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Unveiling the global urban virome through wastewater metagenomics

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Understanding global viral dynamics is critical for public health. Traditional surveillance focuses on individual pathogens and symptomatic cases, which may miss asymptomatic infections or newly emerging viruses, delaying detection and response. Wastewater-based epidemiology has been used to track pathogens through targeted molecular assays, but its reliance on predefined targets limits detection of the full viral spectrum. Here, we analyse longitudinal wastewater samples from 62 cities across six continents (2017–2019) using metagenomics and capture-based sequencing with probes targeting viruses associated with gastrointestinal disease. We detect over 2500 viral species spanning 122 families, many with human, animal, or plant health relevance. The bacteriophage family *Microviridae* and plant virus family *Virgaviridae* dominate the metagenomic dataset, while *Astroviridae* and *Picornaviridae* prevail in the capture-based sequence dataset. Virus distributions are broadly similar across continents at the family and genus levels, yet distinct city-level fingerprints reveal geographical and temporal variation, enabling spatiotemporal surveillance of viruses such as astroviruses and enteroviruses. Global wastewater-based epidemiology enables early detection of emerging viruses, including Echovirus 30 in Europe and *Tomato brown rugose fruit virus*. These findings highlight the potential of wastewater sequencing for the early detection of emerging viruses and population-wide virome monitoring across diverse hosts.

In recent decades, the frequency and scale of infectious disease outbreaks have increased, particularly those emerging after spillover of animal viruses[1]. The emergence and spread of these diseases have been driven by a range of interconnected factors, including urbanization[2], the development of megacities[3], deforestation[4,5], growing food demands[6], and increasing global connectivity. Migration from rural to urban areas has contributed to population growth in cities, resulting in increased close-contact interactions that facilitate disease transmission[3]. By 2017, 55% of the world's population lived in urban areas[7], making these high-density cities critical hubs for the spread of infectious diseases, and therefore strategic sites for monitoring and responding to both novel and established public health threats.

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Current infectious disease surveillance primarily relies on the identification and reporting of symptomatic cases[8], which has limitations, particularly for viruses that frequently cause asymptomatic or mild infections, or diseases that are not reportable. As a result, novel infectious diseases caused by emerging viruses or variants of known viruses can evade detection, spreading silently before being recognized. This phenomenon was exemplified by the introduction of Zika virus in South America[9] and the early undetected spread of SARS-CoV-2 into Europe[10,11] and the USA[12]. To better understand the prevalence and spread of viral infections, alternative surveillance strategies beyond symptom-based reporting are needed to capture the full spectrum of viral transmission dynamics and enhance early detection and response to emerging infectious disease threats.

Wastewater-based epidemiology (WBE) involves the detection and monitoring of chemicals, drugs and substances[13,14], genes[15], pathogens[16] and vectors[17] within wastewater to assess community health or disease risk. Recent detections of poliovirus in wastewater from major urban cities like New York[18], London[19], and poliovirus essential facilities[20] highlight the potential of WBE in identifying circulation hotspots and guiding targeted interventions, such as enhancing surveillance and launching of vaccine campaigns[17]. During the COVID-19 pandemic, quantitative reverse transcription PCR-based methods were used to assess the viral load in wastewater, with viral concentrations following the number of positive cases at the community level and providing insights into infection dynamics over time[19,20]. Amplicon-based sequencing was used in parallel to identify circulating viral variants, enhancing early detection capabilities in wastewater if used in combination with pathogen specific bioinformatic pipelines[21,22]. Utilizing targeted molecular methods has extended WBE to track other pathogenic viruses like influenza[23], respiratory syncytial virus[23], mpox[24], arboviruses[25], and non-polio enteroviruses (EV) such as EV-D68[26].

Among viruses shed into wastewater, enteric viruses pose a significant public health threat. Enteric viruses mainly transmit through the faecal-oral route, cause a wide range of gastrointestinal diseases and are potential sources of community-wide outbreaks[27]. Detection is complicated by their extensive diversity, nonspecific clinical symptoms that hamper diagnosis, high rate of secondary transmission which may mask initial introductions, and gaps in surveillance systems. As the number of viral targets of interest grows, the question arises whether surveillance efforts focused on individual pathogens can be combined in a more comprehensive approach. In this context, metagenomic sequencing can be a promising technique that can address this need[28]. A significant advantage of metagenomic sequencing is its ability to simultaneously detect and characterize multiple viral agents present within a single (wastewater) sample including non-target (novel) viruses[29,30].

In this study, we explore the potential of metagenomic sequencing and analysis as a tool for population level monitoring of virus circulation across hosts in high-density urban areas. We sequenced and characterized the wastewater virome of 62 major cities worldwide using both a shotgun viral metagenomic and a capture based sequencing method targeting enteric viruses, to compare viromes and viral dynamics across the world. Our findings reveal city-specific virome fingerprints and show that wastewater metagenomics enables early detection of emerging viruses and can inform broader epidemic surveillance.

Results

Virome data overview

To investigate the worldwide urban wastewater virome, wastewater samples were collected from 62 cities across 47 countries on 6 continents (Fig. 1)[31]. For all cities, samples were acquired biannually in June and November over a 2 year period spanning 2017–2019 (biannual samples). Additionally, to investigate temporal changes, monthly samples were obtained from eight cities in different countries in six different continents during the same period. Wastewater samples were analysed using both shotgun viral metagenomic sequencing to determine the viral metagenome and sequencing following capture using a custom probe set (GastroCap, Supplementary Fig. 1), designed to enrich for enteric virus sequences. The median number of reads generated after sequencing was 9.8×10^5 (IQR: 6.9 × 105-1.26 × 106) per sample for the metagenomic sequence dataset and 7.4×10^5 (IQR: 4.6×10^5- 1.17 × 106) for the capture sequence dataset (Supplementary Fig. 2). The median percentage of reads per sample that could be annotated as viral was 11% (IQR: 6.5-15.3%) for the metagenomic sequence dataset and 26% (IQR: 11.8-46.9%) for the capture sequence dataset. The remaining reads were annotated as bacterial, human, other eukaryotic, or unclassified (i.e., without any annotation) (Supplementary Fig. 2). Principal component analysis (PCA) of centred log-ratio (CLR) transformed read count data at the superkingdom level revealed distinct clustering between the two datasets and confirmed the enrichment of viral reads using GastroCap (Supplementary Fig. 3).

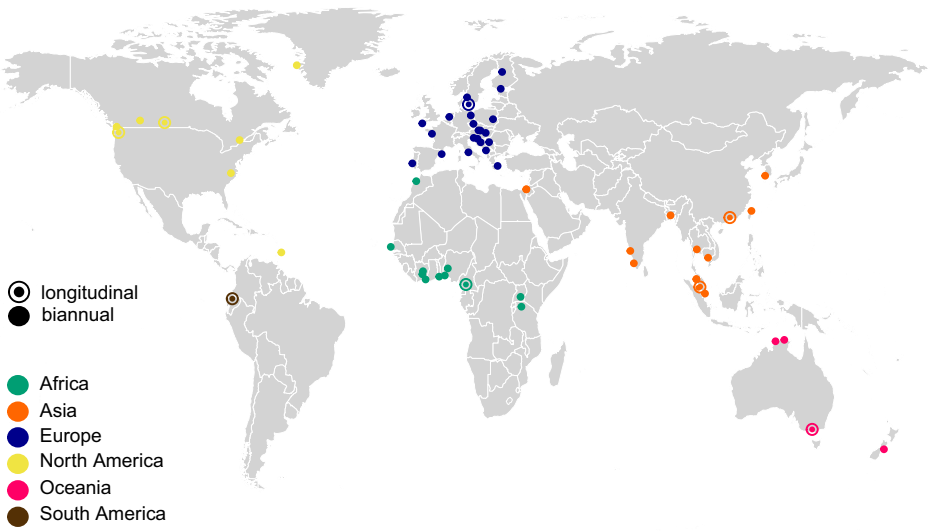

Fig. 1 | Overview of the 62 wastewater collection sites. Cities are coloured by continent and categorized by sampling strategy: colour-filled dots represent cities with biannual sampling, while outlined dots represent cities with longitudinal sampling.

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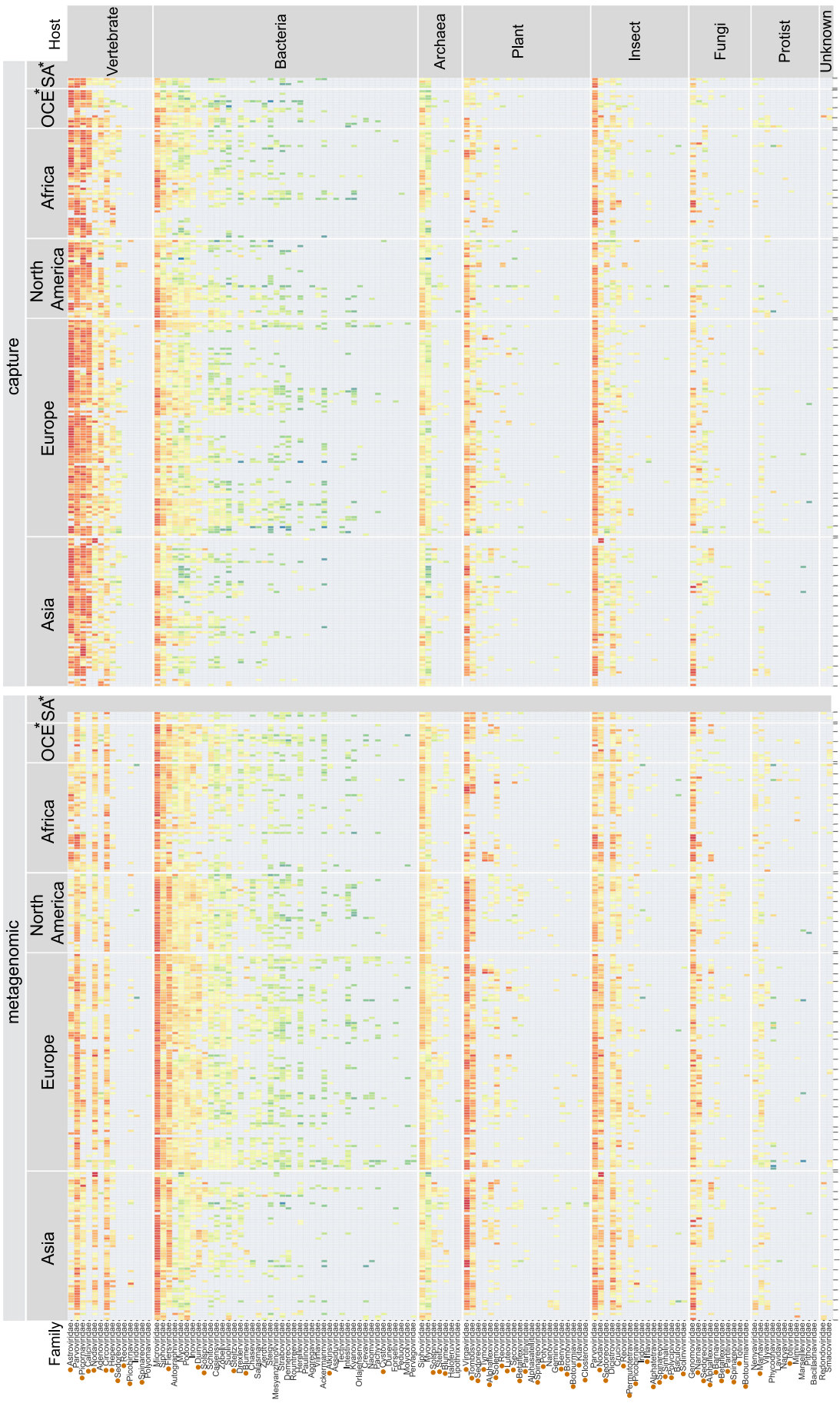

Fig. 2 | Heatmap of the viral family diversity in the global wastewater samples. The heatmap is categorized by continent and host association and ordered by RPM value of the GastroCap sequence data. The colour gradient represents the log-transformed relative abundance of reads normalized to genome length. Viral families marked with an orange dot are RNA viruses; all others are DNA viruses. OCE Oceania, SA South America, Metagenomic shotgun metagenomic dataset, and capture GastroCap sequence dataset.

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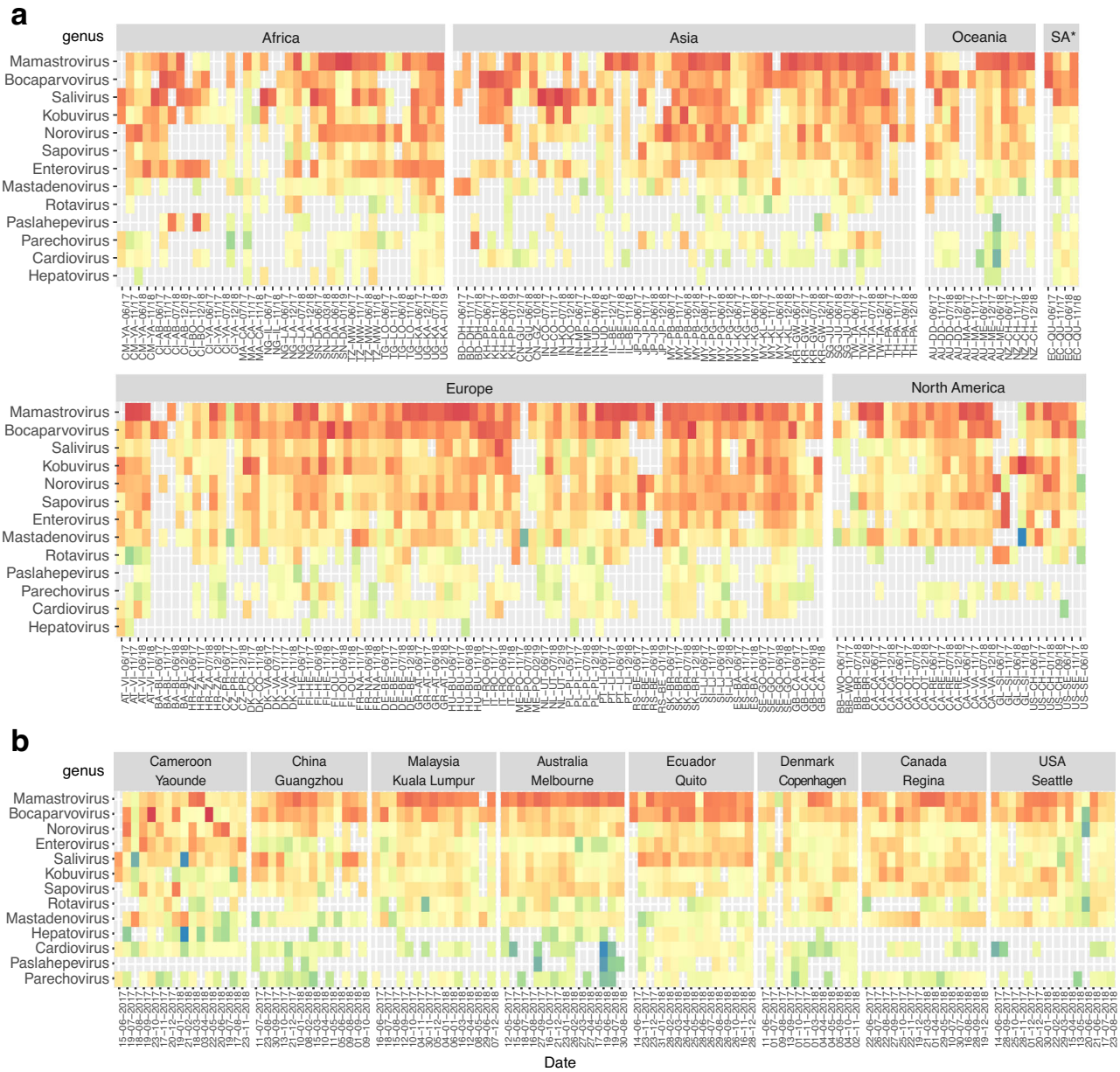

Fig. 3 | Pathogenic enteric viruses were widespread and showed distinct spatiotemporal patterns in urban wastewater. Reads per million viral reads (normalized by genome length) per genus are given for the biannual (**a**) and longitudinal (**b**) GastroCap sample set. The heatmap is ordered by reads per million viral reads (normalized by genome length) and categorized by continent (**a**) and city (**b**). The colour gradient represents log-transformed relative abundance of reads. SA South America.

Composition of the Global Urban Wastewater Virome

To establish a baseline understanding of the urban wastewater virome, we first examined the diversity of viruses at the family level. Overall, viral reads were mapped to a total of 122 families, 1367 genera and 2546 species (Fig. 2). These findings highlight the broad diversity of viruses associated with vertebrates, plants, microbes, and other life forms within urban environments. A large fraction of the metagenomic virome consisted of bacteriophages and plant viruses (Fig. 2). Viral families linked to human disease, such as *Astroviridae, Parvoviridae, and Picornaviridae*, were frequently detected in high abundances. In contrast, other important human-associated families, including *Adenoviridae, Caliciviridae, Hepeviridae, and Sedoreoviridae*, were also detected but less frequently.

Of the eight viral families targeted by GastroCap (*Adenoviridae*, *Astroviridae*, *Caliciviridae*, *Hepeviridae*, *Parvoviridae*, *Picornaviridae*, *Spinareoviridae* and *Sedoreoviridae*) which include 72 genera and 1038 species, a total of 72 genera and 165 species were identified (Fig. 2). GastroCap-based sequencing resulted in a substantial increase in both the total and relative number of reads for the targeted viral families. The median fold increase per sample, based on relative abundance in reads per million (rpm), was as follows: *Adenoviridae* (545-fold), *Astroviridae* (470-fold), *Caliciviridae* (5094-fold), *Hepeviridae* (22-fold), *Parvoviridae* (2-fold), *Picornaviridae* (132-fold), *Sedoreoviridae* (193-fold) and *Spinareoviridae* (21-fold). Viral genera associated with human gastrointestinal disease, including those commonly targeted by surveillance, such as *Mamastrovirus, Bocaparvovirus, Salivirus, Kobuvirus, Norovirus, Sapovirus, Enterovirus, Mastadenovirus, Rotavirus, Paslahepevirus, Parechovirus, Cardiovirus* were detected across all continents. However, while *Mamastrovirus* was highly abundant in most samples across all

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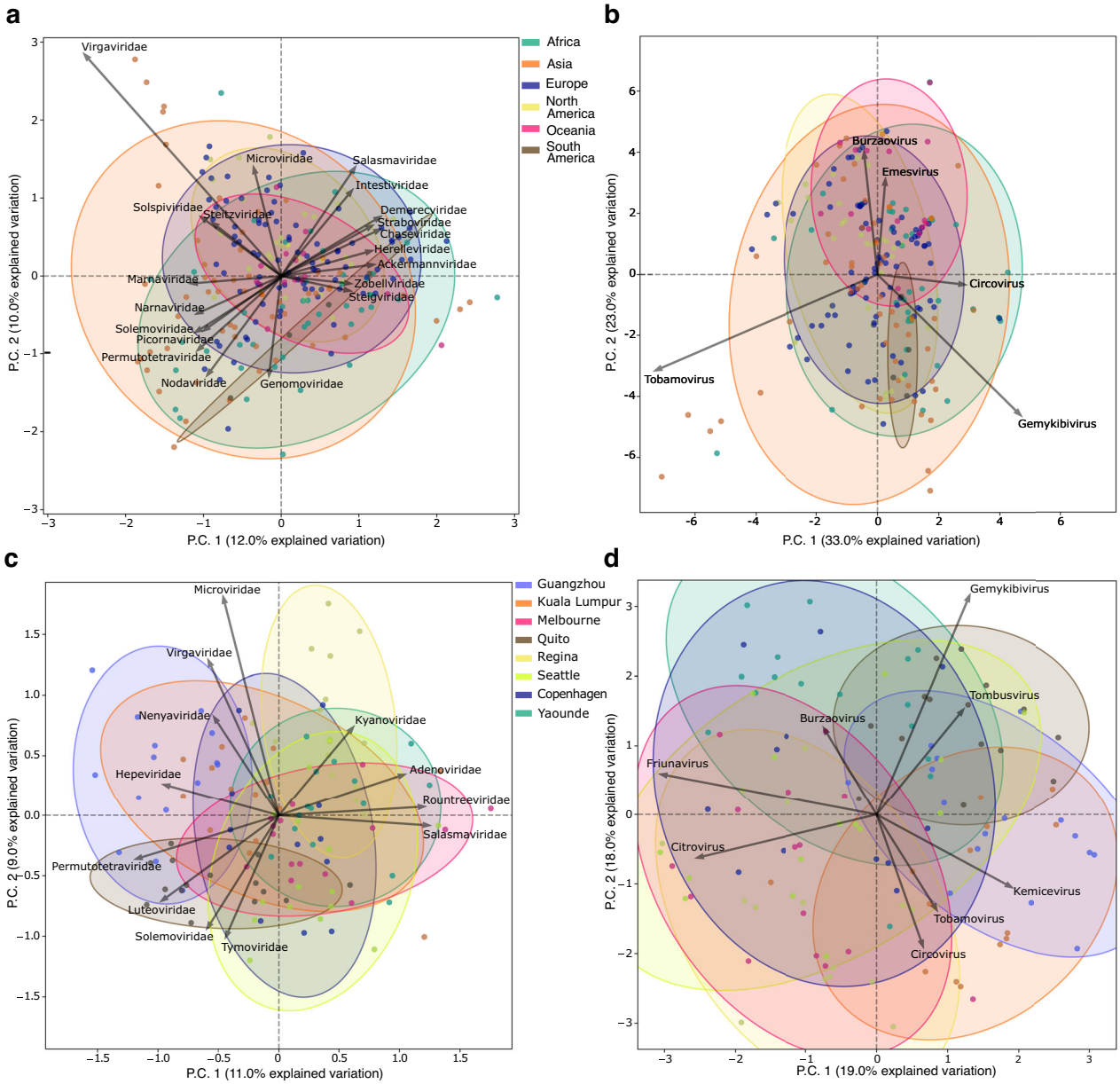

Fig. 4 | PCA clustering of the urban wastewater virome compositions from 62 cities based on the shotgun metagenomic sequence dataset reveals city-level differences without clear continental clustering. The virome compostions of the biannual samples (**a**, **b**) coloured by continent and longitudinal samples (**c**, **d**) coloured by city at the family (**a**, **c**) and genus (**b**, **d**) levels. Principal components were derived from genome size adjusted read counts subjected to a CLR transformation. Arrows represent viral families or genera, with their direction showing their contribution to the principal components and their length indicating the strength of their contribution to the variance in virome composition.

continents (Fig. 3a), *Enterovirus* was mainly detected at high levels in samples from Africa, a few cities in Europe (Belgrade, Bratislava), North America (Sisimiut, Chapel Hill), and South America (Quito) (Fig. 3b). Conversely, *Paslahepevirus*, to which Hepatitis E belongs, was more prevalent in Europe than in other regions. The longitudinal samples further revealed distinct spatial and temporal patterns in the prevalence of certain gastrointestinal viruses. For example, in Regina (Canada), *Enterovirus* levels showed a relative increase during summer months, whereas *Mastadenovirus* abundance was higher in the winter months.

Principal Component Analysis revealed that cities differ in virome composition

To identify broader patterns and potential geographical signatures in virome composition, we applied principal component analysis (PCA) on both the metagenomic and GastroCap sequence datasets. The metagenomic sequence data revealed no clear clustering patterns across continents at either the family or genus level, suggesting that the viral community structures were broadly similar worldwide (Fig. 4a, b). The limited variance between locations was primarily driven by the genera *Tobamovirus* (a group of plant viruses within the *Virgaviridae* family) and *Gemykibivirus* (associated with multiple hosts, including plants, insects, mammals, and occasional human samples[32]), both of which were more prevalent in samples from Asia. However, when comparing the virome composition by city, more pronounced differences were observed in both biannual and longitudinal samples (Supplementary Fig. S4 and Fig. 4c, d). This pattern was consistent when performing the PCA separately for DNA and RNA viruses (Supplementary Figs. 6 and 7), supporting the robustness of these findings across viral genome types.

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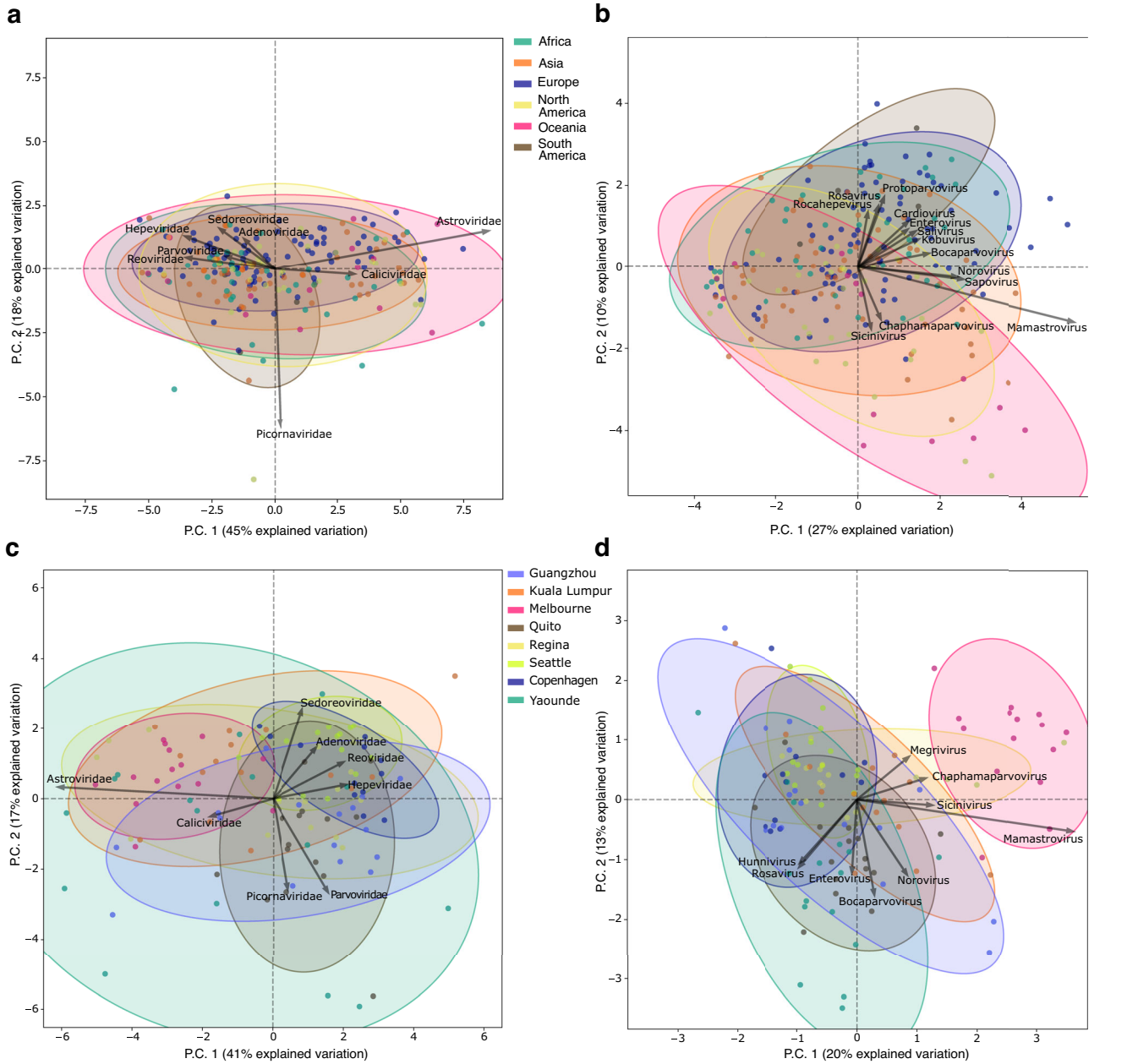

Fig. 5 | PCA clustering of the urban wastewater virome compositions from 62 cities based on the GastroCap sequence dataset highlight city-level clustering, primarily driven by *Mamastrovirus*. The virome compostions of the biannual samples (**a**, **b**) coloured by continent and longitudinal (**c**, **d**) samples coloured by city at the family (**a**, **c**) and genus (**b**, **d**) levels. Principal components were derived from genome size adjusted read counts subjected to a CLR transformation. Arrows represent viral families or genera, with their direction showing their contribution to the principal components and their length indicating the strength of their contribution to the variance in virome composition.

Focusing on the enteric virus families in the GastroCap sequence dataset, PCA revealed a broadly similar composition of these viral families across all continents, as indicated by overlapping clusters in the plot (Fig. 5a). The largest differences were observed for the *Astroviridae* and *Picornaviridae* families. However, at the genus level, more distinct differences in viral composition were observed between continents (Fig. 5b). Notably, Europe and Africa showed overlapping compositions, while the dataset for Oceania was distinctly separated. At the city level, even for the biannually sampled cities, PCA revealed clear distinct clustering, indicating significant variation in viral compositions between cities (Fig. 5c, d, Supplementary Fig. 5). These analyses identified the genus *Mamastrovirus* (*Astroviridae)* as the primary driver of beta-diversity. *Norovirus* and *Sapovirus* showed covariation, as reflected by the small angle between their loading vectors, indicating similar contributions to the observed variation. Within the *Picornaviridae* family, genera such as *Enterovirus*, and *Cardiovirus, also* displayed some degree of covariation. The cities with longitudinal sampling showed that over time, Seattle (USA) and Copenhagen (Denmark) exhibited tightly clustered sample groupings at the family level, suggesting a stable composition of enteric virus families throughout the study period. In contrast, the virome composition in Regina (Canada), Kuala Lumpur (Malaysia), Guangzhou (China), and Yaoundé (Cameroon) showed greater variability, reflecting a more dynamic and heterogeneous composition over time. At the genus level, the enteric virome composition in Melbourne (Australia) was distinct from that of all other cities, primarily due to a high abundance of *Mamastroviruses* which may reflect differences in interregional connectivity (Fig. 5d).

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Fig. 6 | Global prevalence and abundance of species within the *Tobamovirus* genus in urban wastewater. Biannual (**a**) and longitudinal data (**b**) are shown. In each panel, stacked bar plots display the number of reads per million viral reads assigned to the *Tobamovirus* genus (green) and to specific species (red), followed by the relative abundances of *Tobamovirus* species. The biannual samples were grouped using hierarchical clustering based on species-level composition. YoMV Youcai mosaic virus, TVCV Turnip VeinClearing virus, TSAMV Tropical soda apple mosaic virus, ToMV Tomato mosaic virus, ToMMV Tomato mottle mosaic virus, TMV Tobacco mosaic virus, TMGMV Tobacco mild green mosaic virus, TBRFV Tomato brown rugose virus, RMV Ribgrass mosaic virus, RheMV Rehmannia mosaic virus, PMMoV Pepper mild mottle virus, PaMMV Paprika mild mottle virus, HLSV Hibiscus latent Singapore virus, HLFPV Hibiscus latent Fort Pierce virus, CMoV Cucumber mottle virus, CGMMV Cucumber green mild mottle virus, BPMV Bell pepper mottle virus, SA South America.

Species and genotype-level analysis of selected viral genera

We further explored the value of wastewater for monitoring viruses at finer taxonomic resolution, at the level of species and genotypes to capture fine-scale spatiotemporal patterns of viral diversity. The genera *Tobamovirus (Virgaviridae)* from the metagenomic sequence data and *Mamastrovirus* (*Astroviridae)* and *Enterovirus (Picornaviridae*) from the GastroCap data had the largest influence on virome compositions across the biannual samples in the PCA. Due to their relevance to plant, public, and animal health, these genera were selected for further investigation.

Shotgun metagenomic sequencing reveals Tobamovirus diversity in wastewater

Tobamoviruses are of major concern because they can cause disease in a wide range of agriculturally important plant species, affecting tomato, pepper and cucumber plants. We identified a total of 5204 *Tobamovirus* contigs in the metagenomic dataset of which 32% (1653 contigs) were assigned to 17 different species (Fig. 6), including both widely distributed, well-known viruses and emerging viruses with increasing agricultural and environmental relevance. Analysis of the sequences at the species level showed large compositional differences between samples and locations. Overall *Cucumber green mild mottle virus* (CGMMV) and *Pepper mild mottle virus* (PMMoV) – both well-studied and widely distributed viruses - were highly prevalent in urban sewage samples worldwide. PPMoV did not exhibit temporal variation, but its relative abundance was lower in Asia (Fig. 6a, b). The relative abundance of CGMMV was highest in samples from Europe, Asia and North America (Fig. 6a, b). *Tobacco mosaic virus* (TMV) was predominantly found in high abundance in samples from Asia and Africa, while *Tobacco mild green mosaic virus* (TMGMV) was primarily observed in samples from Asia (Fig. 6a, b). Additionally, we detected emerging viruses of concern, such as the rapidly spreading *Tomato brown rugose fruit virus* (ToBRFV), which was detected in wastewater samples from multiple locations including Rome (Italy) in 2017 and Athens (Greece), Vancouver (Canada), Be'er Sheva (Israel) and Seattle (USA) in 2018 (Fig. 6a, b).

Global diversity and phylogenetic analysis of Mamastrovirus in wastewater

Variation in the relative abundance of the genus *Mamastrovirus* contributed most to the differentiation of samples in the PCA in the GastroCap sequence dataset (Fig. 5b). To explore if these differences were associated to specific *Mamastrovirus* types, we developed a custom typing workflow using reference sequences from a phylogenetic study on astrovirus diversity[33]. Our approach identified 10,668 *Mamastrovirus* contigs of which 14% (1512 contigs) could be assigned a genotype, resulting in the detection of 24 different types. Our analysis revealed both well-established (endemic) and less commonly observed *Mamastrovirus* genotypes across various global wastewater samples. There was substantial variation in *Mamastrovirus* genotype

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Fig. 7 | Global prevalence and abundance of *Mamastrovirus* genotypes in urban wastewater. Biannual (**a**) and longitudinal data (**b**) are shown. In each panel, stacked bar plots display the number of reads per million viral reads assigned to the *Mamastrovirus* genus (green) and to specific genotypes (red), followed by the relative abundances of *Mamastrovirus* genotypes. The biannual samples were grouped using hierarchical clustering based on genotype composition. HAstV Human Astrovirus, RAstV Rat astrovirus, CAstV Canine astrovirus, FAstV Feline astrovirus, ChAstV Cheetah astrovirus, YakAstV Yak astrovirus, Other genotypes detected in fewer than 4 samples.

distribution between samples. Compositional differences in genotypes did not clearly correlate with specific continents or cities in the biannual dataset (Fig. 7a). Human astroviruses (HAstV) formed the major fraction of *Mamastrovirus* genotypes detected, among these, the classical HAstV type 1–5 and 8 were most frequently detected. HAstV-1, a genotype considered endemic worldwide, was frequently detected across all sampled continents and was the predominant genotype in many samples. HAstV-8, a genotype rarely reported in clinical surveillance, was also detected in high relative abundance across all continents. Additionally, recently discovered 'non-classical' astroviruses, including MLB1, VA1/HMO-C, VA2/HMO-A and VA3/HMO-B were detected across multiple continents. Human astrovirus BF34, first identified and exclusively detected in Burkina Faso in 2010 (Africa), was detected in wastewater samples from Cameroon (February and March 2018), Austria (June and November 2018) and Uganda (July 2018) indicating its presence across multiple geographic regions in 2018. While less common in wastewater samples, animal astroviruses like, canine- (CAstV), rat astrovirus (RAstV) and feline astrovirus (FAstV) were detected across continents, with surprisingly high prevalence of CAstV in Regina, Canada. For the cities with longitudinal sampling, temporal shifts in the genotype distribution were observed. PCA analyses showed that *Mamastrovirus* was a major driver for the distinct clustering of Melbourne's virome (Fig. 5d) with consistently high proportions of *Mamastrovirus* reads detected. In Melbourne HAstV-5 was more prevalent during 2017 and in early 2018, while HAstV-1 was mainly prevalent during the remainder of 2018. Other cities also exhibited peaks in *Mamastrovirus* reads over the observation period, but the timing of these peaks varied across locations and was not linked to a specific genotype.

To investigate the global diversity and circulation patterns of the abundant 'classical' human astroviruses in greater detail, we conducted phylogenetic analysis. The retrieval of partial capsid sequences for HAstV type 1, 2, 3, 4, 5 and 8 (Fig. 8 and Supplementary Fig. 8) from wastewater considerably expands the number of available capsid sequences from previously underrepresented regions. For several types we observed wastewater-specific clusters, indicating that current clinical surveillance does not fully capture the diversity of HAstVs. Additionally, phylogenetic analysis revealed that integrating global wastewater sequences within a limited timeframe provided insights into country and city specific virus circulation. Viral sequences from the same city often clustered together, as did sequences from samples collected closer in time suggesting periodic circulation and replacement of viral lineages over time. Notably, evidence suggestive of region-specific virus circulation was observed for some African HAstV type 4, 5, and 8 clusters. For example, HAstV-5 wastewater sequences from Cameroon (2018), Senegal (2019), and human faecal sequences from Cameroon (2014) formed a distinct cluster. Similarly, a separate HAstV-8 cluster comprised of sequences from clinical samples from Cameroon from 2014 and wastewater sequences from Nigeria (2018) was observed.

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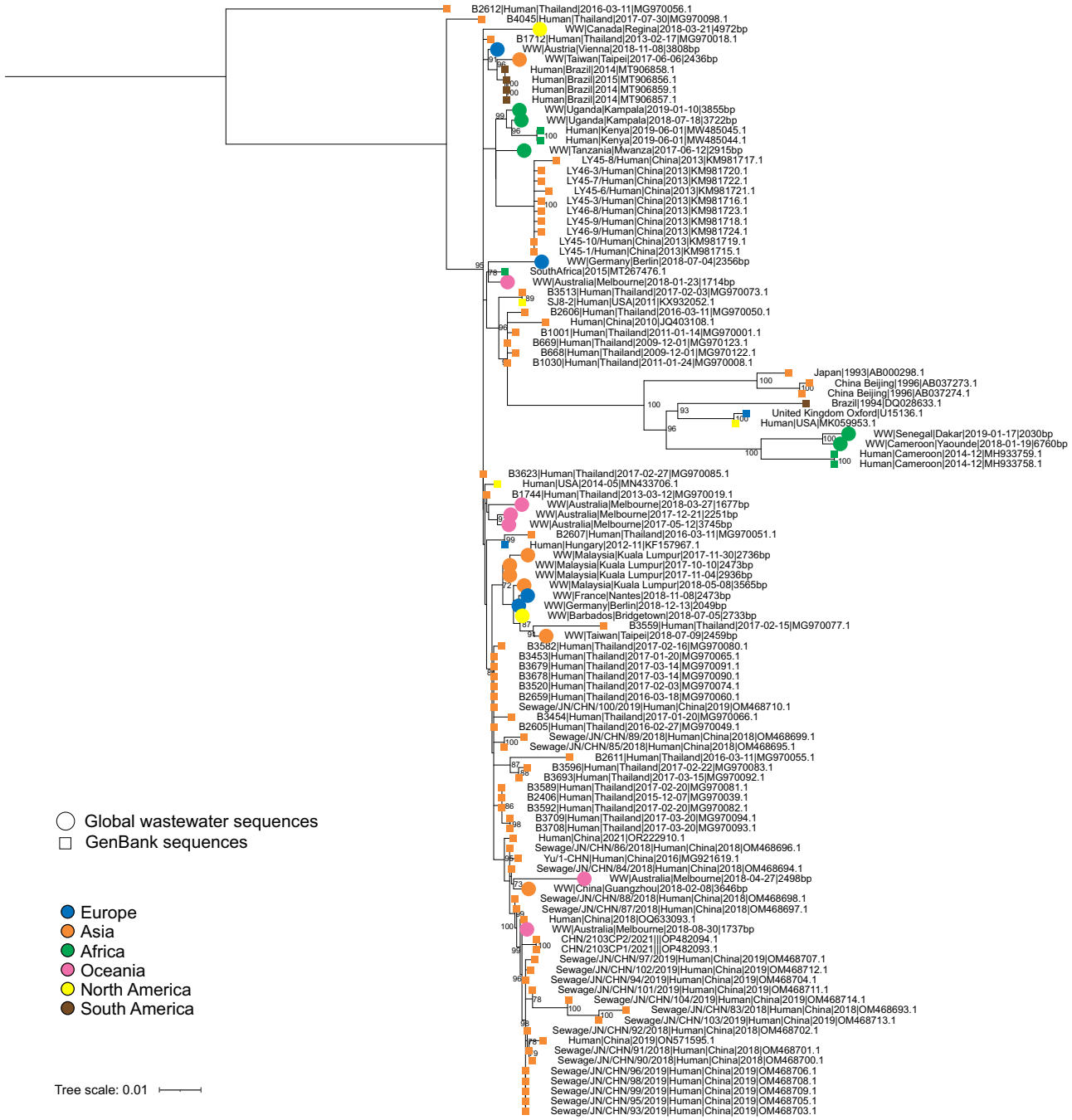

Fig. 8 | Phylogenetic analysis of Human astrovirus type 5 (HAstV-5) from global wastewater and publicly available reference sequences. Maximum likelihood phylogenetic tree of HAstV-5 based on partial ORF2 gene (capsid) sequences. Sequences obtained from GenBank are indicated in coloured squares, while sequences derived from wastewater with a minimum length of 500 bp are denoted in coloured dots. Colours represent the continent of origin (Europe, Asia, Africa, Oceania, North America, and South America). Bootstrap values > 70 are shown.

Global diversity and phylogenetic analysis of enterovirus in wastewater

The abundance of *Picornaviridae* varied across continents, with enteroviruses frequently detected using GastroCap, significantly influencing PCA clustering patterns (Fig. 5d). Enteroviruses, a major public health concern due to their potential to cause a wide range of illnesses, including respiratory infections and neurological diseases, were analysed in wastewater to evaluate their surveillance potential in metagenomic datasets. A total of 2331 contigs were classified within the *Enterovirus* genus, of which 37% (868 contigs) could be genotyped using the RIVM Enterovirus genotyping tool, corresponding to 62 distinct *Enterovirus* genotypes.

Analysis of prevalence and relative abundance revealed differences in enterovirus distribution across continents (Fig. 9a, b). Samples from Africa showed the highest enterovirus abundance compared to other regions. *Enterovirus B* was the most prevalent species worldwide, with Coxsackievirus (CV) B5 and CV-A9 being the most prevalent types across all regions, suggesting widespread endemicity. In North America *Enterovirus A* was the most prevalent species, with Coxsackievirus CV-A6 and CV-A4, CV-A10 – endemic enteroviruses and common causes of hand, foot, and mouth disease- among the most detected types. *Enterovirus C*, though less prevalent, was frequently detected in the African region and was the dominant enterovirus species in longitudinal samples from Ecuador, Malaysia, and

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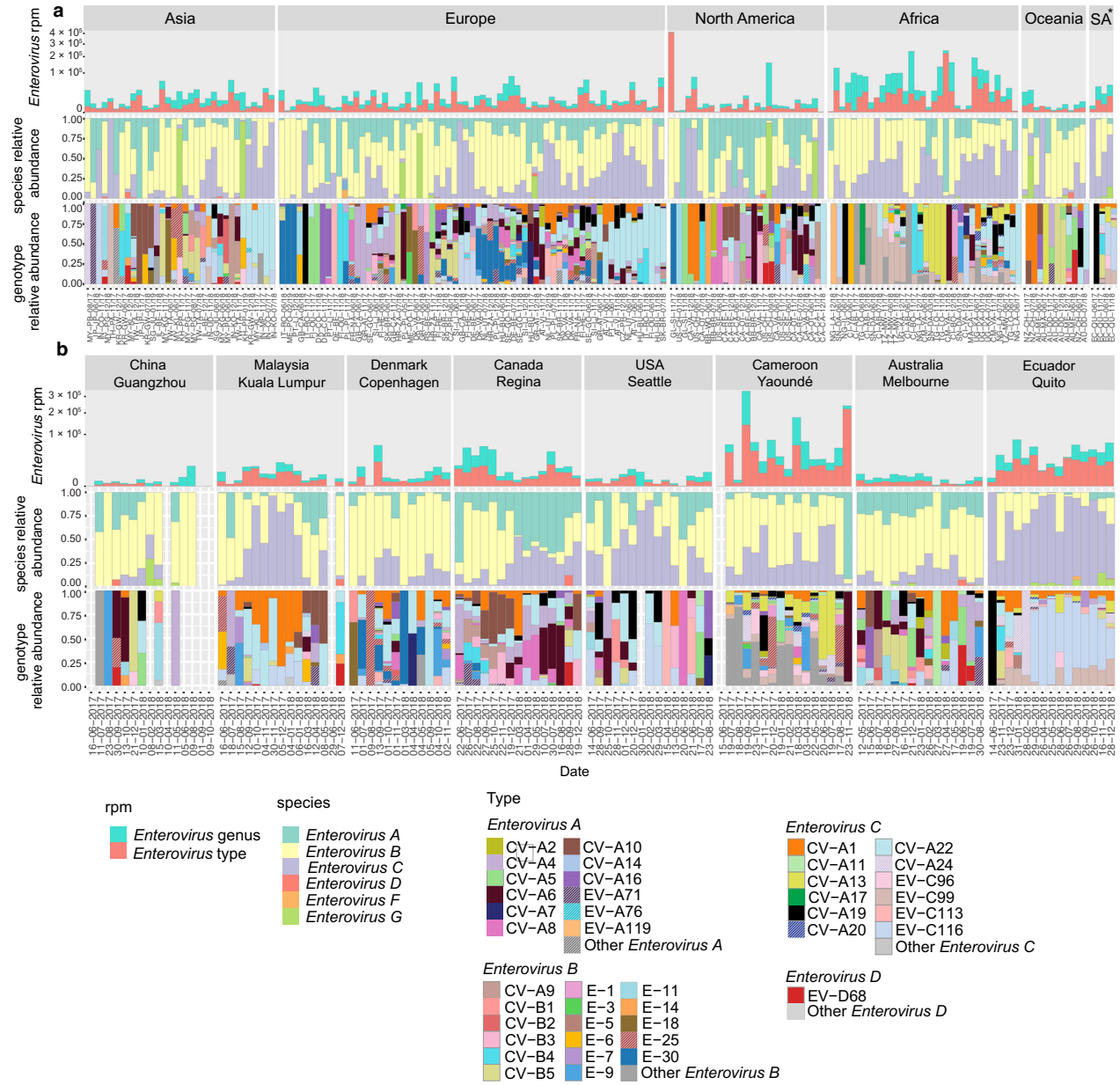

Fig. 9 | Global prevalence and abundance of *Enterovirus* genotypes in urban wastewater. Biannual (**a**) and longitudinal data (**b**) are shown. In each panel, stacked bar plots display reads per million viral reads assigned to the *Enterovirus* genus (green) and to specific genotypes (red), followed by the relative abundances of *Enterovirus* species and of genotypes. The biannual samples were grouped using hierarchical clustering based on genotype composition. Other = genotypes detected in fewer than 10 samples, including (vaccine-derived) poliovirus type 3. SA South America.

Cameroon. While *Enterovirus D* was detected sporadically, Enterovirus D68—a clinically significant genotype due to its association with respiratory illness outbreaks and acute flaccid myelitis—was primarily identified toward the end of 2017 and in 2018. In most locations, Enterovirus D68 Clade B3 was predominant, while Clade A2 was detected in Athens and Taipei. *Enterovirus G*, associated with infection in pigs, was detected in high abundances in several cities across all continents.

Geographical specificity was evident for certain enterovirus genotypes. For example, CV-A20 was exclusively found in Africa and South America, while CV-A1 and CV-A22 were detected across all continents. In Europe, Echovirus type 30 (E-30) displayed an unusually high prevalence, which was not observed in other regions, possibly indicating a recent outbreak or re-emergence after a period of lower endemic circulation. Phylogenetic analysis of partial VP1 sequences revealed that European wastewater E-30 sequences from 2018 were generally more closely related to each other than to sequences from 2017 (Fig. 10).

Longitudinal analysis revealed dynamic patterns in enterovirus genotype distribution across cities over time (Fig. 9b). For instance, Yaoundé (Cameroon) exhibited a broad diversity of circulating genotypes, including vaccine-derived poliovirus type 3. No sequences of the typing region VP1 for other polio types were detected. Longitudinal analysis across sites identified periods of genotype dominance, with CV-C116 – a lesser-known member of *Enterovirus C* with unknown clinical relevance - prevailing in Quito in 2018 and CV-A1 in Kuala Lumpur during 2017 and early 2018. In Regina, seasonal variations in overall enterovirus presence could be observed, with elevated levels occurring primarily during the summer and fall. Additionally, while multiple genotypes circulated in 2017, CV-A10 was present in higher

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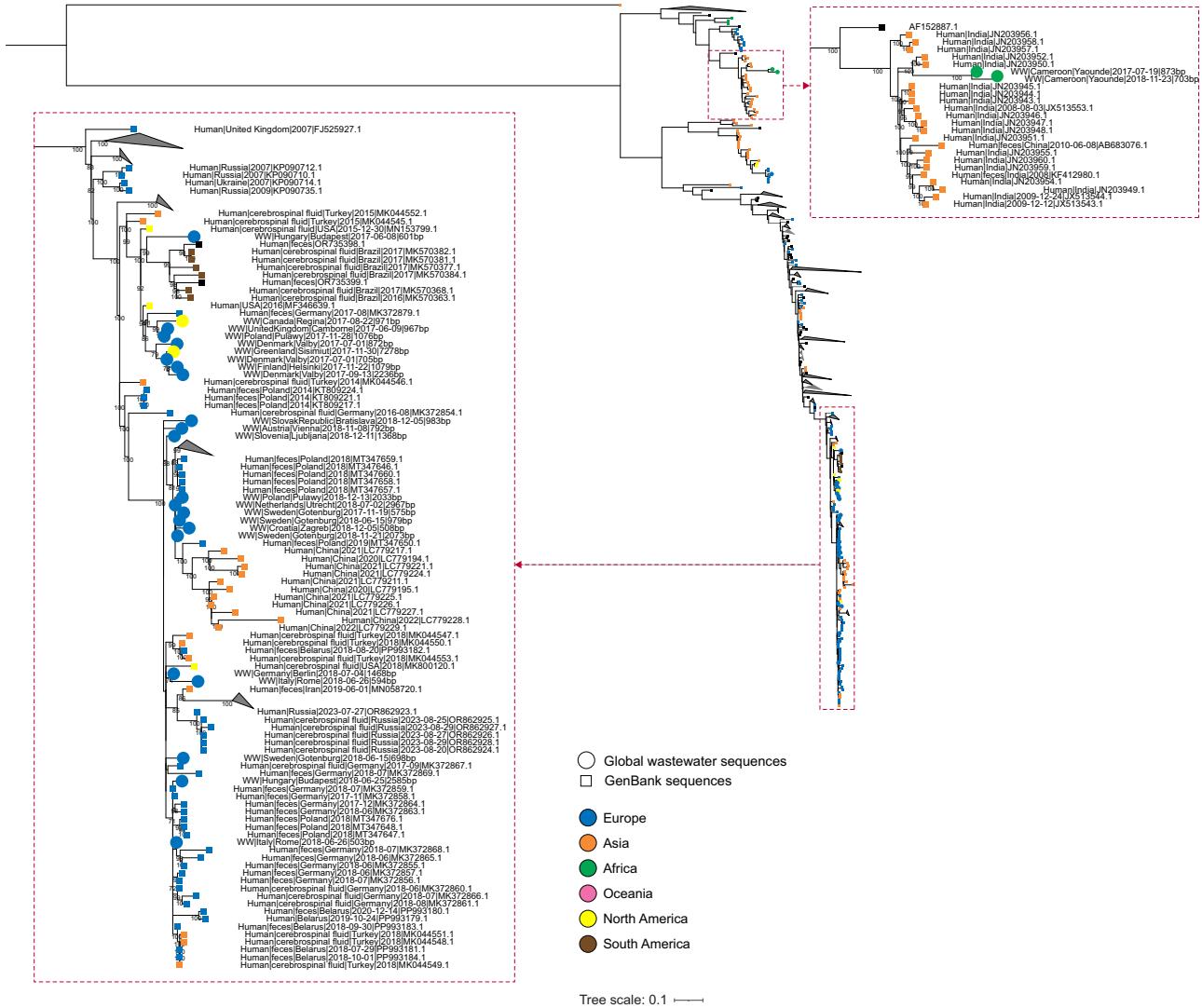

Fig. 10 | Phylogenetic analysis of Echovirus 30 (E30) from global wastewater and publicly available reference sequences. Maximum likelihood phylogenetic tree of E30 based on partial VP1 gene (capsid) sequences. Reference sequences obtained from GenBank are indicated with coloured squares, while sequences derived from wastewater with a minimum length of 500 bp are denoted in coloured dots. Colours represent the continent of origin (Europe, Asia, Africa, Oceania, North America, and South America). Bootstrap values > 70 are shown.

relative abundance during specific periods, and shifted to CV-A6 in 2018, indicating dynamic changes in circulating enterovirus populations over consecutive years. Additionally, EV-A71 –one of the leading causes of hand, foot and mouth disease and associated with neurological complications - was detected in Regina in July, August and November 2017, coinciding with a period of elevated laboratory confirmed enterovirus/rhinovirus cases in Canada that year according to national surveillance data[34,35].

Discussion

This study provides a comprehensive analysis of the urban wastewater virome of 62 major cities worldwide using shotgun metagenomic and capture-based (GastroCap) sequencing. By incorporating both biannual and longitudinal samples, we assessed viral diversity and temporal dynamics. While biannual sampling provided a snapshot of viral diversity within a particular location, more frequent sampling revealed a higher viral diversity per location and enabled the detection of gradual shifts in composition. Shotgun metagenomics, at least with the sequencing depth used in our study, yielded a relatively low proportion of enteric virus reads, making it difficult to effectively monitor enteric viruses in complex wastewater samples. The GastroCap probe set significantly enriched clinically relevant enteric viruses, whereas metagenomic sequencing primarily detected bacteriophages and plant viruses. Thus, for the surveillance of human pathogenic enteric viruses, which are stable in aquatic environments[36], wastewater is a valuable source when combined with GastroCap enrichment. Additionally, a large proportion of reads could not be assigned to known virus taxa, highlighting the potential for future discovery of novel viruses. Our findings and those of others demonstrate the utility of wastewater metagenomics as a powerful One Health surveillance tool, capturing the diversity and dynamics of viruses associated with human, animal, and environmental health.

Cross-continental comparisons revealed homogeneity in viral community composition at high taxonomic levels, consistent with findings that viral communities are often more specific to environmental habitats (e.g. wastewater or marine) rather than geographical location[37]. At the city level, however, we observed geographical partitioning at both the family and genus levels, likely influenced by local factors such as climate, weather, demographics, agricultural practices, diet, population density and previous exposure history[30]. Sampling in

South America, Central America and Oceania was limited, making the findings less generalizable for these regions. Additionally, an expanded dataset of these wastewater samples -sequenced using a strategy optimized for bacterial and AMR profiling- showed distinct regional variations in the resistome and bacteriome across continents. Specifically, bacterial composition divides the world's regions into roughly two major groups (Europe, Central Asia & North America versus Africa & Middle East), while the resistomes showed more unique regional profiles[31]. Our analysis revealed very limited antibiotic resistance gene (ARG) presence in the viral fraction, which may be due to the virus-enrichment protocol and the infrequent occurence of ARGs by bacteriophage genomes[38] (Supplementary Table 1).

By increasing taxonomic resolution to the species and genotype level, our results and those of others demonstrate that metagenomic and phylogenetic analysis of viral communities in wastewater can reveal distinct temporal and geographical patterns[30]. *Tobamoviruses* were highly prevalent in urban wastewater, consistent with their detection in various water sources[39–41] The widespread detection of CGMMV and PMMoV in our study align with their documented widespread prevalence[42–44]. Notably, PMMoV has been proposed as a potential viral indicator for human faecal contamination in water and wastewater[37,42,43]. However, our data showed significant regional variability, with lower proportions in Asia, suggesting that regional differences should be considered when interpreting PMMoV in water quality assessments. Variations in the composition of *Tobamovirus* species might reflect differences in agricultural practices or diet. For instance, the detection of TMGMV in China could be related to their substantial tobacco production and consumption[45–47]. Importantly, early evidence of the emerging plant viruses like ToBRFV could also be detected. ToBRFV, which causes severe infections in tomatoes and peppers, was first described in Jordan in 2015, and retrospectively traced to Israel in 2014[48]. Since then, it has spread widely, with our data indicating its presence in Canada as early as July 2018, more than a year before its first official report in 2019. Similarly, detections in Italy in November 2017 and in Greece in June 2018 precede their reported outbreaks by nearly a year[49,50]. These findings highlight the utility of wastewater monitoring for the early detection and tracking of emerging plant pathogens.

Extending our study to viruses impacting animal and human health, we were able to characterize the diversity of astroviruses on a global scale. The prevalence and distribution of HAstV genotypes in our study generally align with trends observed in environmental, epidemiological, and serological studies. The detection of emerging viruses is an important focus of wastewater surveillance. In our study, 'non-classical' astroviruses, such as HAstV-MLB1, HAstV-VA1/HMO-C, HAstV-VA2/HMO-A, and HAstV-VA3/HMO-C which are sometimes associated with neurological disease in immunocompromised individuals[51] were detected. Consistent with clinical reports indicating that non-classical astroviruses generally have lower detection rates than classical HAstVs, our wastewater data reflect this pattern with fewer samples positive for HAstV-MLB1 and a lower relative abundance compared to classical HAstVs[51,52]. However, in certain samples, non-classical astroviruses were the predominant type, which may contribute to the high seroprevalences observed in seroepidemiological studies[53,54]. An earlier metagenomic wastewater study reported higher abundances of astroviruses in the northern hemisphere during winter compared to the southern hemisphere[55], and seasonal peaks in HAstV infections have been observed in colder months in regions such as China, Spain, and the USA[56–58]. However, comprehensive understanding of astrovirus types and their seasonality remains limited, and it is unclear whether types are constantly prevalent throughout the year or if they disappear and re-emerge over time[52]. Our findings showed consistent detection of some astroviruses like HAstV-1 in most locations with longitudinal sampling, while other types such as MLB1, were only detected in specific months (Fig. 7b). These findings suggest that astrovirus seasonality may be type specific. The type specific differences observed in our study may reflect a complex interplay of viral characteristics (e.g. shedding duration, tropism), host population characteristics (e.g, population immunity, age distribution), and environmental factors (e.g. temperature, humidity, rainfall)[57–60].

Like astroviruses, enteroviruses have a major impact on public health, and polioviruses were the earliest viruses to be monitored in wastewater. Across our globally distributed wastewater samples, enterovirus abundance consistently showed a dominance of *Enterovirus B* across most regions, and high abundance of *Enterovirus C* types in African wastewater samples, aligning with trends from clinical surveillance[60,61]. In contrast, *Enterovirus A* types, such as CV-A6 and CV-A10, which are frequently reported in clinical samples from Asia, were not dominant in wastewater from this region[60], potentially reflecting biases in clinical surveillance efforts that are targeted towards detection of the enterovirus types causing hand, foot, and mouth disease. EV-D68, another focus of clinical surveillance, was infrequently detected, possibly due to its respiratory transmission route and limited faecal shedding[62]. Its biennial circulation patterns, with even-numbered years from 2014 to 2018 in temperate climates[63,64], were reflected in our data, in which we detected EV-D68 primarily in 2018. Unlike some other viruses, wastewater detections of EV-D68 coincided with, rather than preceded, the timing of clinically recognized circulation. These findings suggest that the potential of wastewater metagenomics for early detection varies by virus and is influenced by biological factors, such as shedding dynamics, stability, and transmission mode, as well as clinical surveillance-related factors. Dynamic changes in enterovirus populations over time could be observed, including for genotypes not commonly detected in clinical surveillance, giving a broader perspective on circulating diversity. In many cases we only recovered partial genomes, complicating taxonomic annotation and limiting the ability to determine the clade, type, subtype or lineage which are often necessary to investigate outbreaks[65,66]. For example, poliovirus type 3 detected in Cameroon likely reflects shedding of oral polio vaccine (OPV3) strains, which can, in rare cases, mutate into vaccine-derived polioviruses (VDPVs) capable of causing outbreaks. However, insufficient genomic resolution in critical regions (e.g., VP1, 5'UTR) prevented confirmation of whether the detected sequences represented vaccine strains or VDPVs.

Wastewater monitoring also captured emerging enteroviruses, exemplified by Enterovirus E30. High proportions of E30 were observed in European samples, aligning with increased reporting of E30 across European countries from 2015–2017[67], and a subsequent rise in meningitis and meningoencephalitis cases from April to September 2018[68]. Notably, the detection of E30 in European wastewater as early as June 2017 preceded the clinical observations, suggesting undetected circulation before its recognized outbreak. These findings demonstrate that wastewater monitoring provides a comprehensive view of enterovirus diversity, geographical spread, and temporal changes, enabling the detection of both clinically targeted and undersurveilled types and the (re)emergence of specific genotypes.

Interestingly, animal-associated viruses were frequently detected in our dataset, including canine- and feline astrovirus and porcine enterovirus G, likely reflecting contamination from pet waste or animal industry runoff entering sewage systems. The high abundance of canine astrovirus in Regina (Canada) may be linked to local practices encouraging the flushing of dog waste into sewage systems, a method recommended in several Canadian cities since 2017 to reduce environmental pollution[69].

In conclusion, in this study, shotgun metagenomic and capture-based sequencing of wastewater provided insights into the genetic diversity and spatiotemporal patterns of both endemic and emerging viruses. In resource-limited settings, where clinical testing facilities and surveillance infrastructure may be constrained, centralized metagenomic wastewater-based monitoring could provide a cost-effective

and scalable alternative to detect viral threats. These findings underscore the value of wastewater metagenomic analysis as a One Health resource, offering insights into infectious disease dynamics.

Methods

Urban wastewater sample collection

Raw wastewater samples were collected from 62 sites from 6 continents as previously described[70]. Briefly, unfiltered urban wastewater samples prior to the inlet of the wastewater treatment plant or main outlet to rivers were acquired. Where possible, flow proportion sampling over 24 h or otherwise three crude point samples were obtained for representative sampling. Samples were stored at −80 °C and shipped. Additional metadata containing details on the sampling location and the conditions of the samples such as temperature and sample consistency were gathered. The biannual samples were obtained around June and November in 2017 and 2018 (and in some cases early 2019). Monthly longitudinal samples were collected from eight locations across six continents during the same period. The map showing the sampling locations was generated in R using the tidygeocoder package (version 1.0.6)[71], and geocoded coordinates were obtained via the OpenStreetMap Nominatim API[72].

Sample processing

Samples were thawed at room temperature and 50 ml of raw wastewater was centrifuged at 3744 × g for 15 min to remove large debris. The supernatant was filtered using 0.45 μm pore diameter filter to remove bacterial and eukaryotic cells. Although 0.22 μm filters are also used in virome studies, they have been associated with reduced recovery of certain viral families and were therefore not used in this study[73]. A volume of 30 mL of filtered supernatant was concentrated using 30 kDa Pierce™ Protein Concentrators PES (Thermo Fisher Scientific) by sequential centrifugation at 6000 × g at 4°C (15 min for the initial 15 mL, followed by 30 min for the second 15 mL added to the same concentrator). The filtrate was treated with OmniCleave™ Endonuclease (Lucigen Corporation) for the removal of extracellular nucleic acid (Supplemental Fig. S9). Total nucleic acid content was extracted with the High Pure Viral Nucleic Acid Kit, without the use of DNAse treatment (Roche).

Metagenomic and capture-based sequencing

Library preparation was done using the KAPA HyperPlus kit (Roche) using Superscript IV and random priming to synthesize cDNA from RNA. Subsequently, double-stranded DNA (dsDNA) was generated using Klenow and the samples were subjected to enzymatic fragmentation. End repair and A-tailing was performed and KAPA Dual Indexed adaptors were ligated. A post-ligation cleanup step was done using 0,8 x AMPure XP Beads (Beckman Coulter). After that, a double-sided size selection was performed according to the manufacturer's instruction. Library PCR amplification was conducted using a total of 24 cycles. After purification, the quantity and quality assessment of the libraries was carried out using a Qubit 4 Fluorometer (Thermo Fisher Scientific) and an Agilent 2100 Bioanalyzer, following the respective manufacturers' protocols. The samples were pooled in equimolar proportions and sequenced directly or used as input for the GastroCap following sequencing on the Illumina MiSeq platform to generate 2 × 300 nt paired end sequences.

For target enrichment the GastroCap probe set was used. This probe set was designed to target a manually curated list of vertebrate virus species belonging to the *Adenoviridae*, *Astroviridae*, *Caliciviridae*, *Hepeviridae*, *Parvoviridae*, *Picornaviridae*, *Sedoreoviridae and Spinareoviridae* families (Supplementary Fig. 1). The probes were based on all sequences available in GenBank >500 nucleotides for the selected species. To minimize redundancy, sequences were clustered at 95% nucleotide identity. To enhance representation of less abundant viral species, sequences from genera other than the highly abundant *Enterovirus, Mastadenovirus*, and *Rotavirus* were 'boosted' by multiplying their sequence abundance by a factor of three in the input dataset.

The GastroCap probe set was designed by Roche using their proprietary Nimble Design workflow. Six library samples were pooled in equimolar amounts and purified using AMPure XP Beads of the KAPA HyperCapture Bead kit (Roche). The GastroCap probes were diluted 1:10, and 4,5 mL was added to 10,5 mL of the sample pools. Hybridization was performed by incubating the samples at 95 °C for 5 min and then at 47 °C for 72 h. Following hybridization, 50 mL capture beads from the KAPA HyperCapture Bead kit (Roche) were washed according to the manufacturer's protocol, added to the hybridized sample pools (15 mL) and incubated at 47 °C for 15 min. The sample pools were then washed, and a post-capture PCR was performed using 14 cycles before purification by AMPure XP Beads. The quantity and quality of the captured library pools were assessed using the Qubit 4 Fluorometer and the Agilent 2100 Bioanalyzer following the manufacturers' protocols. The capture reactions were then pooled equimolarly and sequenced on the Illumina MiSeq platform to generate 2 × 300 nt paired end sequences. To monitor potential contamination and technical consistency, we included negative controls and resequenced one sample as a technical replicate for each batch. As shown in Supplementary Fig. 9, replicates with similar read depth exhibited comparable taxonomic composition.

Sequence data analysis

The sequence analysis workflow was generated using Snakemake (version 7.19.1)[74]. Raw fastq files were quality trimmed using FastP (version 0.23.4)[75]. Read ends were trimmed to a mean quality Phred score of 25 with a sliding window of 5. Reads shorter than 30 nucleotides were discarded as well as reads with an average Phred score below 25. To identify and remove PCR duplicates, sequencing reads were deduplicated using CD-HIT[76], which groups and removes duplicate reads that are identical within the first 150 nucleotides. Reads were aligned to the GRCh38 human genome reference (version GCF_000001405.26) using BWA-MEM (version 0.7.17)[77] and filtered using SAMtools (version 1.6)[78] before the reads were assembled using metaSPAdes (version SP)[79]. Taxonomic annotation was performed using DIAMOND v2.1.10.164[80] using the NCBI non-redundant protein reference database. Taxonomic assignment of each assembled contig was done based on the highest bit score normalized by the length of the contig. Finally, reads were mapped to the contigs using BWA-MEM to obtain read counts. Additionally, viral contigs ≥300 bp were screened for antimicrobial resistance genes (ARGs) and prophages using geNomad (version 1.11.0)[81] with default settings. No prophages were detected, and only three contigs contained ARG annotations (Supplementary table 1).

Quantitative analysis: heatmaps of viral diversity

For further quantitative analysis, annotated contigs with a length ≥300 nucleotides and an average depth of coverage ≥3 were selected to ensure the inclusion of more reliable hits. The total number of reads assigned to the superkingdom "Viruses" per sample was used to normalize read counts. The total number of reads mapping to a contig (forward and or reverse) were adjusted by dividing by the average genome size per individual viral family. For viral families with large variation in genome sizes the average genus level genome size was used.

Relative viral abundance was calculated as reads per million (RPM) according to Eq. 1:

$$\text{Relative abundance} = \frac{\text{virus reads}}{\text{average genome length} * \text{total viral reads}} * 10^6 \quad (1)$$

The classification of viral families based on their hosts range was obtained from Virus-Host DB, which pairs viruses and hosts using NCBI taxonomy IDs, integrating host information from genome databases like RefSeq and GenBank, along with additional sources including UniProt, ViralZone, and literature[82].

Comparison of viral composition

We compared viral composition across different locations using PCA, accounting for the compositional nature of metagenomic data. Therefore, the Centred Log-Ratio (CLR) coefficients were computed for distinct data subsets to preserve the relative relationships between viral taxa[83]. PCA was conducted on the viral fraction of the dataset using genome-size adjusted read counts to calculate CLR values. This normalization was not applied to plots at the superkingdom level (Archaea, Bacteria, Eukaryota (human separate), Viruses and Unknown) due to significant variability in genome sizes at this taxonomic level. For the virome data at family, genus and species level (metagenomic sequence data), only viral taxa with a high CLR median and high variance were retained, excluding those with low abundance and minimal variability between samples. PCA was also performed separately for DNA and RNA viruses to explore potential differences in community composition between viral genome types.

For enteric viruses, all viral families targeted by the GastroCap probe set were included without additional filtering. Zero values were replaced using an Aitchison mean point estimate before applying the CLR transformation. The transformed data were then visualized in a biplot using the pyCoDaMath package in Python (version 3.8).

Taxonomic analysis at the species and genotype level for selected viruses

For selected viruses, the dataset was filtered to extract contigs belonging to specific genera using DIAMOND annotation with a contig length ≥300 and average depth of coverage of ≥3x. These contigs were then processed using custom workflows to determine species and genotype. These workflows involved mapping contigs with a blast search against a database of reference sequences from literature or existing typing tools, and assigning species and type based on virus specific ICTV classification criteria.

Tobamovirus species assignment

A custom blastn species assignment database was generated using sequences from all 37 ICTV-recognized *Tobamovirus* species. Contigs were assigned to specific species based on the criterion that strains within the same species must share >300 bp and 90% nucleotide sequence identity across the whole genome, following the species demarcation criteria proposed by ICTV[84].

Mamastrovirus and Enterovirus genotyping and phylogenetic analysis

The typing workflow for *Mamastrovirus* used a nucleotide blast database of complete ORF2 sequences sourced from Donato et al.[33]. Annotated *Mamastrovirus* contigs were aligned against this database using blastn. Contigs that covered at least 10% of ORF2 and exhibiting ≥85% nucleotide identity, in line with classification criteria proposed by the ICTV Astroviridae Study Group[52,85], were included for further analysis. *Enterovirus* contigs were assigned using the RIVM enterovirus typing tool, which performs a blastn search and phylogenetic analysis of the (partial) VP1 gene, with a minimum overlap of 100 bp required for classification[86]. For phylogenetic analysis, sequences covering >500 bp of ORF2 were considered sufficient for reliable alignment and tree-building. Complete and partial Human astrovirus (type 1-8) ORF2 sequences and E-30 VP1 sequences were retrieved from NCBI, combined with our sequences and aligned using MAFFT (version 7.508)[87]. Phylogenetic trees were generated using IQ-TREE (version 2.3.6), using automated model selection, the -czb option, and 1000 bootstrap replicates[88].

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The raw sequencing datasets, metagenome assemblies and analysis results are available on the European Nucleotide Archive under project: PRJEB87273. Source data are provided with this paper.

Code availability

The metagenomic workflow used in this study is made available at https://github.com/EMC-Viroscience/Worp_etal_Global_Sewage_Virome[89].

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Author contributions

M.P.G.K. and F.M.A. conceived the study, secured funding and provided overall supervision. M.G. & N.W. drafted the initial manuscript with input from M.P.G.K., M.G & B.B.O.M. supervised manuscript preparation and data analysis. C.M.E.S and N.W performed NGS. N.W. and D.F.N performed bioinformatic data analyses. E.E.B.J. and C.B. contributed to the PCA and provided methodological guidance. N.W. produced the figures. D.F.N, R.W.I.L., C.M.E.S., C.B., E.E.B.J., P.M., R.S.H., F.M.A., M.P.G.K, M.G. & B.B.O.M., critically read and contributed to the manuscript. The Global Sewage Consortium authors carried out sewage sampling, filled in metadata and shipped the samples.

Competing interests

The authors declare no competing interests.

Additional information

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