal wastewater

Abstract

Due to high diurnal water quality fluctuations in raw municipal wastewater, the use of proportional auto-sampling over a period of 24 h at municipal wastewater treatment plants (WWTPs) to evaluate carbon, nitrogen and phosphorus removal has become a standard in many countries. Microbial removal or load estimation at municipal WWTP however is still based on manually recovered grab samples. The goal of this study was to establish basic knowledge regarding the persistence of standard bacterial fecal indicators and *Bacteroidetes* genetic microbial source tracking markers in municipal wastewater in order to evaluate their suitability for automated sampling, as the potential lack of persistence is the main argument against such procedures. Raw and secondary treated wastewater of municipal origin from representative and well-characterized biological WWTPs without disinfection (organic carbon and nutrient removal) was investigated in microcosm experiments at 5°C and 21°C with a total storage length of 32 h (including a 24-h auto-sampling component and an 8-h post-sampling phase). Vegetative *Escherichia coli* and enterococci as well as *Clostridium perfringens* spores were selected as indicators for cultivation-based standard enumeration. Molecular analysis focused on total (AllBac) and human-associated genetic *Bacteroidetes* (BacHum UCD, HF183 TaqMan) markers using quantitative PCR, as well as 16S-rRNA-gene based next generation sequencing (NGS). The microbial parameters showed high persistence in both raw and treated wastewater at 5°C during the defined storage conditions. Surprisingly, and in contrast to results from treated wastewater, persistence of the microbial parameters in raw wastewater was also high at 21°C. Based on our results, 24-h auto-sampling procedures with 5°C storage conditions can be recommended when investigating fecal indicators or *Bacteroidetes* genetic markers at municipal WWTPs. Such auto-sampling procedures will contribute to better understanding and monitoring of municipal WWTPs as sources of fecal pollution in water resources.

2.1 Introduction

Microbial fecal contamination of aquatic systems by municipal wastewater represents a significant threat to public health (Stevens et al. 2009). Thus, appropriate wastewater disposal technologies and fecal pollution monitoring programs are critical for safeguarding our water resources. Standard fecal indicators, as well as recently developed genetic microbial source tracking (MST) markers, are used to monitor the microbial fecal emission loads from wastewater treatment plants (WWTPs) and their impact on receiving waters (Ahmed et al. 2013, Harwood et al. 2014, Passerat et al. 2011, Schoen et al. 2011, Tambalo et al. 2012). Microbiological sampling of WWTPs is commonly based on manually recovered samples (ISO). However, the concept behind these methods neglects temporal fluctuations in water quality. Large diurnal variations have been reported for key chemical parameters, such as nutrients, in raw wastewater (Henze M. 2008). Determination of the efficacy of carbon, nitrogen and phosphorus removal at WWTPs is thus frequently based on automated diurnal sampling. For example, in Austria automated sampling procedures for chemical parameters are required for the official performance testing of WWTPs with more than 1000 population equivalents (PE), and these procedures use sampling volumes that are proportional to observed water influx levels over a period of 24 h (ÖWAV 2010).

Automated sampling is infrequently used for monitoring microbial fecal pollution. A key argument against the use of automated sampling procedures is the unknown, low, or differential persistence of microbial targets, especially when longer storage periods occur (i.e., > 8 h). This deficiency can potentially lead to false negative results or the underestimation of target concentrations (Green et al. 2011, Hoglund et al. 1998, Liang et al. 2012, Sokolova et al. 2012). Nonetheless, several studies have demonstrated the potential of automated sampling procedures for pollution microbiology (Converse et al. 2011, Ferguson 1994, Passerat et al. 2011, Roser et al. 2002, Stadler et al. 2008). For example, auto-sampling was used to elucidate previously unobserved microbial fecal pollution dynamics in alpine water resources, results that had significant implications for water quality management (Stadler et al. 2010, Stadler et al. 2008). To keep the effects of microbial die-off within a negligible range, batches of collected samples were recovered from an automated sampling device within 24 h and analyzed immediately (Stadler et al. 2008).
The goal of this study was to establish basic knowledge regarding the persistence of standard bacterial fecal indicators and *Bacteroidetes* genetic MST markers in wastewater of municipal in order to evaluate their suitability for automated sampling procedures. Raw and treated wastewater samples from representative

municipal WWTPs were investigated in microcosm experiments at 5°C and 21°C during a period of 32 h. This time span reflects the 24-h auto-sampling period required for WWTP performance testing in the European Community and an 8-h post-sampling phase (equivalent to one working day) that includes sample transport and processing. Surprisingly, in contrast to natural systems such as rivers and lakes, no information is available for raw and treated wastewater of municipal origin regarding the persistence of fecal indicators and genetic markers (Aulenbach 2010, Darakas et al. 2009, Dick et al. 2010, Lessard and Sieburth 1983, Liang et al. 2012, Schulz and Childers 2011). Here, the fecal indicator bacteria *Escherichia coli*, enterococci and *Clostridium perfringens* spores were selected as representatives for cultivation-based standard determination, while molecular quantification by qPCR was used to elucidate total and human-associated genetic *Bacteroidetes* markers. Additionally, 16S rDNA-based next generation sequencing (NGS) was applied to selected samples to further evaluate the recovered results on a more general screening level of the investigated microbial communities. We hypothesized that only the spores of *C. perfringens* are appreciably stable in raw and treated wastewater of municipal origin, whereas vegetative cells of *E. coli* and enterococci as well as genetic markers of *Bacteroidetes* exhibit significant reductions in concentration at 5°C and 21°C during the selected storage period.

2.2 Materials and methods

2.2.1 Investigated WWTPs

Three municipal WWTPs (no. 2, 3, and 4) in the area of Vienna, with sizes ranging from 23000 to 140000 PE, were selected as representative plants for the Austrian/European region (BMLFUW 2012). Detailed information on the characteristics of the WWTPs, the chemical analysis of the raw and treated wastewater, and the methodology is provided in table 1. Samples were taken in both summer and winter to account for potential seasonal differences. Industrial influence at the selected plants was moderate, and thus no inhibitory or toxic effects were expected. The annual mean concentrations of chemical oxygen demand (COD), total nitrogen (TN), and total phosphorus (TP) in the raw municipal wastewater investigated ranged from 460 to 560 mg L^{-1}, 45 to 55 mg L^{-1}, and 4 to 10 mg L^{-1}, respectively. At the time of the study, WWTPs 3 and 4 were using activated sludge treatment with nitrification and denitrification. Phosphorus removal was achieved by chemical precipitation, which is required for sensitive areas in the European Union (Commission 1998). Overall, elimination rates for COD, TN, and TP were ≥ 94%, ≥ 90%, and approx. 80%, respectively. In contrast to

WWTPs 3 and 4, WWTP 2 was overloaded without showing denitrification, and it therefore displayed low rates of nitrogen removal. No disinfection was applied at the investigated WWTP.

TABLE 1: Investigated wastewater treatment plants

WWTP	design capacity [PE]	actual average loading [PE]***	inhabitants connected	sludge age (average) [d]	wastewater treatment	COD** In/Ef [mg L^{-1}]	TN** In/Ef [mg L^{-1}]	TP** In/Ef [mg L^{-1}]
2	40,000	48,700*	23,500	8-10	M, C, N, P	500/45	54/26	10/1.0
3	23,000	13,600	10,800	22-57	M, C, N, D, P	560/20	54/12	9/0.4
4	140,000	44,000	30,800	13	M, C, N, D, P	460/14	45/9	4/0.3

Abbreviations: *overloaded WWTP, **Annual mean values, *** Annual mean chemical oxygen demand (COD) load (kg/a) divided by a COD load per person of 110 g COD/d; TN: Total Nitrogen; TP: Total Phosphorus; In, influent; Ef, effluent; M, primary treatment: mechanical treatment step; C, secondary treatment: biological carbon removal; tertiary treatment: nutrient removal including nitrification (N), denitrification (D) and phosphorus removal (P)

2.2.2 Analysis of chemophysical parameters

The chemical oxygen demand was measured as described in DIN 38409-43 (DIN 1981). Total phosphorus and total nitrogen were investigated with a Merck 500 microwave + SKALAR segment flow analyzer (Skalar, Netherlands) according to the ISO standards (ISO 1997, 2004).

2.2.3 Sampling and microcosm experiments

Grab samples from the influent and effluent sites of the WWTPs being investigated were collected in sterile 5 L plastic bottles (Azlon, Great Britain). Samples were kept cold in the dark and immediately transported to the laboratory. There, samples were thoroughly shaken, sub-divided in two 2 L bottles, carefully temperature equilibrated within 3 to 5 hours (required time depended on sampling temperature), and incubated at 5 ± 2°C or 21 ± 1°C for batch culture microcosm experiments spanning a minimum of 168 h. Although the main focus of the experiments was on the persistence during short-term storage (≤ 32 h), some points of observation were also selected at incubation times > 32 h to achieve a reference to long-term storage. After defined intervals (Table 2), 70 ml sub-fractions were recovered from the microcosms, homogenized in an ultrasonic bath (Bandelin, SONOREX, Germany) for 5 min and subjected to microbiological analyses (analyses were performed in several dilutions and duplicates). Before sub-fractions were removed from microcosms, they were thoroughly shaken, including inversion of

bottles. The remainder of each 5 L municipal wastewater sample was used for chemical analysis (Table 1). The extent of statistical variation at the experimental trial level of the microcosms was also estimated. This was done during four persistence experiments using replicate measurements for AllBac, BacHum and HF183 TaqMan quantitative PCR (qPCR) determinations. Results did not reveal any detectable systematic effect on the regression coefficients due to the replication effort (Mann-Whitney U, $p > 0.5$, $n=4\times12$).

2.2.4 Microbiological and molecular analysis

Cultivation-based enumeration of *E. coli,* enterococci and *C. perfringens* spores was performed by membrane filtration using appropriate dilutions as previously described (Farnleitner et al. 2010, Vierheilig et al. 2013). For quantification of *C. perfringens* spores, 5 ml (influent) and 15 ml (effluent) aliquots from the batch sample were pasteurized at 60 ± 2°C for 15 min. *C. perfringens* was analyzed according to ISO standard 14189 (ISO 2013), based on selective growth on TSC agar (Scharlau, Spain) at 44°C and subsequent colony identification by acid phosphatase reaction (Ryzinska-Paier et al. 2011). Enumeration of presumptive *E. coli* was based on ISO standard 16649-1 (ISO 2001a) using chromogenic TBX agar (Oxoid, Thermo Fisher Scientific Inc., Cheshire, United Kingdom) at 44°C. Enumeration of enterococci was based on the ISO standard 7899-2 (ISO 2000), using Slanetz–Bartley medium (Oxoid) and dry heat incubation at 44 ± 0,5°C for 44 ± 4 h. Appropriate control strains were used to ensure the quality of the media.

Detection of genetic MST markers was based on total and human-associated *Bacteroidetes* assays. Respective 16S rDNA markers for AllBac (Layton et al. 2006), BacHUM-UCD (Kildare et al. 2007) and HF183 TaqMan (Haugland et al. 2010) were quantified by qPCR. For DNA extraction we used polycarbonate membrane filtration (0.2 µm Millipore, Isopore Membrane Filter – GTTP, Cork, Ireland) of 10 ml (influent) and 50 ml (effluent) batch sample aliquots, as previously described (Griffiths et al. 2000, Reischer et al. 2006) followed by phenol/chloroform DNA extraction. Cell lysis was carried out with a FastPrepR-24 Instrument (MP Biomedicals Inc., Irvine, USA) with a speed setting of 6 m/s for 30 s each. The extracted DNA was stored at -20°C prior to analysis of two dilutions (10- and 100-fold) to test for PCR inhibition. The rotor-discs were loaded with mastermix and sample by a Qiagility Robot (Qiagen, Hilden, Germany), and measurements were subsequently performed on a Rotorgene Q Cycler (Qiagen). For the AllBac qPCR assay, we used 2.5 µl of the appropriate DNA sample dilution, 600 nM primer AllBac296f, 600 nM primer AllBac412r, 25 nM TaqMan MGB probe AllBac375Bhqr (Layton et al. 2006), 0.4 g L^{-1} bovine serum albumin (Roche Diagnostics, Mannheim,

Germany), and 7.5 µl of iQ Supermix (Biorad, Hercules, USA) in a total reaction volume of 15 µl. Additionally, we added 5 mM $MgCl_2$ to obtain a total Mg^{2+}concentration of 8 mM (Layton et al. 2006). For the BacHUM assay we used 2.5 µl of the respective DNA sample dilution, 400 nM primer BacHUM-160f, 400 nM primer BacHUM-241r, 80 nM TaqMan MGB probe BacHUM-193p (Kildare et al. 2007), 0.4 g L^{-1} bovine serum albumin, and 7.5 µl of iQ Supermix in a total reaction volume of 15 µl. For the HF183 TaqMan assay we used 2.5 µl of the respective DNA sample-dilution, 100 nmol L^{-1} primer HF183, 100 nmol L^{-1} primer BFDREV, 80 nmol L^{-1} TaqMan MGB probe BFDFAM (Haugland et al. 2010), 0.4 g L^{-1} bovine serum albumin, and 7.5 µl of iQ Supermix in a total reaction volume of 15µl. The PCR program for AllBac was 95°C for 3 min, 45 cycles of 95°C for 30 s and 60°C for 45 s. For BacHum, the PCR program was 95°C for 3 min, 45 cycles of 95°C for 15 s and 60°C for 1 min and for the HF183 TaqMan assay 95°C for 3 min, 45 cycles of 95°C for 15 s and 60°C for 30s. Real-time data were collected during the primer-annealing step at 60°C. Quantification was based on appropriate standard dilutions of plasmid DNA (Reischer et al. 2007) and presented as marker equivalents per volume (ME/vol) according to Reischer et al. 2006 (Reischer et al. 2006).

2.2.5 Next generation sequencing

The DNA extracts (n = 16) of the one representative microcosm series from the WWTP2 effluent, which was chosen for additional 16S rDNA 454 pyrosequencing analysis, were used as templates in PCR to amplify the variable regions V1-V2 of the 16S rRNA gene for 25 cycles. All reactions were run in triplicate with the bacterial specific primers S-D-Bact-0008-a-S-20 (5´-AGAGTTTGATCCTGGCTCAG-3´), as described by Edwards et al. (Edwards et al. 1989), and S-D-Bact-0338-a-A-19 (5´-TGCTGCCTCCCGTAGGAGT-3´), as described by Etchebehere and Tiedje (Etchebehere and Tiedje 2005), the latter equipped with a distinct 12-nucleotide error-correcting Golay barcode for each extract as a multiplex tag (Fierer et al. 2008, Golay 1949, Hamady M. 2008). The nomenclature for the PCR primers was standardized according to Alm et al. (Alm et al. 1996). The sample amplicons (n = 16) were purified, pooled in equimolar amounts and sent to Selah Clinical Genomic Center, formerly EnGenCore (Columbia, SC, USA) for 454 pyrosequencing (titanium chemistry).

2.2.6 Bioinformatic analysis

Sequence analysis was done using the software package Quantitative Insights Into Microbial Ecology, QIIME (Caporaso et al. 2010b). Raw sequences (n = 214,978) were quality filtered and assigned to the samples according to their barcodes. The

flowgrams were denoised to reduce sequencing noise (Reeder and Knight 2009). After removing the primers, chimeric sequences identified by de novo (abundance based) and reference based chimera detection with UCHIME were filtered out (Edgar 2010, Edgar et al. 2011). Remaining sequences (n = 185,374) were binned into Operational Taxonomic Units (OTUs) using USEARCH, with a minimum pairwise identity of 97% (Edgar 2010). Greengenes OTUs (97%; version May 2013) were specified as a reference database at the previous two steps (DeSantis et al. 2006). Rare OTUs represented by less than four sequences were filtered out, leading to 182,914 remaining sequences for further analysis. The most abundant sequence in each OTU was chosen as a representative and aligned using PyNAST (Caporaso et al. 2010a) and the Greengenes reference alignment (DeSantis et al. 2006) trimmed to the V1-V2 region of the 16S rRNA gene (Werner et al. 2012) with a minimum percent identity of 75%. The hyper variable regions were filtered out with the V1-V2 trimmed version of the lanemask and a phylogenetic tree was constructed using FastTree (Price et al. 2009). Taxonomy was assigned with the Ribosomal Database Project (RDP) classifier (Wang et al. 2007) with a minimum confidence of 80% and the greengenes taxonomy of May 2013 (McDonald et al. 2012). The sequences assigned to the phylum *Bacteroidetes* were filtered out. Subsequently, 515 *Bacteroidetes* sequences (i.e. smallest number of taxon-specific sequences per sample) were randomly selected from each sample for further analyses (rarefaction). To compare the diversity within this taxon between the samples, we calculated the unweighted UniFrac distance metric (Lozupone and Knight 2005) for the phylum *Bacteroidetes* and clustered the resulting metric using principle coordinate analysis to visualise the phylogenetic relatedness of these communities.

2.2.7 Data analysis and statistics

All microbial data were expressed as $\log_{10}$ (x+1). Regression analysis and descriptive statistics in this chapter were calculated with IBM SPSS Statistics Version 20.0.0 (IBM, Germany). To account for the multiple tests that were carried out, statistical significance levels were Bonferroni corrected (Bonferroni 1936). All graphs were prepared using Sigma Plot 11.0 (SPSS Inc., Chicago, USA) and CorelDraw X5 (Corel, Canada).

2.3 Results

TABLE 2: Full data set for the persistence of standard fecal indicators and microbial source tracking markers in raw and treated municipal wastewater at 5°C recovered from the microcosm experiments.

		Microcosm Experiments							
		Sampling Design		Descriptive Statistics log_{10} [(ME+1) 100 ml^{-1}] or log_{10} [(CFU+1) 100 ml^{-1}]			Regression Analysis on Microcosm Data		
		S.E[p] (WWTP)	Time[f]	Mean	Min	Max	d[g]	k[g]	Reduction Log / %[o]
AllBac (qPCR)	Influent	1 (2)	a	10.0	9.3	10.3	10.0	0.003	-
		3 (4)	b	10.3	10.1	10.4	10.2	0.004	-
		7 (3)	c	10.6	10.0	11.2	10.2	0.034	-
		9 (3)	d	10.7	10.6	10.8	10.8	-0.005	-
		11 (4)	i	10.1	9.7	10.2	10.0	0.001	-
		13 (2)	i	10.5	10.3	10.6	10.5	-0.003	-
	Effluent	2 (2)	a	7.7	7.2	7.9	7.8	-0.003	-
		4 (4)	b	7.9	7.7	8.2	8.1	-0.007	-
		8 (3)	c	7.8	7.1	9.8	7.2	0.057	-
		10 (3)	d	7.7	7.5	8.0	7.7	0.001	-
		12 (4)	i	8.8	8.7	8.8	8.8	0.001	-
		14 (2)	i	8.2	8.0	8.5	8.3	-0.008	-
BacHum-UCD (qPCR)	Influent	1 (2)	a	8.9	8.4	9.2	8.9	-0.001	-
		3 (4)	b	8.7	8.5	8.9	8.6	0.002	-
		7 (3)	c	9.1	8.9	9.5	9.2	-0.011	-
		9 (3)	d	9.0	8.8	9.0	9.0	-0.004	-
		11 (4)	i	8.7	8.4	9.2	8.6	0.015	-
		13 (2)	i	9.5	9.0	9.7	9.3	0.010	-
	Effluent	2 (2)	a	6.7	6.1	6.9	6.7	-0.002	-
		4 (4)	b	6.1	5.8	6.6	6.4	-0.019	-
		8 (3)	c	6.6	6.1	8.1	6.1	0.042	-
		10 (3)	d	5.6	5.4	5.9	5.6	0.000	-
		12 (4)	i	8.0	7.7	8.2	7.7	0.018	-
		14 (2)	i	7.0	6.7	7.1	6.9	0.004	-

		Microcosm Experiments							
		Sampling Design		Descriptive Statistics log_{10} [(ME+1) 100 ml^{-1}] or log_{10} [(CFU+1) 100 ml^{-1}]			Regression Analysis on Microcosm Data		
		S.E[p] (WWTP)	Time[f]	Mean	Min	Max	d[g]	k[g]	Reduction Log / %[o]
HF183 TaqMan (qPCR)	Influent	1 (2)	a	8.5	7.5	9.1	8.4	0.006	-
		3 (4)	b	8.4	8.2	8.6	8.3	0.003	-
		7 (3)	c	8.0	7.6	8.8	7.8	0.019	-
		9 (3)	d	9.4	9.2	9.4	9.4	-0.002	-
		11 (4)	i	8.5	8.2	8.7	8.3	0.010	-
		13 (2)	i	9.0	8.7	9.3	8.9	0.007	-
	Effluent	2 (2)	a	6.5	6.1	6.8	6.6	-0.008	-
		4 (4)	b	5.7	5.5	6.1	6.0	-0.015*	0.48 / 66
		8 (3)	c	5.6	4.9	7.1	4.7	0.074	-
		10 (3)	d	6.1	5.8	6.9	6.1	0.003	-
		12 (4)	i	7.5	7.3	7.7	7.3	0.013	-
		14 (2)	i	6.5	6.4	6.6	6.5	0.000	-
E. coli (cultivation based)	Influent	1 (2)	a	6.8	6.8	6.8	6.8	0.000	-
		3 (4)	b	6.4	6.2	6.6	6.5	-0.004	-
		5 (4)	e	6.9	6.9	7.0	6.9	0.000	-
		7 (3)	c	6.2	6.2	6.3	6.3	-0.003	-
		9 (3)	d	6.7	6.6	6.9	6.7	-0.002	-
		11 (4)	i	7.8	7.7	8.1	8.1	-0.015	-
		13 (2)	i	6.1	6.1	6.2	6.2	-0.003	-
	Effluent	2 (2)	a	4.4	4.3	4.6	4.5	-0.005	-
		4 (4)	b	4.7	4.6	5.0	4.9	-0.010	-
		6 (4)	e	4.6	4.5	4.6	4.6	0.000	-
		8 (3)	c	3.7	3.6	3.8	3.6	0.006	-
		10 (3)	d	3.6	3.5	3.8	3.7	-0.002	-
		12 (4)	i	5.2	5.2	5.3	5.3	-0.004	-
		14 (2)	i	4.0	3.9	4.1	4.1	-0.005	-

		Microcosm Experiments							
		Sampling Design		Descriptive Statistics log_{10} [(ME+1) 100 ml^{-1}] or log_{10} [(CFU+1) 100 ml^{-1}]			Regression Analysis on Microcosm Data		
		S.E[p] (WWTP)	Time[f]	Mean	Min	Max	d[g]	k[g]	Reduction Log / %[o]
C. perfringens spores (cultivation based)	Influent	1 (2)	a	5.0	4.9	5.1	5.0	0.005	-
		3 (4)	b	4.9	4.7	4.9	4.8	0.003	-
		7 (3)	c	4.6	4.5	4.7	4.6	-0.003	-
		11 (4)	i	4.5	4.4	4.6	4.5	0.000	
		13 (2)	i	4.7	4.6	4.8	4.7	0.003	
	Effluent	2 (2)	a	3.9	3.8	4.0	3.9	0.006	-
		4 (4)	b	3.1	3.1	3.2	3.2	-0.002	-
		8 (3)	c	3.0	2.9	3.0	3.0	0.003	-
		12 (4)	i	3.9	3.7	4.0	3.8	0.004	-
		14 (2)	i	3.9	3.7	4.0	3.9	-0.002	-
Enterococci (cultivation based)	Influent	11 (4)	i	5.4	5.3	5.6	5.3	0.006	-
		13 (2)	i	5.7	5.6	5.9	5.6	0.006	-
	Effluent	12 (4)	i	4.3	4.3	4.3	4.3	-0.001	-
		14 (2)	i	4.1	4.0	4.2	4.0	-0.001	-

Abbreviations: Mean, arithmetic mean; Min, minimum value; Max, maximum value, ME, marker equivalents

p: Sampling event number. In brackets is the number of the investigated WWTP given.

f: Time of analysis during microcosm experiments; a (n=5): 0 h, 4 h, 8 h, 20 h, 24 h; b (n=6): 0 h, 7 h, 19 h, 24 h, 27 h, 43 h; c (n=6): 0 h, 4 h, 8 h, 12 h, 22 h, 24 h; d (n=5): 0 h, 5 h, 18 h, 27 h, 35 h; e (n=5): 0 h, 5 h, 11 h, 17 h, 25 h and i: (n=5) 0 h, 9,5 h, 20 h, 24 h, 29 h

g: d and k, linear regression coefficients; d is the intercept with the y–Axes log_{10} [(ME+1) 100 ml^{-1}] or log_{10} [(CFU+1) 100 ml^{-1}], k is the slope (log_{10} [(ME+1) 100 ml^{-1}] or log_{10} [CFU 100 ml^{-1}]) per hour; asterisks mark statistically significant coefficients ($p \leq 0.05$, Bonferroni corrected).

o: Log_{10} reduction calculated from regression model for a sample storage time of 32 h at 5°C (calculated for significant regression coefficients only). Numbers after the slash refer to percent reduction, relating to the delogarithmized absolute values.

All experiments with raw municipal wastewater samples, including influents from WWTP2, WWTP3, and WWTP4, revealed a high stability of the investigated microbiological parameters at 5°C and 21°C during the selected storage period of 32 h (Table 2/3, Fig. 1). Only two of 64 regression coefficients of microcosm experiments using raw wastewater displayed a negative value that deviated significantly from zero ($p \leq 0.05$, Bonferroni corrected). This statistically significant regression coefficients were from the human-associated *Bacteroidetes* marker BacHUM and HF183 TaqMan, accounting for a maximum 0.5 $\log_{10}$ decrease in concentration in the regression model during storage of 32 h at 21°C (Table 3). All measurements for vegetative *E. coli, enterococci* and the genetic *Bacteroidetes* markers resulted in more pronounced decreases in concentration at the time points at 96 h and 264 h. *C. perfringens* spores did not show any relevant decrease in concentration during the whole observation periods (Fig. 1, Table 2/3).

The persistence of the investigated microbial parameters in treated wastewater samples at 5°C was also high (Fig. 1). With the exception of one experiment, regression analysis did not detect any statistically significant changes over the investigated time frame (Table 2). In contrast, 9 of the microcosm experiments carried out with treated wastewater at 21°C revealed significant negative regression coefficients for *E. coli* and the genetic *Bacteroidetes* markers ($p \leq 0.05$, Bonferroni corrected, Table 3). Decreases in concentration of up to 1.9 $\log_{10}$ for a 32 h storage period were apparent using the regression model (Table 3). Additionally, all measurements taken at 96 h and 264 h yielded large and significant reductions for *E. coli*, enterococci and the genetic *Bacteroidetes* markers; again, no notable decrease in *C. perfringens* spores was found in any of these storage experiments (Table 2; Fig. 1).

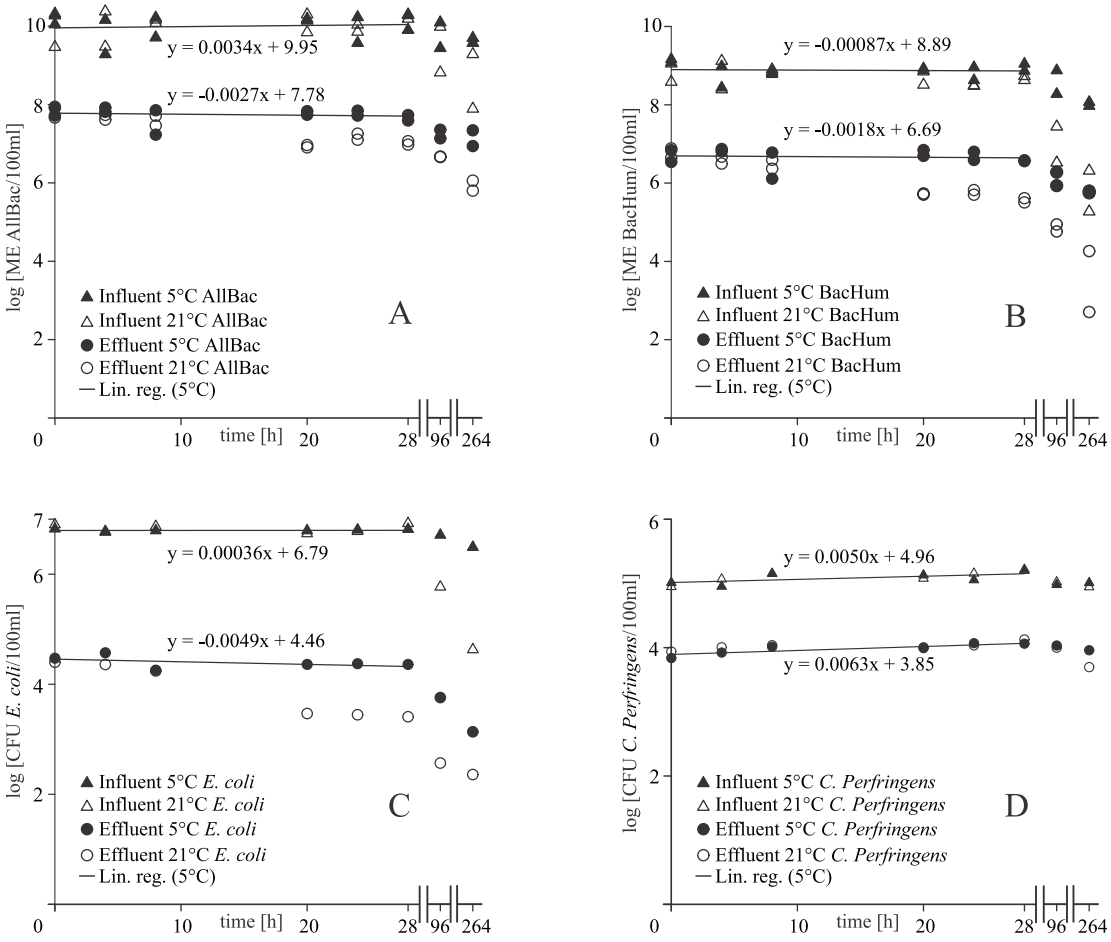

Figure 1: Persistence of standard fecal indicators and genetic MST markers in raw (influent) and treated (effluent) municipal wastewater at 5°C and 21°C.

Data shown is a representative set; for full data, see Table 1 & 2. Linear regression analysis was performed for 28 hours and is only shown for the 5°C storage conditions (values for samples taken at 96 h and 264 h are given as control measurements). (A) AllBac: genetic fecal marker for the total Bacteroidetes populations; (B) BacHum: genetic fecal marker for human-associated Bacteroidetes populations; (C) cultivation-based enumeration of *Escherichia coli*; (D) cultivation-based enumeration of *Clostridium perfringens* spores.

To further evaluate our results regarding the 16S rRNA gene bacterial community composition and the persistence of *Bacteroidetes* populations at the phylum scale, one representative microcosm series from the WWTP2 effluent was chosen for additional 454 amplicon pyrosequencing analysis. Taxonomic pyrosequencing analysis of the 16S rRNA gene microbial community composition revealed a clear predominance of the phyla *Proteobacteria* and *Bacteroidetes*, with average relative abundances of 60% (+/- 5%) and 27% (+/- 6%), respectively. The next most predominant phyla were *Actinobacteria* and *Firmicutes* with an average abundance of 2% (+/- 0.6% and +/- 0.7%) each. Microbial community structure analysis, using an unweighted UniFrac-algorithm combined with principal coordinate analysis (PCoA), did not detect any notable changes in the *Bacteroidetes* community

composition during the investigated short-term storage period at 5°C (Fig. 2). In contrast, major changes in the total *Bacteroidetes* community structure became apparent at 21°C incubation conditions and also at the later time points (96 h and 264 h) of the 5°C microcosms experiments (Fig. 2).

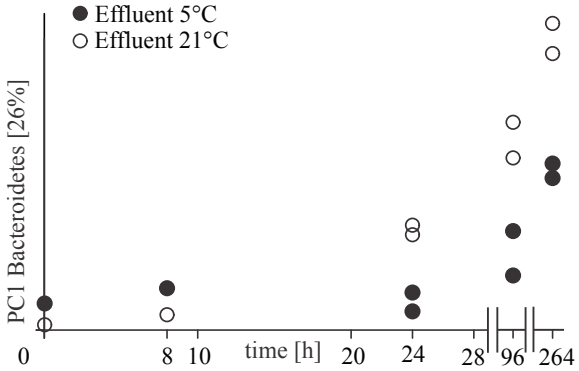

Figure 2: 16S rRNA gene based qualitative UniFrac community structure dynamics for the microcosm experiments with WWTP2 effluent.
The first principal coordinate (PC1) vs. time is shown for the phylum Bacteroidetes (26% of the total variance is explained by PC1) on the x- and y-axis, respectively. Black and open dots relate to microcosm experiments at 5°C and 21°C, respectively. Analysis at time point 0 h and 8 h are given as single analysis, whereas analysis at time points 24 h, 96 h, and 264 h are given as duplicate analysis.

TABLE 3: Full data set for the persistence of standard fecal indicators and microbial source tracking markers in raw and treated municipal wastewater at 21°C recovered from the microcosm experiments

		Microcosm Experiments							
		Sampling Design		Descriptive Statistics log_{10} [(ME+1) 100 ml^{-1}] or log_{10} [(CFU+1) 100 ml^{-1}]			Regression Analysis on Microcosm Data		
		S.E[p] (WWTP)	Time[f]	Mean	Min	Max	d[g]	k[g]	Reduction Log / %[o]
AllBac (qPCR)	Influent	1 (2)	a	10.0	9.5	10.4	9.9	0.007	-
		3 (4)	b	10.2	10.1	10.4	10.1	0.004	-
		7 (3)	c	10.3	10.1	10.6	10.3	0.004	-
		9 (3)	d	10.9	10.7	11.1	10.8	0.003	-
		11 (4)	i	10.0	9.5	10.3	9.7	0.017	-
		13 (2)	i	10.7	10.4	10.9	10.5	0.006	-
	Effluent	2 (2)	a	7.4	7.0	7.9	7.8	-0.030[*]	0.96 / 89
		4 (4)	b	7.8	7.3	8.2	7.9	-0.002	-
		8 (3)	c	7.22	6.95	7.52	7.3	-0.011	-
		10 (3)	d	7.40	7.2	7.6	7.5	-0.006	-
		12 (4)	i	8.75	8.62	8.98	8.9	-0.011	-
		14 (2)	i	8.02	7.65	8.30	8.3	-0.014	-

		Microcosm Experiments					Regression Analysis on Microcosm Data		
		Sampling Design		Descriptive Statistics log_{10} [(ME+1) 100 ml^{-1}] or log_{10} [(CFU+1) 100 ml^{-1}]					
		S.E[p] (WWTP)	Time[f]	Mean	Min	Max	d[g]	k[g]	Reduction Log / %[o]
BacHum-UCD (qPCR)	Influent	1 (2)	a	8.7	8.4	9.1	8.8	-0.009	-
		3 (4)	b	8.3	8.1	8.6	8.5	-0.010*	0.30 / 50
		7 (3)	c	9.2	8.9	9.3	9.1	0.008	-
		9 (3)	d	8.9	8.7	8.9	8.9	-0.002	-
		11 (4)	i	8.4	8.1	8.6	8.5	-0.002	-
		13 (2)	i	9.5	9.1	9.7	9.3	0.011	-
	Effluent	2 (2)	a	6.1	5.5	6.9	6.8	-0.045*	1.89 / 99
		4 (4)	b	5.2	4.2	6.3	6.2	-0.049*	1.57 / 97
		8 (3)	c	6.1	5.7	6.4	6.3	-0.023	-
		10 (3)	d	5.0	4.0	5.5	5.2	-0.013	-
		12 (4)	i	7.9	7.8	7.9	7.9	0.001	-
		14 (2)	i	6.7	6.5	7.1	7.0	-0.014	-
HF183 TaqMan - (qPCR)	Influent	1 (2)	a	8.4	7.5	8.9	8.3	0.009	-
		3 (4)	b	8.0	7.7	8.4	8.3	-0.015*	0.48 / 66
		7 (3)	c	8.1	7.8	8.8	7.7	0.032	-
		9 (3)	d	9.2	9.1	9.4	9.4	-0.007	-
		11 (4)	i	8.2	7.9	8.5	8.3	-0.005	-
		13 (2)	i	9.0	8.5	9.3	8.8	0.009	-
	Effluent	2 (2)	a	6.1	5.3	7.0	6.7	-0.046*	1.42 / 96
		4 (4)	b	6.0	4.7	3.9	5.8	-0.054	-
		8 (3)	c	5.0	4.5	5.6	5.0	0.002	-
		10 (3)	d	5.4	4.0	6.0	5.7	-0.016	-
		12 (4)	i	7.4	7.3	7.5	7.4	0.000	-
		14 (2)	i	6.3	6.0	6.6	6.5	-0.015	-
E. coli (cultivation based)	Influent	1 (2)	a	6.8	6.7	6.9	6.8	-0.001	-
		3 (4)	b	6.3	6.2	6.5	6.4	-0.006	-
		5 (4)	e	6.8	6.6	6.9	6.8	-0.006	-
		7 (3)	c	6.4	6.3	6.4	6.4	0.000	-
		9 (3)	d	6.7	6.6	6.9	6.8	-0.004	-
		11 (4)	i	8.0	7.9	8.2	8.1	-0.009	-
		13 (2)	i	6.1	6.1	6.1	6.1	0.001	-
	Effluent	2 (2)	a	3.9	3.4	4.4	4.5	-0.042*	1.35 / 96
		4 (4)	b	4.1	3.3	5.1	5.0	-0.041*	1.31 / 95
		6 (4)	e	4.2	3.9	4.6	4.5	-0.024	-
		8 (3)	c	3.6	3.5	3.8	3.8	-0.009*	0.29 / 49
		10 (3)	d	3.7	3.6	3.8	3.7	-0.002	-
		12 (4)	i	5.2	5.2	5.3	5.3	-0.005	-
		14 (2)	i	3.8	3.5	4.0	4.0	-0.017	-

		Microcosm Experiments					Regression Analysis on Microcosm Data		
		Sampling Design		Descriptive Statistics log_{10} [(ME+1) 100 ml^{-1}] or log_{10} [(CFU+1) 100 ml^{-1}]					
		S.E[p] (WWTP)	Time[f]	Mean	Min	Max	d[g]	k[g]	Reduction Log / %[o]
C. perfringens (cultivation based)	Influent	1 (2)	a	5.1	4.9	5.2	5.0	0.006	-
		3 (4)	b	4.9	4.8	5.0	4.8	0.001	-
		7 (3)	c	4.5	4.4	4.6	4.5	-0.002	-
		11 (4)	i	4.5	4.3	4.6	4.4	0.005	-
		13 (2)	i	4.7	4.6	4.8	4.6	0.003	-
	Effluent	2 (2)	a	4.0	3.9	4.1	3.9	0.004	-
		4 (4)	b	3.1	3.1	3.2	3.2	-0.002	-
		8 (3)	c	3.0	2.9	3.1	3.0	0.004	-
		12 (4)	i	3.8	3.7	3.9	3.9	-0.004	-
		14 (2)	i	3.8	3.7	3.9	3.8	0.001	-
Enterococci (cultivation based)	Influent	11 (4)	i	5.4	5.3	5.5	5.3	0.003	-
		13 (2)	i	5.68	5.46	5.81	5.6	0.005	-
	Effluent	12 (4)	i	4.2	4.1	4.3	4.3	-0.005	-
		14 (2)	i	3.96	3.87	4.03	4.2	-0.007	-

Abbreviations: Mean, arithmetic mean; Min, minimum value; Max, maximum value, ME, marker equivalents

p: Sampling event number. In brackets is the number of the investigated WWTP given.

f: Time of analysis during microcosm experiments, a (n=5): 0 h, 4 h, 8 h, 20 h, 24 h; b (n=6): 0 h, 7 h, 19 h, 24 h, 27 h, 43 h; c (n=6): 0 h, 4 h, 8 h, 12 h, 22 h, 24 h; d (n=5): 0 h, 5 h, 18 h, 27 h, 35 h; e (n=5): 0 h, 5 h, 11 h, 17 h, 25 h and i: (n=5) 0 h, 9,5 h, 20 h, 24 h, 29 h

g: d and k, linear regression coefficients, d in log_{10} [(ME+1) 100 ml^{-1}] or log_{10} [CFU 100 ml^{-1}], k is the difference of (log_{10} [(ME+1) 100 ml^{-1}] or log_{10} [CFU 100 ml^{-1}]) per hour between data points; asterisks mark statistically significant coefficients ($p \leq 0.05$, Bonferroni corrected).

o: log_{10} reduction calculated from regression model for a sample storage time of 32 h at 21°C (calculated for significant regression coefficients only). Numbers after the slash refer to percent reduction, relating to the delogarithmized absolute values.

2.4 Discussion

The data obtained from the microcosm experiments clearly falsified the initial hypothesis regarding the low persistence of the microbial indicators investigated in municipal wastewater during short-term storage (32 h) at 5°C. In addition to the highly resistant *C. perfringens* spores (Davies et al. 1995, John and Rose 2005,

Vierheilig et al. 2013), the vegetative *E. coli* cells and the genetic *Bacteroidetes* markers displayed remarkable stability at 5°C during the defined time frame. Although qPCR-based detection of a genetic DNA marker does not indicate cell viability (Wuertz et al. 2011), a significantly increasing or decreasing trend in DNA target concentration, due to either cell growth, degradation or grazing effects, would have been detected by the molecular quantification methods used here (Bae and Wuertz 2009, Harwood et al. 2014). Furthermore, the stability of the molecular signatures of *Bacteroidetes* cells was supported by data on the differing taxonomic levels investigated, which were quantified by the BacHum, HF183 TaqMan, and AllBac qPCR assays (Haugland et al. 2010, Kildare et al. 2007, Layton et al. 2006) and qualitatively screened by the 16S rDNA NGS community structure analysis (Shanks et al. 2013).

Strong decreases in the representative bacteria were only observed for the microcosm experiments at 21°C using untreated wastewater samples, with *E. coli* and genetic *Bacteroidetes* markers displaying losses of up to 99% of their original populations (Table 3). However, not all of these experiments yielded such a marked decrease, most likely because storage periods longer than 32 h would have been needed to reach these levels. No signs of toxicological inhibition of the microbial community in the activated sludge, which generally manifests as inhibition of aerobe/anaerobe heterotrophy or specific inhibition of nitrification, were discernible at the WWTPs. Measurements at 96 h and 264 h also revealed a clearly decreasing response, further supporting the absence of inhibiting substances. Very surprisingly, no decreasing effect was detectable in the microcosm experiments using raw municipal wastewater samples at 21°C. Extremely high levels of organic substrates (up to 680 mg COD L^{-1} was measured in raw municipal wastewater) and the absence of oxygen may have contributed to this short-term stability effect. This is only a preliminary speculation, and further investigations beyond the scope of the current study are needed to clarify the actual reason of our observation.
The selected effluent and influent characteristics represent a typical range of municipal wastewater occurring at WWTPs in Austria (Table 1) with respect to catchment type, wastewater channels, and treatment plant performance (BMLFUW 2012). The results can be taken as a strong indication that microbial persistence is not a limiting factor for short-term storage at 5°C of raw and treated municipal wastewater samples. It is important to emphasis, that disinfection was not applied at the investigated WWTP. Disinfection is not required for biological treated wastewater according to Austrian and European regulations. Disinfection is only considered in sensitive areas used for bathing or drinking water production, but not for receiving waters without a particular use. Furthermore, the proportion of industrial wastewater input was low to moderate at the investigated WWTPs. No

specific inhibitory effects or toxic substances have been reported for these WWTPs (e.g. for respiratory or nitrification measurements). Recovered results thus relate to non-disinfected raw and biological treated wastewater from municipal origin, without the occurrence of microbicidal substances from industrial effluents. Pyrosequencing-based 16S-rRNA-gene community analysis also demonstrated the typical bacterial community composition as expected for wastewater of municipal origin (Ranasinghe et al. 2012, Wang et al. 2012). The investigation of effects from disinfection or toxic compounds on the persistence of indicators or fecal marker was not the aim of this study. However, in future, it might also be interesting to elucidate the effect of microbicidal conditions on microbiological parameters with different endpoints during short term storage (e.g. cultivation-based enumeration vs. direct detection of nucleic acids Further studies may also focus on the analysis of the activity of the considered bacterial community at 5°C.

In conclusion, we can recommend 24-h auto-sampling procedures at 5°C storage conditions, not only for chemical analysis, but also for representative microbiological investigations in raw and biological treated waste water of municipal origin, when applying bacterial standard fecal indicators or *Bacteroidetes* genetic MST markers. Such auto-sampling procedures will contribute significantly to a better understanding and monitoring of municipal WWTPs as sources of fecal contamination of water resources (Stevens et al. 2009, WHO 2004).

3 Occurrence of fecal indicators and human-associated genetic fecal markers in Austrian wastewater treatment plants

Abstract

This was a detailed investigation of the seasonal occurrence, dynamics and removal of human-associated genetic *Bacteroidetes* fecal marker compared with ISO-based standard fecal indicator bacteria, human-specific viral fecal markers and one human-associated *Bacteroidetes* phage in raw and treated wastewater of municipal and domestic origin. Characteristics of the selected activated sludge wastewater treatment plants from Austria and Germany were described in detail (WWTPs, n = 13, connected populations from 3.0 to 49,000 individuals). Water quality analysis was supported by volume-proportional automated 24-h sampling. Human-associated genetic *Bacteroidetes* fecal marker were consistently detected in high concentrations in raw (median $\log_{10}$ 8.6 molecular equivalents (ME) 100 ml^{-1}) and biologically treated sewage samples (median $\log_{10}$ 6.2-6.5 ME 100 ml^{-1}), irrespective of size, type and time of the season (n = 53-65). Genetic *Bacteroidetes* fecal marker, *E. coli*, and enterococci concentrations revealed the same range of statistical variability for raw (multiplicative standard deviations s* = 2.3-3.0) and treated wastewater (s* = 3.7-4.5). *C. perfringens* spores matched the variability of chemical parameters (s* = 1.5 in raw sewage). Except for genetic *Bacteroidetes* fecal marker, *C. perfringens* and JC Polyomavirus, correlations amongst microbiological parameters were not observed in raw sewage. Statistical associations amongst microbial parameters increased during wastewater treatment. Two plants with advanced treatment were also investigated, revealing a median $\log_{10}$ 4 reduction of genetic fecal *Bacteroidetes* markers in the activated sludge membrane bioreactor, but no reduction of the genetic markers during UV irradiation (254 nm). This study highlights the potential of human-associated genetic fecal *Bacteroidetes* markers to complement wastewater impact monitoring based on the determination of SFIB. In addition, human-specific JC Polyomavirus and Adenovirus seem to be a valuable support if highly specific markers are needed.

3.1 Introduction

Contamination of aquatic systems by sewage of human origin can pose a serious threat to public health because it frequently contains high numbers of intestinal pathogens (Stevens et al. 2009). Appropriate disposal systems combined with efficient fecal pollution monitoring techniques for municipal and domestic sewage are thus essential for safeguarding our water resources. Wastewater treatment plants based on primary (mechanical), secondary (biological), and tertiary (enhanced biological and chemical) treatment are designed to remove organic carbon (C), nitrogen (N) and phosphorus (P) out of wastewater to a great extent. Although providing a first essential barrier, conventional WWTPs are not built to sufficiently remove microbial fecal loads to support the safe use of effluent wastewater for human related activities, such as recreational purposes or irrigation. Disinfection of sewage effluents has not yet become a common standard in most regions of the world, and such advanced treatment is often restricted to the discharge of wastewater into sensitive aquatic areas. Rainfall events may also lead to a bypass of WWTPs (i.e., combined sewer overflows) and the contamination of water resources with raw wastewater (Molina et al. 2014, Shibata et al. 2014, Tryland et al. 2014).

Routine monitoring of microbial fecal pollution in the aquatic environment is still based on the selective cultivation of standard fecal indicator bacteria, including *Escherichia coli* and intestinal enterococci (ISO 2005). Without doubt, water quality testing based on the application of SFIB has contributed to a fundamental improvement in water safety management since the end of the 19th century (Tallon et al. 2005). However, the application of SFIB has also recently been subjected to increasing criticism (Ishii and Sadowsky 2008). Several studies suggested that SFIB in aquatic habitats also originate from non-enteric compartments, such as soil, sediment and algae (Byappanahalli et al. 2012, Desmarais et al. 2001, Whitman et al. 2003). In addition, SFIB hardly support microbial source tracking and require the formation of unrealistically large catchment-specific strain libraries for source identification (Domingo et al. 2007). These limitations obviously call for additional indicators and tools to complement the existing standard methods to obtain a more detailed and certain view on the existing fecal pollution patterns to support MST and risk assessment (Harwood et al. 2014).

Amongst the vast number of alternative parameters (Hagedorn et al. 2011, Wuertz et al. 2011), PCR-based assays for the analysis of genetic *Bacteroidetes* fecal marker have gained increasing popularity in the field of fecal pollution analysis and MST during recent years (Harwood et al. 2014). Quantitative PCR-based genetic fecal marker assays for general-, human-, sewage-, or animal-associated fecal sources

have been developed (Kildare et al. 2007, Layton et al. 2006, Reischer et al. 2006, Shanks et al. 2009). Several evaluation studies including various aquatic environments successfully demonstrated the value of genetic fecal *Bacteroidetes* marker diagnostics (Boehm et al. 2009, Reischer et al. 2011, Ridley et al. 2014, Riedel et al. 2014, Sauer et al. 2011, Tambalo et al. 2012). However, the application of qPCR-based genetic fecal *Bacteroidetes* marker assays is not yet standardized. It requires careful study design and background information on the catchment to create unbiased results and to recognize methodical limits (Boehm et al. 2013, Reischer et al. 2011).

A useful parameter for the analysis of general- or host-associated microbial fecal pollution in water has to fulfil several basic performance criteria, including source-sensitivity and source-specificity (Wuertz et al. 2011). Considerable effort has been dedicated to sensitivity and specificity testing of genetic fecal marker qPCR assays during recent years, most frequently based on individual sampling strategies covering various sources of animal and human excreta or sewage (Ahmed et al. 2013, Boehm et al. 2013, Keity et al. 2012, Reischer et al. 2013, Reischer et al. 2011, Riedel et al. 2014, Shanks et al. 2009). Emphasis has also been put on sampling techniques, DNA extraction, and PCR quantification procedures (Cankar et al. 2006, Karlen et al. 2007, Shanks et al. 2012, Siefring et al. 2008, Stoeckel et al. 2009). However, information on the occurrence of genetic fecal markers in sewage regarding the characteristics of the disposal system (combined and separate sewer systems), its seasonal variability, and its relationship to standard and alternative fecal indicators is scarce (Srinivasan et al. 2011).

The aim of this study was to investigate the prevalence and abundance of human-associated genetic fecal markers by qPCR determination in raw and treated sewage of well-characterized municipal wastewater treatment plants over one year. Emphasis was put on municipal WWTPs with primary, secondary, and tertiary treatment, as such systems are of the highest importance for the Central European Region (CER). Small domestic WWTP (dWWTPs) were also included in our investigation, as they are frequently implemented in remote areas, where the connection to municipal sewer systems is not possible. Although advanced treatment was not the main focus of this study, the investigation of UV disinfection at one selected WWTP was included, as such treatment is becoming increasingly important. The TaqMan HF183 qPCR assay (Haugland et al. 2010) and the BacHUM UCD qPCR assay (Kildare et al. 2007) were selected for the determination of human-associated genetic fecal *Bacteroidetes* marker concentrations, following recommendations of recent evaluation studies (Boehm et al. 2013, Layton et al. 2013, Reischer et al. 2013). To support methodical cross-comparisons, cultivation-based SFIB using ISO standard methods and viral fecal markers for human-specific fecal pollution were simultaneously determined. Among these, JC Polyomavirus

(JCPyV) as well as human adenoviruses (HAdV), which have been used as human fecal viral indicators and highly specific MST tools (Bofill-Mas et al. 2000, Pina et al. 1998), and bacteriophages infecting *Bacteroides thetaiotaomicron,* which have been proposed as a human fecal indicator, were tested.

3.2 Materials and methods

3.2.1 Selection criteria and parameters to characterize the sewer disposal systems and WWTPs

The overall aim was the selection of municipal and domestic WWTPs that represented the situation of Austria and CER. The Danube Region and other parts of the CER are defined as sensitive areas with respect to water bodies. In terms of nutrients, strict discharge limits for WWTPs according to the EU urban wastewater directive have been established (EC 1991). Sewage disposal is caused by very small (i.e., a few inhabitants) up to large treatment systems (> 100,000 persons connected), as rural areas and large cities characterize this region. Activated sludge is the common process to treat the wastewater. In Austria, WWTP effluent concentrations are restricted by certain removal efficiency related to the influent load (%) and by a maximum effluent concentration (mg L^{-1}). These limits depend on the plant size (AEV 1996). For organic carbon (C) parameters COD/TOC/BOD_5 limits vary from 90/30/25 mg L^{-1} for plants with up to 500 population equivalents to 75/25/15 mg L^{-1} as daily averages for plants with more than 50,000 P.E. Nitrogen (N) removal requirements vary from nitrification without denitrification for WWTPs with < 5,000 P.E. to > 70% N removal as a yearly average for all WWTPs > 5,000 P.E. For phosphorus removal (P), no requirements exist for plants < 1,000 P.E., limits for WWTPs with 1,000 – 2,000 P.E. are < 2 mg L^{-1}, and < 1 mg L^{-1} is the yearly average for WWTPs with > 2,000 P.E. A general disinfection of WWTP effluents is not required (restricted to areas used for recreation or ground water protection only).

Only municipal WWTPs providing data for a basic characterization over the investigation period were selected. Essential information on WWTP design, including design capacity (P.E., population equivalent), actual average loading inhabitants connected, type of treatment (mechanical (M), carbon removal (C), nitrification (N), denitrification (D), phosphorus removal(P)), advanced treatment available (UV irradiation, membrane filtration), removal efficiency of nutrients (C, N and P), and sludge age, were required. To characterize the raw wastewater quantity and quality over the investigation period, volumetric flow rate (Q), chemical oxygen demand (COD) or biological oxygen demand (BOD), total nitrogen (TN), total phosphorus (TP), temperature (°C), and pH were requested. In addition to these

parameters, ammonium (NH_4), nitrate (NO_3), and total suspended solids (TSS) were included for the treated wastewater. All of these flow and chemical data were provided on a daily basis because they were available from representative sampling by automated and cooled sampling devices (24-hour proportional-flow sampling). All provided chemical information was cross-checked by own investigations.
In contrast to municipal WWTP, data availability for dWWTPs (< 150 P.E.) was very low. The basic requirements were information on the type of treatment system, the total number of persons connected, and the effluent concentrations of COD, NH_4 and pH. Only information based on grab sample analysis was available.

TABLE 4: Investigated municipal wastewater treatment plants

			WWTP					
			2	3	4	5	6	7
	design capacity [PE]		40,000	23,000	140,500	20,000	45,000	21,000
	Type		M, C, N, P	M, C, N, D, P	M, C, N, D, P	M, C, N, D, P	M, C, N, D, P	M, C, N, D, P
Influent	Q [m³ d^{-1}]	mean	7,086	3.217	18.681	1.549	3.901	2.246
		90%	8.887	4.646	30.888	2.464	5.648	4.399
	COD [mg L^{-1}]	mean	503	557	462	472	565	844
		90%	731	813	664	749	774	1181
	TN [mg L^{-1}]	mean	54	54	45	39	60	43
		90%	71	71	62	55	75	57
	TP [mg L^{-1}]	mean	10.2	8.9	4.1	5.4	8.8	8.4
		90%	10.7	13.2	7.4	8	11.5	11.7
Effluent	COD [mg L^{-1}]	mean	44	16	17	16	25	19
		90%	58	19	29	19	33	23
	TN [mg L^{-1}]	mean	26	12	9	3	14	7
		90%	32	22	12	4	19	16
	TP [mg L^{-1}]	mean	0.8	0.3	0.4	0.3	0.9	0.6
		90%	1.5	0.5	0.7	0.2	1.8	1.1
	SS [mg L^{-1}]	mean	17	12	7	5	4	-
		90%	33	26	11	7	5	-
	NH_4-N [mg L^{-1}]	mean	0.9	0.3	0.3	0.4	1.1	2.2
		90%	2.4	0.8	1	0.8	2.6	7.4
	NO_3-N [mg L^{-1}]	mean	21	5	4	1	10	4
		90%	28	10	6	2	15	8
	nutrient	COD	94	97	96	9	96	98

			WWTP					
			2	3	4	5	6	7
	design capacity [PE]		40,000	23,000	140,500	20,000	45,000	21,000
	Type		M, C, N, P	M, C, N, D, P	M, C, N, D, P	M, C, N, D, P	M, C, N, D, P	M, C, N, D, P
Effluent	removal efficiency [%]	TN	52	78	80	93	77	83
		TP	92	96	90	98	90	93
	Sludge age [d]		8-10	22-57	~13	~8	~17	20-25
	T [°C]	10%	9.5	-	9.3	8.2	10.5	6.8
		mean	14.9	-	14.4	14.8	15.5	12.2
		90%	20.5	-	20.6	21.3	21.2	17.4
	pH	10%	7.1	7.2	7.3	6.8	7.3	7.2
		90%	8.3	8.6	7.9	7.2	8.2	7,9

Abbreviations: WWTP: wastewater treatment plant; PE: population equivalent, M: primary treatment: mechanical treatment step; C: secondary treatment: biological carbon removal; N,D,P: tertiary treatment: nutrient removal including nitrification (N), denitrification (D) and phosphorous removal (P); Q: discharge water; COD: chemical oxygen demand; TP: total phosphorous; TN: total nitrogen; NO_3-N: nitrate nitrogen; NH_4-N: ammonium nitrogen; SS: suspended solids

TABLE 5: Investigated domestic wastewater treatment plants

			Domestic WWTP							
			8	9	10	11	12	13	14	15
	design capacity [PE]		130	20	6	20	74	8	6	100
	Type		Dr. Renner R-130/70	Dr. Renner A-20	Dr. Renner A-6	Dr. Renner A-20	Putox 25-27-76	Puratox 1-20-22-8	Putox	Puratox
Effluent	COD [mg L^{-1}]	mean	31	<15	16	56	29	<15	<15	41
		90%	-	-	-	-	-	-	-	-
	TP [mg L^{-1}]	mean	-	-	-	7.6	-	1.2	-	-
		90%	-	-	-	-	-	-	-	-
	NH_4	mean	0.8	0.2	0.3	1.7	0.7	1	0.6	-
		90%	-	-	-	-	-	-	-	-
	pH	mean	7.41	6.98	6.89	6.88	7.63	6.9	7.3	6.8
		90%	-	-	-	-	-	-	-	-

Abbreviations: WWTP: wastewater treatment plant; PE: population equivalent; COD: chemical oxygen demand; TP: total phosphorous; NH_4: ammonium nitrogen.

3.2.2 Sampling for chemical and microbiological analysis

24-h volume-proportional composite samples were recovered by fix installed and cooled (4°C) automated sampling devices from raw and treated municipal wastewater (Mayer et al. 2015). Samples were collected in sterilized 2 L glass bottles and immediately transferred to the laboratory at 5 ± 3°C for analysis after the automated sampling process. To cover seasonal variations, samples were taken in 4- to 6-week intervals over an annual cycle. Following a homogenization by manual shaking, 1 L of the sample volume of each was used for chemical and microbiological analysis.

Sampling of dWWTPs proved complicated, as the systems belonged to private persons. Grab sampling could be realized in the course of the annually performed control measures of the plants. Only effluent samples could be taken. Due to the small size of the systems, a constant influent flow did not exist for dWWTPs.

3.2.3 Chemical analysis

For COD, BOD_5, TP, TN, pH, SS, and conductivity analysis, the first preparation step included the homogenization of the sample. A pre-filtration step, applying a 0.45 µm membrane (sterilized cellulose-nitrate filter), was needed to analyse the dissolved parameters PO_4-P, NH_4-N, NO_2-N, NO_x-N. All selected parameters were performed according to standardized methods, as given in detail in table 6.

TABLE 6: Chemical analysis of waste water samples

Parameter	Method	Norm	Equipment
COD (short-time method)	Extraction with sulphuric acid lead– and silver sulphate–containing potassium dichromate solution and determination of surplus dichromate with ammonium iron (II) sulphate against Ferroin-indicator.	DIN 38409-43(DIN 1981)	
BOD_5 (biochemical oxygen demand, 5 days)	Determination with dissolution of the sample (inflow), difference of oxygen content at beginning and after 5 days (20°C)	DIN EN 1899-1(DIN 1998a)	LDO HQ 40 Lange (Hach Lange, Germany)
BOD_5 (biochemical oxygen demand, 5 days)	Determination without dissolution of the sample (effluent), difference of oxygen content at beginning and after 5 days (20°C)	DIN EN 1899-2(DIN 1998b)	
PO_4-P (orthophosphate)	Photometric determination with ammonium molybdate	DIN EN ISO 6878(ISO 2004)	SKALAR segment flow analyzer (AutoAnalyzer) (Skalar, Netherlands)
NH_4-N (ammonium nitrogen)	Photometric determination with ammonium nitrate by sodium dichloroisocyanurate and sodium salicylate	DIN 38406-5(DIN 1983)	

Parameter	Method	Norm	Equipment
NO_x-N (nitrogen oxide nitrogen)	Photometric determination, reduction to nitrite by cadmium	DIN EN ISO 13395(ISO 1996)	SKALAR segment flow analyzer (AutoAnalyzer) (Skalar, Netherlands)
NO_2-N (nitrite nitrogen)	Photometrical determination with sulphanilamide and N-(1-naphthyl)-ethylendiamine	DIN EN ISO 13395(ISO 1996)	
TP (total phosphorus)	Photometrical determination with ammonium molybdate after extraction with peroxodisulphate in microwave (MW)	DIN EN ISO 6878(ISO 2004)	Merck 500 microwave + SKALAR segment flow analyzer (AutoAnalyzer) (Skalar, Netherlands)
TN (Total Nitrogen)	Photometrical determination after extraction with peroxide sulphate in microwave (MW)	DIN EN ISO 11905-1(ISO 1997)	
pH	Potentiometric method	DIN EN ISO 10523(ISO 2008)	Radiometer table pH-meter (Radiometer analytical, France)
conductivity	Conductometric method	ISO 7888(ISO 1985)	WTW LF 323 (Hach Lange, Germany)
SS (suspended solids)	Gravimetric analysis (weight of the dried filter before and after filtration of a defined volume)	DIN 38409-2(DIN 1987)	Water-jet pump

3.2.4 Quantification of genetic fecal *Bacteroidetes* markers by qPCR

Genetic fecal markers were quantified based on total- and human-associated *Bacteroidetes* qPCR assays. For DNA extraction, polycarbonate membrane filtration (0.2 µm Millipore, Isopore Membrane Filter – GTTP, Cork, Ireland) based on phenol/chloroform extraction of 20 ml influent, 50 ml effluent and 1500 ml membrane-filtrated effluent, was used as previously described (Griffiths et al. 2000, Reischer et al. 2006). Cells were lysed with a FastPrepR-24 Instrument (MP Biomedicals Inc., Irvine, USA) with a speed setting of 6 m/s for 30 s. The extracted DNA was stored at -20°C. Respective 16S-rRNA-gene markers for AllBac (Layton et al. 2006), BacHUM-UCD (Kildare et al. 2007), and HF183 TaqMan (Haugland et al. 2010) were quantified by qPCR. The rotor-discs and 96-well plates were loaded with the mastermix and the sample by a Qiagility Roboter (Qiagen, Hilden, Germany). The measurements were subsequently performed on a Rotorgene Q Cycler (Qiagen, HILDEN, Germany). For the AllBac qPCR assay we used 2.5 µl of the respective DNA sample-dilution, 600 nmol L^{-1} primer AllBac296f, 600 nmol L^{-1} primer AllBac412r, 25 nmol L^{-1} TaqMan MGB probe AllBac375Bhqr

(Layton et al. 2006), 0.4 g L^{-1} bovine serum albumin (Roche Diagnostics, Mannheim, Germany), 7.5 µl of iQ Supermix (Bio-Rad, Hercules, USA) in a total reaction volume of 15 µl; additionally, 5 mmol L^{-1} $MgCl_2$ was added to obtain a total Mg^{2+} concentration of 8 mmol L^{-1} (Layton et al. 2006). For the BacHUM assay we used 2.5 µl of the respective DNA sample dilution, 400 nmol L^{-1} primer BacHUM-160f, 400 nmol L^{-1} primer BacHUM-241r, 80 nmol L^{-1} TaqMan MGB probe BacHUM-193p (Kildare et al. 2007), 0.4 g L^{-1} bovine serum albumin, and 7.5 µl of iQ Supermix in a total reaction volume of 15 µl. For the HF183 TaqMan assay, we used 2.5 µl of the respective DNA sample-dilution, 100 nmol L^{-1} primer HF183, 100 nmol L^{-1} primer BFDREV, 80 nmol L^{-1} TaqMan MGB probe BFDFAM (Haugland et al. 2010) 0.4 g L^{-1} bovine serum albumin, and 7.5 µl of iQ Supermix in a total reaction volume of 15 µl. The PCR program for the AllBac assay was 95°C for 3 min and 45 cycles of 95°C for 30 s and 60°C for 45 s; for the BacHUM assay, 95°C for 3 min and 45 cycles of 95°C for 15 s and 60°C for 1 min; for the HF183 TaqMan assay, 95°C for 3 min and 45 cycles of 95°C for 15 s and 60°C for 30 s. The real-time data were collected during the primer annealing step at 60°C. Quantification was based on appropriate plasmid standard dilutions and given as molecular equivalent targets per volume (ME vol^{-1}) as previously described (Reischer et al. 2006).

Each DNA sample was analysed in two dilution steps (10- and 100-fold dilution) with each dilution in duplicate to check for a possible PCR inhibition. No signs of PCR inhibition could be detected for any of the applied qPCR assays. Inhibition tests resulted in a very good correlation (R^2) amongst the concentrations as revealed for the different dilutions (Fig. 3). In addition, to monitor the filtration and extraction step of *Bacteroidetes* DNA targets from the sewage samples, a defined genetic target number cell standard (DeTaCs) was directly spiked into 50% of the collected wastewater samples as previously described (Kaiblinger 2008). The low variability of the concentrations obtained from the DeTaCs spikes by qPCR further proved the reliability of the filtration and extraction process. The multiplicative standard deviation for the lumped data set resulted in a value of 2.1.

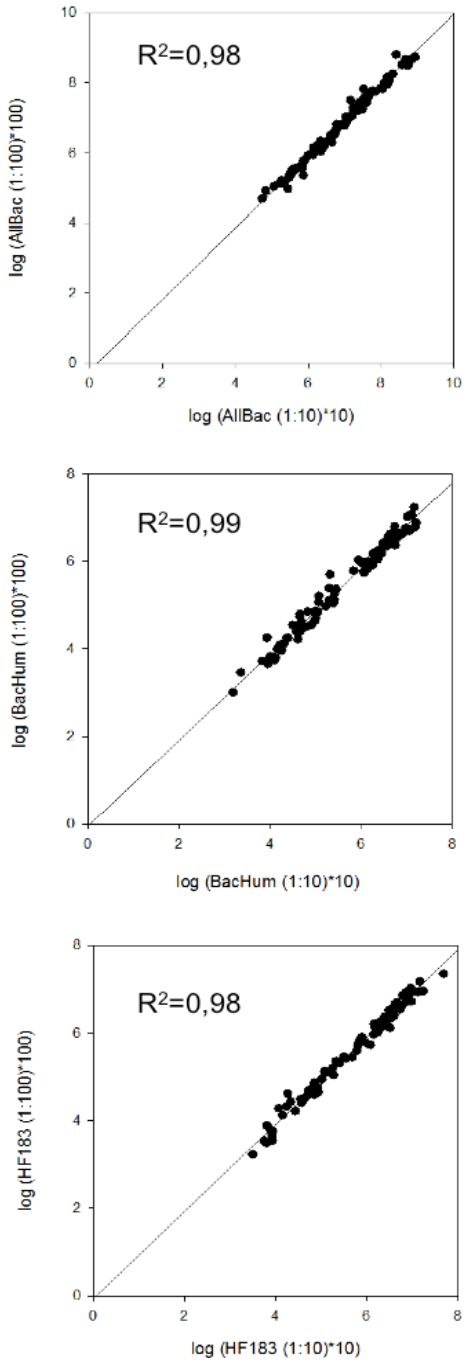

Figure 3: Inhibition control scatter plot of the data calculated out of dilutions.
Data shown are a pooled set. AllBac: genetic fecal marker for the total Bacteroidetes populations; BacHum, HF183: genetic fecal marker for human-associated Bacteroidetes populations.

3.2.5 Cultivation-based enumeration of standard fecal indicator bacteria

Cultivation-based enumeration of SFIB (i.e., *E. coli,* enterococci and *Clostridium perfringens* spores) was performed in the frame of our ISO 17025 accreditation. Before analysis, the samples were homogenized in an ultrasonic bath for 5 min. For membrane filtration, appropriate dilutions were performed (Farnleitner et al. 2010,

Vierheilig et al. 2013). Enumeration of presumptive *E. coli* was based on the ISO standard 16649-1 (ISO 2001a), using the chromogenic TBX agar (Oxoid, Thermo Fisher Scientific Inc., United Kingdom) and incubation at 44 ± 0.5°C for 24 ± 0.5 h. Enumeration of enterococci was based on the ISO standard 7899-2 (ISO 2000), using Slanetz–Bartley medium (Oxoid) and incubation at 37 ± 2°C for 44 ± 4 h. For quantification of *C. perfringens* spores, 5 ml influent and 15 ml effluent were pasteurized at 60 ± 2°C for 15 minutes. *C. perfringens* was analysed according to the established ISO method 14189 (ISO 2013), based on selective cultivation using TSC agar (Scharlau, Spain) at 44 ± 0.5°C for 21 ± 3 h and subsequent identification of colonies by acid phosphatase reaction (Ryzinska-Paier et al. 2011). For quality assurance, control strains *E. coli* NCTC 9001, *Enterococcus fecalis* NCTC 775 and *Clostridium perfringens* NCTC 8237 were used.

3.2.6 Quantification of human-specific viral fecal indicators by qPCR

To concentrate the desired viral DNA, 50 ml of influent and 500 ml of effluent were used. For membrane bioreactor 5000-10000 ml of effluent grab samples, were used for the skimmed milk flocculation process as established by Calgua (Calgua et al. 2013, Calgua et al. 2008). Viral concentrates were resuspended in 1 ml of phosphate buffer. A control spike (adenovirus type 35) was also added as a process control. Tap water was used as negative control of the process. Viral DNA was extracted from all samples using the QIAamp Viral RNA kit (Qiagen, Inc.). Nucleic acid eluates were sent at room temperature to the laboratory in Barcelona for quantification by qPCR. Specific real-time qPCR assays were used to quantify HAdV and JCPyV as previously described in detail (Bofill-Mas et al. 2006, Hernroth et al. 2002, Pal et al. 2006). Amplifications were performed in a 25-µl reaction mixture containing 10 µl of DNA and 15 µl of TaqMan Environmental Master Mix (Life Technologies). After activation of the AmpliTaq Gold for 10 min at 95ºC, 40 cycles (15s at 95°C and 1 min at 60°C) were performed in a Stratagene Mx3000P detection system. A ten-fold dilution of the DNA suspensions was run in duplicate (4 runs/sample) to analyse environmental samples, whereas each dilution of standard DNA suspensions from 10^0 to 10^7 (pBR322 plasmid containing HAdV 41 hexon or Mad1 JCPyV full genome) was run in triplicate. In all of the qPCRs carried out, the amount of DNA was defined as the mean of the data obtained. A non-template control (NTC) and a non-amplification control (NAC) were added to each run. The presence of enzymatic inhibitors within the samples was studied by adding known amounts of target DNA as an external control to environmental samples assayed.

3.2.7 Enumeration of human-associated bacteriophages infecting *Bacteroides thetaiotaomicron*

Bacteriophages infecting *Bacteroides thetaiotaomicron* were enumerated according to the standard method ISO 10705-4 (ISO 2001b) as described for *Bacteroides fragilis* RYC2056 or HSP40 infecting phages. The host strain applied for the phage analyses was *Bacteroides thetaiotaomicron* (GA17), kindly provided by Prof. Maite Muniesa, University of Barcelona, Spain. To reduce the high concentration of background flora, samples were filtered through a low protein-binding membrane (0,2 µm; Minisart 16534, Sartorius). For enumeration of the phages, 1 ml of the host strain (inoculum culture) was added to tubes containing 2.5 ml of semisolid agar and aliquots of the samples to be tested, gently mixed, and poured onto solid agar plates. The plates were incubated under anaerobic conditions (AnaeroGen AN0025A, Oxoid) at 36 ± 2°C for 21 ± 3 h. The results are expressed as the number of plaque-forming units (pfu) per sample volume.

3.2.8 Data analysis and statistics

All microbial data are expressed as $\log_{10}(x+1)$, after having performed all the needed calculations on the untransformed data. Reductions were calculated as $\log_{10}$ (effluent) minus $\log_{10}$ (influent). Microbial loads were calculated as numbers per inhabitant and day. To achieve this, the respective microbiological concentrations were multiplied by the amount of discharge and divided by the number of connected people. Visual and statistical data were analysed with Visplore 2.0 (Piringer et al. 2010) (VRVis GmbH, Austria, Vienna) and Sigma Plot 11.0 (SPSS Inc., Chicago, USA). To account for multiple testing, statistical significance levels were corrected according to Bonferroni (Bonferroni 1936). All graphs in this chapter were prepared using Sigma Plot 13.0, Visplore 2.0 and CorelDraw X5 (Corel, Canada). To support correct comparisons of the variability of log-normal distributed variables, the multiplicative standard deviation s* was calculated for the recovered results according to Limpert et al 2001 (Limpert et al. 2001). The multiplicative standard deviations s* ranged from 1.5 to 6.4 and from 1.5 to 1.7 for microbiological and chemical parameters, respectively.

3.3 Results

3.3.1 Characteristics of selected municipal WWTPs and chemical sewage quality

Five municipal activated sludge WWTPs (WWTP2-6) in the metropolitan area of Vienna, Austria, with design capacities ranging from 20,000 to 140,000 P.E. and

actual average loadings of 6,600 to 78,400 P.E. were selected. The number of connected inhabitants ranged from approx. 2,000 up to approx. 31,000 (Table 4). The catchments could be described as a mix of rural and urbanized areas. Sewers were constructed as combined systems, with a pressure pipe as inflowing sewer in case of WWTP4. Industrial influence in the catchment was evaluated as low to moderate. A potential impact on raw sewage quality due to seasonal events relating to crop harvest and processing (i.e., wine production in the catchments of WWTPs 2, 4, 5, 6) and tourism (i.e., summer tourism in the catchment of WWTP5) could not be excluded. The average discharge at the influent varied from 1,600 m³ per day at WWTP5 to 18,700 m³ per day at WWTP4 (Table 4).

All WWTPs were using an activated sludge process with mechanical treatment and carbon removal. WWTPs 3-6 performed also nitrification and denitrification, whereas WWTP2 was overloaded and not designed for denitrification, resulting in significant lower nitrogen removal rates compared to other WWTPs (Table 4). The average sludge age ranged from 8 to 57 days (Table 4). P removal was achieved at all WWTPs by chemical precipitation with iron salts and/or aluminium salts, which is required for sensitive areas in the European Union (EC 1991) WWTP5 was also equipped with additional UV-disinfection (48 Trojan Technologies 302418 UV Lightspots, UV Transmission UVT 10 mm: 65%, max discharge: 135 m^3 h^{-1}) at the effluent, which was operated only during summer. One additional activated sludge plant (WWTP7) was selected in the rural area of Bavaria, Germany. WWTP7 is a membrane bioreactor with a system of ultra-filtration membranes submerged within the aeration tank. The three vacuum rotation membrane units, each having a membrane surface of 2,264 m^2 and a pore size of approximately 38 nm, are used to separate the activated sludge flocks from the treated wastewater by means of a pressure difference. (physical solid-liquid separation process, Table 4).

In raw wastewater of WWTPs 2-7, average COD, TN, and TP yielded values from 460 to 850 mg L^{-1}, from 40 to 60 mg L^{-1}, and from 4 to 10 mg L^{-1}, respectively (Table 4). Observed elimination rates were 94-98%, 77-93% (except WWTP2 with ~50%), and 90-96%, respectively, for COD, TN, and TP (Table 4). The average water temperature at the sewage effluents was 12.2°C to 15.5°C (further details in Table 4).

3.3.2 Characteristics of selected domestic WWTPs and chemical sewage quality

Eight small dWWTPs, numbers 8-15, with a design capacity of 6 to 130 P.E., were selected in the metropolitan area of Vienna, Austria (Table 5). Two types of dWWTPs were discovered, including Dr. Renner® technology (also known as Gallé wastewater technique®) and Putox® technology (also known as Purator®). Two dWWTPs were linked to little taverns in the mountainous area of Vienna. Up to 50 persons were contributing their excreta to these sewer systems. Fecal load were

strongly fluctuating. One dWWTP was localized at a horse barn with approximately 25 persons as permanent fecal sources. The rest of the dWWTPs was connected to individual households, with up to 5 contributing persons. Determined COD in the treated sewage of dWWTPs 8-14 ranged from 15 to 56 mg L^{-1}; the settle-able solids were generally less than 0.1 mg L^{-1}. Ammonium and pH yielded values of 0.2-1.7 mg L^{-1} and 6.9-7.6, respectively, in the treated sewage (see Table 5 for more details).

3.3.3 Does sewage from different municipal WWTPs show differences in genetic fecal *Bacteroidetes* markers and SFIB concentrations?

One of the aims was to evaluate whether human-associated genetic fecal *Bacteroidetes* markers and SFIB concentrations in raw and treated sewage show significant differences with regard to the investigated municipal disposal systems or background conditions. The genetic fecal *Bacteroidetes* markers BacHUM-UCD, HF183 TaqMan and AllBac as well as *E. coli*, enterococci and *C. perfringens* spores were considered as test parameters (Table 7). Statistical comparisons were performed between all the individually investigated municipal WWTPs, covering all WWTP2 to WWTP6 combinations (type 1 comparisons). Comparisons were also performed for the concentrations of microorganisms in sewage from cool vs. warm seasons for the lumped results from WWTP 2-6 (type 2 comparisons). The results of type 1 and type 2 comparisons revealed no significant differences (Mann-Whitney Rank Sum Test, $p < 0.05$, Bonferroni-corrected). Hence, the results from WWTP2 to WWTP6 were pooled for further analysis. The results from WWTP5 (UV-disinfection) are shown separately in section 3.3.6.

TABLE 7: Logarithmic concentration of genetic microbial source tracking markers in raw (influent) und treated (effluent) sewage of WWTP 2-.

	AllBac						BacHum UCD						HF183					
	Influent			Effluent			Influent			Effluent			Influent			Effluent		
	n	Median	min/max	n	Median	min/max	n	Median	min/max	n	Median	min/max	n	Median	min/max	n	Median	min/max
WWTP2	8	9,7	9.3/10.7	9	7,8	6.5/9.5	8	8,8	8.4/9.2	7	6,5	4.8/7.9	6	8,8	8.5/9.7	6	6,3	5.1/6.7
WWTP3	10	9,8	9.1/10.9	10	7,9	6.7/8.6	10	8,7	8.3/9.2	10	6,4	5.8/7.3	9	8,7	8.2/9.2	8	6,2	6.0/6.8
WWTP4	9	9,6	9.0/10.9	9	7,6	6.9/8.8	9	8,3	8.0/8.8	9	6,2	5.6/7.3	8	8,2	7.5/8.6	7	6,1	5.4/7.6
WWTP5	8	9,4	8.7/10.7	9	7,7	7.2/8.4	9	8,3	8.1/9.2	9	6,3	6.0/6.7	7	8,3	7.8/9.2	7	6,5	5.5/7.1
WWTP6	10	9,6	9.3/10.8	10	8	6.3/9.1	9	8,6	8.2/9.0	10	7	5.6/7.7	9	8,7	7.8/9.0	9	6,7	5.5/7.3
WWTP2-6	45	9,6	8.7/10.9	47	7,8	6.3/9.5	45	8,6	8.0/9.2	45	6,5	4.8/7.9	39	8,6	7.5/9.7	37	6,4	5.1/7.6
t< 15°C	22	9,5	8.7/10.7	24	7,6	6.8/9.5	23	8,7	8.0/9.2	22	6,6	5.8/7.9	21	8,5	7.5/9.2	20	6,5	5.9/7.3
t> 15°C	23	9,8	9.1/10.9	23	8	6.3/9.1	22	8,4	8.0/9.2	23	6,2	4.8/7.3	18	8,6	7.7/9.7	17	6	5.1/7.6
WWTP2-6 strati	-	-	-	16	7,7	6.5/9.5	-	-	-	16	6,4	5.1/7.3	-	-	-	16	6,5	5.1/7.0
WWTP 8-14	-	-	-	16	8,1	7.2/10.6	-	-	-	16	6,3	4.9/8.9	-	-	-	16	6,2	5.1/8.9

Abbreviations: WWTP2-6 strati: is a stratified sample set to compare domestic WWTP 8-14 with municipal WWTP2-6 treated wastewater. AllBac: genetic fecal marker for the total *Bacteroidetes* populations; BacHum, HF183: genetic fecal marker for human-associated *Bacteroidetes* populations.

3.3.4 Occurrence of microbial indicators in raw and biological treated sewage from municipal systems

Prevalence and abundance of genetic fecal *Bacteroidetes* markers and comparison with SFIB and human viral fecal markers

All investigated genetic fecal *Bacteroidetes* markers showed 100% occurrence in raw and biologically treated sewage with primary, secondary and tertiary treatments. The human-associated fecal marker BacHUM UCD and HF183 TaqMan revealed remarkably similar concentrations, with medians of $\log_{10}$ 8.6, $\log_{10}$ 8.6 (raw) and $\log_{10}$ 6.5, $\log_{10}$ 6.4 (treated) ME per 100 ml wastewater, respectively (Fig. 4). The AllBac maker showed concentrations one order of magnitude higher, with medians of $\log_{10}$ 9.6 (raw) and $\log_{10}$ 7.8 (treated) ME per 100 ml sewage.

SFIB also proved 100% prevalent in the investigated raw and treated sewage, but with concentrations 2 to 3 orders of magnitude lower compared to genetic fecal *Bacteroidetes* markers. The median concentrations for *E. coli,* enterococci, and *C. perfringens* spores were $\log_{10}$ 6.6, $\log_{10}$ 6.1, $\log_{10}$ 4.8 (raw) and $\log_{10}$ 4.2, $\log_{10}$ 3.8, and $\log_{10}$ 3.4 (treated) CFU per 100 ml of sewage, respectively (Fig. 4). The analysed bacteriophages and human viruses could not be detected in all samples. The prevalence of the human fecal-associated bacteriophage BtioPh was 97% in raw and treated sewage. Prevalence rates for the human viruses HAdV and JCPyV were 92%, 98% (raw) and 85%, 60% (treated). Median concentrations for BtioPh, HAdV, and JCPyV revealed $\log_{10}$ 4.5, $\log_{10}$ 3.9, $\log_{10}$ 4.2 (raw) and $\log_{10}$ 2.5, $\log_{10}$ 2.5, and $\log_{10}$ 2.1 (treated) PFU or GC per 100 ml of sewage (Fig. 4).

Variability of genetic fecal *Bacteroidetes* markers concentrations in sewage and comparisons with SFIB and human viral markers

In raw sewage, the s* were remarkably low for the genetic marker concentrations of the BacHUM-UCD and HF183 TaqMan assay and were comparable with the variability of *E. coli* and enterococci, ranging from s* = 2.3 to s* = 3.0 (Fig. 4). *C. perfringens* spores proved to be the most constantly occurring indicator in raw sewage (s* = 1.5).

A general increase in the variability of indicator concentrations between influent and effluent samples, irrespective of the considered parameter and WWTP, was obvious ($p<0.05$, n = 9, Kruskal-Wallis). The multiplicative standard deviation s* increased by an average factor of 1.5 (range 0.9 – 2.0) during wastewater treatment (Fig. 4). In treated sewage the variability of genetic fecal *Bacteroidetes* markers concentrations matched the variability of SFIB concentrations as well (s* = 3.6 to 5.5).

The concentration variability of viral markers was higher, ranging from s* = 3.8 to 6.4 in raw sewage and s* = 4.4 to 6.4 in treated sewage. The investigated chemical

parameters showed a statistical variability in the range of *C. perfringens* spores in raw sewage (s* = 1.5, 1.8, 1.6 and 1.7 for COD, BOD, TN and TP, respectively). In treated sewage the chemical parameters revealed lower variability compared to the microbiological parameter (s* = 1.6, 2.4, 3.1, 2.4 for COD, BOD, TN, and TP, respectively).

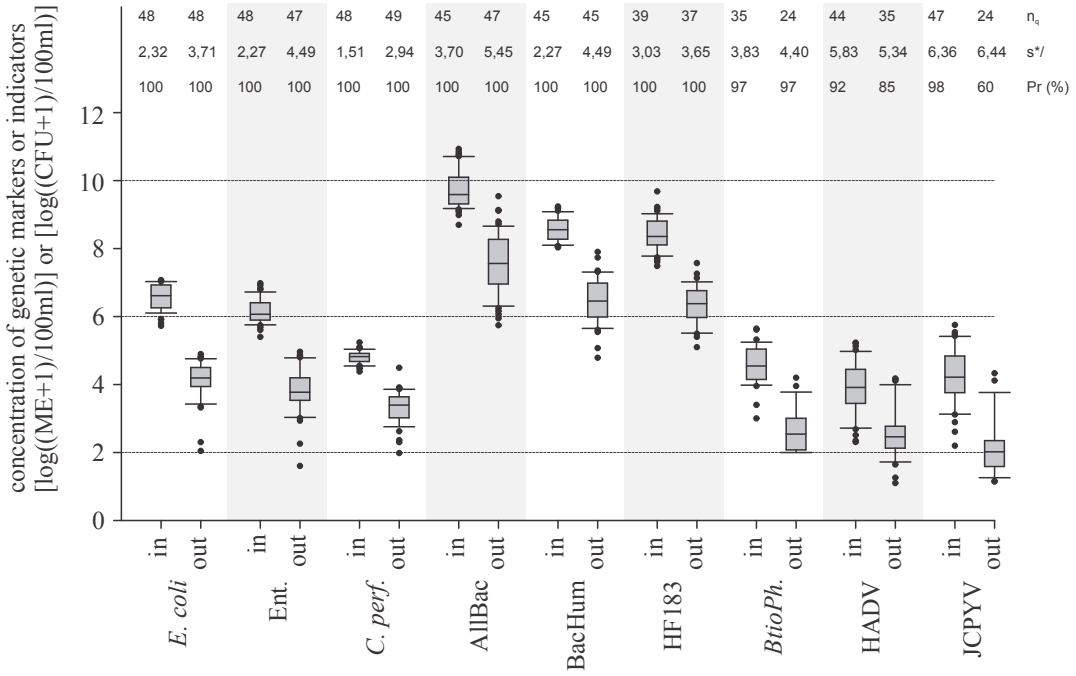

Figure 4: Concentration of standard fecal indicators and genetic microbial source tracking markers in raw (in) and treated (out) sewage in lumped data from WWTP 2-6.

Data shown are a pooled set. AllBac: genetic fecal marker for the total Bacteroidetes populations; BacHum, HF183: genetic fecal marker for human-associated Bacteroidetes populations; C. perf: Clostridium perfringens spores, Ent: enterococci, BtioPh: bacteriophages infecting Bacteroides thetaiotaomicron, HAdV: human adenovirus, JCPyV: JC Polyomavirus, nq: number of quantifiable samples, s*: estimated multiplicative standard deviation, PR (%): Prevalence of investigated markers. Boxes cover the 25th to 75th percentile, whiskers the 10th to 90th percentile

Establishing genetic fecal *Bacteroidetes* markers loads per connected person and day

Medians for the calculated fecal marker loads AllBac, BacHUM-UCD, and HF183 TaqMan resulted in $\log_{10}$ 13.2, $\log_{10}$ 12.2, and $\log_{10}$ 12.2 (raw sewage) and $\log_{10}$ 11.5, $\log_{10}$10.1, $\log_{10}$ 9.9 (treated sewage) ME per connected persons and day (Fig. 5). Quantitative relationships and statistical variability between genetic fecal *Bacteroidetes* markers, SFIB and human viral fecal markers loads were similar to the obtained relationships regarding the concentrations (Fig. 5).

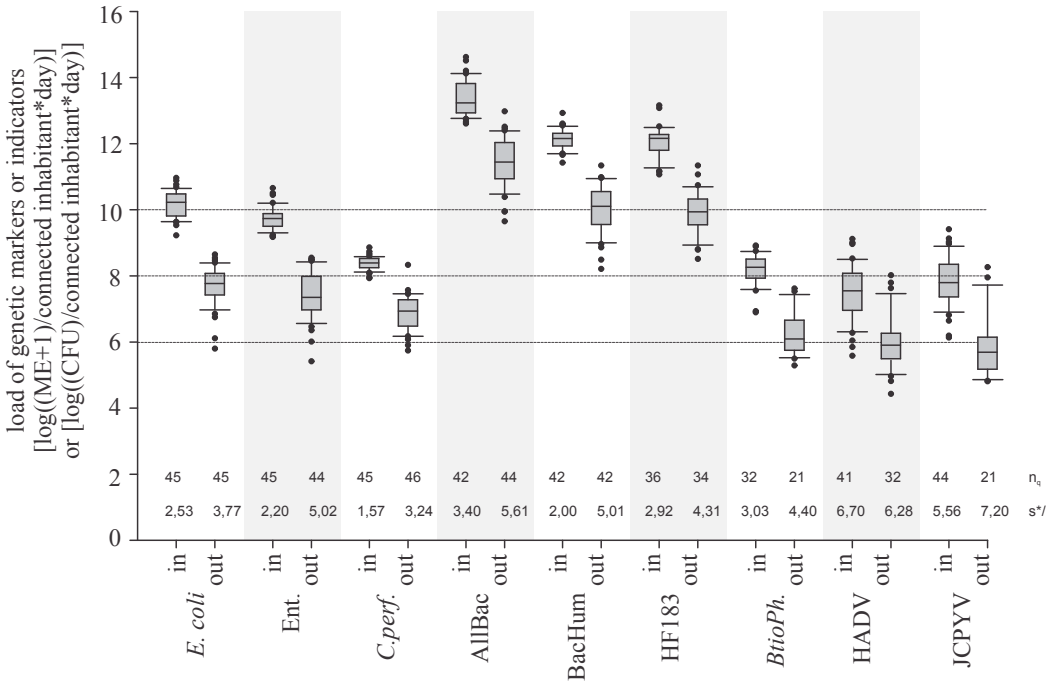

Figure 5: Load of standard fecal indicators and genetic microbial source tracking markers in raw (in) and treated (out) sewage of WWTP 2-6.

Data shown are a pooled set. AllBac: genetic fecal marker for the total Bacteroidetes populations; BacHum, HF183: genetic fecal marker for human-associated Bacteroidetes populations; C. perf: Clostridium perfringens spores, Ent: enterococci, HAdV: human adenovirus, JCPyV: JC Polyomavirus, nq: number of quantifiable samples, s*/: estimated multiplicative standard deviation. Boxes cover the 25th to 75th percentile, whiskers the 10th to 90th percentile.

Achieved microbiological reductions by sewage treatment

C. perfringens revealed significantly lower reductions compared with the other SFIB and genetic fecal *Bacteroidetes* markers. HAdV achieved lower reductions compared with *E. coli* (Kruskall Wallis, $p<0.001$) during wastewater treatment (including primary, secondary and tertiary treatment in WWTPs 2-6). Some basic trends were obvious. *E. coli* achieved the highest reduction and showed a 10th-percentile value of $-\log_{10}1.6$. Genetic fecal *Bacteroidetes* markers, enterococci, and bacteriophage BtioPh revealed very similar 10th-percentile values that ranged from $-\log_{10}1.0$ to $-\log_{10}1.2$ (Fig. 6). The lowest 10th-percentile reduction values were achieved by the human-specific viral fecal indicator HAdV and the bacterial fecal indicator *C. perfringens* spores, at $-\log_{10}0.5$ and $-\log_{10}0.9$ reductions, respectively (Fig. 6).

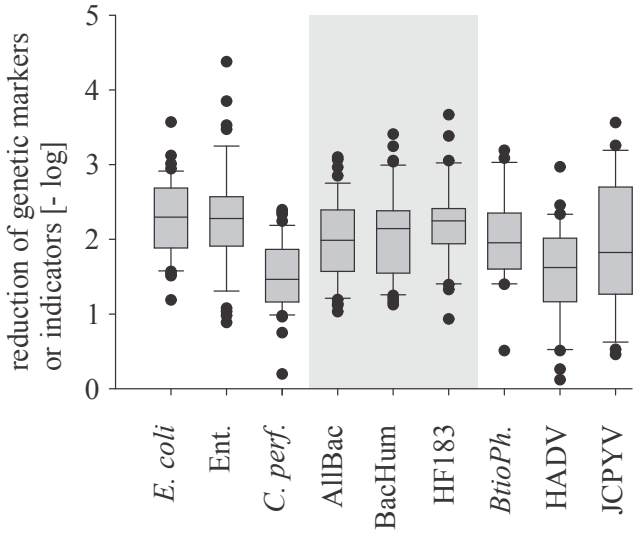

Figure 6: Reduction of standard fecal indicators and genetic microbial source tracking markers in municipal WWTPs 2-6 during wastewater treatment (lumped data).
AllBac: genetic fecal marker for the total Bacteroidetes populations; BacHum, HF183: genetic fecal marker for human-associated Bacteroidetes populations; Ent: enterococci; C. perf: Clostridium perfringens spores,; BtioPh: bacteriophages infecting Bacteroides thetaiotaomicron, HAdV: human adenovirus, JCPyV: JC Polyomavirus. Boxes cover the 25th to 75th percentile, whiskers the 10th to 90th percentile

The reduction of genetic fecal *Bacteroidetes* markers at the activated sludge membrane bioreactor (WWTP7) revealed a median 2.8 to 3.6 orders of magnitude increase in treatment efficacy of genetic fecal *Bacteroidetes* markers compared to the conventional activated sludge treatment plants WWTPs 2-6 (Fig. 7).

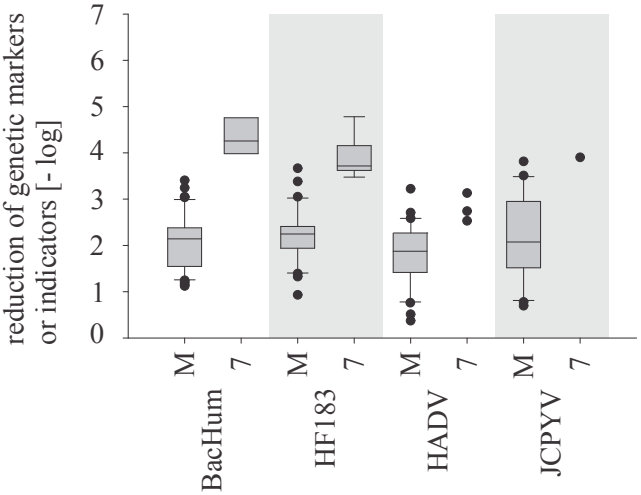

Figure 7: Reduction of standard fecal indicators and genetic microbial source tracking markers in municipal (M) WWTP 2-6 and membrane reactor (7) WWTP 7 during treatment.
BacHum, HF183: genetic fecal marker for human-associated Bacteroidetes populations; HAdV: human adenovirus, JCPyV: JC Polyomavirus. Boxes cover the 25th to 75th percentile, whiskers the 10th to 90th percentile.

Elucidating the relationships amongst genetic fecal *Bacteroidetes* markers and other microbial/chemical variables

Except for total suspended solids (TSS), statistical analysis of the pooled data set from WWTPs 2-6, including information from raw and treated sewage, resulted in significant relationships amongst all parameters (correlation coefficients rho = 0.73-0.95, $p<0.0045$; Table 8). Due to the inhomogeneous distribution of the raw vs. the treated sewage data, such correlation analysis with pooled data led to a statistically biased relationship. Separate analysis was thus performed for the raw and treated sewage data. Viral data were not included in this correlation analysis because the replicate number was considered too low.

Correlation analysis for the raw sewage data from catchments WWTPs 2-6 indicated a tight relationship between the human-associated fecal marker BacHUM-UCD and the HF183 TaqMan (rho = 0.80, $p<0.0045$). In sharp contrast, the AllBac marker did not show a discernible relationship with the human-associated genetic fecal *Bacteroidetes* markers or SFIB (Table 8). Interesting but non-significant correlation coefficients were obtained amongst the SFIB (rho = 0.39, $p > 0.0045$). Remarkably, a relationship between the human-associated genetic fecal *Bacteroidetes* markers and the SFIB with the biological oxygen demand, the nitrogen content, and the phosphorus content in raw sewage became evident. This was indicated by a range of significant correlations, including BacHUM-UCD, *E. coli* and enterococci with one or several components of the chemical parameters (rho = 0.46 - 0.51, $p< 0.0045$). Amongst the microbiological parameters, *C. perfringens* spores had the most pronounced relationship with the chemical quality characteristics of raw sewage (rho = 0.49 - 0.65, $p< 0.0045$). Except for TSS, a general interrelationship between all investigated chemical variables was obvious (rho = 0.49 – 0.76, $p < 0.0045$).

A contrasting situation could be found for the data set of the treated sewage. Except for *C. perfringens* spores, a general increase in correlations amongst the microbiological variables during the treatment process was observed (Holm Sidak, $p<0.005$, Table 8). Unlike for the raw sewage, a correlation between the genetic AllBac and the human-associated BacHUM-UCD marker became discernible after the treatment (rho = 0.62, $p < 0.0045$). In contrast, relationships between the human-associated genetic fecal *Bacteroidetes* markers and SFIB with the chemical quality characteristics of the sewage disappeared during the treatment process (Holm Sidak, $p<0.001$). Again, the only exception was *C. perfringens* spores, showing significant relationships with the COD and TSS (Table 8).

TABLE 8: Correlation analysis table showing the Spearman rank coefficient for the pooled influent and effluent (in & out), the influent (in) and the effluent (out) data of investigated WWTP 2-6.

* = significant Bonferroni P=0,0045

AllBac		BacHum		HF183		*e. coli*		Ent		C.P.		COD		TN		NH4		TP		TSS	
									in & out												
									in	out											
0.85*																					
0,25	0.62*																				
0.81*		0.95*																			
0,19	0,45	0.80*	0.84*																		
0.77*		0,83		0.78*																	
0,11	0.42*	0,06	0.59*	0,08	0.58*																
0.76*		0.83*		0.78*		0.86*															
0,24	0,17	0,14	0.49*	0,04	0,46	0,39	0.52*														
0.73*		0.82*		0.77*		0.80*		0.82*													
0,26	-0,06	0.41*	0,15	0,22	0,18	0,39	-0,01	0,39	0,22												
0.75*		0.81*		0.76*		0.80*		0.82*		0.89*											
0,29	0,03	0,36	0,17	0,19	0,23	0.43*	-0,03	0,39	0,13	0.65*	0.47*										
0.60*		0.71*		0.67*		0.63*		0.65*		0.77*		0.80*									
0,27	0,01	0.51*	0,14	0,47	0,01	0,38	0,07	0.46*	0,08	0.62*	0,36	0.62*	0.66*								
0.74*		0.83*		0.79*		0.77*		0.82*		0.81*		0.85*		0.71*							
0,29	-0,04	0.48*	0,22	0,32	0,21	0,01	0,15	0,43	0,2	0.60*	-0,04	0.49*	0,32	0.73*	0,37						
0.73*		0.80*		0.77*		0.78*		0.78*		0.84*		0.90*		0.82*		0.87*					
0,1	0,04	0,44	0,03	0,37	0,04	0,28	0,04	0,24	0,07	0.49*	0,28	0.58*	0.59*	0.76*	0.83*	0.61*	0,42				
0,06		0,23		0,24		-0,03		0,2		0.52*		0,34		0,22		-0,03		0,13			
n.a	0,06	n.a	0,23	n.a	0,24	n.a	-0,03	n.a	0,2	n.a	0.52*	n.a	0,34	n.a	0,22	n.a	-0,03	n.a	0,13		

AllBac: genetic fecal marker for the total *Bacteroidetes* populations; BacHum, HF183: genetic fecal marker for human-associated *Bacteroidetes* populations; C. perf: *Clostridium perfringens* spores, Ent: Enterococci; COD: chemical oxygen demand; TN: total nitrogen; NH_4-N: ammonium nitrogen; TP: total phosphorus; TSS: total suspended solids.

3.3.5 Occurrence of genetic fecal *Bacteroidetes* markers and SFIB in treated sewage of small domestic WWTPs and comparison to municipal WWTPs

Occurrence of genetic fecal *Bacteroidetes* markers, SFIB and human viral fecal markers in treated sewage of dWWTPs.

Genetic fecal *Bacteroidetes* markers in treated sewage from dWWTPs showed 100% prevalence. The human-associated fecal marker BacHUM UCD and HF183 TaqMan revealed similar concentrations, with medians of $\log_{10}$ 6.3 and $\log_{10}$ 6.2 ME per 100 ml sewage (Fig. 8). The AllBac maker showed concentrations two orders of magnitude higher, with a median of $\log_{10}$ 8.0 ME per 100 ml sewage. FIB markers in treated domestic sewage also resulted in 100% prevalence. The medians for *E. coli*, enterococci, and *C. perfringens* spores were $\log_{10}$ 3.9, $\log_{10}$ 3.6 and $\log_{10}$ 3.9 CFU per 100 ml treated sewage (Fig. 8). JCPyV were detected in 3 of 6 dWWTPs ($\log_{10}$ 2.0-$\log_{10}$ 3.0 ME per 100 ml of sewage) evaluated that treated wastewater from 6-130 P.E.

Comparing concentrations of genetic fecal *Bacteroidetes* markers and SFIB in treated sewage from small domestic and municipal WWTPs

Genetic fecal *Bacteroidetes* markers and SFIB in treated sewage had very similar concentrations for both the small domestic and the municipal WWTPs (Fig. 8). No differences in concentration could be detected (Man Whitney, $p<0.008$, n from WWTPs randomly adjusted to number of dWWTPs).

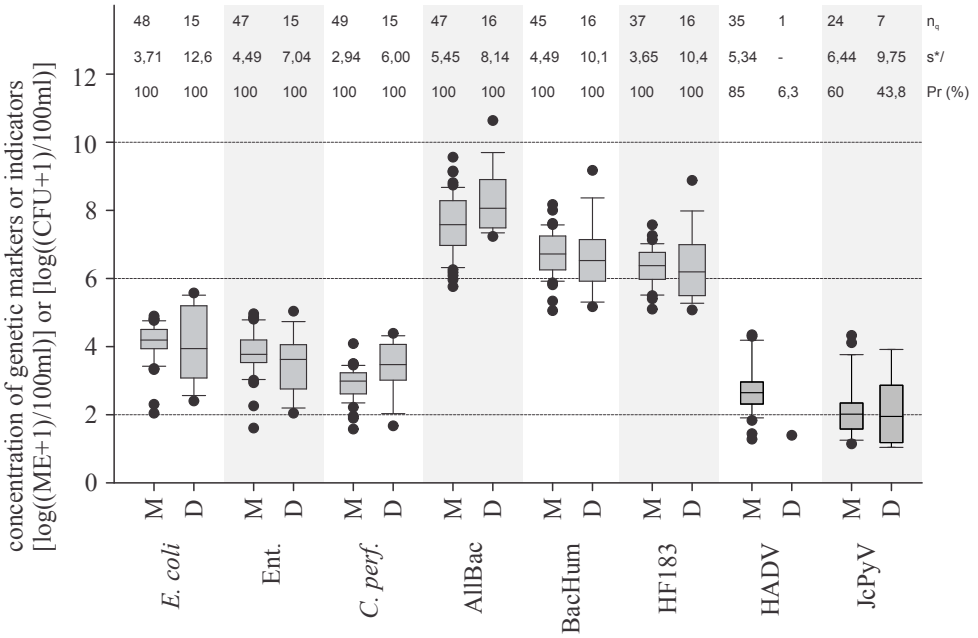

Figure 8: Concentration of standard fecal indicators and genetic microbial source tracking markers in lumped data of municipal (M) WWTPs 2-6 versus lumped data from domestic (D) dWWTPs 8-14 effluents.

AllBac: genetic fecal marker for the total Bacteroidetes populations; BacHum, HF183: genetic fecal marker for human-associated Bacteroidetes populations; C. perf: Clostridium perfringens spores, Ent: Enterococci, nq: number of quantifiable samples, s*: estimated multiplicative standard deviation, PR (%): Prevalence of investigated markers. Boxes cover the 25th to 75th percentile, whiskers the 10th to 90th percentile.

3.3.6 Observed reductions due to UV irradiation (254 nm) (WWTP5)

To evaluate the effect of UV irradiation on genetic fecal *Bacteroidetes* markers and other microbiological indicators, 10 additional pairs of samples (before and after the UV system) were taken at the WWTP5 throughout the season (low pressure UV system, 48 lamps, UV 200, Trojan, Canada). After UV irradiation, the observed reductions, given as 5th-percentile values (i.e., only 5% of the values showed a lower reduction), were $\log_{10}$ 3.4, $\log_{10}$ 3.0, and log 2.7 for enterococci, *E. coli*, and somatic coliphages, respectively (Fig 9). *C. perfringens* spores were only slightly inactivated, resulting in a 5th-percentile reduction of $\log_{10}$ 0.69. In contrast, no statistically significant reduction of genetic fecal *Bacteroidetes* markers was detectable (one-way ANOVA, $p<0.05$).

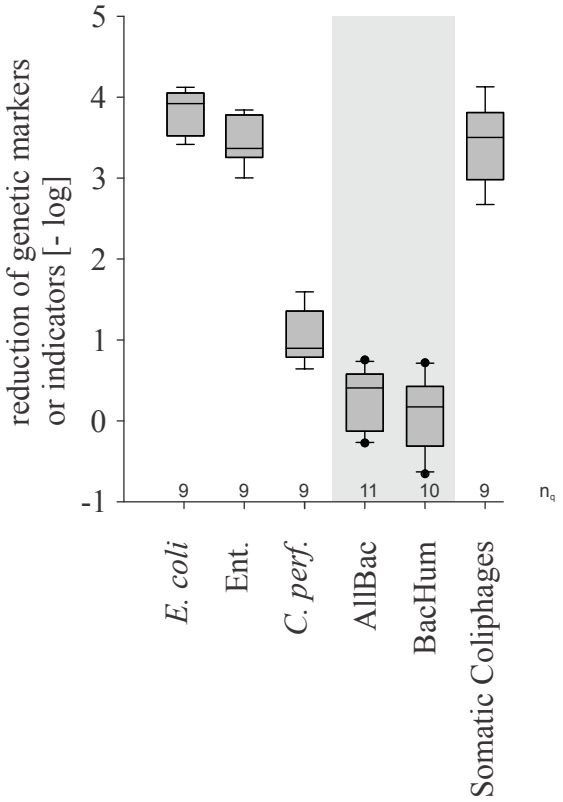

Figure 9: Reduction of standard fecal indicators and genetic microbial source tracking markers in WWTP 6 during treatment and UV disinfection.
AllBac: genetic fecal marker for the total Bacteroidetes populations; BacHum: genetic fecal marker for human-associated Bacteroidetes populations. Boxes cover the 25th to 75th percentile, whiskers the 10th to 90th percentile

3.4 Discussion

Human-associated genetic fecal *Bacteroidetes* markers were consistently detected in high concentrations in the investigated samples from raw and biological treated sewage. The size of the studied wastewater systems varied over 4 orders of magnitude, with populations ranging from as few as 3 individuals up to 49,000 inhabitants connected. Statistical analysis also demonstrated that genetic fecal *Bacteroidetes* markers concentrations did not reveal differences regarding the type of the sewage system or the time of the season investigated. Our results thus provide strong empirical evidence of the ubiquitous and abundant occurrence of genetic fecal *Bacteroidetes* markers in raw and biological treated sewage, regardless whether the sewage is derived from single households, larger settlements, or towns. Information on the quantitative occurrence and dynamics of genetic fecal *Bacteroidetes* markers in sewage alongside the wastewater and sanitation pathway has been limiting so far. The few studies available have focussed

on individual samples or single systems (Ervin et al. 2013, Silkie and Nelson 2009, Srinivasan et al. 2011, Stapleton et al. 2009). To our knowledge, our results provide the first comprehensive information on the occurrence and dynamics of genetic fecal *Bacteroidetes* markers in raw and biologically treated sewage from several well-characterized wastewater systems and treatment plants.

The selected systems were predominately influenced by wastewater from households. No signs of significant influence from agriculture or industry could be found, and chemical analysis did not show any deviations from quality characteristics as expected for raw sewage of municipal or domestic origin (Gujer 2002). Data on the discharge dynamics also indicated that large rain events did not happen during the seasonal sampling campaigns (Table 4). A relevant influence on the sewage quality due to surface runoff in the catchment area, potentially leading to strong dilution effects or to the input of fecal material from non-human sources, was thus not expected.

This study further supports the fact that fecal pollution based on genetic fecal *Bacteroidetes* markers qPCR quantification can be performed with at least equal precision compared with traditional ISO-based cultivation techniques (Stapleton et al. 2009). The determined concentrations of genetic fecal *Bacteroidetes* markers and SFIB (*E. coli* and enterococci) indicated equal statistical variability in raw and treated sewage. This finding is of special interest regarding the current evaluation of genetic fecal *Bacteroidetes* markers as a potentially new means to complement routine water quality testing (Betancourt and Fujioka 2006, McQuaig et al. 2012, Molina et al. 2014). The statistical variability of genetic fecal *Bacteroidetes* markers and SFIB concentrations was lowest for raw sewage and, interestingly, increased during biological wastewater treatment. Raw sewage apparently underlies sufficient mixing in the sewer channels, to balance potential differences of input concentrations from the connected households (Limpert et al. 2001). Daily variations were accounted for by volume-proportional 24-h automated sampling. Interestingly, C*. perfringens* showed a statistical variability in the range of the measured chemical parameter, which was far lower than the rest of the microbiological parameters. A very low variability of *C. perfringens* in water has been reported previously (Byamukama et al. 2005).

It has to be mentioned that the statistical comparison of variability was supported by the implementation of two methodical innovations. To obtain representative samples from the influent and effluent of WWTPs, an automated 24-h volume-proportional and cooled sample was taken. In contrast to this study, most studies dealing with microbiological investigations rely on randomly chosen grab samples. To obtain an appropriate measure of statistical variability from log-normal distributed parameter, the multiplicative standard deviation s* was introduced to the field of pollution microbiology (Limpert et al. 2001).

Unlike chemical load calculations, load calculations for microbial source tracking marker have not been popular (Wilkes et al. 2014, Wilkes et al. 2013). The current results suggest the future use of human-associated genetic fecal *Bacteroidetes* marker loads as a valuable metric to estimate the impact of municipal and domestic sewage input into the environment. The established median loads for raw and biological treated sewage of approximately 10^{12} and 10^{10} molecule equivalents of human-associated genetic fecal *Bacteroidetes* marker per person and day, respectively, demonstrate the sensitivity of genetic fecal *Bacteroidetes* markers as a general measure of fecal pollution from municipal sewage. Assuming a defecation rate of 100 g to 1000 g of fecal excrement per person and day (Cummings et al. 1992, Geldreich 1978), the estimated median load for raw sewage can be converted back to a concentration range of 10^9 to 10^{10} human-associated genetic fecal *Bacteroidetes* markers per g of faeces. This estimated range of their concentration compares well with concentrations in human faeces measured by qPCR (Haugland et al. 2010, Kildare et al. 2007).

The recovered genetic fecal *Bacteroidetes* marker concentrations were in good agreement with previously reported levels (Reischer et al. 2013, Silkie and Nelson 2009, Stapleton et al. 2009). Genetic fecal *Bacteroidetes* markers had concentrations at least two orders of magnitude higher than SFIB (Fig. 4). Given the reported occurrence of intestinal microbiota in intestinal systems and human faeces, the dominance of genetic fecal *Bacteroidetes* markers over SFIB is well known and expected (Ley et al. 2008, Reischer et al. 2007). This quantitative dominance of genetic fecal *Bacteroidetes* markers in raw and biological treated sewage is of high practical importance, regarding the sensitivity of molecular fecal pollution detection in comparison with cultivation-based standards. SFIB enumeration in water requires only minimal processing efforts. Samples are either directly applied (MPN procedures) or subjected to membrane filtration before cultivation is started (ISO 2000, 2005). PCR analysis involves several additional manipulation steps, including nucleic acid extraction, purification, and partial analysis of the extracted volumes (Ervin et al. 2013). Molecular detection methods thus have to apply higher sampling volumes or have to focus on more abundant targets to achieve comparable sample limits of detection (SLOD). The highly abundant nature of genetic fecal *Bacteroidetes* markers in sewage supports equal to superior sensitivity in comparison to SFIB methods, without the need for largely increased sampling volumes. This fact is the basis for the generation of large comparative sampling sets to appropriately cover pollution dynamics in aquatic systems (Ervin et al. 2013, Reischer et al. 2008, Reischer et al. 2011, Riedel et al. 2014).

A high statistical association between the concentrations of the two human-associated genetic fecal *Bacteroidetes* marker assays, the HF183 Taqman and the

BacHUM UCD, was observed for raw and treated sewage (Table 8). A possible explanation for this tight relationship can be found in the nature of the targeted human-associated *Bacteroidetes* populations. The most widely used human-associated genetic fecal *Bacteroidetes* marker assays (including the above-mentioned ones) still focus on the same or similar phylogenetic sequence targets originally described by Bernhard and Field in 2000 (Bernhard and Field 2000). Recent research indicates that the HF183 Taqman and the BacHUM UCD assay target populations within the species of *B. dorei* (McLellan and Eren 2014). Although these assays revealed quite different specificity and sensitivity characteristics in a recent multi-laboratory study (Layton et al. 2013), our data elucidate the redundant nature of the HF183 Taqman and BacHUM UCD assays for the detection of human-associated fecal pollution along the pathway of sewage disposal. However, the tight association between these two independently performed assays proved the analytical precision of the recovered results within our study.

Correlations analysis for the whole set of microbiological variables revealed contrasting results (Harwood et al. 2005). Statistically significant correlations between concentrations of genetic fecal *Bacteroidetes* markers and SFIB could not be detected, although significant associations between the concentrations of fecal indicators and chemical parameters became obvious. A remarkable exception for raw sewage was the slight but significant relationship between the human-associated BacHUM UCD and the human-specific JCPyV (rho = 0.45, $p<0.02$), pointing to the human-associated fecal pollution indication capacity of these molecular targets. Additionally, *C. perfringens* showed a slight correlation with BacHUM UCD (rho = 0.41, $p <0.02$).

The process of biological wastewater treatment had an increasing effect on the relationship amongst the microbiological variables, whilst the correlation of microbiological to chemical parameters, except for a few cases, totally disappeared. To our knowledge, such a shift of correlation between microbiological parameters during wastewater treatment has not been reported so far and needs further verification.

Although the prevalence and abundance of the human-specific viruses HAdV and JCPyV found in the Austrian WWTP are lower than the range previously reported (Bofill-Mas et al. 2006, Rusinol et al. 2014), the still high prevalence of HAdV (92%) and JCPyV (98%) in the raw sewage of the investigated municipal disposal systems at all seasons, and the detection of JCPyV in small dWWTPs, suggests the usefulness of these specific tools as markers to trace human fecal pollution from WWTPs. The low abundance and prevalence were probably due to differences in the protocol applied (for instance, 500 ml of effluent municipal and domestic wastewater were concentrated instead of the 10 L tested in other studies). JCPyV and HAdV have

been described as highly stable in the environment and present in nearly 100% of raw sewage samples with concentrations up to $\log_{10}$ 7.0 ME 100 ml^{-1} (Bofill-Mas et al. 2013). JCPyV is a highly specific human marker excreted in urine, and the detection of HAdV has been recently described as particularly useful as a prediction risk in bathing waters (Marion et al. 2014). These parameters seem to be suitable tools to complement genetic fecal *Bacteroidetes* markers and SFIB-based surface water monitoring for selected sampling locations or situations when higher sampling volumes can be taken. HAdV and JCPyV have been successfully applied for the identification of the source of contamination in river catchments covering various geographical areas (Rusinol et al. 2014). These viruses can also support verification of MST results in situations when the specificity level from human-associated genetic fecal *Bacteroidetes* markers is deemed insufficient (Reischer et al. 2013).

Genetic fecal *Bacteroidetes* markers, *E. coli* and enterococci revealed similar reductions rates in the representatively chosen municipal activated sludge WWTPs (Fig. 6). Only *C. perfringens* spores demonstrated a lower reduction, most likely due to its conservative nature (Vierheilig et al. 2013). These results clearly demonstrate that genetic fecal *Bacteroidetes* markers emission from municipal WWTP is to be expected in similar concentrations, and treatment just eliminates 2 log orders of magnitude from raw wastewater. The activated sludge membrane bioreactor removed approximately 5.4 and 5.0 log orders (10th percentile) of magnitude of BacHUM-UCD and HF183 TaqMan from wastewater, which is in line with previously reported data on bacterial removal in a membrane bioreactor (van den Akker et al. 2014). Data on UV irradiation (254 nm) indicated no discernible effect on the PCR detectable concentrations of genetic fecal *Bacteroidetes* markers in wastewater, which is in agreement with a recently published study (Chern et al. 2014). It should be mentioned that the effect of chlorination was not investigated. This type of disinfection is not applied in European WWTPs.

In conclusion, the results strongly support the application of human-associated genetic fecal *Bacteroidetes* markers to complement fecal pollution monitoring programs in water resources based on *E. coli* and enterococci. This study focussed on the occurrence and dynamics of genetic fecal *Bacteroidetes* markers in point sources along the human wastewater pathway as expected in the Central European Region. This study did not focus on other important factors, such as mobility, persistence, or specificity, which also have to be considered for monitoring applications (Reischer et al. 2013).

4 Investigation of human-associated genetic fecal markers in raw and treated wastewater from 13 countries on six continents

Abstract

Human-associated genetic fecal markers are interesting candidates to investigate and allocate fecal pollution from municipal WWTP in water resources. The lack of knowledge about the worldwide occurrence, specificity and sensitivity of these markers in wastewater is a major obstacle for the broad application of these methods. This study focused on 4 widely used human-associated genetic *Bacteroidetes* fecal markers, HF183 TaqMan, HF183 II, BacHum and BacH and the *Firmicutes* marker Lachno2, all detected by qPCR. The occurrence of these markers was investigated in raw and treated wastewater of 29 sites in 13 countries on six continents. Results suggest that these markers are ubiquitous in raw and treated wastewater. Concentrations and data variability were comparable across all countries, seasons and across all population sizes contributing to the wastewater treatment plants investigated. Furthermore source-sensitivity and -specificity of the assays were tested on a previously established fecal DNA sample collection. This data showed satisfactory results for the source-sensitivity of the tested genetic markers. In contrast the source-specificity may be critical for certain applications. Therefore it remains advisable to evaluate the markers under the local circumstances in order to choose the best assays for the corresponding study.

4.1 Introduction

The disease burden from water, sanitation and hygiene is estimated to be responsible for 4% of all deaths worldwide (Pruss et al. 2002). The lack of access to safe drinking water led the World Health Organization (WHO) to focus their strategies on managing water quality with a view to protecting and promoting human health (WHO 2013). To provide information about water quality, standardized determination of fecal indicator bacteria, such as *E. coli* and enterococci, is done in trained laboratories (ISO 2000, 2001a). But the measurement of SFIB does not provide information about the origin of fecal pollution, because feces of most animals also contain SFIB concentrations that are great enough to affect water quality (Stalder et al. 2011). The innovative discipline of microbial source tracking allows discrimination between the many possible sources of fecal pollution (Hagedorn et al. 2011). Cultivation-based and molecular methods are used to detect several host-associated bacteria, viruses or chemicals. As in any diagnostic assay two crucial criteria for such MST methods to provide useful information are the host-specificity and -sensitivity (Hagedorn et al. 2011). Source specificity describes the diagnostic ability of an assay to exclude false positive results, which in the context of MST means positive results in non-target fecal material, while sensitivity enumerates the percentage of individuals from the targeted group that yields positive results. For example Boehm et al. compared the analysis data for 41 microbial source tracking methods applied in 27 laboratories. They were able to show that while a number of the tested markers showed a reasonably high specificity, they at the same time lacked in sensitivity (Boehm et al. 2013). But up to date most assays among the large number of assays available in literature have not been tested for their source-specificity and -sensitivity beyond the regional level (Ahmed et al. 2013, Green et al. 2014, Shanks et al. 2010). For this reason it is difficult to choose the appropriate MST tools when planning an MST application in a new area.

To improve this situation Reischer et al. compared five PCR assays targeting human-, cattle- or ruminant-associated *Bacteroidetes* populations by testing 280 different defined fecal samples from 16 countries and six continents to investigate the source-specificity and sensitivity. The obtained results suggested that the tested markers show satisfactory occurrence no matter where the samples were taken. The study also emphasized that the quantitative distribution of the marker concentrations in the sample setup is essential for the assessment of assay performance (Reischer et al. 2013). The results were obtained from single defined fecal samples, but did not include wastewater.

The aim of this study was to provide a snapshot insight into the occurrence and abundance of human-associated genetic fecal markers in raw and treated wastewater on a global level. Three widely applied qPCR assays (BacH, BacHum and HF183 TaqMan) and two recently developed assays (HF183II and Lachno2) were tested on wastewater samples from 29 sites in 13 countries on six continents. Sites were divided between urban biological wastewater treatment plants serving large numbers of populations and rural treatment plants serving lower numbers of populations. Special efforts were undertaken to ensure standardized sampling, sample processing and logistics. In addition the fecal DNA sample collection of a previous study (Reischer et al. 2013) was used to investigate the source-specificity and -sensitivity of the new qPCR assays on human and animal fecal samples. This is the first co-operational study attempting to assess the capability of human-associated genetic fecal markers as indicators of wastewater contamination across multiple countries.

4.2 Materials and methods

4.2.1 Collection and processing of wastewater samples

The requirements for cooperation partner in this MST evaluation project were defined in 2013. A detailed standard operating procedure (SOP) for sampling and filtration was distributed to all partners and a demonstrational video on the important filtration and filter packing steps was also provided online to ensure that sampling procedures are equal. Partners were required to use an online sampling protocol to collect all the necessary metadata. To improve comparability the polycarbonate membran filters (0.2 µm Millipore, Isopore Membrane Filter – GTTP, Cork, Ireland), preprinted labels and vials for transportation of the filters were provided to all partners by the lead lab. Partners were instructed to select one WWTP with a pollution load greater than 500.000 population equivalents (urban wastewater) and one with less than 50.000 P.E. (rural wastewater) both with preferably low industrial influence in their country of residence in order to cover municipal wastewater with different characteristics (Table 9). The plants were equipped with mechanical treatment followed by either activated sludge or fixed film treatment. Basic data on plant capacity (P.E.), sewer system (separate, combined) and possible influence from industry or livestock, had to be provided by the plant operators.

TABLE 9: Investigated wastewater treatment plants

Country	Sewer System	Influence		P.E.
		Industry	Livestock	
Argentina (R)	seperate sewer system	slight	strong	350,000
Argentina (U)	combined sewer system	strong	strong	600,000
Australia (U)	seperate sewer system	slight	no	500,000
Australia(R)	seperate sewer system	-	-	50,000
Brazil (R)	seperate sewer system	no	no	19,054
Brazil (U)	seperate sewer system	slight	-	4,400,000
Canada (R)	separate sewer system	slight	slight	20.000
Canada (U)	combined sewer system	no	no	500.000
Germany (R)	combined sewer system	slight	-	16,800
Germany (U)	combined sewer system	moderate	slight	1,000,000
Japan (R)	seperate sewer system	no	no	10,165
Japan (U)	seperate sewer system	slight	slight	300,000
New Zealand	n.a.	-	-	-
New Zealand	n.a.	-	-	-
Singapore	separate sewer system	moderate	slight	1,700,000
Spain (R)	seperate sewer system	slight	no	45,134
Spain (U)	seperate sewer system	slight	no	384,000
Tanzania (R)	combined sewer system	no	no	3,000
Tanzania (U)	combined sewer system	no	no	10,000
Uganda (R)	separate sewer system	no	no	750
Uganda (U)	separate sewer system	no	no	320,000
UK (R)	combined sewer system	no	slight	14,554
UK (U)	combined sewer system	strong	slight	3,500,000
USA (R)	seperate sewer system	no	no	3,500
USA (R)	combined sewer system	no	no	16,000
USA (R)	combined sewer system	moderate	slight	29,779
USA (U)	combined sewer system	strong	no	142,022
USA (U)	combined sewer system	moderate	slight	3,000,000
USA (U)	combined sewer system	moderate	no	480,000

Abbreviations: P.E.: population equivalent; U: urban, R: rural, n.a.: not available

To obtain comparable results, grab samples at all sites were taken in the morning hours during dry weather conditions when the load of human fecal matter in the wastewater was expected to be highest. Raw wastewater samples were taken after the mechanical screen, mechanical-biological treated wastewater samples after the secondary sedimentation/clarifier before any advanced treatment (e.g. UV, chlorination, coagulation). Sampling points were chosen in turbulent zones to guarantee good mixing and the samples were collected about 20 cm below the water table. Samples were stored in dark cooling boxes at 4°C and immediately transported to the local partner laboratory for filtration. From every sample 50 ml were filtered on 4 replicate filters and immediately frozen. Two of these filters stayed with the cooperation partner as a backup. For every filtration session a blank filter control was also stored. Postal service was carried out with qualified logistics partners and under controlled frozen conditions with dry ice.

4.2.2 Collection and processing of fecal DNA samples

The fecal DNA samples used in the investigation of assay specificity and sensitivity were collected and extracted in the course of a previous study (Reischer et al. 2013). In short the sample set consisted of a total of 280 fecal samples including 61 human fecal samples and 219 animal fecal samples from various sources (mammalian and avian livestock and wildlife) (Reischer et al. 2013). The dataset included samples of fecal DNA from the following sources: *Homo sapiens* (human, n=61); ruminant animals (n=79) comprised of *Bos taurus* (cattle, n=47), *Ovis aries* (sheep, n=16), *Cervus elaphus* (deer, n=8), *Capra hircus* (goat, n=6), *Rupicapra rupicapra* (chamois, n=1) and *Lama sp.* (llama, n=1); non-ruminant animals (n=140) covering *Equus caballus* (horse, n=17), *Macropus sp.* (kangaroo, n=4), *Oryctolagus cuniculus* (rabbit, n=2), *Lepus timidus* (mountain hare, n=1), *Equus asinus* (donkey, n=2), *Equus quagga* (zebra, n=1), *Marmota marmota* (groundhog, n=1), *Sus scrofa domesticus* (swine, n=21), *Sus scrofa* (wild boar, n=8), *Canis lupus familiaris* (dog, n=29), *Felis catus* (cat, n=7), *Canis latrans* (coyote, n=1), *Didelphis* sp. (opposum, n=1), *Lontra canadensis* (river otter, n=1), *Gallus gallus* (chicken, n=19), *Anas platyrhynchos* (duck, n=5), members of the subfamily *Anserinae* (geese, n=3), members of the family *Columbidae* (pigeons, n=3), members of the family *Sturnidae* (starlings, n=2), *Meleagris gallopavo* (turkey, n=2), *Larus* sp. (gull, n=1) and other wild birds including parrot, coot, grebe, owl and pelican (n=9). For sampling and DNA extraction of the mentioned DNA samples refer to Reischer et al 2013.

4.2.3 DNA extraction and molecular analysis

DNA from wastewater filters was recovered by phenol/chloroform extraction as previously described (Griffiths et al. 2000, Reischer et al. 2006). Cell lysis was done with a FastPrepR-24 Instrument (MP Biomedicals Inc., Irvine, USA) with a speed setting of 6 m/s for 30 s. The concentration of the extracted DNA was measured with Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermo Fisher Scientific, United Kingdom) and DNA samples were stored at -80°C. For two samples the DNA concentration was below the detection limit. These samples were discarded.

The following host-associated fecal genetic 16S-rRNA-gene markers were quantified by qPCR: HF183 Taqman (in this chapter called HF183I) (Haugland et al. 2010), HF183II (Green et al. 2014) BacH (Reischer et al. 2007) and BacHUM-UCD (in this chapter called BacHum) (Kildare et al. 2007), all of which target human-associated Bacteroidetes, and Lachno2 (Newton et al. 2011) targeting a human-associated *Firmicutes* genus. In addition the general Bacteroidetes marker AllBac was used for quality control purposes (Layton et al. 2006). The qPCR measurements were done on a Rotorgene Q Cycler (Qiagen, Hilden, Germany). A Qiagility liquid handling robot (Qiagen) was used to load the rotor-discs with the mastermix and the sample DNA. All qPCR reactions were run 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 mg 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 AllBac375Bhqr were used (Layton et al. 2006). Additonally 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 10^3 copies of IAC Template IPC-ntb2 plasmid DNA (Anderson et al. 2010) were added to every Allbac qPCR assay run. For the BacHUM 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 (Kildare et al. 2007). For the HF183I assay 100 nmol L^{-1} primer HF183, 100 nmol L^{-1} primer BFDREV and 80 nmol L^{-1} TaqMan MGB probe BFDFAM were used (Haugland et al. 2010). For the HF183II assay 1000 nmol L^{-1} primer HF183, 1000 nmol L^{-1} primer BacR287, and 80 nmol L^{-1} TaqMan MGB probe BacP234MGB were used (Green et al. 2014). For the BacH assay 200 nmol L^{-1} primer BacH_f, 200 nmol L^{-1} primer BacH_r, 100 nmol L^{-1} TaqMan MGB probe BacH_pC and 100 nmol L^{-1} TaqMan MGB probe BacH_pT were used (Reischer et al. 2007). For the Lachno2 assay 1000 nmol L^{-1} primer Lachno2F, 1000 nmol L^{-1} primer Lachno2R and 80 nmol L^{-1} TaqMan MGB probe Lachno2P were used (Newton et al. 2011).

Quantification was based on plasmid standard dilutions. The respective plasmid stock for each assay was diluted in an unspecific 500 ng ml^{-1} poly(dI-dC) background to avoid adsorption of plasmid DNA to reaction vials at low plasmid concentrations

(Roche Diagnostics, Mannheim, Germany). A total of at least seven tenfold serial dilutions of plasmid standard (10^0 to 10^6 gene copies) were run in each qPCR experiment. Every run also included several no-template and DNA extraction controls.
Each DNA sample was analyzed in two dilution steps (10- and 100-fold dilution) and each dilution in duplicate reactions in order to check for a possible PCR inhibition. Additionally, an IAC was run in duplex with the AllBac assay to control for PCR inhibition. All qPCR runs in this study revealed a calculated PCR efficiency of between 90% and 105% and the no-template controls were consistently negative.

4.2.4 Data analysis and statistics

All microbial data were expressed as $\log_{10}$ (x+1) and reductions were calculated as $\log_{10}$ (effluent) minus $\log_{10}$ (influent). Visual and statistical data analysis were done with Visplore 2.0 (Piringer et al. 2010)(VRVis GmbH, Austria, Vienna) and Sigma Plot 13.0 (SPSS Inc., Chicago, USA). To account for multiple testing statistical significance levels were corrected according to Bonferroni (Bonferroni 1936). All graphs were prepared using Sigma Plot 13.0, Visplore 2.0 and CorelDraw X5 (Corel, Canada). To support correct comparisons of the variability of the log-normally distributed data produced by the MST marker assays the multiplicative standard deviation s* was calculated for the recovered results (Limpert et al. 2001). The multiplicative standard deviation is a measure for the variation of log normal distributed data and describes the shape of the distribution. The median of the data is multiplied or divided with it and the result is the confidence interval which covers 68.3% of the data (Limpert et al. 2001).

4.3 Results

Five different human-associated genetic fecal *Bacteroidetes* markers were quantified in 29 raw and 29 treated municipal and rural wastewater samples from 13 countries on six continents. The commonly used markers HF183 TaqMan (HF183I), BacHum and BacH were evaluated and compared to each other as well as the improved HF183 Version II (HF183II) and the newly established *Firmicutes* assay Lachno2.

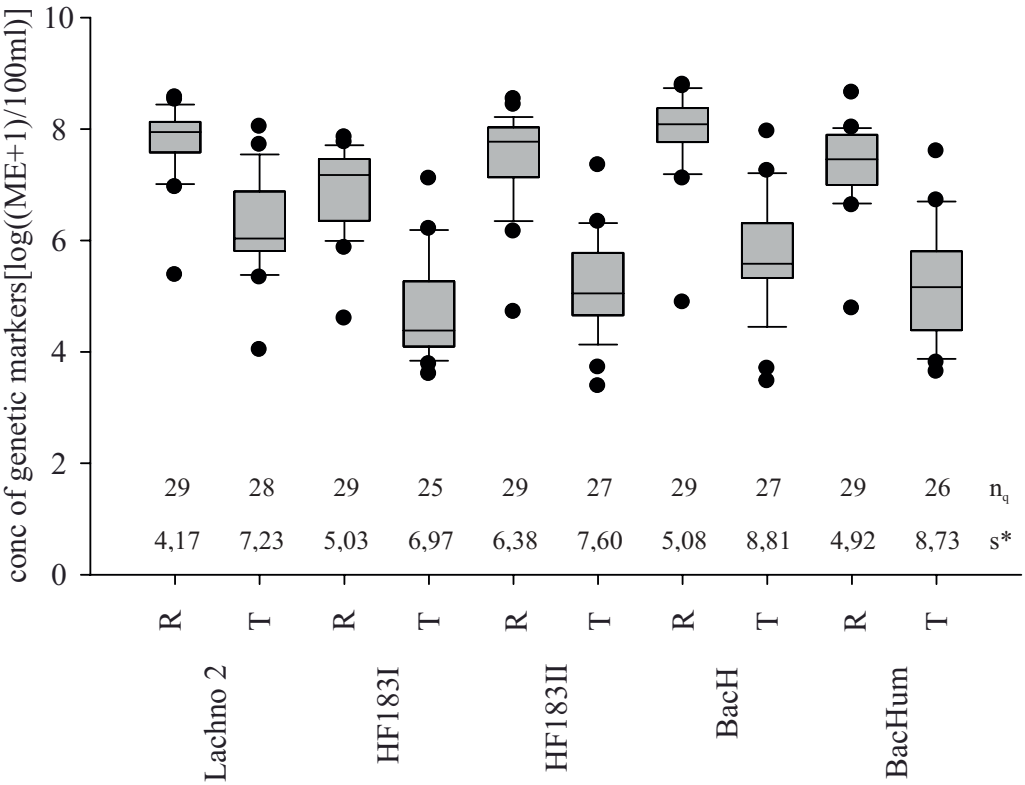

Figure 10: Concentration of human-associated genetic microbial source tracking markers in raw (R) and treated (T) wastewater.

n_q: number of quantifiable samples, s*: estimated multiplicative standard deviation, boxes cover the 25th to 75th percentile; line within the boxes, median; whiskers the 10th to 90th percentile

The investigated human-associated assays showed 100% prevalence in all raw and treated wastewater samples (Fig. 10). For statistical tests the results of the concentration of the human-associated assays were grouped into rural and urban sites and compared.

Results of the comparison showed no statistical differences (Mann-Whitney Rank Sum Test, $p < 0.05$, Bonferroni corrected). Hence, results from rural sites and results from urban sites from all countries were pooled for further analysis. The amount of different country specific samples was not big enough to perform country specific comparisons and reveal reliable results.

Among all assays HF183I showed the lowest median concentration in raw wastewater of $\log_{10}$ 7.2 ME 100 ml^{-1} (Figure 10). The HF183II and BacH markers showed similar values with medians of $\log_{10}$ 7.8 and $\log_{10}$ 7.8 ME 100 ml^{-1}, respectively. The BacHum marker 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}.

In treated wastewater the HF183I again showed the lowest quantities with a median of $\log_{10}$ 4.6 ME 100 ml^{-1}, and the BacHum marker, the HF183II and BacH group together with medians of $\log_{10}$ 5.2, $\log_{10}$ 5.3 and $\log_{10}$ 5.3 ME 100 ml^{-1},

respectively (Fig. 10). The Lachno2 assay had a median concentration $\log_{10}$ 6.0 ME 100 ml^{-1} in treated wastewater.

To describe the variance of our data set and compare it correctly we calculated the multiplicative standard deviation s*. In raw sewage the multiplicative standard deviation s* for the assays BacHum, Hf183I and BacH was similar and between s* = 4.9 and s* = 5.0. Only for HF183II the variability was higher with a value of s* = 6.4. A general increase in the variability of indicator concentrations between influent and effluent samples was observed. The multiplicative standard deviation s* increased by an average factor of 1.5 (range 1.2 – 1.8) during wastewater treatment (Fig. 10).

To detect if there was any relationship between the applied genetic markers in the investigated wastewater samples we did a correlation analysis. It showed a statistically significant connectivity between all investigated markers. The corresponding Spearman rank coefficients for the human-associated genetic markers ranged from 0.83 to 0.91 in raw and from 0.86 to 0.93 in treated wastewater. (Table 10)

TABLE 10: Correlation analysis table showing the Spearman rank coefficient for the pooled influent and effluent (in & out), the influent (in) and the effluent (out) data of investigated WWTP

AllBac				in & out	
				in	out
0,91*				*=significant	
0,72*	0,78*	**BacHum**			
0,93*		0,96*			
0,77*	0,77*	0,86*	0,92*	**HF183I**	
0,92*		0,96*		0,97*	
0,74*	0,81*	0,84*	0,93*	0,90*	0,90*
0,91*		0,95*		0,97*	0,97*
0,69*	0,79*	0,83*	0,86*	0,91*	0,91*

HF183II	**BacH**

Abbreviations: AllBac: genetic fecal marker for the total *Bacteroidetes* populations; BacHum, HF183I, HF183II and BacH: genetic fecal marker for human-associated *Bacteroidetes* populations;

The reductions of the marker concentrations (Monte Carlo Simulation) achieved by wastewater treatment were in the same range as Mayer et. al (submitted) showed for the central European region, namely -$\log_{10}$ 2,143 for BacHUM, -$\log_{10}$ 2,205 for HF183I, -$\log_{10}$ 2,346 for HF183II (Fig. 11) and -$\log_{10}$ 2,20 for BacH. Only the Lachno2 assay showed a lower reduction with a median of -$\log_{10}$ 1,68.

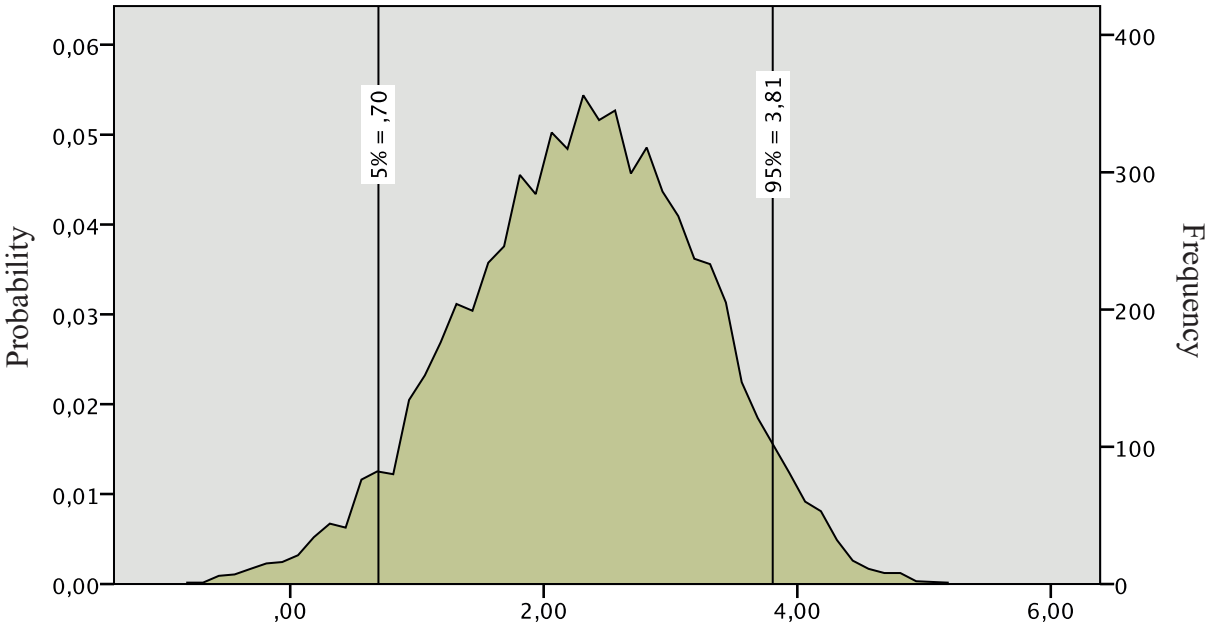

Figure 11: Monte Carlo simulated probability density function of HF183II reduction, n = 6548

To complement the data provided by Reischer et al. 2013 (Reischer et al. 2013) and to investigate the source-specificity of the newly applied MST marker assays used in this current study HF183I, HF183II and Lachno2 were measured in the fecal DNA extracts of the previous study.

The quantitative analysis of the fecal sample setup provided interesting insights into the source-specificity and -sensitivity of the new investigated markers in comparison to the prior published data on the BacH and BacHUM markers (Reischer et al. 2013). The concentration of the newly investigated markers in the human fecal samples was widely distributed, up to 3.2 log steps for the 75% percentile. Among the newly tested assays the Lachno2 assay showed the highest median concentration of $\log_{10}$ 2.0 in the human target samples.
However Lachno2 also showed very high concentrations in the animal fecal samples, with a 75% percentile of $\log_{10}$ 1.9, the highest level of concentrations in false positives for any of the human-associated assays. In contrast the newly investigated human associated Bacteroidetes assays HF183I and Hf183II showed a 75% percentile of $\log_{10}$ 0 and $\log_{10}$ 0.3, respectively, which was lower than the 75% percentiles of the prior investigated BacH and BacHUM marker in the animal sample collection (Fig. 12)

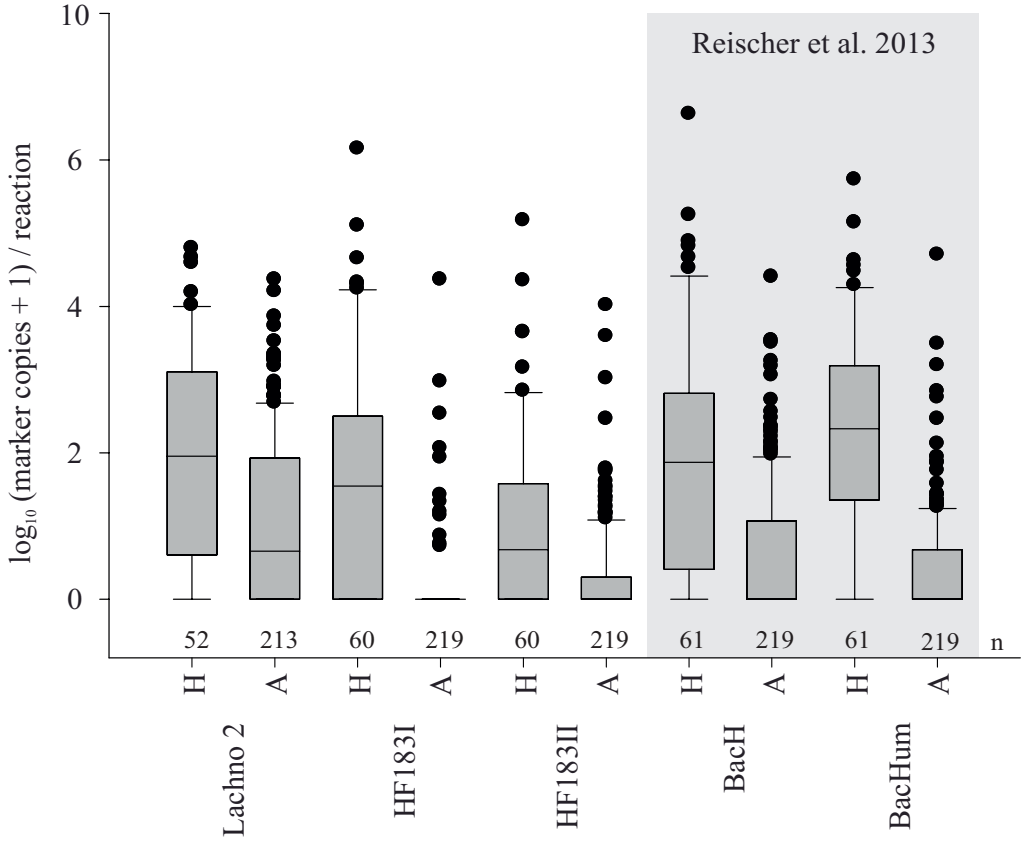

Figure 12: Concentrations of markers measured in human (H) and animal (A) fecal DNA extracts for each assay (grey box previously published data (Reischer et al. 2013)).
Results were measured in the 1:4 dilution of the DNA samples and transformed into logarithmic format after addition of 1 to each value. Boxes, 25th and 75th percentile; lines within the boxes, median; whiskers, 10th and 90th percentile, respectively; n, number of samples in each category.

4.4 Discussion

The results of the qPCR MST marker evaluation study published by Reischer et al. (Reischer et al. 2013) demonstrated that microbial source tracking tools for the detection if source-associated bacterial markers can be used in human and ruminant fecal samples from all over the world. One important aspect that was missing from this study was the most important source or to be more precise, the most important pathway for human fecal contamination of the environment: wastewater. Therefore many of the erstwhile partners and several new ones joined in the effort of collecting a representative set of wastewater samples from 13 different countries on six continents. While the previous investigation could not provide data on the actual concentration of host-associated marker in fecal material due to logistical and methodical restrictions, gaining quantitative levels of markers in wastewater was a central aim of this study. To that end it was absolutely critical

to impose strict measures for quality control and assurance on all partners. These included clearly formulated guidelines for the selection of appropriate sampling sites (cf. Appendix), the collection of metadata of the treatment plants, harmonized standard operating procedures for sampling, sample filtration and shipment (including an instructional video of the filtration procedure), provided identical sampling material and the centralized DNA extraction and measurement of all parameters in the lead laboratory. This procedure was optimized to allow the maximum comparability of the results from the highly divers sampling locations (Bustin 2010).

The five human-associated markers proved to be 100% prevalent in all wastewater samples investigated. This was true irrespective of sample type (raw or biologically treated waste water), provenance (all countries) or site location (urban or rural). This in itself is quite astounding considering the variety of locations from rural raw wastewater from developing countries such as Tanzania to urban treated wastewater from highly industrialized countries such as the UK or Singapore. Other studies have also reported high prevalence of commonly used genetic markers in wastewater (Ahmed et al. 2012, Ahmed et al. 2008, Layton et al. 2013), but never before was it demonstrated on such a broad geographical level.

Even more striking is the fact that the concentrations of the markers in the investigated wastewater samples were very similar across all countries, seasons and across all population sizes contributing to the plants. These results are highly complementary to and in agreement with a study recently conducted by Mayer et al. (submitted) about the dynamics of fecal indicators (cultivation-based and genetic) throughout waste water treatment in a large range of Austrian wastewater treatment plants spanning from very small, household sized plants to plants serving more than a million inhabitants. In that study concentrations of human-associated genetic markers also proved to be highly stable throughout a whole year and across all sampling sites.

Wastewater as investigated in the current study is a diluted and highly homogenized suspension containing among other sources of pollution a high load of human fecal material. Therefore it is not completely surprising that concentrations of fecal indicators at a certain site turn out to be fairly constant but to observe these highly similar levels across different countries and even continents has huge implications for the applicability of genetic fecal markers. The results also showed that biological wastewater treatment resulted in a fairly constant reduction rate in marker concentrations between raw and treated wastewater (Fig. 11).

However, the determination of marker concentrations in wastewater does only provide insight into the question of source-sensitivity (Does the marker occurs in a wastewater as a pollution source? At which concentration does it occur?), but gives no indication of source-specificity (Do other potential sources of pollution which are

not associated with human feces also contain the marker?). To answer this last question, the newly established human-associated assays HF183I, HF183II and Lachno2 were tested against the previously compiled global fecal DNA sample collection (Reischer et al. 2013). The results indicated that all the tested assays have a substantial rate of false positive results in animal fecal DNA. Although the used fecal DNA sample set does not allow determination of marker concentrations in the fecal material, the relatively small differences between the true positive results in human fecal material and the false positives highlight a certain lack in source-specificity of the investigated assays. While the HF183I and HF183II assays exhibit a higher level of source-specificities than the previously tested assays BacH and BacHum (data from Reischer et al 2013) the Lachno2 assay seems to have a very low source-specificity with very little difference in marker concentrations between true positives and false positives.

The results of this study demonstrate that the currently available broadly used human– associated genetic markers are highly prevalent and abundant in raw as well as treated wastewater. In that context they satisfy the requirement of source-sensitivity to a very high degree when compared to the prevalence and distribution of marker concentrations in human fecal samples around the world. Nevertheless even the improved or newly developed assays have less than perfect levels of source-specificity. Therefore it is recommended to perform an investigation of local source-specificity and -sensitivity on the locally occurring sources relevant for a new study area (Reischer et al. 2011). After that an informed choice should be made whether the objectives of the study in question can be achieved under the local circumstances (variety of possible sources, relative importance, level of fecal pollution) with the available MST tools (Astrom et al. 2015). For settings with relatively low levels of fecal pollution dominated by animal sources, current genetic bacterial markers might not be able to reliably detect or exclude the possibility of human fecal contamination.

5 Conclusions

The discipline of microbial source tracking (MST) is gaining increasing importance as a valuable tool to investigate the origin of fecal pollution. MST can complement water quality monitoring based on standard fecal indicators (SFIB), providing useful pollution source related information, not given by SFIB. Host-associated genetic *Bacteroidetes* fecal markers have been increasingly used over the last years as promising tools in MST applications. However, information on the occurrence, persistence, treatment resistance, and methodical performance characteristics (e.g. specificity or sensitivity) of human-associated genetic fecal *Bacteroidetes* marker along the communal or domestic wastewater path is still limiting.

The aim of this thesis was to evaluate the occurrence, fate and applicability of human-associated genetic *Bacteroidetes* fecal marker in raw and treated wastewater of communal or domestic origin. The obtained data were compared with results from ISO based SFIB and alternative human-associated *Bacteroidetes* or human-specific viral fecal markers. Investigations were performed on well-characterised wastewater disposal and treatment systems of different size. Automated sampling techniques for the microbial parameters under investigation were established in order to generate representative data from the influent and effluent of the selected WWTPs. The research was realised within a highly interdisciplinary research network, supported by scientists from the Medical University of Vienna (Unit Water Hygiene), the Vienna University of Technology (Institute for Water Quality Resources and Waste Management) and the University of Barcelona (Laboratory of Virus Contaminants of Water and Food).

In chapter two the stability of human-associated *Bacteroidetes* fecal markers detected by the BacHUM-UCD and HF183 TaqMan assays and the SFIB *E. coli,* enterococci and *C. perfringens* spores in wastewater during short term storage was investigated to obtain basic information on the applicability in cooled and automated sampling procedures. Volume-proportional automated sampling devices have already become a common standard for chemical wastewater quality analysis of WWTP. As described in chapter two, it could be successfully demonstrated that automated sampling provides a reliable technique to obtain representative samples also for microbiological analysis. It was shown by microcosm experiments that SFIB and genetic *Bacteroidetes* fecal markers in raw and treated wastewater show high persistence at 5°C for at least 32 hours, which is equivalent to a 24-h sampling procedure and an 8-h working day. The results obtained in this second part of the thesis demonstrate the suitability of volume-proportional automated sampling for the investigated microbiological parameters in municipal WWTP. Such automated sampling procedures provide a unique opportunity to generate reliable and

representative results on the microbial emission characteristics and treatment efficacy of WWTPs, because daily dynamics are accounted for. This sampling technique also allows an estimation of the fecal emission load, which is being discharged into receiving waters.

The findings of the work described in chapter two were applied for the work described in chapter three, in which the abundance and the prevalence of the human-associated *Bacteroidetes* fecal markers BacHum-UCD and HF183 TaqMan, the SFIB *E. coli*, enterococci and *C. perfringens* spores and the human-specific viral markers JC Polyomavirus and Adenovirus were investigated. 14 well-characterized Austrian and one Bavarian domestic and municipal WWTPs were investigated over a period of one-year (i.e. 2012-2013). The size of the studied wastewater systems ranged from 3 individuals up to 49,000 inhabitants, who were connected to the WWTPs. The results showed that the human-associated genetic *Bacteroidetes* fecal markers occur consistently in raw and biologically treated sewage, irrespective of catchment size and season. For the first time, spiking with a newly developed Defined genetic Target Cell standard (DeTaCs) proved the comparability of results gained after waste water filtration, DNA-extraction and qPCR analysis. The multiplicative standard deviation (s*) as a measurement for statistical variability (based on log-normal distributions) was introduced for fecal markers. Equal statistical variability between the SFIB and the human-associated genetic fecal markers in raw and treated wastewater could be demonstrated by s*. Furthermore, recovered concentrations were independent from seasonality and size of the investigated WWTP.

Whereas the work described in chapter three of the dissertation is based on raw and treated wastewater mainly from Austrian WWTPs (i.e. local level), work described in chapter four focused on the occurrence and abundance of human-associated genetic *Bacteroidetes* fecal markers in waste water on a global level. For this purpose, raw and treated wastewater from 29 sites in 13 countries (i.e. Argentina, Australia, USA, Spain, Japan, United Kingdom, Brazil, Germany, Tanzania, Canada, New Zealand, Uganda and Singapore) and six continents was analysed for the occurrence of human-associated fecal genetic markers HF183 TaqMan, HF183II, BacH, BacHUM UCD and Lachno2. The WWTPs' size ranged from 3,000 to 4,400,000 Population equivalents. Due to the complex nature of the study and the high efforts of sampling transport, it was only possibly to rely on single sampling events ("generate a first snapshot"). To ensure comparable WWTP selection and sampling, an innovative online sampling protocol was established and a video, showing the standard operating procedure for water filtration, was provided. Based on these procedures every partner lab was able to perform sampling, sample processing and shipment in a defined and comparable way. The results impressively demonstrate that the analysed genetic markers ubiquitously

occur in WWTP all over the world in comparable and abundant concentrations, independent of the size and the location of the WWTP. Results based on Monte Carlo simulations also showed that the reductions of genetic *Bacteroidetes* fecal markers through primary and secondary wastewater treatment are in good agreement with the results from Austrian WWTP (as presented in chapter three). In addition, the specificity of the investigated genetic *Bacteroidetes* fecal markers was further evaluated on a previously established fecal-DNA-sampling-collection from various animal and human fecal sources also derived from around the globe.

In this thesis it could be demonstrated that human-associated genetic *Bacteroidetes* fecal markers are ubiquitous and occur worldwide in raw and treated wastewater in high concentrations. These markers thus appear as very promising candidates to complement fecal pollution monitoring of aquatic programs in case additional information on the sources of fecal pollution is needed. It could be shown that these human-associated genetic *Bacteroidetes* fecal markers are highly sensitive for communal and domestic wastewater pollution.

The evaluation based on the global fecal DNA sample collection of the genetic Bacteroidetes fecal markers also indicated that their source specificity might be critical for certain applications. If genetic *Bacteroidetes* markers shall be applied in a new catchment, their local fecal specificity characteristics thus have to be tested in order to evaluate whether the methodical performance is sufficient for the selected monitoring application. Complementing *Bacteroidetes* markers with human-specific Adenovirus and JC Polyomavirus marker may be used to verify results. For MST applications where high sensitivity and specificity is required, a tiered approach is suggested by combining highly sensitive human-associated bacterial genetic marker for high throughput screening, followed by a verification step, using highly human-specific viral markers, in order to verify the influence from human fecal pollution at selected locations.

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Appendix:

Contributions of the author

Chapter 2 of this PhD Thesis is based on the manuscript **"High persistence of bacterial fecal indicators and genetic microbial source tracking markers in sewage during short-term storage at 5°C supports automated sampling procedures."** by Mayer RE, Vierheilig J, Egle L, Reischer GH, Saracevic E, Mach RL, Kirschner AKT, Zessner M, Sommer R, Farnleitner AH.

My main contributions to this paper were:

- development of the study design with the support from Andreas Farnleitner
- to manage the sampling campaigns, including logistics and communication
- to measure the genetic markers for total and human-associated fecal pollution by quantitative polymerase chain reaction (qPCR)
- to process and analyse the resulting data, including statistical and graphical analysis
- to prepare the manuscript with the support of the co-authors, mainly from Andreas Farnleitner

Chapter 3 of this PhD Thesis is based on the manuscript **" Occurrence of human-associated *Bacteroidetes* genetic source tracking marker in raw and treated wastewater of municipal and domestic origin and comparison to standard and alternative indicators of faecal pollution"** by Mayer RE, Bofill-Mas S′ Egle L, Reischer GH, Schade M., Fernandez-Cassi X, Mach RL, Kirschner A, Brunner K, Gaisbauer M, Piringer H, Blaschke A. P, Girones R, Zessner M, Sommer R and Farnleitner AH. My main contributions to this paper were:

- development of the study design with the support from Andreas Farnleitner
- to manage the sampling campaigns, including logistics and communication
- to establish the alternative viral marker „Skimmed Milk flocculation" process in the lab
- to measure the genetic markers for total and human-associated fecal pollution by quantitative polymerase chain reaction (qPCR)
- to process and analyse the resulting data, including statistical and graphical analysis
- to prepare the manuscript with the support of the co-authors, mainly from Andreas Farnleitner

Chapter 4 of this PhD Thesis is based on the manuscript **"Genetic fecal Bacteroidetes markers in worldwide wastewater streams"** by Mayer RE, Mach R.L, Reischer G.H. and Farnleitner AH. My main contribution to this manuscript were:

- development of the study design with the support from Andreas Farnleitner
- to manage the sampling campaigns, including logistics and communication with support from the international cooperation partner: Tom Edge (Canada), Margit Schade (Germany), Anicet R. Blanch (Spain), Huw Taylor and James Ebdon (United Kingdom), Orin C. Shanks, Joan Rose and Asli Aslan (USA), Maria Inês Zanoli Sato (Brazil), Veronica Rajal (Argentina), Stefan Würtz (Singapore), Yoshifumi Masago (Japan), Warish Ahmed (Australia), Marion Savill (New Zealand), Douglas Mushi (Tanzania) and Denis Byamukama (Uganda)
- to measure the genetic markers for human-associated fecal pollution sensitivity tests by quantitative polymerase chain reaction (qPCR)
- to process and analyse the resulting data, including statistical and graphical analysis
- to prepare the manuscript with the support of the co-authors, mainly from Georg H. Reischer and Andreas Farnleitner.

Appendix:

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Automated Sampling Procedures Supported by High Persistence of Bacterial Fecal Indicators and *Bacteroidetes* Genetic Microbial Source Tracking Markers in Municipal Wastewater during Short-Term Storage at 5°C

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Because of high diurnal water quality fluctuations in raw municipal wastewater, the use of proportional autosampling over a period of 24 h at municipal wastewater treatment plants (WWTPs) to evaluate carbon, nitrogen, and phosphorus removal has become a standard in many countries. Microbial removal or load estimation at municipal WWTPs, however, is still based on manually recovered grab samples. The goal of this study was to establish basic knowledge regarding the persistence of standard bacterial fecal indicators and *Bacteroidetes* genetic microbial source tracking markers in municipal wastewater in order to evaluate their suitability for automated sampling, as the potential lack of persistence is the main argument against such procedures. Raw and secondary treated wastewater of municipal origin from representative and well-characterized biological WWTPs without disinfection (organic carbon and nutrient removal) was investigated in microcosm experiments at 5 and 21°C with a total storage time of 32 h (including a 24-h autosampling component and an 8-h postsampling phase). Vegetative *Escherichia coli* and enterococci, as well as *Clostridium perfringens* spores, were selected as indicators for cultivation-based standard enumeration. Molecular analysis focused on total (AllBac) and human-associated genetic *Bacteroidetes* (BacHum-UCD, HF183 TaqMan) markers by using quantitative PCR, as well as 16S rRNA gene-based next-generation sequencing. The microbial parameters showed high persistence in both raw and treated wastewater at 5°C under the storage conditions used. Surprisingly, and in contrast to results obtained with treated wastewater, persistence of the microbial markers in raw wastewater was also high at 21°C. On the basis of our results, 24-h autosampling procedures with 5°C storage conditions can be recommended for the investigation of fecal indicators or *Bacteroidetes* genetic markers at municipal WWTPs. Such autosampling procedures will contribute to better understanding and monitoring of municipal WWTPs as sources of fecal pollution in water resources.

Microbial fecal contamination of aquatic systems by municipal wastewater represents a significant threat to public health (1). Thus, appropriate wastewater disposal technologies and fecal pollution monitoring programs are critical for safeguarding our water resources. Standard fecal indicators, as well as recently developed genetic microbial source tracking (MST) markers, are used to monitor the microbial fecal loads emitted from wastewater treatment plants (WWTPs) and their impact on receiving waters (2–6). Microbiological sampling of WWTPs is commonly based on manually recovered samples (7). However, the concept behind these methods neglects temporal fluctuations in water quality. Large diurnal variations have been reported for key chemical parameters, such as nutrients, in raw wastewater (8). Determination of the efficacy of carbon, nitrogen, and phosphorus removal at WWTPs is thus frequently based on automated diurnal sampling. For example, in Austria, automated sampling procedures for chemical parameters are required for the official performance testing of WWTPs with more than 1,000 population equivalents (PE), and these procedures use sampling volumes that are proportional to the observed water influx levels over a period of 24 h (9).

Automated sampling is infrequently used for monitoring of microbial fecal pollution. A key argument against the use of automated sampling procedures is the unknown, low, or differential persistence of microbial targets, especially when longer storage periods (i.e., >8 h) are used. This deficiency can potentially lead to false-negative results or underestimation of

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TABLE 1 Full data set for the persistence of standard fecal indicators and MST markers in raw and treated municipal wastewater at 5°C recovered from microcosm experiments

Method, sample, and SE (WWTP)[a]	Time[b]	Microcosm experiment descriptive statistics[c]			Regression analysis of microcosm data		
		Mean[d]	Min[e]	Max[f]	d[g]	k[g]	Log/% reduction[h]
AllBac qPCR							
Influent							
1 (2)	a	10.0	9.3	10.3	10.0	0.003	
3 (4)	b	10.3	10.1	10.4	10.2	0.004	
7 (3)	c	10.6	10.0	11.2	10.2	0.034	
9 (3)	d	10.7	10.6	10.8	10.8	−0.005	
11 (4)	i	10.1	9.7	10.2	10.0	0.001	
13 (2)	i	10.5	10.3	10.6	10.5	−0.003	
Effluent							
2 (2)	a	7.7	7.2	7.9	7.8	−0.003	
4 (4)	b	7.9	7.7	8.2	8.1	−0.007	
8 (3)	c	7.8	7.1	9.8	7.2	0.057	
10 (3)	d	7.7	7.5	8.0	7.7	0.001	
12 (4)	i	8.8	8.7	8.8	8.8	0.001	
14 (2)	i	8.2	8.0	8.5	8.3	−0.008	
BacHum-UCD qPCR							
Influent							
1 (2)	a	8.9	8.4	9.2	8.9	−0.001	
3 (4)	b	8.7	8.5	8.9	8.6	0.002	
7 (3)	c	9.1	8.9	9.5	9.2	−0.011	
9 (3)	d	9.0	8.8	9.0	9.0	−0.004	
11 (4)	i	8.7	8.4	9.2	8.6	0.015	
13 (2)	i	9.5	9.0	9.7	9.3	0.010	
Effluent							
2 (2)	a	6.7	6.1	6.9	6.7	−0.002	
4 (4)	b	6.1	5.8	6.6	6.4	−0.019	
8 (3)	c	6.6	6.1	8.1	6.1	0.042	
10 (3)	d	5.6	5.4	5.9	5.6	0.000	
12 (4)	i	8.0	7.7	8.2	7.7	0.018	
14 (2)	i	7.0	6.7	7.1	6.9	0.004	
HF183 TaqMan qPCR							
Influent							
1 (2)	a	8.5	7.5	9.1	8.4	0.006	
3 (4)	b	8.4	8.2	8.6	8.3	0.003	
7 (3)	c	8.0	7.6	8.8	7.8	0.019	
9 (3)	d	9.4	9.2	9.4	9.4	−0.002	
11 (4)	i	8.5	8.2	8.7	8.3	0.010	
13 (2)	i	9.0	8.7	9.3	8.9	0.007	
Effluent							
2 (2)	a	6.5	6.1	6.8	6.6	−0.008	
4 (4)	b	5.7	5.5	6.1	6.0	−0.015[i]	0.48/66
8 (3)	c	5.6	4.9	7.1	4.7	0.074	
10 (3)	d	6.1	5.8	6.9	6.1	0.003	
12 (4)	i	7.5	7.3	7.7	7.3	0.013	
14 (2)	i	6.5	6.4	6.6	6.5	0.000	
E. coli (cultivation based)							
Influent							
1 (2)	a	6.8	6.8	6.8	6.8	0.000	
3 (4)	b	6.4	6.2	6.6	6.5	−0.004	
5 (4)	e	6.9	6.9	7.0	6.9	0.000	

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TABLE 1 (Continued)

Method, sample, and SE (WWTP)[a]	Time[b]	Microcosm experiment descriptive statistics[c]			Regression analysis of microcosm data		
		Mean[d]	Min[e]	Max[f]	d[g]	k[g]	Log/% reduction[h]
7 (3)	c	6.2	6.2	6.3	6.3	−0.003	
9 (3)	d	6.7	6.6	6.9	6.7	−0.002	
11 (4)	i	7.8	7.7	8.1	8.1	−0.015	
13 (2)	i	6.1	6.1	6.2	6.2	−0.003	
Effluent							
2 (2)	a	4.4	4.3	4.6	4.5	−0.005	
4 (4)	b	4.7	4.6	5.0	4.9	−0.010	
6 (4)	e	4.6	4.5	4.6	4.6	0.000	
8 (3)	c	3.7	3.6	3.8	3.6	0.006	
10 (3)	d	3.6	3.5	3.8	3.7	−0.002	
12 (4)	i	5.2	5.2	5.3	5.3	−0.004	
14 (2)	i	4.0	3.9	4.1	4.1	−0.005	
C. perfringens spores (cultivation based)							
Influent							
1 (2)	a	5.0	4.9	5.1	5.0	0.005	
3 (4)	b	4.9	4.7	4.9	4.8	0.003	
7 (3)	c	4.6	4.5	4.7	4.6	−0.003	
11 (4)	i	4.5	4.4	4.6	4.5	0.000	
13 (2)	i	4.7	4.6	4.8	4.7	0.003	
Effluent							
2 (2)	a	3.9	3.8	4.0	3.9	0.006	
4 (4)	b	3.1	3.1	3.2	3.2	−0.002	
8 (3)	c	3.0	2.9	3.0	3.0	0.003	
12 (4)	i	3.9	3.7	4.0	3.8	0.004	
14 (2)	i	3.9	3.7	4.0	3.9	−0.002	
Enterococci (cultivation based)							
Influent							
11 (4)	i	5.4	5.3	5.6	5.3	0.006	
13 (2)	i	5.7	5.6	5.9	5.6	0.006	
Effluent							
12 (4)	i	4.3	4.3	4.3	4.3	−0.001	
14 (2)	i	4.1	4.0	4.2	4.0	−0.001	

[a] SE, sampling event number. In parentheses is the number of the WWTP investigated.
[b] Analysis times during microcosm experiments: a ($n = 5$), 0, 4, 8, 20, and 24 h; b ($n = 6$), 0, 7, 19, 24, 27, and 43 h; c ($n = 6$), 0, 4, 8, 12, 22, and 24 h; d ($n = 5$), 0, 5, 18, 27, and 35 h; e ($n = 5$), 0, 5, 11, 17, and 25 h; i ($n = 5$), 0, 9,5, 20, 24, and 29 h.
[c] Values obtained by qPCR are in $\log_{10}$ ([ME + 1] 100 ml^{-1}) (where ME is marker equivalents), and those obtained by cultivation are in $\log_{10}$ ([CFU + 1] 100 ml^{-1}).
[d] Mean, arithmetic mean.
[e] Min, minimum value.
[f] Max, maximum value.
[g] d and k are linear regression coefficients. d is the intercept with the *y* axis $\log_{10}$ ([ME + 1] 100 ml^{-1}) or $\log_{10}$ ([CFU + 1] 100 ml^{-1}). k is the slope {$\log_{10}$ ([ME + 1] 100 ml^{-1}) or $\log_{10}$ [CFU 100 ml^{-1}]} per hour.
[h] Log_{10} reduction calculated from regression model for a sample storage time of 32 h at 5°C (calculated for significant regression coefficients only). The value after the slash is the percent reduction, relating to the delogarithmized absolute values.
[i] Statistically significant coefficient ($P \leq 0.05$, Bonferroni corrected).

target concentrations (10–13). Nonetheless, several studies have demonstrated the potential of automated sampling procedures for pollution microbiology (2, 14–17). For example, autosampling was used to elucidate previously unobserved microbial fecal pollution dynamics in alpine water resources, results that had significant implications for water quality management (14, 18). To keep the effects of microbial die-off within a negligible range, batches of collected samples were recovered from an automatic sampling device within 24 h and analyzed immediately (14).

The goal of this study was to establish basic knowledge regarding the persistence of standard bacterial fecal indicators and *Bacteroidetes* genetic MST markers in municipal wastewater in order to evaluate their suitability for automated sampling procedures. Raw and treated wastewater samples from representative municipal WWTPs were investigated in microcosm

TABLE 2 Full data set for the persistence of standard fecal indicators and MST markers in raw and treated municipal wastewater at 21°C recovered from microcosm experiments

Method, sample, and SE (WWTP)[a]	Time[b]	Microcosm experiment descriptive statistics[c]			Regression analysis of microcosm data		
		Mean[d]	Min[e]	Max[f]	d[g]	k[g]	Log/% reduction[h]
AllBac qPCR							
Influent							
1 (2)	a	10.0	9.5	10.4	9.9	0.007	
3 (4)	b	10.2	10.1	10.4	10.1	0.004	
7 (3)	c	10.3	10.1	10.6	10.3	0.004	
9 (3)	d	10.9	10.7	11.1	10.8	0.003	
11 (4)	i	10.0	9.5	10.3	9.7	0.017	
13 (2)	i	10.7	10.4	10.9	10.5	0.006	
Effluent							
2 (2)	a	7.4	7.0	7.9	7.8	−0.030[i]	0.96/89
4 (4)	b	7.8	7.3	8.2	7.9	−0.002	
8 (3)	c	7.22	6.95	7.52	7.3	−0.011	
10 (3)	d	7.40	7.2	7.6	7.5	−0.006	
12 (4)	i	8.75	8.62	8.98	8.9	−0.011	
14 (2)	i	8.02	7.65	8.30	8.3	−0.014	
BacHum-UCD qPCR							
Influent							
1 (2)	a	8.7	8.4	9.1	8.8	−0.009	
3 (4)	b	8.3	8.1	8.6	8.5	−0.010[i]	0.30/50
7 (3)	c	9.2	8.9	9.3	9.1	0.008	
9 (3)	d	8.9	8.7	8.9	8.9	−0.002	
11 (4)	i	8.4	8.1	8.6	8.5	−0.002	
13 (2)	i	9.5	9.1	9.7	9.3	0.011	
Effluent							
2 (2)	a	6.1	5.5	6.9	6.8	−0.045[i]	1.89/99
4 (4)	b	5.2	4.2	6.3	6.2	−0.049[i]	1.57/97
8 (3)	c	6.1	5.7	6.4	6.3	−0.023	
10 (3)	d	5.0	4.0	5.5	5.2	−0.013	
12 (4)	i	7.9	7.8	7.9	7.9	0.001	
14 (2)	i	6.7	6.5	7.1	7.0	−0.014	
HF183 TaqMan qPCR							
Influent							
1 (2)	a	8.4	7.5	8.9	8.3	0.009	
3 (4)	b	8.0	7.7	8.4	8.3	−0.015[i]	0.48/66
7 (3)	c	8.1	7.8	8.8	7.7	0.032	
9 (3)	d	9.2	9.1	9.4	9.4	−0.007	
11 (4)	i	8.2	7.9	8.5	8.3	−0.005	
13 (2)	i	9.0	8.5	9.3	8.8	0.009	
Effluent							
2 (2)	a	6.1	5.3	7.0	6.7	−0.046[i]	1.42/96
4 (4)	b	6.0	4.7	3.9	5.8	−0.054	
8 (3)	c	5.0	4.5	5.6	5.0	0.002	
10 (3)	d	5.4	4.0	6.0	5.7	−0.016	
12 (4)	i	7.4	7.3	7.5	7.4	0.000	
14 (2)	i	6.3	6.0	6.6	6.5	−0.015	
E. coli (cultivation based)							
Influent							
1 (2)	a	6.8	6.7	6.9	6.8	−0.001	
3 (4)	b	6.3	6.2	6.5	6.4	−0.006	
5 (4)	e	6.8	6.6	6.9	6.8	−0.006	

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TABLE 2 (Continued)

Method, sample, and SE (WWTP)[a]	Time[b]	Microcosm experiment descriptive statistics[c]			Regression analysis of microcosm data		
		Mean[d]	Min[e]	Max[f]	d[g]	k[g]	Log/% reduction[h]
7 (3)	c	6.4	6.3	6.4	6.4	0.000	
9 (3)	d	6.7	6.6	6.9	6.8	−0.004	
11 (4)	i	8.0	7.9	8.2	8.1	−0.009	
13 (2)	i	6.1	6.1	6.1	6.1	0.001	
Effluent							
2 (2)	a	3.9	3.4	4.4	4.5	−0.042[i]	1.35/96
4 (4)	b	4.1	3.3	5.1	5.0	−0.041[i]	1.31/95
6 (4)	e	4.2	3.9	4.6	4.5	−0.024	
8 (3)	c	3.6	3.5	3.8	3.8	−0.009[i]	0.29/49
10 (3)	d	3.7	3.6	3.8	3.7	−0.002	
12 (4)	i	5.2	5.2	5.3	5.3	−0.005	
14 (2)	i	3.8	3.5	4.0	4.0	−0.017	
C. perfringens spores (cultivation based)							
Influent							
1 (2)	a	5.1	4.9	5.2	5.0	0.006	
3 (4)	b	4.9	4.8	5.0	4.8	0.001	
7 (3)	c	4.5	4.4	4.6	4.5	−0.002	
11 (4)	i	4.5	4.3	4.6	4.4	0.005	
13 (2)	i	4.7	4.6	4.8	4.6	0.003	
Effluent							
2 (2)	a	4.0	3.9	4.1	3.9	0.004	
4 (4)	b	3.1	3.1	3.2	3.2	−0.002	
8 (3)	c	3.0	2.9	3.1	3.0	0.004	
12 (4)	i	3.8	3.7	3.9	3.9	−0.004	
14 (2)	i	3.8	3.7	3.9	3.8	0.001	
Enterococci (cultivation based)							
Influent							
11 (4)	i	5.4	5.3	5.5	5.3	0.003	
13 (2)	i	5.68	5.46	5.81	5.6	0.005	
Effluent							
12 (4)	i	4.2	4.1	4.3	4.3	−0.005	
14 (2)	i	3.96	3.87	4.03	4.2	−0.007	

[a] SE, sampling event number. In parentheses is the number of the WWTP investigated.
[b] Analysis times during microcosm experiments: a ($n = 5$), 0, 4, 8, 20, and 24 h; b ($n = 6$), 0, 7, 19, 24, 27, and 43 h; c ($n = 6$), 0, 4, 8, 12, 22, and 24 h; d ($n = 5$), 0, 5, 18, 27, and 35 h; e ($n = 5$), 0, 5, 11, 17, and 25 h; i ($n = 5$), 0, 9,5, 20, 24, and 29 h.
[c] Values obtained by qPCR are in $\log_{10}$ ([ME + 1] 100 ml^{-1}) (where ME is marker equivalents), and those obtained by cultivation are in $\log_{10}$ ([CFU + 1] 100 ml^{-1}).
[d] Mean, arithmetic mean.
[e] Min, minimum value.
[f] Max, maximum value.
[g] d and k are linear regression coefficients. d is $\log_{10}$ ([ME + 1] 100 ml^{-1}) or $\log_{10}$ (CFU 100 ml^{-1}). k is the difference in $\log_{10}$ [(ME + 1) 100 ml^{-1}] or $\log_{10}$ (CFU 100 ml^{-1}) values per hour between data points.
[h] Log_{10} reduction calculated from regression model for a sample storage time of 32 h at 21°C (calculated for significant regression coefficients only). The value after the slash is the percent reduction, relating to the delogarithmized absolute values.
[i] Statistically significant coefficient ($P \leq 0.05$, Bonferroni corrected).

experiments at 5 and 21°C for a period of 32 h. This time span reflects the 24-h autosampling period required for WWTP performance testing in the European Community and an 8-h postsampling phase (equivalent to 1 working day) that includes sample transport and processing. Surprisingly, in contrast to natural systems such as rivers and lakes, no information is available for raw and treated wastewater of municipal origin regarding the persistence of fecal indicators and genetic markers (19–23). Here, the fecal indicator bacteria *Escherichia coli*, enterococci, and *Clostridium perfringens* spores were selected as representatives for cultivation-based standard determination, while molecular quantification by quantitative PCR (qPCR) was used to elucidate total and human-associated genetic *Bacteroidetes* markers. Additionally, 16S rRNA gene-based next-generation sequencing (NGS) was used to selected samples to further evaluate the results recovered from the microbial communities investi-

gated on a more general screening level. We hypothesized that only the spores of *C. perfringens* are appreciably stable in raw and treated wastewater of municipal origin, whereas vegetative cells of *E. coli* and enterococci, as well as genetic markers of *Bacteroidetes*, exhibit significant concentration reductions at 5 and 21°C during the storage period selected.

MATERIALS AND METHODS

WWTPs investigated. Three municipal WWTPs (no. 2, 3, and 4) in the area of Vienna, Austria, with sizes ranging from 23,000 to 140,000 PE, were selected as representative plants for the Austrian/European region (24). For detailed information on the characteristics of the WWTPs, the chemical analysis of the raw and treated wastewater, and the methodology, see Table S1 in the supplemental material. Samples were taken in both summer and winter to account for potential seasonal differences. Industrial influence at the selected plants was moderate, and thus no inhibitory or toxic effects were expected. The annual mean chemical oxygen demand (COD) and total nitrogen (TN) and total phosphorus (TP) concentrations in the raw municipal wastewater investigated were 460 to 560, 45 to 55, and 4 to 10 mg liter^{-1}, respectively. At the time of this study, WWTPs 3 and 4 were using activated sludge treatment with nitrification and denitrification. Phosphorus removal was achieved by chemical precipitation, which is required for sensitive areas in the European Union (25). Overall, elimination rates for COD, TN, and TP were ≥94%, ≥90%, and approximately 80%, respectively. In contrast to WWTPs 3 and 4, WWTP 2 was overloaded without showing denitrification, and it therefore displayed low rates of nitrogen removal. No disinfection was applied at the WWTPs investigated.

Sampling and microcosm experiments. Grab samples from the influent and effluent sites of the WWTPs investigated were collected in sterile 5-liter plastic bottles (Azlon, Great Britain). Samples were kept cold in the dark and immediately transported to the laboratory. There, samples were thoroughly shaken, subdivided between two 2-liter bottles, carefully temperature equilibrated within 3 to 5 h (the time required depended on the sampling temperature), and incubated at 5 ± 2 or 21 ± 1°C for batch culture microcosm experiments spanning a minimum of 168 h. Although the main focus of the experiments was on persistence during short-term storage (≤32 h), some points of observation were also selected at incubation times of >32 h to achieve a reference to longer-term storage. At defined intervals (Table 1), 70-ml subfractions were recovered from the microcosms, homogenized in an ultrasonic bath (SONOREX; Bandelin, Germany) for 5 min, and subjected to microbiological analyses (analyses were performed with several dilutions and duplicates). Before subfractions were removed from microcosms, they were thoroughly shaken with inversion of the bottles. The remainder of each 5-liter municipal wastewater sample was used for chemical analysis (see Table S1 in the supplemental material). The extent of statistical variation at the experimental trial level of the microcosms was also estimated. This was done during four persistence experiments by using replicate measurements for AllBac, BacHum-UCD, and HF183 TaqMan qPCR determinations. The results did not reveal any detectable systematic effect on the regression coefficients due to the replication effort (Mann-Whitney U test, $P > 0.5$, $n = 4 \times 12$).

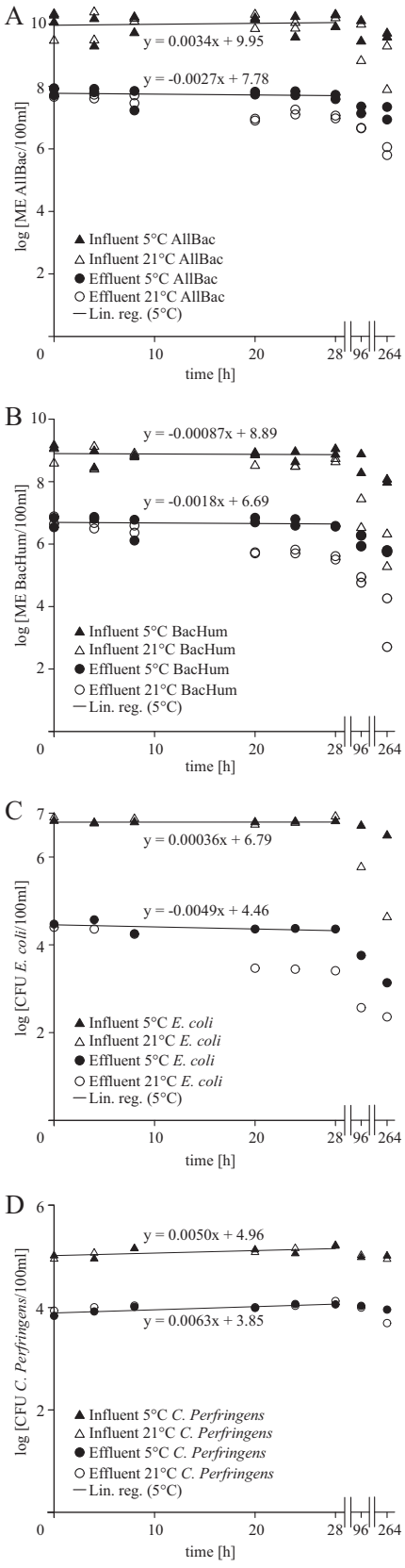
Microbiological and molecular analyses. Cultivation-based enumeration of *E. coli* bacteria, enterococci, and *C. perfringens* spores was performed by membrane filtration using appropriate dilutions as previously described (26, 27). For quantification of *C. perfringens* spores, 5-ml (influent) and 15-ml (effluent) aliquots from the batch sample were pasteurized at 60 ± 2°C for 15 min. *C. perfringens* was analyzed according to ISO standard 14189 (28), on the basis of selective growth on tryptose sulfite cycloserine agar (Scharlau, Spain) at 44°C and subsequent colony identification by acid phosphatase reaction (29). Enumeration of presumptive *E. coli* bacteria on the basis of ISO standard 16649-1 (30) was done with chromogenic tryptone bile agar with X-glucuronide (Oxoid, Thermo Fisher Scientific Inc., Cheshire, United Kingdom) at 44°C. Enumeration of enterococci on the basis of ISO standard 7899-2 (31) was done with Slanetz-Bartley medium (Oxoid) and dry-heat incubation at 44 ± 0.5°C for 44 ± 4 h. Appropriate control strains were used to ensure the quality of the medium.

Detection of genetic MST markers was based on total and human-associated *Bacteroidetes* assays. Respective 16S rRNA gene markers for AllBac (32), BacHum-UCD (33), and HF183 TaqMan (34) were quantified by qPCR. For DNA extraction, we used polycarbonate membrane filtration (0.2-μm Isopore membrane filter GTTP; Millipore, Cork, Ireland) of 10-ml (influent) and 50-ml (effluent) batch sample aliquots, as previously described (35, 36), followed by phenol-chloroform DNA extraction. Cell lysis was carried out with a FastPrepR-24 Instrument (MP Biomedicals Inc., Irvine, CA) at a speed setting of 6 m/s for 30 s each. The extracted DNA was stored at −20°C prior to analysis of two dilutions (10- and 100-fold) to test for PCR inhibition. The rotor discs were loaded with Master Mix and sample by a Qiagility Robot (Qiagen, Hilden, Germany), and measurements were subsequently performed on a Rotorgene Q Cycler (Qiagen). For the AllBac qPCR assay, we used 2.5 μl of the appropriate DNA sample dilution, 600 nM primer AllBac296f, 600 nM primer AllBac412r, 25 nM TaqMan MGB probe AllBac375Bhqr (32), 0.4 g liter^{-1} bovine serum albumin (Roche Diagnostics, Mannheim, Germany), and 7.5 μl of iQ Supermix (Bio-Rad, Hercules, CA) in a total reaction volume of 15 μl. We also added 5 mM $MgCl_2$ to obtain a total Mg^{2+} concentration of 8 mM (32). For the BacHum-UCD assay, we used 2.5 μl of the respective DNA sample dilution, 400 nM primer BacHum-160f, 400 nM primer BacHum-241r, 80 nM TaqMan MGB probe BacHum-193p (33), 0.4 g liter^{-1} bovine serum albumin, and 7.5 μl of iQ Supermix in a total reaction volume of 15 μl. For the HF183 TaqMan assay, we used 2.5 μl of the respective DNA sample dilution, 100 nmol liter^{-1} primer HF183, 100 nmol liter^{-1} primer BFD REV, 80 nmol liter^{-1} TaqMan MGB probe BFDFAM (34), 0.4 g liter^{-1} bovine serum albumin, and 7.5 μl of iQ Supermix in a total reaction volume of 15 μl. The PCR program for AllBac was 95°C for 3 min and 45 cycles of 95°C for 30 s and 60°C for 45 s. For BacHum-UCD, the PCR program was 95°C for 3 min and 45 cycles of 95°C for 15 s and 60°C for 1 min. For the HF183 TaqMan assay, the PCR program was 95°C for 3 min and 45 cycles of 95°C for 15 s and 60°C for 30 s. Real-time data were collected during the 60°C primer-annealing step. Quantification was based on appropriate standard dilutions of plasmid DNA (37) and presented as marker equivalents per volume (ME/vol) according to Reischer et al. (36). For a detailed description of the NGS methodology used here, which was based on the V1-V2 region of the 16S rRNA gene, see the supplemental material.

Data analysis and statistics. All microbial data were expressed as $\log_{10}(x + 1)$. Regression analysis and descriptive statistics were calculated with IBM SPSS Statistics version 20.0.0 (IBM, Germany). To account for the multiple tests that were carried out, statistical significance levels were Bonferroni corrected. All graphs were prepared with SigmaPlot 11.0 (SPSS Inc., Chicago, IL) and CorelDraw X5 (Corel, Canada).

RESULTS

All experiments with raw municipal wastewater samples, including influents from WWTPs 2, 3, and 4, revealed high stability of the microbiological parameters investigated at 5 and 21°C during the 32-h storage period (Tables 1 and 2; Fig. 1). Only 2 of 64 regression coefficients of microcosm experiments using raw wastewater displayed a negative value that deviated significantly from zero ($P \leq 0.05$, Bonferroni corrected). These statistically significant regression coefficients were from the human-associated *Bacteroidetes* marker BacHum-UCD and HF183 TaqMan, accounting for a maximum 0.5-$\log_{10}$ concentration decrease in the regression model during storage for 32 h at 21°C (Table 2). All measurements of vegetative *E. coli* and enterococci and the genetic *Bacteroidetes* markers resulted in more pronounced concentration decreases at the 96- and 264-h time points. *C. perfringens* spores



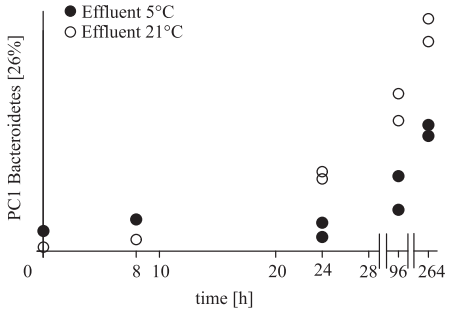


FIG 2 16S rRNA gene-based qualitative UniFrac community structure dynamics in the microcosm experiments with WWTP 2 effluent. The first principal coordinate (PC1) versus time is shown for the phylum *Bacteroidetes* (26% of the total variance is explained by PC1) on the *x* and *y* axes, respectively. Black and open dots represent microcosm experiments at 5 and 21°C, respectively. Analyses at the 0- and 8-h time points are shown as a single analysis, whereas analyses at the 24-, 96-, and 264-h time points are shown as duplicate analyses.

did not show any relevant concentration decrease during the whole observation period (Fig. 1; Tables 1 and 2).

The persistence of the microbial parameters investigated in treated wastewater samples at 5°C was also high (Fig. 1). With the exception of one experiment, regression analysis did not detect any statistically significant changes in the time frame investigated (Table 1). In contrast, nine of the microcosm experiments carried out with treated wastewater at 21°C revealed significant negative regression coefficients for *E. coli* and the genetic *Bacteroidetes* markers ($P \leq 0.05$, Bonferroni corrected, Table 2). Concentration decreases of up to 1.9 $\log_{10}$ for a 32-h storage period were apparent when the regression model was used (Table 2). Additionally, all measurements taken at 96 and 264 h yielded large and significant reductions for *E. coli*, enterococci, and the genetic *Bacteroidetes* markers; again, no notable decrease in *C. perfringens* spores was found in any of these storage experiments (Table 1; Fig. 1).

To further evaluate our results regarding the 16S rRNA gene bacterial community composition and the persistence of *Bacteroidetes* populations at the phylum scale, one representative microcosm series from the WWTP 2 effluent was chosen for additional 454 amplicon pyrosequencing analysis. Taxonomic pyrosequencing analysis of the 16S rRNA gene microbial community composition revealed a clear predominance of the phyla *Proteobacteria* and *Bacteroidetes*, with average relative abundances of 60% ± 5% and 27% ± 6%, respectively. The next most predominant phyla were *Actinobacteria* and *Firmicutes*, with average abundances of 2% ± 0.6% and 2% ± 0.7%, respectively. Microbial community structure analysis with a unweighted UniFrac algorithm combined with principal-coordinate analysis did not de-

FIG 1 Persistence of standard fecal indicators and genetic MST markers in raw (influent) and treated (effluent) municipal wastewater at 5 and 21°C. The data shown are a representative set; Tables 1 and 2 contain the complete data. Linear regression analysis was performed for 28 h and is shown only for the 5°C storage conditions (values for samples taken at 96 and 264 h are given as control measurements). Panels: A, AllBac analysis of genetic fecal markers for the total *Bacteroidetes* populations; B, BacHum-UCD analysis of genetic fecal markers for human-associated *Bacteroidetes* populations; C, cultivation-based enumeration of *E. coli* bacteria; D, cultivation-based enumeration of *C. perfringens* spores. Lin. reg., linear regression.

tect any notable changes in the *Bacteroidetes* community composition during the short-term period of storage at 5°C investigated (Fig. 2). In contrast, major changes in the total *Bacteroidetes* community structure became apparent under 21°C incubation conditions and also at the later time points (96 and 264 h) of the 5°C microcosms experiments (Fig. 2).

DISCUSSION

The data obtained from the microcosm experiments clearly contradicted the initial hypothesis regarding the low persistence of the microbial indicators investigated in municipal wastewater during short-term storage (32 h) at 5°C. In addition to the highly resistant *C. perfringens* spores (26, 38, 39), the vegetative *E. coli* cells and the genetic *Bacteroidetes* markers displayed remarkable stability at 5°C in the defined time frame. Although qPCR-based detection of a genetic DNA marker does not indicate cell viability (40), a significantly increasing or decreasing trend in the DNA target concentration due to cell growth, degradation, or grazing effects would have been detected by the molecular quantification methods used here (6, 41). Furthermore, the stability of the molecular signatures of *Bacteroidetes* cells was supported by data on the differing taxonomic levels investigated, which were quantified by the BacHum-UCD, HF183 TaqMan, and AllBac qPCR assays (32–34) and qualitatively screened by 16S rRNA gene NGS community structure analysis (42).

Strong decreases in the representative bacteria were observed only in the microcosm experiments at 21°C using untreated wastewater samples, with *E. coli* and genetic *Bacteroidetes* markers displaying losses of up to 99% of their original populations (Table 2). However, not all of these experiments yielded such a marked decrease, most likely because storage periods longer than 32 h would have been needed to reach these levels. No signs of toxicological inhibition of the microbial community in the activated sludge, which generally manifests itself as inhibition of aerobe/anaerobe heterotrophy or specific inhibition of nitrification, were discernible at the WWTPs (see the WWTP data in the supplemental material). Measurements at 96 and 264 h also revealed a clearly decreasing response, further supporting the absence of inhibiting substances. Very surprisingly, no decreasing effect was detectable in the microcosm experiments with raw municipal wastewater samples at 21°C. Extremely high levels of organic substrates (CODs of up to 680 mg liter^{-1} were measured in raw municipal wastewater), and the absence of oxygen may have contributed to this short-term stability effect. This is only a preliminary speculation, and further investigations beyond the scope of this study are needed to clarify the actual reason for our observation.

The effluent and influent characteristics selected represent a typical range of municipal wastewaters occurring at WWTPs in Austria (see Table S1 in the supplemental material) with respect to catchment type, wastewater channels, and treatment plant performance (24). Our results can be taken as a strong indication that microbial persistence is not a limiting factor in short-term storage at 5°C of raw and treated municipal wastewater samples. It is important to emphasize that disinfection was not applied at the WWTPs investigated. Disinfection is not required for biological treated wastewater according to Austrian and European regulations. Disinfection is considered only in sensitive areas used for bathing or drinking water production and not for receiving waters without a particular use. Furthermore, the proportion of industrial wastewater input was low to moderate at the WWTPs investigated. No specific inhibitory effects or toxic substances have been reported for these WWTPs (e.g., for respiratory or nitrification measurements). The results obtained thus relate to nondisinfected raw and biological treated wastewater of municipal origin, without the occurrence of microbicidal substances from industrial effluents. Pyrosequencing-based 16S rRNA gene community analysis also demonstrated the typical bacterial community composition expected of wastewater of municipal origin (43, 44). The investigation of effects of disinfection or toxic compounds on the persistence of indicators or fecal markers was not the aim of this study. However, in future, it might also be interesting to elucidate the effect of microbicidal conditions on microbiological parameters with different endpoints during short-term storage (e.g., cultivation-based enumeration versus direct detection of nucleic acids). Further studies may also focus on analysis of the activity of the bacterial community considered at 5°C.

In conclusion, we can recommend 24-h autosampling procedures under 5°C storage conditions not only for chemical analysis but also for representative microbiological investigations of raw and biological treated wastewater of municipal origin when using bacterial standard fecal indicators or *Bacteroidetes* genetic MST markers. Such autosampling procedures will contribute significantly to a better understanding and monitoring of municipal WWTPs as sources of fecal contamination of water resources (1, 45).

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Appendix:

Sampling form

TECHNISCHE UNIVERSITÄT WIEN
Vienna University of Technology

ICC Interuniversity Cooperation Center Water & Health

IC-SEWAGE
http://www.cws.msu.edu/ic-sewage/

Sampling and Shipment Protocol

Global *Bacteroidetes* evaluation study 2013+ (WWFWII)

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Information on Cooperation Partner:

Name of Institution		Name of Cooperation Partner (contact):	
Address		Country/State	
Email		Phone	

Information on Sampling and Shipment WWTP 1

(please refer to SOPs & treatment plant selection criteria before starting activities)

Person in charge of sampling		e-mail		tel	
Person in charge of filtration		e-mail		tel	
Person in charge of shipment		e-mail		tel	
Sampling date					
Sampling time (influent, hh:mm)					
Sampling time (effluent (hh:mm)					
Filtration date		Starting time filtration (hh:mm)			

Used label influent ☐ Used label effluent ☐

Additional information on sampling site/ filtration ☐

Pictures on
- ☐ Treatment plant
- ☐ Influent sampling location
- ☐ Effluent sampling location

Shipment company ☐

Shipment date ☐

Shipment time ☐

Additional information on shipment ☐

Basic Information on Wastewater Treatmant Plant 1

Plant at ☐ urban area ☐ rural area

WWTP Name ☐ Location (nearest city/village) ☐

Latitude: ☐ Longitude: ☐

Operating company (if possible) ☐

Communal sewage (household) probably influenced by:

Industry: ◯ no ◯ slight ◯ moderate ◯ strong

Livestock: ◯ no ◯ slight ◯ moderate ◯ strong

other (please specify) ☐
- ◯ slight
- ◯ moderate
- ◯ strong

WWTP Population Equivalents: ☐ daily volume influent ☐

Sewer system ☐ combined sewer system ☐ seperate sewer system

Chemical Data WWTP I

Influent: (all values mg per litre)

Data from ◯ own investigation ◯ treatment plant company

Data retrieved
◯ directly out of the grab sample (recommended)
◯ different sample but same day
◯ average data for a longer period of wwtp investigation

Date of analysis		
Chemical oxygen demand		applied method:
Biological oxygen demand (BOD5)		applied method:
Ammonium Nitrogen (NH4-N)		applied method:
Nitrate Nitrogen (NO3-N)		applied method:
Phosphate Phosphorus (PO4-P)		applied method:
Total Phosphorus:		applied method:
Total Nitrogen:		applied method:

Effluent: (all values mg per litre)

Data from ◯ own investigation ◯ treatment plant company

Data retrieved
◯ directly out of the grab sample (recommended)
◯ different sample, same day, same samplepoint
◯ average data for a longer period of sampling

Date of analysis		
Chemical oxygen demand (COD)		applied method:
Biological oxygen demand (BOD5)		applied method:

Ammonium Nitrogen (NH4-N)		applied method:	
Nitrate Nitrogen (NO3-N)		applied method:	
Phosphate Phosphorus (PO4-P)		applied method:	
Total Phosphorus:		applied method:	
Total Nitrogen:		applied method:	
Additional information:			

Information on Sampling and Shipment WWTP 2

(please refer to SOPs & treatment plant selection criteria before starting activities)

Person in charge of sampling		e-mail		tel	
Person in charge of filtration		e-mail		tel	
Person in charge of shipment		e-mail		tel	
Sampling date					
Sampling time (influent, hh:mm)					
Sampling time (effluent (hh:mm)					
Filtration date		Starting time filtration (hh:mm)			
Used label influent		Used label effluent			
Additional information on sampling site/ filtration					

Pictures on
- ☐ Treatment plant
- ☐ Influent sampling location
- ☐ Effluent sampling location

Shipment company

Shipment date

Shipment time

Additional information on shipment

Basic Information on Wastewater Treatmant Plant 2

Plant at ☐ urban area ☐ rural area

WWTP Name | Location (nearest city/village)

Latitude: | Longitude:

Operating company (if possible)

Communal sewage (household) probably influenced by:

Industry: ◯ no ◯ slight ◯ moderate ◯ strong

Livestock: ◯ no ◯ slight ◯ moderate ◯ strong

other (please specify) ◯ slight ◯ moderate ◯ strong

WWTP Population Equivalents: | daily cubic meter influent

Sewer system ☐ combined sewer system ☐ seperate sewer system

Chemical Data WWTP II

Influent: (all values mg per litre)

Data from ◯ own investigation ◯ treatment plant company

Data retrieved ◯ directly out of the grab sample (recommended)
◯ different sample, same day, same samplepoint
◯ average data for a longer period of sampling

Date of analysis			
Chemical oxygen demand (COD)		applied method:	
Biological oxygen demand (BOD5)		applied method:	
Ammonium Nitrogen (NH4-N)		applied method:	
Nitrate Nitrogen (NO3-N)		applied method:	
Phosphate Phosphorus (PO4-P)		applied method:	
Total Phosphorus:		applied method:	
Total Nitrogen:		applied method:	

Effluent: (all values mg per litre)

Data from ◯ own investigation ◯ treatment plant company

Data retrieved ◯ directly out of the grab sample (recommended)
◯ different sample, same day, same samplepoint
◯ average data for a longer period of sampling

Date of analysis			
Chemical oxygen demand (COD)		applied method:	
Biological oxygen demand (BOD5)		applied method:	

Ammonium Nitrogen (NH4-N)		applied method:	
Nitrate Nitrogen (NO3-N)		applied method:	
Phosphate Phosphorus (PO4-P)		applied method:	
Total Phosphorus:		applied method:	
Total Nitrogen:		applied method:	
Additional information:			

Danke! Gracias! Thank YOU! Dankie!

Appendix:

Curriculum vitae

Curriculum Vitae

Mag. rer. nat. **René Mayer**

Persönliche Daten

Geburtsdatum, Ort:	23. April 1985, Wien
Nationalität:	Österreich
Adresse:	Schwendergasse57/8, A- 1150 Wien
Telefon:	0676/63 73 009
e-Mail:	rene.mayer@tuwien.ac.at

Ausbildung

09/2011 -	**Technische Universität Wien** **Dr.-Studium der Naturwissenschaften Technische Chemie**
10/2003 -	**Technische Universität Wien** **Diplomstudium der Technischen Chemie**
10/2003 – 08/2011	**Technische Universität Wien** **Diplomstudium des Lehramtes Chemie/Mathematik**
09/1995 – 06/2003	**Allgemeinbildende Höhere Schule** Bundesrealgymnasium Marchettigasse, A-1060 Wien Abschluss: Matura - Juni 2003

Wissenschaftliche Arbeiten und Erfahrungen

09/2011 -	**Projektassistent (FWF) Farnleitner Lab, TU Wien** Fäkales Sourcetracking entlang des kommunalen Abwasserpfades (GeBaM)
01/2012	**Auslandsaufenthalt** Laboratory of Virus Contaminants of Water and Food, Faculty of Biology, University of Barcelona, Spain Erlernen neuer Methoden zur Virendetektion und Etablierung im Labor Wien