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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	

(Continued on following page)

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	

(Continued on following page)

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 post-sampling 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, $P > 0.5$, $n = 4 \times 12$).

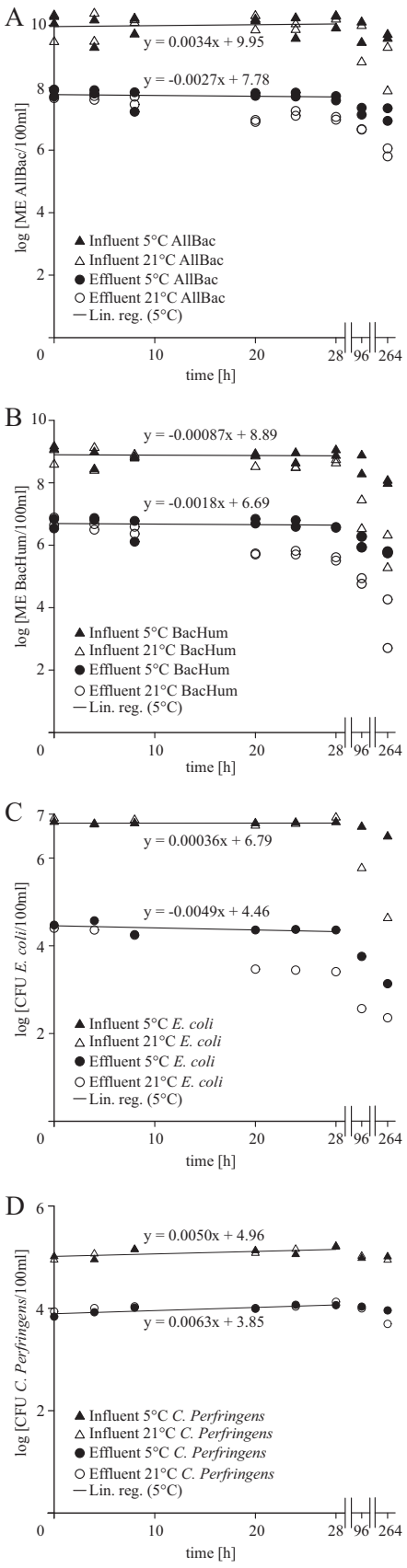
Microbiological and molecular analyses. Cultivation-based enumeration of *E. coli* bacteria, enterococci, and *C. perfringens* spores was performed by membrane filtration using appropriate dilutions as previously described (26, 27). For quantification of *C. perfringens* spores, 5-ml (influent) and 15-ml (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



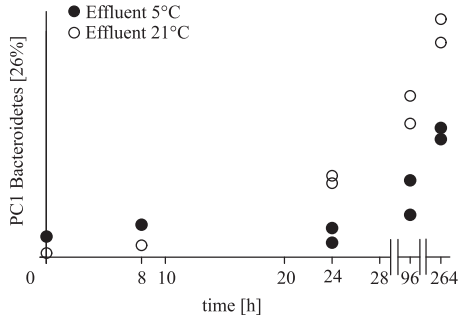


FIG 2 16S rRNA gene-based qualitative UniFrac community structure dynamics in the microcosm experiments with WWTP 2 effluent. The first principal coordinate (PC1) versus time is shown for the phylum *Bacteroidetes* (26% of the total variance is explained by PC1) on the *x* and *y* axes, respectively. 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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