



Full length article

Simultaneous biomass concentration and subsequent quantitation of multiple infectious disease agents and antimicrobial resistance genes from community wastewater

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ABSTRACT

Wastewater-based surveillance (WBS) of infectious disease agents is increasingly seen as a reliable source of population health data. To date, wastewater-based surveillance efforts have largely focused on individual pathogens. However, given that wastewater contains a broad range of pathogens circulating in the population, a more comprehensive approach could enhance its usability. We focused on the simultaneous detection of SARS-CoV-2, sapovirus, *Campylobacter jejuni*, *Campylobacter coli*, *Salmonella* spp., pathogenic *Escherichia coli*, *Cryptosporidium* spp., *Giardia* spp. and antimicrobial resistance genes (ARGs) of clinical relevance. To achieve this goal, biomass concentration and nucleic acid extraction methods were optimized, and samples were analyzed by using a set of (RT)-qPCR and (HT)-qPCR methods. We determined the prevalence and the spatial and temporal trends of the targeted pathogens and collected novel information on ARGs in Finnish wastewater. In addition, the use of different wastewater concentrates, namely the ultrafiltered concentrate of the supernatant and the centrifuged pellet, and the effect of freezing and thawing wastewater prior to sample processing were investigated with the indicator microbe crAssphage. Freeze-thawing of wastewater decreased the gene copy count of crAssphage in comparison to analyzing fresh samples ($p < 0.001$). *Campylobacter* were most abundant in two of the four studied summer months (30 % detection rate) and in wastewaters from regions with intensive animal farming. *Salmonella*, however, was detected in 40 % of the samples without any clear seasonal trends, and the highest gene copy numbers were recorded from the largest wastewater treatment plants. Beta-lactamase resistance genes that have commonly been detected in bacteria isolated from humans in Finland, namely *bla*_{CTX-M}, *bla*_{OXA48}, *bla*_{NDM}, and *bla*_{KPC}, were also frequently detected in wastewaters (100, 98, 98, and 70 % detection rates, respectively). These results confirm the reliability of using wastewater in public health surveillance and demonstrate the possibility to simultaneously perform WBS of multiple pathogens.

Abbreviations: AMR, antimicrobial resistance; ARG, antimicrobial resistance gene; CT, cycle threshold; HT-qPCR, high-throughput quantitative polymerase chain reaction; LOD, limit of detection; LOQ, limit of quantification; NA, nucleic acid; rcf, relative centrifugal force; RT-qPCR, reverse-transcriptase quantitative polymerase chain reaction; WBS, wastewater-based surveillance; WWTP, wastewater treatment plant.

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1. Introduction

Wastewater is rich in viruses, bacteria, and protozoa of environmental, human, and animal origin, but also particles and chemical compounds that may interfere with and inhibit molecular analyses (Amoah et al., 2022; Sinclair et al., 2008). This makes urban wastewater both an interesting and a challenging data source for disease surveillance. Moreover, it contains anonymous health-related biomarkers such as RNA and DNA of infectious disease agents circulating in the population. Therefore, the use of wastewater-based surveillance (WBS) to acquire health information is considered cost-effective, ethically less controversial, and a rapid alternative for population-level surveillance. WBS can be applied as an independent or complementary method to more conventional, clinical-based surveillance (Levy et al., 2023; Tiwari et al., 2022b; Wu et al., 2022). A proactive approach in WBS is to use the information as an early-warning tool for rapidly progressing infection outbreaks, such as the SARS-CoV-2 pandemic (Lämsivaara et al., 2023a) or other highly contagious viruses. However, wastewater has also been used to detect and estimate the magnitude of slower pandemic threats, such as antimicrobial resistance (AMR) (Hendriksen et al., 2019; Kim and Cha, 2021; Pärnänen et al., 2019). Moreover, WBS of infectious disease agents can be used to measure the impacts of infection prevention practices (Institute of Medicine, 2000), as well as in setting priorities and allocating resources.

Ideally, multiple emerging and existing epidemic-prone threats could be monitored effortlessly from a single wastewater sample (Levy et al., 2023; O'Brien and Xagorarakis, 2019). An all-in-one method to fully utilize the potential of wastewater and to yield simultaneous surveillance data on multiple targets from influent wastewater sample is presently lacking (Rao et al., 2024; Singh et al., 2024). For instance, waterborne disease agents, such as *Salmonella* spp., *Campylobacter* spp., and pathogenic *Escherichia coli*, pose a major threat to public health globally (Toze, 1999; Zahedi et al., 2021), but their surveillance efforts are mainly limited to data collection from clinical isolates. Moreover, the rapid evolution of AMR is a global crisis threatening the lives of humans and animals (Murray et al., 2022). Communicable disease surveillance is important for public health, and in the case of AMR, a holistic approach is needed (Tiwari et al., 2022a). However, current AMR surveillance actions largely rely on clinical antimicrobial susceptibility data (European Centre for Disease Prevention and Control and World Health Organization, 2022). Environmental AMR surveillance can provide important baseline data that would be a useful addition to current surveillance of population health.

The COVID-19 pandemic revealed the potential of wastewater surveillance and established an imminent need to develop rapid, sensitive, and more accurate monitoring methods for various potential pathogens (Kilaru et al., 2023; Lämsivaara et al., 2023b). WBS of infectious diseases with quantitative polymerase chain reaction (qPCR) and other molecular methods relies on the assumption of pathogen DNA or RNA shedding (Barceló, 2020; Sinclair et al., 2008). The conjecture is that even in the absence of symptoms, pathogens can be detected from feces or other excreta (Barceló, 2020; Sinclair et al., 2008) that eventually end up in a wastewater treatment plant (WWTP) via the sewerage system. PCR-based approaches have multiple advantages for WBS (Toze, 1999), as they enable the rapid detection of pathogens, regardless of their viability in sewage, and are also suitable for unculturable microbes. However, they may suffer from a lack of tolerance of inhibitors as well as comparability between research laboratories when working with complex sample matrices such as wastewater (Barceló, 2020). The physicochemical characteristics of wastewater (e.g. temperature and the content of solids), the size of the sewerage network and WWTP, the proportion of runoff waters, and flow can all affect the pathogen detection rate (Amoah et al., 2022; Tiwari et al., 2022b). Therefore, independent of the surveillance targets, WBS requires comprehensive method optimization.

Our main goal was to implement a combination of molecular and

microbiological methods for the simultaneous monitoring of viruses, bacteria, protozoa, and antimicrobial resistance gene (ARG) targets in 24-h composite influent wastewater samples collected for infectious disease surveillance purposes. To achieve this goal, biomass concentration and nucleic acid extraction methods for centrifuged wastewater pellet and ultrafiltered supernatant aliquots were optimized, and the extracted nucleic acids were analyzed by using a set of (reverse transcriptase) quantitative polymerase chain reaction (RT)-qPCR and high-throughput (HT)-qPCR methods to determine the prevalence and the spatial and temporal trends of pathogenic viruses, bacteria, and protozoa, and to collect novel information on ARGs in Finnish wastewater. In addition, the effect of freezing and thawing wastewater prior to biomass concentration and nucleic acid extraction was investigated. As an outcome, the repeatability and sensitivity of in-house qPCR and HT-qPCR methods and their relevance for WBS of pathogens and ARGs were evaluated as a proof of concept of the possibility to simultaneously perform WBS for multiple targets. Our results support and aid the establishment of an easy-to-use platform for the surveillance of relevant human pathogens and ARGs in community wastewater.

2. Materials and methods

To collect health-relevant information carried by community wastewater, we optimized a method that uses two types of wastewater fractions, namely the centrifuged pellet (solid fraction) and ultrafiltered concentrate of the supernatant designed for WBS of SARS-CoV-2 (Hokajärvi et al., 2021; Tiwari et al., 2022b), for the surveillance of multiple viruses, bacteria, protozoa, and AMR.

2.1. Sample collection

Samples were collected from WastPan consortium study sites ($n = 10$; Lehto et al., 2023; Tiwari et al., 2024), i.e., WWTPs in the cities of Helsinki, Espoo, Turku, Tampere, Oulu, Kuopio, Seinäjoki, Pietarsaari, Lappeenranta, and Rovaniemi. The study sites covered the sewerage network areas of both larger and smaller municipalities around Finland (Table S1), representing different geographical areas in Finland and corresponding to 40 % of the Finnish population, including the capital region of Helsinki. The sewerage network areas of these ten WWTPs include regions with seasonal international tourism, e.g., Rovaniemi, intensive animal husbandry, namely Seinäjoki and Pietarsaari, and the city of Lappeenranta, located near a checkpoint on the border with Russia.

About one-liter volumes of 24-h composite influent wastewater samples were collected once per month from Sunday to Monday at participating WWTPs and transported in cool boxes with ice packs and temperature loggers to the Water Microbiology Laboratory of the Finnish Institute for Health and Welfare (THL), Kuopio, Finland, as described earlier (Hokajärvi et al., 2021; Tiwari et al., 2022b). The total number of samples analyzed during the study period in two consecutive years 2020 and 2021 from May to August was 80. The arrival time and temperature of the samples were recorded, each wastewater sample was divided into aliquots upon arrival at the laboratory and stored at 4 °C in case that the analysis did not start immediately upon arrival.

2.2. Sample processing

An aliquot of each sample was processed fresh within 24 h after arrival at the laboratory (Fig. 1). Another aliquot was stored for four to six months at -20 °C, and after storage, the frozen sample aliquots were slowly thawed at 4 °C within 24 h prior to processing. A total of 80 mL of fresh sample was centrifuged, and the supernatant was concentrated with ultrafiltration, the pellet was discarded according to the protocol of national SARS-CoV-2 wastewater monitoring at the time (Tiwari et al., 2022b). In addition, 0.3 mL of unconcentrated control was analyzed. From each freeze-thawed wastewater sample (á 80 mL), the supernatant

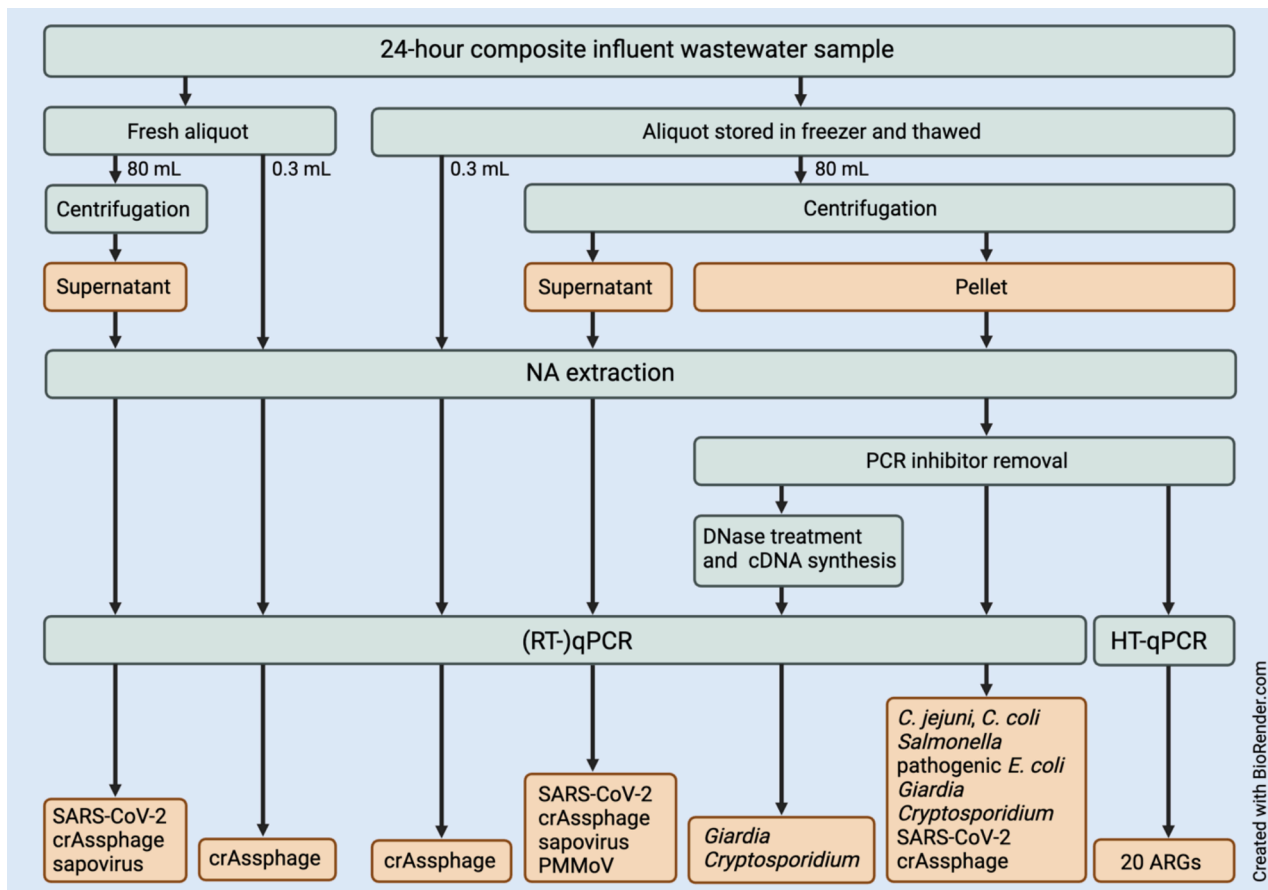


Fig. 1. Flowchart of influent wastewater (n = 80) sample processing steps and microbial targets of the analysis. The abundance of 20 antimicrobial resistance genes (ARGs) was investigated from all the samples. The prevalence of other targets was only determined from samples collected in 2021 (n = 40).

concentrate, pellet, and 0.3 mL of unconcentrated control were analyzed.

Supernatant samples were concentrated with ultrafiltration (Medema et al., 2020), as previously described (Hokajärvi et al., 2021). Known quantities of mengovirus were spiked to the samples before ultrafiltration as an internal process control, as was performed by Hokajärvi et al. (2021). In brief, 70 mL of pre-centrifuged (4654 relative centrifugal force (rcf) for 30 min without brake) supernatant was pipetted onto 10 kDa Centricon Plus-70 centrifugal filters (Millipore Corporation, Germany) and centrifuged for 25 min at 3000 rcf. Furthermore, concentrates were collected after 2 min of centrifugation (1000 rcf). The volumes of the concentrates prepared from fresh aliquots varied from 0.26 mL to 1.45 mL and from freeze-thawed aliquots from 0.19 mL to 0.55 mL. Ultrafiltered supernatant was added to low volume concentrates to reach a minimum volume of 0.7 mL. Prior to nucleic acid extraction, 0.3 mL of the concentrate was lysed with an equal amount of Lysis Buffer 1 (Perkin-Elmer). From the freeze-thawed sample aliquots, nucleic acids from duplicates of 0.3 mL concentrates were extracted. Negative process controls, i.e., 80 mL of sterile filtered ultra-pure water, were processed similarly to the supernatant samples.

The pellet samples were collected after the pre-centrifugation step. To ease the pipetting, the pellet was resuspended in 0.5 mL of the pre-centrifugation supernatant. Pellet volumes varied from 0.7 mL to 2.0 mL. Duplicate 0.3 mL fractions of the resuspended pellets were lysed similarly to supernatant samples. In addition, 0.3 mL of nuclease-free water (HyClone™ Water, Cytiva, Thermo Fisher Scientific) was lysed with 0.3 mL of lysis buffer as a negative process control for the pellets. After lysis, the lysed pellets were placed into tubes containing 212–300 µm glass beads (Merck KGaA, Darmstadt, Germany) and mechanically disrupted for 1 min at the maximum speed with a bead beater (Mini

BeadBeater, Biospec Products). After cell disruption, the samples were centrifuged at 12 000 rcf for 5 min. Finally, the lysed supernatant of the pellet was collected for nucleic acid extraction.

In addition to the supernatant and pellet fractions, 0.3 mL of unprocessed wastewater sample was lysed with 0.3 mL of Lysis Buffer 1 prior direct extraction. The samples were labeled as direct extraction samples.

2.3. Extraction and purification of nucleic acids

Nucleic acids (NAs) from the samples and process controls were extracted with the Chemagic Viral DNA/RNA 300 Kit H96 (Perkin-Elmer) using a Chemagic-360D instrument (Perkin-Elmer, Germany), as described earlier (Hokajärvi et al., 2021; Tiwari et al., 2022b). Each 96-well extraction plate had one SARS-CoV-2-positive control (Hokajärvi et al., 2021) and two negative extraction controls containing nuclease-free water (HyClone™ Water, Cytiva, Thermo Fisher Scientific). The NAs of the pellets were eluted into 100 µL, and supernatant concentrates and direct extraction samples were eluted into 50 µL of elution buffer. Duplicates that were prepared from the freeze-thawed samples were pooled together after extraction prior to downstream analysis.

The NAs from pellet samples and controls were purified from PCR inhibitors with the OneStep PCR Inhibitor Removal Kit (Zymo Research, USA) by following the manufacturer's instructions. Prior to two-step RT-qPCR of protozoan parasites, an additional DNase treatment was performed with the TURBO DNA-free™ kit (ThermoFisher Scientific) for 30 µL of nucleic acids, as previously described (Rytönen et al., 2021), and the purified RNA was synthesized into complementary DNA (cDNA) using 0.8 µL and 8 µL template volumes with SuperScript™ IV VIL0™ Master Mix (ThermoFisher Scientific), as instructed by the

manufacturer. DNA and RNA concentrations were measured with Qubit 3.0 Fluorometer and Qubit™ dsDNA and RNA HS kits (ThermoFisher Scientific), after which the nucleic acids were stored at -80°C . The cDNA was stored at -20°C prior to the qPCR runs.

2.4. qPCR and RT-qPCR for microbial targets

By using the (RT-)qPCR method, the prevalence of viruses, bacteria, and protozoa in Finnish wastewaters was investigated from samples collected in 2021 ($n = 40$). The viral targets, namely SARS-CoV-2 and sapovirus, and indicators of fecal contamination, namely crAssphage (Sabar et al., 2022; Stachler et al., 2017) and pepper mild mottle virus (PMMoV) (Rosario et al., 2009), were studied from the supernatant and direct extraction fractions (Fig. 1, Table S2). SARS-CoV-2 and crAssphage were also quantified from the pellet fraction. The internal process control, mengovirus, was studied from the supernatant fraction. To determine the effect of freezing and thawing of wastewater on virus numbers, the quantities of SARS-CoV-2, sapovirus, and crAssphage in freeze-thawed samples were compared with their quantities in freshly analyzed samples. The bacterial targets, namely *Campylobacter jejuni*, *Campylobacter coli*, *Salmonella* spp., and pathogenic *Escherichia coli*, and the protozoan targets, *Giardia* spp. and *Cryptosporidium* spp., were investigated from the pellet fraction only (Fig. 1, Table S2). For the two protozoan parasite targets, both ribosomal RNA (rRNA) and ribosomal RNA gene (rDNA) were used as templates for two-step RT-qPCR and qPCR, respectively, as previously described (Pitkänen et al., 2013; Inkinen et al., 2019; Rytönen et al., 2021). Pellet fractions were prepared from freeze-thawed sample aliquots. All RT-qPCR and qPCR assays were performed using the QuantStudio 6 Flex real-time PCR system (Applied Biosystems, ThermoFisher Scientific) (Tiwari et al., 2022b).

For the RNA viruses, one-step RT-qPCR was performed, and for crAssphage, which was the only DNA virus in this study, the qPCR method was employed (Tiwari et al., 2022b). The reaction volume for viruses was $25\ \mu\text{L}$, including $5\ \mu\text{L}$ of DNA or RNA, and all runs included at least one reaction with the no-template control (NTC). Reaction mixes and standard curves for assays to detect and quantify SARS-CoV-2, crAssphage, and mengovirus were performed similarly as in Tiwari et al. (2022b). Reaction mixes for sapovirus and PMMoV were prepared in the same manner as for SARS-CoV-2. Duplicates of both non-diluted and 10-fold-diluted nucleic acids were quantified with primers and probes (Table S2) and by using the TaqMan Fast Virus 1-step Master Mix (Applied Biosystems, ThermoFisher Scientific) to detect SARS-CoV-2, sapovirus, and PMMoV. Mengovirus was similarly quantified, but without duplicate reactions. To quantify crAssphage, 10- and 100-fold-diluted nucleic acids were used with TaqMan Environmental Master-Mix 2.0 (Applied Biosystems, ThermoFisher Scientific).

The thresholds of reactions for viral assays were 0.1 for N2 (SARS-CoV-2) and PMMoV, 0.04 for sapovirus and mengovirus, and 0.05 for crAssphage. The reactions were considered successfully amplified when the CT value was below 40. The results for the SARS-CoV-2 N2 gene, crAssphage, and mengovirus were interpreted similarly as in Tiwari et al. (2022b). SARS-CoV-2 was interpreted as non-detected, detected, or detected and quantified. The quantities of sapovirus and PMMoV were calculated as the weighted mean of results from diluted and undiluted samples. However, if inhibition was observed in the undiluted sample, the results were calculated using the diluted sample alone. Further information on qPCR reaction mixes, thermal cycling conditions, (RT-)qPCR amplification efficiencies, R2 values, slopes, and Y-intercept values, and the detection and quantification limits is provided in Supplemental Table S3.

The reaction mixtures in (RT-)qPCR for bacterial and protozoan targets consisted of template NA or cDNA, TaqMan Environmental MasterMix 2.0 (Applied Biosystems, ThermoFisher Scientific), primers, probe, and nuclease-free water (Table S3). The cDNA templates were used in a two-step RT-qPCR for activity (18S ribosomal RNA, rRNA) analysis of the two protozoan targets. The NAs and cDNAs were used as

templates both non-diluted and 10-fold diluted. The final volume of the qPCR and two-step RT-qPCR reaction mixes was $20\ \mu\text{L}$, and, as with viral targets, all runs included at least one NTC and standard curves. The standard curves had six points, which ranged from 2 to 200 000 gene copies, and the four middle points were duplicated. The thresholds for reactions were 0.1 for *C. jejuni*, *C. coli*, and *Salmonella* spp., 0.04 for *stx1*, *stx2*, and *eae*, and 0.03 for protozoa. The limit of detection (LOD) was set as three copies per reaction (Bustin et al., 2009), whereas the limit of quantification (LOQ) was the LOD plus the lowest copy number in the standard curve that was detected (depending on the assay, either 2 or 20). To calculate the gene copy numbers in 100 mL of wastewater, the sample volume and volume of NA after extraction, possible cDNA synthesis, and the qPCR reaction (amount of template) were acknowledged (Rytönen et al., 2021). The LOD, LOQ, and gene copy numbers in 100 mL were calculated as presented in Rytönen et al. (2021). If the results were below the LOQ and above the LOD, the results were interpreted as detected only, and the value was set to half of the LOQ (Table S3).

All plots were drawn and statistical analyses were carried out in R Studio version 4.2.3 (2023-03-15). Results from the statistical tests were considered statistically significant when the p-value was < 0.05 . The Shapiro-Wilk normality test was used to assess whether the (RT-)qPCR data followed a normal distribution. The non-parametric paired Wilcoxon test was used to test for statistically significant differences in crAssphage and sapovirus abundance between fresh samples and samples analyzed after freezing and thawing, and with crAssphage also between results generated from the pellet fraction, supernatant, and direct extraction sample aliquots.

2.5. High-throughput qPCR for antimicrobial resistance genes

Relative abundances of ARGs were investigated with HT-qPCR, namely the SmartChip Real-Time PCR system (Takara Bio, Mountain View, CA, USA), from the pelleted samples ($n = 80$). The HT-qPCR was carried out by Resistomap Oy (Helsinki, Finland), a specialized commercial service provider. Twenty clinically relevant ARGs conferring resistance to beta-lactams were selected for analysis (Table S4). Briefly, $30\ \mu\text{L}$ of NA of each sample was diluted to $10\ \text{ng}/\mu\text{L}$ with nuclease-free water and shipped in a cool box with ice packs within 24 h from Kuopio to Helsinki. The samples were fitted to three chips, each containing 36 samples or controls. Each chip contained at least one process negative control, one NTC, and one positive control. The positive control was Resistomap Oy's own wastewater sample, known to contain a high abundance of beta-lactam resistance genes (Majlander et al., 2021). Takara Bio's recommended qPCR reagents were used, and qPCR cycling conditions were as described in Wang et al. (2014). According to Resistomap Oy, to minimize the possibility of false-positive or false-negative results, each sample was run in three replicates, and amplification in at least two out of the three replicates was considered as a positive reaction. The cycle threshold (CT) was set to 27 and the mean value of the replicates was used for analysis.

In total, 48 assays were performed, including 7 taxonomic assays, namely Firmicutes, enterococci, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, staphylococci, and *Campylobacter*. The ARG-specific assays targeted the following gene groups: *bla*_{CMY}, *bla*_{MOX}/*bla*_{CMY}, *ampC*/*bla*_{DHA}, *bla*_{ACT}, *bla*_{OXA-10}, *bla*_{OXA1}/*bla*_{OXA-30}, *bla*_{SHV}, *bla*_{TEM}, *bla*_{VEB}, *bla*_{CTX-M}, *bla*_{GES}, *bla*_{OXA-58}, *bla*_{OXA-48}, *bla*_{NDM}, *bla*_{KPC}, *bla*_{OXA-23}, *bla*_{IMP}, *bla*_{VIM}, *bla*_{IMI}, and *bla*_{SME} (Table S4). As listed in Supplemental Table S4, some of the ARG assays targeted the same gene, and the mean of these results was therefore considered in the analysis. The overall amplification success was assessed with crAssphage assays crAss64 and crAss56, and finally, to obtain relative abundance data, the CT results were normalized to 16S rRNA gene assay ($\Delta\text{CT} = \text{CT detected gene} - \text{CT 16S rRNA gene}$). Another assay, 16S rRNA2 was also included but not used in normalization.

The vegan package from R was used to create NMDS plots and to analyze similarities (ANOSIM) within the resistomes. The indicpecies

package was used for multilevel pattern analysis to determine whether certain ARGs were found significantly more often at a particular WWTP as compared to other WWTPs. Correlations within the ARGs were examined with the corrplot package and the Spearman method. The plots for microbial and ARG data were created using the packages ggplot2, colorspace, ggpubr, and scales.

3. Results

3.1. (RT-)qPCR-based detection and quantification of pathogenic viruses, bacteria, and protozoa in wastewater in summer 2021

The presence of the novel coronavirus SARS-CoV-2 was analyzed from pellet and supernatant fractions of the wastewater samples collected in 2021 ($n = 40$) (Fig. 2a). The pathogen RNA was quantified from freshly processed samples and from water samples that had been stored prior to processing at $-75\text{ }^{\circ}\text{C}$ or lower and then thawed (“pellet after freeze–thaw” and “supernatant after freeze–thaw”). Overall, regardless of the concentration method, more SARS-CoV-2 virus particles were detected in the last two studied months, July and August 2021. The *N2* gene of SARS-CoV-2 was most frequently quantified from freshly processed supernatant samples, and this assay target could not be quantified from the supernatant fraction of the samples that had undergone a freeze–thaw cycle. Moreover, although the results obtained from freeze–thawed pelleted samples mirrored the results obtained from freshly treated supernatant samples, the gene copy count remained lower (Wilcoxon signed rank exact test, $p < 0.0001$). More SARS-CoV-2 virus gene copies per 100 mL of influent wastewater were detected from

larger municipalities.

In addition to the respiratory virus SARS-CoV-2, the prevalence of sapovirus in different municipal wastewaters was also determined (Fig. 2b). The quantities of sapovirus RNA in wastewater increased in Finland during the summer of 2021. The highest peaks in sapovirus numbers were detected in August 2021 in wastewater of Kuopio, Espoo, and Helsinki. Interestingly, while freeze–thawed samples showed higher level for inhibition in RT–qPCR analysis compared to the fresh samples, the calculated sapovirus numbers were higher in the freeze–thawed than the fresh samples. This difference in abundance was significant according to the Wilcoxon signed-rank exact test ($p < 0.05$).

Freezing and thawing of wastewater prior processing significantly lowered the gene copy counts determined for the fecal indicator crAssphage as compared to the analysis of freshly processed samples (Fig. 3, $p < 0.001$). This was verified with the Wilcoxon signed-rank test when comparing the crAssphage gene copies quantified from the unconcentrated (direct extraction) fraction and supernatant fraction, before and after freezing the wastewater. In addition, the method of wastewater concentration influenced the measured gene copy quantities. The highest quantities of crAssphage were assayed from directly extracted wastewater and the lowest quantities from supernatant that had been concentrated with ultracentrifugation (Fig. 3). Samples that had been stored in a freezer and later concentrated as pellets had significantly ($p < 0.001$) higher quantities of crAssphage in comparison to supernatant samples, but not compared to samples extracted directly ($p > 0.05$).

The copy numbers of fecal indicator PMMoV were determined only from the supernatant fractions of the freeze–thawed wastewater samples. The PMMoV counts, the wastewater sample temperature, and the

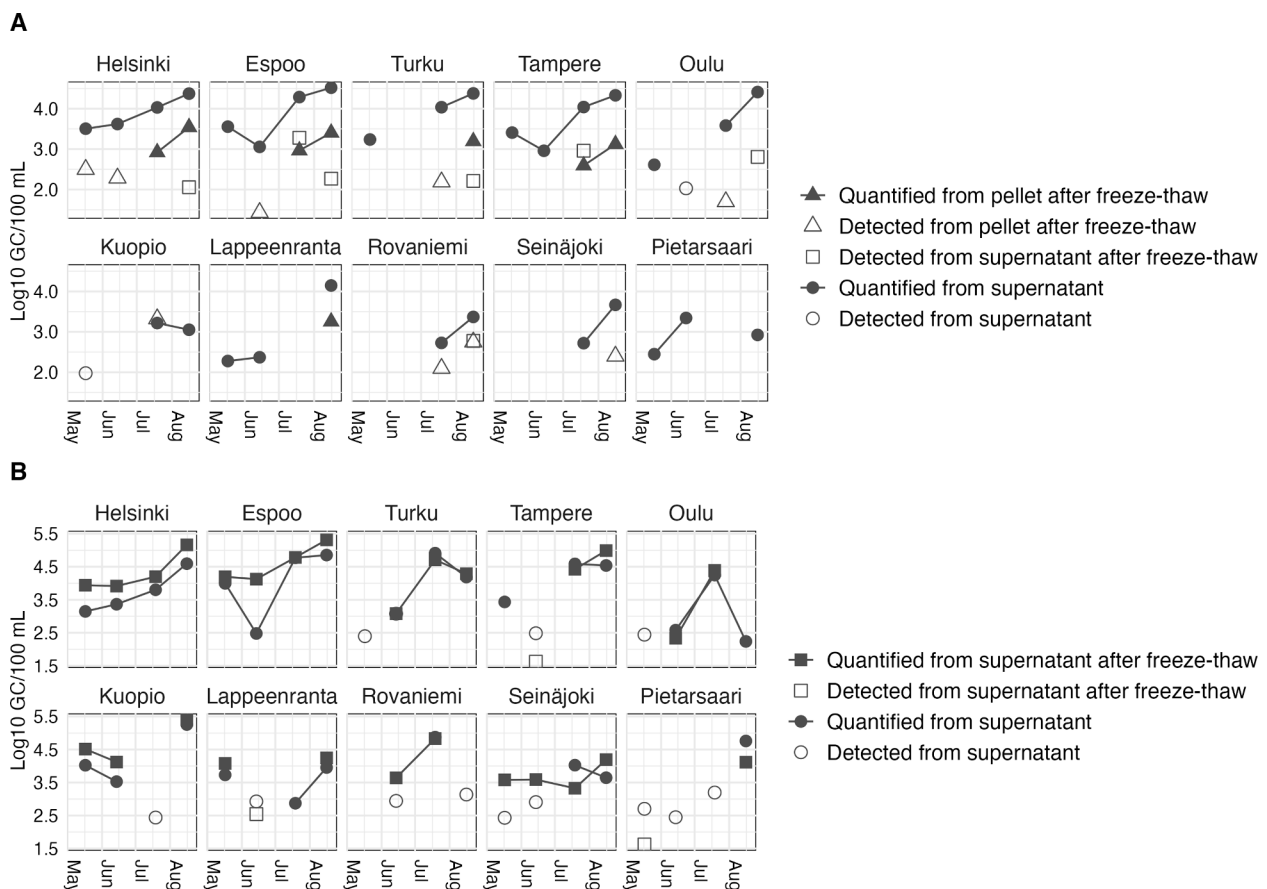


Fig 2. Detection and quantification of A) SARS-CoV-2 virus and B) sapovirus from 10 WWTPs in Finland during the summer months of 2021. The symbol shape indicates the sample processing method used (A) and whether the wastewater sample had been frozen and thawed prior to concentration (A and B). Sapovirus was only analyzed from the supernatant fraction. The results above the LOQ are marked with filled symbols, while the results below the LOQ are marked with hollow symbols.

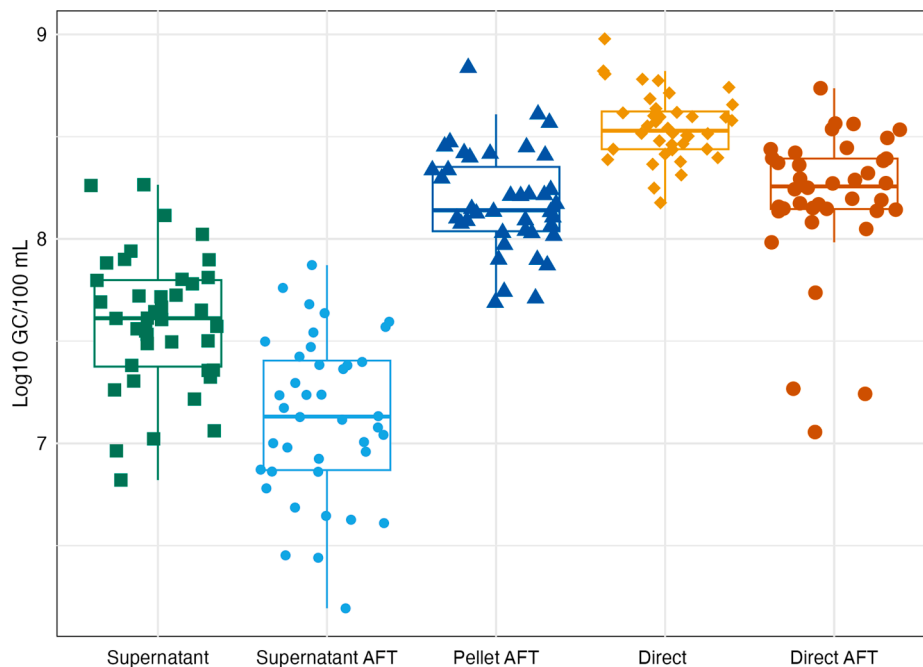


Fig. 3. Effect of freeze–thawing and the sample processing method on crAssphage abundance (log₁₀ GC/100 mL). CrAssphage was quantified from wastewater samples (n = 40, May–August 2021) before and after freeze–thawing with three different methods: pellet and supernatant concentration and direct NA extraction (pellet fraction only after freeze–thawing). Direct = direct NA extraction, AFT=after freeze–thawing. Effective sample volumes before NA extraction: supernatant AFT=60 mL, pellet AFT=69 mL, direct NA extraction (AFT) = 0.3 mL.

influent flow during the composite sampling events were dependent on the size of the WWTP, i.e., the size of the population served by the corresponding WWTP (Fig. S1). Measured with Kendall’s rank correlation tau, the gene copies of PMMoV correlated with the population size of the sewershed (tau = 0.36, p < 0.01), except for the WWTPs in Kuopio and Seinäjoki. The sample temperature upon arrival at the laboratory was lowest for samples from the largest regions (the largest WWTPs), and similarly to PMMoV abundance, temperature correlated significantly with population size (tau = -0.34, p < 0.01). The total flow was highest for the WWTP in Helsinki and, once again, significantly correlated with the population size (tau = 0.82, p < 0.001). The smaller WWTPs, namely from Kuopio, Lappeenranta, Rovaniemi, Seinäjoki, and

Pietarsaari, displayed very little variation in flow during the sampling event (Fig. S1).

The gene copy quantities of *Campylobacter jejuni*, *Campylobacter coli*, and *Salmonella* spp. were analyzed from the pellet fractions of the freeze-thawed samples (n = 40) collected in summer 2021 (Fig. 4). Neither of the *Campylobacter* species were detected in samples collected in May or June. In fact, *C. coli* was only detected twice: once at the WWTP in Pietarsaari and once at the WWTP in Oulu. *C. jejuni* was a more common finding, with seven out of ten results above the limit of quantification. The highest quantities of *C. jejuni* gene copies per 100 mL were recorded in wastewater samples collected from Seinäjoki and Pietarsaari, which are regions with intensive animal husbandry and the lowest population

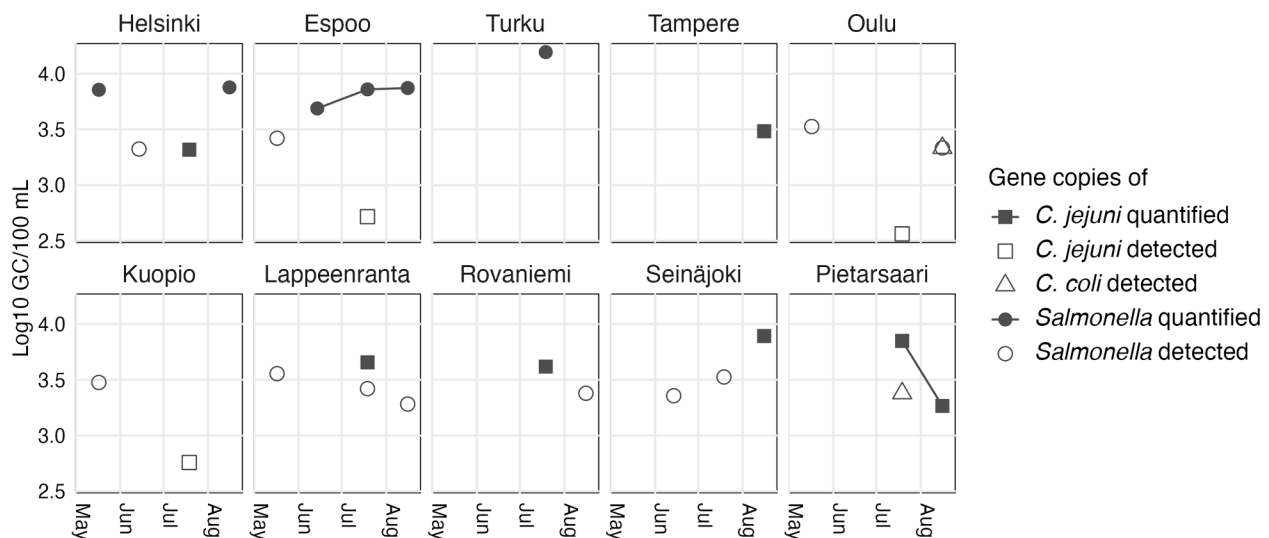


Fig. 4. Occurrence of zoonotic bacteria in wastewater samples (n = 40) collected in 2021. Gene copy numbers of the *hipO* gene of *Campylobacter jejuni*, *glyA* gene of *Campylobacter coli*, and *invA* gene of *Salmonella* spp. are presented on a logarithmic scale. The results above the LOQ are marked with filled symbols, while the results below the LOQ are marked with hollow symbols.

sizes of the ten studied regions. *C. jejuni* was not detected in the samples from the Turku WWTP.

On the contrary to the prevalence of *Campylobacter* species, no clear differences in temporal distribution were observed for *Salmonella* spp. The pathogen was only quantified from the sewerage systems with the largest population size, namely in Helsinki, Espoo, and Turku. *Salmonella* spp. was detected with the qPCR method at least once in wastewater samples collected from other WWTPs except for Tampere and Pietarsaari.

To estimate the trends and prevalence of pathogenic *E. coli* in wastewater, the Shiga toxin genes *stx1* and *stx2* and the intimin gene *eae* were investigated (Fig. 5). The *eae* gene was found in all studied months and WWTPs (100 %, in 40 out of 40 pelleted after freeze–thaw samples). The highest gene copy numbers in the studied period were quantified towards the end of the summer, in July and August. The Shiga toxin gene *stx2* was detected in 30 out of 40 samples (75 %), while the *stx1* gene was only detected in 8 out of 40 samples (20 %). Both Shiga toxin genes were absent from samples collected from Lappeenranta and *stx1* was further absent from Tampere, Kuopio, and Pietarsaari. The *stx1* gene was mostly detected in July.

The prevalence and activity of zoonotic enteric protozoa, namely *Giardia* spp. and *Cryptosporidium* spp., were determined by quantification of rDNA and rRNA, respectively, from the pellet fraction of wastewater after freeze–thaw ($n = 40$, Fig. 6). In general, the rDNA of *Giardia* spp. exhibited a higher abundance than rRNA (Wilcoxon signed rank exact test, $p < 0.001$), and according to Pearson's product-moment correlation test, the abundances of the two nucleic acids were significantly correlated ($cor = 0.64$, $p < 0.001$). Based on visual inspection, the quantities of the nucleic acids especially correlated in wastewaters collected from Oulu, Lappeenranta, and Pietarsaari. However, no mutual temporal trend in the quantities of *Giardia* spp. DNA and RNA was observed across the municipalities.

With the genus-level *Cryptosporidium* assay, rRNA of *Cryptosporidium* spp. was identified in every sample and in higher quantities than *Giardia* spp. rRNA or rDNA. Nonetheless, rDNA of *Cryptosporidium* spp. was rarely detected with this method (5 samples out of 40 were positive), and its trends did not follow those of rRNA. Specifically, DNA was only detected in the last two studied months, July and August, at which time rRNA quantities appeared to decrease.

3.2. Antimicrobial resistance gene abundance in wastewater in summer 2020 and 2021

All targeted 20 beta-lactam resistance genes were detected in wastewater samples collected in 2020 and 2021 ($n = 80$, Fig. 7). The ARGs were analyzed from freeze–thawed wastewater samples that had been concentrated as pellets. The five most abundant genes were *bla*_{OXA-10}, the carbapenemase gene *bla*_{GES}, the AmpC genes *bla*_{ACT} and *bla*_{OXA-1}/*bla*_{OXA-30}, and the carbapenemase gene *bla*_{OXA-58}, respectively. The five least abundant genes were the carbapenemase genes *bla*_{SME}, *bla*_{IMP}, *bla*_{OXA-23}, *bla*_{IMI}, and *bla*_{KPC}, respectively. Genes that were detected in all samples were *bla*_{SHV}, *bla*_{OXA-1}/*bla*_{OXA-30}, *bla*_{TEM}, *bla*_{CTX-M}, *bla*_{ACT}, and *bla*_{GES}.

Analysis of similarities revealed a statistically significant difference in ARG abundances between WWTP network areas ($R=0.41$, $p < 0.001$) but not between months ($R=0.03$, $p > 0.05$) or years ($R=0.02$, $p > 0.05$). It appears that the smaller the population size served by the WWTP, the more fluctuation there was in resistomes (Fig. 8A). As demonstrated in the non-metric multidimensional scaling (NMDS) plot in Fig. 8A, WWTPs with the smallest population served, namely Pietarsaari, Seinäjoki, and Rovaniemi, had the most differing resistomes. Resistomes from Helsinki, Espoo, Turku, and Tampere, on the other hand, had a high similarity in comparison with each other. Samples from Oulu were clustered slightly separately in the NMDS plot and displayed a low diversity in terms of temporal trends.

Spearman's rank correlation coefficient was used to analyze the statistical dependence between the abundances of the studied ARGs (Fig. 8B). Interestingly, the most abundant carbapenemase genes, *bla*_{GES} and *bla*_{OXA-58}, mostly only correlated with each other, with the exception of *bla*_{OXA-10}. Other carbapenemase genes that showed a weak correlation with all other genes were *bla*_{OXA-23} and *bla*_{IMP}. AmpC genes correlated with each other but also with many other genes, such as with the ESBL gene *bla*_{CTX-M} and carbapenemase genes *bla*_{OXA-48}, *bla*_{NDM}, *bla*_{KPC}, *bla*_{VIM}, and *bla*_{IMI}. The abundances of the genes *bla*_{SHV} and *bla*_{CTX-M} strongly correlated with each other but not with the other potential ESBL genes, *bla*_{TEM} and *bla*_{VEB}. The genes *bla*_{SHV} and *bla*_{CTX-M} also correlated with many clinically relevant carbapenemase genes.

Multilevel pattern analysis revealed statistically significant spatial differences in the abundances of 11 out of the 20 studied ARGs. For instance, the carbapenemase genes *bla*_{IMP} and *bla*_{OXA-48} were significantly ($p < 0.01$ and $p < 0.05$, respectively) more abundant in the wastewater of the Oulu WWTP as compared to the other WWTPs

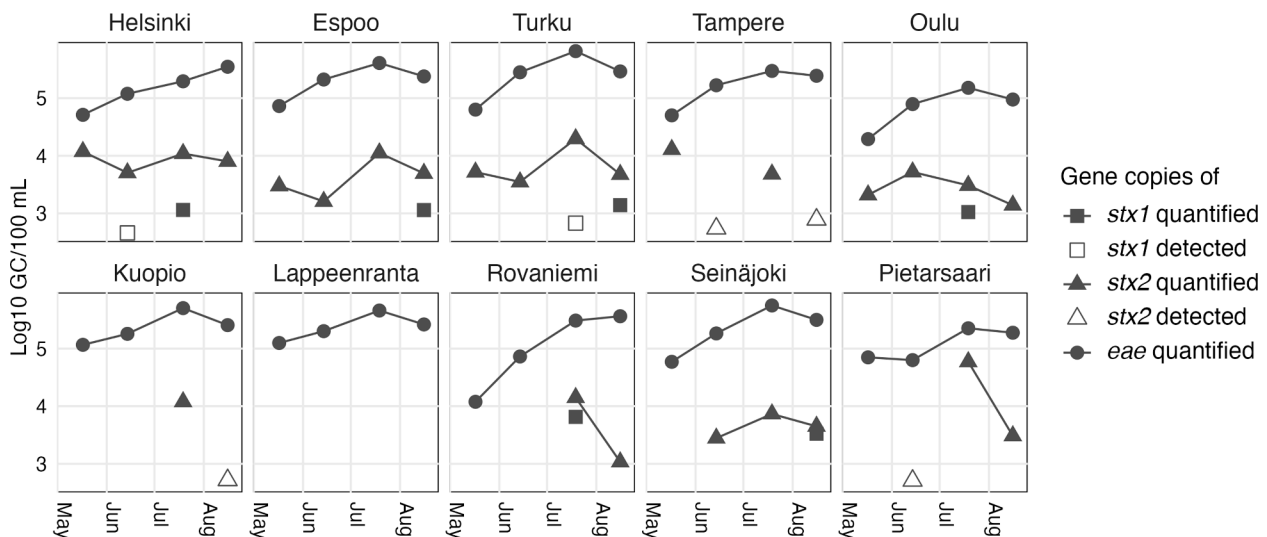


Fig. 5. Prevalence of pathogenic *Escherichia coli* in wastewater samples ($n = 40$) collected in 2021. Gene copy numbers of the virulence genes *stx1*, *stx2*, and *eae* of EHEC/STEC/EPEC are presented on a logarithmic scale. The results above the LOQ are marked with filled symbols, while the results below the LOQ are marked with hollow symbols.

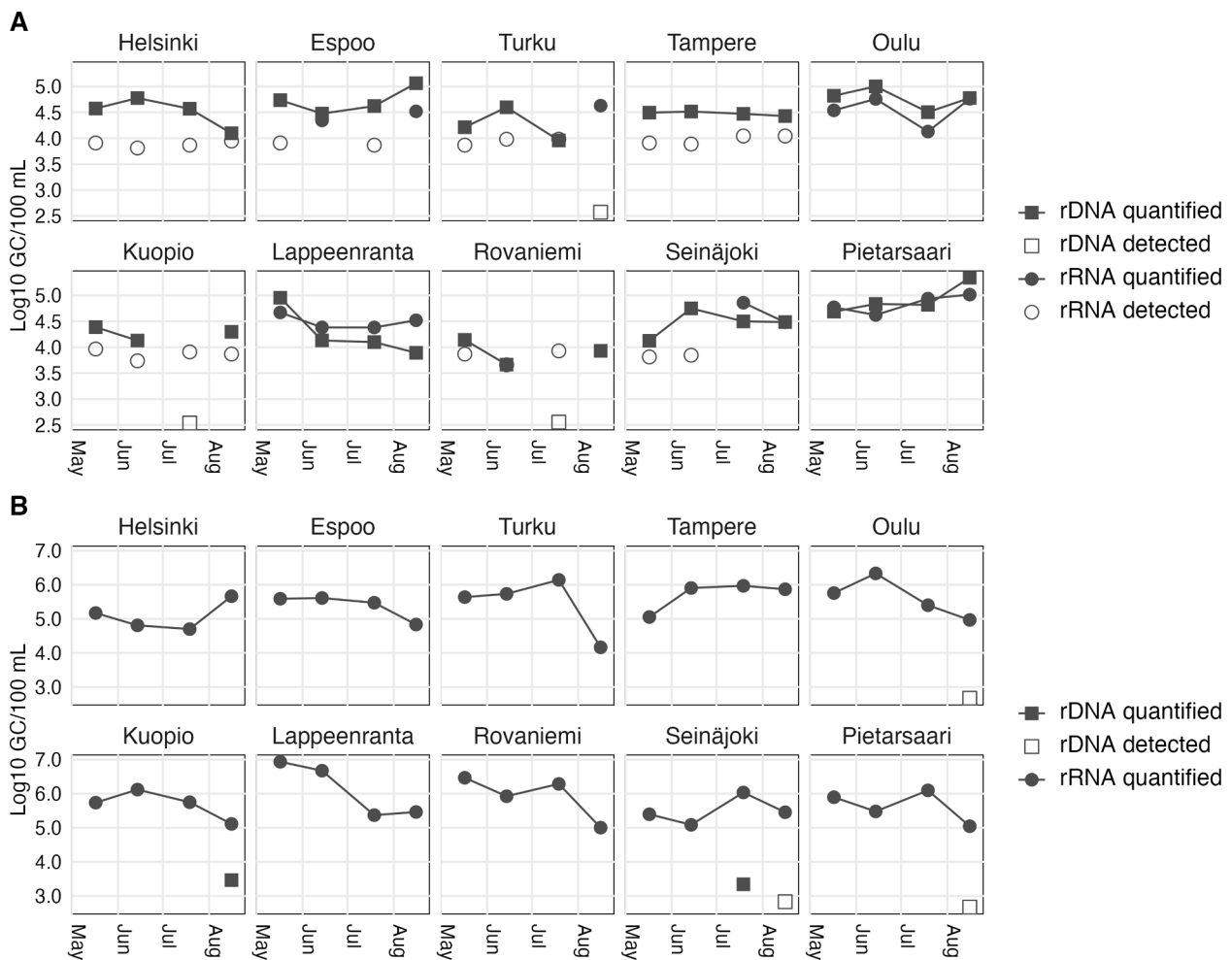


Fig. 6. Prevalence of zoonotic enteric protozoa measured as ribosomal DNA (rDNA) and ribosomal RNA (rRNA) copy numbers of (A) *Giardia* spp. and (B) *Cryptosporidium* spp. The results above the LOQ are marked with filled symbols, while the results below the LOQ are marked with hollow symbols.

(Table 1). Furthermore, the highly abundant gene *bla*_{OXA-1}/*bla*_{OXA-30} and one of the rarest carbapenemase genes, *bla*_{IMI}, were significantly more abundant at the Pietarsaari WWTP in comparison to other WWTPs. No spatial difference was observed in the abundances of the 9 ARGs not listed in Table 1, namely *bla*_{MOX}/*bla*_{CMY}, *ampC*/*bla*_{DHA}, *bla*_{SHV}, *bla*_{CTX-M}, *bla*_{NDM}, *bla*_{KPC}, *bla*_{OXA23}, *bla*_{VIM} and *bla*_{SME}.

The taxonomic assays of HT-qPCR were not able to detect *Campylobacter* spp., whereas staphylococci were only detected in one sample (from Rovaniemi in May 2020). The genes of *Firmicutes*, *A. baumannii*, *K. pneumoniae*, enterococci, and *P. aeruginosa* targeted with the assays were highly abundant, as they were detected in 80, 79, 75, 70, and 46 samples out of 80, respectively.

4. Discussion

Environmental surveillance of communicable diseases is not dependent on clinical testing strategies, the asymptomatic features of infections, or the carriage of ARGs, or on the willingness or capability of an infected person to seek help from health care services. This study demonstrated the possibility of using wastewater to detect multiple pathogens and ARGs at the population level. We analyzed supernatant and pellet fractions of wastewater with (RT)-qPCR and HT-qPCR and were able to detect all the targeted pathogens, namely SARS-CoV-2, sapovirus, *C. jejuni*, *C. coli*, *Salmonella* spp., genes of pathogenic *E. coli*, *Giardia* spp., *Cryptosporidium* spp., and 20 ARGs of clinical relevance. The ten sampling locations comprehensively covered Finland and included the capital region of Helsinki.

During the study years 2020 and 2021, work, travel and the free-time habits of people were exceptional. Starting from March 2020, the Finnish government introduced a variety of recommendations and restrictions aimed at preventing the spread of COVID-19, including restrictions on large public events, traveling to and within Finland, and on the opening hours of restaurants and other public spaces (Tiirinki et al., 2020). Similarly, in 2021, lockdowns and face mask requirements greatly affected the meeting habits of the public. These events are likely to have also affected the spread of other communicable diseases (Räsänen et al., 2021). Thus, the WBS results presented herein reflect the communicable disease situation during the pandemic years. Continuation of wastewater monitoring actions is required to determine the pathogen and ARG loads in community wastewaters of Finland during non-pandemic times. Further, to enable population level interpretation of any monitoring result from wastewater influents, normalization is needed to correct the findings taking into account the variable fecal content over time. Influent flow and reference microbe numbers are commonly used for normalization in wastewater surveillance (Tiwari et al., 2022b, Zhan et al., 2022), but currently there is no consensus of the best normalization method.

4.1. The potential of WBS of multiple pathogens

Routine WBS is largely focused on individual pathogens, especially on viral diseases with a high pandemic potential, such as SARS-CoV-2. Therefore, the majority of methods used in WBS have been optimized for virus surveillance (Levy et al., 2023; Rao et al., 2024). With a

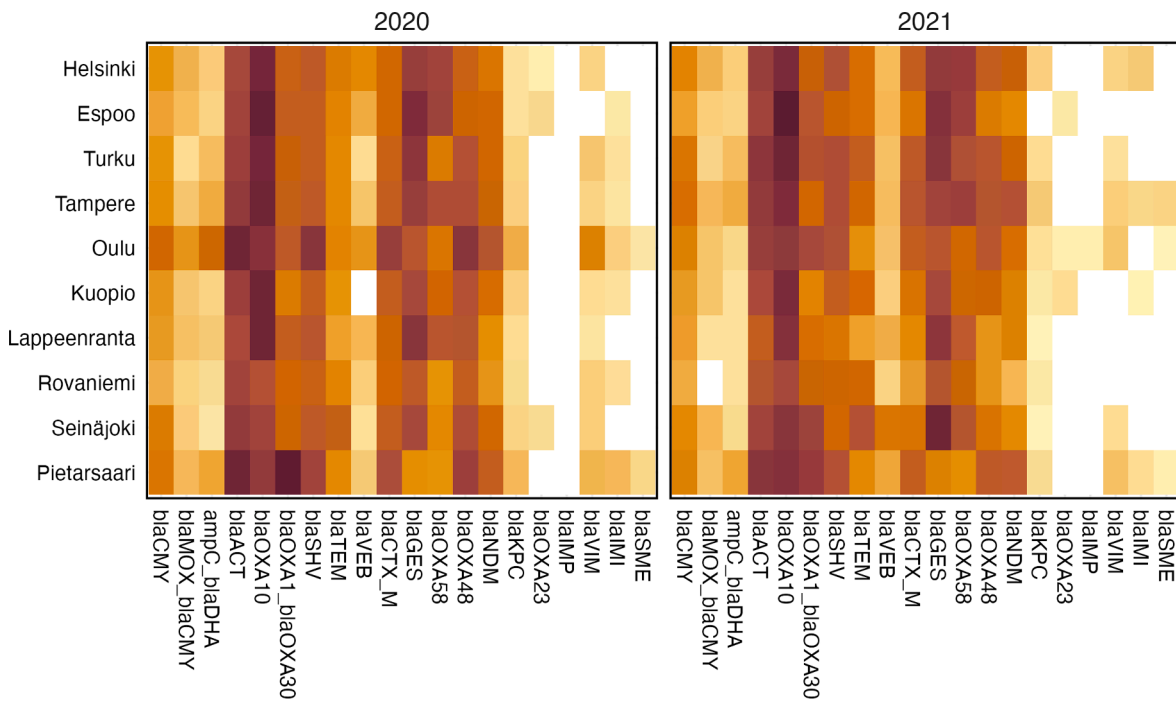


Fig. 7. Relative abundance of antimicrobial resistance genes in wastewater in May, June, July, and August in 2020 and 2021 (n = 80). In the heatmap, the darker the color, the more abundant the target gene is. The Y-axis presents the municipality of the WWTP, listed in order of the size of population served by the WWTP. In 2021, the WWTP in Helsinki served a population of 860 000 and the WWTP in Pietarsaari a population of 30 000.

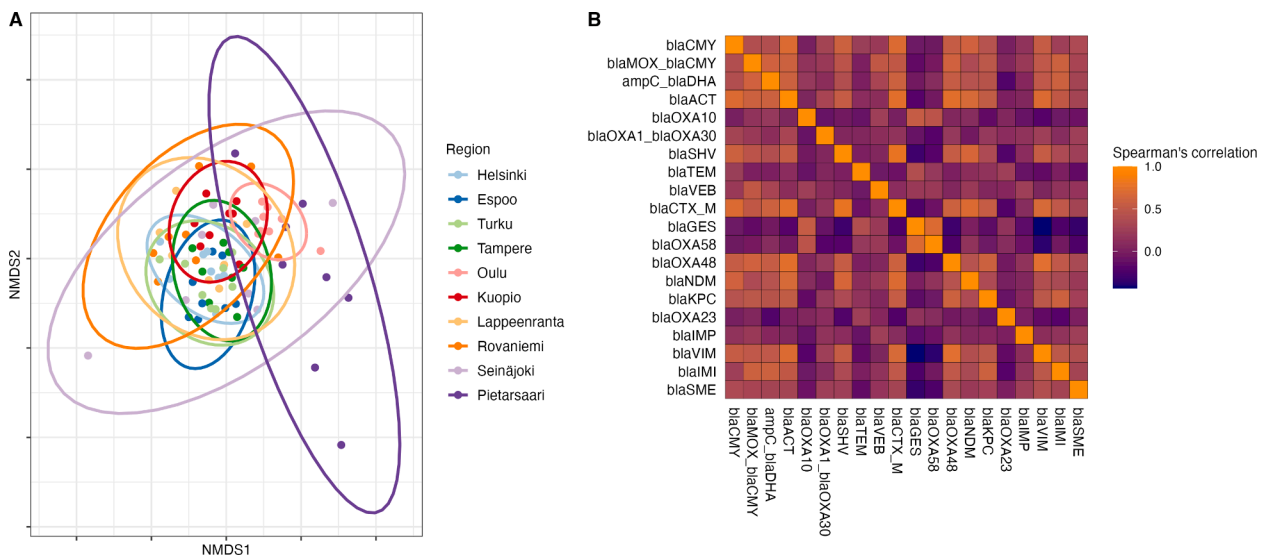


Fig. 8. A) An NMDS plot demonstrating the differences ($p < 0.001$) in wastewater resistomes in each WWTP sewershed region and B) a heatmap of the correlations of ARG abundances. The dependencies were measured with Spearman's rank correlation coefficient (1.0 = highest correlation). In both figures, the wastewater samples were collected monthly from May to August in 2020 and 2021 (n = 80). The municipalities of the WWTPs are presented in order of the population size served by the WWTP. The WWTP in Helsinki serves a population of 860 000 and the WWTP in Pietarsaari a population of 30 000.

scalable method, however, wastewater can also be used for the simultaneous surveillance of other pathogens. By optimizing our pre-established method for SARS-CoV-2 surveillance (Tiwari et al., 2022b), we expanded our method to simultaneously cover WBS of multiple pathogens. Ultrafiltration is a widely used method for the concentration of viruses from large sample volumes (ENETWILD-consortium et al., 2022) and in times of low disease prevalence, which is also useful for WBS of SARS-CoV-2. Ultrafiltration includes a pre-centrifugation step in which the pelleted solids are discarded. We retained the pellet and used it to detect bacterial and protozoan

pathogens and ARGs. The concentration of wastewater solids as a pellet is an inexpensive, simple, and rapid method to simultaneously capture and concentrate bacteria and protozoa, although further studies are still required to determine the effect of storage and freeze-thaw of the pelleted biomass on the counts of these multiple targets.

The simultaneous detection of multiple pathogens increases the utility and cost-effectiveness of WBS (Rao et al., 2024) and complements existing surveillance systems. For instance, zoonotic bacteria and protozoa are generally only studied from clinical samples. However, a more holistic approach through environmental surveillance could be

Table 1

Table of the 11 assays detecting ARGs with significant geographical differences. Significance at the level: *** < 0.001, ** < 0.01, * < 0.05. Municipalities of the WWTPs are presented in order of the size of the population served. The WWTP in Helsinki serves a population of 860 000 and the WWTP in Pietarsaari a population of 30 000.

Assay	Helsinki	Espoo	Turku	Tampere	Oulu	Kuopio	Lappeenranta	Rovaniemi	Seinäjoki	Pietarsaari
<i>bla</i> _{IMP}					**					
<i>bla</i> _{OXA48}					*					
<i>bla</i> _{OXA1} / <i>bla</i> _{OXA30}										***
<i>bla</i> _{IMI}									*	
<i>bla</i> _{CMY}					*				*	
<i>bla</i> _{TEM}			***						***	
<i>bla</i> _{OXA58}	***	***		***				***		
<i>bla</i> _{OXA10}	***	***	***	***		***	***			
<i>bla</i> _{ACT}		**	**	**	**				**	**
<i>bla</i> _{VEB}	*	*		*	*		*		*	*
<i>bla</i> _{GES}	***	***	***	***			***	***	***	

advantageous. The need for an effective surveillance system that includes environmental monitoring of foodborne diseases, such as zoonotic campylobacteriosis and salmonellosis, has been highlighted in the most recent One Health Joint Plan of Action (FAO, UNEP WHO, and WOA, 2022).

4.2. Pathogenic viruses and fecal indicator crAssphage

The rate of detection and quantitation of SARS-CoV-2 varied according to the use of the supernatant or pellet fraction from biomass concentration and the use of fresh or freeze-thawed samples for this concentration. The present results are consistent with our earlier study (Hokajärvi et al., 2021) and highlight the effect of the concentration method on virus gene copy numbers and suggest that when using freeze-thawed samples, virus detection is more sensitive when conducted on the pellet fraction. During the study period (May–August 2021), the incidence of COVID-19 cases in Finland was generally low, being lowest in June 2021 and highest in August 2021 (Finnish Institute for Health and Welfare, 2023a), which is in line with the results obtained from wastewater. Our results, together with previous literature, also support the analysis of SARS-CoV-2 from fresh samples rather than from water samples that have been frozen and thawed prior to concentration (Robinson et al., 2022). Especially in periods with low community infection rates, the concentration method used for WBS and the amounts of PCR inhibitors that co-concentrate in the selected method can cause disparity in the detection rates of SARS-CoV-2 (Ahmed et al., 2020; Philo et al., 2021).

Sapovirus infections can be asymptomatic and tend to be milder than norovirus infections, which might lead to underdiagnosis of sapoviruses in clinical settings (McCall et al., 2020). In addition, a person infected with sapovirus might continue to shed viral particles in feces for weeks after the resolution of symptoms (McCall et al., 2020; Oka et al., 2015). In fact, wastewater-originated sapovirus has already been identified twice as the main causative agent of outbreaks in Finland caused by drinking water (Kauppinen et al., 2019). In our study, the RNA quantities of sapovirus in wastewater suggest a rise in sapovirus prevalence in Finland during the summer of 2021. Seasonal peaks of sapoviruses in wastewater have also been noted in other studies (Fioretti et al., 2016; Haramoto et al., 2008). Our results support the idea that due to an estimated underdiagnosis of the burden of sapovirus infections (McCall et al., 2020), health care systems could greatly benefit from WBS of sapovirus. Surprisingly, the freeze-thaw step positively affected the recorded abundance of sapovirus ($p < 0.05$), which is likely to be due to methodological differences that were introduced to address the high level of inhibition in freeze-thawed samples. Based on CT values, the undiluted NAs in the freeze-thawed samples systematically displayed more inhibition than the diluted NAs, forcing us to use only diluted samples in the calculations. This could have led to a slightly higher estimation of the gene copy numbers in 100 mL of wastewater and caused the observed discrepancy between fresh and freeze-thawed

samples.

CrAssphage is a highly abundant bacteriophage found nearly exclusively in the human gut, making it suitable as a fecal marker for wastewater-based pathogen studies (Sabar et al., 2022; Stachler et al., 2017). Even though its genome is in the robust form of DNA, freezing and thawing of wastewater was demonstrated to bring about a significant reduction in crAssphage abundance. This is important, as a reduction in crAssphage abundance may reflect a general reduction in viral levels in the sample (Sabar et al., 2022). In addition, the quantities of crAssphage gene copies varied markedly depending on the fraction of wastewater used (supernatant or pellet). The crAssphage results confirmed our earlier findings with SARS-CoV-2 (this study and Hokajärvi et al., 2021) that it is advisable to use the wastewater pellet fraction instead of the supernatant for virus quantitation when the water samples have been stored in the freezer prior to NA extraction. As crAssphage is ubiquitous in wastewater and PCR inhibitors are likely to co-concentrate in concentration processes, our finding that the highest crAssphage quantities were measured from the directly extracted, i.e., unconcentrated sample fraction, was expected.

4.3. Trends in the prevalence of bacterial pathogens in wastewater

The prevalence of multiple zoonotic bacterial pathogens, namely *Campylobacter jejuni*, *C. coli*, and *Salmonella* spp., and the virulence genes of EHEC, namely *stx1*, *sxt2*, and *eae*, were investigated from wastewater pellets from ten WWTPs in Finland in 2021. The campylobacters were only detected in two of the four studied summer months, namely July and August, indicating a seasonal trend in their occurrences. These findings are supported by earlier studies conducted in northern Europe and the Czech Republic, according to which both the *Campylobacter* cases in humans (Kuhn et al., 2020) and the quantities of *Campylobacter* spp. in wastewater (Strakova et al., 2022) rise during summer and peak in late summer.

Gene copies of *C. jejuni* in 100 mL of wastewater were most abundant in wastewaters from regions with intensive animal farming but a lower population size, namely Seinäjoki and Pietarsaari. Campylobacters are abundant but rarely cause disease in warm-blooded animals (World Health Organization, 2020a), which could explain the higher *Campylobacter* abundances in these regions. However, the number of samples positive for either *C. jejuni* or *C. coli* was rather low (30 %; 12 out of 40). The effect of freeze-thawing of samples prior to NA extraction on bacterial pathogen abundance was not investigated in the present study.

In contrast to *C. jejuni*, *Salmonella* spp. was most abundant at the WWTPs serving the largest populations, namely Turku, Helsinki, and Espoo. No seasonal trend in wastewater was noted, even though *Salmonella* cases are expected to peak in the summer (Akil et al., 2014). The detection rate of *Salmonella* (*invA* gene) in the studied wastewater samples (43 %; 17 out of 40) was slightly higher than that for *Campylobacter* spp. However, the rate and duration of shedding for different gastrointestinal pathogens, as well as the survival

characteristics of these pathogens, can vary considerably, and these need to be considered when comparing *Campylobacter* and *Salmonella* detection rates in wastewater (Rukambile et al., 2019). Notably, and presumably due to the precautions and travel restrictions associated with the COVID-19 pandemic, *Salmonella* cases in Finland in 2021 were the lowest in nine years (Finnish Institute for Health and Welfare, 2023b). *Salmonella* is rarely found in animals in Finland (Finnish Food Authority, 2022), and most of the *Salmonella* infections in the country are of foreign origin (Kääriäinen et al., 2022). Nevertheless, Yanagimoto et al. (2020) reported a rise in the concentration of *invA* in wastewater as being related to increased human *Salmonella* cases, and that WBS of *Salmonella* could assist in estimating the magnitude of an epidemic.

The *eae* gene, which is important for the infectivity of EHEC and enteropathogenic *E. coli* (EPEC), was ubiquitous in wastewater and detected in every sample in our study. The Shiga toxin gene *stx2* was notably more abundant (75 %, 30 out of 40) than the *stx1* gene (20 %, 8 out of 40), and the copy numbers of all three EHEC genes peaked in July. Yang et al. (2014) noted similar differences between the *eae* and Shiga toxin gene quantities and seasonal trends in the occurrence of the *eae* gene in sewage samples collected in Hawaii. Our results suggest a greater abundance of EPEC than EHEC in wastewater. However, based on virulence genes, the presence of EPEC and EHEC in wastewater can only be speculated. The information gathered from emerging infectious pathogens such as EHEC via wastewater surveillance is nonetheless important, as the data can be used, for example, to estimate the spread of epidemics.

4.4. Protozoan pathogens and the use of rRNA and rDNA templates

Our investigations into the prevalence and activity of the emerging zoonotic protozoa *Giardia* spp. revealed high quantities of both rDNA and rRNA in wastewater. The nucleic acids detected with the *Giardia* spp. assay correlated significantly with each other. *Giardia* cysts have previously been reported to be highly prevalent in wastewater (Hamilton et al., 2018; Nasser et al., 2012). *Giardia* infections have been acknowledged globally as underdiagnosed and underreported due to their higher prevalence in low- and middle-income countries with inadequate disease reporting systems. Furthermore, most human *Giardia* infections are asymptomatic. Therefore, the results obtained from wastewater have the potential to reflect the infection rates in humans even better than clinical notifications. However, the genus-level assay used in this study detects both pathogenic and non-pathogenic *Giardia* species, and the prevalence of *Giardia* spp. might therefore be biased towards the overestimation of infections.

When detected with the *Cryptosporidium* spp. assay, the rDNA of this pathogenic genus was almost nonexistent in comparison to the quantities measured when rRNA was used as a template. Humans are mostly infected with either anthroponotic *C. hominis* or zoonotic *C. parvum* (Hashim et al., 2006; Sekikawa and Toshiki, 2015). The results collected from wastewater might better mirror the zoonotic cycle infections in humans, as *C. parvum* has been found to dominate in wastewaters in Europe (Zahedi et al., 2021). However, if present in community wastewater, the genus-level assay is also likely to detect environmental, non-pathogenic species of *Cryptosporidium*.

The use of an rRNA template that had originated from fecal and wastewater samples has been shown to increase the sensitivity of multiple microbial source tracking (RT)-qPCR assays in comparison to the use of a corresponding rDNA template (Rytkönen et al., 2021). On the contrary, in the study conducted by Rytkönen et al. (2021), the specificity of the assays was lower with an rRNA template. The lower specificity with an rRNA template could explain the discrepancy in our results regarding the quantities of *Cryptosporidium* spp. rRNA and rDNA. Previously, rRNA approach has been used to identify the active members of the eukaryotic communities in water (Inkinen et al., 2019), but rRNA templates have not previously been used for specific detection and quantification of protozoan parasites. Thus, the specificity of these two

protozoan assays used in our study would require additional method validation prior further use.

4.5. Antimicrobial resistance genes in Finnish wastewaters

Twenty antimicrobial resistance genes conferring resistance to clinically important beta-lactams were selected for investigation. Carbapenems are the broadest spectrum beta-lactam antibiotics, they are stable against hydrolysis by ESBLs and AmpCs, and are used in the treatment of severe resistant infections. Fortunately, resistance to carbapenems is still relatively rarely encountered in healthcare settings in Finland (Ilmavirta et al., 2022). However, some carbapenemase genes that have been detected in bacteria isolated from humans in Finland, namely *bla_{OXA48}*, *bla_{NDM}*, and *bla_{KPC}* (Finnish Institute for Health and Welfare, 2023c), were frequently detected in wastewaters of the present study. For instance, *bla_{OXA-48}* and *bla_{NDM}* were found in 98 % of the samples (78 out of 80), *bla_{VIM}* in 69 %, and *bla_{KPC}* in 70 % of the samples (55 and 56 out of 80). Other clinically relevant carbapenemase genes such as *bla_{OXA-23}* and *bla_{IMP}* were infrequently detected (10 out of 80 and 6 out of 80, respectively).

The second most abundant gene, the resistance gene *bla_{GES}*, was detected in every wastewater sample (80 out of 80). The gene has been reported as abundant in hospital and municipal wastewater in Helsinki, Finland (Majlander et al., 2021; Tiwari et al., 2022c), although it is a rare finding in bacteria isolated from humans in Finland (Finnish Institute for Health and Welfare, 2022). It is possible that as a clinically rare finding and sometimes as a phenotypically weak carbapenemase gene, *bla_{GES}* can be underdiagnosed (Ellington et al., 2019). Our results confirm its omnipresence in Finnish wastewaters and also in locations other than Helsinki. The role of environmental and non-clinical bacteria as carriers of *bla_{GES}* in wastewater could be significant.

The *bla_{CTX-M}* genes are the most prevalent ARGs in ESBL-producing *Enterobacteriaceae* in humans (Castanheira et al., 2021; Kurittu et al., 2022) and animals (Ejaz et al., 2021; Zhang et al., 2021), and also among the most prevalent ESBL genes in wastewater (Zaatout et al., 2021). Our results demonstrating a high abundance of the ESBL gene *bla_{CTX-M}* and the potential ESBL genes *bla_{SHV}* and *bla_{TEM}* in Finnish wastewaters are likely to reflect the level of ESBL carriage in the asymptomatic population and patients in hospitals. In a study conducted in 2016, the asymptomatic carriage of ESBL *E. coli* and *K. pneumoniae* isolates in the Finnish population was higher than expected and had increased during a decade (Rintala et al., 2018). An increase in the carriage of ESBL *E. coli* in patients and healthy individuals is emerging globally and is alarming, as ESBL infections significantly increase infection mortality and morbidity rates (European Centre for Disease Prevention and Control, 2022; Bezabih et al., 2022). Moreover, the eminent global spread of plasmid-mediated *bla_{CTX-M}* genes in the 21st century is concerning, as CTX-M-producing bacteria causing infections lead to the increased use of carbapenem drugs and further contribute to the emergence of carbapenemase-producing *Enterobacteriaceae* (Bevan et al., 2017). It is important to keep monitoring ESBL genes in wastewaters.

In terms of co-existence and co-abundance, the rare carbapenemase genes *bla_{OXA-23}* and *bla_{IMP}* only correlated with each other, as did the abundant resistance genes *bla_{GES}* and *bla_{OXA-58}*. The co-abundance of the latter genes *bla_{GES}* and *bla_{OXA-58}* is potentially expected due to their shared host range, namely *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (Haghighi and Reza Goli, 2022; Xin et al., 2019), which also were highly frequently found in our samples (79 and 46 out of 80, respectively). Of the four ESBL-related genes studied here, the abundances of the genes *bla_{SHV}* and *bla_{CTX-M}* correlated with each other, but not with the other two genes, *bla_{TEM}* or *bla_{VEB}*. The first two also correlated better with the relative abundances of the carbapenemase genes *bla_{OXA-48}*, *bla_{NDM}*, and *bla_{KPC}*. The third most abundant gene, the AmpC gene *bla_{ACT}*, correlated with the other studied AmpC genes, but not with the other most abundant genes, *bla_{OXA-10}*, *bla_{OXA1}/bla_{OXA-30}*, *bla_{GES}*, or *bla_{OXA-58}*. These similarities in relative abundances may be

explained by the genes having the same bacterial host range and mechanisms of spread, e.g., by being carried by the same plasmid.

Within our study period (summers 2020 and 2021), overall temporal changes within ARG resistomes in wastewaters were undetectable, but differences within WWTPs were significant. Interestingly, the smaller the population size, the more fluctuation in resistomes was observed within a single WWTP. Similar fluctuation was observed in a 4-year retrospective surveillance study of clinical cases of ESBL *E. coli* and *K. pneumoniae* in Finland (Tiwari et al., 2024). Moreover, in comparison to sampling 28 WWTPs, the whole of Finland, or just sampling the capital region of Helsinki, the 10 WWTPs that were examined in this study were found optimal for the surveillance of AMR and infectious agents in terms of cost efficiency. The five WWTPs serving the largest populations, namely Helsinki, Turku, Tampere, Kuopio, and Oulu, also have the largest hospitals in Finland (university hospitals) within their sewersheds.

Importantly, our results only considered summer months (May–August), so seasonal variation in wastewater between summer and winter remains unknown. A recent study conducted in Japan in 2019 reported more ARGs in WWTP influent during summer compared to winter (Honda et al., 2023). In fact, Honda et al. (2023) found seasonality to be the main factor determining the wastewater resistomes, which, in turn, was a consequence of seasonality in antimicrobial use. Indeed, antimicrobial usage and the prevalence of antimicrobial resistance in clinics are reflected in ARG profiles in wastewater (Pärnänen et al., 2019). Pärnänen et al. (2019) discovered that European wastewater resistomes correlate significantly with the phenotypic resistance of clinical isolates of *E. coli*, *K. pneumoniae*, *P. aeruginosa*, or *S. aureus*. In addition, the use of antimicrobials in animal farming contributes to the abundance of ARGs in wastewaters (Hu et al., 2017; Pärnänen et al., 2019).

4.6. Advantages and challenges of molecular-based WBS

In comparison with culture-based methods, molecular-based methods overcome the challenge that not all bacteria resistant to antimicrobials survive in the extreme conditions of wastewater (Tiwari et al., 2022a). In wastewater-based surveillance of pathogens and AMR, it is not necessary to know whether the microbes carrying the targeted genes are alive. Moreover, molecular methods in WBS outperform conventional culture-based methods in terms of cost-efficiency, rapidness, quantitiveness, scope, and the ability to detect genes from unculturable bacteria. Compared with other molecular methods, HT-qPCR allows the simultaneous detection of multiple, even hundreds, of genes and it is customizable (Waseem et al., 2019). Moreover, the extremely low reaction volume of HT-qPCR (100 nL) is superior and brings a clear advantage in terms of consumable and reagent costs. Importantly, (HT-)qPCR has a lower detection limit and simpler data analysis in comparison to the metagenomic sequencing approach. These are necessary factors to consider when operating continuous surveillance of AMR or taxonomic targets (Waseem et al., 2019).

Despite the prominent advantages of molecular-based methods, there are still challenges. Firstly, molecular methods are sensitive to inhibitors that wastewater is saturated with. Method sensitivity can be difficult to establish, and it can vary markedly across molecular-based methods (Waseem et al., 2019). For instance, *Campylobacter* spp. were not detected with HT-qPCR but were detected multiple times with our in-house qPCR method. The lower sensitivity and specificity of HT-qPCR has been addressed by using technical replicates and a low CT threshold value. Secondly, the use of primers is a clear disadvantage for all qPCR-based research, forcing the target sequences to be of known origin. Finally, the method reproducibility of HT-qPCR is not clear due to the high number of reactions per run, and it could be affected by the small amount of template DNA required for each well.

4.7. Environmental surveillance of AMR

AMR has been stated by WHO as one of the top ten threats to global public health (World Health Organization, 2020b). Despite AMR being recognized as one of the six key health challenges requiring a One Health approach, environmental surveillance of AMR has not been implemented in the 2022–2026 One Health Joint Plan of Action (OHJPA) (FAO, UNEP WHO, and WOA, 2022). The integration of environmental knowledge, data, and evidence in decision making, however, has been noted as one of the high-level actions in the action plan. Moreover, the European One Health Action Plan against Antimicrobial Resistance, published by the European commission in 2017, includes statements that support the environmental monitoring of antimicrobials and microorganisms resistant to antimicrobials (European Commission, 2017).

There are several reasons to continue environmental surveillance of AMR in Finland, even though in our study, the changes in ARG composition over the period of one year were not significant. The status of AMR in Finland might deteriorate due to climate warming, as bacteria thrive in warmer conditions (Kaba et al., 2020; MacFadden et al., 2018; Pärnänen et al., 2019). Trends in international travel and movement, agriculture, and potentially antibiotic use are likely to change and contribute to the level of resistance in Finland. Urban wastewater is a useful source of population-level information on changes in AMR abundance over time and enables the detection of new emerging ARGs. WBS of AMR also provides information on the burden of AMR on the environment and humans, and potentially also other animals. This information brings added value to intervention decision making and is important in AMR risk assessment.

5. Conclusions

Ideally, wastewater samples should be processed without freezing and thawing, but it is not always possible to process the samples on the day of arrival, especially with large sample numbers or multiple analyses to perform. Our results demonstrated that freezing and thawing of wastewater did not prevent the detection of bacteria or protozoa. The detection of SARS-CoV-2 and crAssphage, however, was affected by freezing and thawing, and based on our results, viral analyses should be conducted on fresh samples. In terms of pathogen surveillance, our investigations into the prevalence and activity of protozoa in wastewater yielded promising results, but assay specificity still requires further method validation.

The HT-qPCR-based ARG results provided herein, together with existing literature, highlight the slow and silent nature of the pandemic threat of AMR, given that no temporal difference between summer 2020 and summer 2021 was observed. Regardless of the lack of temporal changes, a high abundance of ARGs conferring resistance to critical carbapenems was recorded in wastewaters across Finland. These results, together with clinical data, can be beneficial in estimating the burden and risks of AMR carriage in the Finnish population.

This study demonstrated the possibility to simultaneously detect and quantify viral, bacterial, and protozoan pathogens and ARG from wastewater with the use of biomass concentration methods together with (RT-)qPCR and HT-qPCR. The outcomes of our research support possible future initiatives to expand the national WBS scheme from individual pathogens to multiple infectious agents and encourage the establishment of an electronic surveillance platform for relevant human pathogens and antimicrobial resistance genes in wastewater. Such an easy-to-use platform could be utilized by public health authorities, such as epidemiologists, and researchers from different fields of science.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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