

Development of a new RT-PCR multiplex assay for poliovirus detection

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ABSTRACT

Potent vaccines to protect individuals from poliomyelitis are available and great achievements have been reached by the Global Polio Eradication Program (GPEI). Different objectives (i.e. surveillance, outbreak events) require scalable screening of samples. Although cell culture-based analysis is still needed due to the benefits regarding detailed characterization of the isolated virus, it is also time consuming and intensive lab work is required, limiting the number of samples that can be analyzed. Reliable and sensitive molecular methods would increase the capacity for high-throughput testing. In this study we designed a poliovirus type-specific multiplex RT-PCR assay allowing swift screening of various samples by real-time or digital RT-PCR. The validation data demonstrate a reliable assay in respect of sensitivity and specificity for different clinical specimens, wastewater samples as well as virus isolates. The limit of detection of the multiplex assay ranged from 7 to 50 copies/reaction, depending on the PV serotype and PCR system used. Dilution series of spiked wastewater samples showed a 10–100-fold higher detectability of PV by digital RT-PCR compared to the real-time RT-PCR technique using the designed assay. The designed assay allows rapid PV testing for different matrices to prioritize subsequent analysis.

1. Introduction

During the progress of the Global Polio Eradication Initiative (GPEI) initiated by the World Health Organization (WHO) and their partners in 1988, the number of poliovirus (PV) infections worldwide has been reduced by more than 99.99% (WHO, 1988; Rachlin et al., 2022). The eradication of wild poliovirus type 2 and type 3 has already been declared in 2015 and 2019 (WPV2 and WPV3), respectively (Chard et al., 2020; WHO, 2015). To date, only two countries are still endemic for WPV1 (Pakistan and Afghanistan) (Rachlin et al., 2022). Nevertheless, the risk remains that WPV1 can be reintroduced into countries that are polio-free (Anis et al., 2013). Another threat for the eradication program is the circulation of vaccine-derived poliovirus (VDPV), which originate from continued transmission of attenuated oral poliovirus vaccine (OPV) strains or due to long-term shedding. Replicating OPV strains recover their neurovirulence by reversion of their attenuation sites (Burns et al., 2014). The number of poliomyelitis cases caused by VDPV currently exceeds the number caused by WPV (Alleman et al., 2021).

The WHO Region Europe was declared polio-free in June 2002 (CDC, 2002). To maintain the polio-free status, high vaccination rates and sufficient surveillance systems are required. The surveillance of acute flaccid paralysis (AFP) is still the gold standard. Due to the success of the GPEI and the low manifestation rate of poliovirus infections (<1% infected unvaccinated persons develop an AFP) (Howe, 1949; Nathanson and Kew, 2010), the detection of poliovirus infections based solely on syndromic surveillance systems becomes less likely. Therefore, the importance of environmental surveillance (ES) as a supplementary tool is increasing, since it also allows PV detection regardless of the manifestation status in individuals. This has been shown in several events in polio-free countries, where VDPV2 could be detected in wastewater samples taken in UK, US, Israel and Canada in 2022 and in Spain, Poland, Germany, UK and Finland in 2024 (Böttcher et al., 2025; Klapsa et al., 2022; WHO, 2022; Ryerson et al., 2022; Wiczorek et al., 2025; Zuckerman et al., 2022).

Currently, the detection of PV in stool samples from patients with AFP and wastewater samples is mainly based on cell culture techniques. However, cell culture workflows require trained staff, special laboratory

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equipment and the process is time consuming. This leads to a bottleneck regarding the number of samples that can be analyzed. Furthermore, the handling of PV isolates should also be minimized with regards to containment requirements.

During a research project funded by the German Federal Ministry of Health a multiplex type-specific PV RT-PCR assay for the analysis of clinical and wastewater samples was designed and implemented on a real-time (qRT-PCR) and digital PCR (dPCR) system. As far as is currently known, this is the first description of a dPCR approach for PV detection. The assay was checked for specificity, cross-reactivity with other non-polio enteroviruses (NPEV) using clinical and wastewater samples and the limit of detection (LOD) was determined. Results of spiked wastewater testing indicated a close sensitivity to the established cell culture workflow.

2. Methods

2.1. Design of poliovirus type specific real-time RT-PCR multiplex assay

Primer and probes used for the real-time RT-PCR assay were designed by using the qPCR mode of varVAMP, a free online tool to design pan-specific degenerated oligonucleotides for tiled-PCR and qPCR (Fuchs et al., 2025). In brief, alignments of reference sequences (PV1 = 241, PV2 = 494, PV3 = 209) were used for the design of the assays. Primer and probe combinations, binding to the genome region encoding for structural proteins (P1) and having the best score were selected. Synthesized DNA fragments (gBlocks) comprising the corresponding amplicon region were used for initial tests. The gBlocks contained the genomic region from nucleotide position 1563–2040 for PV1 (NCBI Accession: AY184219), 1858–2332 for PV2 (NCBI Accession: AY184220) and 1560–1979 for PV3 (NCBI Accession: AY184221). The RT-PCR oligomers are summarized in Table 1.

2.2. Multiplex real-time RT-PCR

All three designed PV type specific real-time RT-PCRs were combined in a multiplex-RT-PCR using the 4X CAPITAL™ 1-Step qRT-PCR Probe Master Mix chemistry (BR0502002, Biotectrabbt, Germany) on a Roche Light Cycler 480 instrument according to the manufacturer's instruction with 5 µl template volume in a 20 µl reaction mix. Final concentrations of primers (0.4 µM) and probes (0.2 µM) were identical for each PV assay. The reaction was initiated by reverse transcription at 50 °C for 10 min followed by an activation step at 95 °C for 3 min. Target amplification was carried out in 45 cycles (95 °C for 10 s, 59 °C for 30 s). A color compensation run was executed to reduce cross talk signals between the probes. Real-time data were analyzed using the software provided by the Light Cycler 480 instrument. A sample was considered positive if at least one of the technical replicates was positive ($C_p \leq 40$).

2.3. Digital RT-PCR

Digital RT-PCR (RT-dPCR) of the poliovirus multiplex assay was

carried out using the QIAcuity Probe PCR Kit on a QIAcuity One instrument following the manufacturer's instructions. The reaction mix contained 0.4 µM of each primer and 0.2 µM of each probe. Final reaction volume was 40 µl containing 20 µl of the template. The cycling profile consisted of 50 °C for 30 min followed by 95 °C for 2 min and target amplification for 45 cycles (95 °C for 10 s and 59 °C for 1 min). Imaging the nanoplate was done using the green, yellow and crimson detection channels. Experiments were done using 26 K 24-well nanoplates. All samples were measured in triplicates and data were analyzed with the QIAcuity Software Suite 3.1.1.0.

2.4. Wastewater sampling, concentration and spiking experiments

One liter of wastewater 24h-composite samples was taken and shipped to the German Environment Agency (UBA), while maintaining the cold chain. Concentration of the wastewater was performed using a polyethylene glycol 6000 (PEG)/NaCl precipitation-based workflow. Briefly, wastewater samples were homogenized by head-over-head mixing at room temperature and were then adjusted to pH 7.2. Next, 80 g/L PEG6000 and 17.5 g/L NaCl were added and the solution was stirred for 1 h at room temperature. The sample was incubated overnight at 5 °C and subsequently centrifuged for 15 min at 6800 g at 10 °C. Then, the sample supernatant was discarded and a second centrifugation step was carried out for 20 min at 3500 g. The resulting pellet was resolved in 7 ml PBS buffer, vortexed thoroughly, and subsequently 7 ml chloroform and 70 µl Tween 80 were added. After incubation for 15 min at room temperature under agitation, an additional centrifugation step was performed for 15 min at 1400 g at 10 °C. The supernatant was transferred into a collection tube and the chloroform phase was removed. The remaining pellet was solved in 3 ml 3% beef extract (pH 7.2) to release potential residuals. Finally, following 15 min of shaking and a final centrifugation step for 15 min at 1400 g at 10 °C, the resulting supernatant was merged with the supernatant collected earlier. The prepared concentrates had a final volume between 10 and 15 ml and were stored at –20 °C.

Spiked samples were used to test the sensitivity of the different PV detection workflows. For conducting the spiking experiments, defined amounts of vaccine poliovirus type 1 and type 3 (Sabin 1 and Sabin 3 strains; 10^{-10^2} , 10^{-10^3} , 10^{-10^4} , 10^{-10^5} , 10^{-10^6} , 10^{-10^7} , 10^{-10^8} PFU/L), were spiked to batches of 24h-composite wastewater samples and stirred for 1 h for homogeneity.

For real-life testing of wastewater matrices, 40 samples from one sampling site were taken between week 23/2024 and week 14/2025.

2.5. Cell culture

Cell culture workflow was mainly carried out according to the polio laboratory manual and virus isolation from clinical samples was performed as described elsewhere (Keeren et al., 2021; WHO, 2004).

For PV wastewater testing, wastewater concentrates were centrifuged for 10 min at 14,500 g. The resulting supernatants were either directly used for cell culture or stored at –20 °C. Suspension cultures of

Table 1
Used PV-specific oligonucleotides.

Name	Primer and Probe Sequence 5'-3'	Binding region	Reference genome nucleotide position	NCBI genome accession number
NRZ 533	TGAACACCCCTGGTAGYAATCA	VP3	1779–1800	AY184219 (Sabin 1)
NRZ 534	TGTCAATAGGTGGGGTCACATCAA	VP3	1871–1848	
TM-21	FAM-ACAACCTCCAGTCACCGTGTGCGCT-BHQ1	VP3	1815–1839	
NRZ 515	CCRATCTTGTGTCTCTCGTTGT	VP3	2008–2029	AY184220 (Sabin 2)
NRZ 516	AACITTCGCGTGGCCATCATTTG	VP3	2156–2135	
TM-18	JOE-CGCTTCAGACCCAGATTGGCACACA-BHQ1	VP3	2034–2059	
NRZ 535	CAATWGCCCAATGTGTAGCGA	VP2	1660–1682	AY184221 (Sabin 3)
NRZ 536	GGATTGCGCATGGTGAITGGT	VP3	1798–1777	
TM-22	CY5-AACGGCCTTCGCAACGTGACTGC-BBQ650	VP2	1686–1709	

Degenerate nucleotide code according to IUPAC nomenclature: Y = C/T, R = A/G, W = A/T

five L20B and three RD-A T25 flasks were inoculated with 500 µl of the wastewater concentrate and assessed frequently over a period of seven days for the presence of a cytopathic effect (CPE). Aliquots of RD-A cell culture supernatants showing a CPE were transferred to a fresh L20B cell culture. All L20B cell cultures showing a CPE were analyzed for the presence of poliovirus regardless of whether they resulted from a L20B flask only (L-arm) or RD-A cell culture (RL-arm).

All experiments were conducted in separate laboratories (BSL3**) to prevent parallel handling of clinical and wastewater samples.

2.6. RNA extraction of wastewater samples

RNA extraction using 1.2 ml of the wastewater concentrate was done using the MagMAX Viral RNA Isolation Kit based on a protocol for large volume RNA extraction (Bujaki et al., 2024) with slight modifications (available upon request). Elution was performed using 200 µl of the elution buffer. Extraction was carried out using the automated extraction mode of the King Fisher Duo Prime device.

2.7. Samples used for validation

The supernatants of positive L20B cultures were collected and RNA was extracted using the QIAmp viral RNA Kit (cat. number 52906, Qiagen, Hilden, Germany) according to the manufacturer's protocol. PV confirmation and intratypic differentiation (ITD) was performed using VP1 Sanger sequencing as described previously (Kilpatrick et al., 2011; WHO, 2004).

RNA from clinical samples that were either positive for PV or NPEV or showed no evidence of EV presence, together with RNA from selected NPEV prototype strains and other gastrointestinal viruses (rota-, noro-, astro- and sapovirus), were used for PCR assay validation.

2.8. RT-PCR amplification and nanopore sequencing

Amplification of the VP1 region of PV by semi-nested RT-PCR for subsequent nanopore sequencing was performed according to the direct detection of poliovirus by nanopore sequencing (DDNS) protocol (Shaw et al., 2020). Barcoding was done using the Native Barcoding Kit 96 V14 (SQK-NBD114.96). Sequencing was performed on GridION with a FLO-MIN114 flow cell. Sequencing runs were executed for 24 h with super-accurate basecalling 400 bps using the MinKNOW software version 25.05.14.

2.9. Bioinformatic analysis of sequencing results

Nanopore reads were analyzed and consensus sequences were generated by a bioinformatic pipeline using QIAGEN CLC Genomics Workbench 25.01.1. Mapping was done with the Map Long reads to Reference 1.3 tool, using Sabin 1–3 as references (NCBI accession number: AY184219, AY184220, AY184221).

3. Results

3.1. Design and development of a multiplex RT-PCR poliovirus detection assay

Three single PV RT-PCR assays for PV1, PV2, and PV3 were designed using varVAMP (Fuchs et al., 2025) and the multiplex assay developed following the optimal temperature profile provided by the tool. In a first attempt gBlocks comprising the corresponding target regions were used in a dilution series (10^7 – 10^{-1} cp/µl) in the real-time RT-PCR. Lowest template concentrations that could be detected in at least one of the replicates were 10^0 copies/µl (corresponding to 5 copies/reaction). The PV multiplex assay was also adapted to the QIAquity One dPCR system.

3.2. In-vitro sensitivity and specificity

The LOD was determined on a qPCR and dPCR system using dilution series of Sabin 1 and Sabin 3 viral RNA. For containment reasons, we used the designed gBlock for the validation of the PV2 assay. Copy numbers were directly quantified in the dPCR software. Five different concentrations were tested on three different days with eight replicates per run. The LOD95 was determined as the lowest tested concentration showing 95% positive results. For the PV1 assay, the LOD95 was determined to 8.5 copies/reaction (1.7 copies/µl RNA) in the qPCR system and to 7 copies/reaction (0.35 copies/µl RNA) in the dPCR system. For the PV2 assay, the LOD95 was determined to 50 copies/reaction (10 copies/µl RNA) in the qPCR system and to 24.9 copies/reaction (1.25 copies/µl RNA) in the dPCR system. For the PV3 assay, the LOD95 was determined to 16.5 copies/reaction (3.3 copies/µl RNA) in the qPCR system and to 16 copies/reaction (0.8 copies/µl RNA) in the dPCR system.

To rule out cross reactivity between different PV types, RNA extracted from cell culture isolates of PV 1–3 (Sabin) were tested in serial dilution as well. Cross-reactivity was not observed.

Due to the absence of PV cases in Germany, only a limited number of positive clinical specimens have been available to assess PV multiplex assay performances. Therefore, stored RNA extracted from stool samples that had been tested positive for PV1 and/or PV3 collected between 2013 and 2024 were used (Böttcher et al., 2015). The multiplex qPCR assay detected all PV positive samples with the previously assigned PV type. One sample showed a positive amplification result for both PV1 (Ct 32.72) and PV2 (Ct 35.49) (Table 2). To verify the PV2 result NGS was performed using DDNS (Shaw et al., 2020). Using our bioinformatic pipeline, 13,575 reads mapped to PV1, 129 reads to PV2 and 0 reads to PV3, confirming the presence of PV1 and PV2 in a stool sample detected by the PCR-based approach. All reads covered the complete VP1 region.

To test for cross-reactivity with NPEV, RNA extracted from 60 high-titer virus isolates (17 selected prototype strains, 23 NPEV isolates from wastewater samples, 20 NPEV isolates obtained from clinical samples) and RNA of other gastrointestinal viruses (rota-, noro-, astro- and sapovirus) were tested by the multiplex qPCR assay.

A weak fluorescence signal below the positive control (10^{-4} dilution of PV1–PV3 RNA extract originating from cell culture isolates) according to the Cp value and the maximum fluorescence was observed for three prototype isolates: in the PV1 assay for coxsackievirus A13 (CVA13) and CVA1 and in the PV2 assay for CVA21. There was no cross-reactivity detected from current circulating NPEV isolates originating from wastewater or clinical samples.

Additionally, to account for various sample matrix interactions, 152 clinical specimens (99 stool samples, 41 CSF and 32 throat swabs) were analyzed of which 101 samples were tested positive for EV (68 stool, 31 CSF and 22 throat swabs). None of the samples showed a signal for any PV type in our multiplex assay (detailed data available on request).

These findings indicate a technical sensitive and specific PV multiplex assay regardless of the specimen source, PV or NPEV type.

Table 2
Human PV positive samples tested in multiplex assay.

Sample	ITD/VP1 result	PV multiplex RT-qPCR assay result	specimen
sample 1	PV3	PV3	stool
sample 3	PV3	PV3	stool
sample 4	PV1	PV1/PV2	stool
sample 10	PV3	PV3	stool
sample 13	PV1/PV3	PV1/PV3	stool
sample 14	PV3	PV3	stool
sample 15	PV3	PV3	stool

3.3. Comparison of cell culture- and PCR-based workflows for PV detection of environmental samples

Two sets of wastewater samples were spiked separately with serial log10 dilutions of Sabin 1 and Sabin 3 isolates and tested for poliovirus in a cell culture- and a RT-PCR-based workflow (Fig. 1). Sabin 2 was not included due to containment reasons. The cell culture-based workflow was able to detect PV1 and PV3 down to the lowest spiking level (10–100 Pfu/L) showing at least one positive cell culture flask. In the RT-PCR-based workflow PV1 was detected down to 10^{−4} (10⁴–10⁵ Pfu/L) and PV3 down to 10^{−5} (10³–10⁴ Pfu/L) on the real-time RT-PCR system. Using the dPCR system, the sensitivity increased 10-fold (PV3) to 100-fold (PV1) in comparison to the real-time RT-PCR, allowing to detect 100–1000 Pfu/L. Samples without spiked PV were not tested positive by any of the workflows.

Based on the spiking experiment results, 40 wastewater samples were analyzed using the new designed RT-dPCR assay. In 16 out of 40 samples at least one positive dPCR partition was recorded in one of the triplicates for at least one PV type (data not shown). Ten samples with two or more positive partitions were classified as “suspected PV positive” and analyzed in the cell culture-based workflow and with RT-qPCR for potential result confirmation (Table 3). PV were isolated by cell culture from four of the ten “suspected PV positive” wastewater samples. Only one sample, showing the highest dPCR signal, could be confirmed by RT-qPCR. Subsequent typing of the isolates confirmed the respective PV type previously detected by RT-dPCR. Sequencing of the virus isolates revealed that all obtained isolates were classified as Sabin-like PV.

4. Discussion

In this study, we established a multiplex RT-PCR workflow for the direct molecular detection of PV in clinical and environmental samples, enabling scalable prescreening on both real-time and digital PCR system.

To prove suitability of both workflows to be used for testing wastewater samples for poliovirus presence, spiked and real-life wastewater samples were used. Results showed that the sensitivity of the workflow can be increased by 10-fold to 100-fold for the PV1 and PV3 assay by using the dPCR system compared to the qPCR system. This suggests an additional value of the dPCR technology to enhance direct detection of PV from wastewater samples. This observation is in line with the results of the 40 screened wastewater samples, where only one sample showing the strongest RT-dPCR signal, could be confirmed by RT-qPCR. A sensitive detection of other pathogens by RT-dPCR was also reported for SARS-CoV-2 and measles from wastewater samples (Ahmed et al., 2022; Roman et al., 2025). An increased sensitivity would be a strong benefit for ES analysis as early warning system allowing to detect the introduction or silent circulation of PV in a population earlier than other surveillance systems. That would give more time to react with proper public health measures to prevent clinical cases.

The designed multiplex format does not show relevant cross-

Table 3
Comparison of RT-dPCR workflow of “suspected PV positive” wastewater samples with cell culture and RT-qPCR workflows.

Sample-ID	Number of positive partitions (RT-dPCR)			Number of Poliovirus isolates (cell culture)			RT-qPCR result
	PV1	PV2	PV3	PV1	PV2	PV3	
24-492	3	0	0	0	0	0	n. d.
24-561	2	0	1	0	0	0	n. d.
24-667	2	0	2	1	0	0	n. d.
24-770	0	0	81	0	0	8	PV3
24-818	8	0	0	0	0	0	n. d.
24-929	4	0	12	0	0	0	n. d.
24-1053	2	0	0	0	0	0	n. d.
25-0036	6	0	0	1	0	0	n. d.
25-0059	2	0	0	0	0	0	n. d.
25-0218	6	0	1	1	0	0	n. d.

n. d. = not detected

reactivity between the PV types and only weak signals were observed by selected NPEV prototype strains (CVA1, CVA13, CVA21). Other viruses shed via feces (rota-, noro-, astro- and sapovirus) and sample matrices background (stool, CSF, throat swabs and wastewater), that were tested did not induce any relevant cross-reactivity as well. Notably, we detected PV2 in a PV2 cell-culture negative tested stool sample from 2013 (sample 4, Table 2), that could be confirmed by NGS using the DDNS approach. The lower fluorescence signal in the real-time RT-PCR and the reduced number of mapped reads indicate a double infection where PV2 shedding was already decayed or noninfectious particles excreted. As the PV2 was not detected by WHO standard protocols, the RT-dPCR and DDNS (Shaw et al., 2020; Shaw et al., 2023; Ueno et al., 2023) combination could be an alternative cell culture free approach for the analysis of stool samples for PV infection. This is crucial for routine PV surveillance overall, as every PV detection needs to be further characterized as WPV, vaccine strain (Sabin or Sabin-like) or VDPV. As cell culture is time consuming and DDNS needs special equipment and bioinformatic pipelines, a sensitive and specific PCR-based assays for sample screening enables a more targeted sample processing. In contrast to cell culture or NGS, standard real-time PCR assays can be performed in hours and can be easily implemented in routine labs, increasing the test capacities.

Confirmation of RT-dPCR positive tested wastewater samples by cell culture underlines the suitability of our PV multiplex approach. As detection of PV in wastewater is mainly performed by cell culture, it is limited by the number of samples that can be tested simultaneously and in a timely manner. To increase PV test capacities for ES, the RT-dPCR workflow can be applied to screen a higher number of wastewater samples for relevant PV presence. As wastewater analysis is already established for different pathogens worldwide, same logistics can be used to screen for PV in routine laboratories. This would lower the costs for high-throughput analytics and minimize processing times because RT-dPCR “suspected PV positive” samples could be prioritized for further confirmation and analysis in specialized PV certified laboratories

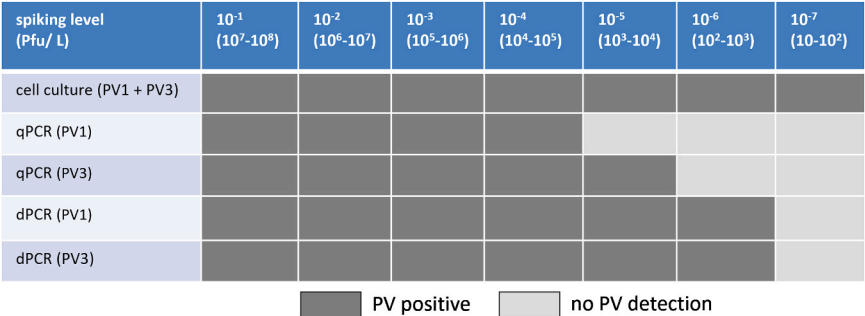


Fig. 1. Comparison of cell culture- and PCR-based workflow in PV spiked wastewater samples.

using the cell culture workflow.

Not all RT-dPCR-positive wastewater samples could be confirmed by cell culture. This could be explained by the putative detectability of noninfectious or degraded viral RNA. In addition, low-level background signals cannot be fully excluded in complex wastewater matrices.

Further studies are therefore required to define meaningful interpretation thresholds for RT-dPCR positive wastewater samples and to determine at which signal strength additional analyses, such as cell culture or DDNS are helpful.

The use of PV type-specific multiplex qRT-PCR for clinical samples and dRT-PCR for wastewater samples enables sensitive high-throughput screening and may support prioritization of samples for downstream analyses such as cell culture and sequencing as a meaningful supplement to existing poliovirus surveillance systems. Further studies will show the comparability between cell culture and dPCR.

CRediT authorship contribution statement

Julian Kreibich: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sophia Beyer:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Jonas Fuchs:** Writing – review & editing, Software, Methodology. **René Kallies:** Writing – review & editing, Methodology, Conceptualization. **Hans-Christoph Selinka:** Writing – review & editing, Methodology. **Birgit Walther:** Writing – review & editing, Conceptualization. **Sabine Diedrich:** Writing – review & editing, Funding acquisition, Conceptualization. **Sindy Böttcher:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Ethical statement

No ethical approval was needed for this study because the analyzed wastewater cannot be tracked back to specific persons.

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Data availability

Data will be made available on request.

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