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Research needs for optimising wastewater-based epidemiology monitoring for public health protection

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ABSTRACT

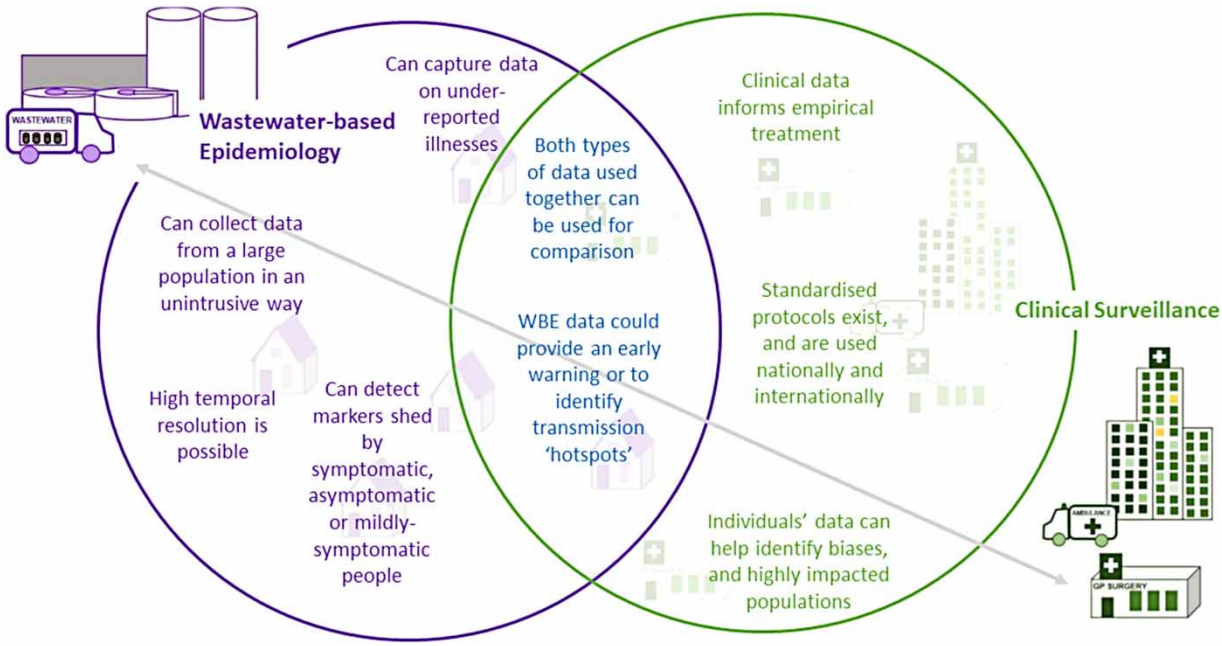
Wastewater-based epidemiology (WBE) is an unobtrusive method used to observe patterns in illicit drug use, poliovirus, and severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2). The pandemic and need for surveillance measures have led to the rapid acceleration of WBE research and development globally. With the infrastructure available to monitor SARS-CoV-2 from wastewater in 58 countries globally, there is potential to expand targets and applications for public health protection, such as other viral pathogens, antimicrobial resistance (AMR), pharmaceutical consumption, or exposure to chemical pollutants. Some applications have been explored in academic research but are not used to inform public health decision-making. We reflect on the current knowledge of WBE for these applications and identify barriers and opportunities for expanding beyond SARS-CoV-2. This paper critically reviews the applications of WBE for public health and identifies the important research gaps for WBE to be a useful tool in public health. It considers possible uses for pathogenic viruses, AMR, and chemicals. It summarises the current evidence on the following: (1) the presence of markers in stool and urine; (2) environmental factors influencing persistence of markers in wastewater; (3) methods for sample collection and storage; (4) prospective methods for detection and quantification; (5) reducing uncertainties; and (6) further considerations for public health use.

Key words: antimicrobial resistance, chemicals, metabolites, pathogens, public health wastewater-based epidemiology

HIGHLIGHTS

- Wastewater-based epidemiology (WBE) has been successfully used to track SARS-CoV-2.
- WBE can monitor populations which are not captured through routine clinical surveillance.
- WBE has the potential for monitoring many additional indicators of public health.
- Research is required to validate the use of WBE in public health management.
- Considerations include integration of WBE with existing public health systems and ethics.

GRAPHICAL ABSTRACT



INTRODUCTION

The COVID-19 pandemic has had a major impact on public health, well-being, and economic systems globally (Kolahchi *et al.* 2021). The causative agent, severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has spread rapidly since 2019. Clinical presentation has ranged from asymptomatic cases to hospitalisation or death (Weiss *et al.* 2020; Zheng *et al.* 2020; Park *et al.* 2021). One method for monitoring levels of SARS-CoV-2 that is inclusive, prone to fewer population biases, and less invasive than testing individuals is surveillance of wastewater in sewer systems, where the level of infection in the catchment community is associated with viral copies in sewage (Polo *et al.* 2020; Sims & Kasprzyk-Hordern 2020). This approach, termed wastewater-based epidemiology (WBE), has been used to complement existing public health data and clinical monitoring procedures (Naughton *et al.* 2021), with most data coming from high-income countries, for example, in the United Kingdom (Wade *et al.* 2020), the Netherlands (Medema *et al.* 2020), and the United States (Kirby *et al.* 2021). There are WBE studies in low and middle-income countries (LMICs), such as Tunisia (Jmii *et al.* 2021), Morocco (Amahmid *et al.* 2021), Brazil (Barbosa *et al.* 2022), where challenges for conducting this work are greater and complex (Pandey *et al.* 2021). The response to the pandemic has led to rapid acceleration of WBE research and development, and monitoring is now being performed at national, regional, and local scales (University of California 2022). Additionally, WBE has provided strategically important information through the development of new bioinformatics pipelines for variant detection and advanced algorithms for data interpretation, all of which have helped reduce uncertainty in the data (Karthikeyan *et al.* 2021; Habtewold *et al.* 2022). WBE has also provided early warning of local disease outbreaks, thus facilitating public health actions, such as focusing subsequent deployment of face masks, mobile testing, and vaccination facilities (e.g., surge testing; Hillary *et al.* 2021) and increasing bed capacity at hospitals (Brown *et al.* 2021; Crits-Christoph *et al.* 2021; Herold *et al.* 2021; Swift *et al.* 2021).

WBE is not a new concept, having been used to monitor illicit drug consumption as early as 2005 (Zuccato *et al.* 2005; Biello 2007) and the global distribution of poliovirus over the last decade (Hovi *et al.* 2012). Aside from these applications and monitoring of SARS-CoV-2, WBE has been used on a more *ad hoc* basis, with the implementation of a wide range of temporally and spatially distinct use cases, such as consumption of the antiviral oseltamivir (Tamiflu) during the 2009 H1N1 pandemic (Singer *et al.* 2013), the presence of influenza A and H1N1 in wastewater in the Netherlands (Heijnen &

Medema 2011), consumption of illicit and licit drugs (including antidepressants) during the 2010–2014 Greek economic crisis (Thomaidis *et al.* 2016), and the global distribution of antimicrobial resistance (AMR) (Hendriksen *et al.* 2019).

WBE has demonstrated potential in monitoring different human health and behaviour markers to provide valuable information for public health activities. However, this remains a relatively novel epidemiological tool, and to facilitate the expansion of WBE to other health targets and integration into public health surveillance activities research gaps need to be identified, so further study can be directed in these areas.

The aim of this paper is to review the applications of WBE for public health surveillance, focusing on the following three broad areas: (1) pathogenic viruses; (2) AMR; and (3) chemicals. It describes the additional value WBE contributes to public health surveillance, evaluates the evidence base supporting these applications, and identifies research gaps that need to be addressed to permit large-scale adoption of WBE as a central pillar in public health surveillance and decision-making.

PATHOGENIC VIRUSES

Added value of WBE monitoring

Viruses that cause diseases in humans significantly impact life quality and expectancy, put strain on healthcare settings, and negatively impact economies (Shang *et al.* 2021). Many novel pathogens to emerge in the past 100 years have been zoonotic in origin, including SARS-CoV-1 and Middle East respiratory syndrome coronavirus (MERS-CoV); human immunodeficiency virus (HIV); novel influenza viruses H1N1, H3, N2, and H2N2; Zika virus; and Ebola virus (Sharp & Hahn 2011; Morse *et al.* 2012; Recht *et al.* 2020). Furthermore, the most recent pandemic virus, SARS-CoV-2, is likely to have originated in a zoonotic reservoir (although the zoonotic origin has not been conclusively identified) (Holmes *et al.* 2021). The conditions for the emergence of novel viruses with pandemic potential remain (Woolhouse *et al.* 2008; Plowright *et al.* 2021) and the development of robust plans and resources to monitor the progress of pathogen spread and inform public health interventions is essential to adequately prepare for subsequent pandemics. Endemic pathogens, such as influenza virus, non-influenza RNA respiratory viruses (e.g., respiratory syncytial virus (RSV)), and enteric viruses (e.g., norovirus, rotavirus, etc.) also have a significant health burden, particularly among children, the elderly, and the immunocompromised (Modjarrad *et al.* 2016; Paget *et al.* 2019). Enhanced monitoring for pathogenic viruses imposing high burdens of disease by testing wastewater could help to reduce the number of respiratory infections and sequelae (Jartti *et al.* 2006; Ruotsalainen *et al.* 2013).

Currently, clinical surveillance for pathogenic viruses is limited to those who seek treatment, which excludes those who are asymptomatic, mildly symptomatic, or who do not present for treatment for other reasons (e.g., lack of access to healthcare or mistrust in healthcare systems; Tsimtsiou *et al.* 2021). There have been attempts to use data collection methods that would include information on populations not seeking clinical treatment, as well as people who do. This has been done using surveys of symptoms, such as the FluSurvey in Europe (FluSurvey 2017), or Flu Near You in the United States and Canada (FluSurvey 2017; Baltrusaitis *et al.* 2017), and analysis of digital search engine data on influenza-like illness by FluModel (i-sense-flu) (Lampos *et al.* 2015; Wagner *et al.* 2018). However, the results of surveys and search engine data analyses are also affected by biases such as a greater proportion of participants from more affluent and educated backgrounds than the population baseline tend to take part in voluntary surveys (Baltrusaitis *et al.* 2017; FluSurvey 2017). Models using digital search engine data to track search trends are biased towards populations with regular internet usage, and for whom surveillance data exists to train the model.

Clinical diagnostics, such as PCR-based methods or rapid antigen testing of individuals' samples, allow correct identification of an infective agent to inform empirical treatment of symptomatic patients, which may reduce the consumption of antimicrobials and allow the infected patients to isolate and limit the spread of the disease (Chen *et al.* 2011). However, the lack of community-level surveillance means infected patients are not identified until they reach clinical settings, at which point the disease may have spread significantly. WBE offers the opportunity to provide community-level data on pathogen presence to supplement and/or enhance existing clinical surveillance methods and public health activities (Figure 1), namely through (1) detecting the spread of viruses in individuals who have not interacted with healthcare systems; (2) identifying strategic locations for implementation of disease interventions and preventative measures; and (3) tracking changes in the epidemiology of the target that warrant further attention (Tang *et al.* 2017).

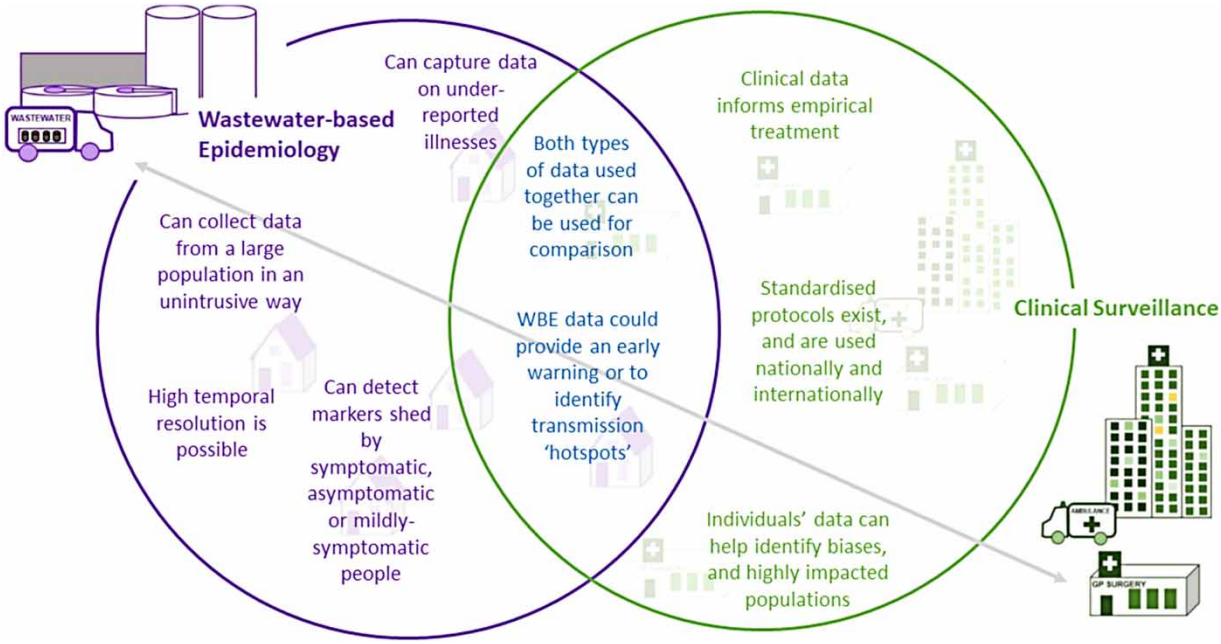


Figure 1 | Diagram showing the advantages of WBE and clinical surveillance techniques for pathogenic virus detection, and the potential benefits of integrating WBE with clinical surveillance data for public health.

Presence of pathogens in human stool and urine

For WBE to be useful for viral pathogen surveillance, markers of specific viral infection must be present in faeces, urine, or vomit (Jones *et al.* 2020). The shedding patterns of viral particles are likely to be affected by several factors, including virus-specific factors (e.g., infection site, incubation period) and host-specific factors (e.g., symptom severity, susceptibility to infection, and age).

The evidence base for faecal shedding of enteric viruses is well established (Supplementary Material, S1), (Allard *et al.* 1992; Papapetropoulou & Vantarakis 1998; Chitambar *et al.* 2001; Maunula *et al.* 2009; Lion *et al.* 2010; Jeulin *et al.* 2011; Ganesh & Lin 2013), since the transmission of such viruses is understood to be via the faecal–oral route. However, quantitative estimates of faecal shedding of respiratory viruses are less well explored (Supplementary Material, S1) since their primary transmission route is through respiratory aerosol droplets, rather than water or food contamination.

Interest in WBE for the pandemic respiratory virus, SARS-CoV-2, has resulted in numerous studies producing quantitative and temporal information related to faecal shedding of SARS-CoV-2 RNA (Cheung *et al.* 2020; Jones *et al.* 2020; Hoffmann & Alsing 2021). Evidence suggests SARS-CoV-2 shedding is greatest early in the course of the infection cycle and then decreases and continues for several days after symptoms and respiratory shedding have ceased (Gupta *et al.* 2020b; Van Doorn *et al.* 2020; Xu *et al.* 2020; Cavany *et al.* 2022; Hoffmann & Alsing 2021; Zhang *et al.* 2021b). For most non-enteric viruses, shedding studies are limited to a binary positive/negative result and are conducted when gastrointestinal symptoms are also experienced and may only be detected in a minority of confirmed cases (von Listow 2006; Minodier *et al.* 2015). Furthermore, the faecal and urinary shedding dynamics of respiratory viruses may not align with shedding at other sites. For example, prolonged shedding of H7N9 Avian Influenza has been observed in faecal and urine samples after respiratory samples became negative and viral detection was more common in the urine of severely ill patients (Hu *et al.* 2013a; Zhu *et al.* 2015).

For most viral pathogens, including SARS-CoV-2, our knowledge of shedding rates in faeces and urine have been obtained from hospitalised individuals, thus biasing the results towards cases presenting with more severe symptoms or comorbidities. Additionally, there is scant understanding of how shedding varies with the age of the patient, comorbidities, vaccination status, or re-infection. Data are therefore not representative of the general population, and future research needs to address

this bias by ensuring diversity in symptom phenotypes and immunological states is represented in study cohorts. Limited available evidence (e.g., from norovirus, Sabrià *et al.* 2016) underscores the importance of understanding the differences in shedding of different viruses in faeces and urine from both symptomatic cases and asymptomatic carriers (Jones *et al.* 2020), who will both contribute waste to sewer systems. Further data are also needed on shedding dynamics, as existing evidence rarely captures the pre-symptomatic phase when shedding rates may be high (Hoffmann & Alsing 2021). The ability to detect outbreaks before cases present in healthcare settings is extremely valuable for public health planning and infection control. Information is also needed on the persistence of faecal shedding following the infectious period to improve the ability to detect active cases (Santos *et al.* 2020). Understanding how immunity (whether attained through vaccination or previous infection) impacts shedding is also necessary, as this could potentially influence the interpretation of epidemiological estimates through different phases of an outbreak.

In general, urine samples provide lower sensitivity for viral detection, particularly in enteric pathogens such as Enterovirus and Parechovirus (>90% in faeces vs. <60% in urine) (Crom et al. 2013). However, variability exists and for some pathogens shedding in other excretions may be the predominant source: the measles virus (measles morbillivirus) has been found in the urine of ~88% of infected patients in one study (van Binnendijk *et al.* 2003), and viral markers may be present in body fluids which also enter sewer systems (Crank *et al.* 2022), such as sputum (e.g., SARS-CoV-2 (Li *et al.* 2022)), saliva (e.g., human herpes virus (Fox *et al.* 1990), Epstein–Barr virus (Niederman *et al.* 1976)), semen (e.g., HIV-1 (Kariuki *et al.* 2020), hepatitis C virus (Cavalheiro *et al.* 2008), and Zika virus (Atkinson *et al.* 2016)). This is particularly relevant when developing the sampling framework to account for differences where samples may be more heterogenous (e.g., building-level surveillance; Crank *et al.* 2022).

Impacts of wastewater environments on viral detection

The stability of pathogen markers in the environment and in wastewater are highly variable (Bofill-Mas *et al.* 2006). In general, DNA targets are more stable than RNA targets, although physiochemical characteristics of the environment influence the persistence of both (Ward & Ashley 1980; Bofill-Mas *et al.* 2006; Muirhead *et al.* 2020). Enveloped viruses (viruses with a lipid envelope that include influenza viruses, coronaviruses, and parainfluenza viruses) are usually less stable in the environment than non-enveloped viruses (without a lipid envelope, including enteroviruses, noroviruses, and adenoviruses). Noroviruses and adenoviruses can survive up to months in the environment (Kotwal & Cannon 2014) and have frequently been detected in wastewater and surface waters (Lago *et al.* 2003; La Rosa *et al.* 2010; Rajko-Nenow *et al.* 2013; Guillois *et al.* 2016; Farkas *et al.* 2018; Falman *et al.* 2019; Mancini *et al.* 2019; McCall *et al.* 2021). However, very limited information currently exists on the persistence of the genetic material of enveloped viruses in the environment, especially in wastewater. It is only recently that Ebola (Zaire ebolavirus) was found to persist in water for 8 days (Bibby *et al.* 2015), whereas it was previously assumed that enveloped viruses were quickly deactivated in the environment (Wigginton *et al.* 2015; Ye *et al.* 2016).

Due to the requirement for biosafety level (BSL) 3 laboratories to perform infectivity studies, surrogate viruses such as Phi6 bacteriophage have been used to assess the potential for survivability (Aquino de Carvalho *et al.* 2017). The persistence of Phi6 bacteriophage under different temperatures, biological activity, and liquid media was found to vary significantly, with temperature being particularly important (Aquino de Carvalho *et al.* 2017). Even though temperature greatly affects viral survival, most WBE studies do not take temperature or seasonality into account and risk mis-quantifying results at different times of the year, particularly in temperate regions (Hart & Halden 2020). The presence of stormwater in combined sewers can also result in dilution which can limit the accurate quantification of microbial communities (Wade *et al.* 2022). Even in separated systems, fluctuating groundwater infiltration has the potential to impact observed results, in a way that is not immediately apparent (Choi *et al.* 2018).

Matrix composition has repeatedly been found to influence virus detection efficiency and survival in wastewater. For example, organic matter has a great impact on molecular detection as they inactivate the enzymes used for DNA amplification (Matheson *et al.* 2010; Hata *et al.* 2015). Ammonia can inactivate viruses through genome degradation and viruses could be aggregated or adsorbed into liquid media (Bibby *et al.* 2015; Aquino de Carvalho *et al.* 2017). A study found that aggregation of viruses in the solid fraction of the wastewater was higher for enveloped viruses than non-enveloped viruses at 26 and 6% of virus adsorbed, respectively (Ye *et al.* 2016). Therefore, sampling only the liquid fraction may exclude a large proportion of the viral target.

Biotic factors may also impact detection of viral targets in wastewater. Wastewater is a complex and dynamic mix of microbial communities and competition, predation and selective pressures are not well understood (Choi *et al.* 2018). In some locations, urban wastewater may also contain animal viruses (e.g., influenza A; Heijnen & Medema 2011), which can confound the signal due to difficulties in separating human and animal contributions without sequencing-based approaches.

It is evident that mechanisms of virus marker inactivation in wastewater environments for different viruses of interest require further investigation. The observed differences between faecal and sewage samples indicate environmental conditions that alter microbial communities and further research is required to be able to interpret viral signals in WBE accurately.

Sampling methodology and storage requirements for viral pathogens

Wastewater sampling for viral pathogens may use flow-based interval approaches, but is typically achieved through grab or composite sampling. Grab samples are collected at a single point in time, often at a time of day that is estimated to be 'peak flow' to maximise the target load from human sources (Polo *et al.* 2020). However, this can vary greatly depending on the location of sampling sites as network travel times of wastewater can typically range between 2 and 12 h (Castiglioni *et al.* 2013) and peak shower usage may dilute the signal (Ahmed *et al.* 2021). Some markers can have greater daily variability than others (Ahmed *et al.* 2021). Due to the temporal variability of wastewater concentrations, this is often not representative of the entire population input over 24 h, therefore a composite sample may be used. Composite sampling is a means to try and overcome this, typically via the use of autosamplers, which sample at set intervals (ranging from minutes to hours) and can be used to develop a time series or mixed to make a composite sub-sample. Grab samples appear to be suitable for enteric viruses in wastewater effluent (i.e., treated wastewater) (Sidhu *et al.* 2017; Farkas *et al.* 2018), however, variation in wastewater influent means daily composite samples are the best approach for enteric viruses (~4 samples per day; Farkas *et al.* 2018) and SARS-CoV-2 detection in influent wastewater (24 samples per day; Ahmed *et al.* 2021). Further temporal studies to determine enteric pathogen variation over 24 h are recommended (Farkas *et al.* 2018) and whether these patterns are consistent across various wastewater systems. There is a lack of research comparing sampling techniques for other respiratory viruses. The use of autosamplers requires specific infrastructure and is associated with a higher operational workload and cost, as well as the need for on-site sample refrigeration. These facilities are not always available, particularly in resource-poor areas.

Passive samplers are absorbent devices that can accumulate markers of interest when submerged in wastewater and may offer a good alternative to composite sampling. They do not require specialised equipment to be deployed (Habtwold *et al.* 2022; Schang *et al.* 2021), and can be used for sampling in the sewer network where autosampler use is impractical (Corchis-Scott *et al.* 2021) and are considerably less costly (Petrie *et al.* 2016). Passive samplers have been successfully used to detect viruses, such as hepatitis A and E, norovirus (Vincent-Hubert *et al.* 2017), poliovirus (Sattar & Westwood 1977; Cassemiro *et al.* 2016), SARS-CoV-2 (Schang *et al.* 2021). However, they may saturate quickly, and daily exposure times may be too long to obtain representative results. Furthermore, as passive samples are submerged and accumulate biological material over time, often to the point of saturation, viral concentration data obtained from passive samplers cannot be easily normalised for factors such as wastewater flow. Thus, the results are considered semi-quantitative. Overall, insight into wastewater dynamics and temporal variation of the marker of interest is needed for different pathogens to determine the most accurate and cost-effective sampling method. This could vary significantly between different sites depending on the pathogen of interest, the availability of infrastructure to permit composite sampling, and whether quantitative data are required for public health use.

There is currently limited standardised guidance on wastewater sample storage conditions and duration prior to molecular marker testing for viruses. It has been suggested that samples be kept refrigerated and analysed as soon as possible after collection, although temperatures and duration of storage vary, depending on the type of viral analyte (e.g., RNA/DNA, enveloped vs. non-enveloped viruses). For example, the US Centers for Disease Control and Prevention (CDC) recommends immediate sample refrigeration at 4 °C, and processing with 24 h (CDC 2022), whereas the US Environment Protection Agency (USEPA) recommends that samples be stored and chilled for up to 48–72 h prior to viable bacteriophage testing (USEPA 2001). For enterovirus viability and norovirus RNA testing in drinking water, the USEPA suggests storage for up to 24 h prior to analysis (Fout *et al.* 2010). Standardised methods need to be developed and agreed upon to allow comparability between studies, times and locations, and improve quality assurance.

Laboratory analysis methodologies for viral pathogens

Different methods for optimising RNA extraction, concentration, and quantification of SARS-CoV-2 have been explored. Concentration methods for viral pathogens in wastewater, primarily developed for non-enveloped enteric viruses, have since been applied for SARS-CoV-2 surveillance (Ahmed *et al.* 2021). These include precipitation with salt or polyethene glycol (PEG) (Farkas *et al.* 2021; Kevill *et al.* 2022), electrostatically charged membrane filtration (Ahmed *et al.* 2021), ultrafiltration (Izquierdo-Lara *et al.* 2021), adsorption-extraction (AE) with aluminium chloride, silica (Randazzo *et al.* 2020) or magnesium chloride ($MgCl_2$) (Ahmed *et al.* 2021), or using an automatic concentrating pipette (CP) instrument (Ahmed *et al.* 2021; McMinn *et al.* 2021). Concentration methods have been found to be one of the greatest sources of variation in the quantification of SARS-CoV-2 in the English wastewater monitoring programme, based on the recovery of Phi6 bacteriophage, which was used as a process control (Wade *et al.* 2022).

To improve the efficiency of wastewater sampling, testing samples for the presence of multiple viral markers would necessitate optimising viral recovery for multiple pathogens. However, a study by Ahmed *et al.* (2021) evaluated two concentration techniques (AE supplemented with $MgCl_2$ and CP) against concentration of SARS-CoV-2 and enteric pathogens. They found the CP method achieved the greatest overall recovery of SARS-CoV-2, however recovery did vary and was not consistent across all viral targets. Another study found viral recovery was reduced when CP was applied to high turbidity samples (Kevill *et al.* 2022). Further research should investigate concentration methods for different pathogens in wastewater and recovery efficiencies. This is key to optimising multi-pathogen recovery methods, minimising equipment requirements, and streamlining lab throughput. Further method development could additionally focus on optimisation in terms of concentration methods to enable both RNA and DNA extraction, where the latter could also be analysed using culture-independent methods for AMR surveillance, for example.

The gold standard for the targeted detection of viral pathogens in wastewater is quantitative polymerase chain reaction (qPCR). Although ubiquitous in much of WBE programmes, the method is sensitive to biological and chemical inhibitors, which are often present in wastewater extracts (Volkmann *et al.* 2007). Commercially available reaction mixes provide different levels of resistance to inhibitors, which may result in false-negative results and generate inter-laboratory variation (Farkas *et al.* 2017). The validation and implementation of strict quality control measures for each qPCR assay are crucial to achieving reliable results (Bustin *et al.* 2009). For example, one meta-analysis found that only 26% of studies published on SARS-CoV-2 detection in wastewater reported qPCR quality control details and where they were reported did not meet the minimum criteria (Bivins *et al.* 2021), questioning the reliability of the results.

Emerging targeted technologies such as digital droplet PCR (ddPCR), isothermal amplification (e.g., LAMP) assays and biosensors have also been developed; however, some of these are often less sensitive than qPCR (Haramoto *et al.* 2018; Farkas *et al.* 2020b). To date, besides qPCR, only ddPCR has been routinely used for WBE (Jahne *et al.* 2020; Al-Duroobi *et al.* 2021; Bivins & Bibby 2021), as this technique may be more sensitive than qPCR (Zhao *et al.* 2016), particularly for low viral loads or in matrices with more variable contamination (Taylor *et al.* 2017; Falzone *et al.* 2020).

Sequencing-based approaches have been trialled and implemented in WBE to generate data describing viral communities, including variant-level identification of circulating viruses (Fernandez-Cassi *et al.* 2018; Adriaenssens *et al.* 2018, 2021; Guajardo-Leiva *et al.* 2020) and identification of novel viruses and their variants (Rios *et al.* 2021). However, existing methods are time-consuming and often biased, tending to over-represent abundant or large DNA genomes and miss rare, shorter genomes (Garner *et al.* 2021). Amplicon sequencing increases sensitivity and accuracy for known viruses and has been effective in wastewater research to describe certain viral groups, including astroviruses (Hata *et al.* 2018), adenoviruses (Ogorzaly *et al.* 2015; Iaconelli *et al.* 2017), saproviruses (Mancini *et al.* 2019), enteroviruses (Tao *et al.* 2020), and noroviruses (Fumian *et al.* 2019). A similar approach has been used for SARS-CoV-2 by amplifying its whole genome or certain genes using the Illumina ARTIC pipeline (Avgeris *et al.* 2021; Dharmadhikari *et al.* 2021; Hillary *et al.* 2021).

Ultimately, the methodology for detection and quantification of viral pathogens will depend on the requirements of the data for public health. Research on the application of these technologies to wastewater samples for various pathogens is necessary to demonstrate their detection and quantification capability. Additionally, if it is necessary to detect several pathogens in one sample, development of high-throughput quantification tools for multiple targets would be necessary.

Comparison of WBE and public health data for viral pathogens

Clinical data, such as microbiological diagnostic data, may be used to assess how well markers in sewers correlate temporally and spatially with specific health outcomes in a community of interest. Comparison of WBE data should encompass two

broad aspects: (1) that the markers reliably measure the intended target of surveillance (i.e., sensitivity and specificity) and (2) that data are collected at a temporal and spatial resolution that enable public health decision-making.

For SARS-CoV-2 in the UK, WBE data could be compared with public health data, including hospital admissions (Kaplan *et al.* 2020; Peccia *et al.* 2020; Galani *et al.* 2022), case data from the national and regional surveillance programmes (Wade *et al.* 2022), the Coronavirus Infection Survey (CIS) (Pouwels *et al.* 2020), and Real-time Assessment of Community Transmission studies (REACT) (Elliott *et al.* 2021). Comparisons between datasets also reveals potential population behaviours driving transmission, including social response to the pandemic, temporally resolved variations in population size (e.g., commuting, weekend breaks), sampling biases in wastewater, or biases, gaps, or reporting lags in clinical testing (Galani *et al.* 2022).

While the extensive clinical testing available throughout the pandemic meant comparable data was readily available (noting limitations within these data (Elliott *et al.* 2021)), similar comparisons might not be as feasible for pathogens like norovirus or RSV, where monitoring is dependent on data from healthcare settings. Therefore, to ensure the data collected through WBE is an accurate reflection of infection rates, future research must ensure these comparisons are designed into studies (e.g., by collecting samples or data from healthcare institutions, the wider community, and open data sources, such as social media).

Summary and suggestions for further research

Asymptomatic carriage of pathogenic viruses by members of the community is currently under-estimated but can play an important role in disease transmission. WBE has the potential to provide additional information on viral transmission dynamics that are currently missed by existing surveillance programmes, which are biased towards symptomatic and severely ill patients. However, knowledge gaps remain that must be addressed before WBE can be useful as a stand-alone surveillance tool to inform public health practice.

- Further quantitative studies on the shedding of viral pathogens (particularly for enveloped viruses such as influenza) are needed. Studies should ideally monitor patients throughout the entire course of the infection, with a range of variants, and recruit diverse cohorts to ensure generalisability of results. Shedding should be evaluated in faeces and urine. Impacts of immunity against viruses (achieved through vaccination or infection) on viral shedding dynamics need to be elucidated.
- Further studies on the persistence of various viruses in wastewater are necessary (particularly enveloped viruses), including evaluation of the impact of factors such as temperature, matrix composition, and biotic processes on viral detection. Researching this within the context of WBE may inform data interpretation for public health.
- Different sampling procedures and analytical techniques should be explored for pathogens of interest to optimise methods. The extent to which viral pathogen detection in wastewater samples can pre-empt or match clinical and community-level data must be evaluated to ensure that WBE data are an accurate reflection of clinical outcomes of interest.

ANTIMICROBIAL RESISTANCE

Added value of WBE for AMR

AMR, where microbes develop resistance against antimicrobial drugs, poses a threat to public health that is only likely to worsen. Antimicrobials are becoming less effective over time with continued overuse (Prestinaci *et al.* 2015) and fewer are being brought to market. Economic impacts are expected due to reduced productivity, resulting from lost days-of-work and longer hospital admissions (WEF 2021).

Compared to infectious viral pathogens described in the previous section, monitoring AMR is complicated given there is no one singular infectious agent responsible, nor single reservoirs for resistant organisms. There are numerous pathogenic antibiotic-resistant bacteria (ARBs) of concern, and antibiotic-resistant genes (ARGs), which can also be released in faecal matter and transferred between even distantly related bacteria by horizontal gene transfer (HGT). Beyond bacteria, resistance to antimicrobial treatment arises in other microorganisms such as azole antifungal resistance in pathogenic fungi, like *Candida albicans* (Whaley *et al.* 2017), antiviral resistance, including resistance to antivirals for hepatitis B (Locarnini 2004) and influenza virus (Lampejo 2020; Van Poelvoorde *et al.* 2020) and resistance among parasites, like Plasmodium spp.

AMR is an issue that is now recognised as extending beyond healthcare settings, with resistance determinants emerging in environmental settings (Larsen *et al.* 2022; Poirel *et al.* 2002) and selected for by antimicrobial use in agriculture systems (Woolhouse *et al.* 2015; Cheng *et al.* 2019; Pollock *et al.* 2020), the release of resistance in sewage into waterways and general

environmental pollution (Harris *et al.* 2014; Agga *et al.* 2015; Murray *et al.* 2018; Hiller *et al.* 2019; Mughini-Gras *et al.* 2019). Therefore, surveillance and management of AMR are often considered through a One Health lens including environmental and animal monitoring (Mendelson & Matsoso 2015; WHO 2021a).

With current monitoring focussing on healthcare settings and a small group of key pathogens (e.g., English Surveillance for Antimicrobial use and Resistance (ESPAUR), (UKHSA 2021); European Antimicrobial resistance surveillance (EARS-Net), and WHO Global AMR Surveillance System (GLASS), (WHO 2021a)), the true scale and potential burden of AMR may be under-estimated (Burnham *et al.* 2019). ARGs, which are abundant in stool and wastewater samples, are the main way of indirectly monitoring AMR prevalence at large scales. The most comprehensive resistance monitoring study quantified ARGs in wastewater metagenomes in over 70 countries and found major differences in relative AMR abundances and classes in community sewage, reflecting large regional differences in 'local guts' (Hendriksen *et al.* 2019). Furthermore, a recent review by Pruden *et al.* (2021) described a range of applications of WBE for AMR monitoring including the following: (1) identification of hotspots to guide policy action on interventions; (2) inform medical and veterinary practitioners of the best antibiotics to use to avoid therapeutic failure; (3) inform risk assessments for discharge of antibiotics/ARB/ARGs into the wider environment; and (4) provide data for forecasting models and key drivers. Overall, the use cases for AMR surveillance using wastewater demonstrate the potential to add significantly to public health monitoring and therefore warrant further exploration.

Presence of AMR in human stool and urine

AMR is represented in stool by a diverse array of ARGs and mobile genetic elements (MGEs), primarily associated with bacterial hosts. Resistance carriage in humans varies substantially internationally due to different environmental exposures, diet, and antimicrobial use (Hu *et al.* 2013b; Pal *et al.* 2016; Feng *et al.* 2018). The abundance and diversity of ARGs also varies between body sites/tissues, with one study using metagenomic sequencing on oral and stool samples in different countries and finding a higher abundance, but lower diversity, of ARGs present in oral samples compared to stool samples (Carr *et al.* 2020). Studying stool samples may provide information not captured from data such as antibiotic prescription rates, which do not always correlate with ARGs present in faeces (Collignon *et al.* 2018; Hendriksen *et al.* 2019; Carr *et al.* 2020).

Quantification of ARGs present in stool using metagenomics and qPCR has been well studied (Hu *et al.* 2013b; Rose *et al.* 2017; Feng *et al.* 2018) and in urine mostly studied in the limited context of urinary tract infections (Li *et al.* 2020; Kaye *et al.* 2021). However, the clinical relevance of ARG detection in metagenomic data is poorly understood since the presence of ARGs in faecal samples does not necessarily result in AMR infections, even in high-risk patients (Tacconelli *et al.* 2009; Wryes *et al.* 2021). Furthermore, some genes (e.g., tetracycline genes) perform multiple functions unrelated to AMR, including signal tracking and hydrophobic protein transport (Martínez 2008; Dantas & Sommer 2012; Feng *et al.* 2018). There is also the question as to whether ARGs present in the gut and stool are an accurate indication of resistance at other parts of the body (Piddock 2016; Carr *et al.* 2020), the duration of colonisation, and risk factors extending shedding in faeces (Leonard *et al.* 2018). Further research in this area is also required for ARGs in urine samples, since there is evidence to suggest enhanced resistance following kidney transplantation is reflected in urine (Rani *et al.* 2020). Overall, the link between ARG detection in stool and urine to treatment-resistant infections among various at-risk groups needs further exploration.

Impact of wastewater on AMR detection

Like viral markers, the stability and abundance of AMR indicators in wastewater are complicated by many abiotic and biotic factors that influence microbial populations and resistomes (collection of AMR genes within the microbiome). As with viral pathogens, animal hosts of AMR can contribute ARGs to wastewater, making it difficult to precisely identify which AMR genes in a sample originated from humans (McCann *et al.* 2019). Biofilms present in sewers may also serve as a source of ARGs in wastewater independent of human waste inputs (Medina *et al.* 2020), and dispersion of ARG-bearing microbes may be triggered by the conditions in sewer environments (Kaplan 2010). In addition, bacteria and ARGs are capable of multiplying in the environment, and the presence of antibiotic residues, heavy metals, and biocides in wastewater can influence the selection of certain bacteria and their genes, and therefore their abundance, outside the human body (Baquero *et al.* 2008; Knapp *et al.* 2008; Martinez 2009; Gullberg *et al.* 2011; Murray *et al.* 2018).

There are many additional factors in wastewater that have an impact on the microbial community and resistome dynamics, including local residence times, geographic area, antibiotic prescribing, temperature, salinity, dissolved oxygen, micro-ecological factors such as predation, and the presence of organic matter (Caucci *et al.* 2016; Sun *et al.* 2016; Jiao *et al.* 2018;

Lamba *et al.* 2018; Jong *et al.* 2020; Pallares-Vega *et al.* 2021). Additionally, further research on different wastewater treatment plant types and maintenance and impacts of combined sewer overflows (CSOs) on AMR detection is critical in places with aging or poorly maintained sewer systems.

Due to the higher antibiotic usage in hospital settings, previous studies have investigated the impact of hospital wastewater mixing with community wastewater on resistomes, with mixed results (Islam *et al.* 2017; Lamba *et al.* 2017; Buelow *et al.* 2018, 2020; Quintela-Baluja *et al.* 2019, 2021; Li *et al.* 2021). Future work should focus on evaluating the factors that affect the influence of sewer conditions and inputs to the sewer systems that impact measures of AMR. Such research will aid interpretations of AMR data derived from wastewater samples.

Sampling methodology and storage requirements for AMR

As with viral markers, methods for the collection and storage of samples must be evaluated to ensure robust data are extracted from samples. A systematic review by Chau *et al.* (2021) compared composite and grab samples to evaluate performance according to association with clinical AMR data and found greater agreement with composite samples. The authors observed that several studies did not report their sampling methodologies, limiting comparability of studies and highlighting the urgent need for standardisation of approaches (Hassoun-Kheir *et al.* 2020). There are also few studies on the use of passive samplers for AMR surveillance, which may provide a more cost-effective and practical approach to sampling than composite samplers. Further research should seek to determine the most accurate and cost-effective sampling methodologies to inform standard practice, to allow data to be compared regionally and internationally. This has commenced through the EMBRACE-WATERS (rEporting antiMicroBial ResistAnCE in WATERS) initiative, which is aiming to develop reporting standards for AMR research in wastewater and other aqueous environments (Hassoun-Kheir *et al.* 2021).

Poulsen *et al.* (2021) compared storage conditions on the microbiome and resistome of sewage samples and pig faeces. They observed that sample storage conditions had an influence on microbial community and resistance genes, where freezing wastewater changed the microbiome in comparison to processing the samples immediately. AMR abundance in frozen sewage samples was also overall significantly higher than samples stored at 22 or 5 °C (Poulsen *et al.* 2021). Therefore, it was recommended that sewage samples are processed the same day, frozen immediately, or stored in the fridge and processed during the day or on the following day. However, the opposite finding was observed for pig faeces, indicating that stool samples, or perhaps even near-source samples have different storage requirements (Poulsen *et al.* 2021). Further research on the storage requirements of near-source samples would inform standard practice and standard protocols.

Access to storage facilities and appropriate alternatives need to be considered, to ensure sampling is consistent and samples are comparable.

Laboratory analysis methodologies for AMR

AMR detection can be conducted through culturing and gene-based methods (or a combination) (Figure 2). Culturing faecal indicators or pathogens, such as extended-spectrum beta-lactamases (ESBL) *Escherichia coli* is recommended by the WHO Tricycle Protocol (WHO 2021a, 2021b). Quantifying ESBL-producing Enterobacteriaceae (Marano *et al.* 2020) and carbapenem-resistant bacteria (Blaak *et al.* 2021) is useful as they are important but relatively rare phenotypes and can be effective indicators of clinically relevant bacteria displaying resistance to essential and last-resort antibiotics (WHO 2017). Antimicrobial susceptibility test methods can also indicate levels of resistance and have been well studied and standardised (Jorgensen & Turnidge 2015). Culturing is cost-effective and technically easy, and therefore more accessible for LMICs (Pruden *et al.* 2021). However, it provides comparatively narrow information about the phenotypic characteristics of target organisms. Therefore, combining genotypic methods, such as qPCR, extends monitoring to gene-specific targets to accompany phenotypic data. Targeting MGEs which may be high-risk also can be valuable (Zhang *et al.* 2021a), or have been found to closely reflect anthropogenic resistance, using the Intl1 (Gillings *et al.* 2015) or aintl1 (Quintela-Baluja *et al.* 2021) assays. Genotypic methods like qPCR can provide accurate quantitative data on AMR abundance which can be normalised to cell abundance through co-quantification of genes like 16S rRNA. However, to be meaningful in a health protection context, such analyses should be corroborated a priori by parallel culturing methods to ensure they mirror health consequences.

The use of multi-array high-throughput qPCR arrays has shown promise because they can be designed to capture many different indicator genes at once (Wang *et al.* 2014). These have already been used successfully for wastewater applications (Quintela-Baluja *et al.* 2019).

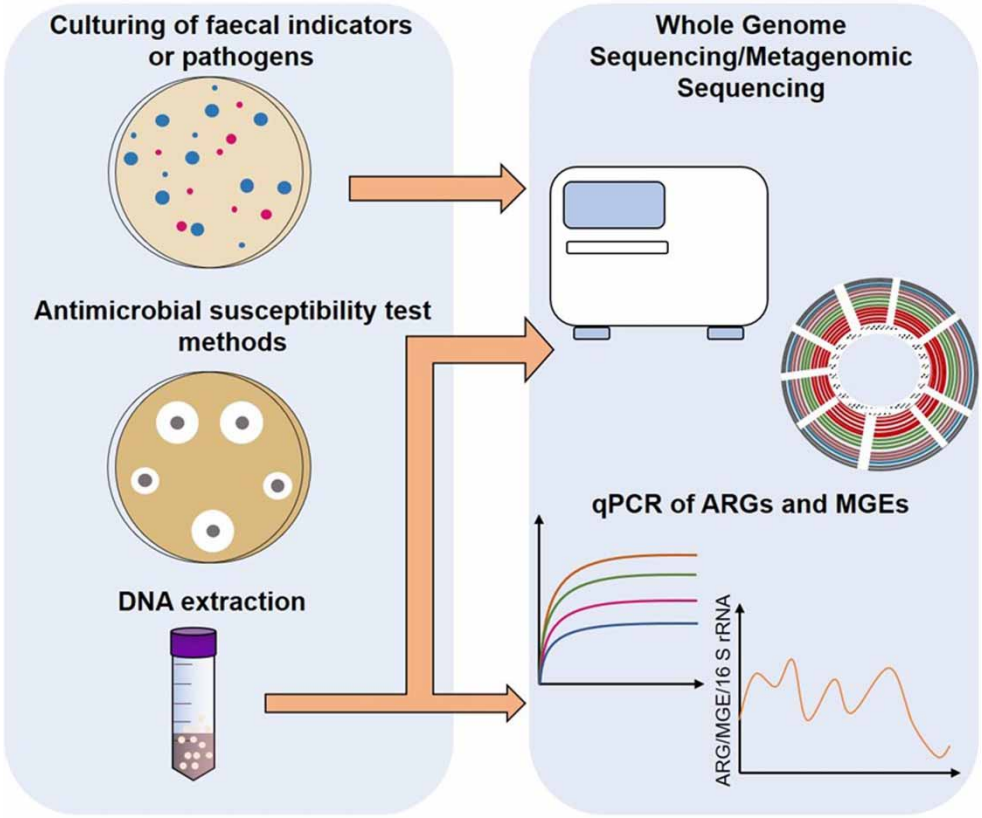


Figure 2 | Summary of laboratory techniques for analysis of AMR that could be used for WBE.

In recent years, metagenomic sequencing has proved to be a powerful approach for the detection of AMR. Metagenomic sequencing has the potential to reveal the diversity of ARGs, from natural ocean and soils to samples from the guts of animals, humans, and sewage (Hendriksen *et al.* 2019; Zeng *et al.* 2019). Factors included in metagenomic pipelines such as the Meta-Compare's relative resistome risk (Martínez *et al.* 2015), can assign a value to the mobility of ARGs or the potential to occur in pathogens which could be useful for determining clinical risk using metagenomic data. Sequencing-based approaches can also be used with and without a culture phase: targeted metagenomics permits the quantification of thousands of ARGs in culturable and potentially pathogenic bacteria (Leonard *et al.* 2018). As with viruses, detection of novel and rare genes will be limited.

Multiple pipelines for detection and enumeration of AMR exist (Gupta *et al.* 2020a), but a standardised method is needed for WBE if opportunities to compare data internationally are to be realised. With the high cost of sequencing-based approaches, this approach may not be accessible to some regions, therefore centralised sample processing and data analysis resources and funding to LMICs is needed (Aarestrup & Woolhouse 2020). Development of combined sequencing with multiplex qPCR and culturing methods would make monitoring more accessible across different socioeconomic settings.

Comparison of WBE and public health data for AMR

There is some evidence that WBE AMR data are correlated with data on regional clinical infections. Hutinel *et al.* (2019) demonstrated strong and significant correlations between *E. coli* isolated from wastewater samples and patient samples from Gothenburg, Sweden. Significant correlations were also found between samples taken from 10 wastewater treatment plants in Europe and clinical samples from EARS-Net, where resistance of *E. coli* to four antibiotics was analysed (Huijbers *et al.* 2020). Genotypic data gathered via qPCR arrays have also been found to broadly reflect clinical resistance in European sites (Pärnänen *et al.* 2019). Chau *et al.* (2021) identified a strong association with phenotypic and genotypic data from WBE to data gathered from clinical settings, indicating WBE provided insight into the resistance profiles of a community.

Comparisons between clinical and wastewater data have also shown WBE can be used to inform colonisation of specific pathogens, such as carbapenem-producing Enterobacteriaceae (CPE). CPEs are a major threat to healthcare settings and screening is currently limited to those who are most at risk (i.e., who have travelled, came into contact with a positive case, or visited a hospital abroad (Brown & Fry 2020)). Studies have found wastewater CPE cultures are reflective of what is observed in clinical samples (Meir-Gruber *et al.* 2016; Ludden *et al.* 2017; Flach *et al.* 2021). Isolates have been identified in WBE prior to identification in clinical samples, demonstrating its potential use as an early warning tool to prevent hospital outbreaks (Flach *et al.* 2021). WBE has also identified strains which were not detected in clinical samples, interpreted to be due to a carrier that was missed from clinical screening, or a result of persistence in wastewater after previous exposure (White *et al.* 2016).

While there are correlations between WBE and clinical resistance datasets, there is currently too much uncertainty to enable public health response. An improved understanding of the connection between clinical colonisation and wastewater could be achieved through more comprehensive studies including participants who are deemed low risk, and further investigation into the impact of wastewater on the persistence of resistance.

Summary and suggestions for further research

Surveillance of AMR using wastewater is complicated by the ubiquity of AMR across the One Health triad. Detection of AMR in wastewater may therefore not be from human carriers experiencing or at risk of treatment-resistant infection, but may be there as a result of zoonotic reservoirs, or persistence of AMR in the environment. Further research is needed before this tool can be used to support public health decision-making.

- The link between ARG detection in stool and urine to treatment-resistant infections among various at-risk groups warrants further exploration.
- Further studies should consider the effect of abiotic and biotic factors, selection processes, the influence of seasonality, and different types of wastewater treatment plants as this will aid interpretation of WBE data. The influence of wastewater from healthcare settings should be reviewed on a case-by-case.
- Further research should determine the best sampling and storage methodologies with consideration to resource constraints to inform standard practice and facilitate data comparisons.
- A standardised methodology for wastewater sample laboratory analysis is needed to allow international collaboration and comparisons. This approach needs to be accessible for LMICs.
- Improved understanding of the link between WBE and clinical resistance could be achieved through a comprehensive study incorporating participant and wastewater sampling and further investigation into the impact of wastewater on the persistence of resistance.

CHEMICALS

Added value of WBE for chemical surveillance

Chemical analysis of wastewater provides a broad range of information that can be used to understand multiple parameters influencing public health. Potential insights include but are not limited to (1) illicit drug use (Thomas *et al.* 2012; Ort *et al.* 2014; González-Mariño *et al.* 2020), (2) alcohol and tobacco consumption (Baz-Lomba *et al.* 2016), (3) consumption of over-the-counter medicines (Harman *et al.* 2011; Fattore *et al.* 2016; Melchor-Martínez *et al.* 2021; Rice *et al.* 2020), (4) consumption of antimicrobials (Castrignanò *et al.* 2020; Elder *et al.* 2021), (5) exposure to chemicals such as pesticides (Rousis *et al.* 2020), toxicants from everyday products including parabens, UV filters, bisphenols (BPAs), and phthalates (Lopardo *et al.* 2019a; Senta *et al.* 2020), and (6) biomarkers for certain non-communicable diseases, for example, cocaine, F2-isoprostanes, Prostaglandin E2 (Thomas & Reid 2011; O'Brien *et al.* 2019).

WBE has long been recognised as an approach for monitoring illicit drug use. Illicit drug use data are especially challenging in terms of surveillance, due to the behaviour being illegal, and highly stigmatised (Huizer *et al.* 2021). For this purpose, it fills significant gaps in other monitoring techniques such as surveys, drug-related hospital admissions, and arrest data, which do not capture the extent of consumption and are not temporally and spatially sensitive (González-Mariño *et al.* 2020; Huizer *et al.* 2021). This approach has been used widely, including an international study in 2011, where five illicit drugs (cocaine, cannabis, amphetamine, methamphetamine, and methylenedioxymethamphetamine (MDMA)) were analysed in the wastewater of initially 19 European cities, increasing to a total of 73 cities globally by 2017, including locations in LMICs

(Thomas *et al.* 2012; Gonzalez *et al.* 2020). Ongoing monitoring in 80 European cities is supported by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) which hosts an interactive website (EMCDDA 2021). Antibiotic consumption has also been investigated within the context of ARG prevalence in wastewater, showing strong correlations between antibiotic levels, ARGs, and population size (Castrignanò *et al.* 2020; Elder *et al.* 2021).

Data gap exist in understanding consumption of pharmaceuticals being prescribed and purchased online or in pharmacies. Patients often do not follow dosage or disposal recommendations, stockpiling leftover doses, or self-prescribing (Escolà Casas *et al.* 2021). One benefit of WBE for pharmaceutical use is the ability to differentiate between metabolised vs. discarded medication levels. The example of carbamazepine (Figure 3) shows the importance of measuring metabolic products compared to drugs themselves as direct disposal of unused drugs might result in overestimation of intake. Furthermore, information on public health trends tend to rely on individual data collection and biological samples or surveys, such as NHS England's health survey (Rice & Kasprzyk-Hordern 2019). These data are often not available in real-time due to the need to collate data before assessing and formulating appropriate responses.

Chemical analysis of wastewater could therefore provide spatially and temporally comprehensive epidemiological information to aid public health and healthcare practitioners. Longitudinal analysis would allow regional and temporal comparisons to be made and help to understand principal mechanisms driving public health trends. WBE could also provide information in data-poor regions, where antimicrobials, prescription drugs, and exposure to pollutants may be less regulated.

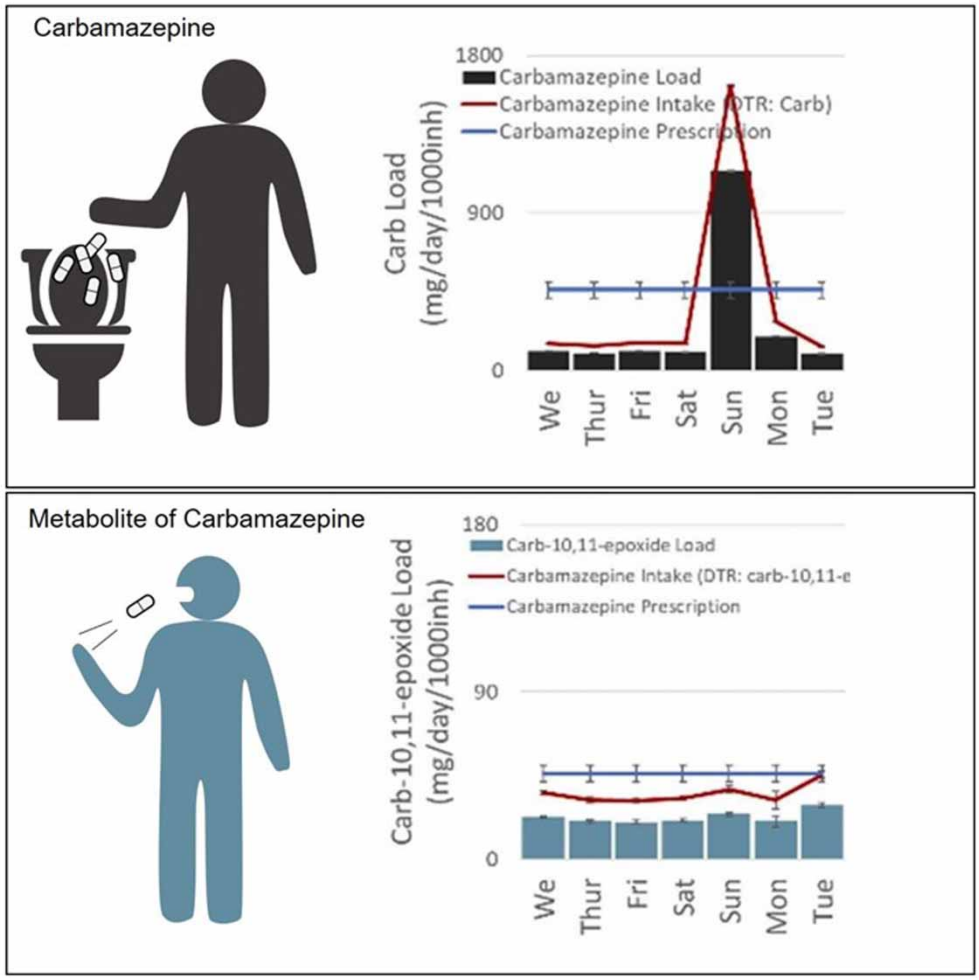


Figure 3 | Comparison of carbamazepine loads and intake calculated using carbamazepine-1-,11-epoxide and prescribed over a 7-day sampling week (adapted from Wade *et al.* 2022).

Presence of chemicals in faeces and urine

Chemical residues are excreted through urine and stool and can be detected in wastewater, usually reported as a percentage of the target residue (Baker *et al.* 2014). Understanding of human pharmacokinetics including excretion rates are therefore essential to back-calculate the approximate total exposure or consumption of chemicals (Zhang *et al.* 2019).

A comparison between the parent compounds and their metabolites for WBE found a higher certainty using metabolic markers (Baker *et al.* 2014), while other studies went further to suggest that only metabolic markers should be used in calculations where possible (Kasprzyk-Hordern *et al.* 2021).

An extensive list of excretion rates of biomarkers for lifestyle (e.g., illicit drugs, alcohol), exposure (e.g., pesticides), health (e.g., pharmaceuticals), and population (e.g., endogenous compounds) is available from Gracia-Lor *et al.* (2017). These estimates are from studies published after 2000, and primarily after 2010. However, some metabolites only have urine or faecal excretion percentage reported, and rarely both. This means some studies estimating exposure have only used excretion rates from urine in back-calculations (van Nuijs *et al.* 2015), likely underestimating exposure since often only phase I metabolites of chemicals are excreted in urine. A more accurate approach would be to include both phase I and II metabolites, which can be found in urine and faeces (Daughton 2018; Escolà Casas *et al.* 2021).

Further research is needed to understand excretion data for metabolites, which are often less complete than for parent compounds and varies depending on individual and medical conditions (Escolà Casas *et al.* 2021). Unfortunately, further pharmacokinetic studies on excretion rates are not always feasible, due to high costs, time requirements, and ethical considerations (Gracia-Lor *et al.* 2017). Alternative approaches include *in vitro* studies using liver enzymes (Mardal *et al.* 2016) and computer modelling (Reid *et al.* 2014). However, such data provide information on metabolic products and general insight into metabolism, but not quantitative information on excretion rates that could be used for WBE. Clinical proteomics have also led to the identification of new biomarkers. However, due to the time and expense required for clinical trials, very few are being confirmed clinically (Nkuipou-Kenfack *et al.* 2017; Rice & Kasprzyk-Hordern 2019). Nevertheless, the identification of human proteins in wastewater offers an opportunity for community-wide monitoring of diseases (Carrascal *et al.* 2020), and further studies on key biomarkers including metabolites as well as other endogenously formed entities such as proteins are needed. Emphasis should be put on improving understanding of pharmacokinetics to help quantify excretion factors for stool and urine, particularly where population variation is likely.

Impact of wastewater on chemical detection

Chemical stability in wastewater varies depending on the original compound and the conditions in wastewater. The stability of illicit drug biomarkers has been reviewed elsewhere (McCall *et al.* 2016a). A study investigating 43 pharmaceuticals showed that the majority remained stable in a pressurised sewer of 7.6 km, with a concentration error of $\pm$10% for most pharmaceuticals, with some at ~60% (Jelic *et al.* 2015). Temperature impacts biomarker stability, with one study showing that half-lives of biomarkers in colder temperatures were significantly longer than in warmer temperatures (Senta *et al.* 2014). McCall *et al.* (2016b) also found that temperature, pH, hydraulics, residence times, sorption processes, and biodegradation rates affect drug stability in sewers. Biofilms may also influence the stability of illicit drug biomarkers (McCall *et al.* 2016a; Li *et al.* 2018) and particulate matter in sewage has been shown to affect results, where a large proportion of the analyte may partition onto particulate matter (Baker *et al.* 2012). A study from Tang *et al.* (2020) found phthalate metabolites in wastewater were in much higher concentrations than predicted from urinary excretion, therefore whether metabolites are formed from parent compounds within wastewater environments should be investigated.

Further studies are required to estimate correction factors for metabolites that are to be used in WBE, particularly through understanding the conditions in wastewater that may influence interpretations, including the amount and type of sewage biofilm and different environmental conditions (e.g., temperature, pH and biodegradation) (McCall *et al.* 2016a).

Sampling methodology and storage requirements for chemicals

Analysis of chemicals and drugs in wastewater is most accurately conducted through 24-h flow-proportional composite sampling. While both time- and flow-proportional sampling is commonly applied, flow-proportional instead of time-proportional samples (e.g., every 20 min), have been found to provide an accurate indication of peak input of chemicals and biomarkers excreted into wastewater (Ort *et al.* 2010; Petrie *et al.* 2017). Grab or spot sampling does not allow for full analyte capture due to common diurnal variabilities in wastewater composition linked with variable human excretion patterns as well as external factors, like rainfall. To preserve chemicals for accurate analysis, storage time and temperature are crucial.

Samples need to be transported to laboratories refrigerated (if over a short period of time, e.g., a few hours) or frozen (for overnight transport). If chilled, they must be immediately frozen on arrival to prevent analyte degradation. Acidification can improve the stability of chemicals, by preventing some drugs from partitioning to the water fraction (Baker & Kasprzyk-Hordern 2011).

Passive sampling has been investigated (Moschet *et al.* 2015; Townsend *et al.* 2018; Rimayi *et al.* 2019). A comparison of passive and active sampling undertaken by Petrie *et al.* (2016), found the concentration of chemicals captured from passive sampling was within a factor of 2 of the known concentration. However, passive samplers might not achieve the same resolution as composite samples (Jones *et al.* 2015), but may nevertheless be used when analyte detection alone is a suitable measure for public health purposes. Further research is needed before it can be used as a standard alternative sampling method where there is insufficient infrastructure for composite sampling.

To preserve chemicals, understanding storage time and temperature for chemical stability is crucial. Chemicals are likely to degrade under repeated freeze–thawing cycles and over long-term storage. In a study by Fedorova *et al.* (2014), influent wastewater samples were spiked with 124 pharmaceuticals and personal care products to determine the impact of different storage conditions on analyte detection. Storage conditions at 4 °C resulted in greater amounts of analyte being detected compared to samples kept at −18 °C, with 15 analytes undetected after a freeze/thaw cycle. An understanding of the stability of target compounds and biomarkers, and appropriately designed standards for storage is necessary, to ensure consistency and comparability among studies.

Laboratory analysis methodology for chemicals

Chemical markers are present in wastewater at low concentrations, spanning from sub-ppt to ppm levels. Ultra-performance liquid chromatography is the method of choice for the separation of structurally wide-ranging groups of analytes with variable polarities, but gas chromatography can be also utilised for more volatile targets, also with common derivatisation steps utilised for more polar analytes.

In order to enable analysis at trace ppt levels, analyte enrichment is required. Solid phase extraction with wide-ranging materials is widely utilised to both clean-up and enrich liquid samples. QuEChERS method as well as pressurised liquid extraction or accelerated solvent extraction are also suitable for the extraction of analytes from solid samples such as suspended particulate matter in wastewater (Nieto *et al.* 2010; Kachhawaha *et al.* 2017; Pang *et al.* 2017). High-throughput methods are increasingly being used and within the context of WBE can provide more insight into multiple targets. Triple quadrupole mass spectrometry is usually applied to undertake multi-residue targeted analysis of >100 markers with one analytical run and is highly useful for large catchment studies (Proctor *et al.* 2019). High-resolution mass spectrometry can also allow untargeted screening for novel chemicals, with subsequent data mining allowing the potential to expand and develop analytes of interest (Lopardo *et al.* 2019b).

Further research on high-resolution mass spectrometry approaches is required for data mining and identifying further steps in sensing approaches. High-resolution analysis approaches would enable detection of an expanded biomarker suite, and allow multilevel estimation of public exposure to stressors and resulting effects (e.g., disease status).

Further studies are also necessary on chemical population markers. Population normalisation is necessary for all aspects of WBE, where inputs into the sewer may vary over the course of the day or be diluted by stormwater. Estimation of population may also provide insights into population mobility and travel. Metabolic markers for population normalisation are being explored, such as metabolites for caffeine or nicotine (Senta *et al.* 2015), or prescription pharmaceuticals such as atenolol (Lai *et al.* 2011). Further application of these biomarkers in WBE studies, and more information on how these products are metabolised, are necessary to use these to quantitatively estimate population.

Comparison of chemical WBE data

Several studies have compared prescription and WBE data (Carballa *et al.* 2008; Lai *et al.* 2011; Verlicchi *et al.* 2014; Escolà Casas *et al.* 2021). Data on illicit drug market trends and confiscated drugs have been used to sense-check WBE illicit drug data (Kankaanpää *et al.* 2016; Krizman-Matasic *et al.* 2019; Gonzalez *et al.* 2020). Considerations for evaluating WBE for pharmaceutical consumption include ensuring prescription data are at an appropriate temporal and spatial scale, where national averaged data are insufficient for comparison (Carballa *et al.* 2008; Lai *et al.* 2011; Verlicchi *et al.* 2014). An example of a study with a higher temporal resolution is Escolà Casas *et al.* (2021), which compared pharmaceutical detection via WBE

against a monthly aggregated pharmaceutical dataset. Such datasets need to be explored and compared against WBE data to ensure data are correct, identify and explain discrepancies, and account for any variation.

Summary and suggestions for further research

Chemicals can also act as indicators of human health. However, these are known to undergo changes in the body and/or the sewer environment, affecting the choice of chemical markers for surveillance, collection and storage of samples, and data interpretation.

- Further research is needed to understand excretion data for metabolites and endogenously formed entities, such as proteins. Improved understanding of pharmacokinetics is necessary to help find excretion factors in stool and urine, and how these may vary due to treatment received or age group.
- There is a lack of information on biomarker stability, particularly for metabolites. Further studies should investigate conditions in the sewer which may impact interpretations of WBE data, including sewage biofilm and different environmental conditions.
- Improved understanding of passive sampling could support WBE in areas where composite sampling is not feasible. Further understanding of the stability of various biomarkers is necessary to design appropriate standards, to ensure consistency and comparability among studies.
- Expansion of the biomarker suite coupled with high-resolution mass spectrometry approaches could allow multilevel estimation of public exposure and resulting effects. Exploration and application of population level biomarkers in WBE studies are necessary to have an improved estimated of population.
- WBE data need to be compared to appropriate datasets at an appropriate temporal and spatial scale to identify and explain discrepancies and account for any variation.

ADDITIONAL CONSIDERATIONS FOR IMPLEMENTATION

Integration into public health monitoring

Looking forward, WBE can be used for strategic and urgent public health surveillance needs, and to support environmental surveillance (Figure 4). Consideration should be given to the detection approach necessary for each area of interest and the subsequent response to an increase in infection or positive detection.

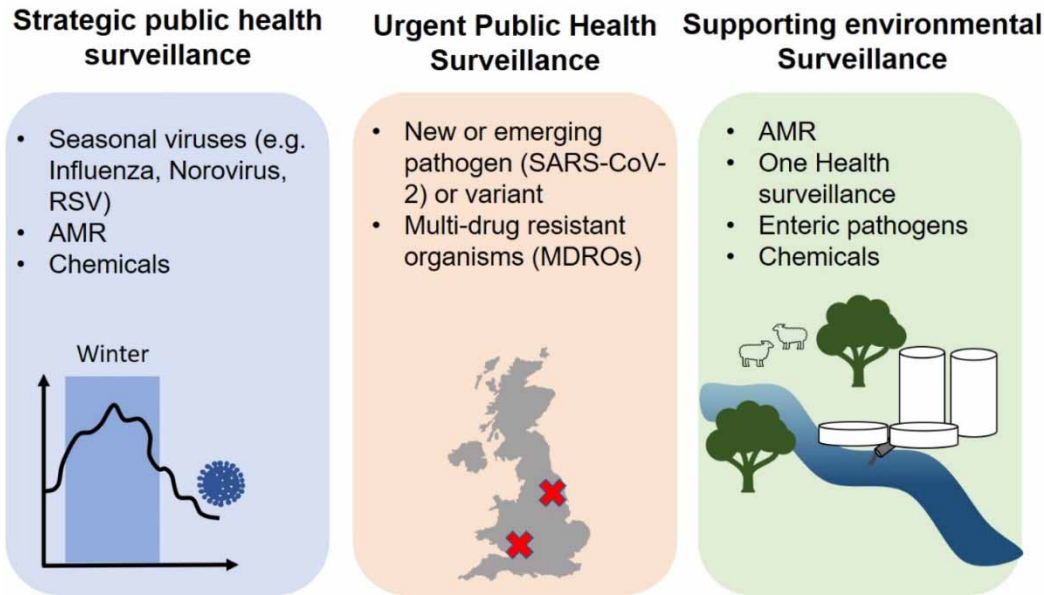


Figure 4 | WBE could be used to support public health surveillance through strategic public health surveillance, urgent public health surveillance, and by supporting environmental surveillance.

To be useful for public health monitoring, WBE needs to be integrated and add value to other public health initiatives, including clinical case reports, self-reporting systems, or social media tracking systems like FluDetector (Lampos 2016) or ZoeApp (Menni *et al.* 2021). Current epidemiological systems typically do not integrate clinical and environmental monitoring together. However, such an approach is necessary to ensure both groups are openly communicating, sharing insights, and harmonising approaches. Communication and collaboration with clinical and public health teams are also necessary for ensuring the data collected are useful in informing practice (e.g., identification of clinically relevant resistance within a community would need to be communicated to veterinary and clinical practitioners). Likewise, people working in healthcare might share with WBE teams requirements for novel targets. Collaboration with organisations collecting animal and environmental data could also be beneficial for developing a One Health surveillance framework.

Further research is needed between people working in WBE and those in healthcare settings to develop improved ways for public health users to receive these data and the best communication tools. Further discussions across clinical and environmental sectors is crucial for the design of WBE to ensure this is integrated into existing monitoring and provides a practical application. In addition, communication across the general public warrants further consideration. Publicly available dashboards during the COVID-19 pandemic showing daily case rates should be considered for additional WBE applications, allowing the public to have transparent access to information, raise awareness of health trends and provide information to make informed decisions. An interactive website or centralised dashboard providing clear, accessible summarised/non-identifiable data, integrating environmental, wastewater, and clinical data would be a potential way to communicate this information.

Having an overall understanding and ability to communicate uncertainties is also important. Within the UK, sewage networks are regionally diverse and can be publicly and privately owned, leading to differences in the way sampling is delivered (Wade *et al.* 2022). Some of the challenges may be especially evident in smaller rural communities, which may have hundreds of small wastewater treatment sites (Farkas *et al.* 2020a). Being able to communicate this uncertainty to relevant clinical stakeholders, the media and the general public is necessary to ensure data transparency and guide informed interpretations and responses.

Ethical implications

Despite wastewater being considered an ethical way of collecting data, further consideration and engagement of social scientists and the public is required to assess the ethics of WBE in applications beyond the pandemic. It is widely assumed that WBE uses aggregate measurements and cannot be used to identify individual persons. However, concerns have been raised over the privacy of data obtained from small populations or marginalised communities (McClary-Gutierrez *et al.* 2021), as well as over how the data will be used. Individual consent for the collection of wastewater samples for SARS-CoV-2 analysis has not been deemed necessary (Kwiatkowska *et al.* 2021). While data should be made publicly available and transparent, it is necessary to navigate how best to ethically and legally handle data, as well as communicate this information. Further research is required to gain community feedback and support open access information and prevent disagreements and stigmatisation (Polo *et al.* 2020). Regions or marginalised communities may be associated with negative health, and this could have a negative impact on well-being, local economies, and potentially insurance policies (Wang *et al.* 2020; Harris *et al.* 2021). These discussions are particularly urgent as the technology and applications for WBE advance.

CONCLUSIONS

Research on WBE has proven it is a potentially useful tool in monitoring the presence of health markers, providing a theoretically constant stream of information on the health status of whole communities. Proposed uses include tracking viral pathogens, AMR, and chemicals (i.e., drug and pharmaceutical metabolites, markers of human disease or pollution exposure). However, each application of wastewater has its own challenges and research needs, including varying disease dynamics, non-human reservoirs, instability of markers in sewers and storage and interpretation for public health. Research into these areas would allow WBE to be developed as a useful tool for public health to supplement existing surveillance. However, WBE methods and protocols have been developed in high-income countries with improved sanitation. With greater globalisation, emerging threats to health can occur anywhere, and spread around the world rapidly. International cooperation and coordination is necessary to monitor for future threats, and if WBE is to play a role in this, comparable datasets across communities and countries are needed. In particular, we should consider and support the development and validation of these techniques for lower income settings to ensure improved global health surveillance.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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