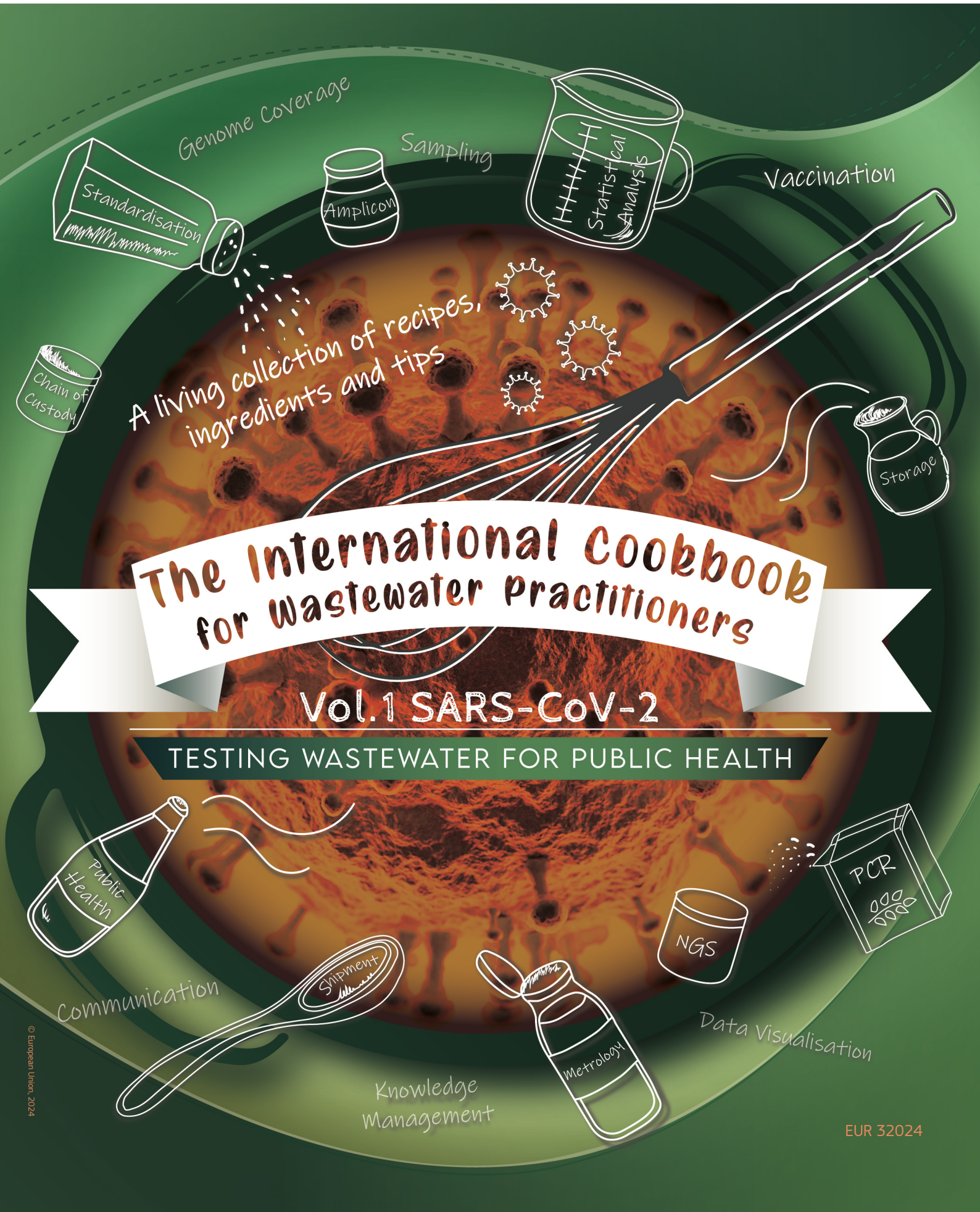
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The International Cookbook for Wastewater Practitioners

Vol.1 SARS-CoV-2

TESTING WASTEWATER FOR PUBLIC HEALTH

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SARS-COV-2 WASTEWATER-BASED SURVEILLANCE COOKBOOK

High-Level Overview and Best Practice Guide on how to undertake wastewater-based surveillance of SARS-CoV-2 and its variants for practitioners & operators

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Abstract

During the 2020-2023 COVID-19 Global Public Health Emergency of International Concern, rapid progress was made with the application of Wastewater-Based Surveillance (WBS) (also known as Wastewater-Based Epidemiology (WBE)) to provide population-scale evidence of SARS-CoV-2 shedding into wastewater. The information gained was used to help monitor, communicate, and manage the COVID-19 pandemic.

Rapid and significant innovations in the field of WBS were realised in parallel in multiple jurisdictions globally. Global collaboration resulted in step-change improvements in multiple aspects of WBS simultaneously, including: health sector leadership; integration with public health surveillance; social and ethical guidance; high-level tactical wastewater catchment sampling strategies; wastewater sampling techniques; transport and storage of samples; laboratory analysis; normalisation and standardisation; data analysis and interpretation; and communication of results.

Innovations in WBS relating to COVID-19 continue to take place, and the benefits of those improvements are now being applied to other targets, such as other pathogens and genes. There are many journal articles and other publications describing these innovations. However, valuable lessons from the COVID-19 pandemic for WBS have not been systematically documented into a practical and readily digestible format.

The purpose of this 'Cookbook' is to provide practical and proven 'recipes' for WBS for SARS-CoV-2 for two reasons: firstly to help those considering new or extended WBS programmes targeting SARS-CoV-2; and secondly to provide a valuable record of what has been learned from the 2020-2023 period to help inform WBS programmes to respond to future pandemics and for surveillance of other targets.

The Cookbook is structured into eight parts: 1. Introduction; 2. High-level Overview; 3. Health Sector Perspectives; 4. Methods and Methodologies - From data to action; 5. Methods and Methodologies - Measurement and testing; 6. Methods and Methodologies - Sampling; 6. Methods and Methodologies - Transportation protocols; 8. Conclusions. Multiple authors have contributed to each part, primarily from the European Union, as well as others from the global community of practice.

The Cookbook has been drafted by a global team of authors, with one or more lead authors identified for each chapter and working with their co-authors. The chapters were then subjected to extensive peer review from the external review group. These authors and reviewers are acknowledged in the Cookbook.

Foreword

DR PETER GREVATT, CHAIR, GLOBAL WATER RESEARCH COALITION, AND CEO, WATER RESEARCH FOUNDATION, DR STEPHANIE RINCK-PFEIFFER, MD GLOBAL WATER RESEARCH COALITION

The EC has been supported in this effort by both European and international experts, covering the global body of expertise. The Global Water Research Coalition (GWRC)'s is proud to have played a central role in supporting the development of this Cookbook. The GWRC provided access to its network to identify collaborators to help lead, co-author, and review the chapters.

During the COVID-19 Global Public Health Emergency of International Concern, extensive global collaboration took place between GWRC partner organisations and the many organisations that support those partners (such as water utilities, research organisations, health agencies, and other sector professionals). The GWRC played a major role in connecting practitioners to help jointly learn and work together to support the COVID-19 response. That same professional network has contributed to this Cookbook.

The global water research sector quickly mobilised at the onset of the COVID-19 pandemic apply their understanding of wastewater flows and catchments to help identify sampling points that provided actionable information on SARS-CoV-2 shedding from defined populations. Established and innovative sampling methods were developed and employed to collect and transport samples to laboratories. Environmental and clinical microbiologists repurposed their analytical methods to perfect testing of wastewater, environmental water, and sludge, to provide timely and actionable information on concentrations of SARS-CoV-2 genetic material present, including developing population normalisation markers to help improve quantification for trend analysis. Molecular genetic methods were further enhanced to permit variant monitoring. Epidemiologists and bioinformatics professionals provided mechanisms to communicate the information from WBS to both health professionals responsible for managing COVID-19 and to the public via internet-hosted dashboards. Guiding principles were developed for collaboration, and ethical guidance was developed for the use of WBS.

The GWRC is continuing to collaborate on WBS to further explore and enhance its value for SARS-CoV-2 another targets. These collaborative efforts include day-to-day interactions between professionals, connected via the GWRC member organisations, as well as through webinars, conferences, and communities of practice, including those coordinated by the EC in collaboration with the GWRC and its partner organisations.

Preface

On behalf of the EU Health Emergency Preparedness and Response Authority (HERA) and the European Commission's Joint Research Centre (JRC), it is with great pleasure that we introduce this comprehensive guide, referred to as the 'Cookbook'. This publication is the fruit of an extensive collaboration with the Global Water Research Coalition, and has been bolstered by a public-private alliance that we co-led with VEOLIA, SUEZ, RIVM, KWR, and WATER FUTURES. Together, we have endeavored to bridge a significant gap in the literature on wastewater-based surveillance (WBS) for SARS-CoV-2, with a particular focus on its role in public health.

The genesis of the Cookbook was sparked by an acute awareness that, despite the abundance of scientific research and the growing community of professionals in the global water and health sectors, there was a distinct lack of a unified, comprehensive resource for those entering the field or seeking to expand their knowledge of WBS. The COVID-19 pandemic brought this need into sharp relief, highlighting the urgency for such a resource. As the Commission's Science Service, the JRC spearheaded the creation of this guide, with the goal of crafting a practical and accessible compendium that would serve both novices and experts alike. The Cookbook encapsulates the wealth of collective knowledge and experience garnered throughout the pandemic, offering critical insights for managing future public health emergencies.

The Cookbook is structured into five distinct sections, providing a high-level overview, insights from the health sector, in-depth methods and methodologies, illustrative use cases and case studies, and practical recommendations. Authored by a global consortium and subjected to rigorous peer reviews, this guide stands as a testament to quality and applicability. The guide's purpose is manifold. It not only offers practical advice in an easily digestible format, guiding readers through the entire WBS process for SARS-CoV-2—from programme design to technical execution and the utilisation of results—but it also serves as a historical record of the field's progress, setting a benchmark for future pandemic responses.

Furthermore, the Cookbook is designed to inspire engagement and co-creation across all sectors of society. It challenges us to re-envision wastewater from being merely a by-product to being a critical source of public health data. Achieving this paradigm shift requires a concerted effort to educate and involve communities, ensuring that the full potential of WBS is communicated in an effective and accessible manner. The advent of SARS-CoV-2 and the ensuing global health crisis have underscored the critical need for innovation in public health surveillance. As traditional methods of disease moni-toring struggled to keep pace with the rapid dissemination of the virus, the necessity for scalable, cost-effective, and timely surveillance strategies became clear. WBS emerged as a formidable tool in this landscape, offering a non-invasive means to gauge the prevalence of the virus within populations and to track its trends over time.

This Cookbook is a testament to the power of international cooperation in overcoming public health challenges. It marks a significant achievement in the collaborative efforts led by HERA and the JRC to establish a standardised global guidance framework for the implementation of WBS for SARS-CoV-2. Recognising the diverse contexts in which it will be applied, this guide is intended to be a practical resource rather than a rigid standard, allowing for local adaptations that yield equivalent outcomes.

Our ambition with the Cookbook is to standardise best practices in sampling, analytical techniques, and result interpretation of results, ensuring data reliability and comparability. We are committed to establishing guidelines for transparent communication, fostering trust with the public and stakeholders through the clear dissemination of WBS findings. Additionally, we advocate for strategies that integrate WBS data into public health policy, thereby enhancing evidence-based decision-making.

In essence, the Cookbook is more than just a manual; it is a call for public health officials, researchers, and practitioners to adopt WBS as a vital surveillance tool for SARS-CoV-2. It serves as an invitation to join a global movement dedicated to leveraging the capabilities of wastewater surveillance to advance public health initiatives and protect communities from the extensive impacts of pandemics. We trust that this guide will become a cornerstone for practitioners around the world, supporting the ongoing battle against the COVID-19 pandemic and equipping us for the challenges that lie ahead.

Warm regards,

Laurent Muschel

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We extend our profound gratitude to all individuals and groups who contributed to the creation of this pivotal resource. This Cookbook represents a collective undertaking that would not have been possible without the commitment and expertise of each participant.

EXPERT CONTRIBUTORS

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We recognise the efforts of the teams behind each contributor, whose collaborative spirit and unwavering support were instrumental in the development of this guide. Their contributions, often behind the scenes, enabled the smooth progression of this project from concept to completion.

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- European Bank for Reconstruction and Development (EBRD).

Their support underscores the importance of international cooperation in addressing public health challenges.

DESIGN AND EDITORIAL TEAM

Special recognition is owed to the overall document editor, Sara Comero, and the designer Tanja Casado, who pulled the many chapters of the Cookbook together from its diverse global author consortium, and brought clarity and visual appeal to the guide, making complex information accessible and engaging. Their creative talent has made this Cookbook not only informative but also a pleasure to use.

We also wish to thank the proofreaders, whose keen eyes and attention to detail have ensured the accuracy and readability of the content. Their dedication to linguistic precision has greatly enhanced the quality of this guide.

FINAL WORD OF THANKS

Lastly, we express our collective thanks to everyone who has played a part in bringing this Cookbook to fruition. Your contributions are a testament to the power of collaboration and shared vision in advancing the field of public health surveillance. It is our hope that this guide will serve as a cornerstone for practitioners worldwide, aiding in the ongoing fight against the COVID-19 pandemic and preparing us for future challenges.



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Executive summary

CONTEXT AND IMPERATIVE FOR INNOVATION IN PUBLIC HEALTH SURVEILLANCE

In the face of the COVID-19 pandemic, public health systems across the globe have encountered unprecedented challenges. The need for effective surveillance to monitor and predict outbreaks has never been more critical. Traditional testing methods, limited by capacity and timeliness, are complemented by the innovative approach of Wastewater-Based Surveillance (WBS). This report, produced by a consortium led by the European Commission's Joint Research Centre and including prominent organisations such as the Global Water Research Coalition and RIVM, introduces a comprehensive guide for the application of WBS in tracking SARS-CoV-2.

SCOPE AND AMBITION OF THE COOKBOOK

The Cookbook is a culmination of the collective wisdom from numerous experts, who have distilled their knowledge into practical "recipes" for WBS implementation. Unlike rigid standards, this guide is designed to be adapted and interpreted according to local conditions, promoting flexibility and creativity in public health responses. The report provides a compendium of methodologies, ranging from sample collection to data interpretation, tailored to guide public health sector leaders in both established and emergent surveillance programmes.

OBJECTIVES AND BENEFITS

The Cookbook is structured to achieve the following core objectives:

- To standardise a global guidance framework for the implementation of WBS for SARS-CoV-2, adaptable to diverse contexts.
- To detail best practices in sampling, analytical techniques, and result interpretation, ensuring reliability and comparability of data.
- To establish guidelines for transparent communication, building trust with the public and stakeholders through clear dissemination of WBS findings.
- To recommend strategies for the integration of WBS data into public health policy, enhancing evidence-based decision-making.

By addressing these objectives, the guide equips public health leaders with an essential resource, enabling them to leverage WBS as a potent tool for pandemic monitoring and management. The comprehensive approach outlined within the report is envisioned to fortify preparedness and response strategies for current and future infectious disease threats.

DETAILED OUTLINE OF THE COOKBOOK

The report unfolds with an initial chapter that outlines the importance of WBS in the context of SARS-CoV-2 surveillance. It progresses through a series of meticulously structured chapters, each akin to a culinary recipe, providing step-by-step guidance on:

- Navigating leadership challenges and fostering collaboration within the public health sector.
- Designing sampling programs that are robust, representative, and sensitive to community dynamics.
- Applying detailed methodologies for the collection, processing, and analytical examination of wastewater samples.
- Interpreting results to draw meaningful conclusions for public health implications.
- Crafting communication strategies to effectively share results with stakeholders, policymakers, and the general public.
- Utilising WBS data to inform, refine, and adjust public health policies and intervention strategies.

Each chapter is crafted to be self-contained, allowing users to navigate directly to sections most pertinent to their needs, while also providing comprehensive insights when read in sequence.

A CALL TO ACTION

The Cookbook is more than a manual; it is a call to action for public health officials, researchers, and practitioners to embrace WBS as a surveillance tool for SARS-CoV-2. It encourages a proactive stance in public health surveillance, offering a blueprint for action that is scientifically grounded and practically feasible. The report is an invitation to join an ongoing global effort to harness the power of wastewater surveillance, advancing public health initiatives and safeguarding communities against the far-reaching impacts of the pandemic.



Chapter 1

Introduction



1 INTRODUCTION

1.1 POLICY CONTEXT AND BACKGROUND

The emergence of the SARS-CoV-2 virus, which causes COVID-19, has led to a global health crisis of unprecedented scale. Governments and health organisations worldwide were forced to rapidly adapt and innovate in response to the pandemic. One of the most significant challenges in managing the spread of the virus has been the ability to effectively monitor and predict outbreaks. To address this, one innovative approach that has gained traction is WBS. WBS is the monitoring of infectious disease markers in wastewater to provide insights into the health status of a population. The use of WBS for SARS-CoV-2 offers a non-invasive way to estimate the prevalence of the virus within a community and to monitor trends over time.

This report has been crafted as a comprehensive guide for implementing WBS for SARS-CoV-2. It is the result of a collective effort of an alliance led by the European Commission's Joint Research Centre and the EU Health Emergency Preparedness and Response Authority (DG HERA) as well as by the Global Water Research Coalition, RIVM, VEOLIA, Suez, Water Future and the European Bank for Reconstruction and Development. Numerous experts embraced the challenge to share their combined knowledge and experience in a novel and uncomplicated way facilitating further dissemination. Each "recipe" is led by a "master chef", and organised as collection of ingredients and instructions. The "Science behind" links the instructions to latest research findings.

Similar to a real cookbook, the collection is meant to be a hands-on guidance and NOT a strict standard. In embracing this philosophy, the collective of authors acknowledges that the different variations and interpretations, which consider better local elements, are to be allowed and will lead to equivalent results.

The Cookbook is also a response to the urgent need for alternative surveillance strategies that complement clinical testing and provide broader community-level insights. By capturing data from a collective sewage system, WBS can detect signals of the virus shed by asymptomatic as well as symptomatic individuals, thus offering an inclusive and timely indicator of community infection levels.

The policy problem addressed by this report is the need for a scalable, cost-effective, and less resource-intensive method of surveillance that can act as an early warning system for SARS-CoV-2 spread. Traditional individual testing approaches are often hampered by testing capacity, accessibility issues, and delays in results. WBS, on the other hand, can be a crucial tool in the public health arsenal, providing policymakers with near real-time data to inform decisions on public health interventions, resource allocation, and easing or tightening of restrictions. Its significance lies in the potential to enhance preparedness and response to not only the current pandemic but also future infectious disease outbreaks.

MAIN OBJECTIVES

The main objectives of this Cookbook are to:

- Establish a guidance framework for WBS of SARS-CoV-2 that can be adapted and applied globally.
- Develop a set of best practices for sampling, analysis, and data interpretation to ensure reliable and comparable results.
- Create guidelines for the effective communication of WBS data to stakeholders and the public, ensuring transparency and trust.
- Provide evidence-based recommendations on how WBS data can be integrated into public health decision-making.

By achieving these objectives, the report aims to empower public health leaders with a robust tool to enhance the monitoring and management of the COVID-19 pandemic.

This report is crafted as a "Cookbook," providing a collection of recipes and ingredients necessary to perform WBS for SARS-CoV-2. It is intended to guide public health sector leaders through the intricacies of establishing and managing a WBS programme. The scope is strictly limited to WBS involving monitoring SARS-CoV-2, with the intent to provide information that aids in COVID-19 pandemic monitoring, response, communication, and management. It does not extend to other targets of WBS or other methods of public health surveillance.

The report covers essential components of WBS, including:

- Leadership and coordination within the public health sector.
- Design of robust and representative sampling programmes.
- Detailed methodologies for sample collection, processing, and analysis.
- Interpretation of results in the context of public health implications.
- Communication strategies to effectively disseminate findings to stakeholders and the public.
- Utilisation of WBS data to inform and adjust public health policies and interventions.

The first chapter of this cookbook provides an overview of WBS, its importance, and the rationale behind using this approach for SARS-CoV-2 surveillance. Subsequent chapters are structured as a comprehensive guide to executing WBS, from the initial steps of planning and design to the final stages of data interpretation and policy application. Each chapter is akin to a recipe, detailing the necessary "ingredients" (procedures, equipment, and considerations) and the "method" (step-by-step instructions) required to successfully conduct WBS for SARS-CoV-2.

In conclusion, this report serves as a foundational resource for public health officials, researchers, and practitioners interested in implementing WBS as a surveillance tool for SARS-CoV-2, ultimately contributing to more informed and effective public health strategies.

Chapter 2

High-Level Overview



2 HIGH-LEVEL OVERVIEW

2.1 BACKGROUND

MASTER CHEF:

OLEKSIY SHEVCHENKO

CREW:

DANIEL DEERE

The primary objective of sanitation is to capture faecal material, safely contain it, then adequately treat it for safe reuse or disposal, to limit transmission of infectious faecal-oral pathogens. Access to sanitation is a basic human right under the United Nations Sustainable Development Goals (SDG) (United Nations, 2023).

In larger population centres faecal material is typically captured in engineered wastewater infrastructure that collects water from water-washed flush toilets that capture faecal matter and urine, as well as cleansing water from bidets and sanitary sprays. In most cases, the same infrastructure captures wastewater from showers, baths, sinks, and laundry. As a result, wastewater contains, as a minimum, water-washed faecal excreta and urine from domestic settings and in most contexts also contains wastewater from other domestic uses, such as water-washed exfoliated and secreted materials from personal hygiene activities, and materials from food preparation. Wastewater may also contain a range of other commercial and industrial inputs, as well as stormwater that may be captured into combined sanitary sewers. In unanswered areas, stormwater may contain similar materials due to open defecation and urination and open disposal of wastes and wastewater.

As a result, wastewaters that are captured in both sewered and unsewered settings capture and transport material that has the potential to serve as targets for epidemiological surveillance. Determination of a pathogen or its genetic material by qualitative methods indicates its circulation in a given area, while quantitative methods make it possible to assess the dynamics and intensity of the epidemic process.

2.1.1 HUMAN VIRUSES IN WASTEWATER AND OTHER VIRAL PATHOGENS AS CONTAMINATION INDICATORS

The most common forms of treatment used for wastewater are designed to meet targets set for gross pollutants, such as suspended solids, biological oxygen demand, and in some cases nutrients such as phosphorus, and/or nitrogen. Such treatment typically provides only incidental reductions in the concentrations of human-infectious viruses before wastewater is discharged into the environment or applied for reuse (Osuolale & Okoh, 2017). Additionally, in many regions of the world, the respective standards for wastewater treatment are either missing, or inadequately adhered to, and often do not include virus reduction requirements. As a result, transmission of viruses via wastewater that has been inadequately contained and treated can occur.

Several types of human viruses are known to be present in wastewater, causing so-called waterborne illnesses. These typically include noro-, sapo-, rota-, astro-, picorna-, and adeno- viruses (Hellmér et al., 2014; Ramírez-Castillo et al., 2015; Rodríguez-Díaz et al., 2009; Symonds et al., 2009). These waterborne human enteric viruses are sufficiently stable in water that they can persist adequately to permit transmission between hosts via wastewater, with some being relatively resistant to commonly used disinfectants such as chlorination and ultraviolet irradiation (Fong & Lipp, 2005; Okoh et al., 2010).

In addition to viruses that can be readily transmitted via wastewater, others can be present in wastewater, even if they are attenuated or partially degraded and of limited or no risk of causing infection. For instance, the proteinaceous antigenic structures or genetic material that forms part of viruses can persist in wastewater even if transmission via the waterborne route is not a significant risk. For instance, studies have found genetic material from polyomaviruses and human coronaviruses (such as SARS-CoV-2) at high concentrations in wastewater (Albinana-Gimenez et al., 2009; Lastra et al., 2022; D. Zhang et al., 2022; J.-B. Zhou et al., 2021).

In 2020, the United States Centers for Disease Control and Prevention (CDC) launched the National Wastewater Surveillance System (NWSS) in response to the COVID-

19 pandemic (CDC tracker) using quantitative polymerase chain reaction to quantify SARS-CoV-2 RNA. With this method, only the virus RNA level (i.e., number of virus genome copies) is measured, not the virus itself. Until now, there are no proven cases of people being infected with SARS-CoV-2 due to exposure to wastewater (Lastra et al., 2022; D. Zhang et al., 2022; J.-B. Zhou et al., 2021).

Contaminated wastewater is very rich in microorganisms. *E. coli*, *Clostridium* spp., *Legionella* spp., *Mycobacterium* spp., *Shigella* sp., *Vibrio* spp., *Salmonella* spp., *Streptococcus* spp., and *Pseudomonas* spp. are typically common in sewage (Francy et al., 2011; Numberger et al., 2019). The abundance of bacteria in wastewater inevitably indicates even more bacterial viruses (or bacteriophages). Phages form the dominant group of viruses in sewage or any other type of used water. The actual/predicted phage diversity includes ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) coliphages (Hata et al., 2013) as well as phages of *Pseudomonas* spp., *Streptococcus* spp., *Shigella* sp., etc.

The occurrence of plant viruses (tobamo-, cucumo-, poty-, and potexviruses) in wastewater has also been noted. Some stable viruses may eventually survive the hostile environments of the gut. It was demonstrated that such viruses might retain their infectivity. There were reports of tobamoviruses found in environmental waters and treated wastewater (Bačnik et al., 2020; Filipić et al., 2021; Jeżewska et al., 2018, 2019; Oki et al., 2017), and even in drinking water (Haramoto et al., 2013).

High titres of bacterial and plant viruses in wastewater (in parallel to pathogenic human viruses) suggested that they could be used to monitor the efficiency of water treatment in terms of overall virus removal. There are numerous works indicating successful implementation of this idea. Recent data indicates that removal rates of phages were similar to those of human viruses (Amarasiri et al., 2017; Bivins, Crank, et al., 2020; Dias et al., 2018). Plant tobamoviruses were also shown to be efficient indicators of pathogen contamination level in environmental water samples (Kato et al., 2018; Kitajima et al., 2018; Shrestha et al., 2018; Tandukar et al., 2020).

2.1.2 HISTORY OF COVID-19 PANDEMIC

In view of the above, screening wastewater for dangerous human viruses using effective laboratory methods and/or reliable biological indicators has shown great value, particularly in the context of the ongoing COVID-19 pandemic.

As widely known, in December 2019, an outbreak of infection caused by a new severe acute respiratory syndrome coronavirus (SARS-CoV-2) was identified in Wuhan (Hubei, China) (P. Zhou et al., 2020). Since then, the virus (causative agent of the ongoing COVID-19 pandemic) has been found in more than 200 countries and regions around the world, where it had caused approximately 770 million COVID-19 cases and 7 million deaths as of 12 August 2023 (https://covid19.who.int/).

The Coronaviridae family includes a wide range of animal and human coronaviruses. There are currently seven known human coronaviruses.

During the process of adaptation to the human population, several variants of SARS-CoV-2 emerged. The primary ones included Alpha, Beta, and Delta, which became the most transmissible by mid-2021. However, by December 2021, these were superseded by the Omicron variant, the variants of which are still predominantly circulating around the world at the time of writing this document (da Silva Torres et al., 2022).

SARS-CoV-2 is believed to spread primarily through the respiratory tract and through close contact. However, a significant number of patients with COVID-19 have had gastrointestinal symptoms, and SARS-CoV-2 RNA has been detected in faecal samples from almost half of patients with confirmed COVID-19 (Guo et al., 2021).

Wastewater observation confirms the presence of the genetic material of SARS-CoV-2 and its variants (Polo et al., 2020). The importance of integrating wastewater surveillance into the COVID-19 outbreak management system as a scalable, cost-effective and non-invasive surveillance tool is noted, especially for the early detection of asymptomatic or pre-symptomatic individuals (Keshaviah et al., 2021).

2.1.3 APPROACHES FOR VIRUS DIAGNOSTICS IN WASTEWATER

Widely known methods of virus detection are used to diagnose viruses in wastewater. These techniques, however, have undergone certain modifications due to the relatively low concentration of viruses in the samples (Corpuz et al., 2020; Wyn-Jones, 2007).

Viruses are isolated from wastewater by concentrating, mostly by passaging the sample through positively charged cartridge filters followed by elution and identification in cell culture. Nevertheless, this method is quite time-consuming and not always reproducible for many viruses, in particular for SARS-CoV-2, working with which also requires conditions with a certain level of biological protection (Amoah et al., 2020; Hryniszyn et al., 2013).

Therefore, the most common methods for detection of SARS-CoV-2 in wastewater are polymerase chain reaction and nucleic acid sequence-based amplification (NASBA) (Stobnicka-Kupiec et al., 2022; Tiwari et al., 2022; Westhaus et al., 2021), which provide information on the total viral load, but do not allow determination of the infectivity of the virus. Electron fluorescence and atomic force microscopy methods are also used for visual identification of the virus (Y. Zhang et al., 2020). In addition, immunochemical approaches that were originally developed for the detection of other viruses have been adapted for SARS-CoV-2. These include methods such as flow cytometry (Safford & Bischel, 2019), enzyme-linked immunosorbent assay (Ejeian et al., 2018), and biosensors (Yu et al., 2016).

For a comprehensive analysis of the epidemic situation, a combination of methods should be used. Detection of SARS-CoV-2 RNA in wastewater is mainly carried out by molecular polymerase chain reaction (PCR)-based methods by amplifying parts of the viral genome, in particular, genes encoding the proteins of the nucleocapsid or viral envelope (https://www.fda.gov/media/134922/download).

2.1.4 POLICIES FOR WASTEWATER VIRUS MONITORING IN UKRAINE: A COMPARATIVE VIEW

The examination of wastewater for SARS-CoV-2 monitoring is an emerging field of importance in public health. While EU policies provide a broad framework, Ukraine's situation, marked by conflict and healthcare challenges, offers a compelling case for focused study. In addition, the challenging humanitarian situation in Ukraine allowed to explore the applicability of WES in situations of disaster and conflicts. It anticipated the use of WES for other pathogens in conflict areas and crisis situations, e.g. the earthquake which affected large part of Turkey and Syria in 2023. This section examines therefore Ukraine's unique wastewater surveillance policies and their role in maintaining public health surveillance amidst crisis.

In Ukraine, the virological examination of wastewater is regulated by the guidelines 'Sanitary and virological control of water bodies' (https://ips.ligazakon.net/document/MOZ6926). According to these regulations, within the framework of epidemiological surveillance, enteroviruses (including polioviruses), rotavirus, adenoviruses (types 40-41), and hepatitis A virus are monitored. Coliphages are also determined as one of the indicators of the quality of wastewater treatment. The study of wastewater for enteroviruses in the country has been carried out for >60 years. Now this function is strengthened as an additional component of the epidemiological surveillance of poliomyelitis. During 2021 alone, more than 2,300 wastewater samples were tested for enteroviruses. There are 26 virological laboratories (2 reference and 24 regional laboratories) in the system of the Ministry of Health of Ukraine.

The COVID-19 pandemic has triggered major adjustments to the virological screening of wastewater, adding the SARS-CoV-2 virus as a target to provide information to assist with its monitoring and control. The isolation of the virus within faeces was reported near the beginning of the pandemic (Zadorozhna & Vynnyk, 2020). Several publications on the topic were issued by the World Health Organization (WHO) and its partners (https://www.who.int/publications/i/item/WHO-HEP-ECHWSH-2022.1; https://www.who.int/europe/publications/i/item/WHO-EURO-2022-5274-45038-64164).

The importance of this issue can be demonstrated by the example of Ukraine. Before the hostilities caused by Russia's aggression began on 24 February 2022, Ukraine was experiencing a peak in its incidence of COVID-19 caused by a new variant of the virus at that time, Omicron. Military operations, intensive migration of the population, and gathering during air raids in overcrowded train stations and places of temporary accommodation, would have been expected to contribute to a further rise in this incidence. However, the decline in the number of people seeking medical care, and the limited accessibility of the population to medical care, led to a sharp decrease in the official registration of morbidity. For instance, according to the data from the Public Health Centre of the Ministry of Health of Ukraine, on 22 February 2022 (before the hostilities began), the daily incidence of registered cases and hospitalisations due to COVID-19 was 25,062 and 3,140, respectively. Just five days later, on 27 February 2022, these figures decreased rapidly by over 80%, to 3,481 and 674 per day, respectively. The reporting on the incidence of COVID-19 cases in Ukraine had, therefore, rapidly lost much of its informative value. Furthermore, less than six months after hostilities began, during the 28th week of the epidemiological reporting year (11-17 July, 2022), 1,431 cases of COVID-19 were registered in the country, of which 1,038 were hospitalised, so hospitalisations represented 73% of the total cases, compared to 13% prior to the start of hostilities. This indicates that only the more severe cases were being recorded in such a situation, which does not allow an objective assessment of the epidemic situation and timely adoption of appropriate anti-epidemic and preventive measures.

Wastewater screening provided an additional and invaluable component to reliably assess the epidemic situation and trends in the epidemic process in order to

make adequate management decisions aimed at stabilising the epidemic. Currently, in Ukraine, with the support of international organisations, modelled on several regions, a project has been launched to study wastewater samples for SARS-CoV-2 in accordance with WHO Interim Guidance (https://www.who.int/publications/i/item/WHO-HEP-ECHWSH-2022.1).

2.2 ETHICAL, ANTHROPOLOGICAL AND SOCIAL ASPECTS

MASTER CHEF:
CRESTEN MANSFELDT

CREW:
ALICE CAROLLO
NATALIA GŁOWACKA
RICHARD ELELMAN

WBS unites two concepts with wide social diversity in conception, acceptance, and stigmatisation:

- human waste; and
- public observation.

As detailed in the preceding section, WBS provides a flexible public health tool that has been deployed globally in high and low resource settings in both rural and populated regions, altering the type of wastewater sampled and the application of the collected data. Even with this broad range of potential monitoring campaign types, ethical practice is informed by principles and guidelines established in the pro-fession of public health to assist in conforming WBS to local norms. In introducing these principles, this section provides a broad overview of those sociological and ethical considerations to highlight concepts that will be further detailed in sections throughout this Cookbook.

2.2.1 KEY INGREDIENTS



- Wastewater-based epidemiology
- Public health information resource
- Social and cultural perceptions of waste and water
- Ethical frameworks for environmental monitoring and public health
- Socio-political consequences of WBS
- Community involvement and engagement

2.2.2 RECIPE



1. **Understanding Wastewater as a Public Health Information Resource:**
   a. Discuss the shift from viewing wastewater as a by-product to dispose of, to recognising it as a material or information resource to capture.
   b. Explore the interpretative nature of social sciences in addressing the novel environmental, health, and cultural challenges posed by this redefinition of wastewater.
   c. Examine the role of waste and water in social and cultural practices.
2. **Ethical and Sociological Considerations in WBS:**
   a. Analyse the intersection of waste and public observation in the context of WBS.
   b. Discuss the established frameworks for environmental monitoring and public health, and their relevance to WBS.
   c. Examine the unique challenges posed by the composite nature of monitored samples, diverse wastewater types, and multiple objectives within a single sample.
   d. Highlight the potential for stigmatisation in WBS campaigns targeting specific diseases or substance use.
3. **Socio-political Consequences and Community Involvement:**
   a. Discuss the importance of framing WBS within a broader social discourse on water, waste, and public health.
   b. Examine the need for effective communication and public awareness of WBS at various scales (local, national, and international).
   c. Explore the role of the so-called Quintuple Helix model (Section 2.2.4.3) in promoting public engagement, social partnership, and dialogue.
   d. Discuss the benefits of community involvement in the development, implementation, and continuity of WBS programmes.

2.2.3 TIPS

- Contextualise best practices for WBS with socio-political dependencies and consequences.
- Foster open and inclusive communication strategies that cater to diverse audiences and address potential stigmatisation concerns.
- Encourage community-led initiatives and engagement to promote ownership and trust in WBS programmes.
- Emphasise the importance of continuous education and awareness-raising activities to maintain public support and interest in WBS.
- Establish clear guidelines for privacy and anonymity protection in WBS campaigns.
- Encourage collaboration between local, national, and international stakeholders to promote knowledge exchange and the adoption of WBS at a broader scale.



2.2.4 THE SCIENCE BEHIND

2.2.4.1 SOCIOLOGY OF WASTE: WASTE IN WATER

Water and waste both entail complex physical and ideological natures, which shape and challenge the way humans understand their reality. Socially, water and waste provide two often polar opposite concepts. Water traditionally embodies the idea of purity and cleanliness in diverse regions and cultures, whereas waste is culturally associated with concepts of filth, disease, and disorder. Therefore, their points of contact and the cultural landscape they generate remain key areas to explore. Notably, this analysis arrives at their intersection: wastewater.

Social sciences have examined and discussed water and waste in great detail, but usually as independent concepts. With wastewater broadly undergoing a redefinition from a by-product to dispose of to a material or information resource to capture, critical questions common within these analyses should be posed explicitly for our sewage infrastructure. Why should society explore wastewater not only from a material resource point-of-view but as a “total social fact” - as the French anthropologist Marcel Mauss (1990) once defined water? Further, how can the interpretative nature of social sciences be useful in addressing the novel environmental, health, and cultural challenges that this redefinition of wastewater poses? With a multidisciplinary lens focusing mainly through the discipline of anthropology, and deriving from the academic discourses on waste and water, this section provides a brief answer to these questions.

Renewed interest for waste within the social sciences developed rapidly after the publication of ‘Purity and Danger’ by Mary Douglas in 1966. The book, considered a seminal work in the field, set the ground for contemporary waste studies. Douglas’ theory is that dirt, and thereby waste, is fundamentally “matter out of place” because every social order organises itself on ideas of dichotomies of cleanliness/dirtiness and order/disorder. Therefore, the maintenance of social order is strictly related to the separation of waste from daily life (Carollo A. et al., unpublished). Contemporary waste/discard studies evolve this concept further, affirming that waste is more than a cultural product of human societies’ demand for order, but a local and global process, and a social and ecological priority involved in the reshaping of the governance of the world (Reno, 2015). Waste itself is more than “what remains of us”, it is inherently related to our way of being in the world as humans, as biological entities, and as consumers. Waste is that part of our lives that we ditch in the shadow, that we do not want to see, the fundamental other in our existence (Scanlan, 2005). These characteristics of waste inherently raise questions about its nature, its ability to be a tool to reflect ourselves, and its role in public health surveillance (Reno, 2015).

In this framework, wastewater poses novel and challenging questions: how does society balance managing the physical characteristics whilst acknowledging the competing cultural values of both water and waste (Carollo A. et al., unpublished)? Beyond carrying a strong symbolic meaning because of its universal value and absolute necessity for survival

(Wilk, 2006), water holds an exclusive status because of its inherent ability to perform cultural values regarding order and purity, clearly noticeable in diverse traditions around the world: baptism, ablutions, washing of the new-born or the dead, all involve the concept of water as a healing and purifying substance. Wastewater challenges this precise idea of water as one of the holiest substances on earth: water polluted by industrial or household waste is water at the opposite of its purifying cultural proprieties, a water that cannot sustain human life and social order (Carollo A. et. al., unpublished).

Historically, novel engineered approaches to the challenge of handling wastewater emerged in the 19th century. During this period, Thomas Crapper championed the water closet as a means to supersede the more predominant earthen closet. From then on, high gross domestic product per capita nations that could fund large municipal projects adopted and became locked into the technology of sewerage and, later, wastewater treatment. During these 200 years of operation, however, the initial concept of discharging human waste into the water cycle for transportation and treatment has in and of itself created several challenges. First, a substantial volume of potable water is transformed into wastewater that requires management (Larsen et al., 2013; Niemczynowicz, 1993; Thompson & Beck, 2017). Second, the physical collection and treatment system requires substantial upkeep and evolution to evolve with society's changing definition of waste. Third, civil and environmental engineering was additionally challenged to maintain a safe system that removed contact between humans and their waste (Beck, 2011; Thompson & Beck, 2017). This resulted in a system that makes our bodily waste, and waste in general, essentially invisible (Carollo A. et. al., unpublished).

However, both economically and socially, the concepts of waste and wastewater are undergoing a renaissance. For example, as a material source, wastewater is being considered for and applied in agricultural and other water reuse practices, essential for coping with the approaching water crisis caused by climate change and for the recovery of materials and resources.

As an information source, wastewater surveillance gained popularity during the COVID-19 pandemic. At the end of February 2020, Dutch researchers identified SARS-CoV-2 in Amsterdam Schiphol Airport effluent wastewater (W. Lodder & De Roda Husman, 2020). Rapidly, additional countries adopted this practice of surveilling wastewater. By the middle of 2020, wastewater monitoring for SARS-CoV-2 biomarkers became widespread, constituting a useful "early warning signal", complementary to other strategies such as clinical testing (Anderson, 2022; Vaughn, 2022). Wastewater surveillance was and continues to be demonstrated as a powerful tool in monitoring of virus concentrations in communities, cementing the definition of wastewater to include the concept of a "public health information resource". Socially, treating wastewater as an information source uniquely shortens the conceptual distance between humanity and its waste, invoking nuanced and complex ethical concerns.

With the physical nature of waste directly mirroring the individual and the intrinsic ideological position of water in communal life, wastewater surveillance must be framed in the broader and ongoing social discourse on the evolution of waste in the 21st century. This discourse must not exclusively view wastewater as a material problem to solve but as a tool to question fundamental aspects of future life. How is humanity's relationship with the environment, waste and health changing with the rise of new public health tools such as WBS? How do the priorities established around preserving the signal in the waste challenge the objectives of remediating the water? Is this contributing to an ideological re-evaluation of waste? These questions open novel economic, social and cultural considerations for public health, environmental engineering, and environmental sciences that require a strong interdisciplinary collaboration.

2.2.4.2 EARLY ETHICAL CONSIDERATIONS IN WBS

WBS developed through the interdisciplinary practice of deploying an environmental monitoring technique to inform public health intervention. Thereby, WBS unites two practices with established frameworks for ethical applications: environmental epidemiology and public health. Although environmental epidemiology surveils communities to manage environment-induced health hazards (Soskolne & Light, 1996), as mentioned previously, the nature of WBS in context of SARS-CoV-2 primarily is deployed to monitor and track those infected rather than the origin of the infection. Therefore, aspects of previously developed guidelines for environmental monitoring, such as fault assignment, do not directly translate for current WBS applications (Kramer et al., 2012; Soskolne et al., 2021; Soskolne & Sieswerda, 2003).

Best practices in public health monitoring more directly translate to the ethical application of WBS. Surveillance in support of public health maintains robust guidance for ethical application (Fairchild et al., 2017; Fairchild & Bayer, 2004; WHO, 2017), enabling rapid re-contextualisation and adoption for WBS. The distinguishing features of WBS requiring reinterpretation when compared to other forms of surveillance discussed within public health guidance are

the composite rather than individualised nature of the monitored samples (Prichard et al., 2014), the wide diversity in wastewater type monitored (e.g., latrine, within-sewer, wastewater treatment plant inflow), and the potential to monitor multiple objectives within a single sample (e.g., virus circulation, illicit substance use, human DNA). When compared to best practices associated with clinical monitoring of disease propagation in hospitalised patients, wastewater specific ethical guidance should focus on the broad and diverse nature of potential applications and outcomes of the surveillance.

Prior to the emergence of SARS-CoV-2, WBS applications were demonstrated in disease propagation and illicit substance consumption within communities, each emphasising differing ethical imperatives within potential monitoring campaigns. Multiple long-term WBS campaigns have targeted various pathogens of interest such as poliovirus (Alleman et al., 2021; Kroiss et al., 2018) and hepatitis (Iaconelli et al., 2020), raising, on the one hand, the imperative for equal access to a useful technique for public health management across resource settings and access to centralised wastewater collection systems (Street et al., 2020). On the other hand, reporting on the presence of these viruses within specific communities raises the potential for stigmatisation common to emerging or re-emerging infectious diseases (Joffe, 2011). This stigmatisation risk is shared within illicit substance WBS campaigns (Prichard et al., 2014), likely challenging the ultimate public health effort of harm reduction by favouring drug prevalence management instead (Lancaster et al., 2019).

The complexity of these campaigns and outcomes resulted in multiple interpretative frameworks being developed to assist WBS application (Hrudey et al., 2021; Price & Trowsdale, 2022; Prichard et al., 2016; Scassa et al., 2022). Common key elements from these previous studies that are expanded on in these cited texts focus on the following: supporting a public health outcome; enabling equal access to WBS; maintaining consent from the community; establishing clear communication to/with the community; minimising hazard in handling the sample; and supporting privacy and anonymity. Each of the considered frameworks discusses these and other key facets that enable a general successful WBS campaign. However, the diversity in application, target, and sample source challenges overly general statements. WBS provides a public health tool, raising ethical concerns only in the form, intent, and context of the application. Therefore, combining these general recommendations into an ethical and implementable programme requires contextualising these best practices with the experienced, socio-political dependencies and consequences.

2.2.4.3 SOCIO-POLITICAL CONSEQUENCES OF TARGETING THE WASTE INSTEAD OF THE WATER

Long-term sustainability of WBS requires assessing and addressing the potential socio-political consequences of this technique. In addition to addressing these issues promptly and fully, the process is strengthened by performing these reviews openly and inclusively. Seeking this public awareness and participation at a supranational, regional, national, subnational and local scale when incorporating WBS into public health practice enables framing wastewater surveillance successfully within a broader social discourse on water, waste and public health, overcoming the possible stigmatisation of a specific social group when analysing such issues, and guarantee not only a socio-political acceptance of WBS but also the elusive political continuity required by any policy. As has been recognised by the European Union, the United Nations, the Organisation for Economic Co-operation and Development (OECD) and the World Bank, (Akhmouch, A. & Romano, O., 2018) effective communication must take place first on the local community level to truly involve the Quintuple Helix, the intersection and overlap of academia, industry, government, media and civil society, and the environment (Carayannis & Campbell, 2010). This signifies the active instruction and subsequent involvement of the public, private, research, cultural and, above all, citizen sectors of a local community with an emphasis on the inclusion of all genders, age groups, income groups, and ethnic minorities.

The evolution of the common perception of wastewater from an unpleasant by-product to be disposed of into a source of information to be monitored to benefit society as a whole, although not new, transforms wastewater into a new social compact. Maintaining the necessary public consent during this conceptual development demands a concerted effort to not only fully inform a local community about the consequences and benefits of WBS, based on proven scientific fact, but to disseminate such information in a truly accessible manner. For example, the development of awareness and the growth of public interest cannot depend on the passive attendance by members of the general public to a single conference or workshop, where occasionally, a citizen can pose a question to a local expert. Thoughtful accessibility requires multiple modes of citizen contact, including cultural and artistic means that can provoke an emotional response and general understanding, no matter the previous knowledge of the target audience. The overall communication strategy must be designed to lead to that most ambiguous of political concepts: engagement. Engagement is the opportunity for elected

politicians to move from a representative to an open form of governance. It permits, at a local level, the channelling of public interest, itself the consequence of effective communication, into a process of co-creation, thus satisfying a demand which if not addressed, can rapidly mitigate citizen concern, public disaffection, or mistrust. An initiative which represents the opinion of interested members of a community capitalises on public awareness and transforms interest, supported by data and technical capacity into action. The Quintuple Helix, when united, is capable in the words of the United Nations Environment Programme Decision: 27.2 "... of activating a sense of common purpose on behalf of all sectors of society. The chances of forging such a sense of purpose will depend on the willingness of all sectors to participate in genuine social partnership and dialogue, while recognising the independent roles, responsibilities and special capacities of each" (https://www.un.org/esa/dsd/agenda21/res_agenda21_27.shtml).

Services work better when designed and delivered in partnership with citizens. Listening to stakeholders' insights can foster innovation in practices and enhance public trust in both the methodology and the results. As the OECD affirmed, greater social involvement in ostensibly technical programmes permits one to "...legitimise government actions and set a foundation for successful policymaking and implementation, thus allowing a focus on medium- and long-term planning" (Akhmouch, A. & Romano, O., 2018). The capacity of the successful employment of WBS must be maintained. Continuity is an essential feature of all effective water policymaking, and the surveillance of wastewater is no exception. Therefore, the citizen who has become aware of the value of WBS, who has developed an interest with regards to the repercussions for public health and who has contributed their ideas to the practical completion of a programme must also be engaged in the implementation of such an initiative. Not from the analytical perspective but as an overseer of a programme's continuity and as a channel of communication and dissemination to their own community and beyond. Socially and politically, the level of acceptance will always be more elevated if the community itself has been involved in its evolution. The scientist and researcher benefits from a higher probability of continuity, which is not exposed to the changing whims of local party-political strategies.

If applied with a certain degree of pragmatism and flexibility, adjusting to the nuances of the community in question, then the socio-political benefits of such an approach can be notable. As supranational agencies recognise the gaps in their own capacity to monitor and survey, they have, in the examples where this approach has been applied, observed an improved relationship between local community implementation and international necessity. Whether these campaigns were top-bottom or bottom-up in nature, they remained dependent on the willingness of the individual politician or utility manager, scientist or administrator to embrace a more open form of decision-making. Politically, this type of approach is already being examined as a possible means of creating links between different communities in different regions who at a national level would perhaps not find common ground, but at a local level, when discussing wastewater and health, would actually be constructing the basis of an exercise in science or public health diplomacy. From individual municipalities can be born the national, regional, and international capacity to promote knowledge exchange, to support with one's experience, the adoption of WBS at a broader scale, and to replicate not only as the result of formal decrees or published academic papers but because of the testimony of ordinary citizens of local societies who have understood and benefitted from such measures. Social groups wary of being identified with specific diseases, citizens who distrust an authority's capacity for the maintenance of privacy and anonymity are more disposed to accept the word of their peer group than that of an outsider. The employment of WBS depends on it being owned by the community it seeks to inform and protect, and delivering this open-source report advances the flexibility of WBS, enhances the accessibility of the benefits and consequences of the techniques, and strengthens public health diplomacy across the European Union and beyond.

2.3 INTEGRATION OF SARS-COV-2 AND OTHER FORMS OF WBS

MASTER CHEF:

DESPO FATTA-KASSINOS

CREW:

DANIEL DEERE

The concept that wastewater could be used for the estimation of the consumption of chemical substances by communities was proposed for the first time by Daughton in 2001 (Daughton, 2001). Later, in 2005, Zuccato and others (Zuccato et al., 2005) were the first to implement the methodology of WBS to successfully quantify cocaine in wastewater with the aim to investigate its consumption on a community level.

2.3.1 ILLICIT AND OTHER NON-PHARMACEUTICAL DRUGS

According to Zuccato and others (Zuccato et al., 2005), "monitoring of illicit drug consumption would be very important for assessing the actual extent of this phenomenon and detecting changes in trends. A more realistic picture of local use patterns for the most common illicit drugs would also be needed to identify priority problems and plan selective countermeasures. The evidence-based approach, which is in principle adaptable to other illicit drugs, could be refined and further validated to become a general, rapid method to help estimate drug abuse at the local level. This approach, with its unique ability to monitor changing habits in real time, could be helpful to social scientists and authorities for continuously updated appraisal of drug abuse" (Daughton, 2001; Zuccato et al., 2005).

Since then, WBS and research on its methodology have been conducted in various areas globally. While the studies were initially focusing on illicit drugs, such as cocaine, amphetamines, cannabis, various other psycho-active substances, nicotine and alcohol, the methodology continued to expand and clearly proved its usefulness (Du et al., 2015; González-Mariño et al., 2020).

Many national and international organisations, like the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA, 2023) and the Australian Criminal Intelligence Commission (ACIC, 2021), employ WBS to routinely evaluate and report drug use.

2.3.2 POSSIBLE CONFLICTS BETWEEN PROGRAMMES

There may be a reluctance to support WBS for public health applications if WBS is perceived to have roles in monitoring people's personal, private, or criminal activities. The application of WBS for understanding pathogens such as SARS-CoV-2 is focused on public health and community good. In contrast, the use of WBS approaches for the monitoring of things such as the use of illicit drugs is directly related to providing information on criminal activities. There is the potential for such methods to be used, (or at least to be perceived by the public as being intended to be used), to inform criminal investigations; as 'spying' on the public and invading their privacy. Indeed, the English term 'surveillance' is partly synonymous with the term 'spying'. At its worst, this may even lead to persons choosing disconnection from, or reluctance to use, wastewater systems. There may be public opposition to WBS programmes focused on public health if those public health programmes and the participating agencies are also engaged in WBS for other purposes. Therefore, keeping WBS programmes that have primarily public health objectives fully independent of those with criminal intelligence objectives has the potential benefit of reducing the risk of public suspicion or rejection of the public health programmes.

2.3.3 PHARMACEUTICAL DRUGS

WBS can be used to provide information on the use of legally prescribed drugs. For instance, identifying the spatial patterns of metformin use offers new insights into the management of type 2 diabetes and the distribution among populations, for instance, and is one of the novel uses of WBS. In this work (Yang et al., 2022), a WBS strategy was used to estimate metformin use in various Australian populations, and the results were compared to conventional methods using survey and prescription data.

WBS was used in another study (F. Ahmed et al., 2020) to estimate the consumption of painkillers such nonsteroidal anti-inflammatory medications (NSAIDs) and opioids, and it was recently employed in a study by Rousis and others (Rousis et al., 2022) to determine the socioeconomic features of catchment areas. Correlations were examined between mass loads of the compounds determined and socioeconomic and demographic descriptors of the wastewater catchments derived from census data. Public health markers including, for instance, for cardiac arrhythmia, cardiovascular disease, and type 2 diabetes were identified in the wastewater. Amongst other interesting findings, these were positively correlated with descriptors including disadvantage in education, occupation, marital status, and income, and negatively

correlated with descriptors of advantage in education and occupation. This study showed that it is possible to establish links between sociodemographic data for populations, human behaviour and lifestyle, and public health biomarkers. Additionally, the study showed that WBS can pinpoint particular geographic regions and socioeconomic groups that might require more support than others.

2.3.4 WASTEWATER SURVEILLANCE FOR ANTIMICROBIAL RESISTANCE

Of particular importance is the capability of WBS to contribute to the understanding and possibly placing measures against one of the most critical issues of public health; that of antimicrobial resistance (AMR). The rising problem of resistance has resulted in AMR being declared as one of the biggest public health risks threatening societies and medicine in the 21st century (O'Neill, 2014). Increasing concerns over AMR have led to the establishment of the Global Antimicrobial Resistance Surveillance System (GLASS) in 2015 by WHO with the aim of sharing information on a global scale to strengthen data and aid decision-making on national and international actions (World Health Organization, 2018). According to the WHO (World Health Organization, 2018), it is recognised that there is a lack of systematic and objective sampling strategy, leading to selection bias when countries are reporting data and information on AMR, and also patient samples are typi-cally taken from those that have sought out medical care and hence might not be representative for a population.

It is already well established that environmental reservoirs can contribute to the emergence and spread of AMR, including wastewater discharges from cities, hospitals, and pharmaceutical industries. The establishment of methods for environmental AMR surveillance is encouraged by both the WHO and the Food and Agriculture Organization's (FAO) global action plans on AMR (World Health Organization, 2020a). Community sewage proved to be a useful medium for AMR surveillance of populations residing in urban areas as it provides the resolution of tracking sources of AMR from different waste streams within a community (Laccourreye & Maisonneuve, 2019). In this study, antibiotic resistance profiles of four opportunistic patho-gens were compared from three sewage sources: raw community sewage from two housing estates, a foreign workers' dormitory, and a pet centre. Hence WBS was proved to be not only a useful tool to understand emerging trends of AMR at community level and across communities, but can also provide the resolution of capturing AMR data from key sewage sources based on population demographics (Laccourreye & Maisonneuve, 2019).

Chapter 3

Health Sector Perspectives

3 HEALTH SECTOR PERSPECTIVES

3.1 FINANCIAL ASSESSMENT

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3.1.1 KEY INGREDIENTS

- **Programme objectives** (objectives, envisaged duration)
- **Collection of protocols of sampling, measurement and testing**
- **Inventory of existing and needed instrumentation**
- **Staff resources needed and related costs**
- **Overview on laboratory infrastructure needed**
- **Shipment costs**
- **Financial planning tools**

3.1.2 RECIPE

Performing a financial assessment for a wastewater surveillance programme for SARS-CoV-2 involves evaluating the costs associated with the programme determining its financial feasibility and sustainability, considering those against its benefits. Here is a list of necessary information and tools that should be collected and the reasons why they are needed:

1. **Programme Scope and Objectives**: Define the geographical coverage, population served, and specific goals of the wastewater surveillance programme to understand the scale of the financial assessment.
2. **Sampling and Testing Protocols**: Determine the frequency of sampling, the number of sampling sites, and the types of tests to be conducted. This will help estimate the costs for labour, materials, and laboratory analyses.
3. **Equipment and Supplies**: List the required equipment (e.g., autosamplers, refrigeration units) and consumables (e.g., sample containers, reagents) to calculate the initial and recurring costs.
4. **Personnel**: Identify the human resources needed, including their roles, numbers, and the level of expertise required. This will help estimate salaries, benefits, and training costs.
5. **Laboratory Capacity**: Assess the available laboratory infrastructure and any additional investments required to handle any increased workload.
6. **Data Management and Reporting:** Consider the costs of data management systems, software licenses, and personnel needed for data analysis and reporting.
7. **Transportation and Logistics**: Evaluate the costs related to sample collection, storage, and transportation to the laboratory.
8. **Overhead Costs**: Account for indirect costs such as utilities, facility maintenance, and administrative support.
9. **Contingency Funds**: Set aside funds for unexpected costs or emergencies that may arise during the programme.
10. **Funding Sources**: Identify current and potential funding sources, including government grants, private investors, and partnerships.
11. **Cost-Benefit Analysis**: Conduct a cost-benefit analysis to assess the economic value provided by the surveillance programme compared to the costs incurred. This benefit analysis should consider the potential costs of resulting from the lack of information associated with not implementing a wastewater surveillance programme
12. **Financial Planning Tools**:
    a. Utilise budgeting software or spreadsheets to organise and analyse financial data.
    b. Project cash flow to ensure that the programme has sufficient funds to operate over time.

13. **Regulatory and Compliance Costs**: Include any costs associated with meeting regulatory requirements or obtaining necessary permits.
14. **Stakeholder Engagement**: Factor in the costs of communicating with stakeholders, public outreach, and community engagement efforts. This also includes costs for participation in meetings and conferences.
15. **Training and Capacity Building**: Include the expenses for training personnel on new equipment, procedures, or software.
16. **Evaluation and Audit**: Budget for periodic evaluations or audits to ensure financial accountability and programme effectiveness.
17. **Risk Assessment**: Conduct a risk assessment to identify potential financial risks and plan for mitigation strategies.

By collecting this information and utilising appropriate financial tools, you can perform a comprehensive financial assessment that will help decision-makers understand the costs associated with implementing and maintaining a wastewater surveillance programme for SARS-CoV-2. This assessment will be critical for budgeting, securing funding, and ensuring the program's long-term viability.

3.1.3 TIPS

- **Plan with flexibility** and anticipate possible issues. The financial assessment may need to be iterative, adapting in response to feedback from potential funding partners or changing epidemiological priorities.
- **Seek synergies** with other wastewater surveillance applications in reach (one sample - many uses).



3.1.4 THE SCIENCE BEHIND

For understanding incidence and prevalence of infectious agents and targets at the community level, since wastewater surveillance can monitor trends in virus-circulation in whole populations whose wastewater discharges to a single sampling point (typically tens to hundreds of thousands of people), it is generally regarded as more cost-effective than testing individual persons. Obviously, wastewater surveillance cannot replace direct testing of individuals for the purposes of diagnosis and treatment, but it can serve as a population sample to monitor presence of and trends in virus circulation in that population. Several studies have reported on the costs of wastewater surveillance and compared these against the costs of collecting similar information from testing individuals.

Reagent costs were used to calculate order-of-magnitude cost to compare the cost-effectiveness of population surveillance via wastewater versus individual tests (Hart & Halden, 2020). If the total population of Germany (83 million) would need to be monitored via individual tests, the reagent costs would amount to €1.15 billion. It would also take huge testing resources and time. In contrast, all 9,636 wastewater treatment plants in Germany can be sampled and analysed in 1-2 days at a reagent cost of €133,000, or 0.012% of the cost required for clinical testing of the whole population. For speed and economy, surveillance of trends in SARS-COV-2 circulation in the population via wastewater heavily favours wastewater surveillance over clinical testing.

A study in Utah (USA) estimated the cost of weekly surveillance of the larger wastewater treatment plants (covering 79% of the population) to be 0.005 USD per inhabitant for urbanised areas (Weidhaas et al., 2021). For rural areas, shipment costs increased the estimated cost per inhabitant 20-fold, to 0.1 USD per inhabitant. Which costs were included in these cost estimations was not specified.

The World Bank report on wastewater surveillance in Latin America (Manuel et al., 2022) also states that the resource requirements for wastewater testing are typically much lower than for clinical testing. Sampling of smaller treatment plants would reduce the cost efficiency of the wastewater surveillance. For operating costs, using a cost of €280 per test, they estimate €0.3 per person per test for building surveillance (population around 1,000 people), €0.006-0.3 per person per test for sites in sewer catchments or small wastewater treatment plants (population 1,000-50,000) to €0.0003-0.006 per person per test for larger wastewater treatment plants (population 50,000-1,000,000). The report also estimates the costs of clinical testing in Latin America and the Caribbean, with

an average of 29 tests per 100 persons per year, and an average cost of €37 per test, as around €11 per person per year, clearly more than tenfold the costs of a wastewater surveillance programme.

Most of these cost estimates focus on operational costs of the laboratory, but do not include the (significant) costs of sampling, sample transport and laboratory equipment. Sampling costs depend on the type of sampling. Grab sampling is cheap (reusable sterile flask and labour costs of 15-30 mins), but not very representative, while composite sampling with autosamplers is more expensive, particularly for purchase of autosampling equipment (price €3,000 – 10,000 per sampler) and their maintenance. Passive samplers are cheaper alternatives for composite sampling (Schang et al., 2021a), but they do require additional analysis to understand the assay result. Transport costs largely depend on the distribution of the sampling sites, sampling frequency and distribution of laboratory facilities over the country. Most of the wastewater surveillance studies were conducted in settings where laboratory equipment for sample processing and testing were already available, and do not include costs for laboratory equipment and training of laboratory staff.

In addition to costs to generate data, wastewater surveillance programmes need information technology to manage the data and report these into public health surveillance systems. The set-up and operation of such information technology also requires funding. There are many examples of wastewater surveillance dashboards already available, which reduces development costs, but the integration in the national public health reporting system would still require some funds.

In a survey in Europe (European Commission, Joint Research Centre, 2021), several countries (Belgium, Hungary, Italy, Luxembourg, Portugal, Spain, the Netherlands and the UK) reported the running costs of wastewater surveillance at the intake of wastewater treatment plants. The authors extracted the average and range of costs for sampling, shipment and laboratory analysis (Table 1) and, assuming the laboratory to be equipped with adequate instrumentation and a sampling frequency of twice a week, summed these to annual running costs of ≈ €25,000 per wastewater treatment plant. The cost of implementing and maintaining a wastewater testing programme can vary 10-fold or more, depending on the spatial and temporal resolution selected. Differences existed between cost estimates of different countries, due to the differences in design of the surveillance system (sampling frequency), costs of shipment and logistics, type of sampling, salaries, and overhead models. Most of these countries were running a sentinel system targeted at the larger wastewater treatment plants.

Gawlik et al (European Commission, Joint Research Centre, 2021), used these data to make a crude estimation of the cost of an EU-wide wastewater surveillance programme, including sampling, transport, analysis and reporting of the data. If surveillance was focused on the wastewater treatment plants that serve more than 150,000 people (571 in the 27 EU member states, covering approximately 44% of the population of the EU 27), this would cost 571 x 25,000 = €14.3

Table 1. Cost estimates retrieved from EU Survey.

Cost type	Average (€)	Range (€)
Sampling and field work	140	50 - 450
Shipment	88	20 - 400
Laboratory analysis*	236	60 - 550

* Cost estimate by laboratory

Source: (European Commission Joint Research Centre, 2021)

million + around €1 million for the development of an EU-wide database and dashboard and the necessary activities on harmonisation and exchange for the first year of operation, so a total of €15.3 million for the EU 27, or €0.035 per inhabitant. If the 348 wastewater treatment plants in the EU 27 that serve between 100,000 and 150,000 people would be included in the surveillance programme, the coverage would rise to approximately 51%, and the cost would rise to €24 million[1] (€0.055 per inhabitant).
If the 4,853 treatment plants that serve 15,000-100,000 people were to be included, coverage would rise to approximately 67% and costs to €58.8 million (€0.134 per inhabitant), assuming the larger number of samples taken would lead to increases in laboratory efficiency and, therefore 2.5 times lower annual running costs (€10,000).

The World Bank report describes higher set-up and running costs[2] for a programme for the first year of a wastewater surveillance programme on the scale of a province (Ontario, Canada) of €0.63 per person per year (€9.3 million for 14.7 million people). For the national wastewater surveillance program of the Netherlands, where all wastewater treatment plants (>300) are sampled 3-4 times per week, the running costs are €0.86 per person per year (€15 million for 17.5 million people). The World Bank report also compares the resources required for clinical versus wastewater testing, recognising the required resources for clinical testing are proportional to the number of people tested and may lead to a very significant requirement of human (clinical staff) and laboratory staff, equipment and reagent resources to run a clinical surveillance programme, where the required resources for wastewater surveillance are proportional to the number of sites tested, requires much less municipal and environmental staff and environmental laboratory resources, which also fluctuate minimally compared to the resources for clinical surveillance.

In low-income settings, a joint study of researchers from Malawi, Nepal, UK and USA (Ngwira et al., 2022) estimated the cost of SARS-CoV-2 environmental surveillance pilot studies conducted in Blantyre, Malawi (80 sites), and in Kathmandu, Nepal (23 sites). Both pilots used grab sampling and sampled sites every other week. They included the costs of consumables, equipment, and salaries for environmental surveillance from sample selection until pathogen detection and overhead costs for the projects. For the Blantyre pilot site, the average cost[3] per sample ranged from €68 (84 samples per month) to €23 (336 samples per month). For the Kathmandu pilot, the cost per sample ranged from €161 (96 samples per month) to €110 (250 samples per month). The results showed efficiency gains from processing a larger number of samples per month. Human resource and consumables were the most significant components of these cost estimates. Human resources accounted for approximately 30-45% and consumables for approximately 40-50% of the cost when lower sample numbers were taken. In the schemes with more samples per month, the consumables cost share increased to approximately 70%. There were economies of sharing of resources with other laboratory activities. The cost per person in the catchment area per year ranged from €0.063 to €0.092 in Blantyre and €0.063 to €0.12 in Kathmandu. The study concluded that environmental surveillance may be a low-cost early warning signal for SARS-CoV-2 to complement other SARS-CoV-2 surveillance efforts.

The cost of sampling and testing samples from a subset of 210,000 residents of Addis Ababa that were connected to 14 wastewater treatment systems via the wastewater (€3,900) was compared to the cost of conducting a PCR test on each of the residents (€1,000,000 million). Recognising that low income countries face the twin challenges of high infectious disease burden and small health budgets, these authors compared the reported number of conducted COVID19 tests in the first half of 2020 per 1,000 population between low income countries, where they were extremely low: South Sudan (36), Ethiopia (43), Uganda (58) and Kenya (68), with middle and high income countries: South Africa (427), Italy (3,725), UK (7,372), USA (2,741) and Germany (1,560) (data from: (Hasell et al., 2020)). They conclude that in low-income countries, the capacity to detect and monitor SARS-CoV-2 in wastewater gives a better insight in the trends in SARS-CoV-2 circulation in the population at lower cost than clinical testing. These insights were key to the implementation of public health interventions, such as lockdowns, with high impact on economy and society, but in general more reactive than proactive. The authors (Ali et al., 2022) state “effective utilisation of

1 Cost estimate not given in the report but calculated using the same input data

2 To be able to compare studies, the costs have been translated from USD to Euro, using an exchange rate of 1,10

3 To be able to compare studies, the costs have been translated from USD to Euro, using an exchange rate of 1,10

wastewater surveillance of COVID-19 may minimise the large evidence gap between high- and low-income-countries without compromising the limited resources in low- and middle-income-countries".

Overall, cost estimates vary substantially, depending on costs for human resources, spatial and temporal resolution of the wastewater surveillance programme and the sampling and sample processing methods used and the costs noted in the above cited studies are summarised in Table 2.

Table 2. Cost estimates for the first year set-up and operation of a wastewater surveillance programme, indifferent settings and with different resolutions.

COUNTRY	SITES	GEOGRAPHICAL DISTRIBUTION	SAMPLING FREQUENCY PER WEEK	SAMPLING METHOD	€ PER ASSAY	€ PER PERSON PER YEAR	COST CATEGORIES INCLUDED
Malawi	80	City	0.5	Grab	23	0.063	Sampling, transport, analysis
Nepal	23	City	0.5	Grab	110	0.063	Sampling, transport, analysis
Canada	160	Ontario province	?	Composite autosampler	?	0.63	Set-up, sampling, transport, analysis, reporting
Netherlands	320	National	3-4	Composite autosampler	260	0.86	Set up, sampling, transport, analysis, reporting

Source: Malawi and Nepal (Ngwira et al., 2022); Canada and Netherlands (Manuel et al., 2022)

Table 3 provides an overview of the various cost categories that are relevant when setting up and running a wastewater surveillance programme, with the factors that primarily drive the costs and comments about potential for optimisation of these cost factors versus the public health needs.

Table 3. Factors that determine the cost of a wastewater surveillance programme

CATEGORY	COST FACTORS	COMMENTS
Sampling		
Equipment	• Type of sampling (composite autosamplers, passive samplers, and grab sampling) • Number of sites to be sampled • Sampling frequency	High costs for composite autosamplers, low costs for passive samplers, very low costs for grab samples. Composite autosamplers provide the most representative sample, which aids in data interpretation.

CATEGORY	COST FACTORS	COMMENTS
Equipment maintenance	• Type of sampling (composite autosamplers, passive samplers, grab sampling) • Number of sites to be sampled • Sampling frequency	Costs for composite autosamplers, no or low costs for (disposable) passive samplers or grab samples.
Labour	• Number of sites • Geographical distribution of sites • Travel time to sites • Sampling frequency • Type of sampling	Labour time of sampling is 15-30 mins per sample, regardless of the type of sampling. Travel time to sites will largely determine labour costs.
Transport		
Sample transport	• Number of sites • Geographical distribution of sites • Geographical distribution of laboratories • Travel time from sites to laboratory	Ability to group samples from different regional sites for transport to the central (regional) laboratory can significantly reduce transport costs.
Laboratory analysis		
Equipment	• Existing laboratory equipment • Feasibility to process environmental samples in the laboratory • Number of laboratories • Sample processing method	Limited costs if the laboratory is already experienced in the processing and analysing of viruses from wastewater samples.
Equipment maintenance	• Existing laboratory equipment • Number of laboratories • Sample processing method	Limited costs if laboratory is already experienced in processing and analysing viruses from wastewater samples
Staff training	• Existing staff expertise on qPCR and in processing of environmental samples	If the laboratory is already experienced in qPCR: costs for training of processing of wastewater samples
Reagents/kits/consumables	• Number of sites • Sampling frequency • Sample processing method • Laboratory staff experience	Different sample processing methods and kits, with different costs, are available.
Labour	• Number of sites • Sampling frequency	Cost optimisation of the number of samples per laboratory per week increases efficiency

CATEGORY	COST FACTORS	COMMENTS
	• Number of laboratories	
Quality assurance	• Number of laboratories	When multiple laboratories are engaged in analysis, interlaboratory comparison trials are needed
Data reporting		
Development of information technology/dashboard	• Existing public health data management system • Number of sites	Use of best practices/dashboards from wastewater surveillance programmes running elsewhere
Running costs (labour)	• Number of sites • Sampling frequency • Presence of Laboratory Information Management System (LIMS)	Optimise the frequency of updating dashboard: costs versus public health need. Automated updating is possible with LIMS.
Data interpretation	• Number of sites • Sampling frequency • Presence of dashboard and data on clinical testing system results • Presence of contextual data	• Automation of data analysis possible • Frequent interpretation is needed for public health/early warning • Contextual data relevant for identifying errors.
Programme management		
Set-up	• Public health use cases(s) • Number of sites • Number of stakeholders • Pre-existence of public health data management system	Engagement and mobilisation of water and public health sector stakeholders needed. Can be time consuming to convince stakeholders but improves use and sustainability
Operations	• Public health use case(s) • Number of sites • Sampling frequency • Level of automation • Level of troubleshooting	Frequent engagement with stakeholders for public health uptake and response

Source: Developed by the authors for this document.

3.2 GOVERNANCE AND COMMUNICATION

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3.2.1 KEY INGREDIENTS



The governance of a wastewater surveillance programme for SARS-CoV-2 or any other pathogen involves collaboration between various stakeholders and partners to ensure the programme's effectiveness, efficiency, and compliance with relevant policies and regulations. The specific stakeholders and partners can vary based on the context and location, but generally, they include:

- **Public Health Authorities:** National, regional, and local health departments that oversee public health initiatives and respond to infectious disease outbreaks.
- **Environmental Agencies:** Governmental bodies responsible for environmental protection and regulation, including the management of sanitation systems and water resources.
- **Water and Wastewater Utilities:** Entities that manage the collection, treatment, and disposal of wastewater, as well as the supply of clean water.
- **Academic and Research Institutions:** Universities and research centres that contribute scientific expertise, conduct analyses, and develop new methodologies.
- **Laboratories:** Facilities equipped to test wastewater samples for the presence of SARS-CoV-2 (and other pathogens where relevant), providing data for surveillance.
- **Government Officials and Policymakers:** Elected officials and policymakers who allocate resources, enact regulations, and provide oversight for public health initiatives.
- **Non-Governmental Organisations (NGOs):** Organisations that may support the programme with expertise, funding, advocacy, or by facilitating community engagement.
- **Community Representatives:** Stakeholders representing the interests and concerns of the communities served by the wastewater surveillance programme.
- **Healthcare Providers:** Hospitals, clinics, and other healthcare facilities that can use wastewater data to inform clinical decision-making and resource allocation.
- **Epidemiologists and Public Health Experts:** Professionals who analyse surveillance data to inform public health responses and understand disease trends.
- **Private Sector Partners:** Companies that may provide technical solutions, laboratory equipment, or data management services.
- **Emergency Management Agencies:** Entities responsible for coordinating responses to public health emergencies and disasters.
- **Regulatory Bodies:** Organisations that set standards for wastewater testing and ensure quality control and safety.
- **Funding Agencies:** Governmental and private entities that provide financial support for the programme.
- **Citizens and Local Communities:** Engaging with the public to inform them about the programme and its benefits, and to maintain transparency and trust.

Effective governance of a wastewater surveillance programme requires clear communication, defined roles and responsibilities, and a collaborative approach among all these stakeholders. Regular meetings, data sharing agreements, and a coordinated response strategy are essential components for the success of such a programme.

3.2.2 RECIPE



Assessing the use cases of a wastewater surveillance programme and designing a proper governance structure involves a series of steps that ensure the programme is tailored to meet specific public health needs and operates within a framework that encourages collaboration, accountability, and efficiency.

This process requires careful planning and coordination among all stakeholders. Here are some key steps to undertake:

ASSESSING POSSIBLE USE CASES

1. **Identify Public Health Needs**: Determine the key public health challenges that the wastewater surveillance programme aims to address, such as tracking the spread of SARS-CoV-2 (and potentionally monitoring for antimicrobial resistance genes, or other targets).
2. **Evaluate Current Surveillance Capabilities**
   a. Review existing surveillance systems and identify gaps that wastewater surveillance could fill.
   b. Assess the limitations of current approaches, such as individual testing, and the potential benefits of adding wastewater surveillance.
3. **Consult Stakeholders**: Engage with public health officials, epidemiologists, water utility managers, and other stakeholders to understand their perspectives on the utility of wastewater surveillance for various use cases.
4. **Analyse Potential Impact**: Consider how wastewater surveillance data could complement other data sources to improve public health responses, resource allocation, and policy decisions.
5. **Review Case Studies**:
   a. Look at other jurisdictions where wastewater surveillance has been implemented.
   b. Analyse the outcomes, challenges encountered, and lessons learned.
6. **Determine Feasibility**: Assess the technical feasibility, including the availability of laboratory capabilities, logistics of sample collection, and the necessary infrastructure for data management and reporting.
7. **Cost-Benefit Analysis**: Conduct a cost-benefit analysis to compare the investment needed for wastewater surveillance against the potential public health and economic benefits. (See also section 3.1).
8. **Set Priorities**:
   a. Based on the assessment, prioritise use cases that offer the most significant benefits.
   b. Align with public health goals.

DESIGNING A GOVERNANCE STRUCTURE

1. **Define Leadership and Oversight:** Establish clear leadership and oversight bodies, such as a governing board or steering committee, with representation from key stakeholder groups.
2. **Articulate Roles and Responsibilities**: Clearly outline the roles and responsibilities of each stakeholder within the programme, ensuring all necessary functions are covered.
3. **Develop Collaborative Agreements**: Create formal agreements, such as memorandums of understanding or contracts, that specify the terms of collaboration, data sharing, and confidentiality.
4. **Establish Decision-Making Processes**: Define processes for decision-making, including how decisions are made, who is involved, and how disagreements are resolved.
5. **Implement Regulatory Compliance**: Ensure the governance structure adheres to relevant laws, regulations, and ethical guidelines, including those related to privacy and data protection.
6. **Create Communication Channels**: Set up effective communication channels for internal coordination among stakeholders and for disseminating information to the public.
7. **Incorporate Quality Assurance**: Implement quality assurance measures across the programme, including standardised procedures, training, and periodic audits.
8. **Set Up Data Governance**: Establish data governance policies that cover data collection, storage, analysis, sharing, and reporting. Define data ownership and access rights.
9. **Plan for Emergency Response**: Integrate the programme into broader emergency response and public health surveillance frameworks to enable swift action when surveillance data indicate a public health threat.
10. **Monitor and Adapt**:
    a. Regularly review the governance structure for effectiveness.
    b. Adapt as necessary to address evolving challenges.
    c. Incorporate new insights.
11. **Sustainability Planning**: Ensure long-term sustainability by planning for ongoing funding,

stakeholder engagement, and integration into public health infrastructures.

12. **Evaluation Metrics**: Develop metrics to evaluate the performance of the governance structure and the impact of the wastewater surveillance programme.

By methodically assessing use cases and carefully designing a governance structure, you can establish a wastewater surveillance programme that is responsive to public health needs and operates with transparency, accountability, and effectiveness.



3.2.3 THE SCIENCE BEHIND

From a national policy and governance structure for a wastewater surveillance programme through close collaboration between participating stakeholders and partners.

PUBLIC HEALTH AGENCIES

For a national programme this will generally be the Ministry of Health, with (if in place) support of a national institute of public health. For local programmes this could also be local health departments. The Ministry of Health should lead and coordinate the WBS programme and have overall responsibility. Public health authorities should investigate whether a WBS programme is feasible and appropriate in the national context and where it will be of added value in public health decision-making. As these agencies also have the data and data infrastructure for public health surveillance, they should also be responsible for documenting and analysing surveillance data and interpreting the WBS data in light of the other public health information available, to provide the best possible information to support decision-making by policymakers.

WASTEWATER/SANITATION UTILITIES/OPERATORS

The wastewater/sanitation utilities are important stakeholders in a WBS programme as they have information on wastewater catchment areas, (variation in) flows and how they relate to residential locations of populations and to public health districts. Within a wastewater surveillance programme the wastewater sector is responsible for providing this information to support the selection of the best monitoring sites, collecting and transporting wastewater samples to be analysed and providing necessary information on wastewater systems. Wastewater utilities may already have automated composite samplers at the wastewater treatment plant for sampling for their operational parameters; such autosamplers are also very useful for representative samples in a WBS programme. Also, information to normalise the measured concentrations of viruses or other health targets for the (variable) influx of rainwater, snowmelt, groundwater, industrial wastewater, construction water etc. into the sewers is critical for the improved interpretation of the data if the use case involves understanding trends. Sampling and/or sample transport can also be (partially) outsourced to dedicated (commercial) parties. Arrangements need to be in place for collecting the necessary contextual data about the sewer network and also about the actual situation at the time of each sampling (such as wastewater flows, rainfall, sampling quality assurance etc.), and for reporting these results either directly to the public health agency or via the testing laboratories, together with the results of their assays on the sample.

TESTING LABORATORIES

This could either be environmental laboratories that are well equipped and trained in molecular methods for virus and other assays if relevant (qPCR, sequencing) or clinical laboratories that are experienced with these methods and are equipped and trained with methods to process wastewater samples. The existence of commercial kits[4] for processing wastewater samples for

[4] Please note that the European Commission's Joint Research Centre does not endorse or promote any specific commercially available kits mentioned herein. These examples are provided solely for informational purposes to illustrate the types of resources available to laboratories.

virus assays, such as Promega SARS-CoV-2 RT-qPCR Kit for Wastewater | SARS-CoV-2 Variant Detection Kit (promega.com), Bio Rad SARS-CoV-2 / COVID-19 Wastewater Testing | Bio-Rad, and several other suppliers can support the laboratories. Procurement and supply chain continuity of sample processing and virus assay consumables is an important element of a WBS programme. Changes in materials or kits as a result of supply issues may result in different virus recoveries and hence assay results.

The role of testing laboratories is to perform the sample processing (purification and concentration), nucleic acid extraction and (RT)PCR assay (quantitative or digital PCR) and potentially also (amplicon) sequencing. Laboratories should have sufficient skill, organisational capacity, and equipment and consumables to perform the assay. They are responsible for calibration of the method and performing (and reporting) the necessary quality assurance and controls. Good agreements need to be in place about sample receipt, test turn-around times and reporting of results to the public health agency. If multiple laboratories are/need to be involved in the WBS programme, interlaboratory proficiency testing prior to the WBS programme being formalised is warranted, to ensure all participating laboratories are able to perform the sample processing and assays and quality controls adequately. This requires a (ideally independent) dedicated reference laboratory that can arrange the interlaboratory testing. A reporting structure needs to be agreed upon with the laboratories for reporting their results, along with contextual data on the wastewater sample (see above) in a similar format and timeliness to the public health agency. Ideally, this would be a digital reporting system for rapid, consistent and smooth transfer of the results to one central data location.

INFORMATION MANAGEMENT, DATA ANALYTICS AND COMMUNICATION

Data that is collected and generated during design, sampling and assays need to be stored in databases and/or information management systems. As surveillance systems require rapid evaluation, data needs to be collected, reported, documented and interpreted in a time frame that supports public health actions. This means as soon as possible. The practice in countries with experience with the WBS programme is that it takes generally 2-3 days between the day the sample is taken and documentation of the results at the public health agency, where they can be interpreted alongside other public health surveillance data and information. The communication of the WBS programme results from the (national) public health agency to the regional and local public health agencies could (and ideally should) generally flow through the channels that are in use for communicating public health/disease outbreak information between these agencies, such as weekly epidemiological reports, and/or via digital information dashboards. The latter may also serve as information/communication source for decision-makers and for the public. Since wastewater surveillance is novel, communication of the data to health agencies, decision-makers and the public should be accompanied by clear explanations of what can (and cannot) be seen from the data. It is possible to mine appropriate explanatory text from the 176 existing communication dashboards from many countries (COVIDPoops19 Dashboard | COVID19wbec.org).

The experience of many countries that have embarked on wastewater surveillance is that mining wastewater for health data is atypical for authorities, public health agencies and wastewater utilities. For setting-up and running a successful and valuable WBS programme, the stakeholders need time and engagement to experience how to interpret the data and how to extract added value out of wastewater surveillance.

Testing laboratories in research institutes with experience in virus testing of wastewaters in a public health context (such as the public health institutes in the Netherlands, Finland and Hungary) had the advantage of the experience in wastewater data collection, reporting and interpretation in the public health context. This helped in creating a good infrastructure and interpretation of WBS programme. Also, in countries or regions where such institutes were not present, engaged public health agencies working in close collaboration with wastewater utilities and testing laboratories have shown very good set-up and public health use of the WBS programme.

3.3 HIGH-LEVEL TACTICAL SAMPLING STRATEGY



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This section outlines the considerations and necessary elements for developing a high-level tactical sampling

strategy for a WBS programme, particularly in the context of monitoring SARS-CoV-2 and potentially other pathogens or substances. An analysis of the key components and steps required for such a strategy are given below. This strategic approach allows for a structured and flexible surveillance system capable of adapting to the evolving landscape of COVID-19 and other public health threats.

3.3.1 KEY INGREDIENTS



- **Country-Specific Goals and Objectives:**
  - Determine the public health policy goals.
  - Assess local conditions and available resources.
- **Scope and Scale of the Programme:**
  - Define the extent and coverage of the surveillance.
  - Plan for adaptability as information evolves.
- **Evaluation Plan:** Establish criteria for assessing the programme's design and implementation.
- **Sentinel Sites:** Identify strategic locations for monitoring, such as large cities, hospitals, and airports.
- **Sampling Frequency and Locations:** Decide on the frequency or triggers for sampling and the diversity of sites to cover different scenarios.
- **Analytical Methods:** Choose appropriate laboratory techniques for detecting the presence of SARS-CoV-2 (and other targets if relevant), quantifying viral load, and identifying variants.
- **Data Normalisation and System Information:** Standardise data for comparisons and understand the wastewater system dynamics.
- **Additional Targets for Surveillance:** Consider including other targets, such as infectious agents or substances like drug residues.
- **Cost-Effectiveness:** Evaluate the financial implications and strive for cost-effective methods

3.3.2 RECIPE



1. **Design Process Initiation:**
   a. Set the specific goals and objectives of the WBS programme based on the country's needs.
   b. Frame the key questions that the programme aims to answer.
2. **Early Warning Strategy:**
   a. Implement a system for detecting the introduction of SARS-CoV-2 or variants.
   b. Monitor sentinel sites and scale-up monitoring if needed.
3. **Pandemic Tracking Strategy:**
   a. Estimate trends in SARS-CoV-2 circulation for high prevalence settings.
   b. Use quantification of the genetic signal to assess trends.
4. **Post-Pandemic Tracking Strategy:**
   a. Detect rapid re-emergence and prepare public health responses.
   b. Adjust monitoring efforts based on the indications of new pandemic waves.
5. **Viral Evolution Tracking Strategy:**
   a. Detect and analyse Variants of Concern (VOC), Variants of Interest (VOI), and Variants Under Monitoring (VUM).
   b. Sample centralised facilities or sentinel sites and interpret data in conjunction with public health case data.
6. **Incorporation of Additional Surveillance Targets:**
   a. Assess the added value and feasibility of monitoring other diseases or substances.
   b. Adapt the design for each additional target in terms of data interpretation, sampling, and analytical methods.
7. **Implementation and Evaluation:**
   a. Execute the designed surveillance strategy.
   b. Continuously evaluate and adjust the strategy based on the preliminary evaluation plan and the data obtained.

This strategic approach allows for a structured and flexible surveillance system capable of adapting to the evolving landscape of COVID-19 and other public health threats.



3.3.3 THE SCIENCE BEHIND

The primary goal of any wastewater surveillance programme is to inform public health policy decisions and to reduce the transmission of disease. Country-specific goals and objectives can however vary depending on local conditions and resources. The first step in the design process is to decide on country-specific goals and objectives of the WBS programme, which strategy to adopt and to frame the questions that the programme needs to answer (WHO, 2022). This helps determine the scope and scale of the wastewater surveillance programme to be designed, although insights may change during the design process or as more information becomes available. It is advisable to also setup a preliminary evaluation plan so that it is clear how the WBS programme design and implementation will be assessed and evaluated.

The potential objectives and strategies for a WBS programme for SARS-COV-2 are summarised below.

Early warning of SARS-CoV-2 introduction. The main focus is to be able to detect introduction of SARS-CoV-2 in the population. Detection of the of SARS-CoV-2 genetic signal in wastewater (presence/absence) is of importance rather than quantification. This is relevant in no prevalence settings, to confirm absence of virus circulation or warn about introduction of the virus. Monitoring sentinel sites (larger cities) at centralised wastewater treatment plants is most efficient for this strategy. In areas where this is not possible (such as in remote or rural communities) sentinel or at-risk sites such as hospitals, health clinics, care facilities, emergency camps or airports can be monitored to detect first indications of a possible outbreak. Monitoring efforts can be scaled up as necessary if indications of an outbreak are found, for example by sampling more frequently and/or at more locations. It is possible to run this strategy at relatively low costs.

Pandemic tracking of SARS-CoV-2 prevalence in a community. This strategy is used to estimate trends in the circulation of SARS-CoV-2 in a community and to determine whether infection rates are increasing, decreasing or stable. This strategy is mostly adopted in high prevalence settings and focused on the general population, although also specific subpopulations can be targeted. It generally provides a more unbiased indication than people testing, because of (changes in) willingness and/or access to testing. Downward trends can provide confirmation of the effectiveness of management interventions. New peaks can provide early warning of re-emergence. Quantification of the genetic signal is important within this strategy to support the assessment of trends in SARS-COV-2 prevalence. Data normalisation and information on the wastewater system are also more important for this strategy and it is therefore considered more advanced.

Post-pandemic tracking of SARS-CoV-2 prevalence in a community. In the post-pandemic period, SARS-CoV-2 is still circulating, but people testing is scaled down and self-tests are available but rarely used or reported. The traditional surveillance methods turn 'blind' as very little people testing/reporting occurs. In this setting, WBS programme still provides insight in the trends of the SARS-COV-2 circulation, the main focus is to be able to detect a rapid re-emergence in the occurrence of SARS-COV-2 in the population and to prepare and focus a public health response. Monitoring sentinel cities' centralised wastewater treatment plants is most efficient for this strategy. Monitoring efforts can be scaled up or down as necessary if indications of a new pandemic wave are found, for example by sampling more frequently and/or at more locations. It is possible to run this strategy at relatively low costs.

Viral evolution. This strategy focuses on detecting new emerging variants of SARS-COV-2 , called Variants of Concern (VOC), Variants of Interest (VOI), or Variants Under Monitoring (VUM). Variant classification serves as an important communication tool for alerting countries about the emergence of SARS-CoV-2 variants with concerning properties likely to impact the epidemiological situation. For example, to assess their potential impact on vaccines, therapeutics, and diagnostics, their transmission and severity of disease as well as to evaluate their circulation throughout the population between and within countries. Generally, centralised treatment facilities or sentinel sites (such as airports) are sampled. The analytical approach used in the testing laboratories is significantly different to routine wastewater surveillance. These data need to be interpreted in conjunction with reported case data from public health agencies. This strategy is considered the most advanced.

Another consideration to be made during the design phase of a WBS programme is whether simultaneous surveillance of other targets than SARS-COV-2 is of added value. With the surveillance infrastructure in place and the samples in the laboratory, it is relatively easy and cost-effective to add other infectious disease agents (such as influenza A and B, Respiratory Syncytial Virus) and genetic targets (such as antimicrobial resistance genes). Wastewater monitoring to address epidemiological questions has been used to track enteric viruses and other pathogens (Global Water Pathogens Project (GWPP)), including the poliovirus vaccine and wild type strains (Hovi et al., 2012), norovirus, adenovirus, and other pathogens(W. Ali et al., 2021), as well as antimicrobial resistance (Hendriksen et al., 2019), and drugs such as opioids (Burgard et al., 2014) and illicit stimulants (SCORE network – https://score-network.eu/, (Huizer et al., 2021; Thomas et al., 2012). More targets are being added through ongoing scientific research (Choi et al., 2018). Each target will require some degree of adaptation to the design, either in interpretation of the data, sampling, and/or analytical methods. It should therefore be considered what the added value is in relation to the additional costs and effort required.

3.4 THE ROLE OF SEWAGE SURVEILLANCE IN PUBLIC HEALTH SURVEILLANCE

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Public health surveillance is an essential ingredient in maintaining community health and well-being. Among its many tools, sewage surveillance has risen as a powerful method for early detection and intervention in the face of public health threats. This chapter offers a practical guide to integrating sewage surveillance into public health practices, following a framework influenced by the WHO's Essential Public Health Operations (EPHO) and One Health principles.

The chapter explores the key 'ingredients' required, provides a step-by-step 'recipe' for implementation, and offers 'tips' for optimising this surveillance method. From sample collection to data interpretation and policy action, this chapter serves as a concise manual for health professionals navigating the complexities of sewage surveillance to protect public health.

3.4.1 KEY INGREDIENTS



- **Established Public Health Frameworks:** Familiarity with WHO's Essential Public Health Operations (EPHO) and One Health principles.
- **Surveillance Infrastructure:** Access to wastewater collection systems, treatment facilities, and sampling sites.
- **Laboratory Capacity:** Equipment and reagents for pathogen detection, including PCR machines, genomic sequencing tools, and related laboratory supplies.
- **Data Management Tools:** Software for data collection, management, and analysis, such as GIS systems and data visualisation dashboards.
- **Interdisciplinary Collaboration:** A network of professionals spanning public health, environmental science, virology, epidemiology, and water management sectors.
- **Communication Channels:** Mechanisms to disseminate information to stakeholders, policymakers, and the public, including social media, press briefings, and scientific reports.
- **Funding and Resources:** Sustained financial investment for programme establishment, operation, and research.
- **Policy and Legal Frameworks:** Guidelines to ensure privacy, ethical considerations, and compliance with regulations.

3.4.2 RECIPE

1. **Preparation of the Infrastructure:**
   - Identify and map key sampling points within the wastewater network.
   - Establish laboratory protocols for pathogen detection and quantification.
   - Train personnel in sample collection, processing, and data management.
2. **Data Collection:**
   - Implement a routine schedule for wastewater sample collection.
   - Transport samples to laboratories while preserving integrity for analysis.
   - Extract and amplify genetic material to detect target pathogens.

Data Analysis and Synthesis:

   - Analyse the data using statistical and bioinformatic tools to identify trends and variants.
   - Integrate wastewater surveillance data with other public health data sources.
   - Synthesise findings into actionable intelligence for health authorities.

3. **Communication of Findings:**
   - Develop clear reports and visualisations to convey surveillance results.
   - Communicate insights to relevant stakeholders and policymakers for timely action.
   - Engage with the public to inform on risks, prevention measures, and reassure where appropriate.
4. **Response and Action:**
   - Coordinate with health agencies to align surveillance findings with public health interventions.
   - Inform healthcare system preparedness, including hospital capacity and resource allocation.
   - Adjust public health strategies in response to early warning signals from wastewater data.
5. **Feedback and Optimisation:**
   - Continuously gather feedback from the system's stakeholders to improve surveillance strategies.
   - Invest in research and development to refine methods and expand pathogen detection capabilities.
   - Review policy frameworks and adapt as needed to ensure the programme's sustainability and effectiveness.

3.4.3 TIPS

- **Flexibility in Sample Collection:** Be prepared to adjust sampling frequency and locations based on epidemiological needs or in response to specific events (e.g., outbreaks).
- **Quality Control:** Implement rigorous quality control measures at each stage of the process to ensure data reliability.
- **Stakeholder Engagement:** Maintain open lines of communication with all involved parties, including the public, to build trust and enhance collaboration.
- **Continual Learning:** Stay abreast of scientific advancements in wastewater surveillance to incorporate the latest techniques and technologies.
- **Public Education:** Utilise wastewater surveillance data as an opportunity to educate the public on broader public health issues and the importance of preventive measures.



3.4.4 THE SCIENCE BEHIND

3.4.4.1 CONTEXT OF KNOWLEDGE MANAGEMENT IN PUBLIC HEALTH, INCLUDING ONE HEALTH SURVEILLANCE

The World Health Organization (WHO) defines ten "Essential Public Health Operations" (EHPO) for the health policy context and standard in Europe (https://who-sandbox.squiz.cloud/en/health-topics/Health-systems/public-health-services/policy/the-10-essential-public-health-operations). This WHO guidance was adapted to form the basis of this chapter. Based on the WHO definition of sustainable health and human well-being, the core areas are defined as health protection, health promotion, disease prevention and information – fed by information of surveillance, monitoring and (public health) intelligence.

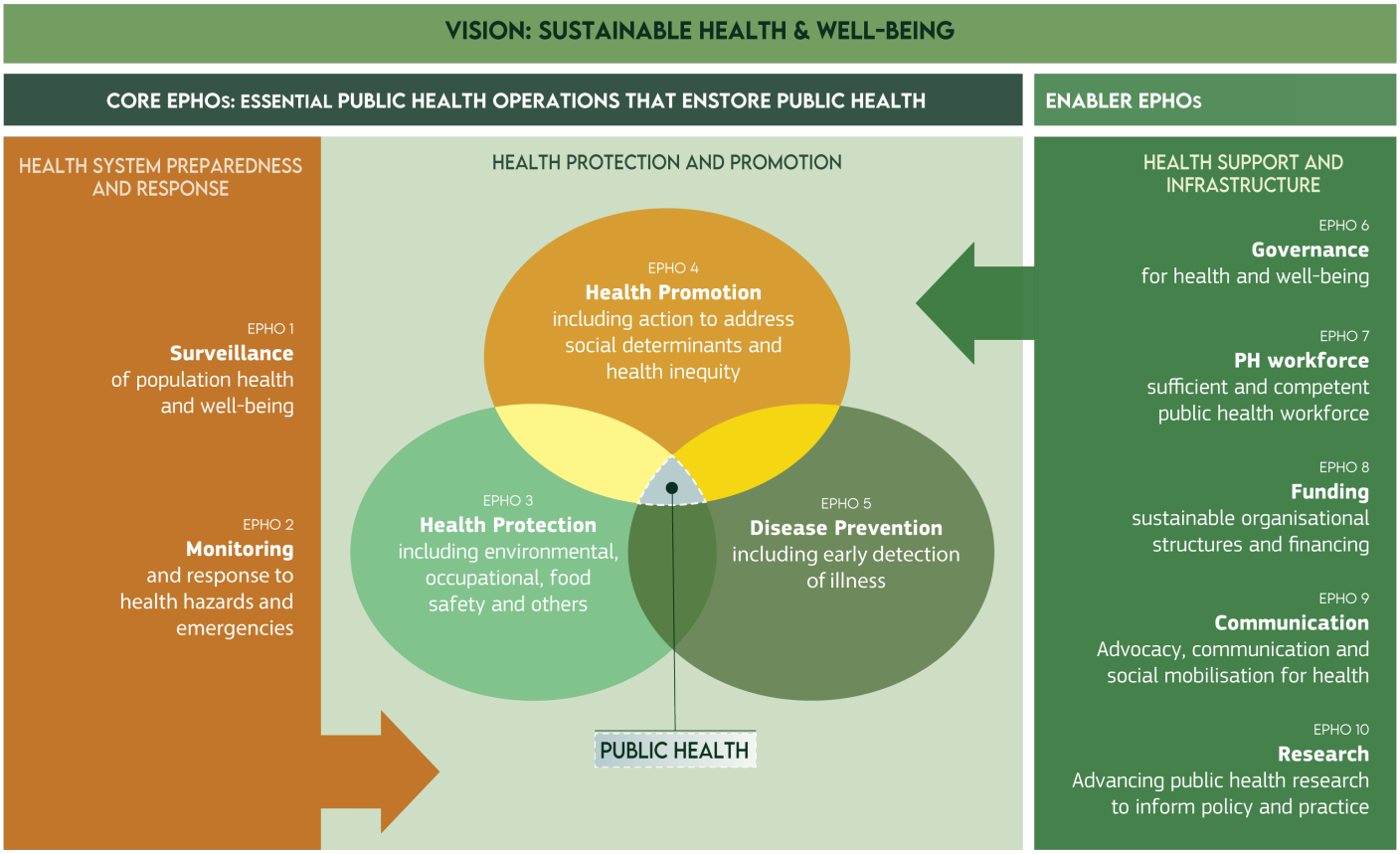
In this context, political will, personnel, financing, communication, and research are important basic requirements for successful implementation of public health programmes, so-called "enablers" (Figure 1). How could Knowledge Management, especially in crisis situations, be framed based on this WHO EPHO-Framework?

The framework depicted in Figure 2 was therefore developed.

Information – as data base for Public health action – is based on different sources, different processes and different methodologies of collection. Differentiated should be Surveillance as well as Public Health/ Medical Intelligence.

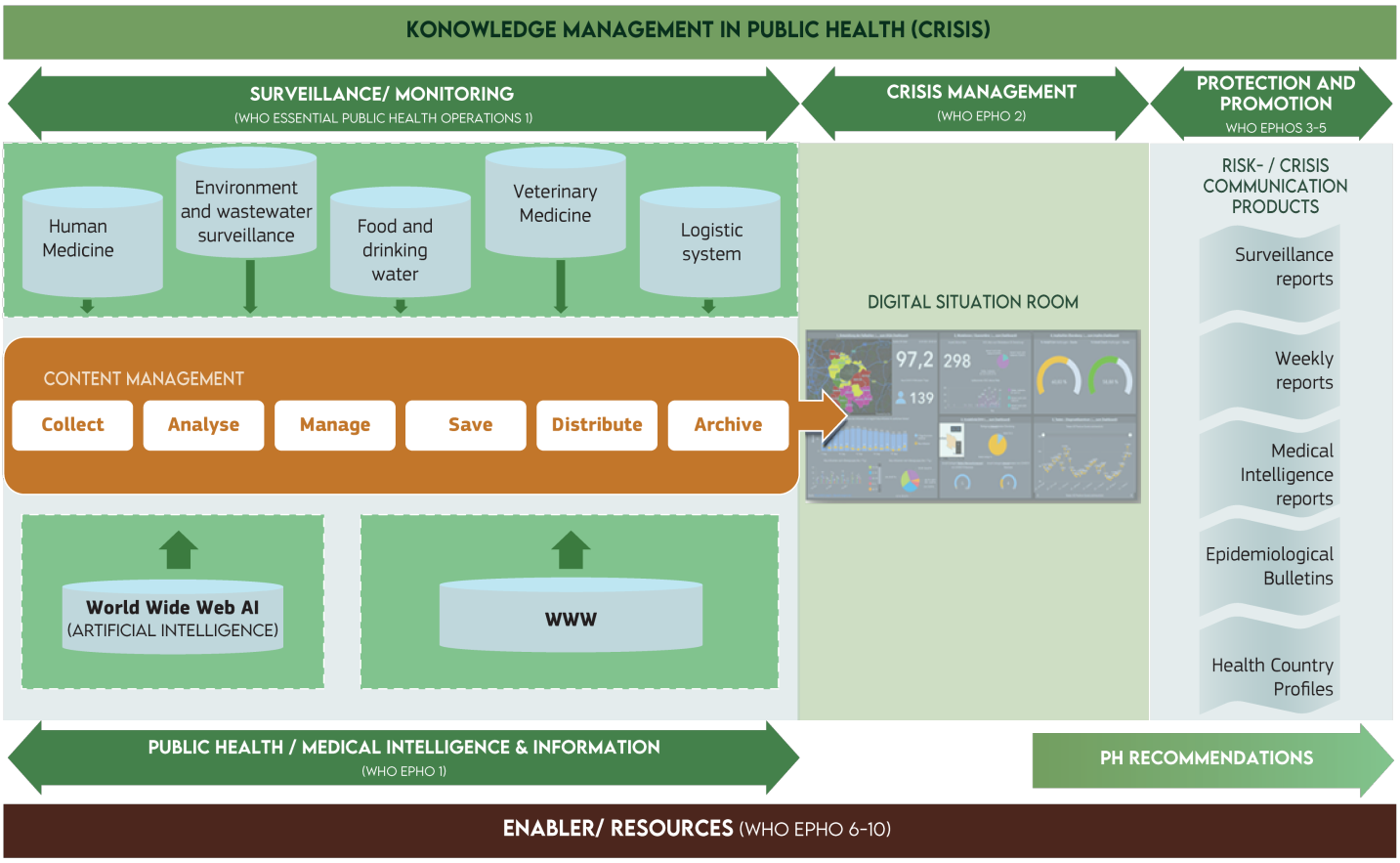
Surveillance/monitoring (WHO EPHO 1): An ongoing process of collecting specific data, preferably including laboratory-confirmed or quality-assured information in

Figure 1. Simplified diagram of the convergence model



Source: https://who-sandbox.squiz.cloud/en/health-topics/Health-systems/public-health-services/policy/the-10-essential-public-health-operations (redesigned)

Figure 2. Tailoring of the WHO EPHO framework to incorporate WBS



Source: A. de Roda Husman unpublished (redesigned)

the spirit of a holistic One Health approach, from the following sources:

- Human medicine, e.g. individual medical outpatient or pre-clinical anonymised or pseudonymised data of patient care or laboratory data according to the German Protection against Infection Act (IfSG), population medicine-oriented data for influenza surveillance.
- Genome sequencing of dangerous pathogens and georeferenced assignment.
- Molecular and epidemiological analyses of antibiotic resistance.
- From the environmental sector, e.g. air and wastewater monitoring, but also radiation measurement data, climate forecasts and weather data.
- From sources of food such as drinking water or even drug safety
- Georeferenced database of dangerous fauna and flora such as vectors, e.g. mosquito register or tick atlas as well as an appropriate rodent monitoring for potential zoonotic diseases.
- Veterinary medicine, e.g. animal disease news or zoonosis data, antibiotic resistance development especially in the livestock sector, but also in the luxury animal sector.
- Last but not least, data from logistics systems, such as antibiotic consumption for antibiotic stewardship, would be necessary to incorporate.

Public Health Intelligence (WHO EPHO 1): Unlike the "Surveillance/Monitoring" data collection, this area contains global information from non-proprietary sources and includes:

- Open internet searches, databases and other sources;

- Results from artificial intelligence (algorithm-based partial/automated searches).

The entire collected information should be visually processed and presented e.g. in dashboards and digital maps for situation rooms in order to optimise crisis management according to WHO EPHO 2.

For the purpose of WHO EPHOS 3-5 (Health Protection, Health Promotion and Disease Prevention), risk/crisis communication products, such as Surveillance reports, Epidemiological Bulletins, Health Risk Analysis/Medical Intelligence reports, Health Country Profiles should be generated which lead to public health measures and action.

Since "data is the basis for action" standardised processes need to be implemented – therefore we developed a framework to start the technical discourse of experts. Without a sufficient situation picture – as the basis of knowledge management – no meaningful and legally permissible decisions can be made on prevention or intervention in the event of (health) crises that have already occurred or will occur in the future.

3.4.4.2 CONTEXT - GENERAL BENEFITS TO THE HEALTH AGENCY – WHY IS HEALTH LEADERSHIP SO IMPORTANT? HOW IS THE HEALTH SECTOR LEADING? WHO ARE THE ACTORS?

A wastewater surveillance programme serves to protect and promote the health of the population. In the opinion of the chapter authors, it should be the public health agencies that lead design and adaptation of the programme to serve current and emergent public health needs, optimise use of complementary clinical and wastewater surveillance systems, and be the end user of results to inform public health decisions. Coordination of all the essential stakeholders is also critical including an interdisciplinary collaboration involving central and decentralised public health actors as well as environmental sampling, laboratory and water utility professionals from the water and sanitation sector.

If there are one health benefits extending to animal or environmental health, other relevant experts should also be included in the interdisciplinary collaboration.

The most important stakeholders in any country's infectious disease surveillance landscape will be context specific, including leadership from national, subnational and decentralised local public health agencies and the lead health agency. They receive and combine surveillance data to enable health care providers, regulators, and policymakers, to decide upon appropriate measures. Intended beneficiaries including those at risk or vulnerable to the infectious pathogen and increased morbidity and mortality are also important stakeholders to consider and engage.

For wastewater surveillance to be efficient, effective, and acceptable, key stakeholders should be engaged in setting up and implementing any wastewater surveillance. In the case of COVID-19 existing infrastructures for public health surveillance and infectious disease prevention and control were in place for influenza and other relevant public health threats to varying extents. Apart from limited polio surveillance and small research projects there was little if any infrastructure to support wastewater surveillance at the onset of the COVID-19 pandemic in 2020. Because of the pandemic threat on lives and livelihoods, there was urgency for additional useful population level tools such as wastewater surveillance to complement existing clinical surveillance tools and there has been rapid development and use of wastewater surveillance for SARS-COV-2.

The COVID-19 experience has provided diverse country experiences in the leadership and coordination of wastewater surveillance and the critical insight that while water sector engagement is essential, maximum value is achieved when programmes are led by public health with effective interdisciplinary collaboration.

The value of the programme in early detection and containment or other beneficial public health actions and/or reassurance of absence of circulating pathogens without causing harm should drive the sustainable funding of a regional, national, or subnational programme. There are also cost efficiencies for additional pathogens, for example a core SARS-CoV-2 wastewater surveillance programme can be leveraged, with collected samples also being tested for other relevant pathogens, such as poliovirus, monkeypox virus and influenza virus, or stored and tested for an emergent as yet unknown threat as part of pandemic preparedness.

3.4.4.3 EARLY DETECTION OF THREAT(S)

It became clear early on in the COVID-19 pandemic that wastewater surveillance could provide geographically defined, population level detections of SARS-CoV-2 viral fragments with large population coverage and with short turnaround times. Moreover, the virus could be detected in wastewater before or in the absence of

clinical symptoms and whether or not the infected individuals sought testing. It has been shown to be a leading indicator of cases or case waves prior to cases being diagnosed in many locations around the world at different geographic scales. This application is relevant to any pathogen which: a) poses a public health threat; b) is not normally present endemically in the population; c) is shed into wastewater whether through urine or faecal excretion or washing (e.g. from skin/fomites) or nasopharyngeal or respiratory secretions; and d) is detectable with a current or newly developed diagnostic assay.

3.4.4.4 RELATIONSHIP TO PUBLIC HEALTH SURVEILLANCE – LINK TO HEALTH PROMOTION IN THE PANDEMIC

WBS is best conceptualised as a complementary tool to other sources of information, such as various clinical surveillance systems, surveys, and qualitative intelligence. All have strengths and limitations. A notable challenge for the global COVID-19 response is the declining case ascertainment which limits visibility of infection trends and availability of PCR samples for genomic surveillance. WBS can provide different information relevant to the context; this ranges from early detection where no virus had been present (now relevant only in localised settings for the most part), quantitative trends showing increase, peaks and declines, and variant surveillance showing incursion, spread and changes of variants, lineages and sublineages and which may include airports/ports and population centres.

There has been a rapid evolution with innovation and growing scientific knowledge of the use, applications, and limitations of WBS which is much documented in preprints as well as peer-reviewed publications and grey literature. One of the main challenges lies in interpretation of results including consideration of current knowledge, limitations and complementary data from other sources to provide intelligence and inform public health decisions. A further challenge has been in providing information to the public and key stakeholders in an accessible and understandable way to inform direct individual and community level health promoting behaviours.

3.4.4.5 PLANNING FOR HOSPITAL CARE REQUIREMENTS

Early warning of case surges or waves through quantitative trends and/or the spread and relative growth of variants is in health system planning. This can be used directly or through modelling to inform health care and workforce planning including hospital bed and intensive care unit (ICU) capacity and alerting public of potential limitations to other health care services such as elective surgery. As emphasised previously waste-water surveillance data would often be triangulated with or complemented by other data sources. This requires trust of stakeholders such as hospital management, health care professionals, Ministry of Health (MOH) and government decision makers.

3.4.4.6 VARIANTS OF CONCERN

Over time at high virus replication rates in large groups in the population including those who are vulnerable in the case of SARS-CoV-2 led to the emergence of new strains. Such novel strains can have a different transmission pattern and pathogenicity. Data from wastewater surveillance may show aberrations making them more difficult to interpret by stakeholders. This is halted when a new variant takes over and trends over time and geographically can again be understood and inform public health policies.

3.4.4.7 VACCINATION

Another challenge was the lack of vaccines for the new SARS-CoV-2. Development has never been that rapid as well as the roll-out of the vaccination campaigns. After vaccines were developed and distributed rapidly, the patterns of infection, and consequently, the data from wastewater surveillance, began to change. This disrupted the previously established trends, making it difficult to interpret the surveillance data. The trend data from wastewater surveillance as well as other indicators such as hospitalisations entered a new dynamic, which affected clear interpretation. For example, a decrease in the virus detected in wastewater could be a result of successful vaccination efforts rather than a natural decline in cases. To be able to recalibrate and then again use the data for public health policies requires a certain time period. And stakeholders must regain their trust in the wastewater surveillance outcomes for COVID-19.

3.4.4.8 FROM LOCAL TO GLOBAL - HOW TO OBTAIN THE BIG PICTURE IN VIEW OF PANDEMIC MANAGEMENT

Wastewater surveillance can contribute to knowledge of pathogen prevalence and infectious disease burden. Local stakeholders such as health nurses or clinics may be better prepared for certain infectious disease outcomes if sufficient resources are available. Moreover, stigma may be avoided that can arise from individual clinical testing among certain vulnerable groups. At a regional level, it may be more clear which health care provisions are most needed because of complemented human surveillance data. Wastewater surveillance may inform national health care facilitators such as policymakers at the MOH on vaccination programmes such as for polio and on the required stewardship for antimicrobial resistance. And last but not least at the global level, governments can prepare for ensuing pandemics that demand different levels of governance in crisis situations.

3.4.4.9 ONE HEALTH CONTEXT

Since wastewater surveillance is directed to human sewage, domestic wastewater treatment plants are largely included in the surveillance strategy. Most of the waste is therefore human and not animal waste. In systems where wastewater is collected separate from rainwater and other runoff waters there may be a small fraction of animal waste from e.g. dogs and wild birds. When virus variants are analysed this waste of multiple origin may hamper variant calling. On the other hand more and more experience is gathered on identifying novel cryptic SARS-CoV-2 lineages not recognised in the Global Initiative on Sharing All Influenza Data's (GISAID) EpiCoV database (Smyth et al., 2022). This is relevant with respect to emerging SARS-CoV-2 variants that may be of concern for causing a new wave and/or vaccine escape.

Chapter 4

Methods and Methodologies- From data to action

4 METHODS AND METHODOLOGIES – FROM DATA TO ACTION

4.1 COMMUNICATION AND ENGAGEMENT

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DOUGLAS MANUEL

This chapter focuses on the importance of effective communication and engagement in wastewater surveillance programmes. It emphasises the need to define the target audience, purpose, data requirements, and communication methods. The chapter highlights the various uses of WBS data and the need for tailored communication strategies for different target groups. It also discusses the importance of ensuring that all stakeholders understand the capabilities and limitations of WBS. The chapter provides examples of effective communication practices and emphasises the need for regular updates, contextualisation of results, and collaboration with relevant authorities and experts.

4.1.1 KEY INGREDIENTS

- **Target Groups:** Identify who will be consuming the information, such as health authorities, decision-makers, and the general public.
- **Purpose:** Clarify why the information is important – for risk assessment, management, or communication.
- **Data Context:** Understand what data is needed and the context it sits within.
- **Communication Channels:** Select how to reach different target groups effectively, including press releases, social media, and direct communication.
- **Transparency:** A clear method for regularly updating and sharing information.
- **Contextualisation:** Techniques for putting results into context to ensure they are meaningful and interpretable.
- **Trend Analysis:** Methods for focusing on and communicating significant trends and alerts.
- **Responsiveness:** Systems for communicating results quickly and efficiently.
- **Engagement:** Forums and methods for engaging with those who use the data.
- **Collaboration:** Working with key stakeholders such as epidemiologists and statisticians.
- **Significance:** Strategies for communicating the importance and relevance of the data.

4.1.2 RECIPE

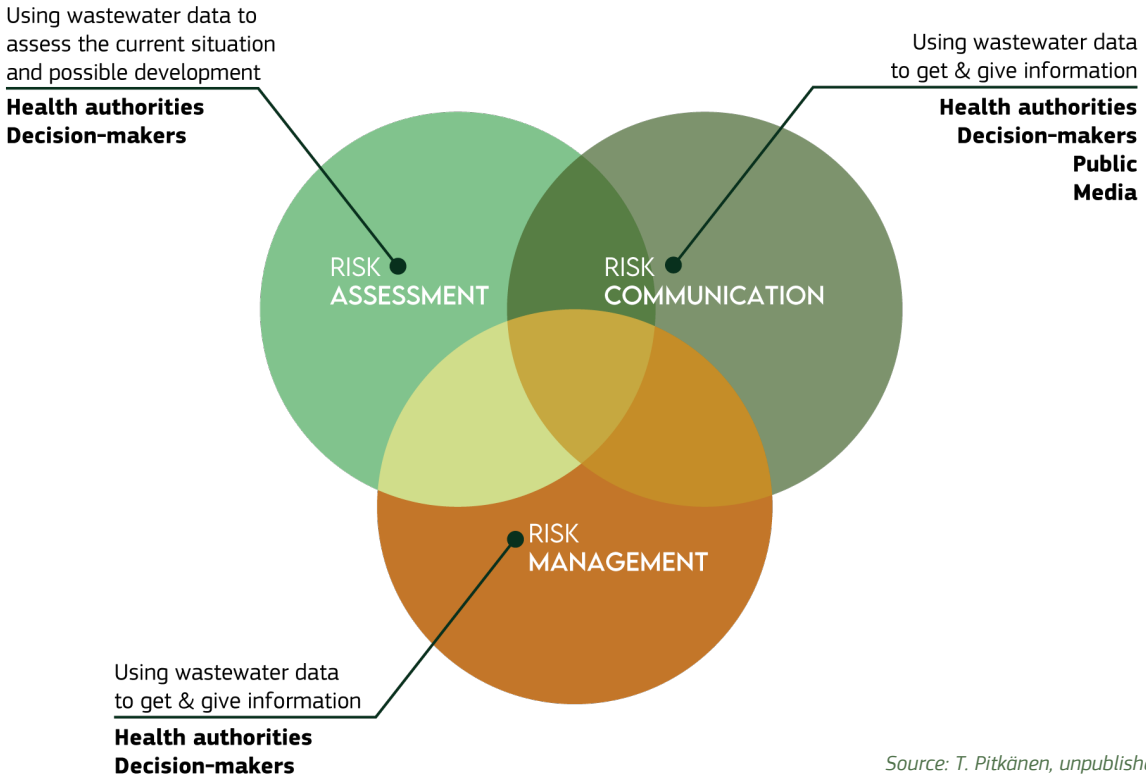
1. **Defining the Diners:** Identify the **who** – your audience. Make a list of all potential stakeholders ranging from health authorities to the general public. Understand their needs, preferences, and the best ways to serve them the information they require.
2. **Crafting the Menu:** Clarify the **why** – the purpose of your communication. Is it to inform, to warn, or to guide? Each goal may require a different approach and tone in the message.
3. **Selecting the Ingredients:** Determine the **what** – the type of data and context needed. Just as a dish requires the right balance of ingredients, ensure the data you present has relevance and is comprehensive.
4. **Cooking Techniques:** Decide on the **how** – the channels for reaching your audience. Will it be through a press release, an interactive website, or direct email? Choose the method that aligns with your audience's habits.

5. **Seasoning Transparency:** Regularly update your website and communication materials. Transparency is key to maintaining trust and credibility. Provide a clear update cycle and make data accessible.
6. **Flavourful Context:** Put results into context. Like a dish that needs explanation, provide visualisation and clear explanations to illustrate trends and make the data digestible.
7. **Sensing the Trends:** Focus on the trends and alerting results. Use your 'Wastewater Epi curves' to serve up meaningful insights that can guide public health actions.
8. **Fast Food or Fine Dining?** Be prepared to serve the results quickly, especially in emergencies. Fast communication can be critical. Ensure you have up-to-date contact information for key players.
9. **Table Discussions:** Engage with users in various forums. Regular meetings and discussions can enrich the understanding and application of the data served.
10. **Acknowledging the Kitchen Staff:** Remember and appreciate the role of the wastewater plant personnel. Their contribution is like that of line cooks in a kitchen – essential for the final product.
11. **Recipe Sharing:** Collaborate and communicate with epidemiologists and statisticians to ensure a comprehensive and comparative data analysis.
12. **Savouring the Significance:** Communicate the significance of your data clearly and transparently. Offer frequent updates and be available for questions to enhance the flavour of trust.

4.1.3 TIPS

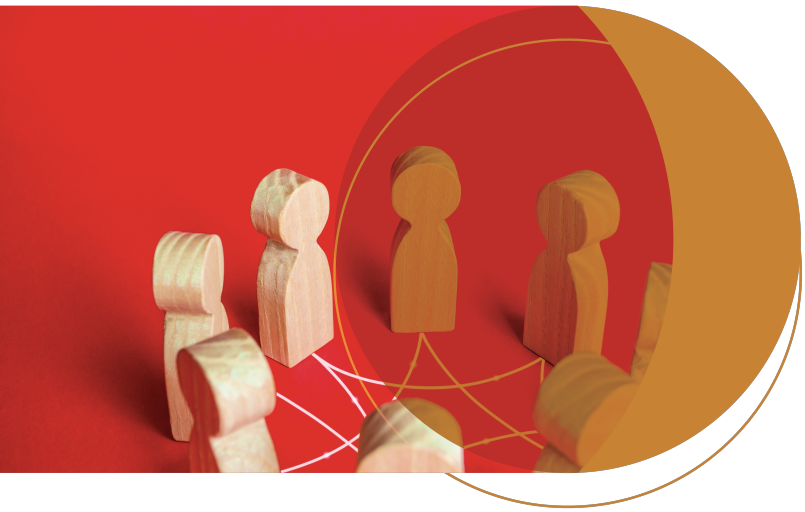
- **Presentation is Key:** Just as plating is important for a chef, visualising data in an understandable way can make a significant difference in how it is received.
- **Taste Test:** Before fully rolling out your communication strategy, test it with a small group to gather feedback.
- **Fresh Ingredients:** Keep your data and contacts up to date to ensure the information you serve is always fresh and relevant.

Figure 3. A masterfully crafted dish of communication and engagement



Source: T. Pitkänen, unpublished. (redesigned)

- **Listen to Your Diners:** Be open to feedback and ready to adjust your strategy based on the needs and responses of your audience.
- **The Secret Sauce:** Collaboration is the secret sauce in this recipe. Engage with all stakeholders to create a well-rounded and effective communication strategy.



4.1.4 THE SCIENCE BEHIND

4.1.4.1 MAKE SURE EVERYONE KNOWS WHAT THE WASTEWATER-BASED SURVEILLANCE IS AND WHAT IT ISN'T

It is important that the information from the wastewater surveillance is understood by all target groups in order to be used for the different goals. Make sure they understand both the possibilities and limitations of wastewater surveillance. For example, what are the main messages that can be drawn from wastewater results about the current situation of the outbreak? Does it bear predictive power? How long does it take before the reductions in the infection rates are seen in the wastewater data?

Therefore, it is important to communicate, for instance, how long the virus can be detected in the samples, whether one can be infected via wastewater or how the surveillance measures the amount of the virus in a large population rather than a singular family or person.

As an example, the very first press release (Tarja Pitkänen, 2020) in Finland about WBS published by the Finnish public health institute that conducts the national wastewater-based SARS-CoV-2 surveillance in the country, attracted much more attention on social media than expected. With a Facebook account followed by a few tens of thousands, the institute's first post about surveillance reached more than 800,000 people. That is approximately 50% of active Facebook users in Finland. This interest was mainly because people thought that bathrooms and tap water could be contagious. Only later did the scientific community produce information about the survival of SARS-CoV-2 and its infectivity in untreated sewage and the institute was then better able to communicate the risk assessment results to the public (Tarja Pitkanen, 2021).

4.1.4.2 UPDATE YOUR RESULTS FREQUENTLY AND MAKE SURE ALL THE DATA IS AVAILABLE

A clear webpage on which the wastewater surveillance data and information are regularly and preferably automatically updated is the backbone of all communication actions. Website update cycles must be communicated transparently. To promote a regular information flow, the link to the webpage could include a short update on key findings each time the page is updated. Dissemination could be through direct emails, social media, newsletters, and press releases.

4.1.4.3 PUT THE RESULTS INTO CONTEXT

As part of effective communication, the results should be put into context – what exactly do the latest results show? Can certain trends be identified? The use of different channels for the message delivery is usually handled by the communication offices of the organisations responsible for the surveillance.

The number of RNA copies found in a sample by itself does not really tell the public or the health authorities and decision-makers much. Therefore, it is recommended to visualise the results to show trends and context (Tarja Pitkanen, 2021). Depending on the current risk situation, the result must be analysed in detail by experts continuously or at longer intervals. These results should be prepared and communicated in an understandable form, including regular press releases.

4.1.4.4 MAKE THE FOCUS ON THE TRENDS AND THE ALERTING RESULTS

Beyond the so-called 'Wastewater Epi curves', i.e. the variation in concentration of the RNA fragments over time in the areas sampled, communicating interpretable results and alerts, to the expert assessing the epidemiological situation, is of importance. In Belgium, a complementary alerting system was developed in order to make the results more understandable and to facilitate the objectivity of the alerts given to the decision-makers. This alerting-system is based on three distinct indicators, two of them screening for the increasing trends (short and long), and one screening for the viral circulation level. Both the trends and the viral circulation level are therefore screened twice each week and officially reported every week to the expert assessing the epidemiological situation and the decision-makers (Raphael Janssens et al., 2023).

4.1.4.5 BE PREPARED TO COMMUNICATE THE RESULTS QUICKLY

To be able to react quickly, the information from wastewater surveillance is particularly important for the health authorities, such as infectious disease doctors at national, regional or hospital/campus level, who are responsible for the pandemic preparedness in the area where the WBS is conducted. They are usually the main target group for the communication measures. It is important to know the exact emergency contacts – who is responsible for what; this information must be always kept up to date. This priority group should receive the results of the wastewater surveillance without delay once the samples have been analysed. In practice, it has been shown that often the most efficient way for a quick information transfer is personal emails with the result figures as an attachment. Where necessary, also a phone call can be useful, especially if a sudden increase in the SARS-CoV-2 levels in wastewater have been detected. It is essential that the wastewater surveillance team has valid email addresses and phone numbers of the responsible persons to ensure a quick flow of information if needed.

Once a week is usually (e.g. Belgium) the frequency at which the epidemiological situation is re-assessed. A central point (instance) where to communicate the results of the WBS facilitates the necessary resulting communication flow. Ideally, to trigger the resulting communication flow, for risk management or broader communication purposes, that central point where to communicate is also reached by the weekly results of the other indicators of health. In Belgium, that central point is the Risk Assessment Group (RAG), which then notably decides on the communication to make to the Risk Management Group (RMG), the authority taking the decisions regarding the measures to implement to better manage the situation. This was done routinely, during the time of pandemic management, on a weekly basis.

4.1.4.6 ENGAGE WITH THOSE WHO USE THE DATA ON VARIOUS FORUMS

The communication actions with health authorities should be complemented by regular (virtual) meetings to present and discuss not only current results from wastewater surveillance, but also other surveillance results and any changes that might be needed in a pandemic response. Such events are usually organised by a pandemic preparedness task force and could involve different stakeholders from health authorities and national and regional administration. It is important that a representative from the wastewater surveillance team attends such meetings to help interpret the results and answer any questions about the wastewater findings.

During pandemic COVID-19 periods, information needed to be adapted very quickly to the changing situation. This required a high alert level of all actors involved in COVID-19 surveillance. One aspect of communication needs is the flow of information between analytical laboratories and scientists. To meet information needs, it can be helpful to offer or participate in training and exchange opportunities, such as webinars or events. All this refers to local, national and international levels.

4.1.4.7 REMEMBER THAT SURVEILLANCE WOULD NOT BE POSSIBLE WITHOUT WASTEWATER PLANT PERSONNEL

In many cases, the actual sampling and sample shipping is done by wastewater treatment plant personnel and the organisations responsible for the sanitation systems, so their work should not be overlooked. Communication about the sampling programme, sending information via newsletters, for example, and acknowledging the valuable participation of staff responsible for sampling are all part of positive public relations for the WBS and might improve the collaboration.

4.1.4.8 COLLABORATE AND COMMUNICATE WITH EPIDEMIOLOGISTS AND STATISTICIANS

Public health surveillance experts responsible for reporting of cases, hospitalisations and deaths are key collaborators of the wastewater surveillance team. It is essentially important to ensure a comprehensive data flow for comparative purposes – WBS is an additional tool to supplement the information obtained from clinical surveillance. The purpose of this communication is to create a common understanding of the situation and interpretation of the surveillance results. It is often beneficial to report the wastewater results together with clinical surveillance results, for example by presenting the SARS-CoV-2 RNA concentrations along with the new laboratory confirmed COVID-19 cases in a figure. Because clinical surveillance and environmental surveillance results are often produced by different disciplines and stakeholders, bringing the two data sources together adds real value.

4.1.4.9 COMMUNICATE THE SIGNIFICANCE OF YOUR DATA

In a nutshell, the key to success is clear, transparent communication of wastewater results. Updates should be offered as frequently as possible, e.g. once a week as a report on the website. In addition, contact with the lead scientists should be offered and they should be available to answer questions from the media, stakeholders, and the public.

It is important that the wastewater team be involved in the interpretation of the results and that health authorities in the geographic region of interest (country, region, city, etc.) also be involved. While the situation at different geographical scales is assessed through wastewater surveillance, when particular areas with alerting trends are noted, the results can be highlighted and both reasons behind and available control measures can be planned. An important aspect is the open access to the results. It is usually perceived very positively when the raw surveillance data have been made available.

Perhaps most importantly, however, wastewater surveillance teams can embed their results into the national and/or local surveillance reports that compile the pandemic situational updates. In Finland, for example, the Ministry of Social Affairs and Health and the Finnish Institute for Health and Welfare organised a weekly public press briefing every Thursday at 10 a.m., explaining in detail the contents of the weekly COVID-19 surveillance report (https://www.youtube.com/watch?v=ZwycIQTl8no). The latest results of the WBS were part of those briefs and reports. Everyone could follow these events on TV and YouTube, and the most important news was published in newspapers (https://www.hs.fi/kotimaa/art-2000008563480.html), for example.

Another important aspect to remember is the commitment of policy-makers and funding organisations. This is essential to ensure that resources remain available for WBS. Environmental surveillance as a whole is not (yet) a priority in the health sector and its importance must therefore be continually reaffirmed in order to remain viable until the necessary long-term resources to conduct wastewater analyses are secured. However, further steps are needed to include wastewater findings into international situational reports such as COVID-19 reports by the European Centre for Disease Prevention and Control (ECDC) or WHO.

4.2 DATA QUALITY, DOCUMENTATION, AND USE

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This chapter explores the critical components of data documentation, quality, and usage within the context of WBS. The rapid expansion of WBS, both geographically and in the array of pathogens monitored, necessitates a rigorous approach to data handling. The complexity of analyses, coupled with the diversity of data collection environments, underscores the importance of establishing standardised practices for data quality and documentation.

This chapter serves as a guide to navigating the multifaceted challenges of WBS data, ensuring that it is not only accurate and reliable but also accessible and actionable for stakeholders.

4.2.1 KEY INGREDIENTS



- **Metadata:** structured information that describes the characteristics of the data collection. Metadata codifies the attributes essential for understanding and analysing the underlying data.
- **Data Quality Attributes:** Criteria that define the integrity and applicability of your data, similar to the selection of choice ingredients for a dish. These criteria are explained in section 4.2.4.3
- **Quality Assurance and Quality Control:** Standardised procedures and practices to ensure the WBS system operates effectively, minimising errors from sample collection to data analysis.
- **Usage Rights and Authorisations:** Policies and principles governing data management, access, and re-use to protect communities and ensure ethical data usage.
- **Data Provenance:** Documented history of data, detailing its origins, processing steps, and the rationale behind its collection and transformation.
- **Data Integration:** The harmonisation of wastewater data with other health surveillance data to enhance overall insights into public health trends.
- **Data Governance and Management:** The overarching framework and processes that ensure data is created, maintained, and utilised effectively and efficiently.

4.2.2 RECIPE



1. **Define Metadata.** Start by meticulously documenting the metadata, which includes the who, what, where, and why of your data. This vital step ensures that anyone who encounters the data understands its origin and context.
2. **Assess Data Quality Attributes.** Evaluate your data against the established quality attributes. Aim for data that is timely, credible, complete, valid, reliable, and reasonable, while also adhering to standards for ease of sharing and comparison.
3. **Implement Quality Assurance and Control.** Integrate quality checks at each stage of data collection and analysis, from sample handling to final reporting.
4. **Define Usage Rights and Authorisations.** Develop clear data policies that outline the management, access, use, and re-use of data, ensuring protection and ethical considerations.
5. **Maintain Data Provenance.** Maintain a detailed record of data origins and processing steps to support transparency and trustworthiness.
6. **Integrate Data.** Combine wastewater data with other health surveillance data to expand its value. Facilitate easy integration through the use of standard formats and clear geographic definitions.
7. **Refine Data Governance and Management Plans.** Regularly review and improve data governance and management practices. This involves setting up review cycles, assessment strategies, and maintaining quality metadata and documentation.
8. **Present Data Accessibly.** Share your data in a way that is readily accessible to both humans and machines, prioritising clarity, open access, and respect for privacy and ethical standards.

4.2.3 TIPS



- **Utilise Existing Frameworks:** Leverage established surveillance and data system evaluation frameworks to guide the creation of data quality attributes.
- **Maintain Flexibility:** Given the dynamic nature of WBS, remain adaptable in your data documentation and quality assurance practices to accommodate new methodologies.
- **Prioritise Transparency:** Ensure all data processes are transparent to foster credibility and trust among stakeholders and the public.
- **Embrace Open Science:** Where appropriate, support open access to data, balancing the need for transparency with privacy and ethical considerations.
- **Engage Communities:** Involve communities in the design and usage of WBS data, particularly vulnerable groups, to ensure their interests are represented and protected.
- **Ensure Interoperability:** Promote data interoperability across different regions and disciplines within WBS to facilitate comprehensive analysis and insights.



4.2.4 THE SCIENCE BEHIND

4.2.4.1 DATA DOCUMENTATION

Data documentation defines the who, what, where and why of data. Collectively, these data attributes are called metadata. Metadata is defined as 'data about data", or data that provide information about other data (Kirch, 2008b). Metadata describes data, but it isn't data itself. Metadata implies that data documentation is coded, organised, and machine-actionable.

4.2.4.2 DATA QUALITY

Data quality is defined as the degree to which data meets its use (Donabedian, 1988). Data quality depends on its use and the criteria applied to it (Donabedian, 1988; Iezzoni, 1997; World Health Organization, 2003; Wyatt & Sullivan, 2005)

Given the many uses of WBS data, there are also varying degrees of quality required. Data quality is a process that spans the data life cycle from collection to analysis. An effort is required to ensure documentation is maintained during data transformation and transmission to others.

4.2.4.3 ATTRIBUTES OF DATA QUALITY

Surveillance and data system evaluation frameworks have a range of attributes that are related to data quality and use (Calba et al., 2015; Centers for Disease Control, 2004; Centers for Disease Control (CDC), 1988; Chen et al., 2014; German, Horan, et al., 2001; German, Lee, et al., 2001; Groseclose & Buckeridge, 2017; Manuel et al., 2022; Paunović, 2008; World Health Organization, 2006). Good data management - discussed later - creates processes to ensure data is collected and maintains these data qualities.

Data quality attributes for WBS include:

- **TIMELINESS:** The time between any two steps in the surveillance process, from data collection to its use by all stakeholders. The timeliness of data within WBS may vary depending on what is being surveilled and its use. SARS-CoV-2 is highly infectious within a short generation time. Wastewater monitoring of SARS-CoV-2 can be used for detecting outbreaks and waves because people shed the virus early during their infection. However, for this use the surveillance system needs to be timely.
- **CREDIBILITY** (trustworthiness, transparency): The extent the provider of data or source of data can be trusted or relied upon to ensure that the data represents what it is intended to represent and that there is no intent to misrepresent the data. Credibility is increased through transparency, clear data governance, and independent compliance audits (Kirch, 2008a). Credible data also has a narrower definition related to validity that describes whether measures were collected and performed under an accepted sampling and analysis plan, including quality control, quality assurance procedures and historical data availability (Donabedian, 1988; Wyatt & Sullivan, 2005).
- **COMPLETENESS:** The proportion of all required data that is recorded. Completeness can be considered on two levels. Within a single database, completeness is the amount of missing data within the data fields. An example of database completeness is whether the collection date is recorded for all measurements. From the perspective of a surveillance system or research programme, completeness describes the degree to which relevant fields are collected. This perspective of completeness is synonymous with data relevance. At a minimum, data should include information regarding the "who, what, where and why" of the measures. Data should also include information about the measurement methods, measurement quality, and important features that may affect the interpretation of reported measures.
- **VALIDITY, RELIABILITY, REASONABLENESS:** How well data measurement conforms to a reference or known values. Valid data accurately and precisely records what was meant to be measured or observed. Reliability is the ability to repeat the measurement

consistently. Valid data conforms to predetermined standard definitions, including data units and ranges. For example, wastewater temperature is measured in degrees Celsius. Reasonableness is assessed at a high-level data audit to ensure values and relationships conform to expectations. Within a WBS system, data is generated when measurements are performed. The data is then transcribed, transferred, transformed, and transmitted – often through many additional steps and systems. At each step, there is the potential for transcription or transformation error. Valid data conforms to how closely the data at any step of the data 'pipeline' conforms to how the measure was collected or intended to be transformed. For a database, data accuracy is related to data integrity (see below). Note that validity and accuracy can also refer to the overall performance of a surveillance system, which has a purpose and meaning distinct from database accuracy.

- **STANDARDS USE** (data management, comparability and sharing): The use of messaging, data exchange and/or other information technology standards to improve its ability to communicate, exchange data, and use information that has already been exchanged within the system. Data should use existing standards or be comparable and translatable to standard data concepts. A challenge for WBS is the wide range of measured biological and physical domains. WBS data need to conform or be able to be translated to all its domains. Data should be interoperable within the field of WBS. Data collected from a site in one world region should be able to be transferred and combined with data from another world region. Data collected at one time should be comparable to another period.

- **AVAILABILITY** (stability): Availability measures, collects, manages, and provides data without failure. Availability is the operational availability of the surveillance system. Reliability and availability can be measured or modelled. Measurement can include the duration of unscheduled outages of the information system that support the surveillance system. Modelling includes assessing processes of continuity and reliability within a data system.

- **ACCESSIBILITY:** The ability (effort or ease) to access and obtain surveillance data by stakeholders. Open Science defines stakeholders at all levels of society, amateur or professional (Vicente-Saez & Martinez-Fuentes, 2018). Open science emphasises transparent and accessible knowledge that is shared and developed through collaborative networks (Vicente-Saez & Martinez-Fuentes, 2018). Accessibility includes the perspectives of whether information can be accessed and from through the information can be accessed. Data must be held confidentially and privately when required. Communities, especially racialised and vulnerable communities, must have control over the data they own, and they must control how this information can be stored, interpreted, used, or shared (Mecredy et al., 2018). See usage rights and authorisation section below. When appropriately accessed, data should be easily accessed by humans (clearly presented data) and machines (machine-accessible and actionable data). Accessible data has few barriers, including technology (open access and open-source software), organisational (straightforward and quick processes), and financial (free or low-cost to access data).

- **COMPLIANCE** (privacy, security, confidentiality, and ethics): The methodologies and procedures to keep surveillance data and information confidential, available, and accurate. Usage rights and authorisations are critical aspects of WBS. Using data for purposes not identified by the data custodian erodes trust in WBS and can violate data usage laws. However, not using or sharing data for purposes identified by the data custodian undermines the potential benefit of WBS. There must be clearly defined data usage rights and authorisation; security levels and procedures for surveillance system data or system access; system safeguards for the privacy of individuals and communities; and access to the surveillance system software application. Before a surveillance system becomes operational, it must conform to all laws, policies, and ethical practices. There must be processes and procedures to enforce security levels and procedures.

There are several additional attributes that may be included as a data quality, but they are also considered from the broader context of the overall surveillance system beyond data.

- **SIMPLICITY:** The surveillance system's structure and ease of operation, from data collection to use and/or dissemination. The surveillance system should be as simple as possible while meeting its objectives. Security levels and procedures for surveillance system data or system access are defined and enforced.

- **RELEVANCE** (usefulness): Data relevance is a measure of the impact of specific data on decisions or actions by the user. Including relevant data improves a surveillance system's usefulness. Data should include relevant details – data with enough detail to support decisions – and relevant

context – data with context to support the appropriate interpretation of the data. As a rapidly developing field, knowing what data is relevant or irrelevant is challenging; however, collecting an extensive amount of data that has low relevancy unnecessarily increases data management effort, contributes to information "overload", and complicates decision-making.

- **REPRESENTATIVENESS:**
  Data representativeness refers to the degree to which a sample of data accurately represents the larger population or universe that it is intended to represent. Representative sampling and data provide a true reflection of the patterns and trends of the surveillance targets within the population served by the surveillance system.

4.2.4.4 QUALITY ASSURANCE AND QUALITY CONTROL

Quality assurance and quality control ensure a surveillance system or research experiment runs smoothly, safely and effectively without errors (European Centre for Disease Prevention and Control, 2014). Quality assurance applies to all stages of a surveillance system, from sample collection to publication of surveillance findings. Data systems should include data quality metrics. Data quality can be reported for each sample or measure: from measuring the flow rate of a wastewater treatment plant to viral genome sequencing. However, there are chiefly concerns in two areas: samples, including collection, transportation, and storage; viral and microbial measurement, including PCR and sequencing. Approaches to recording data quality are emerging, and there is currently no well-established standard.

Table 4. Example of data quality sets from the Open Data Model.

SAMPLE QUALITY SET	frozen	Sample frozen
	leaked	Leaked Sample
	lowVol	Low-volume sample
	noLabel	Sample not labelled
	noTime	Sample missing time stamp
	spill	Sample spilled
	wrongStorage	Sample stored incorrectly
	wrongTemp	Sample stored at wrong temperature
PCR QUALITY SET	AI	Inhibition present but addressed
	B	Trace levels of contamination
	expFail	Experiment Failed
	FI	Inhibition present and unaddressed
	J	Weak signal extrapolation
	ND	Non-detect
	NoConcernPCR	No quality concerns for PCR
	UJ	Trace signal extrapolation
	UQ	Unquantifiable

Source: D. Manual, unpublished.

Laboratories may also have quality control and assurance processes for auditing and regulatory compliance. Consideration can be given to reporting these metrics as part of surveillance system reporting. An Open Data Model (ODM) was developed to allow quality assurance reporting or further extended to address to include additional metrics.

4.2.4.5 USAGE RIGHTS AND AUTHORISATIONS

Using data for purposes not identified by the data generator or custodian can harm communities, erode trust in WBS, and can violate data usage laws (Mecredy et al., 2018). Wastewater monitoring data must include a clear data policy with principles and conditions for management, access, use, and re-use (European Commission, 2022). An unclear data policy also increases the risk of data being used inappropriately (Johnson et al., 2022). Section 0 further discusses the ethical, anthropological and social aspects of wastewater surveillance.

Data without a clear data policy generates friction in discovery and access that impedes the data's legitimate use and benefit. Increasingly, wastewater surveillance systems promote open access to data, including the European Union Digital European Exchange Platform (EU4S-DEEP) and the United States National Wastewater Surveillance System (CDC, 2022; European Commission, 2022). Open access means that data are available to the user without making a specific request or obtaining a specific authorisation. The limitations of controlled access are recognised, including a review of international viral genomic data sharing within the GISAID model (Johnson et al., 2022).

There are four main approaches to ensure widespread benefit from open access with protecting communities against potential harms and stigmatisation. First, wastewater testing programmes fully engage the communities they serve, placing communities at the centre of planning and use of surveillance information. Second, a multi-layered approach to data access is used. The description and methods of a wastewater surveillance programme is transparent and widely available in virtually all settings. Transparency about when, where and why wastewater testing is performed is an important attribute of trust. A minimal dataset with measurement values can be available, but at a level of aggregation that reduces the risk of stigmatisation and harms to racialised communities. More detailed data is available with restricted approval and use. More detail data allows for detailed analyses that is then aggregated or summarised to reduce potential harms. Fourth, data includes clear usage rights, authorisations and data provenance. All data requires strong, clear and representative governance. Vulnerable and racialised communities should be represented, if wastewater surveillance includes these communities.

4.2.4.6 DATA PROVENANCE

Data provenance refers to a record trail from the origin of a piece of data (in a database, document, or repository), together with an explanation of how and why it got to the present place. Data provenance is important for all science disciplines, and WBS is no exception, particularly when WBS data is combined across testing sites and programmes and reused and reanalysed.

Data provenance helps answer several questions about data (Hills et al., 2015). Where did the data originate? How were they generated and processed? What was other data used to normalise, standardise, validate, and process these data? In other words, clear data provenance improves trustworthiness and transparency. Data provenance provides the context of how data was collected, which helps ensure data and subsequent data products are used with the appropriate context. An example is whether the data included vulnerable or marginalised populations where ownership and stewardship need to be led by those communities (Mecredy et al., 2018).

Approaches to maintain data provenance have arisen following the recognition of its importance and expanding the open science 'toolkit' to support transparent, collaborative, and reproducible science. The most important step in data provenance is maintaining a record of the origins of the data, along with sufficient metadata to describe how the data was collected and its measurement quality. All data should include a unique identifier, such as a Digital Object Identifier (DOI), a licence or data usage statement, and contact information about who collected the data and for what purpose.

4.2.4.7 DATA INTEGRATION

Wastewater monitoring data is seldom used in isolation. Data integration is the process that brings these separate data collections together to generate higher data value and insights.

WBS for SARS-CoV-2 is typically used with other forms of disease surveillance, such as clinical case detection, hospitalisations, serology studies and syndromic surveillance. Each form of disease surveillance has its strengths and applications that provide an understanding of disease transmission and burden. Understanding the performance of wastewater monitoring and epidemiology is facilitated when

assessed using reference data. In these studies, trends in data derived from wastewater monitoring are compared to other metrics through normalisation.

Data integration for wastewater monitoring data has three key considerations. First, data should be accessible using machine-actionable processes, such as Application Programming Interfaces (API), which enable applications to exchange data and functionality easily and securely. Second, data elements should use standards when available. For example, dates should be recorded using the ISO 8601 format. Third, the geographic area should be clearly defined. A constraint of wastewater monitoring is the requirement to collect wastewater from existing sewage systems. These boundaries of these systems are typically discontinuous with the spatial resolution used by other surveillance systems (e.g., health authority region or municipal boundaries).

4.2.4.8 PROCESSES FOR ENSURING USEFUL DATA

Data governance and management is the system to ensure usable and interoperable data is effectively and efficiently created, maintained and used. Data governance includes processes, policies, roles, metrics, and standards. Data management refers to the lifecycle of the collection, documentation, storage, sharing, and preservation of data (Research Data Management, n.d.; U.S. Geological Survey, n.d.).

Many organisations and entities directly or indirectly generate, collate and/or use wastewater monitoring data, from individual academic laboratories to multinational data repositories. Large surveillance and research programmes will have formal data governance structures. All programmes should have a clear data management process, including a data management plan.

Typical steps and processes of a data management plan to ensure high-quality data include (U.S. Geological Survey, n.d.; World Health Organization, 2017):

- Establishing data governance and coordination.
- Establishing a data quality review cycle, including a review committee.
- Developing a data assessment strategy.
- Maintaining data-quality metadata and documentation.
- Tracking data changes and implementing version control for the data.
- Performing automated quality checks.
- Comparing new data to historical values.
- Calculating summary statistics for data or displaying data to evaluate for possible anomalies.
- Evaluating the surveillance system, including data quality indicators.
- Appropriate processes for storage and fate of data over its lifecycle, including a plan for deletion or removal of data.

4.2.4.9 MUST DO, SHOULD DO

MUST DO:

1. When required, users must hold data confidentially and privately.
2. Communities, especially racialised and vulnerable communities, must have control over the data they own, and they must control how this information can be stored, interpreted, used, or shared.
3. There must be clearly defined data usage rights and authorisation; security levels and procedures for surveillance system data or system access; system safeguards for the privacy of individuals and communities; and access to the surveillance system software application.
4. Before a surveillance system becomes operational, it must conform to all laws, policies, and ethical practices. There must be processes and procedures to enforce security levels and procedures.
5. Wastewater monitoring data must include a clear data policy with principles and conditions for managing, accessing, using, and re-using wastewater monitoring data.

SHOULD DO:

1. People should benefit from WBS data.
2. Data should include relevant details – data with enough detail to support decisions – and relevant context – data with context to support the appropriate interpretation of the data.
3. All programmes should have a clear data management process, including a data management plan. Vulnerable and radicalised communities should be represented in data governance, if wastewater surveillance includes these communities.
4. At a minimum, data should include information regarding the “who, what, where and why” of the measures.

5. Data should also include information about the measurement methods, measurement quality, and important features that may affect the interpretation of reported measures.
6. Data should use existing standards or be comparable and translatable to standard data concepts.
7. Data should be interoperable within the field of WBS. Data collected from a site in one world region should be able to be transferred and combined with data from another world region. Data collected at one time should be comparable to another period.
8. When appropriately accessed, data should be easily accessed by humans (clearly presented data) and machines (machine-accessible and actionable data).
9. The surveillance system should be simple while meeting its objectives.
10. Data systems should include data quality metrics.
11. All data should include a unique identifier, such as a DOI, a license or data usage statement, and contact information about who collected the data and for what purpose.
12. Data should be accessible using machine-actionable processes such as application programme interfaces (API) that enable applications to exchange data and functionality easily and securely.

4.2.4.10 CONCLUSIONS

The Covid-19 pandemic has reinforced the imperative for collecting and maintaining usable and interpretable data to inform public health practice and increase our understanding of factors that affect our health. The pandemic also demonstrated that collaboration is needed to develop and share data. Widespread public benefit and trust in WBS data is achieved when partners throughout the wastewater monitoring field support each other and acknowledge each other's efforts to collectively develop and maintain data.

4.3 DATA MODELS

MASTER CHEF: **DOUGLAS MANUEL**

CREW:	CRITICS:
DAVID A. CÁCERES	DARREN DAHLY
MARGARITA PALAU	JASMINE GRIMSLEY
JEAN-DAVID THERRIEN	CLAIRE DUVALLET
GEORGIOS NIKOLOPOULOS	
ANNALISA QUATTROCCHI	
SORIN SION	
FRANCESCO PIERANNUNZI	
MATTHEW JOHN WADE	

Data models are critical in WBS, ensuring effective data sharing and management across small-scale collaborations and large-scale international efforts. The challenges of WBS data modelling include a lack of standardisation in data collection, storage, and reporting, coupled with the complexities introduced by the multidisciplinary nature of the field. The rapid expansion of WBS during the COVID-19 pandemic, with increased data complexity and testing for various pathogens, has further highlighted the need for robust data models. This chapter outlines the challenges and describes the construction of effective data models that facilitate interoperability, stakeholder engagement, and efficient data management within the WBS landscape.

4.3.1 KEY INGREDIENTS



- **Data Elements and Relationships.** A clear representation of real-world processes, such as sample collection and testing methods, forms the base of the WBS data model stew.
- **Interoperability.** The capability to exchange information easily among various systems and stakeholders, akin to a versatile kitchen gadget used across different culinary traditions.
- **Stakeholder-Specific Needs.** A blend of requirements from data generators, analysts, and decision-makers that must be balanced in the data model recipe for a palatable outcome.
- **Ecosystem.** A robust combination of organisations, disciplines, processes, and technology that adds

- **Depth to the data management flavour.** Software Tools. The utensils needed for data collection, validation, transformation, storage, and sharing essential for cooking up an efficient data management system.
- **Quality Assurance and Control.** The seasoning of the data model, ensuring that the final dish is of the highest standard and ready for consumption.
- **FAIR Data Principles.** The garnishing of Findability, Accessibility, Interoperability, and Reusability that makes the data model appealing to a wide palate.
- **Open Science.** An invitation to chefs around the world to contribute to and benefit from the data model, fostering a communal culinary experience.
- **Trust and Ethical Use.** The assurance that WBS data is sourced responsibly, maintaining the integrity and reputation of the surveillance kitchen.
- **Metadata.** Structured information that delineates the specifications of the data architecture. Metadata encapsulates the critical elements necessary for comprehending and utilising the data models within the rapidly evolving field of WBS.

4.3.2 RECIPE

1. **Construct the Base.** Start by defining the core entities of your data model – the sample sites, the measures taken, and the testing methods used.
2. **Ensure Interoperability.** Mix information technology and organisational interoperability to create a data model that can easily blend with various systems and stakeholders.
3. **Balance Needs.** Whisk together the common and specific needs of all stakeholders to create a data model that caters to everyone at the table.
4. **Develop a Supportive Ecosystem.** Fold in a network of collaborating organisations and disciplines to provide structure and support for the data model.
5. **Implement Software Tools.** Utilise data management tools to streamline the collection, validation, transformation, storage, and sharing of data.
6. **Add Quality Assurance.** Season with quality assurance and control measures to ensure the data models yield high-quality, reliable results.
7. **Sprinkle FAIR Principles.** Garnish your data model with the principles of findability, accessibility, interoperability, and reusability to make it more appealing and useful.
8. **Embrace Open Science.** Open your kitchen to chefs worldwide by including an open license and encouraging contributions to the development of your data model.
9. **Build Trust.** Secure the ethical use of WBS data by ensuring transparency and controlled access through metadata for provenance and lineage.
10. **Finalise with Metadata.** Complete your data model with robust and extendable metadata to support the richness of the WBS field.

4.3.3 TIPS

- **Keep It Simple:** While complexity is inherent in WBS, strive for simplicity in your data model to maintain usability and efficiency.
- **Stay Flexible:** Allow room for your data model to grow and adapt to new pathogens, technologies, and methodologies.
- **Promote Transparency:** Openly share your data model development process and encourage feedback to build trust and improve quality.
- **Document Thoroughly:** Maintain detailed records of your data model ingredients and preparation steps for easy replication and understanding by others.
- **Test and Validate:** Regularly test your data model with sample data to ensure it meets the needs of all stakeholders and can handle the volume and complexity of WBS data.



4.3.4 THE SCIENCE BEHIND

Good data models are key for WBS implementation and its success. Data models support effective and efficient data sharing regardless of the implementation scale – from academic inter-laboratory research collaborations to large-scale international efforts to develop global pandemic early-warning systems. Science advances and effective public health surveillance are achieved when high-quality data is available and used by many stakeholders.

There are many challenges to collecting, storing, and sharing WBS data in a consistent and interoperable way. As a relatively new field of study, there are no standards regarding measuring and reporting findings or even what data should be collected. As a multidisciplinary field, WBS has different data practices. Environmental surveillance systems such as WBS are more complex than most clinical counterparts.

Early in the COVID-19 pandemic, wastewater monitoring started and quickly expanded worldwide. A key to early rapid adoption was data collected and stored in desktop spreadsheets such as Microsoft Excel. Laboratories performed PCR calculations, quality checks and data cleaning, then generated graphs that could be immediately shared – all within a single computer programme. Initially, many national programmes also collected and stored their data in spreadsheets.

These spreadsheets worked well – for a while – when laboratories used their data model or dictionary and had a limited amount of data (Kelion, 2020).

The need for robust data management increased over the COVID-19 pandemic as data became more complex and data sharing increased, along with the number of pathogens tested. Requirements for quality assurance, control measures and reporting methods increased. Genomic sequencing generated large and complex data. Sharing and comparing data between sites and countries increased as monitoring programmes expanded. Testing expanded from SARS-CoV-2 to other targets, including influenza virus, respiratory syncytial virus, poliovirus and AMR genes.

Over the pandemic, laboratory spreadsheets became longer (as testing for sites has continued or expanded) and wider (as reporting became more complex; additional metadata being included as datasets became more enriched). Using spreadsheets for large surveillance programmes became exponentially more challenging. Data models provide a scalable foundation for complex data landscapes (West, 2011).

4.3.4.1 CHALLENGES COLLECTING AND SHARING INTEROPERABLE WBS DATA

- **A rapidly expanding field:**
  - New methods and measures are rapidly introduced.
  - No consistent approach to reporting findings.
  - Lack of data quality standards.
- **Complex surveillance system:**
  - Results are affected by many system, environmental and lab factors.
  - Viral sequencing and other measures generate complex data.
- **Multidisciplinary field:**
  - Many stakeholders generate WBS data.
  - Many stakeholders analyse and use WBS data and findings.
  - Varying data management and governance practices and experience.

4.3.4.2 WHAT IS A DATA MODEL?

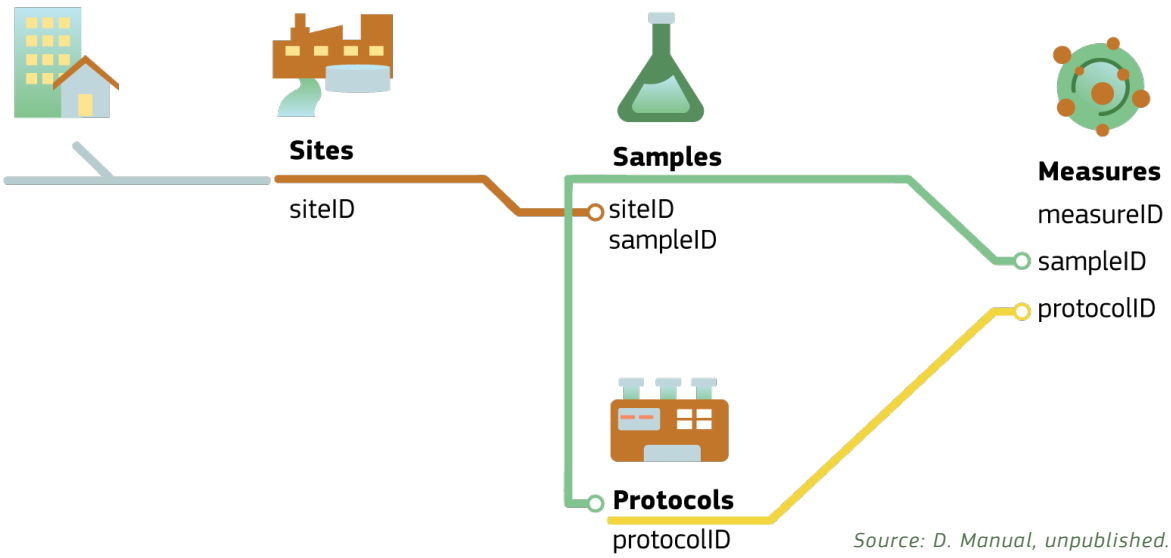
A data model represents real-world processes through data elements and data relationships. In WBS, **samples** are taken from wastewater **sites**, and **measures** are taken using different testing **methods**. These four processes together are the core entities of a WBS data model.

4.3.4.3 WHAT MAKES A GOOD DATA MODEL?

A GOOD DATA MODEL REDUCES THE EFFORT AND FRUSTRATION OF MANAGING WBS DATA

All data stakeholders, from those who generate data to those who use it, benefit from a shared, clear understanding of data processes through a model representation of real-world processes and their relationships. Data management efficiencies are gained through a supportive ecosystem that grows around interoperable and consistent data. Interoperable has two meanings – and good data models support both.

Figure 4. Core entities of a WBS data model



Source: D. Manual, unpublished. (redesigned)

In information technology, interoperability means easily exchanging information between different systems. From an organisational perspective, interoperable considers people and processes working together from a social or political perspective.

BALANCING COMMON AND SPECIFIC DATA NEEDS

WBS data stakeholders include data generators, analysts, data aggregators, knowledge synthesisers and decision-makers. A good data model supports common and specific stakeholders' needs (See Table 5 for examples of stakeholders' specific needs). Common needs for all stakeholders are a clear representation of the entities of WBS. Data generators benefit from a data model well-aligned with their measurement pipeline, with as much automation as possible. Data analysts benefit from a data model with detailed data because they are concerned that many parameters, such as the sampling and the testing method, affect wastewater pathogen measurement. Everyone recognises the benefit of standardisation, but they also want a data model to be flexible and extendable.

BUILDING A SUPPORTIVE ECOSYSTEM FOR EFFICIENT DATA MANAGEMENT

A data model that only describes how to report consistent data is insufficient to yield data management efficiencies. Data models include good documentation systems (See Section 4.2) and an ecosystem of collaborating organisations and disciplines, data processes, and information technology.

Data management tasks are easier when software tools can accompany data models, including:

BUILDING A SUPPORTIVE ECOSYSTEM FOR EFFICIENT DATA MANAGEMENT

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Data management tasks are easier when software tools can accompany data models, including:

- **Data collection** - data input templates and examples.
- **Data validation** - validation schema and applications to validate whether data conform to the data model, including ensuring data is complete and uses the specified data types.
- **Data transformation** - data that does not meet the data model specification can often be transformed or mapped to a data model or from one data model to another.
- **Data storage** - specifications for database and data storage specifications, such as SQL schema.
- **Data sharing** - sharing schema and application to filter or select data based on permissions.

A GOOD DATA MODEL INCREASES WBS VALUE AND USE

Science advances and effective public health surveillance are achieved when high-quality data is available and used by stakeholders representing a range of disciplines.

SUPPORT FAIR DATA PRINCIPLES

FAIR data principles refer to data Findability, Accessibility, Interoperability, and Reusability (Wilkinson et al., 2016). Findable data have persistent identifiers that are registered or indexed worldwide. Accessible data can be easily retrieved. Our World in Data is an example of a large amount of Covid-19 data accessible by the media and public (simple graphs and supporting tables) or scientists (through an application programme interface). An interoperable data model uses a common understanding and ways to access data. Data models support reusable data by including attributes to support data provenance and a clear and accessible data usage license (Mayernik et al., 2013).

SUPPORT OPEN SCIENCE

An open science approach means people worldwide can contribute to and benefit from the data collected using the data model and the data model itself. Data models support open science by including an open license. People and organisations worldwide should be able to contribute to developing data models, including adding new measures and developing applications to support new uses. To foster collaboration, data models can use an open software development process – described below (Von Krogh & Von Hippel, 2006).

BUILD TRUST IN WBS

Trust in WBS and the ethical use of WBS data are paramount to achieving WBS goals. Trust is built when WBS is well known and available for scrutiny. A data model should include provisions for identifying the "who, what, when, where and why" of WBS. Trust and data provenance are linked. Data provenance is the trail from data origins to where it has moved (Viglas, 2013). A data model should support the provision for data updating throughout the entire data life cycle, including recording of data transformation and aggregation by data analysts.

Table 5. Example of data stakeholders and their model needs.

	STAKEHOLDER INCLUDES	EXAMPLE MODEL NEEDS
DATA GENERATORS	Municipal sampling sites and testing laboratories	Alignment with and support for laboratory operations. • Support sample management, instrument integration, audit and compliance management, quality assurance and control, traceability, and analysis workflows.
ANALYSTS	Researchers, modellers, and public health analysts	Ability to transform data during data normalisation, modelling, and reporting.[1] • Support translation from relational databases (how WBS data is stored) into combined unified summary tables (how data is analysed). • Support the calculation of commonly derived variables such as aggregation and normalisation.
DATA AGGREGATORS	Data repositories, including national and international public health organisations and aggregator sites	Ability to easily aggregate data from numerous WBS and other surveillance systems. • Consistent and standard data that is easily accessed using application programme interfaces. • Clearly defined data use statements.
KNOWLEDGE SYNTHESIS AND DECISION-MAKERS	Public health departments, news agencies, citizen scientists, businesses, and individual citizens	Ability to combine WBS data with other health and surveillance data using different standards and models. • A WBS data model that is a good player with other data forms: builds from existing ontologies and modelling approaches.

[1] See sections 4.4 and 4.7

Source: D. Manual, unpublished.

Data models should include entities and metadata to support ownership and controlled access. Data access should be fine-grained and multi-layered. WBS data may be shared with many people and organisations, each with its own data needs or requirements and level of trust by the data custodian.

A GOOD DATA MODEL SUPPORTS THE GENERATION OF HIGH-QUALITY DATA

High-quality data are required to support WBS goals and uses described in the previous sections. Many of the attributes of how data models support the creation of high-quality data also are covered in those sections; however, a few additional organisational and technical considerations round out the discussion.

MODEL DEVELOPMENT AND GOVERNANCE

The development and maintenance of the data model can support open science by using open software development approach, including a process to contribute, identify concerns, provide feedback, identify issues, and obtain support (Von Krogh & Von Hippel, 2006). Models should have clear goals and purposes with processes to ensure that goals are met. Data models should have a governance that represents the community the model serves.

DATABASE PRACTICES

A model should support good database practices. Many data users require detailed and large datasets rather than aggregated or summarised information. However, data models should also support aggregated data. Models with many entities (or tables), such as WBS, commonly use a relational database structure.

ROBUST AND EXTENDABLE METADATA

Models should be able to accommodate new pathogens and health risks. Wastewater (water), air and surfaced-based surveillance and epidemiology are similar, and models should be extendable to these environmental compartments.

There should be sufficient metadata to support WBS as a complex and rapidly expanding field. Metadata should include attributes that affect the interpretation of data uses, such as how samples were collected or how viruses are measured. Quality assurance and control measures (QA/QC) are also included (see next chapter).

4.3.4.4 A GOOD DATA MODEL

REDUCES THE EFFORT AND FRUSTRATION OF MANAGING WBS DATA

Efficient data management is only possible when there is interoperable, consistent data.

- Supports a shared, clear understanding of data processes.
- Supports a data management ecosystem of collaborating organisations, processes, and information technology.

INCREASES THE WBS DATA VALUE AND USE

Science advances and effective public health surveillance are achieved when high-quality data is available and used appropriately by stakeholders.

- Promotes the creation of high-quality data with transparent methods and quality assurance measures.
- Supports FAIR data (Findable, Accessible, Interoperable and Reusable data and metadata).
- Supports open science (the ability to contribute to and benefit from WBS).
- Builds trust and supports the ethical use of WBS data through metadata for provenance, lineage, ownership, and controlled access.

SUPPORTS THE COLLECTION AND USE OF HIGH-QUALITY DATA

Models that support high-quality WBS have additional attributes.

- Follows model development and governance for open software.
- Uses database practices that are standard, consistent, and support a wide range of database and programme applications.
- Includes sufficient metadata to support WBS as a complex and rapidly expanding field.

4.3.4.5 THE OPEN DATA MODEL

The Public Health Environmental Surveillance Open Data Model (ODM) is used by the European Union Digital European Exchange Platform (EU4S-DEEP) and other surveillance programmes worldwide (https://osf.io/49z2b/). The ODM aims to "make data collection and sharing high-quality data as easy as possible." The ODM began development in 2020 to support wastewater-based measurement of SARS-CoV-2 using qPCR. The most recent version of the ODM expanded its scope to support future WBS needs (Therrien et al., 2024).

ODM VERSION 2 OBJECTIVES

- Support FAIR data.
- Use an open science, open software approach.

- Include provision for any biological, chemical or property, including antimicrobial resistance, drugs and toxins. Biologics include variants, sequences, alleles, mutations and proteins.
- Include samples from air, surface samples, and wastewater (water).
- Include protocol reporting, including metadata for methods that affect measurement across laboratories.
- Include reporting of quality control and assurance. Each measure can have multiple quality control and assurance measures.
- Comprehensive - includes measures in other wastewater dictionaries, including those described above.

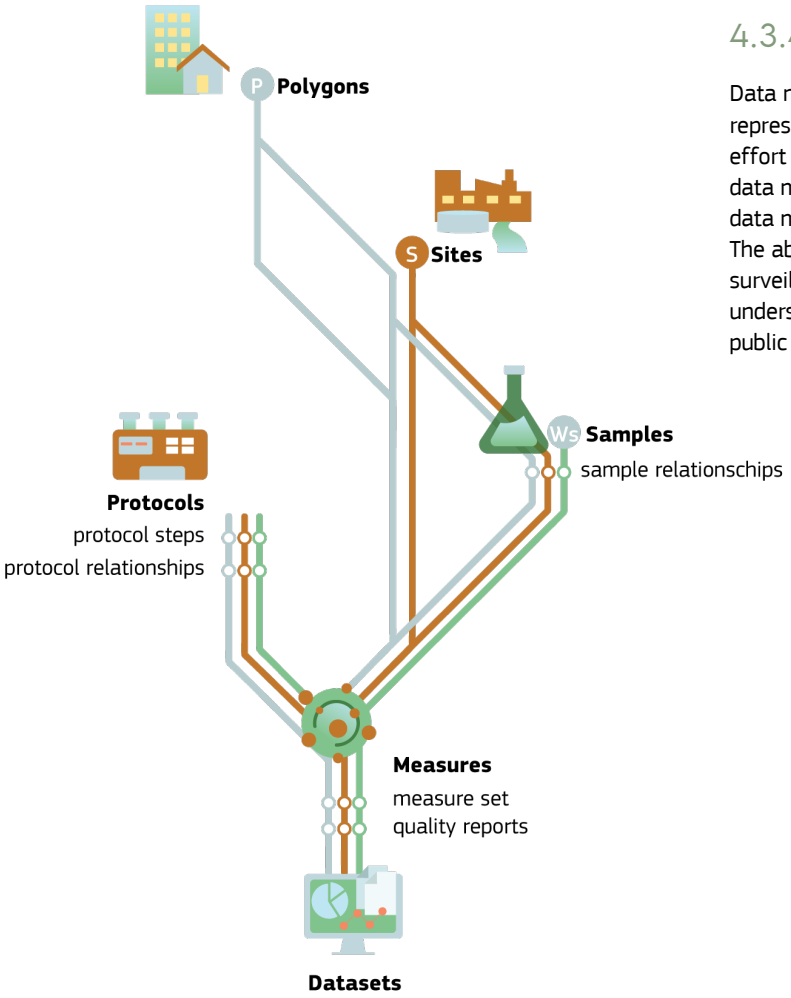
The ODM has two main reporting tables:

- **Samples:** To record information about the sample, such as the location, type of location (e.g., wastewater treatment plant, and material (e.g., raw sewage).
- **Measures:** To record any observation or measure. There are three measure types: population or area (e.g., amount of precipitation), site (e.g. wastewater flow rate), and samples (e.g. SARS-CoV-2 gene region N1 viral copies per ml).

In addition to the samples and measures tables, 12 additional tables store information regarding protocols (methods and instruments), quality measures, and contact information. There is the ability to record the relationship between samples to allow samples to be combined or split. Similarly, protocols can be modified with relationships to previous protocol versions and steps. Together, the 14 tables record the who, what, where, when and why of an environmental testing programme (Figure 5).

4.3.4.6 CONCLUSIONS

Data models for a foundation for describing how to represent the real-world WBS entities as data. The effort is required to develop a robust and easy-to-use data model, but the reward is efficient and effective data management that generates interoperable data. The ability to combine and compare data across surveillance programmes increases the ability to understand health risks and support public health action.



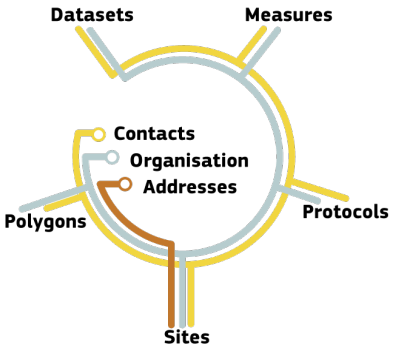


Figure 5. Fourteen tables comprise the entities of WBS. There are three types of measures or observations represented by three lines.

Source: D Manuel, unpublished. redesigned

4.4 DATA NORMALISATION

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Wastewater surveillance is a valuable tool for monitoring the presence and levels of pathogens, such as SARS-CoV-2, in communities. However, various factors, including meteorological events (e.g., rainfall, snowmelt), industrial discharges, or changes in community water use, can dilute or concentrate wastewater samples, leading to fluctuations in pathogen concentration measurements that do not necessarily reflect changes in the prevalence of the pathogen in the population. To address these issues, data normalisation is employed to obtain more representative results.

4.4.1 KEY INGREDIENTS



- **Data for normalisation:**
  - Flow Rate Data: Daily wastewater flow data from the treatment facilities for the times when samples were collected.
  - Normalisation Marker Data: Concentrations of normalisation markers (e.g., faecal indicator bacteria, faecal indicator phage, chemical constituents, or human DNA markers) from the same wastewater samples.
  - Population Data: Estimates of the population size served by the wastewater treatment facilities to allow for per capita calculations.
  - Meteorological Data: If applicable, weather information such as rainfall or temperature that could affect wastewater dilution and pathogen concentrations.
- **Analytical tools:**
  - Statistical Analysis Software: Programmes for processing and analysing data (e.g., R, Python, Excel, SPSS).
  - Data Visualisation Tools: Software to create graphs, charts, and maps to illustrate the normalised data (e.g., Tableau, QGIS).
- **Methodological Framework:**
  - Normalisation Methods: Protocols for how to apply flow rate data and normalisation markers to the pathogen concentration data.
  - Statistical Methods: Established statistical techniques for analysing time series data, managing outliers, and identifying trends.
  - Quality Control Procedures: Methods for verifying the accuracy of the normalisation, such as comparing to known trends or using control samples.
- **Expertise and Collaboration:**
  - Scientific Expertise: Personnel knowledgeable in environmental microbiology, epidemiology, and data analysis to interpret the results.
  - Collaborative Partnerships: Relationships with public health agencies, researchers, and other stakeholders for data validation and response coordination.
- **Documentation and Reporting:**
  - Record-Keeping: Detailed documentation of all methodologies and analyses to ensure transparency and reproducibility.
  - Reporting Formats: Templates or guidelines for how to present the normalised data to various audiences.

4.4.2 RECIPE



1. **Define Objectives and Select Normalisation Methods**
   a. Establish the goals of your surveillance (e.g., tracking SARS-CoV-2 prevalence, or monitoring other targets such as antimicrobial resistance).
   b. Choose appropriate normalisation methods based on the target, (i.e. SARS-CoV-2 or other targets), available data, and local conditions.

2. **Prepare Your Dataset. Ensure your dataset includes the following information for each sample:**

   a. Pathogen concentrations, typically in gene copies per litre or another relevant unit.

   b. The date and time of sample collection.

   c. Daily flow rates of the wastewater at the time of sample collection (if available).

   d. Concentrations of normalisation markers (e.g., faecal indicators, chemical constituents, human genetic markers).

3. **Data Normalisation:**

   a. Normalise pathogen concentrations based on wastewater flow data to account for dilution or concentration effects.

   b. Apply additional normalisation using selected markers (e.g., chemical, faecal, genetic) by calculating the ratio of pathogen concentration to marker concentration.

   c. Adjust pathogen loads for population size if necessary.

4. **Statistical Analysis:**

   a. Analyse the normalised data to identify patterns, trends, or anomalies.

   b. Use statistical methods appropriate for your data structure and objectives, such as time series analysis, regression models, or ANOVA.

5. **Quality Control Checks:**

   a. Review the data for outliers or inconsistencies that may indicate sampling or analytical errors.

   b. Compare the normalised values against any available epidemiological data to validate the normalisation process.

6. **Data Interpretation and Reporting**:

   a. Interpret the results in the context of the surveillance goals. For example, an increasing trend in normalised pathogen levels may suggest a rising prevalence of infection in the community.

   b. Consider other factors that might influence the data, such as population changes or reporting delays.

   c. Prepare reports and communicate findings to relevant stakeholders, including public health officials.

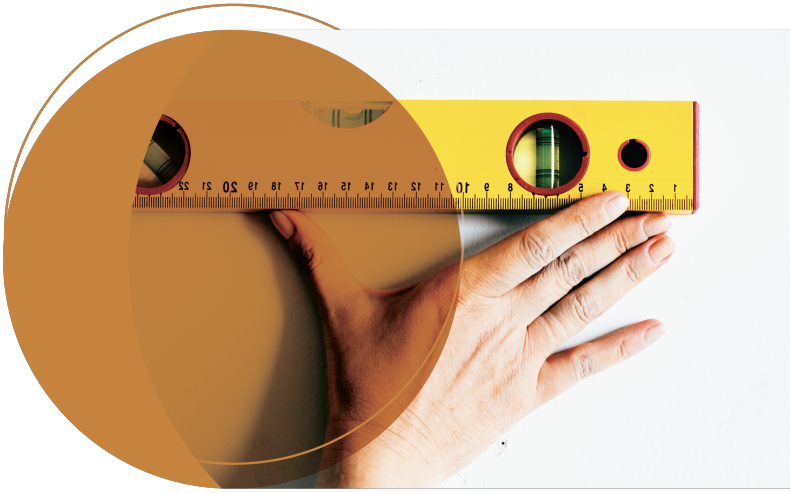
7. **Continuous Evaluation:**

   a. Continuously evaluate the effectiveness of your normalisation strategy.

   b. Adjust your approach based on new insights, feedback from public health officials, or changes in community behaviour that could affect wastewater composition.

4.4.3 TIPS

- **Representativeness:** Ensure the samples are representative of the population and the wastewater system.
- **Temporal Consistency:** Collect samples and data at consistent times to allow for accurate comparisons over time.
- **Marker Selection:** Choose appropriate and reliable markers for normalisation, and ensure they are unaffected by external factors unrelated to the population's health.
- **Collaboration:** Work with public health agencies to correlate findings with clinical data and other surveillance data streams.



4.4.4 THE SCIENCE BEHIND

SARS-CoV-2 RNA concentrations in wastewater may exhibit high variability due to changes in the dilution rates of domestic wastewater in the sewerage network, and differences in the relative contribution of human faecal matter over time, thus leading to misinter-pretation of measurement results in WBS studies. Dilution rates fluctuate mainly due to stormwater runoff (rainfall significantly affects flow rates), industrial discharges, and water infiltration. Variability in faecal

inputs from toilet facilities, on the other hand, may depend on the movement of people (e.g., tourists, daily/weekday travellers, and temporary workers), variability in faecal content among individuals, and spatial heterogeneity. Consequently, sample normalisation is critical in wastewater-based epidemiology if quantification is important. As recommended by both the European Commission (European Commission, 2021) and the CDC (Centers for Disease Control and Prevention, 2022), normalisation of SARS-CoV-2 viral loads by the quantity of water (daily wastewater flow) and the population (number of people served by the sewer system) is essential for a valid comparison of viral concentrations in wastewater samples over space and time. Additional normalisation parameters may be used to improve the trends of SARS-CoV-2 data when quantitative trends are followed over time and compared with public health surveillance data. This is because factors other than the amount of water and the size of the population in the catchment area may influence the variability of SARS-CoV-2 concentrations in wastewater. The source of water (e.g. dilution from inputs other than toilets, such as grey water, rainwater, industrial sewage, groundwater infiltration), for instance, will affect the quantity of faecal matter in wastewater, introducing bias in SARS-CoV-2 concentrations and thus also in COVID-19 trends. To account for the amount of human faecal content, human-specific biomarkers (biological or chemical parameters) can be used as faecal strength indicators. Normalisation is performed by dividing the measured SARS-CoV-2 concentration (gene copies per reaction) by the concentration of these parameters.

It is important to note that the optimal normalisation approach can be different according to the sampling point, given the nature of the catchment. This optimal normalisation approaches depend on the characteristics of the catchment feeding to the sampling point (Population Equivalents (PE), industry and agriculture, weather, etc.). It is important to note that there are no methods that are ideal for all systems, although there are methods that will fit most of the systems. It is also important to note that the storage and transit time from sampling point to the lab can influence the optimal normalisation markers for biological parameters. It is therefore wise to test several normalisation strategies to see which one fits best with your system.

4.4.4.1 FLOW-BASED NORMALISATION

The interpretation of raw pathogen concentrations can be confounded by fluctuations in wastewater flow, which may occur due to weather events, population dynamics, industrial activities, or infrastructure issues. Flow-based normalisation addresses this challenge by adjusting pathogen concentration data to reflect a standardised volume, thereby facilitating more accurate trend analysis and public health decision-making (Langeveld et al., 2023).

Flow-based normalisation relies on the premise that the pathogen load—defined as the total quantity of a pathogen discharged—should remain relatively constant over time, barring any actual changes in infection rates among the population. This approach uses daily flow rates as a denominator to standardise pathogen concentrations, thus enabling comparisons across different time points and conditions.

The process of flow-based normalisation involves several key steps:

- **Data Collection:** Accurate measurement of daily wastewater flow rates at the time of sample collection is crucial. Flow meters and automated logging devices are typically employed for this purpose.
- **Sample Analysis**: Pathogen concentrations are determined through laboratory analysis of wastewater samples, using quantitative PCR methods.
- **Calculation of Pathogen Loads:** The raw concentration data is multiplied by the corresponding daily flow rates to calculate the total pathogen load for each sampling point.
- **Normalisation:** The calculated pathogen loads are then divided by a standard flow rate or averaged over a period to normalise the data, enabling direct comparison between different samples.

Several challenges must be addressed when implementing flow-based normalisation. Flow rates can vary widely throughout the day and in response to external factors (Langeveld et al., 2023). Consistent sampling and accurate flow measurement are essential to mitigate this issue. Moreover, flow-based normalisation must often be integrated with other normalisation approaches, such as constituent-based or genetic marker-based methods, to account for additional sources of variability.

4.4.4.2 WEATHER-BASED NORMALISATION

Weather-based normalisation is a critical method in wastewater surveillance that addresses the impact of meteorological conditions on pathogen concentration data. Weather events such as rainfall, snowmelt, or drought can significantly dilute or concentrate

wastewater, leading to misleading interpretations of pathogen loads (Triggiano et al., 2023).

Meteorological events can introduce substantial variation in wastewater flow, affecting the dilution and transport of pathogens. Rainfall may lead to inflow and the infiltration of stormwater into sewer systems, diluting pathogen concentrations, while dry conditions can result in a concentrated wastewater stream. These weather-induced fluctuations can obscure true changes in pathogen prevalence, necessitating normalisation to account for these environmental factors (Saingam et al., 2023).

Normalisation incorporating weather data typically involves the following strategies:

- **Rainfall Correction:** The correlation of pathogen concentration data with local rainfall records allows for the adjustment of pathogen loads to reflect weather-induced dilution. Models can be developed to estimate the expected dilution factor based on rainfall intensity and duration.
- **Dry Weather Flow Baseline:** Establishing a baseline during periods of minimal precipitation (dry weather flow) provides a reference point for normalising data collected during wet weather events.
- **Hydrological Modelling:** Advanced hydrological models can simulate the effects of various weather conditions on sewer flows, offering a predictive tool for normalisation.

Implementing weather-based normalisation involves several key steps:

- **Data Collection:**
  - Gather detailed, time-matched meteorological data, including precipitation, temperature, and evapotranspiration rates.
  - Record wastewater flow rates and pathogen concentrations concurrently with weather data.
- **Data Analysis:**
  - Use statistical and hydrological modelling to assess the relationship between weather patterns and pathogen data.
  - Apply correction factors or models to normalise pathogen concentrations based on observed or predicted weather impacts.
- **Interpretation and Reporting:**
  - Carefully interpret normalised data, considering the influence of weather on the observed trends.
  - Report findings to public health authorities, including the methods used for weather-based normalisation.

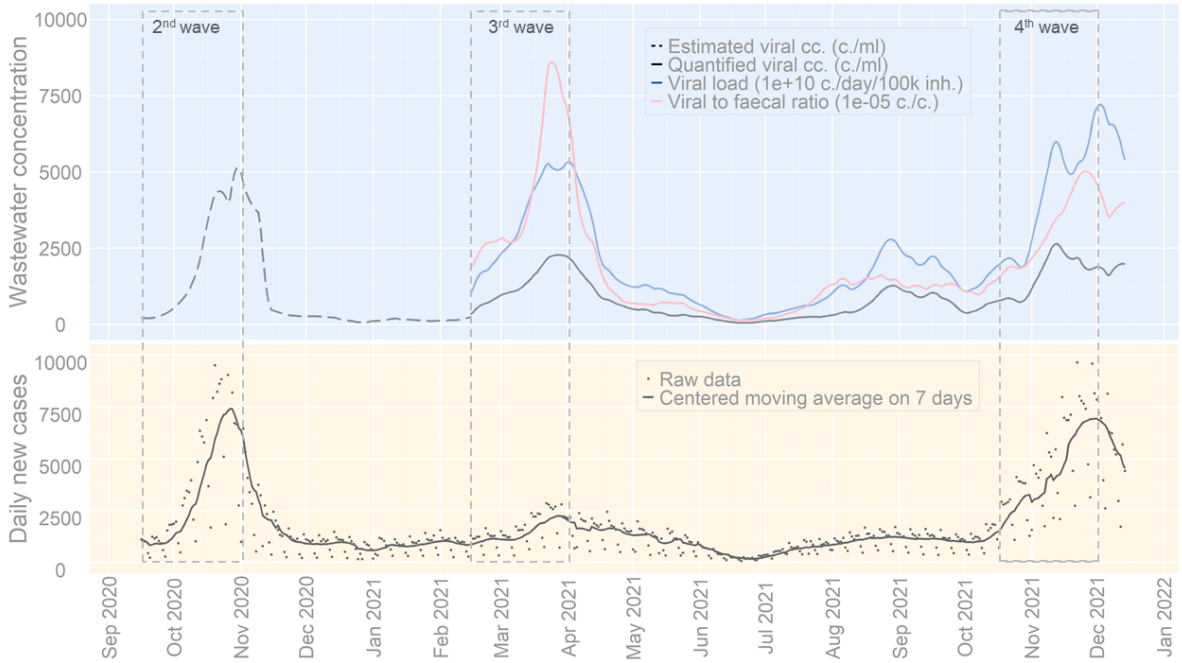
Weather-based normalisation is a challenging process that has to deal with the intricate nature of weather patterns and their diverse impacts on wastewater systems. To ensure accurate normalisation, it is crucial to have access to reliable and high-resolution meteorological data. Furthermore, since the local topography and sewer infrastructure can influence how weather affects wastewater, it is necessary to use tailored normalisation approaches that take into account geographical variation (Lazuka et al., 2021). Additionally, hydrological and statistical models involved in the normalisation process may contain assumptions and uncertainties, which must be accounted for in the analyses.

4.4.4.3 POPULATION-BASED NORMALISATION

Several factors can affect the SARS-CoV-2 concentrations measured at the inlet of wastewater treatment plants, such as stormwater captured in the sewage system, or the variation of the population size connected to the plants. To account for the rain and the population size impacts, the quantified viral concentrations can be expressed as viral load per capita (SARS-CoV-2 RNA copies/day/100,000 inhabitants) by multiplying the quantified viral concentration (SARS-CoV-2 RNA gene copies/ml) with the mean daily inflow rate (m^3/day) provided by regional wastewater agencies and normalised with the number of inhabitants connected to the corresponding treatment plant.

The discrepancies between the viral concentration and the viral load per capita can be seen in several systems. As an example, Figure 6. represents the data measured in the frame of the Belgian wastewater surveillance programme (Janssens et al., 2022).

Figure 6. SARS-CoV-2 concentrations in wastewater and COVID-19 cases aggregated for all areas covered by the Belgian wastewater surveillance over time.



Source: Belgian Public Health Website Available online: https://covid-19.sciensano.be/fr/covid-19-situation-epidemiologique (accessed on 2 January 2022)

Furthermore, the amplitude of the signal measured in wastewater during a wave is time-dependent as it is influenced by the proportion of the population vaccinated, the mix of circulating variants, the mobility restriction measures applied, the analytical method evolution, etc. Therefore, a normalisation procedure can be used to mitigate the impact of the variation of these parameters. For instance, in the Belgian wastewater surveillance of SARS-CoV-2 (Janssens et al., 2022), a normalisation step is performed by expressing the viral concentration in wastewater in percentages of the maximum value recorded during a previous virus wave for each area. This allows for a comparison with a reference period as well as between analyses eventually performed by distinct laboratories.

Although population-based normalisation is a powerful tool, it comes with some challenges that need to be considered. One of the main challenges is related to population dynamics, which can change due to factors such as tourism, events, and commuting patterns that may not be accurately captured in static population estimates (Hou et al., 2021). Another crucial aspect is population estimation, as over- or underestimation can lead to skewed normalisation results (Hsu et al., 2022).

4.4.4.4 NORMALISATION BY PHYSICOCHEMICAL PARAMETERS

Physicochemical wastewater characteristics largely depend on the source and type of wastewater, with around 0.1% of domestic wastewater being (micro) biological or chemical molecules, with water being approximately 99.9%. These molecules can have an impact on the wastewater composition and characteristics, and thereafter on the measurable amount of SARS-CoV-2 gene copies (Amoah et al., 2022; Sweetapple et al., 2022).

To determine fluctuations in investigated population size for the performance of longitudinal or comparative studies, the use of an endogenous normalisation marker present in proportion to the concentration of human excreta and secreta is recommended. The use of such a normalisation parameter may help provide information on fluctuations in contributing population size within a specific catchment, and thus on the SARS-CoV-2 infected individuals within this area during the sampling period. A viral biomarker may not directly correlate to the number of persons served by a specific catchment, while using census-level data for load normalisation

may not provide an adequately high resolution for the comprehension of dynamic individual contributions to wastewater. Moreover, rain contribution to wastewater inflows can increase the normal flow rate. Thus, a suitable normalisation parameter should provide low excretion per capita variance, which should not vary according to season, weather, or geographic location (Tscharke et al., 2019).

Physicochemical water quality parameters that are routinely measured in laboratories such as Chemical Oxygen Demand (COD), Biochemical Oxygen Demand (BOD), total nitrogen (TN) and ammonium (NH_4-N) concentrations may be used to calculate served population and thus normalise SARS-CoV-2 concentrations according to this estimate (also referred to as Population Equivalents, PE) (Been et al., 2014; Hutchison et al., 2022; Polo et al., 2020). This type of measurement may be used as a low-cost indicator approach compared to (micro)biological indicator use, due to the routine measurement of these parameters. When considering the use of these parameters, care should be taken to account for industrial discharges that may alter the population estimated size. Moreover, time or flow-proportional composite sampling may provide more representative data compared with grab sampling, under varying flow conditions.

To be able to estimate the actual served population based on a specific measured physicochemical parameter, the measured parameter concentration (mg/L) must be reported for the total daily wastewater flow that enters the catchment for each sampling day, taking into account the average per capita equivalent production for this parameter, per day (Equation 1) (Been et al., 2014; Reinstadler et al., 2021).

$$P = \frac{M \times F}{E} \text{ (Eq. 1)}$$

Specifically:

P = Total served population (Population Equivalents, PE)

M = Measured parameter concentration (mg/L)

F = 24-hour flow (L) to the sampling point for each sampling day (provided by sewerage system or wastewater treatment plant operators)

E* = Per Capita equivalent physicochemical parameter (mg/person/day)

E* is estimated according to the currently available scientific literature as: COD=110 g/person/day, TN=11 g/person/day and NH_4-N=8 g/person/day

SARS-CoV-2 genetic fragment concentrations can thereafter be estimated for the Total Served Population (P):

$$\text{SARS} - \text{CoV} - 2 \text{ genetic fragments} = \frac{C\ X\ F}{P}\ X\ 1{,}000\ inhabitants \quad \text{(Eq. 2)}$$

Where:

SARS-CoV-2 genetic fragments (gc/day/1,000 Population Equivalents) = The estimated normalised loads of SARS-CoV-2 genetic fragments per day per 1,000 inhabitants

C = Concentration of measured genetic fragments (gene copies (gc)/L)

F = Flow to the sampling point from the catchment (L/day)

P = Population Equivalents calculated in equation 1

The most commonly utilised physicochemical parameter for PE normalisation is NH_4^+, as it has homogeneous excretion within the community and low variance (Been et al., 2014). This is the main form of ammonia (NH_3) in water. As a hydrolysis product of urea, it can be used as an indirect marker of urine in wastewater. Ammonium can be measured directly in the wastewater photometrically or using an ion-selective electrode. Studies conducted so far have shown that this parameter is capable of detecting population fluctuations over large periods of time or during significant events such as the COVID-19 pandemic (Sweetapple et al., 2022).

Exogenous biomarkers that are consistently excreted within specific populations have also been utilised for true population estimation, such as nicotine, caffeine, acesulfame, and atenolol. However, their stability in certain catchments serving populations that have different caffeine consumption or pharmaceutical disposal habits compared to others, is currently a concern (E. Lu et al., 2022). By nature, exogenous biomarkers have the capacity to represent specific catchments and thus should be wisely used only in specific communities.

4.4.4.5 NORMALISATION BY BIOLOGICAL PARAMETERS

FAECAL INDICATOR – VIRAL BIOMARKERS

The two most frequently used human faecal viral indicators applied to WBS for SARS-CoV-2 are Pepper Mild Mottle Virus (PMMoV) and Cross-Assembly phage (crAssphage).

PMMoV, a non-enveloped RNA plant virus, is one of the biomarkers recommended by the European Commission (European Commission, 2021) and CDC (Centers for Disease Control and Prevention, 2022). The virus enters the human host through food items, is constantly excreted by humans, ranging from 10^5 to 10^{10} copies/g of faeces, and is stable in wastewater (Rosario et al., 2009) remaining infective even after conventional wastewater treatment (Bačnik et al., 2020). Concentrations in sewage do not depend on active infection and vary with season and diet, ranging from 5 to 10 $\log_{10}$ copies/L (Kitajima et al., 2018). Several studies have found PMMoV to be a potentially suitable normalisation biomarker capable of strengthening the correlation of data from wastewater or wastewater-settled solids with clinically-based epidemiological parameters (Acosta et al., 2021; D'Aoust, Mercier, et al., 2021; D'Aoust, Towhid, et al., 2021; E. Lu et al., 2022; Scott et al., 2021; Wu et al., 2020). Other studies provided evidence against its usefulness, however (Ai et al., 2021a, p. 19; Feng et al., 2021a; Greenwald et al., 2021; Wolfe et al., 2021; Wurtzer et al., 2022; Zheng et al., 2022). The marker's diet-dependency and the wide range of viral loads found in the literature cast doubt on its applicability to long-term studies and across large geographic regions. Furthermore, the possible existence of a variable fraction of PMMoV that reaches wastewater from solanaceous cropland wash off needs to be taken into consideration.

Cross-assembly phage (CrAssphage) is a bacteriophage specific to the human gut that has been strongly associated with human faeces. Although little information exists on the concentration of CrAssphage on human faeces, Park et al. (Park et al., 2020) have determined that median concentrations ranged from 5.9 $\log_{10}$ copies in stool samples from norovirus outbreaks up to more than 8.0 $\log_{10}$ copies/g of faeces in adults and children under 5 years old of age without acute gastroenteritis. It is globally distributed in wastewater and various faecally-contaminated water bodies at high concentrations without seasonal fluctuations. Concentrations of CrAssphage in sewage typically varies between 4.8 and 9.1 $\log_{10}$ copies/L (Sabar et al., 2022). A few studies applied this normalisation biomarker to SARS-CoV-2 WBS. Some showed adjusted viral loads to improve the correlation to clinical data (E. Lu et al., 2022; Wilder et al., 2021), but others did not (Ai et al., 2021a; Greenwald et al., 2021; Tandukar et al., 2022). Thus, while normalisation by population and wastewater flow is always recommended, the effect of normalisation using viral biomarkers on the correlation with epidemiological metrics is still unclear, and will require further studies.

OTHER MARKERS

The abovementioned normalisation strategies are the most commonly used for SARS-CoV-2 WBS. However, several other biomarkers have emerged some showing promising results. The ideal biomarker should be fairly constant per capita daily excretion, should not be impacted by season, and geographic location.

Faecal bacteria, including thermotolerant coliforms or *E. coli*, and the human-associated *Bacteroides* spp. HF183 bacterial marker, have been applied for SARS-CoV-2 normalisation purposes (Feng et al., 2021a; Zhan et al., 2022). Thermotolerant coliforms are commonly found in faecal samples and sewage in high concentrations and can be easily measured by standardised methods. HF183 is the most frequently used human-associated sewage marker gene belonging to the genus *Bacteroides*, an obligate anaerobe (H. C. Green et al., 2014). Despite being ubiquitous and found in high concentrations in stool samples and sewage both bacterial markers failed to improve the correlation between clinical cases and SARS-CoV-2 normalised loads. What is more, normalisation by HF183 gene marker even decreased the correlations between SARS-CoV-2 viral load and clinical cases.

β-2 Microglobulin (B2M), a protein, has also been used for SARS-CoV-2 normalisation purposes (Zhan et al., 2022). B2M is found upon infection or inflammation in bodily fluids, and is commonly used as a biomarker for patients with HIV or cancers. Relations have been found between SARS-CoV-2 and B2M shed by infected patients (Zou et al., 2021). The authors have found that normalisation with B2M, as well as with PMMoV, provided a more precise tracking of COVID-19 cases at the campus levels and at larger catchment scales collected locally and immediately upon collection.

Studies have evaluated the potential for SARS-CoV-2 normalisation using among others, the human mitochondrial gene NADH dehydrogenase subunit 5 and creatinine (Hutchison et al., 2022). Human mitochondrial DNA is excreted in high abundance in faecal matter (Andréasson et al., 2002; Gerber et al., 2001; Iyengar et al., 1991) and is often used to determine sources of faecal contamination (Caldwell et al., 2007; Martellini et al., 2005; Monteiro et al., 2021). Creatinine is a breakdown product from muscle and protein metabolism and from which the excretion rates are

known (Gracia-Lor et al., 2017). The authors have determined that both markers improved the correlation between cases and SARS-CoV-2 loads in wastewater compared to ammonium and BOD. On the other hand, for Hsu et al. (Hsu et al., 2022), creatinine did not correlate with population. The authors have also tested other biomarkers, including PMMoV, 5-hydroxyindoleacetic acid, caffeine and paraxanthine. 5-hydroxyindoleacetic acid is a serotonin metabolite that has been used as an estimation of the population serviced by a wastewater (Rico et al., 2017). Caffeine is frequently found in food products, such as coffee, energy drinks, and tea, among others, as well as in dietary supplements and some medications and paraxanthine is the major metabolite of caffeine (Crews et al., 2001). Both have been detected previously in wastewater (Driver et al., 2020; Gracia-Lor et al., 2017; O'Brien et al., 2014; Senta et al., 2015). The authors have determined that paraxanthine, and to a lesser extent caffeine, was more appropriate for normalisation of SARS-CoV-2 load per capita as a result of the low variability, high accuracy, and temporal stability. Additionally, normalising SARS-CoV-2 load by the estimated population using this metabolite improved the correlation between clinical cases and SARS-CoV-2 load per capita.

4.5 DATA VISUALISATION

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In this chapter, data visualisation in WBS is explored, where quantitative measurements are transformed into visual representations. The aim is to make complex epidemiological data derived from wastewater analytics easier to understand for a wide range of stakeholders while ensuring that the visualisations are scientifically accurate. The focus is on methods for depicting temporal trends and geographic variations, normalisation and log-scaling for comparative analyses, and the importance of tailoring visual communication to the intended audience. The goal is to create visuals that are not only informative but also drive engagement and understanding, ensuring that the insights gleaned from WBS are effectively communicated and utilised in public health decision-making.

4.5.1 KEY INGREDIENTS

- **WBS Data Types:** Understand the granularity and nature of your data, whether it is continuous (e.g., viral loads) or categorical (e.g., variant detection).
- **Target Audience Insight:** Know the level of expertise and interests of your audience to tailor visual complexity and depth of information.
- **Visualisation Software:** Choose a tool that provides flexibility and supports the creation of both static and interactive visuals.
- **Data Normalisation Methods:** Familiarise yourself with normalisation techniques to ensure comparability and context, such as per capita calculations.
- **Statistical Trendlines:** Select appropriate methods for data smoothing, like moving averages or local regression, to help identify trends.
- **Design Elements:** Understand the impact of colour theory, categorisation, and symbology to enhance clarity and avoid visual clutter.
- **Interactive Features:** For digital platforms, consider elements like sliders, filters, and toggles to allow audience-driven data exploration.
- **Ethical Considerations:** Be aware of how visualisation choices can influence data interpretation and commit to avoiding distortions.

4.5.2 RECIPE

1. **Preparation of Data:** Clean and pre-process your WBS data to ensure accuracy. Choose normalisation techniques that align with your data type and audience, such as inhabitants per catchment area for geographic relevance.
2. **Selection of Visualisation Type:** For time-series data, opt for line graphs or area charts. Integrate trendlines to emphasise movement over time. For geographic distribution, use thematic maps with colour-coded regions or proportional symbols to represent data points.

3. **Designing the Visualisation:** Create a draft layout, selecting colours and symbols that align with commonly accepted standards (e.g., using blue to represent water-related data). Apply data categorisation thoughtfully to simplify interpretation without oversimplifying the data. Incorporate statistical trendlines or moving averages to guide the audience through volatility in the data.

4. **Refinement and Testing:** Review the visualisation for potential misrepresentations, such as misleading axes or inappropriate scaling. Test your visuals with a subset of your target audience to gauge clarity and adjust based on feedback. Ensure the inclusion of necessary context, especially when presenting to a scientific audience.

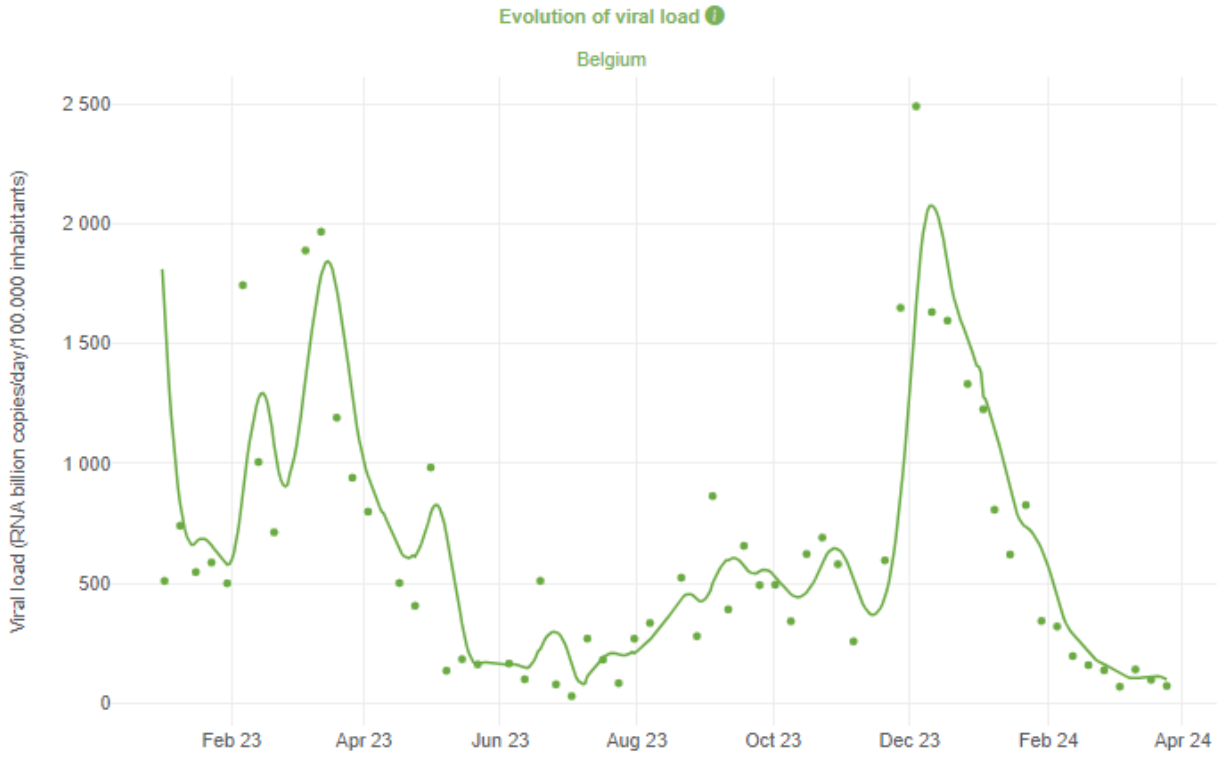
5. **Final Touches and Presentation:** Add annotations, legends, and clear labels to ensure the viewer can understand the data without additional explanation. For interactive elements, provide instructions and ensure functionality across various devices and browsers. Finalise the design by ensuring it is accessible, with alternative text and high-contrast colours for those with visual impairments.

4.5.3 TIPS

- **Simplify complex units** like "RNA copies/day/100k inhabitants" by providing analogies or converting them to more familiar terms when addressing the public.
- **Use dual-axis charts sparingly**, as they can be confusing; ensure that the secondary axis is clearly labelled and justified.
- **Consider the application of logarithmic scales carefully**, as they can be misinterpreted by non-technical audiences.
- When displaying aggregated data, **provide context on the aggregation level** and its implications for the interpretation of trends.
- **Always provide sources and explanations** for any data exclusions or anomalies to maintain transparency.



Figure 7. Viral concentration time series visualisation from the Belgium COVID-19 epidemiological situation, wastewater surveillance



Source: https://wastewater.sciensano.be/dashboard/covid19/en/

4.5.4 THE SCIENCE BEHIND

Considerations regarding WBS visualisations do, naturally, include the main issues to consider when visualising any data. The target audience, the medium of dissemination, the information density versus ease of interpretation trade-off, and potential data distortions resulting from poorly chosen visuals. One area of consideration that merits especially close attention in public health related data is the target audience, since the same data often needs to be presented to a wide range of audiences, from laypersons who are not familiar with many aspects of the results, to health professionals and researchers specialising in the topics at hand.

As different countries have developed different approaches to showing their wastewater-data, in this chapter an effort is made to extract common themes in the visualisations. These commonly align with the goals of presenting temporal trends, geographic differences, their combination, and whether the data is linear in nature (e.g. viral loads), or categorical (e.g. variant detection or some threshold value being reached). Some practical examples on how to approach visualising WBS-data are given, together with some considerations on the target audience.

In addition, some remarks will be made on topics particular to wastewater monitoring that tie in with the choice of statistics to present, given the common need to aggregate results over time or space, and units of measurement that the general public finds hard to interpret, such as log-scaling and normalisation.

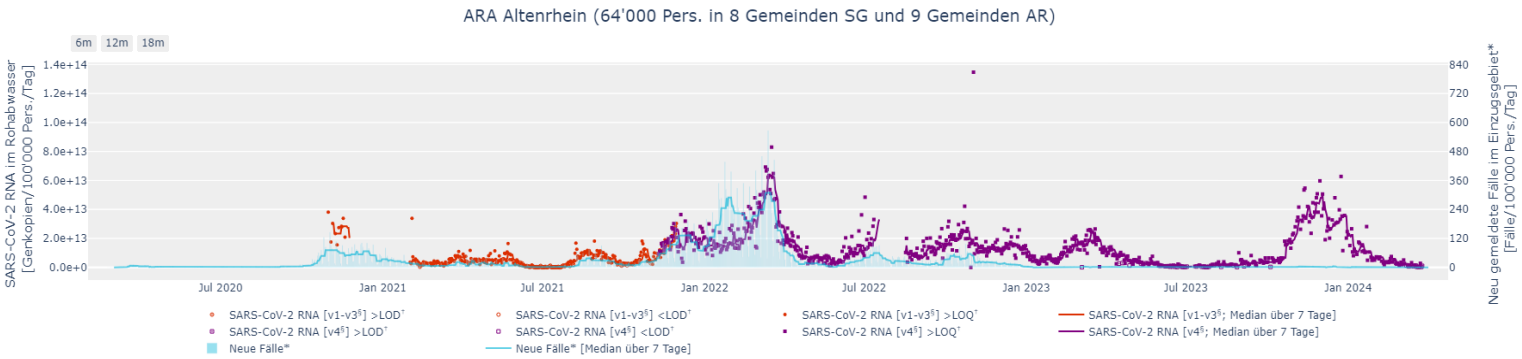
4.5.4.1 OVERVIEW OF DATA AND DATA FORMATS

Generally, two types of data visualisation techniques are common across countries to present information related to the WBS analysis to detect and study SARS-CoV-2 concentration in influent wastewater treatment plant (WWTP) flow.

TIMESERIES

The first and most common type of visualisation is the presentation of a timeseries graph of short-, mid- and long-term SARS-CoV-2 concentration data (Figure 7), on a variety of geographical- or administrative levels, ranging from WWTP to the national level. The virus

Figure 8. Viral concentration time series visualisation from the Swiss eawag SARS-CoV-2 in wastewater

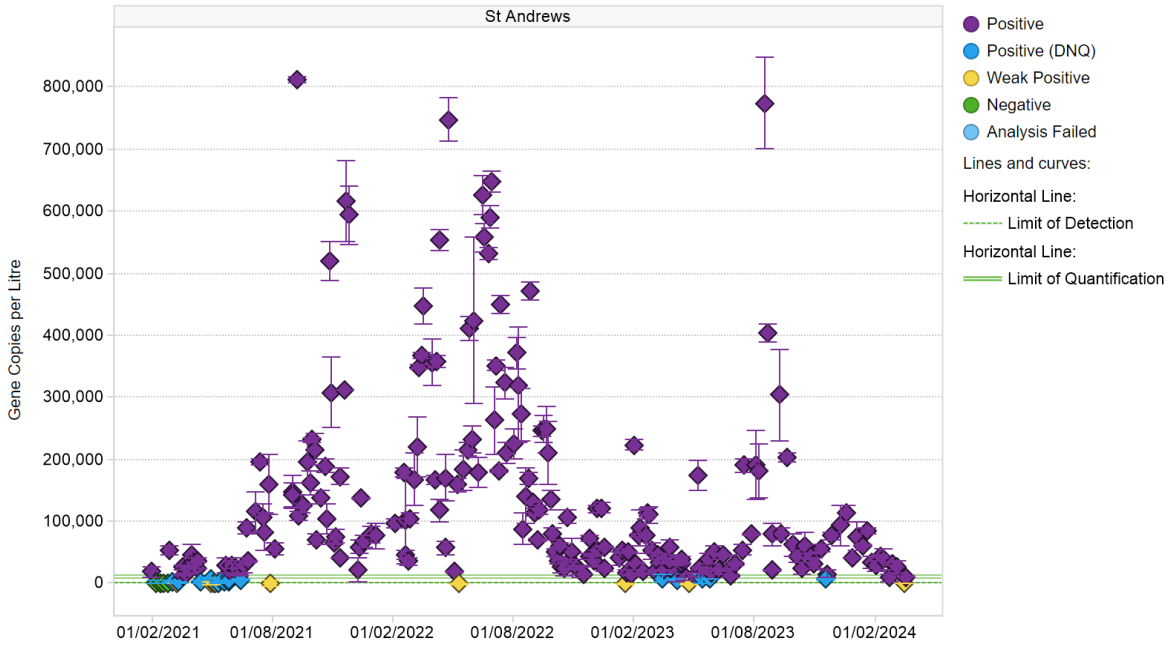


Source: https://informatics.sepa.org.uk/RNAmonitoring/

concentration is often presented in Genome Copies (gc) or viral load (RNA copies) by wastewater volume (e.g. gc/L, gc/ml). Since most measurements are based on 24-hour influent samples, the units are usually presented per day (/day). Sometimes a normalisation to the number of sampled inhabitants is added, leading to a (per 100k- or 1 million inhabitants, e.g. RNA copies/day/100k inhab.) units. When such normalisation is applied, it is recommended to synergise the information as much as possible with any other epidemiological indicators that are reported. Not only does this make results easier to interpret and compare it also reduces the threshold to include the wastewater signal in public reporting and policy considerations. This further allows plotting other indicators, like positive tests or hospitalisations by making use of a secondary Y-axis (Figure 8).

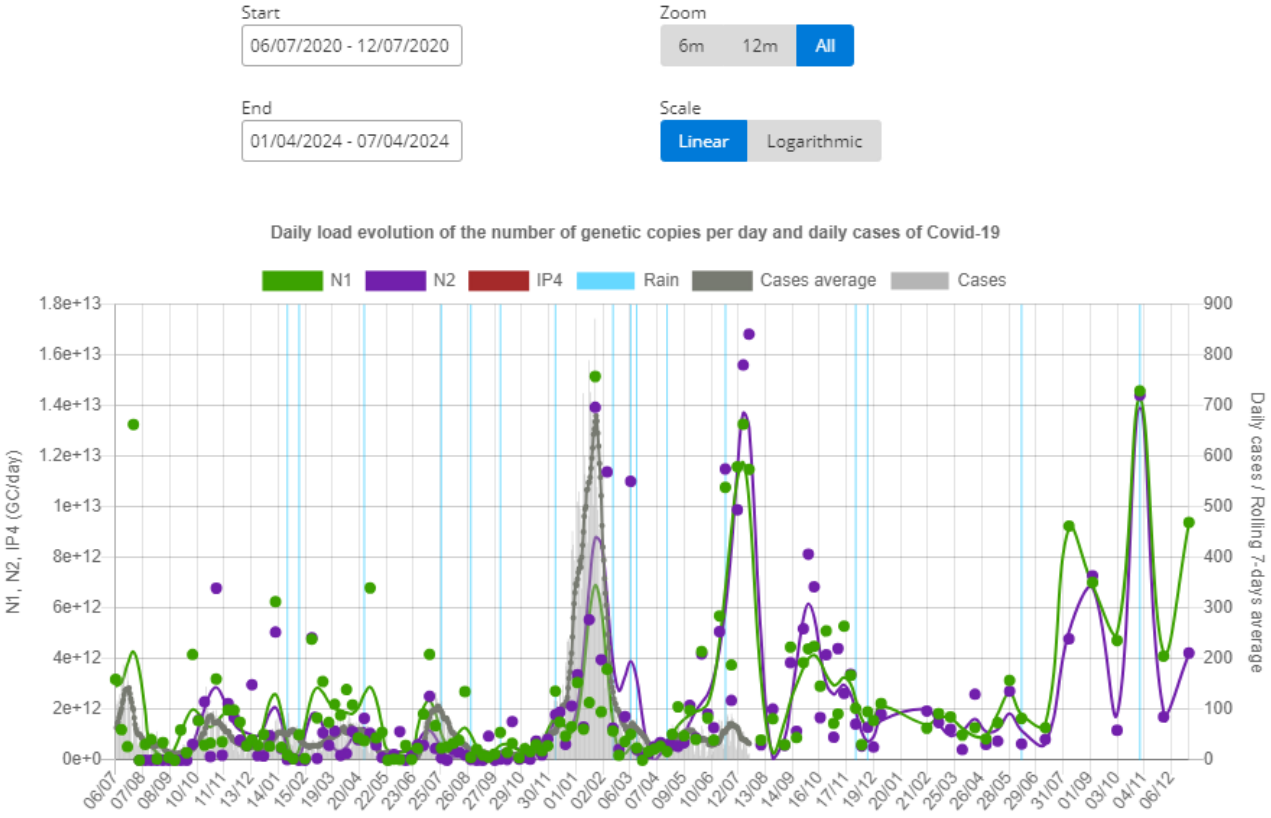
Useful additions to this type of timeseries data are trendlines based on common forms of smoothing of the individual measurements (e.g. moving averages, splines, or local regression smoothing), in order to guide the eye and show a trend through the scatter that is usually inherently present in these types of measurements.

Figure 9. Scottish Environment Protection Agency (SEPA) national RNA monitoring project



Source: https://sensors-eawag.ch/sars/altenrhein.html

Figure 10. Time series representation of virus concentrations of different targets, moving averages and an overlay with reported case-data



Source: https://sarsaigua.icra.cat/

In some cases, virus load is disaggregated by measurements targeting different genes (e.g. N1, N2 and/or IP4 in Figure 10) or results of different laboratory methods (Figure 8 /Figure 10). This categorical information can be represented through different colours of the dots associated with measurement, or e.g. background shading in the case of temporal characteristics. Of course, adding information to one visual increases its complexity and may not be suitable for all target audiences. In the case of interactive graphs, this could be solved by allowing users to toggle between display options. In fixed graphs such as in printed reports, the information may be simplified through categorisations, although these should be used carefully as they reduce the amount of information available to the viewer, or may lead to serious data distortions. The same methodology can be applied to showing data points from different variants of the virus or different levels of severity.

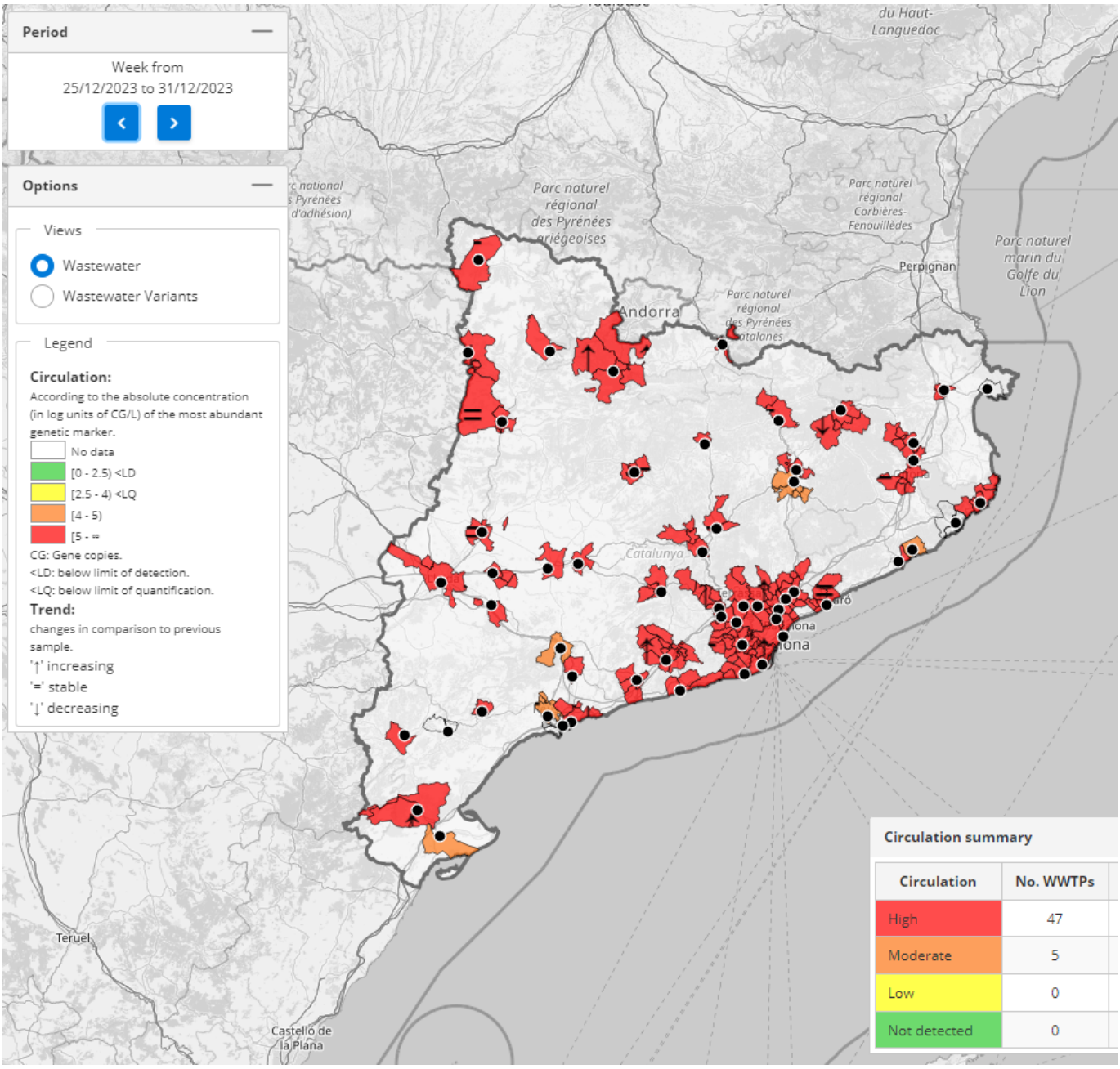
On a few occasions, rain data is also included (blue lines in Figure 10) in order to provide additional insight into interpretation of the data itself, since this may influence virus concentration in wastewater through dilution. In other cases, two additional numerical values are also presented to understanding how much weight should be given to the data. These are known as the Limit of Detection (LoD) and the Limit of Quantification (LoQ) (horizontal green lines in Figure 9). The limit of detection is the value at which the test has been determined to detect the virus material with certainty, and the Limit of Quantification is the value, above which the test has been deemed to measure to virus material with a high degree of accuracy.

Finally, for reporting data to the general public, most countries provide utilities to manage all these functionalities. The time horizon (starting and ending dates) is usually configured through a date time picker (e.g. top left function Figure 10), or through other time-predefined buttons (e.g. 6 months, 12 months, or all data, in top right function in Figure 10). Vertical axis representation can be changed among linear and logarithmic scales, to represent data in absolute or percentage relative values respectively (top right function in Figure 10).

THEMATIC MAPS

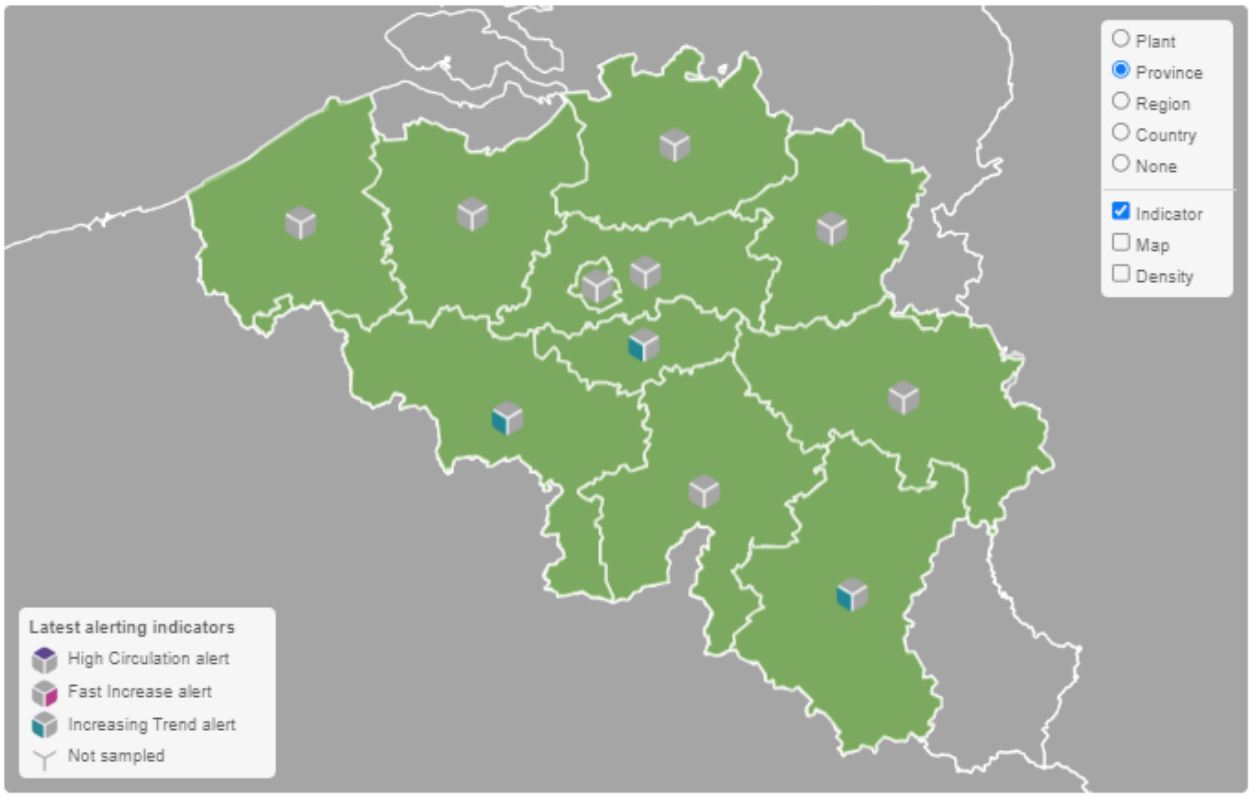
The second most used data visualisation practice provides a geographical representation of areas under monitoring. Geographical data helps to understand the magnitude of the analysis, and the areas and population covered by the analysis. Additionally, categorisation by colours is used to represent different levels of severity. An example is shown in Figure 11. The location of WWTPs can be represented through a dot on

Figure 11. Thematic map of viral concentrations from the Catalan surveillance network of SARS-CoV-2 in sewage



Source: https://sarsaigua.icra.cat/

Figure 12. Thematic map of Belgium, with a dice-shaped, three-part indicator that shows the state of the virus in the sewage for several locations in one map



Source: https://wastewater.sciensano.be/dashboard/covid19/en/

the map, and the covered regions (e.g. municipalities or WWTP catchment areas) are represented by polygons.

Categorisation is preferred over a gradient scale for parameters such as these, as they provide more contrast between the sometimes small areas that are filled in on the maps. The categorisation also lends itself to not only showing absolute concentrations, but derived parameters, such as trends of concentrations or indicating presence or absence of certain variants. When combined, these types of visualisations present a more complete picture of the status of the epidemic than showing solely the absolute concentrations.

4.5.4.2 TAILOR TO TARGET AUDIENCE

As is the case with any type of visualisation, it is vital to adapt it to the target audience and the message that is to be understood by that audience. When informing the general public, policy-makers or professionals in the field, the purpose of their viewing the visualisation, and the knowledge gaps of each group, must be taken into account.

Public dashboards are usually built for informing the general public, meaning special care must be taken to clearly explain graphs and units. Specifically, several authors noted that the unintuitive units that are commonly used in WBS can lead to a lack of understanding in the general public. Nobody is familiar with "RNA copies/day/100k inhabitants", especially if the orders of magnitude go into the billions. Though it may be enough to clearly explain the units in the associated texts or captions, a different solution may be to circumvent the issue by normalising the data to a set scale, though this also leads to subjective choices of scales. Categorisation of data is also an option, as shown in Figure 11. Another option is the use of derived parameters instead of absolute viral loads. For example, in Belgium, beyond the wastewater Epi curves, a complementary alerting system has been developed in order to facilitate the objectivity of the alerts given to the decision-makers. This alerting-system is based on 3 distinct indicators, 2 of them screening for the increasing trends (short and long), and 1 screening for the viral circulation level.

- The first one, the Fast Increase indicator, is positive once a short-term increasing trend is detected (cfr pink legend in Figure 12).

- The second one, the Increasing Trend indicator, is positive once a longer-term trend (cfr blue legend in Figure 12) is observed, confirming that the viral circulation is steadily increasing for at least 7 days.
- The third one, the High Circulation indicator (cfr purple legend in Figure 12), is not trend-associated, but alerts on the level of viral circulation. It gives information on the current location on the epidemic curve, and is positive when the viral circulation level has reached at least half of the level observed for the previous and most comparable wave, epidemiologically speaking.

These indicators are computed on normalised viral loads per treatment plant to allow for a comparison with a reference period as well as between analyses performed by distinct laboratories. Figure 12 shows those alerting results at the local level of the distinct catchment areas covered, for a specific date.

4.5.4.3 USE OF AGGREGATED DATA

Aggregation of data is an important tool in visualising outcomes wastewater-based monitoring, when measurements need to be combined to arrive at a quantitative trend, categorical state-change, and / or a large geographical area than the unit of analysis. We can define aggregation as the combining several individual results to one more clustered result, for example the combination of results from several municipalities to get a result on a provincial or national level (Figure 13). But also, aggregation of data on a temporal scale can be useful in reporting results, for example combining data points to weekly averages.

BENEFITS

Showing aggregated data is usually a necessity, because national policies in Europe up to now have mostly been based on data aggregated to the national level. The same holds for health services, which are often organised regionally and require data that is tailored to their organisational level in order to make optimal use of the data for more local strategies. The benefits of using aggregated data are:

- Reduction of variance (high degree of fluctuation on individual measurement level is averaged out).
- Summary when a problem is composed of too much data.
- Final results better suited for the public.
- Averaging out of seasonality (e.g. weekday) effects.

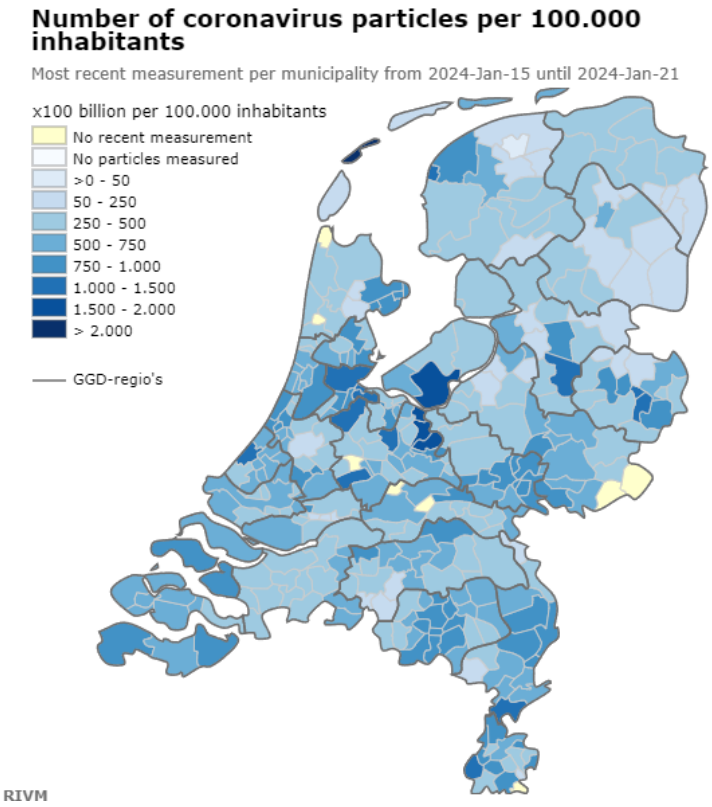


Figure 13. Example of map with aggregated data. The map shows the average number of virus particles in sewage (the viral load) in a selected period, by municipality, per 100,000 inhabitants

Source: https://www.rivm.nl/en/coronavirus-covid-19/current/weekly-update

DRAWBACKS

- With aggregation comes an inescapable loss of information. From a national average viral load for example, you cannot reconstruct how the viral loads are distributed over the country. Interesting phenomena at lower level are not detected.
- Depending on the intended use of the aggregated data, there is a need for additional information in order to be able to calculate the aggregated number. This is especially the case for wastewater-based data, since catchment areas or sewer sheds do not necessarily adhere to the same borders as administrative regions of interest. This generally requires information on the number of inhabitants in the catchment area, the sewer system connection or flow direction of the household wastewater, and potentially a host of confounding issues such as industrial waste proportions, flow rates, or delays between shedding and sampling events. For example, in the Netherlands, in order to aggregate to a provincial level, detailed information on the number of inhabitants of each sewage treatment plant STP/WWTP catchment area and their relation to the municipal borders is provided by Statistics Netherlands. This enables the conversion to the different layers of government (municipality/provincial/national).
- Sometimes aggregation does not make sense; due to the large variation in WWTP sizes, aggregation can distort relevant data; sometimes a single (STP)/WWTP will dominate the values of averaged data to the extent that any other locations effectively do not contribute. This can sometimes be circumvented by using normalisation in order to convert the information of each location into comparable indicators. An example is shown in Figure 12 in the 'Tailor to target audience' section.
- It is also possible that when aggregating data from different locations, regional changes in viral load may average out due to differences in the time a local viral load starts changing. For example; large cities may experience a rise in cases before rural areas are affected, leading to a slower rise of the national average than the regional increases in those large cities.

4.5.4.4 SUMMARY AND TAKEAWAY POINTS

This chapter describes some visualisation features that are commonly used in WBS, alongside some key points that should be taken into consideration when visualising WBS data. WBS measurements are usually presented as a time series or on a thematic map.

When plotting a time-series, it can be beneficial for the target audience to plot normalised data, to make WBS data more readily comparable to other epidemiological indicators. Moreover, inclusion of for example a rolling average, can help with the interpretation of scattered data points. Aggregated data in timeseries plots are commonly used when plotting regional or national averages, the latter is often used in policy making decisions.

Alternatively, geographical visualisations in the form of thematic maps lend themselves for more than plotting absolute values of viral loads. When categorising, derived parameters such as trends and binary 'yes-no' detections of virus variants can be plotted intuitively. Two examples are shown in Figure 11 and Figure 12.

The target audience must be kept in mind when visualising WBS, as the subject and the units are not used by almost anyone in everyday life. Clearly explaining the units is a must, or data can be categorised to add a layer of interpretation. Use of derived parameters, such as info about whether the trend is increasing, can also be used.

4.6 DECISION SUPPORT

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In the kitchen of public health strategy, decision support systems are akin to the central table where all ingredients come together to inform the perfect dish. This chapter offers a step-by-step guide to creating a decision-support system that incorporates wastewater surveillance data, aiding health officials at every level in their fight against pandemics.

The chapter describes dashboard design, trigger values and triggered actions, influencing factors, uncertainties, WBS as part of wider range of tools and information.

4.6.1 KEY INGREDIENTS



- **Wastewater Data** (viral concentrations, variants, operational parameters). The raw data collected from wastewater samples, including the concentration of viral particles and the presence of variants, which serve as indicators of community infection levels, trends and potential outbreaks.
- **Data Platform** (Integration, accessibility). The digital infrastructure that stores, processes, and disseminates data from laboratories to decision-makers, potentially including public dashboards. Data is presented in an accessible and actionable format for health officials at local, national, and international levels.
- **Decision Support System** (Computerised, human-powered, hybrid models). The analytical tools and processes that transform raw data into actionable insights, which can range from fully automated algorithms to human-centred analysis.
- **Trigger Values and Triggered Actions:** Thresholds and response protocols determining when the data indicates that specific actions, such as public health advisories or lockdowns, need to be taken.
- **Influencing Factors and Uncertainties** (Population dynamics, data variability). Variables that can affect the interpretation of data, much like unexpected ingredients that can alter the taste of a dish.
- **Wastewater Surveillance as Part of a Wider Toolkit:** Complementary data sources and integrated approach, recognising that wastewater surveillance is most effective when used alongside other public health tools and information.
- **Health Decision-Makers** (Management, operations, planning). The individuals and teams at various levels of the health sector who utilise the decision support system to inform their actions.

4.6.2 RECIPE



1. **Data Integration and Platform Design**. Develop a system that allows for the seamless transfer of data from the laboratory to the decision support platform. This platform should be designed for ease of use and accessibility. It should cater to the varying needs of health officials at different levels of authority.
2. **Decision Support System Development.** Combine statistical models, predictive analytics, and user-friendly interfaces to create your decision support system. This system should be able to process the wastewater data and provide clear indications of when intervention measures may be necessary.
3. **Implementation and Scaling.** Roll out the decision support system, starting with pilot studies to prove its efficacy. Gradually scale up, integrating it into existing health response and control structures.
4. **Setting Trigger Values and Response Protocols.** Establish clear trigger values based on wastewater data that, when reached, prompt predetermined actions. This step ensures a proactive rather than reactive approach to public health threats.
5. **Accounting for Influencing Factors and Uncertainties.** Integrate methods to adjust for influencing factors and acknowledge uncertainties within the data. This will help avoid misinterpretation and ensure the reliability of the system.
6. **Integrating Wastewater Surveillance with Other Data.** Combine wastewater data with other health metrics and tools to provide a comprehensive view of the public health landscape, allowing for more nuanced and effective decision-making.
7. **Engaging with Health Decision-Makers.** Involve health officials in the design process to ensure the decision support system meets their needs for resource allocation and real-time decision-making.
8. **Feedback Loop and Continuous Improvement.** Establish mechanisms for feedback from health decision-makers to continuously refine and improve the decision support system. This iterative process ensures that the system remains responsive to the needs of its users.

4.6.3 TIPS



- **User Accessibility:** Design the system with the end-user in mind, ensuring that it is user-friendly for those with varying levels of computer proficiency.
- **Transparency and Education:** Clearly communicate the limitations and uncertainties of the data to all stakeholders, including the public, and provide education on how to interpret and act on the information.

- **Flexibility:** Be prepared to adapt the system to meet the evolving needs of the health sector, especially during rapidly changing situations like a pandemic.
- **Holistic Approach:** Always consider wastewater surveillance as a complement to other surveillance methods, never in isolation.

Combining these ingredients and following the recipe will yield a robust decision support system that harnesses the power of wastewater surveillance. When served to health decision-makers, it becomes a critical tool in the public health arsenal, helping to manage the current pandemic and prepare for future health threats.



4.6.4 THE SCIENCE BEHIND

In general, decision support can help people make decisions about problems that may be rapidly changing and are not easily specified in advance such as a pandemic. Decision support empowers health professionals to make informed choices on intervention measures. In case of wastewater surveillance, information usually takes the form of data on the viral load or variants or both plus additional information regarding operational parameters to assure proper sampling procedures. Such data is generally transferred from the laboratory to a data platform that may or may not be a decision support system.

Decision support systems can be either fully computerised or human-powered, or a combination of both, and involve statistical and/ or modelling exercises to lead to trigger values. Health decision-makers will generally have access to the decision support system and in some cases the data may be accessible to the public via an open data platform. Ideally, decision support systems such as COVID-19 dashboards demand to be easy to use by non-computer-proficient people in an interactive mode. More is explained on these platforms such as dashboards in the Data Visualisation Section (Section 4.5). More on communication is described in the Communication and Engagement Section (Section 4.1).

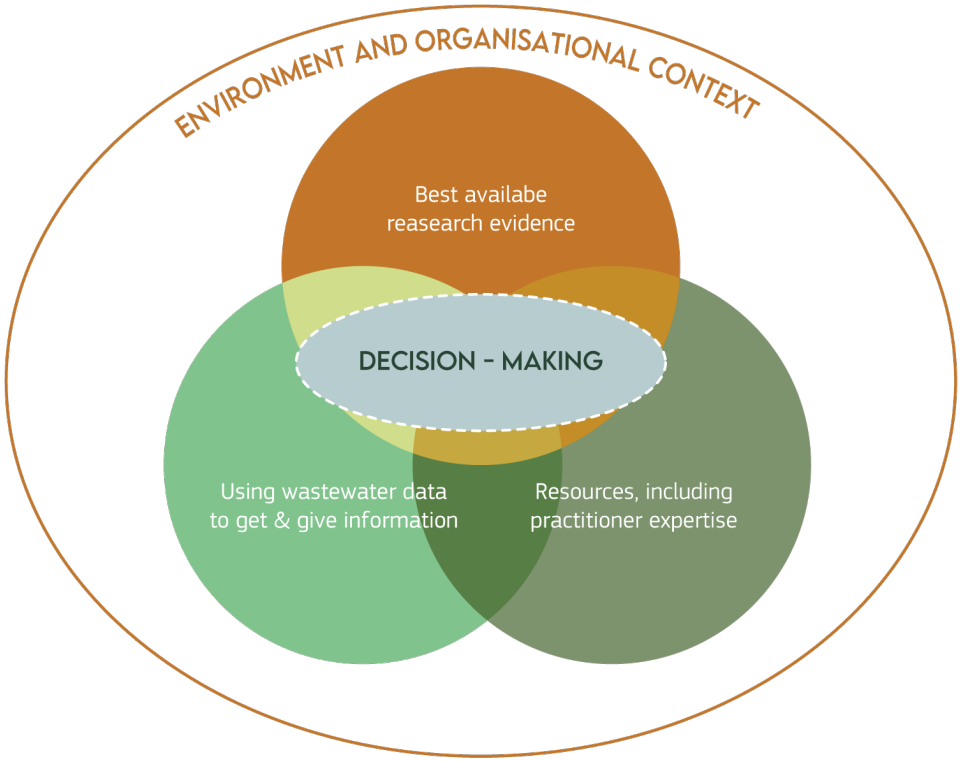
Wastewater surveillance has been largely initiated from academia. While academics have perceived decision support systems as a tool to support decision making processes, users may see it as a tool to facilitate organisational processes and communication.

It has been challenging for many data scientists to integrate SARS-CoV-2 data from wastewater in the COVID-19 response and control structures. Such structures generally exist and had to be scaled up during the pandemic. Timing and scaling of intervention measures such as infection prevention and control (IPC) and lockdowns were informed by incidence rates and hospitalisations. Here, wastewater data can also play a role. It is essential to establish how SARS-CoV-2 data from wastewater will be used in health sector decision-making for the COVID-19 response. On the other hand, it may take time for health and water professionals to understand the value of the data and may, therefore, require stages such as proof-of-principle and pilot studies.

Wastewater surveillance derived decision support can inform health decision-makers at the national, regional and local level depending on the objective and decision level. Health decision-makers can entail the management, operations and planning levels of health care facilities, municipal health centres and health care facilities.

The Venn diagram of Figure 14 shows the relationships among five concepts. Three circles in the middle of the graphic overlap with each other: 1) "Best available research evidence," 2) "Resources, including practitioner expertise," and 3) "Populations characteristics, needs, values, and preferences." Overlapping these three circles and in the centre is the circle "decision-making." This group of 4 circles is surrounded by "Environment and organisational context."

Figure 14. Domains that influence evidence-based decision-making

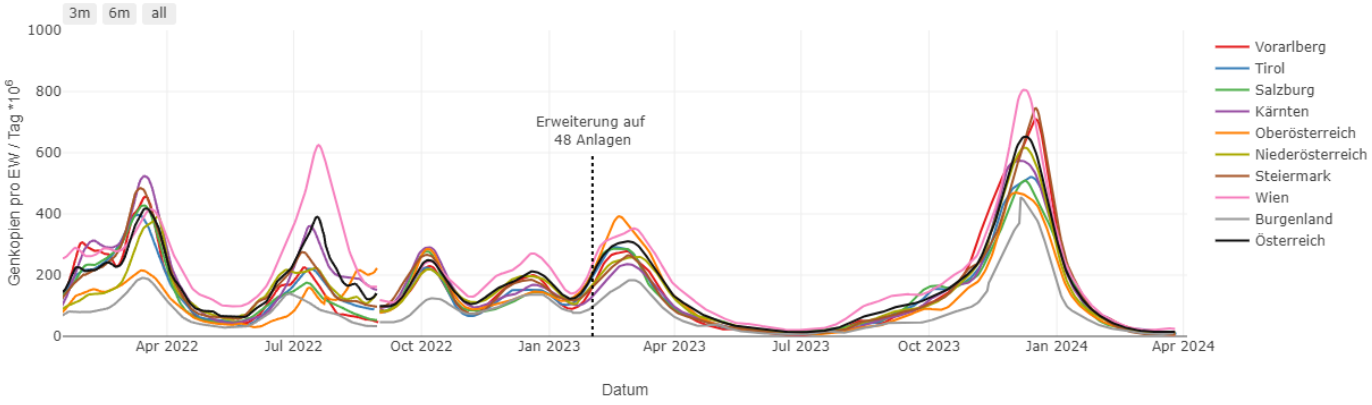


Source: Satterfield et al., 2009
(redesigned)

4.6.4.1 DASHBOARD DESIGN FOR DECISION SUPPORT

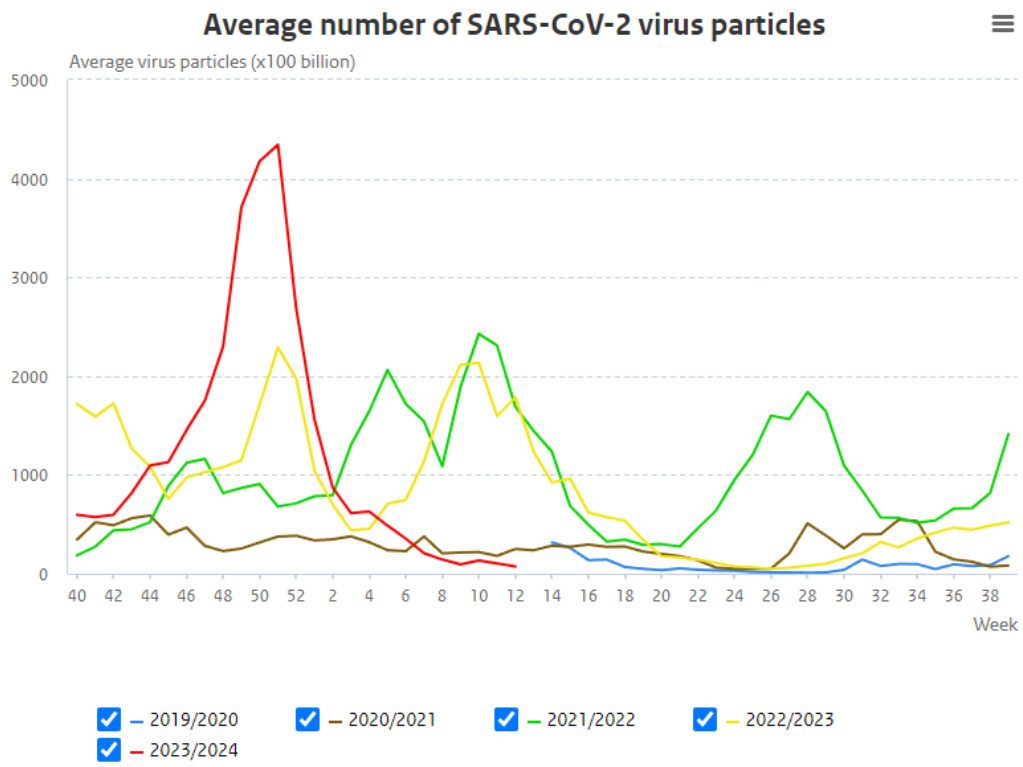
Examples of dashboards for communication are given in Figure 15, Figure 16 and Figure 17. These dashboards can assist with rapid visualisation and presentation of data to help inform decision-makers.

Figure 15. Austrian Dashboard based on total inhabitants per state



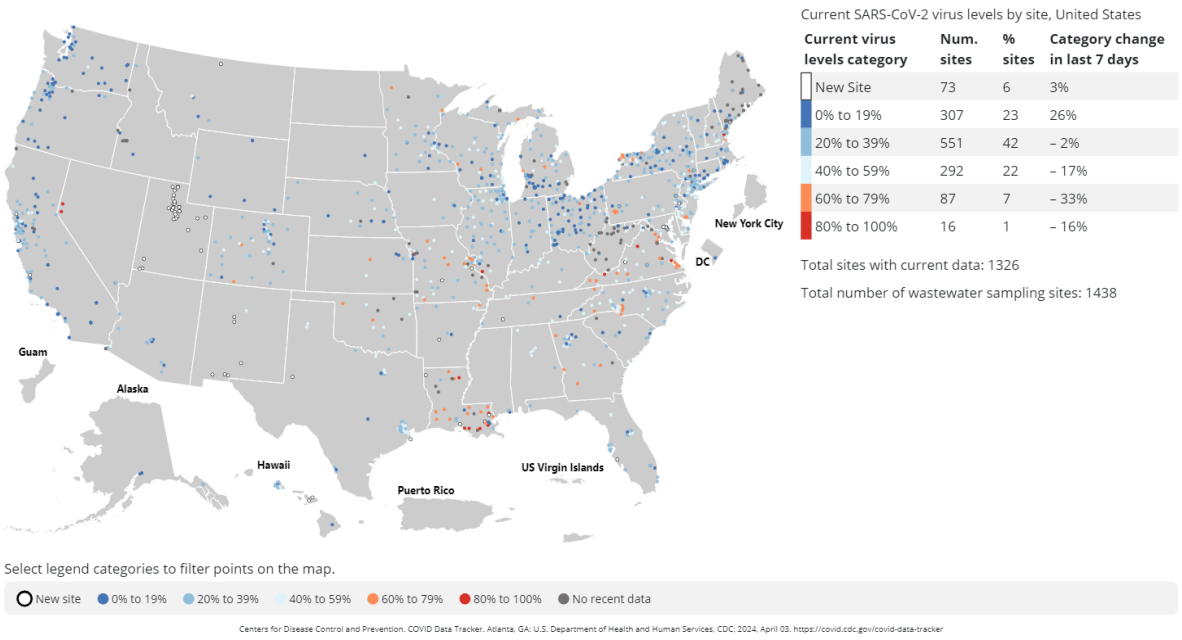
Source: https://abwassermonitoring.at/dashboard/

Figure 16. National average viral load detected in sewage surveillance in the Netherlands



Source: https://covid.cdc.gov/covid-data-tracker/#wastewater-surveillance

Figure 17. National WBS dashboard CDC, USA



Source: https://www.rivm.nl/en/coronavirus-covid-19/current/weekly-update

Table 6. List of common methodologies to calculating trends in virus load.

TREND ANALYSIS METHODS	BACKGROUND	TRIGGERS	LITERATURE
Slope	Linear regression of 7-day values	% change	(Ai et al., 2021a)
Relative strength index (rsi)	Relation of rising/falling values to a baseline	90, 80, 70 | 30, 20, 10	(Chan et al., 2023)
Percent change (rc)	Slope with confidence intervals	99%, 95%, 90%	(Chan et al., 2023), (Centers for Disease Control and Prevention, 2022)
Mann-kendall (mk) trend test	Test for monotonic trends in time-series	Significance 0.01, 0.05, 0.1	Chan et al., 2023
Loess	Deterministic part of variations in the data, point by point	n/a	(Vallejo et al., 2020)

Source: Developed by the authors for this document.

4.6.4.2 HEALTH OFFICIALS AT INTERNATIONAL LEVEL

For larger trends and dashboard comparisons, including research projects and national surveillance, a number of international dashboards have been developed, such as COVIDPoops19(https://www.arcgis.com/apps/dashboards/c778145ea5bb4daeb58d31afee389082) and the US National Wastewater Surveillance System (NWSS) (https://www.cdc.gov/nwss/wastewater-surveillance.html).

4.6.4.3 TRIGGER VALUES AND TRIGGERED ACTIONS

SARS-CoV-2 wastewater monitoring data dashboards visualising individual sample results of biomarkers as well as variants of concern (VoC) can include an indicator for increasing, stable or decreasing trends in virus load. To determine the current trend, various thresholds – so-called trigger values - are in use. Preferably, these calculations allow a valid indication of rising, stable and falling trends for both low and high copy numbers of SARS-CoV-2. Different methods can be used, for example, the German Robert Koch Institute (RKI) uses a locally estimated scatterplot smoothing (LOESS) regression for trend analysis, whereas the US Centers for Disease Control and Prevention (CDC) use percent change methods (Table 6). These methods are not standardised, yet.

4.6.4.4 INFLUENCING FACTORS AND UNCERTAINTIES

Several factors can influence the results of wastewater testing and should be considered when interpreting the results. These include factors relating to the type of sewershed, environment, population, sampling, and analysis. Environmental factors include weather conditions such as rainfall, dissolved oxygen concentration, and temperature. Rainfall can either dilute the viruses in the wastewater or detach biofilms in sewer pipes to which virus particles are attached. Thus, heavy rainfall events may lead to either lower or higher virus concentrations.

Population factors including age distribution, holidays, public events or other festivities, but also pandemic control measures such as lockdowns or travel restrictions can influence the results. Tourism and commuting should also be considered. This means that samples taken on different days can be influenced by factors such as weekends and commutes, or by rare events such as larger public events or festivities. The latter may explain sudden increases in virus concentration due to a higher disease burden compared to a community without such an activity.

Factors regarding sampling concern the size and type of the sewershed (combined sewer vs. stormwater sewer), predominant redox conditions in the sewer, place of sampling (WWTP influent vs. sampling in the sewer system), the type of wastewater (e.g., general population vs. commercial neighbourhoods) the sampling method (grab vs. composite), the sampling duration and time during the day, transport conditions and storage conditions. These factors affect the decay rate of the viral RNA and as a consequence the strength of the signal. The kinetics of viral RNA decay are also affected by various wastewater parameters (e.g., dissolved oxygen concentration, temperature, pH) and by the duration of transport, i.e., by the architecture of the sewer. Different sampling strategies and the sampling duration, storage and transport conditions may further affect viral RNA decay.

Different RNA recovery and extraction methods may have different efficiencies. Methods and equipment for detection and quantification of viral RNA have an error of measurement. Different methods for data analysis may result in slightly different outcomes. For example, in a trend analysis a linear regression compared to a 5-day average may result in slightly different trends over time.

Virus shedding may differ from person to person in probability and magnitude. In addition, virus shedding may be affected by the type of symptoms, immune status and virus variant.

4.6.4.5 WASTEWATER SURVEILLANCE AS PART OF WIDER RANGE OF TOOLS AND INFORMATION

Viral loads in wastewater at times of available test data and willingness-to-test have shown good alignment with positive tests and more so with percentage of positive tests. Moreover, a strong association can be observed with hospitalisations (Van Boven et al., 2022). In the Netherlands, signal values for interventions were initially based on positive tests and hospitalisations and later also on viral loads in sewage. Other early indicators included the non-specific indicator self-reported symptoms (Geubbels et al., 2023).

Table 7. List of common methodologies to calculating trends in virus load

ENVIRONMENT FACTORS	POPULATION FACTORS	SAMPLING AND ANALYSIS
Type and size of sewershed (combined sewer vs. stormwater sewer)	Age distribution, immune status	Place of sampling (WWTP influent vs. sampling in the sewer)
Type of wastewater (general population vs. commercial neighbourhoods)	Rare events (holidays, festivities, public events)	Sample type (grab sample vs. composite sample)
Redox conditions in the sewer	Common events (Tourism, commuting)	Sampling duration
Weather conditions (Rainfall, temperature)	Pandemic control measures (e.g. lockdowns, travel restrictions)	Transport and storage conditions
	Viral shedding, virus variants	• Sample processing • RNA extraction • PCR

Source: de Roda Husman unpublished

4.7 STATISTICAL ANALYSIS AND ASSOCIATIONS

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Wastewater surveillance has emerged as a critical tool in public health, especially highlighted during the COVID-19 pandemic. This chapter delves into the statistical analysis and associations of data derived from WBS, providing a structured approach to interpret the spread and trends of viruses, such as SARS-CoV-2, within communities.

4.7.1 KEY INGREDIENTS



Each ingredient plays a specific role in the overall analysis of wastewater surveillance data. Depending on the specific goals of the study and the available resources, some ingredients might be substituted, or additional ones might be added to enhance the analysis. It is important to choose the right combination of ingredients to ensure that the analysis is robust, accurate, and provides actionable insights for public health decision-making.

- **WBS Data:** This is the core data obtained from wastewater samples, which contains measurements of viral RNA concentrations in wastewater.
- **Clinical Surveillance Data:** This includes epidemiological indicators, such as data on COVID-19 case counts, hospitalisation rates, and other clinical outcomes.
- **Genetic Sequencing Data:** This data provides information on the genetic variants of the virus present in the wastewater. Metagenomic sequencing could offer a broader view of all microorganisms present, which may be useful for comprehensive surveillance beyond a specific virus.
- **Statistical Software:** Programmes/tools for data analysis, such as R, Python, SAS, SPSS, or Stata. Depending on the complexity of the analysis, specialised software for time series analysis (e.g., EViews), machine learning (e.g., TensorFlow), or bioinformatics (e.g., QIIME for microbial community analysis) could be used.
- **Statistical Methods:** These are the techniques used to process and analyse the data, including smoothing, regression, and trend identification methods. In place of traditional statistical methods, one might consider using machine learning algorithms to detect patterns or anomalies in the data. Bayesian statistical methods could also be used to incorporate prior knowledge and quantify uncertainty.
- **Computational Models:** Models that use statistical methods to make predictions or simulate epidemic scenarios.
- **Quality Control Protocols:** These are procedures to ensure the accuracy and reliability of the data, such as the use of controls in laboratory assays. Robustness checks, sensitivity analyses, and interlaboratory comparisons can supplement quality control measures.
- **Normalisation Factors:** Variables used to adjust the viral concentration data to account for dilution factors or variability in wastewater flow. Chemical markers of human waste or population size estimates could provide additional normalisation options.
- **Geographic Information System (GIS) Data:** Geospatial data that allows mapping and spatial analysis of WBS data in relation to the population and infrastructure. Remote sensing data could be used to assess environmental factors that might influence pathogen levels or spread.
- **Epidemiological Models:** These models use WBS and clinical data to estimate transmission dynamics, such as the effective reproductive number (Rt). Network models could be employed to study the impact of human movement and interactions on virus spread

4.7.2 RECIPE 

1. **Data Collection and Preprocessing:** Starting from WBS data, clinical surveillance data, and any additional data sets
   - Conduct initial quality control checks to ensure data accuracy.
   - Use descriptive statistics to get an overview of the data and identify any irregularities or outliers.
   - Apply normalisation factors to adjust for variables like dilution factors or wastewater flow.
2. **Data Exploration and Visualisation.** To obtain a refined dataset with reduced variability and a clearer view of the underlying trends in viral concentrations over time:
   - Create visualisations like time-series plots, scatter plots, and boxplots to examine the distribution and trends in the data.
   - Use GIS data to map viral concentrations and identify spatial patterns related to wastewater infrastructure and population distribution.
3. **Statistical Noise Reduction and Smoothing.** To review the variability and noise present in the WBS data, apply appropriate smoothing techniques such as LOESS/GAM to filter out noise and highlight genuine trends. Select smoothing parameters that suit the data's characteristics and the analysis's goals.
4. **Trend Identification.** Utilise the smoothed data to focus on identifying and characterising trends.
   - Select appropriate time windows and methodologies for assessing trends, such as linear regression or the Mann-Kendall test.
   - Calculate percentage changes, growth rates, or logarithmic returns to quantify recent changes in viral load.
   - Use visual tools like fitted curve plots to illustrate trends and understand their direction and magnitude.
5. **Data Aggregation from Multiple Sites:** Combine and organise data from various WWTPs, considering the differences in methodologies and geographic scope, to represents a regional or national picture of viral levels and temporal trends.
   - Apply weighted averages to merge data points, considering factors like population served or wastewater flow.
   - Ensure comparability of data through normalisation techniques and check for heterogeneity between different WWTPs.
   - Consider additional scaling methods if necessary to enhance the robustness of aggregate indicators.
6. **Association with Clinical Surveillance Data:** Correlate WBS data with clinical surveillance data, including additional clinical outcomes. Explore correlations to assess lead times and predict public health trends. Develop and evaluate regression or machine learning models to further investigate the relationship between WBS and clinical data.
7. **Estimating SARS-CoV-2 Variant Proportion.** To provide insights into the prevalence dynamics and emergence of new variants in the community.
   - Use statistical methodologies to estimate the prevalence of different SARS-CoV-2 variants, considering lineage-defining genetic variations.
   - Solve constrained optimisation problems to derive the most likely proportions of variants present in the community.
8. **Model Development and Forecasting.** Prepare to build predictive models using the cleaned and smoothed data.
   - Develop epidemiological models or machine learning algorithms to forecast trends and simulate epidemic scenarios.
   - Validate and test models using historical data to ensure their predictive accuracy
9. **Interpretation and Reporting.** Synthesise findings into actionable insights, considering the limitations and uncertainties of the models and methods used. Report the results in a format that is accessible and informative for public health decision-makers.

4.7.3 TIPS 

- Always **consider the noisy and scattered nature of WBS data**; pre-processing is essential for accurate analysis.
- When identifying trends, **be aware of the potential for non-monotonic behaviours** and the impact of sampling frequency.
- Ensure that **data from multiple sites are comparable before aggregation**; heterogeneity can lead to misleading estimates.

- **Be cautious when interpreting associations**; the relationship between viral loads and clinical data may not be consistent over time or across locations.
- Remember that **statistical significance in trend analysis is influenced** by both the magnitude of change and the number of observations.
- In forecasting and modelling, **always consider the limitations and uncertainties** inherent in epidemic data and models.



4.7.4 THE SCIENCE BEHIND

The statistical analysis of WBS data serves the purpose of identifying important information that can be used to provide public health authorities with reliable and documented results to support evidence-based decisions, related to preparedness and response. Since the early stages of the COVID-19 pandemic WBS has been widely used to detect and quantify SARS-CoV-2 RNA in various wastewater settings and explore the trends and the temporal evolution of the virus spread within communities. Thus, a major goal is to identify, document and correctly interpret the true signals of viral concentration changes over time, in terms of both direction and magnitude. An important challenge is the often noisy and scattered nature of the data which require appropriate statistical pre-processing to be applied, before analysing and interpreting the results. Statistical methods can also be applied to characterise the extent of virus spread. WBS data are often analysed against conventional clinical surveillance data since WBS is recognised as a complementary surveillance tool. The epidemiological indicators usually include COVID-19 case counts or incidence/prevalence rates, but significant efforts have been made to associate wastewater viral loads with hospitalisation rates. There is a plethora of publications aiming to derive disease incidence/prevalence rate from WBS data using various statistical frameworks. However, caution is suggested since both conventional epidemiological surveillance and WBS have limitations, and changes in practices may affect the temporal consistency of the identified associations. The analysis of WBS against clinical surveillance data can also reveal the temporal aspects of the correlation, determining if changes in viral concentrations precede the corresponding changes in conventional surveillance indicators and to determine if WBS can be used as an early-warning tool to alarm health authorities for upcoming epidemic waves or anticipated pressure to the healthcare system. As changes in the circulation of different variants can have a severe impact on the epidemic curves, especially when new Variants of Concern (VOC) emerge in a community, statistical procedures can be used to more accurately estimate the proportion of prevalent variants in sewage samples and consequently the magnitude of variant circulation within communities. Moreover, appropriate methods to handle data from multiple Wastewater Treatment Plants (WWTPs) are needed aiming to derive regional or nation-wide indicators, to interpret regional or nation-wide time trends and to examine the relationship of WBS with other epidemiological indicators at a larger geographical scale. Handling heterogeneity issues is critical when performing the above-mentioned tasks. Finally, statistical and computational methods can be applied for short-term forecasting of both WBS and conventional epidemiological indicators, although this approach should be performed cautiously and thoroughly consider the limitations and uncertainties of COVID-19 epidemic modelling, when interpreting the results.

4.7.4.1 DATA EXPLORATION, DATA SMOOTHING AND TREND ANALYSIS

DESCRIPTIVE STATISTICS AND EXPLORATORY ANALYSIS

Performing descriptive and exploratory analyses on the raw data will enhance the investigator's understanding of the dataset and its underlying structure, and assist in better designing the appropriate analysis. In this step, the distribution of variables of interest can be determined, the presence of outliers or unusual observations can be observed, and patterns in the time series can be uncovered. When multiple variables are to be examined, insights into the potential relationships can be derived. At this initial data analysis phase, simple summary statistics (means, standard deviations,

medians, percentiles) and graphs (histograms, density plots, scatter plots, boxplots, time-series plots) can be used. Usually, raw WBS data tend to be inherently variable, left-censored and noisy (Van Boven et al., 2022). Since analytical and operational procedures are constantly evolving, and the epidemiological characteristics of the virus are changing, the present patterns may change in the future.

DATA SMOOTHING

As mentioned above, the wastewater signal is expected to contain not only the desired information on the viral load, but also a significant degree of unexplained variability, for reasons that have been reviewed elsewhere (X. Li et al., 2021). This uncertainty can be dealt with by means of Quality Accuracy/Quality Control (QA/QC), by increasing sampling frequency, by analysing multiple replicates and by incorporating normalised SARS-CoV-2 concentrations to the analysis. However, the presence of statistical noise is likely to persist even following the above-mentioned efforts. Moreover, WBS data may be scattered, with missing or irregular spacing of data points. Thus, a data pre-processing step to distinguish between noise and the actual signal is needed to derive a dataset appropriate for subsequent analyses, but also to properly interpret the viral load evolution over time. This can be accomplished with a variety of curve-fitting methods, which usually involve the creation of a mathematical function to capture the temporal patterns (data smoothing). There is an array of available smoothing methods that can be applied to WBS time-series. Simple Moving Average (SMA)

Smoothing is a commonly used parametric method as it is easy to execute and easy to understand. More robust methods can be implemented by applying non-parametric smoothing where the most appropriate functional form is derived from the data. Non-parametric filtering methods include (among others) locally estimated scatterplot smoothing (LOESS) / locally weighted scatterplot smoothing (LOWESS), local polynomial regression, kernel smoothing (with global or local bandwidth) and spline smoothing. Semiparametric methods such as Generalised Additive Models (GAM) have been applied as well (Vallejo et al., 2022). For all these methods, the choice of the smoothing parameters (time window, bandwidth, number of knots, etc.) is a crucial issue. As there is not an undisputedly superior method, the choice of smoothing technique depends on the nature of the data (distribution, frequency of extreme values and outliers, sampling frequency, presence of censored data) and the goals of the analysis (e.g. out-of-sample forecasting, predicting the true underlying function at the most current time point or all time points, and/or predicting current changes in the trend).

4.7.4.2 IDENTIFYING AND CHARACTERISING TRENDS

This section focuses on methods for the analysis and characterisation of short-term trends in wastewater indicators, since the investigation and interpretation of recent temporal patterns is of particular importance for public health policy-makers. The assessment of trends can be achieved by the means of quantitative trend analysis in specified time windows. Although there is no consensus on an optimal length for this 'look-back period', a reasonable time frame of approximately 2 weeks could suffice to assess recent changes. Extending this time window further is possible, but will increase the likelihood of the occurrence of significant non-monotonic trends, which are more challenging to analyse, interpret and communicate. The frequency of measurements is another factor to be considered when determining the above-mentioned time window. Investigating trends may be approached by a series of methods with varying complexity. Fitted curves on WBS data (with methods described in the previous section) offer a visual representation on the temporal evolution and can estimate the underlying functions of recent trends. However, it is often required to generate easily interpretable quantitative metrics of recent changes in viral concentration levels.

The simplest approach is to calculate and report percentage changes of the average concentrations between two discrete consequent time windows (e.g. weeks). This approach can provide rough estimations of recent trends, but it has certain drawbacks, as it does not utilise all available information. A more informative approach is to estimate percent changes per time unit increase (usually per day) for the time window of interest, which practically reflects the growth rate of viral concentrations. This can be achieved by the means of some form of regression using time as the independent variable and viral load as dependent. Choosing to apply a linear regression for this task has certain advantages, as it is simple, easily interpretable and can be universally applied in any short time period with a monotonic trend, as the function is not dictated by the data. When applying linear regression on sewage, log-transforming concentrations/viral loads can often improve the fit, especially in the exponential phases of the epidemics. Equation 1 presents the formula for deducting growth rates from the slope of the linear regression as formulated by the U.S Center of Disease Control and Prevention (Centers for Disease Control and Prevention, 2022). To account for variability in sampling and laboratory procedures, weighted linear regression can be applied.

$$PDC = (10^{slope}-1) \times 100 \text{ (Eq. 1)}$$

Where:

- PDC: Percentage daily change
- Slope: the slope of the linear regression of date against Log^{10} transformed concentrations/viral loads

When non-linear trends persist even after log-transformation alternative approaches should be considered. There are numerous statistical and computational alternatives to linear regression from which growth rates can be deducted, ranging from typical non-linear curve fitting methods to modern Bayesian computational models. When reporting the results, it is essential to provide data or metrics on the viral load's magnitude in addition to percentage changes, since the same relative change corresponds to significantly different absolute changes at low wastewater virus's levels compared to higher levels. An alternative measure to the standard growth rate is logarithmic return, which has been used recently for COVID-19 cumulative incidence data (Barbeito et al., 2023). Opposite to growth rate, logarithmic return tends to be symmetrically distributed and is almost identical to growth rate for moderate and small values.

Another important issue when determining the presence of upward or downward trends is to determine statistical significance. Non-parametric tests such as the Mann-Kendall (MK) test can be used for the detection of monotonic trends in time series data. When interpreting its results, it should be always kept in mind that statistical significance depends not only on the magnitude of change over time but also to the number of the observations included in the analysis. Thus, more frequent sampling is expected to function in a power-enhancing manner.

Quantitative estimations of trends often need to be transformed to ordinal variables to assist the dissemination process to target audiences. Trends should be, at minimum, categorised as increasing, decreasing and steady. Different approaches can be applied to achieve trend characterisation. For example, the Belgian Network uses two binary indicators (Fast increase, Increasing trend) calculated from normalised concentrations of the past 7 and 14 days (Janssens et al., 2022), while US CDC characterises trends as largely decreasing, decreasing, steady, increasing and largely increasing based on percentage changes in the last 15 days (U.S. Centers for Disease Control and Prevention, 22 C.E.).

In infectious diseases epidemiology and in COVID-19 particularly, growth rates are often accompanied with the estimation of the effective reproductive number (R_t), as a real-time indicator of epidemic growth. It is worth mentioning that research efforts have developed methods for estimating the R_t from WBS data with promising results (Huisman et al., 2022).

4.7.4.3 AGGREGATING/MERGING DATA FROM MULTIPLE SITES

For wastewater surveillance networks involving multiple WWTPs, it is often desirable to draw a representative picture on the virus's levels in sewage and the corresponding temporal trends at regional or at national level, based on data from individual sites. Weighted averages can be used to merge data from multiple sites, where weights are determined by the population served, wastewater flow or by the equivalent population:

$$Weighted\ average = \frac{\sum_{i=1}^{n} C_i * w_i}{\sum_{i=1}^{n} w_i}$$

(eq 2)

n: Number of WWTPs

C^i: Wastewater concentration (or indicator) for the WWTP *i*

W^i: Weight for the WTTP *i*

It is important to underline that the above method does not address issues concerning heterogeneity between measurements from different WWTPs, and its application without checking the comparability of measurements from multiple sites can result in misleading estimates. Deriving aggregate indicators is a challenging task, especially when multiple distinct Standard Operating Procedures (SOPs) and laboratory protocols are involved and when technical characteristics of WTTPs vary. Interlaboratory tests have shown that measurements from different laboratories differ significantly (Cluzel et al., 2022; Pecson et al., 2021), while other factors (e.g. differences in sewage network, chemical composition, sampling procedures) can lead to non-comparable results as well. While normalisation (see section 4.4) can mitigate heterogeneity, it might be necessary to perform additional processing to enhance comparability. For example, Biobot Analytics, Inc. who is the current vendor for the CDC's NWSS SARS-COV-2 wastewater testing introduced the concept of the effective concentration as a transparent and future-proof approach to account for technical factors that can impact SARS-CoV-2 concentration in wastewater (Biobot Analytics, 2022). Other groups suggest an additional data-scaling step to further assist the acquisition of more robust aggregate

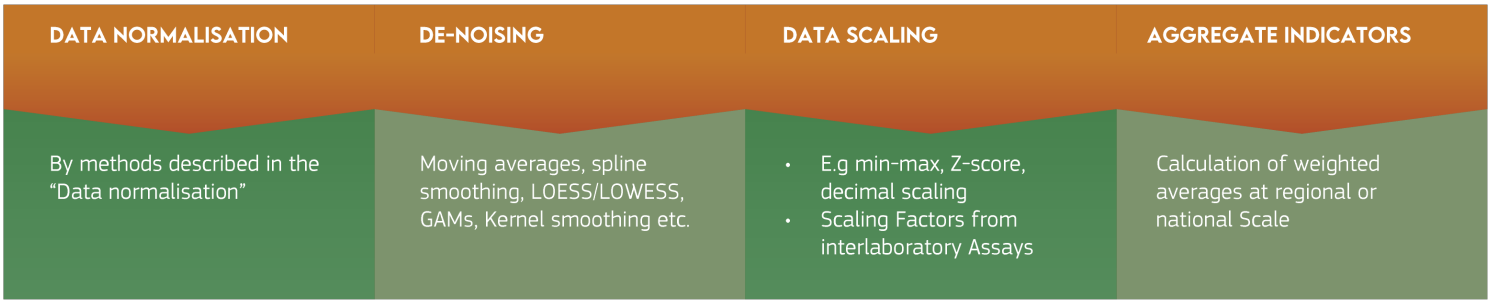
indicators. Cluzel et al. have proposed and tested a normalisation technique to account for various factors including inter-laboratory variability (Cluzel et al., 2022). The approach consists of sequential steps, which include pre-processing to account for hydrological fluctuations, de-noising, and a laboratory specific Min-Max normalisation combined with the application of scaling factors derived from interlaboratory assays. A generic workflow for handling heterogeneous data to calculate aggregate indicators is given in Figure 18.

4.7.4.4 TIME SERIES ANALYSIS TO DETERMINE THE ASSOCIATION WITH CLINICAL SURVEILLANCE DATA

SARS-CoV-2 viral load in sewage has been consistently associated with epidemiological indicators derived from clinical surveillance, by numerous research groups (Shah et al., 2022). Most frequently, reported COVID-19 cases are used as the dependent variable, but several publications demonstrated strong associations with COVID-19 hospitalisations (Galani et al., 2022; Peccia et al., 2020; Schenk et al., 2023). Initially, these findings assisted in the documentation of WBS as a valid surveillance tool and provided evidence on the potential lead indicators. While investigating these associations can be informative, recent research has demonstrated that the relationship between viral loads and clinical data may not always be spatially and temporally consistent but it appears to be site and time specific (Helm et al., 2023; Schill et al., 2023). Consequently, caution is suggested when generalising findings across different areas and time periods. To capture and further explore the dynamic relationship between wastewater signal and reported clinical cases, the wastewater signal to clinical cases (WC) ratio has been proposed, as a diagnostic tool for identifying periods of insufficient clinical testing and the emergence of different variants of concern/interest (VOC/VOI) (D'Aoust et al., 2022; Xiao et al., 2022).

In this section we present methodological approaches that have been applied for analysing wastewater time series against clinical surveillance data aiming to investigate the lead time of wastewater signal, to determine the magnitude of the association and perform short-term forecasting of clinical surveillance indicators.

Figure 18. Processing workflow to handle and aggregate data from multiple Wastewater Treatment Plants



Source: M. Koureas unpublished (redesigned)

INVESTIGATING SARS-COV-2 WASTEWATER LOAD AS A LEAD INDICATOR

SARS-CoV-2 viral load may function as a lead indicator by providing early quantitative information before surveillance systems based on clinical samples, but the exact lead time of wastewater surveillance compared to clinical data has not been precisely characterised yet. A common and relatively simple approach for investigating and estimating lead time is to determine the lag period that yields the strongest correlation between viral load and clinical surveillance indicators. This is achieved by consequent displacements of the viral load time series relative to clinical surveillance indicators, followed by the calculation of correlation coefficients (typically Spearman, Pearson, Kendall). Results can be visualised with the use of cross-correlograms or correlation heatmaps. When interpreting, it should be always kept in mind that these correlation methods do not address the fact that both viral load and clinical SARS-CoV-2 indicators are expected to be strongly autocorrelated, meaning that there are significant dependencies between adjacent observations. This issue can be addressed by adding a "pre-whitening" step into the analysis workflow (John & Ferbinteanu, 2021). Other approaches aiming to explore the lagged relationships include distributed lag models and turning point-based methods (Galani et al., 2022; Peccia et al., 2020).

Table 8. Examples of regression models applied to investigate the association of WBS data with clinical surveillance data

REGRESSION ANALYSIS	DESCRIPTION	EXAMPLES FROM WBS RESEARCH
Linear regression	• The relationship between WBS indicators and clinical indicators is modelled through linear predictor functions. • Log-log transformations are often useful before running and evaluating the performance of the models.	(Anneser et al., 2022; Hoar et al., 2022; Tiwari et al., 2022)
Polynomial regression	Clinical indicators are modelled as an nth degree polynomial of WW indicator	(Aberi et al., 2021; Ai et al., 2021b)
Poisson regression	Usually applied on count data. Assumes that clinical indicators (response variable) follow a Poisson distribution.	(Cohen et al., 2022; Peccia et al., 2020)
Generalised Additive Models	Clinical indicators are modelled as a sum of smooth functions of the predictors.	(Anneser et al., 2022; Vallejo et al., 2022)
Autoregressive models	Time series models that use past values of a variable to predict its future values. In vector autoregression each variable in the system is modelled as a linear combination of its own past values and the past values of the other variables in the system	(Cao & Francis, 2021; Zhao et al., 2022)

Source: Developed by the authors for this document.

DEVELOPMENT AND EVALUATION OF STATISTICAL AND MACHINE LEARNING MODELS

Regression analysis and models are predominately used to investigate the relationship between WBS and clinical surveillance data. Fitting appropriate regression models requires understanding the nature of the data and the research question being investigated. It is important to assess the model assumptions that typically include linearity, normality, independence and homoscedasticity of errors. Appropriate diagnostic methods and to identify violations include Quantile-Quantile plots, residuals versus fitted values plots and Cook's distance. The performance of the models is assessed with the use of various metrics such as the root mean square error (RMSE), the R-squared (R^2) and the adjusted R-squared, the Akaike and Bayesian information criteria (AIC, BIC).

Regression models that have been applied to WBS studies vary, and Table 8 summarises the most frequently used types.

When the research question focuses on the estimation of COVID-19 prevalence from WBS data, there is the option to build models based on back – calculation equations which assume that prevalence is a function of the concentration of SARS-CoV-2 RNA, the catchment area population, wastewater flow, decay ratio of SARS-CoV-2 RNA in wastewater and the excretion rate of SARS-CoV-2 RNA from infected people. Equation 3 (adopted by X. Li et al., 2021) presents its generic form, although several modifications have been proposed.

$$Prevalence = \frac{C*Q}{(1-D)*P*E} \quad \text{(Eq.3)}$$

Prevalence: COVID-19 prevalence in the catchment area

P: population size

C: concentration of SARS-CoV-2 RNA

E: excretion rate of SARS-CoV-2 RNA from infected people

Q: total wastewater flow during the sampling period

D: the decay ratio of SARS-CoV-2 RNA in wastewater

Table 9. Examples of regression models applied to investigate the association of WBS data with clinical surveillance data

COUNTRY	NUMBER OF SITES	CLINICAL VARIABLE EXAMINED	METHODS	FINDINGS	REFERENCE
Austria	123	Hospitalisation occupancy	• Outlier detection, normalisation, regional pooling, data filtering. • Cross-correlation analysis, Multivariate regression models (Linear and Support Vector Regression)	Average lead time of viral load in sewage overs ICU and non-ICU bed occupancy estimated at 14.8-17.7 days and 8.6–11.6 days, respectively	(Schenk et al., 2023)
Netherlands	>300	Hospital admissions	Bayesian multilevel spline model with random effects. Weighting of the relevant posterior virus loads by the number of inhabitants	Estimated national log-transformed SARS-CoV-2 loads aligned with national log-transformed daily number of hospitalisations	(Van Boven et al., 2022)
England	45	Clinical testing and prevalence survey data	Linear regression, linear regression with random effects intercept, linear regression with random effects intercept and slope, gradient boosted regression tree models.	Wastewater signals appeared 4–5 days earlier in comparison to clinical testing data but coincided with prevalence survey data	(Morvan et al., 2022)

Source: de Roda Husman unpublished

As the relationship between WBS indicators and clinical surveillance indicators can be affected by multiple factors, multivariable models including environmental, demographic, policy and WWTP related predictors are recommended to advance the understanding of the complex relationship. Also, most WBS surveillance networks have produced time-series data from a significant number of WWTPs, enabling simultaneous analyses of data from multiple sites. Table 9 provides three examples (from Austria, Netherlands and the United Kingdom) on approaches to analysing data from multiple sites against hospitalisation rates and COVID-19 prevalence data.

Along with methods mentioned above several additional machine learning techniques have been proposed to predict COVID-19 outbreaks (Ai et al., 2022). Although scientific evidence supports the early utilisation of WBS indicators to forecast clinical indicators, this is still a research topic and not yet a standard operating procedure of surveillance systems.

4.7.4.5 STATISTICAL ANALYSES TO ESTIMATE SARS-COV-2 VARIANT PROPORTION

Typically, SARS-CoV-2 genomic surveillance is based on obtaining samples from clinical tests that are subsequently subjected to sequencing. This approach, however, is unable to capture lineage prevalence dynamics due to sampling bias and different operating procedures of the healthcare system in different areas (Brito et al., 2022). The process of sequencing the SARS-CoV-2 genome from wastewater samples can complement the clinical surveillance by enabling the prevalence quantification of VOC/VOI. To achieve this, two distinct statistical methodologies can be applied (Karthikeyan et al., 2022; Valieris et al., 2022).

Both approaches are based on a set of lineage-determining genetic variations (LDGVs) that are typically identified from phylogenetic analysis of SARS-CoV-2 sequenced genomes derived from worldwide clinical surveillance efforts. These sequences are deposited in GISAID: https://gisaid.org/ (Khare et al., 2021) and can be compared to the Wuhan strain (reference sequence) to generate the SARS-CoV-2 phylogeny, identify clades and ultimately characterise VOC and VOI depending on the associated transmissibility, disease severity and immune escape properties. Since the beginning of the COVID-19 pandemic, the Pango Lineage Designation Committee (Rambaut et al., 2020) has spearheaded this effort by manually curating SARS-CoV-2 genome designations into variant groups. Pre-defined lineages (assigned to variant groups) are readily available from the Pango repository (https://github.com/cov-lineages/pango-designation) to researchers that can combine this information with sequences deposited in GISAID and World Health Organization (WHO) defined VOC and VOI to develop customised analytic pipelines.

The most frequently used methodology to estimate variant proportions in wastewater (Freyja) has been proposed by Karthikeyan et al. (Karthikeyan et al., 2022). Freyja utilises UShER (Turakhia et al., 2021) to identify a set of lineage-defining mutations (LDMs) from the global SARS-CoV-2 phylogeny. Based on these LDMs a m x n matrix A is created, where Aij reflects the absence or inclusion of LDMj in lineage i. A is complemented by vectors bn and dn that respectively represent the frequency of reads supporting each LDMj in the wastewater sample and the log-transformed sequencing depth at the LDMj locus. The task is then reduced in estimating the vector of variants prevalence xm, such that each value of xm is constrained between 0 and 1, while their sum equals to 1. The calculation of xm is subsequently formalised as a constrained weighted least absolute deviations problem $x_m = \text{argmin}||A^T x-b||_{1W}$, subject to $xi \geq 0$, $\sum i\ xi=1$, where $||\mu_n||_{1W} = \sum i\ di|\mu i|$.

A similar statistical approach proposed by Valieris et al. (Valieris et al., 2022), leverages the Pango lineage designation and GISAID. Specifically, genome sequences from GISAID are assigned to variant groups based on the Pango clade designation. The sequences are subsequently aligned to the Wuhan reference genome with minimap2 (H. Li, 2018, p. 2) and polymorphisms (mutations, insertions and deletions) are identified with GATK-Mutect2 (DePristo et al., 2011). Polymorphisms with allele frequency higher than 0.8, in at least one variant group, are retained and intersected with LDGVs according to WHO and Pango clade designation to generate a set of polymorphism-based markers (PBM).

The wastewater sequencing samples are pre-processed to remove library preparation contaminants, adapter sequences and low-quality reads. The remaining reads are aligned to Wuhan reference genome with BWA (H. Li & Durbin, 2009) and polymorphisms are identified with GATK-Mutect2 to calculate the coverage of PBM loci. PBMs are subsequently used to create a *m x n* matrix *P*, where P_{ij} reflects the probability (allele frequency) of finding PBM *i* from variant *j*. Given a set of PBM-overlapping read counts derived from a wastewater sequencing sample, Valieris et al. approach estimates vector w_n by decomposing the sample into SARS-CoV-2 variant groups prevalence. Each value of w_n is constrained between 0 and 1, such that their sum equals to 1. The task of estimating w_n is then formulated as a log-likelihood maximisation problem $L(w) = \mathbf{P}(C = c \mid w, P, t)$, where *C* reflects C^r_i and C^a_i that are the sum of latent variables Z^r_{ij} (count of reads with

reference base for PBM_i originating from variant j) and Z^a_{ij} (count of reads with alternate base for PBM_i originating from variant *j*) respectively over *j*, and t_m is the coverage vector such that $t_i = c^a_i + c^r_i$.

López-de-Ullibarri et al. (López-de-Ullibarri et al., 2023) proposed a similar approach that assumes just independence among the number of reads along the genome. Their method is also extended to account for Markovian dependence of counts along loci in the aggregated information coming from wastewater samples. The joint mutation distribution is estimated using GISAID data and the variant proportions are estimated via maximum conditional likelihood estimation using an augmented Lagrangian method.

4.7.4.6 CONCLUSIONS

Statistical analysis of the WBS data is a vital part of the operation of SARS-COV-2 sewage surveillance networks. The available statistical methods and frameworks vary in complexity and the choice of methods depends on the scope of the analysis, data quality, data availability, but also the capacity of the networks in terms of resources. When interpreting and communicating the results it is important to consider the assumptions and limitations associated with statistical methods used in WBS analysis to ensure a proper understanding of the findings. It is advisable for WW networks to employ a statistician, or a group of analysts, depending on the available resources. The study of the relationship between WBS and clinical surveillance can be highly complex and is still a subject of ongoing research. Finally, there is always a need to “translate” the results of statistical analysis to easily interpretable metrics to support evidence-based decisions.

Chapter 5

Methods and Methodologies- Measurement and Testing



5 METHODS AND METHODOLOGIES – MEASUREMENT AND TESTING

5.1 INTRODUCTION

WBS has emerged as a powerful tool in public health for monitoring the spread of SARS-CoV-2, the virus responsible for COVID-19. This non-invasive method provides an aggregated signal of virus shedding from both symptomatic and asymptomatic individuals within a community. As such, it can offer an early warning system for COVID-19 outbreaks and provide insights into the prevalence and dynamics of the disease over time. The integration of measurement and testing of wastewater for SARS-CoV-2 into public health strategies can complement traditional clinical testing by providing data on a larger scale and without the biases associated with individual testing behaviour.

This chapter gives an introduction into the principal techniques in wastewater surveillance for SARS-CoV-2 focussing on the steps after sampling, i.e. concentration, RNA extraction, detection and quantification of the virus and the employment of additional parameters as tracking indicators. This introductory section provides a high-level overview of the workflow and some of the most commonly used methods.

Concentration:

- Ultrafiltration: This method concentrates viruses from large volumes of wastewater through size-exclusion filters.
- Precipitation: Chemicals like polyethylene glycol can be used to precipitate viruses out of wastewater, allowing for concentration.

RNA Extraction:

- The process of isolating RNA from the concentrated wastewater sample, which includes SARS-CoV-2 genetic material if present.

Polymerase Chain Reaction (PCR):

- A highly sensitive technique that amplifies and quantifies specific nucleic acid sequences, providing data on the presence and quantity of SARS-CoV-2 RNA.
- Quantitative PCR (qPCR) and digital PCR (dPCR) are used to provide quantitative data which are converted to an estimate of genome target copies per unit volume or mass.

Sequencing:

- Targeted Sequencing: Focuses on specific regions of the viral genome to identify known variants of concern.
- Next-Generation Sequencing (NGS): Allows for the characterisation of viral genomes directly from wastewater, providing information on the presence of variants and mutations.

Chemical and Biological Indicators:

- Tracking indicators such as ammonia, pH, and temperature, or using biological markers, such as PMMoV, to normalise the data and account for dilution factors.

Each of these techniques plays an essential role in the overall process of wastewater surveillance for SARS-CoV-2. By combining these methods, public health officials can obtain reliable and actionable data to inform policy decisions and public health responses to the COVID-19 pandemic.

5.2 POLYMERASE CHAIN REACTION

MASTER CHEF: DANIEL DEERE

The Polymerase Chain Reaction (PCR) serves as a cornerstone in modern molecular biology, offering the ability to amplify minute quantities of genetic material for various applications. In WBS, PCR is a powerful tool for monitoring the presence of pathogens such as SARS-CoV-2. This chapter outlines the use of PCR in WBS, emphasising its role in detecting and quantifying viral RNA.

5.2.1 KEY INGREDIENTS

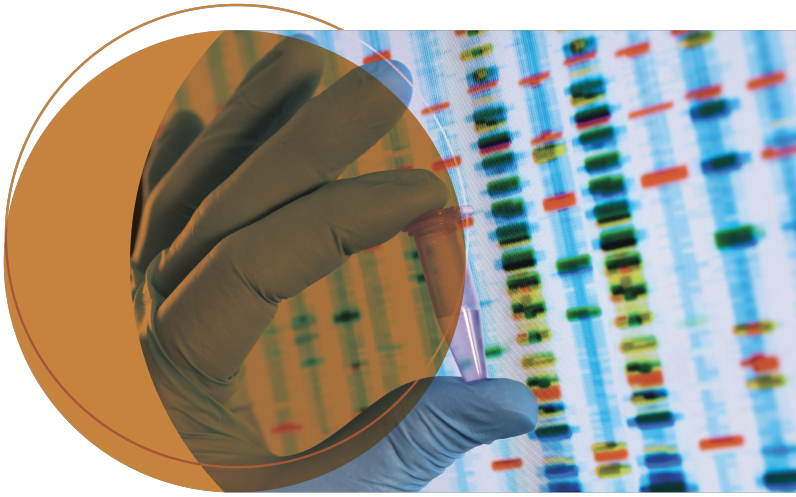
- **Oligonucleotides:** Synthetic DNA fragments, including primers that flank the target sequence and a probe that binds within the target region.
  - Forward Primer: Binds to the upstream boundary of the target sequence.
  - Reverse Primer: Binds to the downstream boundary of the target sequence.
  - Probe: Binds to a portion of the target sequence and carries a fluorescent marker for detection.
- **DNA Polymerase:** The enzyme responsible for synthesising new DNA strands by adding nucleotides to the primer-bound template.
- **Deoxynucleoside Triphosphates (dNTPs):** The building blocks of DNA that are incorporated into the new DNA strand during replication.
- **Buffer Solutions:** Maintain the optimal pH and ionic strength for the PCR reaction.
- **Reverse Transcriptase:** An enzyme used to convert RNA into complementary DNA (cDNA) for RNA-based targets such as SARS-CoV-2.
- **Thermal Cycler:** An automated device that changes temperatures according to programmed cycles for PCR reactions.
- **Quantified Reference Standards and Controls:** Used for calibration and quality control during quantification.

5.2.2 RECIPE

1. **Sample Preparation:** Process wastewater samples to extract RNA while minimising inhibitors.
2. **Reverse Transcription:** Use reverse transcriptase to convert RNA into cDNA.
3. **PCR Setup:** Combine cDNA, primers, probe, dNTPs, DNA polymerase, and buffer in a reaction tube.
4. **Thermal Cycling:** Place the reaction tube in a thermal cycler and run through cycles of varying temperatures to allow for denaturation, annealing, and extension.
5. **Detection:** Monitor the fluorescence emitted by the probe to detect and quantify the target sequence in real-time.

5.2.3 TIPS

- **Ensure the specificity of primers** and probes by comparing target sequences against global databases.
- **Optimise cycle durations** for efficiency without compromising reaction completeness.
- **Include appropriate negative controls** to prevent false positives due to contamination.
- **Follow stringent laboratory practices** to avoid cross-contamination between samples and amplified products.
- **Regularly update protocols based** on empirical optimisations tailored to specific wastewater contexts and target variants.
- **Maintain rigorous quality control** and quality assurance to ensure the reliability of results.

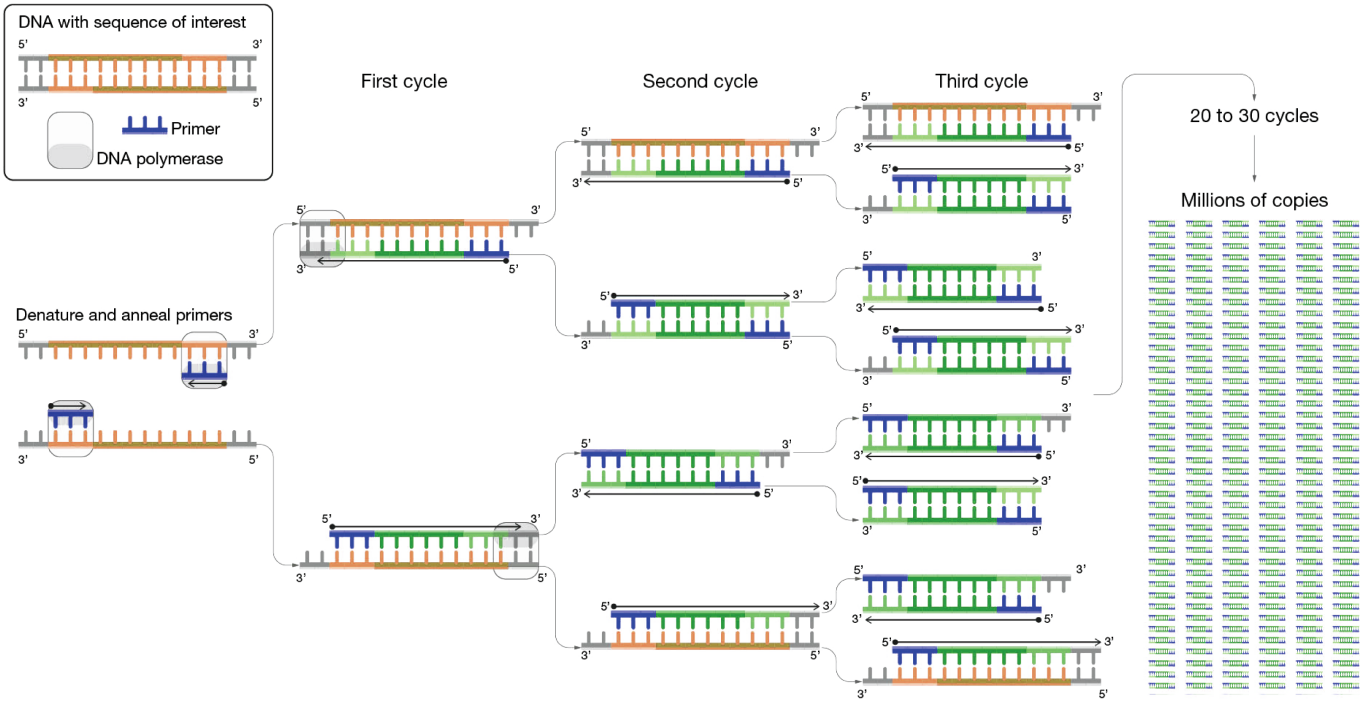


5.2.4 THE SCIENCE BEHIND

5.2.4.1 POLYMERASE CHAIN REACTION OVERVIEW

The polymerase chain reaction (PCR) was first demonstrated 40 years ago (Mullis et al., 1986). PCR is a method of creating billions of copies of a target genetic sequence. The method has been widely used for many applications, including diagnostic testing and environmental surveillance (Heijnen & Medema, 2011).

Figure 19. Overview of PCR – the basics



Source: National Human Genome Research Institute. https://www.genome.gov/ (redesigned)

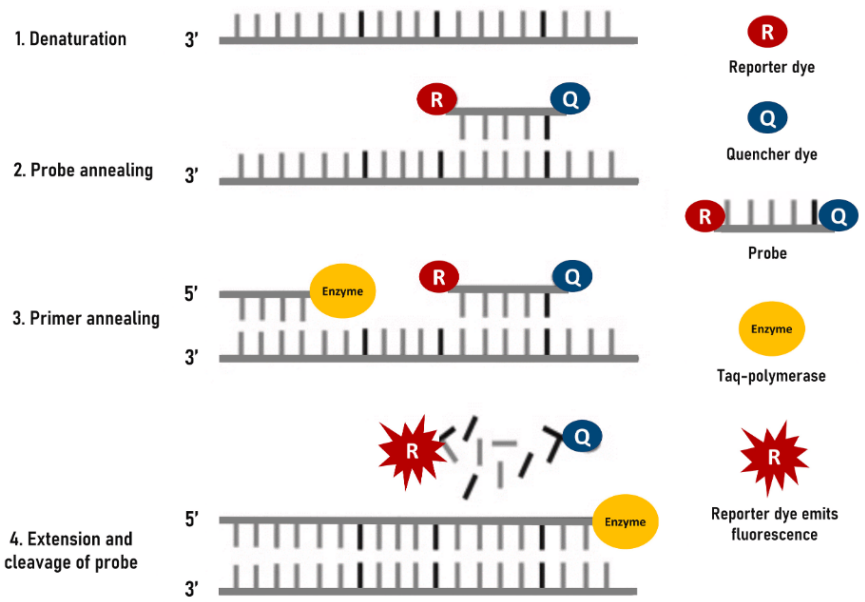
5.2.4.2 POLYMERASE CHAIN REACTION FOR SARS-COV-2 SURVEILLANCE

In the context of PCR as applied for SARS-CoV-2 clinical and environmental surveillance, the process is used to create billions of copies of SARS-CoV-2 target sequences, which in turn enables them to be detected within a few hours of the onset of sample processing at the receiving laboratory. Briefly, the method works as follows:

- Short deoxyribonucleic acid (DNA) fragments, termed "oligonucleotides", are produced synthetically. Oligonucleotides are a polymer consisting of a defined sequence of "bases". There are four of these DNA nucleotide building block bases: adenine (A), cytosine (C), guanine (G), and thymine (T). The latter is replaced with uracil (U) in ribonucleic acid (RNA) sequences; in PCR testing the oligonucleotides are made of DNA.
  - There are two types of oligonucleotides required: "primers" and "probes".
  - Two primers are required for PCR: a "forward" and "reverse primer". These represent the upstream and downstream boundaries of the targeted genetic sequence, or "amplicon".
  - In addition to the two primers, an oligonucleotide probe is used, that binds to a portion of the amplicon.
  - The primer and probe sequences are designed by comparing the genome sequence of the target genetic sequence with other published genetic sequences in global databases. The oligonucleotide sequences are intended to be unique components of the target's genome, whilst being absent from other genomes.
  - To provide a balance between specificity (favouring longer primer and probe sequences) and ease of production and speed of reaction (favouring shorter primer and probe sequences), the oligonucleotides commonly used in SARS-CoV-2 surveillance are typically 18-22 bases in length, but can range from 17-28 bases. The amplicons are typically 60 to 130 bases in length. Examples of primer-probe combinations commonly used in WBS include those recommended by the

- The PCR process proceeds as a series of cycles. The cycles involve holding a small volume (typically 50 to 200 µL) of reaction mixture in a small reaction tube and alternating between lower and higher temperatures: “thermal cycles” where in reactions occur within an automated temperature variable “thermal cycler”.
  - At the lower temperature the primers bind, “anneal”, to the target DNA “template”. DNA bases (in their freely dissolved deoxynucleoside triphosphate form) and DNA-dependent DNA polymerase enzymes are present in the reaction mixture. The polymerase catalyses the sequential addition of the free DNA bases, paired to their matching bases (A-T and C-G) on the opposite DNA strand, to produce new DNA strands as replicated complements of their template.
  - At the higher temperature the DNA polymeric strands remain intact through covalent bonding between bases, but complementary binding of base pairs between the paired DNA strands, bound only by hydrogen bonds, are “denatured” or “melted”, being separated from their counterparts. This frees up those DNA strands to make them available to bind with primers ready to amplify during the next lower temperature cycle.
  - The duration of each higher and lower temperature cycle is optimised to be sufficiently long for the reactions to occur during each cycle but not so long as to make the process slow and inefficient.
- The PCR method only amplifies DNA, not RNA. However, SARS-CoV-2 has a genome made of RNA. Therefore, the first step of PCR for detecting RNA targets is to use a reverse transcriptase enzyme to produce a complementary DNA copy of the RNA, termed “cDNA”. The cDNA is then amplified using PCR. The combination of reverse transcription followed by PCR is termed “Reverse Transcription-PCR” (RT-PCR).
- The amplicons are replicated exponentially. Under ideal conditions the exponential base is 2, i.e. the number of copies of the amplicon doubles with every reaction cycle such that after n reaction cycles the amplicon has been replicated 2^n times. For instance, after a typical number of reaction cycles, 32, a billion-fold amplification can be achieved. That is, for every SARS-CoV-2 genome present at the start of the PCR process, billions of copies can theoretically be yielded.
- Detection is carried in real-time using probes coupled to a quenched fluorescent molecule. Following binding to their target sequence, the fluorescent molecule becomes unquenched following its displacement by a polymerase and can then be detected opto-electronically. In this way the probe serves two functions: to provide an additional level of specificity since it only binds to its complementary DNA sequence; and to enable real-time quantitative PCR (qPCR).
- To enable quantification, the number of thermal cycles required before detection is achieved (the point at which fluorescence from the unquenched fluorescent marker is reliably above the background fluorescence of a negative control sample) is recorded and reported as the “cycle threshold” (Ct). The lower the Ct, the less cycles are required to enable detection of the target amplicon, which is consistent with higher levels of virus in the original sample. Quantified reference standards are used to develop a calibration curve that enables the starting concentration of RNA target copies in a wastewater or sludge sample to be estimated. Therefore, qPCR uses the Ct value as an indirect indicator of virus target genome copies in the sample. Ct values in the 20s are considered representative of high target genome copies; high 20s to high 30s as moderate to high, and high 30s to 40s as being indeterminate and unreliable (such that PCR reactions don’t typically go beyond 40 cycles).
- A less commonly used method for detecting and quantifying the starting concentration of RNA copies in a wastewater or sludge sample is to partition the reaction mixture into multiple parallel replicates (potentially thousands), potentially including one or more serial dilutions for more concentrated samples. This method is analogous to most-probable number assay formats used routinely in microbiology. The presence or absence of the target RNA is measured for each replicate. This “digital PCR” (dPCR) provides similar results to real-time qPCR in comparative trials. However, dPCR can provide more precise quantification than real-time qRT-PCR. In some cases, the digitisation is achieved using multiple replicate reaction vessels set out in plates, and in others using water-oil partitioned emulsions in single reaction vessels

Figure 20. The principle of real-time qPCR based on commercial TaqMan probe



Source: (Meral Yüce et al., 2021), https://doi.org/10.1016/j.bios.2020.112752

(droplet digital PCR). In general, the real-time qRT-PCR is preferred where higher throughput is desired and reduced quantitative precision is acceptable; with the dPCR methods being preferred where more precision is required and/or where the calibration standards required for real-time qRT-PCR are not available.

- Variant-specific primer and probe combinations have been successfully designed and utilised that permit known targeted variants to be detected and quantified in wastewater; and the products of PCR amplification can be sequenced (as discussed in the sequencing chapter) both to assist with confirmation of results, and to identify both known and novel variants (Diamond et al., 2022; Farkas et al., 2023).

5.2.4.3 PRACTICAL APPLICATION OF REAL-TIME QRT-PCR FOR DETECTION OF SARS-COV-2 RNA IN WASTEWATER

The broad principles and typical workflow for real-time qRT-PCR for detection of SARS-CoV-2 RNA in wastewater are largely consistent between laboratories and analytical programmes. However, there are many variables and options available to analysts, resulting in a wide variation between methods at the detailed level.

End-to-end procedures – from the receipt of samples ready for sample processing, to the reading of results – vary widely between laboratories. There is no one standard method, which is why normalisation is important if there is a desire to compare results from different laboratories (as discussed in the normalisation chapter). Variations in the protocols adopted include the following:

- Methods for processing samples received. Typically, wastewater and sludge samples are transported to the laboratory and stored until ready for analysis at refrigeration temperatures (2-8˚C), but not frozen. Freezing is avoided to reduce the risk of ice crystals degrading the protective structures around RNA, and breaking down cells resulting in the release of RNAases that degrade free RNA (as discussed in more detail in the sample transport chapter, chapter 7).
- The methods for extracting RNA. A balance must be achieved between extracting as much of the RNA as achievable, to maximise sensitivity, whilst minimising the extraction of inhibitory substances that reduce sensitivity (as discussed in more detail in the sampling chapter). A wide variety of substances in wastewater can inhibit PCR reactions, and the inclusion of inhibition controls is an essential standard quality control in all assays. A

diverse range of methods for selectively extracting target virus and RNA whilst minimising capture of inhibitory substances has been successfully applied, including precipitation with polyethelyne glycol, flocculation with skim milk, membrane filtration, ultrafiltration, and a wide range of commercial extraction kits (P. Liu et al., 2023; Medema et al., 2020; Pocock et al., 2020)

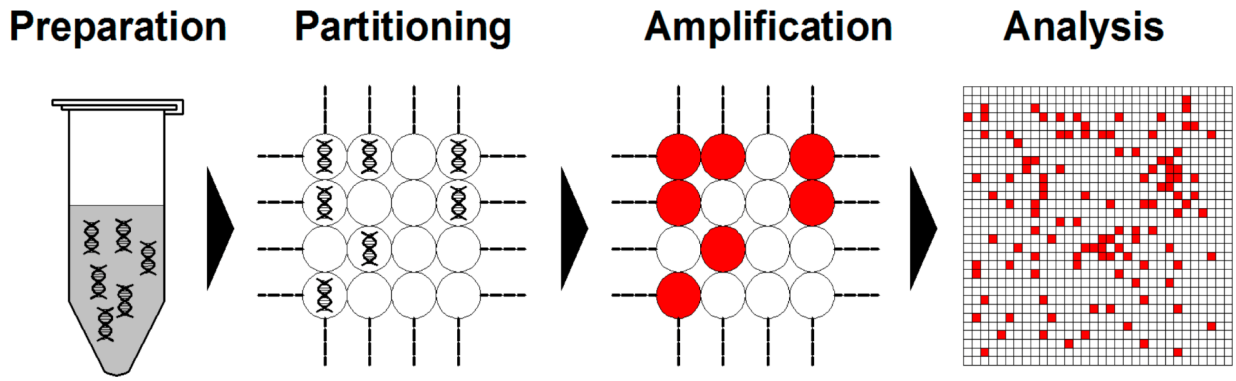
- There are a variety of primers, probes, polymerases, fluorescent markers, reaction mixtures, and reaction conditions (temperatures and durations for each stage of the thermal cycle) that have been successfully used. Where multiple end-to-end workflows have been compared, significant differences have been reported in their performance (Chik et al., 2021; Flood et al., 2021; Griffiths et al., 2022; Länsivaara et al., 2023; Pecson et al., 2021).

5.2.4.4 LIMITATIONS OF PCR METHODS

The end-to-end process of sampling to reporting needs to be carefully controlled to minimise errors (W. Ahmed, Bivins, et al., 2022; Chik et al., 2021). Above all, the most important limitation of PCR methods results when their strength becomes their weakness. Specifically, the impressive sensitivity of PCR means that even tiny traces of contaminating RNA or DNA from non-target sources, such as amplified DNA from other reactions, can lead to false positive results. As a result, the inclusion of negative controls is an essential standard quality control in all assay batches. In addition, any PCR analytical laboratory needs to be set up explicitly to prevent contamination of the test samples with potentially contaminated material containing target RNA or RNA, or carryover DNA from other PCR reactions. This typically involves physically separating sample preparation and RNA extraction and reaction set-up areas from analytical areas, along with methods for the destruction of amplified DNA.

Variations in wastewater and sludge quality, such as the inhibitory substances present, as well as differences between target variant sequences, result in optimal conditions, to achieve the best sensitivity and specificity, varying between and within contexts. Therefore, empirical optimisation through comparison and refining of methods is recommended. Considerations, such as cost, skills levels, familiarity, availability of automation, additional targets, and use cases, can all be relevant to decision-making and method selection. Quality control and quality assurance are critical to ensuring reliable and useful results. Whilst innovation and optimisation of methods is encouraging, any change or difference in methods can have implications for comparability of results over time or location. Full details of methods should be recorded, and changes or differences in methods highlighted when comparing results.

Figure 21. Typical digital PCR workflow



Source: (Cao et al., 2020) *https://doi.org/10.3390/molecules25030706*

5.3 NEXT GENERATION SEQUENCING

MASTER CHEF: **SHELESH AGRAWAL**

CREW:

MIRANDA DE GRAAF

WILLEMIJN LODDER

AARON JEX

BRIGITTA SAUL

CLEMENTINA COCUZZA

ADÈLE LAZUKA

SEBASTIEN LACROIX

The outbreak of COVID-19 brought about an unprecedented global health crisis. In a race against time, scientists and public health officials have relied on innovative and powerful tools, such as Next Generation Sequencing (NGS), to better understand and combat the virus. This chapter aims to provide an overview of NGS, its historical context, its role during the COVID-19 pandemic, the challenges posed by the emergence of variants, and its synergies with quantification methods.

5.3.1 KEY INGREDIENTS



- **High-Quality Nucleic Acid Samples:** The starting material for NGS, representing either DNA or RNA extracted from wastewater samples, which may contain a mixture of genetic material from various pathogens, including SARS-CoV-2.
- **Amplicon Panels and Primers:** Custom-designed short DNA sequences that guide the amplification of specific regions of interest within the genome, targeting SARS-CoV-2 and potentially other pathogens for comprehensive analysis.
- **High-Fidelity Polymerases:** Enzymes that ensure accurate replication of the target sequences during the PCR amplification process, minimising errors.
- **Sequencing Adapters:** Specialised DNA sequences that are attached to the amplicons to facilitate their binding to the sequencing platform.
- **Barcodes/Index Tags:** Unique sequences added to each sample, allowing for the simultaneous sequencing of multiple samples while keeping track of their origin.
- **Sequencing Platforms:** Advanced machines such as Illumina, Ion Torrent, or Oxford Nanopore, each with unique capabilities and applications, tailored to the requirements of the sequencing project.
- **Bioinformatics Tools:** Software for processing the raw sequencing data, including alignment to reference genomes, variant calling, and lineage assignment.
- **Quality Control Reagents:** Solutions and standards used to evaluate the quality and quantity of the extracted nucleic acids and the prepared sequencing libraries.

5.3.2 RECIPE



1. **Sample Preparation:** Begin with extracting nucleic acids from wastewater, ensuring the preservation of RNA integrity and minimising degradation. Quantify and assess the quality of the extracted RNA.
2. **Reverse Transcription:** Convert the extracted RNA into cDNA using reverse transcriptase enzymes, setting the stage for PCR amplification.
3. **PCR Amplification:** Employ the amplicon panel and high-fidelity polymerases to amplify the regions of interest. Monitor the PCR process to ensure specificity and efficiency.
4. **Library Preparation:** Fragment the amplicons if necessary, perform size selection, and purify the fragments. Ligate sequencing adapters and barcode tags to each sample.
5. **Quality Control and Pooling:** Quantify the prepared libraries, validate their quality, and pool them in equimolar amounts for sequencing.
6. **Sequencing Run:** Load the pooled libraries onto the chosen sequencing platform. Adjust the sequencing parameters to achieve the desired coverage and read length.
7. **Bioinformatics Analysis:** Process the sequencing data, demultiplexing the reads, aligning them to the reference genome, and identifying variants. Employ algorithms for lineage assignment and confidence estimation.

5.3.3 TIPS

- **Optimise RNA Extraction:** Use RNA stabilisation solutions during sample collection and employ extraction methods that minimise RNA degradation to ensure high-quality starting material.
- **Primer Design:** Regularly update primer designs to encompass emerging variants and achieve comprehensive coverage of the SARS-CoV-2 genome.
- **Sequencing Depth:** Aim for high sequencing depth to increase the accuracy of variant detection, especially for low-frequency mutations.
- **Avoid Contamination:** Implement strict laboratory practices to prevent cross-contamination between samples, which can lead to erroneous results.
- **Data Interpretation:** Collaborate with bioinformatics experts to accurately interpret the sequencing data, understanding the implications of detected variants for public health.
- **Stay Informed:** Keep abreast of the latest NGS technologies and bioinformatics tools, as the field is rapidly evolving with continuous advancements.



5.3.4 THE SCIENCE BEHIND

5.3.4.1 NEXT GENERATION SEQUENCING AND SARS-COV-2

NGS encapsulates a variety of modern sequencing technologies, which allow for the rapid and high-throughput sequencing of DNA and RNA. These innovative platforms have revolutionised the field of genomics by enabling the sequencing of entire genomes with remarkable speed and precision. Unlike first-generation sequencing, (also known as Sanger sequencing), NGS can process millions of DNA strands in parallel, drastically reducing the time and cost associated with genetic analysis.

HISTORICAL CONTEXT

The legacy of NGS is built upon the pioneering work of Frederick Sanger who, in the 1970s, developed the first method of DNA sequencing (Heather & Chain, 2016). Sanger sequencing became the standard method for sequencing DNA and was used to sequence the human genome in the Human Genome Project, which was completed in 2003 (Heather & Chain, 2016). However, Sanger sequencing is time-consuming and expensive, especially for large-scale genomic projects.

NGS technologies were developed in the mid-2000s to overcome these limitations, with multiple platforms emerging, such as Illumina (formerly Solexa), Roche 454, and Life Technologies' Ion Torrent. These platforms significantly reduced the cost and increased the speed of DNA sequencing, making it feasible to sequence large genomes and perform in-depth studies of genomic variation within populations.

THE NEED FOR NGS DURING COVID-19, RISE OF VARIANTS:

The emergence of the COVID-19 pandemic necessitated a rapid response from the scientific community. NGS became an indispensable tool in tracking the spread and mutation of the SARS-CoV-2 virus. As the virus replicated and spread globally, it accumulated genetic mutations, some leading to the rise of new variants. NGS enabled researchers to quickly sequence viral genomes and identify these mutations, facilitating understanding of the dynamics of the virus's evolution, transmission, and impact on vaccine efficacy (G. John et al., 2021). This real-time genomic surveillance has been crucial in guiding public health decisions and developing therapeutic strategies.

The rapid and global spread of SARS-CoV-2 highlighted the critical need for NGS in the fight against COVID-19. NGS has played a key role in several aspects of the pandemic response, including (G. John et al., 2021):

a. **Viral genome sequencing:** NGS has been instrumental in sequencing the SARS-CoV-2 genome, which provided the basis for developing of diagnostic tests, vaccines, and therapeutics.

b. **Identification of variants:** As the virus evolves, NGS has been used to identify and track emerging SARS-CoV-2 variants with potential implications for transmissibility, disease severity, and vaccine efficacy.

c. **Surveillance and outbreak investigation:** NGS has been used to monitor the spread of SARS-CoV-2 in communities, inform public health measures, and guide targeted interventions (Rios et al., 2021).

The emergence of SARS-CoV-2 variants, such as Alpha, Beta, Gamma, and Delta, has underscored the importance of NGS in understanding viral evolution and monitoring the global spread of the virus. NGS has been used to identify and characterise these variants, assess their potential impact on vaccine efficacy, and inform public health strategies (Jahn et al., 2022).

BENEFITS AND SYNERGIES WITH QUANTIFICATION METHODS:

NGS offers several advantages over traditional quantification methods, such as quantitative PCR (qPCR). While qPCR is excellent for quantifying specific DNA or RNA sequences, it cannot provide a comprehensive view of the genome. NGS fills this gap by sequencing entire genomes, thus providing richer and more detailed genetic information. Moreover, NGS has synergistic applications with qPCR; for instance, variants identified through NGS can be further quantified using qPCR to study their prevalence within a population. Integrating NGS with other molecular techniques forms a powerful toolkit for modern molecular biology and epidemiology (Robin et al., 2016).

NGS advantages over traditional quantification methods like qPCR include:

- **Comprehensive insights:** NGS can sequence entire genomes, providing a complete picture of an organism's genetic makeup rather than targeting specific regions.
- **High sensitivity and specificity:** NGS can detect rare variants and low-abundance sequences that other methods might miss.
- **Quantitative:** NGS can also be used quantitatively, such as in RNA-Seq, to measure gene expression levels across the entire transcriptome.

However, NGS is often used in synergy with other quantification methods:

- **Validation:** NGS findings are often validated using qPCR, a more cost-effective method for specific, targeted studies.
- **Complementary data:** qPCR and other quantification methods can provide quick, targeted information that complements the broad and detailed data obtained from NGS.

STATE-OF-THE-ART:

The current state of NGS is characterised by continual advancements in technology and methodology. Cutting-edge platforms now offer longer read lengths, higher accuracy, and the ability to sequence single cells, opening new frontiers in genomics. Integrating NGS data with bioinformatics and computational biology yields unprecedented insights into complex biological systems. Moreover, portable sequencing devices have democratised NGS, making it accessible for point-of-care diagnostics and in-field genetic analysis. As the technology evolves, NGS is poised to further enhance our understanding of SARS-CoV-2 and other pathogens, cementing its role as an essential instrument in the arsenal of 21st-century science.

Key developments include:

- **Single-cell sequencing:** Analysis of genetic material at a single-cell resolution, providing insights into cellular heterogeneity.
- **Long-read sequencing:** Platforms like Pacific Biosciences (PacBio) and Oxford Nanopore Technologies provide long-read sequencing capabilities that can span complex genomic regions, improving genome assembly and structural variant detection (Barbé et al., 2022; Udaondo et al., 2021).
- **Portable sequencing:** Devices like the MinION from Oxford Nanopore make in-field genomic studies possible, allowing rapid sequencing outside traditional laboratories (Latorre-Pérez et al., 2020).
- **Integration with artificial intelligence (AI):** Using artificial intelligence and machine learning algorithms to analyse and interpret the vast amount of NGS data.
- **Multi-omics:** NGS combined with other 'omics' technologies (proteomics, metabolomics, etc.) to provide a more holistic view of biological systems.

In summary, NGS has been and continues to be instrumental in the fight against COVID-19. Its ability to quickly and accurately sequence the SARS-CoV-2 genome has been vital in monitoring the pandemic's progression, understanding the virus's behaviour, and informing clinical and public health responses. As the technology advances, it is expected to play an even greater role in future efforts to control and prevent outbreaks of this and other infectious diseases.

5.3.4.2 AMPLICON SEQUENCING PANEL

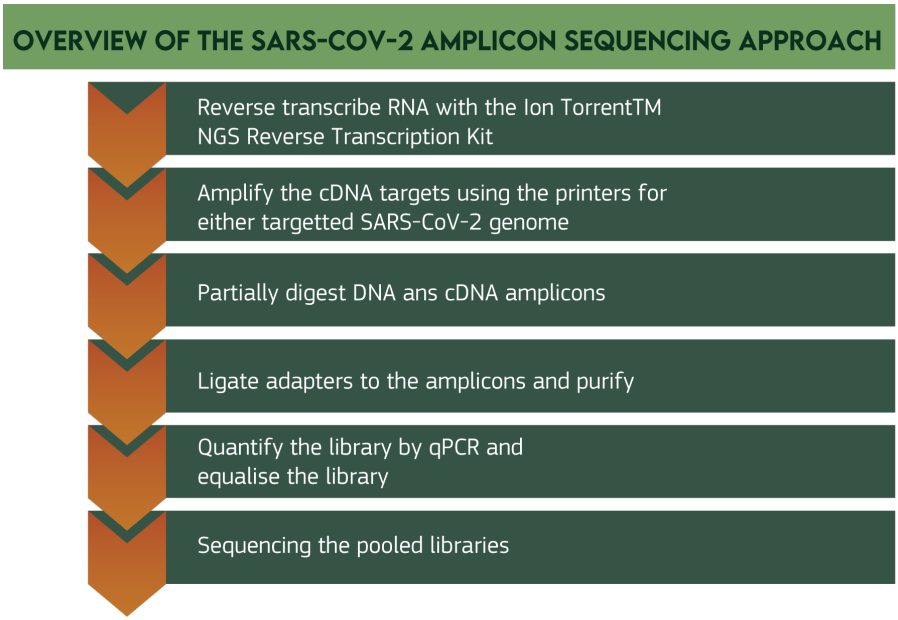
Among the various NGS techniques, amplicon sequencing is a targeted approach that has become essential for studying specific genomic regions of interest. An amplicon panel is a collection of short DNA sequences, called primers, designed to selectively amplify particular segments of a genome using the PCR. This process produces amplicons, the amplified DNA fragments, which are then sequenced to analyse genetic variation within the targeted areas.

The amplicon sequencing workflow (Figure 22) begins with extracting genetic material from the sample. Following extraction, the quality and quantity of the nucleic acids are assessed to ensure that they meet the criteria for successful amplification. Using the primers in the amplicon panel, regions of interest are then amplified through PCR. The resulting amplicons are purified to eliminate unwanted PCR by-products and then prepared for sequencing by attaching specialised adapters compatible with the chosen NGS platform. Once the sequencing libraries are prepared, containing the adapter-ligated amplicons, they are pooled together, often with uniquely identifying sequences (barcodes), allowing for multiple samples to be sequenced simultaneously in a single NGS run. The sequencing data generated is then processed using bioinformatics tools to align the reads, call variants, and perform in-depth analyses of the targeted genomic regions.

During the COVID-19 pandemic, amplicon sequencing panels have been widely used for several purposes:

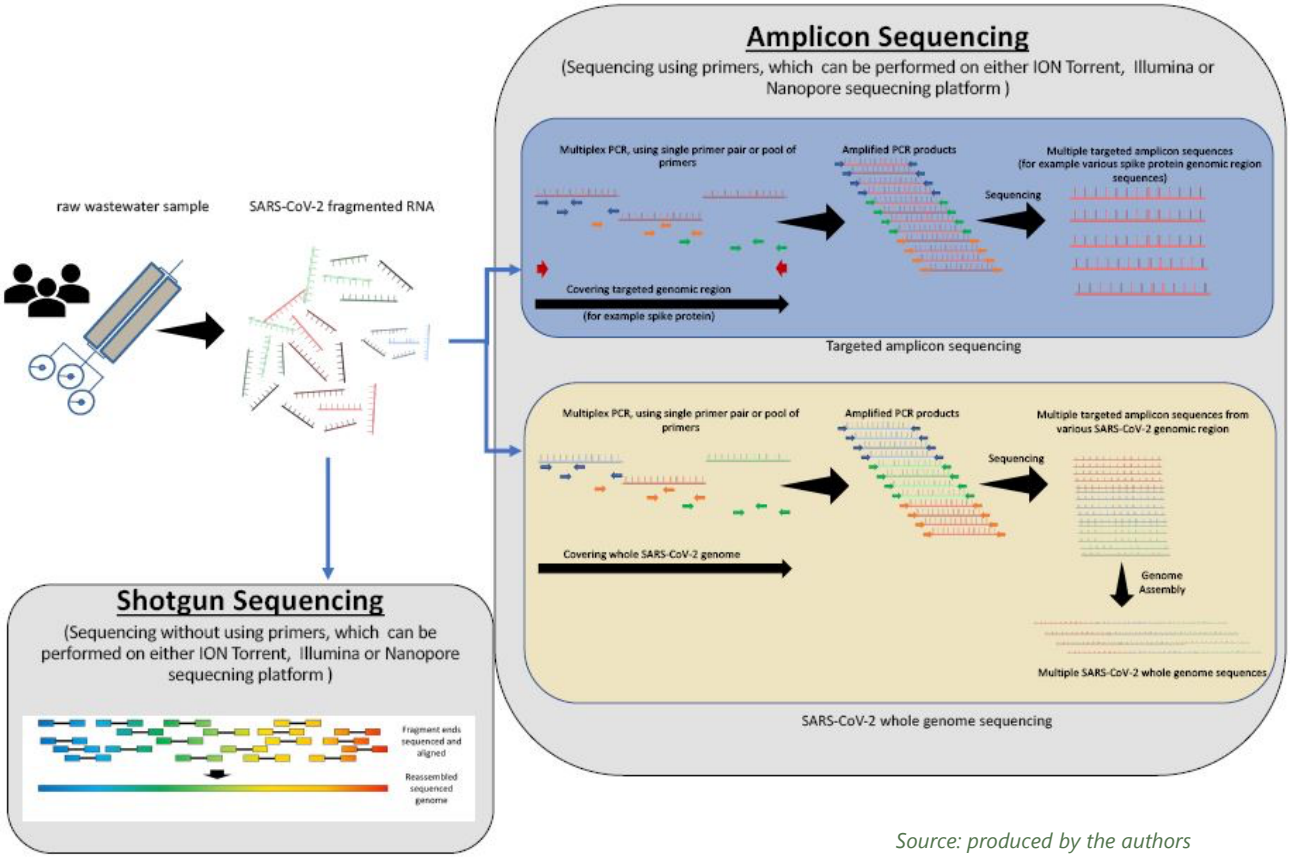
- **Viral Genome Sequencing:** To sequence the genome of SARS-CoV-2 from clinical samples, amplicon panels are designed to cover the entire viral genome. This is essential for identifying and tracking mutations and new virus variants.
- **Variant Identification:** Focused amplicon panels might target specific regions of the viral genome known to mutate frequently, such as the spike protein coding region, which is crucial for virus entry into host cells and is a vaccine target.
- **Surveillance:** Regular sequencing of viral genomes from infected individuals helps to monitior the virus' spread and evolution.

Figure 22. Amplicon sequencing workflow



Source: produced by the authors (redesigned)

Figure 23. Main strategies of amplicon sequencing



Source: produced by the authors

Access to well-designed amplicon panels is crucial for the rapid and accurate sequencing of SARS-CoV-2. However, as the virus evolves, there is a continuous need to optimize these panels. Optimisation may involve:

- **Updating Primer Design:** As the virus mutates, primers may need to be updated to ensure efficient binding and amplification of the target regions, avoiding regions with high mutation rates that could lead to primer mismatch.
- **Improving Coverage:** Ensuring that the entire genome is covered by the amplicons, especially in the face of emerging variants that might have new mutations in previously less variable regions.
- **Enhancing Sensitivity and Specificity:** Optimising the panels to detect low levels of viral RNA in samples and to discriminate between closely related viral strains or other coronaviruses.

The optimisation of amplicon panels is an ongoing process that must keep up with the evolving landscape of the virus to ensure the continued effectiveness of genomic surveillance and research. Collaboration among scientists, sharing sequencing data, and using international databases are critical for the timely update and distribution of optimised panels.

There are two main strategies when designing amplicon panels for SARS-CoV-2 sequencing. The first is to cover the complete viral genome with overlapping amplicons. This comprehensive approach ensures that every part of the genome is sequenced, allowing for a full genetic profile of the virus. It is particularly valuable in the early stages of an outbreak when characterising the pathogen is a priority or when monitoring for the

emergence of new variants across the entire viral genome.

The alternative strategy is to target shorter, specific regions of the genome of particular interest or clinical relevance. These might include regions with known mutations that confer increased transmissibility or resistance to neutralizing antibodies. Targeting such regions can be more cost-effective and may yield quicker results. This approach, however, may miss novel mutations occurring outside the targeted areas, which could also be of significance.

Both strategies have their place in the study of SARS-CoV-2. The choice between them depends on the goals of the research, available resources, and the specific questions being addressed. While complete genome coverage provides a holistic view of the viral genome, targeted sequencing allows for a deeper dive into specific genomic features of interest. As the pandemic evolves, so does the application of amplicon sequencing, adapting to the changing landscape of this formidable virus.

5.3.4.3 SAMPLE PREPARATION AND ANALYSES

In viral genomics, preparing samples for amplicon sequencing is a critical process that requires meticulous attention to detail. This section delves into the intricacies of preparing SARS-CoV-2 RNA samples for NGS, from the initial isolation of RNA to the final steps of preparing sequencing libraries.

FROM ISOLATED RNA TO AMPLICONS

The first step is to assess its quality and quantity. High-quality RNA is essential for reliable reverse transcription and subsequent amplification. RNA integrity can be compromised by enzymatic degradation, physical shearing, or chemical damage, which can occur during sample collection, storage, or processing. Quality control of RNA samples typically involves spectrophotometric analysis to determine the purity of RNA by assessing the absorbance ratio at 260 nm and 280 nm. An ideal A260/A280 ratio is approximately 2.0, indicating minimal protein contamination. Additionally, RNA integrity is evaluated using gel electrophoresis or more advanced techniques such as capillary electrophoresis, which can provide an RNA Integrity Number (RIN) to quantitatively assess quality.

Once the RNA meets QC standards, it undergoes reverse transcription to synthesise cDNA. Reverse transcription is the process of synthesising cDNA from RNA. This step is crucial for RNA viruses like SARS-CoV-2, where the RNA genome must be converted into DNA before amplification. Reverse transcriptase enzymes and primers that anneal to the RNA template are used to generate the first-strand cDNA.

With cDNA in hand, the next stage is to selectively amplify the regions of interest – in this case, the genomic segments of SARS-CoV-2 – using PCR. The amplicon panel, a set of carefully designed primers, is employed to ensure the specificity of the amplification. High-fidelity DNA polymerases are used to minimise the introduction of errors during PCR, and conditions are optimised to promote efficient and specific amplification of the target sequences.

Following PCR, the amplicons may be subjected to fragmentation, a process particularly necessary for platforms requiring shorter reads. Controlled enzymatic or physical methods break the amplicons into smaller, uniformly sized pieces suitable for the sequencing platform. Size selection follows fragmentation to ensure the library consists of DNA fragments within a desired size range. This step is crucial as it can influence the sequencing run's efficiency and the sequence data's quality. Techniques such as gel electrophoresis or magnetic bead-based purification are commonly used for size selection. The final step before library preparation is the purification of the amplicons. This step removes primers, nucleotides, enzymes, and other impurities that could interfere with subsequent library preparation steps. Various purification methods, including spin columns and magnetic beads, are selected based on their compatibility with the downstream processes and the quality of purification required.

FROM AMPLICONS TO SEQUENCING LIBRARIES

The next phase involves the preparation of sequencing libraries from the purified amplicons. This is achieved by ligating platform-specific adapters to the amplicons, which are necessary for the sequencing process and often contain unique index sequences for sample identification. Different steps are required to convert the PCR-generated amplicons into sequencing-ready libraries, focusing on adapter ligation, indexing, purification, quality control (QC), quantification, and pooling.

The ligation of adapters to amplicons is a gateway process, enabling the subsequent sequencing of these DNA fragments on NGS platforms. Adapters are short, synthesized DNA sequences that serve multiple functions: they provide a binding site for the sequencing primers, a scaffold for the DNA to attach to the flow cell, and often contain index sequences that allow for sample identification. To prepare for adapter ligation, amplicons must be compatible in terms of overhangs or blunt ends, depending on the adapter design. The ligation reaction typically uses DNA ligase to covalently attach the adapter sequences to the ends of the amplicons. This step must be conducted with high

precision, as efficient and accurate ligation is critical for the success of the sequencing run.

Indexing is a pivotal step in the NGS workflow, allowing for the simultaneous sequencing of multiple samples known as multiplexing. Each adapter is tagged with a unique index, or barcode, during the ligation process. These indices are crucial for the demultiplexing stage, where sequencing reads are sorted back to their respective samples based on the unique barcode sequences. The use of unique indices for each sample prevents cross-contamination and sample mix-up, enabling researchers to sequence many samples in parallel without compromising the integrity of the results. The design of the barcodes must minimise the potential for misassignment due to sequencing errors or barcode crosstalk.

Following adapter ligation and indexing, the library must be purified to remove unligated adapters, free primers, and other reaction components. This step is crucial to avoid sequencing these unwanted elements, which would reduce the efficiency and accuracy of the sequencing run. Various purification methods, including magnetic beads and spin columns, offer different advantages in yield, purity, and size selection.

Quality control of the prepared libraries is essential to ensure they meet the standards for successful sequencing. QC metrics include the library's size distribution, which can be assessed using capillary electrophoresis or other sizing instruments, and the absence of adapter dimers or other aberrant products. The library concentration must also be accurately determined, as this will affect the quantification and pooling steps.

Quantification of the library is crucial to determine the amount of DNA available for sequencing. Accurate quantification ensures that each library is represented equally in the final pooled sample, which is critical for achieving uniform coverage across all samples. Quantitative methods include qPCR, which is highly sensitive and specific, and fluorometric assays, which measure the fluorescence emitted by DNA-bound dyes.

Once quantified, libraries are pooled together in equimolar amounts. This pooling allows for the efficient use of sequencing capacity and ensures that each sample receives similar sequencing depth. The pooled library is then ready to be loaded onto the sequencer for the final step of the NGS workflow.

SEQUENCING PLATFORMS

The landscape of NGS is dominated by several key platforms, each with its unique chemistry, capabilities, and applications. Here, we delve into three major technologies: Illumina, Ion Torrent, and Oxford Nanopore Technologies.

Illumina sequencing is renowned for its high-throughput and accuracy. It uses a sequencing-by-synthesis approach, where fluorescently labelled nucleotides are incorporated into the DNA template strand, with each addition signalled by a characteristic fluorescence. This technology can produceg large volumes of data with low error rates, making it a staple in genomic research.

Ion Torrent platforms operate on a different principle, detecting hydrogen ions released during DNA polymerisation. This semiconductor-based technology translates chemical information into digital data. While Ion Torrent sequencers may yield faster results and are cost-effective for certain applications, they tend to have shorter read lengths and a higher error rate than Illumina.

Oxford Nanopore Technologies offer a unique approach by sequencing DNA molecules as they pass through a biological nanopore. The technology detects changes in the electrical current as different bases pass through the pore, allowing for real-time sequencing. Nanopore sequencing can generate very long reads, which are ideal for mapping complex genomic regions or identifying structural variants.

The desired output per sample, or sequencing depth, is a critical consideration in NGS. Depth refers to the number of times a nucleotide is sequenced, with higher depths increasing the confidence in variant calls and the ability to detect low-frequency alleles. In the context of SARS-CoV-2, high depth is particularly important due to the potential for a complex mixture of viral populations within a single patient or environmental sample. This complexity may arise from intra-host viral diversity or co-infection with different viral strains. Deep sequencing ensures that even rare variants within the viral population can be detected, which is crucial for understanding the virus's transmission and evolution, as well as for identifying mutations that may impact vaccine and drug efficacy.

After sequencing, the data must be demultiplexed, which sorts the reads back to their respective samples using the unique index sequences introduced during library preparation. This step is essential for multiplexed runs where multiple samples are sequenced together. To ensure the accuracy of sequencing, controls are integrated throughout the NGS workflow. Positive controls, often comprising known sequences, validate the sequencing process, while negative controls detect

contamination and procedural errors. These controls are vital for maintaining the integrity of the sequencing data.

Duplicate reads can arise during PCR amplification and can skew the interpretation of sequencing data. The removal of PCR duplicates can be challenging or impossible due to the lack of unique molecular identifiers and the high similarity between amplicons. This means that identical reads generated during PCR amplification may be indistinguishable from true biological duplicates, potentially leading to inflated variant frequencies or biased results. Bioinformatic tools can help mitigate these duplicates by implementing filtering strategies and statistical models, although they may not completely eliminate the issue.

Barcoding crosstalk occurs when sequencing reads are mistakenly assigned to the wrong sample due to similarities between barcode sequences. This challenge can be minimised by carefully designing barcode sequences, using error-correcting algorithms during demultiplexing, and employing forward and reverse primer barcode pairs. This additional layer of specificity ensures accurate sample identification.

AVAILABLE SOLUTIONS FOR FULL GENOME SEQUENCING

The availability of diverse sequencing solutions has profoundly impacted research and public health. They have enabled rapid genomic surveillance of SARS-CoV-2, identifying variants of concern and tracking transmission pathways. The choice of sequencing solution depends on various factors, including the study's objectives, the resources available, and the expertise of the personnel.

Comprehensive Sequencing Kits: Simplifying the Complex

Various companies have developed sequencing kits to streamline the process of preparing SARS-CoV-2 samples for NGS. These kits are designed to simplify and standardise the workflow, ensuring that laboratories can generate high-quality sequencing data with relative ease. These kits often have detailed protocols and technical support to ensure successful implementation in various laboratory settings. The choice of a kit should be based on the specific needs of the study, the available sequencing platform, and the desired throughput and coverage. It is also important to check the latest information from the manufacturers, as the field of NGS and viral sequencing is rapidly evolving with continuous improvements and updates to existing products.

Following is a comprehensive list of commercially available sequencing kits developed by various companies for preparing SARS-CoV-2 samples for NGS.

- **llumina® COVIDSeq Test (Illumina):** An end-to-end, high-throughput sequencing solution that includes reagents and software for SARS-CoV-2 genome sequencing (Lowry et al., 2023).
- **QIAseq SARS-CoV-2 Primer Panel (Qiagen):** A set of digital PCR primers and probes for targeted enrichment and sequencing of the SARS-CoV-2 genome (T. Liu et al., 2021).
- **NEBNext® ARTIC SARS-CoV-2 Library Prep Kit (New England Biolabs):** Based on the ARTIC Network's protocol, this kit is designed for rapid and comprehensive sequencing of the SARS-CoV-2 genome (Gautreau, 2021).
- **Swift Normalase Amplicon Panels (SNAP) SARS-CoV-2 (Swift Biosciences):** A kit for the rapid generation of amplicon-based libraries for SARS-CoV-2 sequencing (L. Shrestha et al., 2021).
- **Ion AmpliSeq SARS-CoV-2 Research Panel (Thermo Fisher Scientific):** A multiplex PCR-based targeted sequencing solution that provides comprehensive coverage of the SARS-CoV-2 genome (Alessandrini et al., 2020).
- **COVID-19 NGS Assay (BGI):** A next-generation sequencing assay that provides all necessary reagents for SARS-CoV-2 genome sequencing.
- **Paragon Genomics CleanPlex SARS-CoV-2 Panel:** A multiplex PCR-based targeted NGS assay designed to detect and characterise SARS-CoV-2 genomic RNA (Charre et al., 2020).
- **NimaGen EasySeq RC-PCR SARS-CoV-2 Kit:** Uses reverse-complement PCR for amplifying and sequencing of the SARS-CoV-2 genome (Coolen et al., 2021).
- **Agilent SureSelect XT HS2 SARS-CoV-2:** A capture-based NGS library preparation kit for sequencing SARS-CoV-2 genomes (Z. Gerber et al., 2022).
- **Twist Bioscience SARS-CoV-2 Research Panel:** A comprehensive NGS approach using synthetic RNA controls to detect the SARS-CoV-2 virus(Rehn et al., 2021).
- **ArcherDX Archer SARS-CoV-2 Panel:** Designed to detect the SARS-CoV-2 virus, this panel uses Anchored Multiplex PCR (AMP™) for targeted enrichment.

- **SeqOnce Biosciences RhinoSeq SARS-CoV-2 Library Prep Kit:** A streamlined, single-tube library preparation workflow for SARS-CoV-2 NGS.

Community-Driven Protocols: The ARTIC Network

In contrast to commercial kits, the ARTIC protocol represents a community-driven approach to SARS-CoV-2 sequencing (Lambisia et al., 2022). Developed by the ARTIC network, this protocol has gained widespread adoption due to its openness, flexibility, and cost-effectiveness. The ARTIC protocol uses a tiled amplicon strategy to generate overlapping amplicons covering the entire viral genome. This method has been particularly useful for surveillance and research, as it allows for rapid iteration and adaptation in response to the evolving virus. The protocol is platform-agnostic, meaning it can be adapted for use with different NGS platforms and is supported by a robust community of scientists who contribute to its continuous improvement.

5.3.4.4 FINANCIAL IMPLICATIONS OF NGS: GLOBAL PERSPECTIVES AND EQUITY

The application of NGS to analyse SARS-CoV-2 and other pathogens is not uniformly accessible globally (Marais et al., 2023). Economic disparities between high-income countries (HICs) and low- and middle-income countries (LMICs) have created a chasm in scientific capabilities and healthcare outcomes. This section critically examines the financial considerations associated with implementing NGS and explores the challenges and potential solutions for global application.

RESOURCE ALLOCATION AND NGS CAPACITY IN HICS VS LMICS

In HICs, the financial investment in healthcare infrastructure has historically facilitated the adoption of NGS technologies. The cost of NGS, while substantial, is often justifiable by the high throughput, rapid turnaround, and the wealth of genomic data it provides. HICs can invest in NGS platforms, skilled personnel, and the bioinformatics support required for complex genomic analyses.

Conversely, LMICs may face significant financial barriers to entry. The initial costs of NGS equipment, maintenance, consumables, and training can be prohibitive. Additionally, the recurring expenses associated with library preparation kits, reagents, and quality control can strain limited budgets. In many LMICs, resources may be allocated to more immediate and basic healthcare needs, leaving little room for investment in advanced genomic technologies.

RESOURCING IN REGIONS WITHOUT NGS CAPACITY

For countries or regions without inherent NGS capacity, collaborative efforts with international organisations, partnerships with HICs, and the support of global health initiatives become essential. Such collaborations can involve sharing resources, knowledge transfer, and capacity-building programs. Satellite and reference laboratories in HICs may also offer sequencing services to LMICs, although this approach can lead to delays and may hinder local capacity development.

Another strategy is the implementation of portable and more affordable NGS platforms, like the Oxford Nanopore MinION, which, while offering lower throughput, can be a cost-effective option for regions with limited resources. These portable platforms can be pivotal during infectious disease outbreaks, allowing for in-field sequencing without extensive laboratory infrastructure.

APPLICABILITY ACROSS THE WORLD: LIMITATIONS AND SETTINGS

The applicability of NGS across different global settings is fraught with limitations. In addition to financial constraints, logistical challenges include supply chain management, the need for stable electricity and internet connectivity for data transfer and analysis, and a cold chain to preserve reagents and samples.

There are also disparities in technical expertise. Effective use of NGS technology requires skilled personnel who can conduct laboratory procedures and manage and interpret the complex data produced. This necessitates significant investment in education and training.

Despite these limitations, the role of NGS in global health is undeniable. Rapid genomic surveillance using NGS can inform containment strategies and guide public health interventions in outbreak situations. In clinical settings, NGS can lead to better patient outcomes by identifying pathogen resistance patterns and tailoring treatments.

5.3.4.5 PERFORMANCE, CONSTRAINTS, AND STANDARDISATION IN NGS OF SARS-COV-2

The performance of NGS technologies is subject to various constraints that can affect data quality and interpretability. As such, the standardisation of protocols and procedures has become essential to ensure reliable and comparable results across laboratories

Performance in NGS is multifaceted, encompassing accuracy, read length, depth of coverage, and throughput. Accuracy, which reflects the ability to correctly determine the nucleotide sequence, is paramount in detecting SARS-CoV-2 variants. Read length, or the length of the DNA sequence that can be read in a single sequencing run affects the ability to reconstruct the viral genome and identify structural variations. Depth of coverage, the number of times a nucleotide is sequenced, is crucial for identifying low-frequency variants within a viral population. Throughput, the amount of data generated in a sequencing run, determines the scale at which sequencing can be performed.

Despite its transformative potential, NGS is not without limitations. One of the primary constraints is cost, which can be prohibitive, particularly for high-throughput sequencing. Furthermore, the technical complexity of NGS workflows requires specialised equipment and trained personnel, presenting challenges in resource-limited settings. Sample quality is another significant constraint. Degraded RNA, low viral loads in patient samples, and contamination can all compromise the integrity of NGS data. Additionally, the rapid evolution of the virus necessitates continual updates to sequencing protocols and reagents, posing logistical and financial burdens.

The standardisation of NGS protocols is critical to ensure that results are consistent, accurate, and reproducible across different laboratories and over time. This includes standardising sample collection, RNA extraction, library preparation, sequencing, and bioinformatics analyses. Efforts to standardise include developing reference materials, control samples, and guidelines by international consortia and regulatory agencies. For example, the WHO and the CDC have provided protocols and best practices for the sequencing of SARS-CoV-2. Standardising data-sharing formats and repositories is also crucial for collaborative research efforts.

5.3.4.6 THE IMPORTANCE OF GENOME COVERAGE, READ DEPTH, AND READ LENGTH

DEFINITION OF CRITERIA OF READ AND ALIGNMENT QUALITY, AND OF READ TRIMMING CRITERIA

In the high-stakes endeavour of sequencing the SARS-CoV-2 genome, three factors—genome coverage, read depth, and read length—stand out as crucial determinants of data quality and utility. Each plays a vital role in characterising the viral genome and identifying mutations that could affect disease transmission and treatment.

Genome coverage refers to the proportion of the genome sequenced and represented in the reads. High genome coverage is essential to ensure that the entire genetic blueprint of the virus is captured, including all coding and non-coding regions. For SARS-CoV-2, achieving complete or near-complete coverage is critical for identifying all potential mutations, especially those in less conserved regions that might go undetected with lower coverage.

Read depth or sequencing depth, indicates the number of times a given nucleotide is read during sequencing. High read depth enables the detection of low-frequency variants and increases the confidence in variant calling. For SARS-CoV-2, where intra-host viral diversity and mixed infections can occur, sufficient read depth is imperative to identify and quantify minority variants that may have implications for pathogenicity, transmissibility, or resistance to therapeutics and vaccines.

Read length is the length of the nucleotide sequence that can be read in a single continuous sequencing reaction. Longer reads can span larger genomic regions, which aids in the assembly of the viral genome and the resolution of complex structural variants. However, longer reads often come at the cost of reduced accuracy and increased complexity in data analysis. For SARS-CoV-2, balancing read length with the need for precision is a nuanced exercise, particularly when reconstructing the genome from highly fragmented viral RNA.

The quality of reads and their alignment to a reference genome are foundational to the integrity of sequencing data. Quality scores, usually represented on a Phred scale, are assigned to each nucleotide and reflect the probability of a correct base call. Reads with high-quality scores are more reliable and contribute to more accurate consensus sequences. Alignment quality is similarly crucial; it ensures that reads map correctly to the reference, which is especially important for identifying mutations and understanding the virus's evolutionary dynamics.

Read trimming involves the removal of low-quality bases or sequences from reads before alignment and analysis. The criteria for trimming are based on quality scores and the presence of adapter sequences or other contaminants. The goal is to retain high-quality data contributing to accurate genome assembly and variant detection. Trimming must be performed judiciously to avoid the loss of important information, particularly in regions where the virus may have evolved rapidly.

THE ROLE OF READ DEPTH AND FREQUENCY IN SNP CONFIRMATION

Single Nucleotide Polymorphisms (SNPs) are the most common genetic variation among organisms, including viruses like SARS-CoV-2. In the context of NGS, identifying and validating SNPs is crucial for understanding the evolution and spread of the virus. The validation of SNPs through NGS involves several steps, from the initial detection of variants in sequencing reads to their confirmation through stringent analysis. This process ensures that reported SNPs accurately reflect the viral genome and are not products of sequencing errors or biases.

Read depth is a critical factor for SNP validation. Adequate read depth provides multiple observations of the nucleotide at the same position, increasing the confidence that a SNP is a true variant rather than a random error. The required read depth for SNP validation can vary depending on the sequencing platform and the error rate associated with the technology. Still, coverage for reliable SNP calling typically ranges from 30x to several hundred times.

Allele frequency, the proportion of reads displaying the alternative nucleotide at a specific position, is another cornerstone of SNP validation. It indicates the prevalence of the SNP within the viral population in a given sample. For a SNP to be considered valid, it generally must exceed a minimum allele frequency threshold, which helps to filter out low-frequency sequencing artefacts. The threshold can be influenced by factors such as the sample's viral load, the virus's population structure, and the sequencing reads' quality.

DECIPHERING MUTATIONS AND LINEAGES: THE IMPACT OF RAW DATA AND BIOINFORMATICS

The ongoing SARS-CoV-2 pandemic has underscored the importance of NGS in tracking viral mutations and identifying lineages or variants of concern. The quality of raw sequencing data and the robustness of the bioinformatics workflow are pivotal in accurately characterising the viral genome

The quality of raw sequencing data is the foundation for any subsequent analysis. Genome coverage that is comprehensive ensures no part of the SARS-CoV-2 genome is overlooked, allowing for the detection of mutations across the entire viral RNA. Similarly, sufficient read depth is critical for identifying mutations with high confidence, particularly those present at low frequencies in the viral population. Read length can influence the ability to span complex genomic regions and resolve ambiguities in the genome assembly, which may contain mutations of epidemiological significance.

The bioinformatics workflow is a multi-step process that transforms raw sequencing data into interpretable information about the viral genome. The following components of the workflow are instrumental in identifying mutations and classifying lineages:

1. **Quality Control and Pre-processing:** Initial steps involve assessing the quality of raw reads, trimming adapters and low-quality bases, and filtering out poor-quality reads. This pre-processing improves the reliability of the downstream analyses.

2. **Read Mapping and Alignment:** Cleaned reads are aligned to a reference genome, usually the Wuhan-Hu-1 strain for SARS-CoV-2. The accuracy of this step is paramount as it provides the positional context for identifying mutations.

3. **Variant Calling:** Bioinformatics tools analyse the aligned reads to identify single nucleotide polymorphisms (SNPs), insertions, and deletions. The stringency of variant calling parameters, such as the minimum allele frequency and quality score thresholds, affects the sensitivity and specificity of mutation detection.

4. **Consensus Sequence Generation:** A consensus sequence is constructed from the variant calls, representing the most likely sequence of the viral genome in the sample. In cases where multiclonal infections or environmental samples are being analysed, the predominant sequence may also be generated, which represents the most abundant sequence variant in the sample. Both the consensus and predominant sequences are crucial for comparing and classifying lineages.

5. **Phylogenetic Analysis and Lineage Assignment:** The consensus sequence is used for phylogenetic analysis to determine the evolutionary relationships between the sample and known SARS-CoV-2 sequences. Tools such as Pangolin are then used to assign the sample to a specific lineage or variant based on the mutations it harbours. In the context of wastewater surveillance, where samples often consist of a complex mix of variants, specialized tools like Frejya can be employed to tease apart the different lineages and variants present, providing a more comprehensive understanding of the viral diversity in these samples (Karthikeyan et al., 2022).

The robustness of the bioinformatics workflow directly influences the accuracy of mutation identification and lineage classification. Errors or biases at any stage can lead to the misidentification of mutations or incorrect lineage assignments. Furthermore, the rapid evolution of SARS-CoV-2 necessitates continuous updates to

reference genomes, databases, and bioinformatics tools to capture the latest variants and mutations

5.3.4.7 BENCHMARKING WASTEWATER AND CLINICAL GENOMICS

COMPARATIVE GENOMIC SURVEILLANCE: WASTEWATER VERSUS CLINICAL SAMPLING

The genomic characterisation of SARS-CoV-2 through NGS has become a cornerstone in understanding and managing the COVID-19 pandemic. NGS can be applied to various sample types, with clinical samples from individual patients and pooled population samples from wastewater being the primary sources. This section delves into the advantages and disadvantages of genomic characterisation from these two distinct sources, providing insights into their roles and efficacy in public health surveillance.

Genomic Characterisation of Population Samples

Epidemiological surveillance through wastewater provides a macro-level snapshot of the virus's presence in communities. Analysing sewage for viral RNA offers an early detection system that can signal an upcoming outbreak, even before clinical cases emerge. The inclusivity of wastewater sampling stands out, capturing data from symptomatic and asymptomatic individuals, regardless of their access to medical testing. As a cost-effective surveillance approach, it allows for the monitoring of large populations and informs public health strategies by revealing trends in viral prevalence. However, the approach is not without its complexities. The dilution of viral RNA in vast volumes of wastewater presents significant challenges to detection and quantification. Furthermore, the complexity of wastewater as a sample matrix, replete with numerous inhibitory substances, poses additional hurdles to laboratory processing. A notable limitation of this population-level data is the absence of individual health outcomes, which impedes the ability to draw direct correlations with clinical features or to conduct precise contact tracing.

Genomic Characterisation of Individual Samples

Clinical genomics narrows the focus to the individual, providing a high-resolution view of the viral genome from patient-derived samples. This specificity allows for the direct association of genomic variations with clinical manifestations, offering insights into the virus's pathogenicity and informing patient care. The detailed genomic information gleaned from individual samples is indispensable for identifying new mutations and tracking the emergence of variants. However, the individual-focused nature of clinical genomics comes with a set of challenges. The resource-intensive approach requires significant investment in sample collection, processing, and sequencing. The data may also be subject to selection bias, as it typically represents those seeking medical attention and may not reflect the broader community infection dynamics. Additionally, managing individual genomic data raise concerns regarding patient privacy and ethical considerations surrounding data handling.

Benchmarking Wastewater and Clinical Genomics

Benchmarking the performance of wastewater versus clinical genomics involves assessing their capacity to provide actionable intelligence for public health interventions. While wastewater genomics excels in offering a preliminary assessment of community-level viral spread, clinical genomics delivers granular data that is crucial for understanding individual patient responses to the virus. While distinct in their application and scope, the two methods are not mutually exclusive; rather, they are complementary tools in the genomic surveillance toolkit.

LINEAGE ASSIGNMENT CHALLENGES IN POPULATION-LEVEL SARS-COV-2 GENOMIC SURVEILLANCE

The assignment of SARS-CoV-2 lineages is critical to tracking the virus's evolution and the emergence of variants of concern. While NGS has enabled this process, the task is considerably more complex when dealing with population samples, such as those collected from wastewater, than individual clinical samples.

Complexities in Population-Level Lineage Assignment

Wastewater surveillance captures a composite picture of the viral RNA shed by an entire community. This pooled nature of the sample introduces several complexities that can confound lineage assignment:

- **Mixed Viral Populations:** Wastewater samples often contain a mixture of viral genomes from numerous individuals, potentially including several lineages and variants. This heterogeneity complicates determining which sequences are predominant and which are transient or less prevalent.
- **Low-Level Variant Detection:** The sensitivity of NGS in detecting low-abundance variants within a mixed sample is a significant challenge. Assigning lineages to these low-frequency variants requires high read depth and sophisticated bioinformatics tools.

- **Sequence Quality and Coverage:** The fragmented nature of viral RNA in wastewater can lead to gaps in sequence coverage, affecting the confidence in lineage assignment. Incomplete genome coverage may miss critical mutations that define certain lineages, and these recombinant viruses can then be shed into the wastewater, making it challenging to accurately assign lineages.
- **Recombination Events:** Recombination between different viral strains within a host can generate novel genomic combinations that may not align cleanly with established lineage definitions, and these recombinant viruses can then be shed into the wastewater, making it challenging to accurately assign lineages.
- **Temporal Dynamics:** The viral RNA present in wastewater reflects a snapshot of the community infection status at a particular time. The frequency of sampling can impact the ability to capture the dynamics of lineage shifts, with less frequent sampling potentially missing transient lineages or failing to capture the shift in lineage dominance over time, while more frequent sampling may provide a more detailed picture of the evolving viral landscape.

Bioinformatics and Lineage Assignment

The bioinformatics pipeline for lineage assignment from population-level samples must be optimized to address the above challenges. It involves several key steps:

- **Quality Control:** Rigorous quality control measures are essential to ensure that only high-quality, reliable sequences are used for lineage assignment.
- **Deconvolution of Mixed Samples:** Bioinformatics tools must be able to deconvolute mixed sequences into individual variants. This process is computationally intensive and may not always be definitive.
- **Consensus Building:** Generating a consensus sequence from a mixed population requires careful interpretation of the data, with an understanding that some lineage-defining signals may be diluted.
- **Phylogenetic Analysis:** Advanced phylogenetic techniques are employed to place the consensus sequence within the global context of SARS-CoV-2 evolution and to infer the most likely lineage assignments.

The difficulties in lineage assignment from population-level samples directly affect public health surveillance. Misassignment or incomplete identification of lineages can affect the understanding of transmission dynamics and the effectiveness of intervention strategies. Accurate lineage assignment is also crucial for monitoring the emergence of variants that may have implications for vaccine efficacy or therapeutic interventions.

5.3.4.8 ERRORS, LIMITS, AND CONFIDENCE ESTIMATION

ESTIMATING LINEAGES: ERRORS, LIMITS, AND CONFIDENCE

Accurately estimate the proportion of different lineages and variants within a single sample is a challenging endeavour. NGS technologies provide the necessary resolution for detecting multiple viral strains in a single sample, but several factors can influence the accuracy and confidence of these estimates.

Accurately estimating variant proportions in a single sample involves deciphering the complex mixture of viral genomes that can co-exist in an infected individual or a community. This complexity is compounded by several technical and biological factors:

- **Intra-Sample Viral Diversity:** The virus may exist as a heterogeneous population of closely related genomes within a single individual. This intra-host diversity, driven by rapid viral replication and mutation, can make it difficult to disentangle individual variants.
- **Sequencing Depth and Coverage:** The depth of sequencing coverage across the viral genome can significantly influence the detection and estimation of variants. Low coverage areas may yield unreliable estimates, while high coverage is required to confidently identify and quantify minority variants.
- **Biases in Amplification and Sequencing:** PCR amplification biases, sequencing errors, and platform-specific artefacts can skew the observed frequencies of variants. These technical biases must be accounted for to avoid misestimating variant proportions.

Several types of errors can affect the estimation lineage and variant proportions:

- **Random Sequencing Errors:** Sequencing platforms have inherent error rates that can introduce random base substitutions, insertions, or deletions. While error rates are low, they can still affect the variant calls, especially for low-frequency alleles.

- **Systematic Biases:** Certain sequences or motifs may be prone to systematic errors due to PCR amplification or sequencing biases. These errors are not random and can consistently affect certain genomic regions.
- **Sample Preparation Variability:** Variations in sample preparation, such as RNA extraction efficiency or library construction, can result in uneven representation of the viral genome and affect the estimation of variant proportions.

Confidence in variant proportion estimates measures reliably the sequencing data represents the true viral population. Several approaches are used to enhance and measure this confidence:

- **Replicate Sequencing:** Sequencing the same sample multiple times can assess the reproducibility of variant calls and help distinguish true variants from sequencing artefacts.
- **Error Correction Algorithms:** Bioinformatics tools and algorithms are designed to correct for known errors and biases in sequencing data, thereby improving the accuracy of variant estimation.
- **Statistical Modelling:** Statistical models can be employed to estimate the confidence intervals around variant frequencies, considering the depth of coverage and the error rates of the sequencing platform.

IMPACT OF THE PCR CT AND RNA INTEGRITY IN THE SEQUENCING PROCESS

The sequencing process is susceptible to various errors, and recognising the limitations and establishing confidence in the results is crucial. This section analyses the impact of the PCR cycle threshold (Ct) to here values and RNA integrity on the sequencing process, elucidating their roles in the accuracy of SARS-CoV-2 genomic data.

PCR Ct values refer to the number of cycles required for the fluorescent signal of a PCR amplification to cross a certain threshold and become detectable. The Ct value is inversely proportional to the amount of target nucleic acid in the sample—the lower the Ct value, the higher the viral RNA concentration.

Impact on Sequencing:

- **Quantification of Viral Load:** Ct values provide an initial quantification of the viral load in a sample. High viral loads (low Ct values) result in more robust starting material for sequencing, increasing the likelihood of successful amplification and coverage.
- **Influence on Sequencing Depth:** Samples with low Ct values typically require fewer amplification cycles during library preparation, reducing the potential for amplification errors and biases, which can impact sequencing depth and variant detection.
- **Detection of Low-Frequency Variants:** Higher viral loads facilitate the detection of low-frequency variants, as they are represented in greater absolute numbers in the starting material, enhancing the sensitivity of NGS.

Challenges Posed by High Ct Values:

- **Insufficient Viral RNA:** Samples with high Ct values may contain insufficient viral RNA, leading to incomplete genome coverage or failure to generate a sequencing library.
- **Increased Risk of Errors:** Extensive PCR amplification required for samples with high Ct values can introduce errors and chimeric sequences, potentially skewing the data.
- **High Ct values** can lead to challenges in viral genome sequencing, including insufficient viral RNA, increased errors, and primer heterodimer formation, which can result in incomplete or inaccurate data and failed sequencing runs.

RNA integrity is a measure of the extent of degradation of the RNA in a sample. It is a critical quality parameter for sequencing, as degraded RNA can adversely affect the accuracy and completeness of the genomic data.

Influence on Sequencing Success:

- **Fragmentation and Coverage:** Degraded RNA often results in fragmented genomes, which can lead to uneven coverage and gaps in the sequence, affecting the assembly and alignment of the SARS-CoV-2 genome.
- **Bias in Variant Calling:** RNA degradation can lead to overrepresenting or underrepresenting certain regions in the sequencing data, introducing bias in variant calling and potentially missing lineage-defining mutations.

Strategies for Mitigating Risks:

- **Optimised Extraction Methods:** Employing RNA extraction methods that minimise degradation is crucial for preserving RNA integrity.
- **Quality Assessment:** Assessing RNA integrity before sequencing allows for selecting high-quality

samples and adjusting library preparation protocols accordingly.

- **Bioinformatics Corrections:** Bioinformatics tools can partially compensate for low RNA integrity by identifying and filtering out poor-quality reads and regions of low coverage.

The PCR Ct values and RNA integrity are pivotal factors that influence the fidelity of NGS in the genomic analysis of SARS-CoV-2. Low Ct values and high RNA integrity are conducive to more accurate and complete sequencing results. Conversely, high Ct values and compromised RNA integrity pose challenges that can lead to errors, reduced confidence in the data, and potentially misinform public health decisions. Understanding these impacts allows developing strategies to mitigate risks and improve the reliability of sequencing outcomes.

ALGORITHMS FOR CONFIDENCE ESTIMATION IN SARS-COV-2 SEQUENCING

Confidence estimation algorithms function by assessing the probability that a given nucleotide call is correct or that a lineage is truly present in the sample being sequenced. These algorithms must navigate a landscape riddled with potential sources of error, from technical noise and biases in the sequencing process to the biological complexities of the viral populations within a sample.

The process begins with generating raw sequencing reads, which are then aligned to a reference genome. Algorithms assess the quality of these alignments, considering factors such as the number of mismatches, the presence of gaps, and the overall alignment score. High-quality alignments are critical for accurate variant calling, as they reduce the likelihood of false positives arising from misaligned reads or sequencing artefacts.

Lineage calling algorithms analyse the aligned reads for discrepancies from the reference sequence. They consider the depth of coverage at each position and the quality scores of individual nucleotide calls. These algorithms can distinguish between true variants and sequencing errors by aggregating this information across multiple reads covering the same genomic position. The algorithms must also contend with low-frequency variants, which may represent real viral subpopulations or random errors. Statistical models are employed to infer the likelihood that such variants are genuine rather than artefacts.

One critical aspect of confidence estimation is the determination of the lineage allele frequency, which reflects the proportion of sequencing reads that support the presence of the lineage. Confidence in this estimate increases with the number of independent observations—that is, the higher the read depth, the greater the confidence. Nonetheless, algorithms must also consider the context of each lineage, as certain genomic regions may be more prone to errors or may have biological significance that influences the interpretation of the data.

Another layer of complexity comes from the algorithms' need to account for the technical specifications of different sequencing platforms. Error profiles can vary widely between platforms, necessitating tailored approaches to confidence estimation. Confidence estimation algorithms must be calibrated to the unique characteristics of the sequencing technology in use.

The output of these algorithms often includes a confidence score or quality value for each lineage, which can be used to filter the results and prioritize lineages for further investigation. These scores are essential for researchers to navigate the vast output of NGS data and to focus on the most reliable findings.

LOW-FREQUENCY MUTATION ANALYSIS

Low-frequency mutations are alterations in the viral genome present in a small percentage of the viral population within a sample. These mutations may represent minority lineages that could be subject to selective pressures, leading to future dominance, or transient mutations that do not persist. Accurate analysis of these lineages is complicated because the lower the frequency of a mutation, the higher the chance that sequencing errors or biases may obscure it.

One of the primary challenges in low-frequency mutation analysis is the high error rate associated with NGS technologies. While overall error rates are low, they are not negligible, especially when considering that the frequency of some genuine mutations may be similar to the error rate. This issue is compounded by the fact that sequencing errors are not evenly distributed across the genome; some regions are more prone to errors than others.

Another complicating factor is the potential for PCR amplification biases during library preparation. PCR can preferentially amplify certain sequences over others, leading to an overrepresentation or underrepresentation of specific mutations. This bias can mimic the presence of low-frequency mutations or mask their existence.

Given these challenges, rigorous bioinformatics pipelines are employed to analyse NGS data for low-frequency mutations. These pipelines include several steps:

1. Pre-processing of raw sequencing data to remove low-quality reads and bases and trim adapter sequences.

2. Aligning reads to a reference genome, takes care to use algorithms that are optimised for accuracy and that can handle multiple mismatches and indels, which are common in viral sequencing.

3. Variant calling requires algorithms capable of distinguishing low-frequency true variants from sequencing errors. This step often involves using statistical models that incorporate both the depth of coverage and the quality of nucleotide calls at each position.

4. Post-variant calling filters, which apply additional criteria to reduce the likelihood of false positives. These filters might include the requirement for a variant to be observed in both forward and reverse reads or across multiple independent sequencing runs.

Additionally, confidence in low-frequency variant analysis can be bolstered using technical replicates, where the same sample is sequenced multiple times to confirm the presence of variants. Using molecular barcoding, where individual RNA or DNA molecules are tagged with unique sequences before amplification, can also help differentiate between true mutations and PCR or sequencing errors.

THE IMPLICATIONS OF PRIMER CLIPPING

The detection and analysis of SARS-CoV-2 via NGS hinge on our ability to accurately interpret the viral genome. An essential but often understated aspect of this process is the handling of primer sequences used to amplify viral RNA. Primer clipping, the bioinformatics removal of primer sequences from NGS reads, is a crucial step that, if not properly executed, can introduce errors into the dataset.

While preparing sequencing libraries, particularly in targeted amplicon sequencing approaches, primers are used to amplify specific regions of the SARS-CoV-2 genome. These primers are crucial for enriching the genetic material of interest but are not part of the viral genome. Consequently, any primer-derived sequences that remain in the sequencing reads can be mistaken for genuine viral sequences, leading to false variant calls and misinterpretations of the viral genetic landscape. The primer clipping process aims to identify and remove these extraneous primer sequences before downstream analysis. This task is not as straightforward as it may seem, as it requires distinguishing between the primer-derived sequences and genuine viral sequences, especially when the primers are designed to anneal to highly conserved regions of the genome.

If primer sequences are not adequately clipped, several issues can arise:

1. **Artificially Increased Variant Frequency:** Unclipped primers can overestimate the frequency of variants that coincide with primer-binding sites, as the primer sequences may be erroneously read as part of the viral sequence.

2. **Misalignment:** Sequencing reads containing primer sequences may align incorrectly to the reference genome, leading to spurious insertion or deletion (indel) calls and subsequent errors in variant interpretation.

3. **Impact on Coverage Estimates:** The presence of primer sequences in reads can affect the perceived depth of coverage at certain genomic positions, potentially skewing the data used for variant frequency estimation.

Bioinformatics pipelines must, therefore, incorporate efficient and accurate primer clipping algorithms. These algorithms recognise the known primer sequences within the reads and excise them, leaving behind only the sequences that represent the target viral RNA. The accuracy of primer clipping is contingent upon the sequence data quality, the primer's specificity, and the algorithm's sensitivity to identifying primer-derived sequences.

The implications of primer clipping are not limited to the accuracy of variant calls. They also extend to the confidence with which researchers can assert their findings. Confidence estimation algorithms often rely on the uniformity and integrity of the sequence data. When primer sequences are not properly removed, the confidence scores assigned to variants near primer-binding sites may be artificially inflated or deflated, depending on the error introduced.

To mitigate the impact of primer-derived sequences, several strategies can be employed:

- **Molecular Barcoding:** Molecular identifiers can tag individual RNA molecules before amplification, allowing for the differentiation between errors introduced during PCR and true viral sequences.

- **Consensus Sequence Generation:** Generating consensus sequences from multiple overlapping reads can help filter out sequencing artefacts, including those arising from primer contamination.

- **Software Solutions:** Advanced bioinformatics tools are designed to recognise and handle primer sequences, improving the specificity of primer clipping and reducing the likelihood of downstream errors.

5.3.4.9 MITIGATING RNA DEGRADATION

RNA degradation is a persistent challenge in the sequencing of SARS-CoV-2, with the integrity of the viral RNA being paramount for accurate library preparation and sequencing. Degradation can lead to incomplete representations of the viral genome, potentially obscuring critical genomic information and impeding the identification of key mutations.

Preparing a sequencing library from viral RNA involves several stages, each presenting opportunities for RNA degradation. Proper handling and preservation techniques are essential from the point of sample collection through to the final stages of sequencing. The following are key considerations and troubleshooting approaches to prevent RNA degradation during these critical steps:

1. **Sample Collection and Storage:** The stability of RNA begins with proper sample collection. Using RNA stabilisation reagents and ensuring samples are immediately frozen can prevent degradation. Samples should be stored at very low temperatures, ideally in liquid nitrogen or at -80°C, to preserve RNA integrity.
2. **RNA Extraction:** Reagents that inactivate ribonucleases (RNases) are crucial during RNA extraction. Commercial RNA extraction kits often include these reagents, but care must be taken to avoid introducing RNases from external sources, such as contaminated labware or reagents. Consider adding RNAse inhibitors to post-extracted RNA, even for short-term storage, as delays between extraction and testing (even less than 24 hours) can result in significant RNA loss.
3. **RNA Quantification and Quality Assessment:** Prior to library preparation, RNA should be quantified using sensitive methods that distinguish between intact RNA and degradation products. Quality assessment tools, such as the RNA integrity number (RIN) measured by bioanalyser, can provide a clear picture of RNA quality.
4. **cDNA Synthesis and Amplification:** Reverse transcription should be performed promptly after RNA extraction to convert RNA into more stable complementary DNA (cDNA). During PCR amplification, conditions should be optimised to minimise the number of cycles, as excessive cycles can increase the likelihood of degradation and the introduction of errors.
5. **Library Preparation:** Library preparation kits designed for degraded RNA, such as those that use random priming strategies, can be employed to ensure that even partially degraded RNA samples can be used for sequencing. It is also critical to prepare the library in an RNase-free environment to prevent further degradation.
6. **Quality Control Before Sequencing:** After library preparation, a final quality control step should be performed to ensure that the library is free of degradation. Library size distribution can be assessed using capillary electrophoresis, and any signs of degradation should be addressed before sequencing.
7. **Sequencing and Data Analysis:** During sequencing, it is important to use a platform and chemistry suited to the RNA sample's quality. Platforms that can handle shorter read lengths may be more appropriate for degraded samples. In data analysis, algorithms that can compensate for the uneven coverage resulting from degraded samples should be used.

By systematically addressing the potential for RNA degradation at each stage of the sequencing workflow, researchers can enhance the quality of the sequencing data generated. This is particularly important for SARS-CoV-2, where accurately detecting the full spectrum of viral variants is critical for monitoring the pandemic and developing therapeutic and preventive measures.

5.3.4.10 CONCLUSIONS

NGS has revolutionised the field of virology, particularly in the context of SARS-CoV-2 research. The ability to rapidly and accurately sequence the viral genome has enabled researchers to track the evolution of the virus, identify new variants, and inform public health responses. The amplicon sequencing panel has proven to be a valuable tool in this effort, allowing for targeted analysis of specific genomic regions. However, the accuracy of NGS data relies on careful attention to detail throughout the entire workflow, from sample preparation to bioinformatics analysis. By optimising each step of the process, researchers can increase the confidence in their results and ensure that they are accurately representing the viral population. Ultimately, the continued development and refinement of NGS technologies will be crucial in the fight against COVID-19 and future pandemics.

5.4 METROLOGY AND STANDARDISATION

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5.4.1 KEY INGREDIENTS



Standardisation and metrology play crucial roles in wastewater surveillance of SARS-CoV-2 by ensuring the reliability, reproducibility, and comparability of testing results across different laboratories and time periods. The key ingredients in standardisation and metrology for this type of surveillance include:

- **Sampling Protocols**
  - Detailed procedures for wastewater sampling, including timing, frequency, site, and methods for collecting and storing samples.
- **Sample Processing Methods**
  - Detailed protocols for processing samples, including concentration methods
- **Analytical Methods**
  - Agreed methods for detecting and quantifying SARS-CoV-2 RNA, including polymerase chain reaction (PCR)-based methods such as reverse transcription quantitative PCR (RT-qPCR), digital PCR (dPCR), or digital droplet PCR (ddPCR) as well as next-generation sequencing (NGS) technologies.
  - Validation of analytical methods to ensure their efficacy and reliability in different wastewater matrices.
- **Quality Control and Quality Assurance**
  - Use of positive and negative control samples, blanks, and spikes to monitor the precision and accuracy of the analytical workflow and viral RNA recovery rate.
  - Implementation of internal and external quality control procedures, including participation in proficiency testing programmes where available
  - Development and use of certified reference materials (CRMs) and/or reference materials (RMs) for the target or surrogate analyte to calibrate instruments, validate methods and assess their performance.
  - Establishment of standard reference materials that mimic the matrix of wastewater to ensure consistency in detection and quantification.
  - Regular interlaboratory comparisons to assess the performance of different laboratories, identify and share best practices and identify potential areas for improvement.
- **Data Interpretation and Reporting**
  - Guidelines for data processing, interpretation, and reporting to ensure that results are communicated clearly and consistently.
  - Standard units of measurement and agreed-upon thresholds for detection and quantification.
- **Traceability**
  - Ensuring traceability of measurement results to national or international standards to facilitate comparability of results.
- **Uncertainty Evaluation**
  - Assessment and reporting of measurement uncertainty to help inform the confidence to have in the results and their potential variability.
- **Standardisation of Results Across Different Matrices**
  - Accounting for differences in wastewater composition (e.g., raw sewage, influent, effluent) and physical and chemical parameters (e.g. temperature, pH, presence of PCR inhibitors, etc.) and the impact on virus detection and quantification.
- **Ethical and Privacy Considerations**
  - Ensuring that surveillance activities respect individual privacy and comply with ethical standards and regulations, particularly when reporting data that could be linked to specific populations or locations.

- **Collaboration and Harmonisation**
  - Promoting collaboration among standardisation bodies, metrology institutes, regulatory agencies, and the scientific community to develop and harmonise standards and best practices.
- **Responsiveness to Change**
  - Flexibility to update and adapt standards and metrology practices as new scientific knowledge emerges and as the virus and its variants evolve.

The development and implementation of these elements are critical for building confidence in wastewater surveillance data and for using the information derived from this surveillance to inform public health decisions. Standardisation bodies like ISO, CEN, and others, along with national metrology institutes, play pivotal roles in establishing and maintaining these standards and practices.

5.4.2 RECIPE



Implementing the key elements of standardisation and metrology in a wastewater surveillance program for SARS-CoV-2 involves a series of coordinated steps. Here is a general outline of how to proceed:

1. **Establish Goals and Objectives**
   a. Define the purpose and scope of the surveillance program (e.g., early warning, trend analysis, hot spot identification).
   b. Determine the target population and the geographic extent of the surveillance effort.
2. **Develop a Sampling Strategy**
   a. Decide on sampling locations, frequency, and timing based on the goals of the program.
   b. Ensure that the sampling plan is representative of the community or target area.
3. **Select and Validate Analytical Methods**
   a. Choose appropriate methods for SARS-CoV-2 detection and quantification, such as RT-qPCR, dPCR, or ddPCR.
   b. Validate the selected methods against established standards, if available, and adapt the methods as needed for the specific matrix of wastewater. In the absence of established standards, alternative approaches can be used (see section 5.4.3)
4. **Implement Quality Control and Assurance**
   a. Establish laboratory protocols for handling and processing samples to avoid contamination and degradation.
   b. Use control samples and perform regular calibrations to maintain the accuracy and precision of measurements.
5. **Acquire and Use Reference Materials**
   a. Obtain CRMs and RMs suitable for wastewater surveillance.
   b. Utilise these materials for method validation, viral RNA recovery rate quantification, instrument calibration, and ongoing quality assurance.
6. **Participate in Proficiency Testing**
   a. Engage in proficiency testing programs to benchmark against other laboratories and identify potential improvements.
7. **Ensure Data Quality**
   a. Implement data management practices to ensure the integrity and confidentiality of surveillance data.
   b. Analyse data using standard units and thresholds, and estimate measurement uncertainty.
8. **Interlaboratory Comparisons and results harmonisation**
   c. Collaborate with other laboratories to compare methodologies and results, striving for consistency and reproducibility.
9. **Establish Traceability**
   a. Make sure that all measurements can be traced back to national or international standards.
10. **Interpret and Report Data**
    a. Develop standardised procedures for data interpretation and reporting.
    b. Share results with relevant stakeholders in a clear and timely manner, while respecting privacy concerns.
11. **Engage with Regulatory and Standardisation Bodies**
    a. Work with regulatory agencies and standardisation bodies to ensure compliance with relevant guidelines and to contribute to the development of new standards.

12. **Train and Educate Staff**

a. Provide training for all personnel involved in the surveillance program to ensure they understand the protocols and standards.

13. **Monitor and Update Protocols**

a. Continuously monitor the effectiveness of the surveillance program and make adjustments as needed.

b. Stay informed about new technologies, methods, and standards that may improve the program.

14. **Collaborate and Communicate**

a. Foster collaboration between public health officials, scientists, and wastewater management entities.

b. Communicate the goals and findings of the surveillance program to the public and stakeholders.

15. **Ethical Considerations and Community Engagement**

a. Address ethical considerations in data collection and reporting.

b. Engage with the community to maintain transparency and trust.

By following these steps and emphasising the importance of standardisation and metrology, a wastewater surveillance programme can provide valuable insights into the prevalence and spread of SARS-CoV-2, thereby informing public health decision-making and interventions.

5.4.3 TIPS

If no established proficiency testing programmes, reference materials, or standards are available for wastewater surveillance of SARS-CoV-2, you can take several steps to ensure the quality and reliability of your surveillance data:

- **Develop In-House Quality Control Procedures**
  - Create your own internal quality control samples by spiking wastewater with known concentrations of SARS-CoV-2 RNA or surrogate viruses.
  - Establishing a consensus on using commercial surrogate viruses and standardised protocols is crucial for ensuring comparability and consistency across laboratories in wastewater surveillance for SARS-CoV-2.
  - Use these control samples to monitor the consistency and accuracy of your assays.
- **Collaborate with Other Laboratories**
  - Reach out to other laboratories conducting similar work to compare methodologies and results.
  - Consider organising an informal interlaboratory comparison or ring trial to evaluate the performance of different methods or to evaluate reproducibility across laboratories.
- **Leverage Existing Materials and Methods**
  - Use reference materials and proficiency testing schemes from related fields, such as clinical virology, adapting them to the wastewater matrix as needed.
  - Adapt existing standard methods for similar applications to your needs.
- **Synthetic Controls and Non-Pathogenic Surrogates**
  - Employ synthetic RNA controls or non-pathogenic surrogate viruses that mimic relevant features of SARS-CoV-2 to validate your assays.
  - These controls can help establish assay performance characteristics such as sensitivity, specificity, and limit of detection.
- **Implement the Requirements of a Quality Management System (e.g. ISO 17025)**. Establish a quality management system that ensures the reliability and accuracy of wastewater surveillance testing results by:
  - Conducting rigorous method validation studies to demonstrate method fitness for purpose and contributing to the body of knowledge.
  - Maintaining detailed records of all procedures, including sampling, sample processing, and analysis, and documenting any modifications to standard procedures
  - Developing and following Standard Operating Procedures (SOPs) to ensure consistency within the laboratory and regularly reviewing and updating SOPs to reflect changes in best practices or methodologies.
- **Seek Expertise and Guidance**
  - Consult with experts in environmental virology, public health, and standardisation bodies for guidance on best practices.

- Attend workshops, webinars, and conferences to stay informed about the latest developments in the field.

- **Continuous Improvement**
  - Implement a continuous improvement process to refine methods and procedures based on new information and feedback.
- **Transparency**
  - Be transparent about the limitations and uncertainties of your data when reporting results.
  - Clearly communicate the methods used and any non-standard approaches that were necessary due to the lack of established resources.
- **Participate in Scientific Communities**
  - Engage with scientific communities and networks focusing on wastewater surveillance to share experiences and learn from others.
- **Advocate for Resources**
  - Work with relevant stakeholders, including governmenta agencies and professional organisations, to advocate for the development of proficiency testing programmes, reference materials, and standards for wastewater surveillance of SARS-CoV-2.

By taking these proactive steps, you can help ensure the credibility of your wastewater surveillance program and contribute to the broader effort to establish validated methods and resources for monitoring SARS-CoV-2 in wastewater.



5.4.4 THE SCIENCE BEHIND

In the context of wastewater surveillance for public health, particularly when monitoring for pathogens like SARS-CoV-2, the terms "standardisation" and "metrology" have distinct meanings, although they are interrelated within the broader scope of ensuring accurate, reliable, and comparable measurements.

Standardisation refers to the process of developing and implementing guidelines, protocols, and specifications to ensure that methods and practices are consistent and can be replicated. In wastewater surveillance, standardisation might include:

- Compiling consensus-based best practices as guidelines for the wastewater surveillance workflow.
- Defining how samples are to be collected (e.g., composite vs. grab sampling).
- Establishing the methods for virus concentration and RNA extraction.
- Specifying the types of assays and controls to be used in the detection and quantification of the target pathogen.
- Detailing how data and metadata should be analysed, reported and interpreted.

The goal of standardisation is to ensure that the results obtained from different laboratories or at different times are comparable. This comparability is crucial for public health surveillance, where data from various sources need to be integrated to assess the spread of a disease across different regions or over time.

Metrology, on the other hand, is the science of measurement. It encompasses all theoretical and practical aspects of measurement, whatever the measurement uncertainty and field of application. In wastewater surveillance, metrology includes:

- Calibrating instruments to ensure accuracy in measurements (e.g., PCR machines).
- Validating methods to confirm that they provide results with fit-for-purpose accuracy and precision.
- Evaluating and expressing measurement uncertainty.
- Developing reference materials for calibration purposes.
- Designing and developing reference materials that enable trueness checks, which can be designed to

challenge and evaluate either a portion or the entirety of the wastewater surveillance workflow.

Metrology provides the foundation for generating reliable and traceable measurement results. In the context of public health, accurate measurements are critical for decision-making, such as determining the concentration of a virus in wastewater to assess the level of community spread.

In summary, while standardisation in wastewater surveillance aims to harmonise procedures and practices to achieve consistency in data collection and reporting, metrology provides the tools and principles to ensure that the measurements underpinning this data are accurate, reliable, and comparable at the highest level. Both are essential for effective wastewater surveillance programs that inform public health policy and interventions.

5.4.4.1 DOCUMENTARY STANDARDS

SAMPLING STANDARDS

Wastewater sampling is a critical component of environmental monitoring, including surveillance for pathogens like SARS-CoV-2. While there may not be standards specifically for SARS-CoV-2 wastewater sampling, there are existing standards that outline general procedures for collecting wastewater samples. These can be applied or adapted for the purpose of SARS-CoV-2 surveillance. Some standards and committees relevant to wastewater sampling include but are not limited to:

- **ISO 5667-10:2020 - Water quality - Sampling - Part 10: Guidance on sampling of wastewater:** this part of ISO 5667 provides guidance on the design of sampling programmes, techniques, and the handling and preservation of wastewater samples.
- **ISO 5667-3:2018 - Water quality - Sampling - Part 3: Preservation and handling of water samples:** this standard gives instructions on how to preserve and handle water samples to ensure their integrity until analysis, which is also applicable to wastewater.
- **ISO 5667-1:2023 - Water quality - Sampling - Part 1: Guidance on the design of sampling programmes and sampling techniques:** this part of ISO 5667 includes guidance on planning sampling programmes and choosing appropriate sampling techniques, which can be relevant to the surveillance of SARS-CoV-2. · EPA Method 1693: Sewage Sludge, Biosolids, and Manure (Bacteroidales and Human Bacteroidales) by Quantitative Polymerase Chain Reaction (qPCR): although this EPA method is specific to certain bacterial groups, it includes details about sample collection that might be pertinent to viral surveillance in wastewater.
- **EPA Method 1697: Characteriation of Human Fecal Pollution in Water by HumM2 TaqMan Quantitative Polymerase Chain Reaction (qPCR) Assay**: although this EPA method is specific for human faecal pollution in waterways it provides vital QA/QC for qPCR and sampling.
- **ASTM D4448-01(2019)** - Standard Guide for Sampling Ground-Water Monitoring Wells:** while focused on groundwater, some of the principles and techniques could potentially be applied to wastewater sampling.
- **CEN/TC 230 - Water analysis:** this technical committee (TC) develops European Standards for the analysis of water, including wastewater. They address various aspects of water sampling that could be relevant for SARS-CoV-2 surveillance.

These standards offer a framework that can help in designing and implementing wastewater sampling strategies for the detection of SARS-CoV-2. They provide guidance on various aspects of the sampling process, including the selection of sampling sites, frequency, methods for sample collection, preservation, transport, and analysis.

It is important to note that due to the specific nature of SARS-CoV-2 and the need for RNA-based detection methods, additional protocols and best practices specific to the surveillance of this virus in wastewater have been developed by researchers and public health agencies. These are often based on current scientific studies and may evolve as new knowledge and techniques become available.

MEASUREMENT AND TESTING STANDARDS

So far, there were no specific international standards (ISO, CEN, or others) dedicated solely to the surveillance of SARS-CoV-2 in wastewater. However, the following standards, guidelines, and committees not specific to COVID-19 but relevant to the surveillance of pathogens in wastewater could be adapted or used as a framework for developing methodologies for SARS-CoV-2 surveillance:

- **ISO 11731:2017 - Water quality - Enumeration of Legionella:** this standard outlines the detection and enumeration of Legionella, which could provide a methodological framework for detecting other pathogens in water samples.

- **ISO/TC 147/SC 4 - Microbiological methods:** this technical committee develops standards for the enumeration and detection of microorganisms in water. Their work could be adapted for monitoring SARS-CoV-2 in wastewater.
- **ISO 16649-3:2015 - Microbiology of the food chain - Horizontal method for the enumeration of beta-glucuronidase-positive Escherichia coli:** while specific to food chain microbiology, the principles could be informative for wastewater applications.
- **ISO/TS 12869:2019 - Water quality - Detection and quantification of Legionella spp. and/or Legionella pneumophila by concentration and genic amplification by quantitative polymerase chain reaction (qPCR):** this technical specification provides methods that could be adapted for SARS-CoV-2.
- **ISO 20395:2019 - Biotechnology - Requirements for evaluating the performance of quantification methods for nucleic acid target sequences - qPCR and dPCR**: while focused for general nucleic acid target sequences quantification, its principles and guidelines can be adapted for analysing SARS-CoV-2 in wastewaters.
- **EPA Method 1602: Male-specific (F+) and Somatic Coliphage in Water by Single Agar Layer (SAL) Procedure:** while this is an EPA method rather than an international standard, it could aid in the development of methods for wastewater surveillance.
- **Guidelines for Environmental Surveillance of Poliovirus Circulation (WHO) (2023):** these guidelines from the World Health Organization provide a framework for environmental surveillance that could be relevant for SARS-CoV-2 surveillance.
- **Centers for Disease Control and Prevention (CDC) guidelines:** the CDC may provide methodologies or guidelines for monitoring and analysing viruses in wastewater, which can be applied to SARS-CoV-2.

Wastewater surveillance for SARS-CoV-2 is a relatively new area of interest that has gained significant attention during the COVID-19 pandemic. While there are already many protocols in use for wastewater surveillance of SARS-CoV-2, the development of standardised protocols and common guidelines is necessary to ensure consistency, comparability, and quality of data, and to maximise the effectiveness of this surveillance approach. Researchers and public health agencies have been working on optimising methods for the detection, quantification, and monitoring of SARS-CoV-2 RNA in wastewater as a non-invasive surveillance tool to track the spread of the virus within communities. As the field evolves, we can expect to see more formalised standards and guidelines from standardisation bodies.

OTHER SPECIFIC GUIDANCE DOCUMENTS

Specific guidance for wastewater sampling from aircraft or detailed protocols for sampling in particular locations (e.g., specific types of buildings, facilities, or communities) were not widely standardised at the level of ISO, CEN, or similar standardisation bodies. However, various organisations, including public health agencies, environmental agencies, and research institutions, have developed their own protocols and guidance for targeted sampling in response to the COVID-19 pandemic and other public health needs.

For SARS-CoV-2 surveillance, guidance and protocols have been developed by:

- **Centers for Disease Control and Prevention (CDC):** the CDC has provided guidance for wastewater surveillance for SARS-CoV-2, which includes information on sample collection, handling, and testing.
- **World Health Organization (WHO):** the WHO has issued guidelines and technical briefs on environmental surveillance for SARS-CoV-2, which may include information on various types of sampling.
- **European Commission:** the European Commission has supported research and provided recommendations on the monitoring of SARS-CoV-2 in wastewater, which may include specific guidance for sampling in different contexts.
- **Scientific Publications and Research Consortia:** research groups and consortia may publish their methods and findings in scientific journals or on their websites, which can serve as a guide for others conducting similar surveillance efforts.
- **National Environmental or Public Health Agencies:** national agencies may have developed their own guidance documents or methodologies for SARS-CoV-2 wastewater surveillance and sampling strategies for specific locations or circumstances.

For example, in the context of aircraft wastewater sampling, agencies responsible for public health and transportation safety within specific countries or regions may establish their own protocols, often in collaboration with airlines and airports. These protocols would likely be

tailored to the unique challenges of sampling in such environments, balancing public health goals with operational considerations.

If you are looking for guidance on a specific situation, such as sampling from aircraft or in a particular type of location, it would be best to consult with the relevant public health authorities or the environmental agencies responsible for wastewater management in the region of interest. They may have developed tailored protocols or can provide recommendations based on the latest scientific understanding and regulatory requirements.

5.4.4.2 METROLOGY

PROFICIENCY TESTING AND (CERTIFIED) REFERENCE MATERIALS

Since the field of SARS-CoV-2 surveillance in wastewater is rapidly evolving, and efforts are being made to develop and improve quality assurance and quality control measures, including proficiency testing programmes and the development of reference materials.

- **Proficiency Testing Programmes**
  - Some organisations and networks have initiated proficiency testing programmes for SARS-CoV-2 in wastewater. For instance, the Water Environment Federation (WEF), in collaboration with other institutions, has been known to offer proficiency testing programmes to help laboratories assess their SARS-CoV-2 detection and quantification methods. Additional examples include the Ontario Clean Water Agency in Canada, which conducts regular proficiency testing for wastewater, and the CDC NWSS, which is carrying out proficiency testing through the Wisconsin Wastewater Surveillance Program.
  - National and international laboratory networks may also conduct interlaboratory comparison studies and proficiency testing as part of their quality assurance processes for SARS-CoV-2 wastewater surveillance. For example, in 2021, the Consultative Committee for Amount of Substance - Metrology in Chemistry, responded to the Covid-19 pandemic by finalising two pilot studies, CCQM-P199.b and CCQM-P216 (Mi et al., 2022, 2023; Milavec et al., 2022).
- **Reference Materials and Certified Reference Materials**
  - RMs and CRMs are essential for method validation, calibration, and quality control. Due to the novelty of SARS-CoV-2 wastewater surveillance, the availability of CRMs was limited initially, but efforts have been made to produce and distribute such materials.
  - Organisations like the National Institute of Standards and Technology (NIST) in the United States and the Joint Research Centre (JRC) of the European Commission work on developing reference materials for various applications, including environmental and public health.
  - Specific to SARS-CoV-2, research institutions and biotechnology companies may offer synthetic RNA controls or inactivated virus particles that can be used to spike samples for testing the efficacy of the analytical workflow and of the specific detection/quantification methods.

Given the ongoing nature of the COVID-19 pandemic and the critical role of wastewater surveillance in public health responses, the development of proficiency testing programs and CRMs is a dynamic area with new resources likely to become available over time. Laboratories and agencies involved in wastewater surveillance should be proactive in seeking out the latest resources and participating in available proficiency testing programmes to ensure the accuracy and reliability of their surveillance data.

For the most current information, it would be beneficial to reach out directly to organisations involved in environmental testing and public health, such as the International Water Association (IWA), Water Research Foundation (WRF), environmental protection agencies, and national metrology institutes, as they may have more up-to-date information on available proficiency testing programmes and CRMs for SARS-CoV-2 in wastewater.

CLINICAL REFERENCE MATERIALS

RMs designed for clinical surveillance of SARS-CoV-2 can potentially be used as a starting point or as part of the quality control process for wastewater surveillance. However, it is important to consider the differences in matrix composition and concentration levels between clinical samples (like nasal swabs or saliva) and wastewater. Additionally, the large sample sizes typically required for wastewater analysis present another challenge, as adapting analytical procedures to accommodate these larger volumes can be difficult, further highlighting the need for tailored RMs and methods for wastewater surveillance.

Clinical reference materials are typically designed to mimic the matrix and viral load found in human biological samples, which can be quite different from those in wastewater. Wastewater has a more complex matrix that includes a variety of organic and inorganic

substances, and the viral load is usually much lower and more diluted.

When adapting clinical reference materials for use in wastewater surveillance, laboratories should consider the following:

- **Matrix Effects:** The complex nature of wastewater might affect the recovery and detection of the virus. Clinical RMs may not account for these matrix effects. Laboratories may need to validate that the clinical RMs behave similarly to the actual wastewater samples during the detection process.
- **Concentration Levels:** The concentration of SARS-CoV-2 in clinical RMs might be higher than what is typically found in wastewater. Labs may need to adjust the concentration of the virus in these materials to more accurately reflect the levels expected in wastewater.
- **Extraction and Detection Methods:** The methods used to extract and detect SARS-CoV-2 RNA from clinical samples might require modifications to accommodate the characteristics of wastewater.
- **Regulatory and Guidance Standards:** Laboratories should ensure that any reference materials they use, whether from clinical or environmental sources, meet the standards and guidelines set by regulatory and oversight organisations.

Despite these challenges, clinical RMs can still be valuable for certain aspects of method validation, such as evaluating the performance of molecular based detection, which are commonly used in both clinical and environmental surveillance. If clinical RMs are used, they should be part of a broader quality control strategy that may also include wastewater-specific spiked \samples, interlaboratory comparisons, and participation in proficiency testing programs.

Ultimately, for the most accurate and reliable results, it is preferable to use reference materials specifically designed for wastewater matrices. As the field of wastewater surveillance continues to grow, it is expected that more wastewater-specific reference materials will become available, supporting the needs of laboratories engaged in this type of environmental monitoring.

Chapter 6

Methods and Methodologies-
Sampling

6 METHODS AND METHODOLOGIES – SAMPLING

6.1 GENERAL ASPECTS OF SAMPLING AND STRATEGY

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This chapter delves into the fundamental aspects of sampling and strategic planning in the context of wastewater surveillance. We aim to elucidate the critical 'ingredients' that underpin effective sampling procedures and to articulate a methodical 'recipe' for the development and execution of a comprehensive surveillance strategy. Through a systematic examination of the various facets of sampling - including types, locations, frequency, preservation, and quality control - this chapter provides a framework for researchers, practitioners, and policymakers to design and implement rigorous and informative wastewater surveillance programmes. Whether the reader is an established expert in environmental science or a novice in the field, the principles set forth in this chapter are intended to guide the collection of data that is both scientifically robust and contextually relevant.

6.1.1 KEY INGREDIENTS



- **Sample Types** (Grab vs. Composite vs. Passive vs. Sludge):
  - Grab samples are individual samples taken at a specific point in time, offering a snapshot of wastewater composition.
  - Composite samples are mixtures of multiple samples collected over a designated period, providing an averaged representation of wastewater constituents.
  - Passive samples are collected by placing a medium into wastewater for a period of time followed by its removal.
  - Sludge samples are collected, typically from primary settled sludge, as grab samples, but are inherently composites of the wastewater from which the sludge settled.
- **Sampling Locations:**
  - Influent points of the wastewater treatment plants to assess the load entering the facility.
  - Locations within the sewerage or drainage system to identify sources of contamination or community health indicators in the sewer catchment.
  - Sensitive areas such as hospitals or industrial zones for targeted surveillance.
- **Sampling Frequency:** determines how often samples are collected and is crucial for capturing temporal variations in the wastewater stream.
- **Preservation and Transport:** methods to prevent degradation of analytes during storage and transit, including temperature control and chemical stabilisers.
- **Equipment and Supplies:** autoclaved bottles, coolers, ice packs, and automatic samplers are among the essentials for proper sample collection and preservation.
- **Personal Protective Equipment (PPE):** Wear gloves, safety goggles, and face masks to prevent exposure to pathogens.
- **Quality Control:** protocols to ensure data integrity, including the use of blanks, replicates, and standard reference materials.

6.1.2 RECIPE



1. **Preparation Stage**
   a. Identifying Objectives: Decide what you are monitoring for (e.g., pathogens, chemical contaminants, drug usage) and define the scope of your surveillance.
   b. Choosing Locations: Select strategic sampling points that will provide the most relevant data for your objectives. This may involve mapping the wastewater network and identifying key points of

interest (for example, consider population size and geography).

c. Establishing Frequency: Decide on the frequency of sampling that balances resource availability with the need for temporal resolution in data.

d. Select Appropriate Sample Types. Based on your objectives, choose between grab, composite, and passive samples, or a combination, to best represent the community's wastewater profile.

2. **Collection Process**

a. Gather Materials: Ensure all materials used in sampling are prepared and suitable for the types of analyses to be conducted.

b. Sample Collection: Follow the established protocols to collect wastewater samples, ensuring consistency and reliability.

3. **Transportation and Storage**

a. Secure Samples: Immediately after collection, secure samples in controlled environments to maintain integrity.

b. Transport Safely: Move samples to the analysis site while preserving the temperature and containment conditions.

c. Implement Quality Control Measures. Use quality control samples to check for cross-contamination, sample integrity, and analytical accuracy throughout the process.

6.1.3 TIPS

- Always **wear appropriate personal protective equipment** (PPE) when handling wastewater samples to protect against biohazards.
- **Consider the daily and weekly patterns of wastewater flow** when scheduling sample collection to capture representative data.
- **Maintain detailed records** of sampling times, locations, and conditions to support data analysis and interpretation.
- **Stay informed of local and regional regulations** regarding wastewater sampling to ensure compliance with legal requirements.
- **Collaborate with sanitation and wastewater treatment personnel** who can provide insights into the system's dynamics and potential sampling points.
- **Be flexible and ready to adjust your strategy** as new information or technologies become available that may improve the efficacy of your surveillance efforts.
- **Use standardised methods** to allow for comparability of data over time and across different locations.
- **Keep meticulous records** at every stage of the process to ensure reproducibility and transparency.
- **Ethical considerations:** Protect privacy and adhere to ethical standards in all aspects of surveillance.



6.1.4 THE SCIENCE BEHIND

6.1.4.1 SAMPLE TYPES

Wastewater surveillance relies on the collection of samples that can accurately represent the constituents of interest within the sewerage system. The three primary types of samples used in this context are grab, composite, and passive samples. The choice between these sampling methods is influenced by the goals of the surveillance programme, the nature of the analytes of interest, and the temporal variations of the wastewater being examined. These methods of sampling have recently been reviewed and compared (Bivins et al., 2022; Cha et al., 2023; Chan et al., 2023; Schang et al., 2021b; Wilson et al., 2022). The following provides a summary of the key findings from these studies.

GRAB SAMPLES:

Grab samples are single samples taken at a specific moment in time and provide a snapshot of the wastewater composition at that instant. Grab sampling

is simpler and less resource-intensive compared to composite sampling but may not capture the variability present throughout the day. It is beneficial when the timing of discharge is known or when assessing peak concentrations of certain constituents, such as industrial discharges that follow a predictable schedule.

COMPOSITE SAMPLES:

Composite samples are collected over a set period, typically ranging from several hours during the morning peak flow to 24 hours, and represent an averaged sample of the wastewater. These samples are often collected using automatic samplers that can take small volumes of wastewater at predetermined intervals, which are then combined to produce a sample representative of the collection period. Composite sampling is particularly useful for analytes that exhibit significant temporal variability, as it smooths out fluctuations and provides a more stable representation of the wastewater characteristics during the sampling window.

PASSIVE SAMPLES:

Passive samples involve placing a medium that is attractive to viruses and/or their RNA, within a porous container, and after a set period, typically ranging from one to several days, removing the device. The results provide a composite of the wastewater that flowed passed the device during that period. A wide range of porous housings and attractive media have been compared. These devices have similar advantages to composite sampling and are particularly useful in situations where lack of power or security preclude the use of composite samplers.

SLUDGE SAMPLES:

Whilst less commonly collected, grab samples of sludge can be successfully used as a sampling matrix for SARS-CoV-2 collection (Câmara et al., 2023). This can be useful in situations where sludge samples are readily available but liquid wastewater samples are not. Whilst these are grab samples, they are likely to be collected from primary settled sludge and as such represent a form of composite sample of what was in the contributing settled wastewater.

6.1.4.2 SAMPLING FREQUENCY

Determining the appropriate sampling frequency is a crucial component of wastewater surveillance, as it directly influences the ability to capture temporal trends and detect transient events (Feng et al., 2021b). The sampling frequency must balance the need for temporal resolution with the practicalities of resource availability, including time, equipment, and budget constraints.

High-frequency sampling allows for the detection of short-term fluctuations in the concentration of target analytes and can provide insights into diurnal patterns and peak events. This is particularly important when monitoring contaminants that are expected to vary significantly throughout the day or when attempting to identify specific discharge events. However, high-frequency sampling can be resource-intensive and may produce large datasets that require complex statistical analyses.

In contrast, low-frequency sampling is more resource-efficient and may suffice for monitoring long-term trends and changes in the community's exposure to stable contaminants. This approach may miss short-term peaks but can still provide valuable information on average exposure over time.

The optimal frequency often depends on the objective of the surveillance programme and the behaviour of the target analytes. For example, in the surveillance of infectious diseases such as COVID-19, a higher sampling frequency might be necessary during an outbreak to monitor the spread and prevalence of the virus in the community, with two samples per week being considered necessary to follow trends and provide early warning (Feng et al., 2021b). For substances that do not vary significantly over time, such as certain pharmaceuticals with consistent usage patterns, a lower frequency may be adequate.

Ultimately, the sampling frequency should be determined based on a risk assessment that considers the expected variability of the analytes, the objectives of the surveillance, and the resources available for sampling and analysis.

6.2 METHODS AND METHODOLOGIES

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Wastewater surveillance has emerged as a powerful tool to monitor the presence and trends of pathogens in a community. This approach can often provide an early warning for public health responses, as pathogens are shed in bodily fluids before individuals show symptoms. This chapter serves as a guide to the essential procedures for collecting and analysing samples effectively, ensuring reliable and useful data for surveillance purposes.

6.2.1 KEY INGREDIENTS



- **Sampling Containers:** Sterile, autoclavable, or single-use containers that can hold the sample volume without leaking or contaminating the contents.
- **Personal Protective Equipment (PPE):** Gloves, safety goggles, lab coat, and, if necessary, face masks to ensure personal safety while handling wastewater.
- **Composite Sampler:** An instrument that collects wastewater samples over a set period, providing a representative sample of the wastewater stream.
- **Refrigeration Unit:** To preserve the integrity of the samples during and after collection until analysis can be performed.
- **Transport Cooler:** Insulated and equipped with ice packs or gel packs to maintain the samples at 4°C during transportation.
- **Field Data Sheet:** For recording sampling data including date, time, location, volume, temperature, and any observations.
- **Global Positioning System (GPS) Device:** To record the precise locations of sample collection for accurate geospatial analysis.
- **pH Meter and Thermometer:** To measure the pH and temperature of the wastewater at the time of sampling.
- **Flow Meter:** If quantification of pathogen load is necessary, a flow meter can help estimate the volume of wastewater passing through the system for a given time.

6.2.2 RECIPE



1. **Identify the objective:** Determine the purpose of the sampling, whether it is to monitor pathogens, detect emerging concerns, or investigate a specific outbreak.
2. **Choose the sampling location:** Select a location that is representative of the population or area of interest. For example, inlets of wastewater treatment plants, hospitals, schools, transport vessels, and hubs.
3. **Preparation:** Use sterile equipment such as bottles, tubes, and gloves to minimise contamination. Calibrate all instruments prior to use. Label all sampling containers with the date, time, and location of collection.
4. **Safety Measures:** Wear personal protective equipment before beginning the sampling process.
5. **Sample Collection:** Depending on the method, collect grab samples directly from the wastewater stream or use an automatic, passive or sludge sampler for composite samples. Collect samples at various locations to account for flow variability.
6. **Documentation:** Accurately fill out the field data sheet with all necessary information. Record any anomalies or issues during sampling.
7. **Transportation:** Securely transport the samples to the laboratory in a cooler, ensuring they remain at the correct temperature.
8. **Analysis:** Analyse the samples as soon as possible to minimise changes in pathogen concentration or composition.

6.2.3 TIPS



- **Consistency is Key:** Follow the same procedures and use the same equipment for all samples to ensure comparability.
- **Avoid Contamination:** Never touch the inside of a sample container or lid and ensure the sampling area is free from external contaminants.
- **Timeliness:** Analyse samples as quickly as possible after collection, as pathogen levels can decrease rapidly.

- **Record Keeping:** Maintain detailed records of all sampling procedures and results for future reference and analysis.
- **Cross-Training:** Ensure that multiple individuals are trained in the sampling process to prevent disruptions in data collection.
- **Quality Control:** Include field blanks and duplicates to verify the absence of contamination and the reproducibility of results.



6.2.4 VARIANTS OF THE RECIPE - SAMPLING AT SPECIFIC LOCATIONS

Please be aware that the instructions provided here serve as foundational guidelines and should be tailored to accommodate the unique characteristics and needs of each sampling location, as well as the specific objectives of the analysis to be conducted. Additionally, adherence to stringent safety protocols is paramount during the sample collection process, particularly when operating in confined spaces or during emergency situations. It is essential to conduct a thorough risk assessment and implement appropriate safety measures to protect the health and wellbeing of the sampling personnel. Always consult with local safety regulations and public health authorities to ensure compliance with current standards and best practices.

6.2.4.1 SAMPLING AT INLETS OF WASTEWATER TREATMENT PLANTS

When sampling at the inlets of wastewater treatment plants, the approach must be tailored to capture the complex and dynamic nature of incoming wastewater. The following refined steps can enhance the quality and representativeness of the samples collected:

1. **Location Precision:** Target the influent location where raw wastewater first arrives at the treatment plant. This point should be upstream of any treatment or mixing processes to ensure the sample represents the community's wastewater.
2. **Advanced Equipment Usage:** If available, deploy an automated composite sampler or a peristaltic pump designed for wastewater sampling. These devices can be programmed to collect periodic sub-samples, ensuring a composite sample that reflects diurnal variations in flow and pollutant load.
3. **Strategic Sampling Protocol:** If available, implement a time-weighted composite sampling strategy over a 24-hour cycle to account for peak usage times and flow rate fluctuations. This approach will yield a sample that represents the average pathogen load entering the treatment plant. Alternatively, utilise grab or passive samplers, or collect sludge samples.
4. **Sample Stabilisation:** Stabilise the samples immediately upon collection using appropriate preservatives. Ensure the preservative is compatible with downstream analysis methods and does not interfere with pathogen detection.
5. **Climate-Controlled Transportation:** Ensure the integrity of the collected samples by transporting them in a chilled environment, ideally at 4°C, to mimic refrigeration conditions. Use robust, leak-proof coolers and ample coolant packs or gel ice to maintain the required temperature throughout transit.
6. **Documentation and Record-Keeping:** Meticulously document the sampling process, including the exact time and location of sample collection, the volume of wastewater sampled, the type and volume of preservative used, and any anomalies or deviations from standard procedures.
7. **Sample Handling:** Minimise sample handling and exposure to the environment. Transfer the sample to the laboratory as swiftly as possible, following a chain-of-custody protocol to ensure sample integrity and traceability.

6.2.4.2 SAMPLING AT SPECIFIC LOCATIONS SUCH AS HOSPITALS, NURSERY HOMES, OR SCHOOLS

Sampling at targeted locations such as hospitals, nursing homes, or schools requires careful consideration to ensure that the collected samples accurately reflect the health status of these specialised populations.

1. **Targeted Location Sampling:** Identify and select strategic sampling points within hospitals, nursing homes, or schools that will provide a concentrated and representative wastewater profile of the facility's inhabitants. Consider points of convergence in the wastewater system before any potential dilution or mixing with larger sewage systems.
2. **Sterile Equipment Protocol:** Utilise pre-sterilised containers or bottles specifically designed for wastewater sampling. Ensure these containers are properly sealed and capable of withstanding the chemical and biological constituents of wastewater without reacting or leaching contaminants into the sample.
3. **Optimised Sampling Strategy:** Determine the optimal time and location for grab or passive sample collection to capture the most accurate representation of the wastewater effluent. For instance, sampling immediately after peak usage periods, such as morning routines or cleaning times, can provide a concentrated sample that is indicative of the facility's pathogen load.
4. **Preservation and Safety:** Employ safe and effective preservatives that are compatible with subsequent laboratory analyses. Always handle preservatives with care, adhering to Occupational Safety and Health Administration (OSHA) guidelines to protect the sampler and the environment.
5. **Controlled Transport Conditions:** Arrange for immediate and secure transportation of samples to the laboratory. Maintain refrigeration conditions at 4°C with appropriate coolants to prevent biological changes within the sample. Ensure that the transport container is sturdy, well-insulated, and capable of preventing sample spillage during transit.
6. **Detailed Documentation:** Complete comprehensive documentation for each sample, including the specific location, time of collection, methods used, volume of wastewater collected, type and amount of preservative added, and any notable observations during sample collection.
7. **Rapid Analysis:** Strive for prompt delivery to the laboratory to allow for timely analysis. The sooner the sample is analysed, the more reliable the data on pathogen presence and concentration will be.

6.2.4.3 SAMPLING FROM TRANSPORT VESSELS AND HUBS (AIRCRAFTS, TERMINAL BUILDINGS, SHIPS)

Sampling from transport vessels and hubs, such as aircraft, terminal buildings, or ships, presents unique challenges due to their transient populations and the need for rapid response. Improvements to the process can enhance sample quality and the reliability of resulting data:

1. **Strategic Sampling Locations:** Identify critical points within the transport hubs or vessels that are most likely to yield a representative sample of the transient population. This could involve coordinating with facility management to access maintenance areas or waste handling facilities where effluent is consolidated from multiple sources.
2. **Specialised Equipment Selection:** Deploy sterilised, durable containers or bottles designed to withstand the rigours of transport conditions. Make sure the sampling equipment is also compliant with transport security regulations, especially in the case of aircraft.
3. **Calculated Sampling Plan:** Develop a sampling plan that takes into account the operational schedules of the transport hubs or vessels. Timing the grab sample collection during or immediately after peak occupancy periods can ensure a more concentrated and representative sample.
4. **Appropriate Preservation Techniques:** Choose preservatives that do not interfere with the detection of pathogens of interest.
5. **Secure and Expedited Transport:** Implement a system to quickly and securely transport the collected samples to the laboratory. Given the often tight security and logistical constraints of transport hubs, pre-arrange the transfer process to avoid delays. Use insulated coolers with sufficient coolant packs to maintain the necessary refrigerated conditions.

6. **Rigorous Documentation:** Maintain precise records of each sample, including the exact location of the vessel or hub, specific collection point, date and time of collection, methods and equipment used, and any observations or deviations from the standard procedure.
7. **Adherence to Regulations:** Ensure compliance with local and international regulations regarding the transport of biological samples, especially when crossing borders or entering secure areas such as airports or seaports.

6.2.4.4 SAMPLING IN CONFINED LOCATIONS

Sampling in confined locations like sewer tunnels or underground vaults requires a meticulous approach to ensure the safety of personnel and the accuracy of the collected samples. Here are the refined steps for this specific scenario:

1. **Safety-First Location Access:** Prioritise safety when accessing confined locations. Obtain necessary permits, conduct a hazard assessment, and equip personnel with safety gear, including helmets, visibility vests, and confined space entry equipment. Ensure that the sampling team is trained in confined space entry procedures and emergency response.
2. **Specialised Equipment for Confined Spaces:** Use appropriate sample collection equipment that is compact, easy to sterilise, and suitable for use in confined spaces. A telescoping or extendable sampling pole with a securely attached sterile container may be beneficial to safely reach the wastewater without the need to enter the most hazardous areas.
3. **Strategic Sampling Points:** Identify the sampling points that are most likely to yield a sample representative of the confined space's contributing population. In sewer tunnels, opt for upstream locations before mixing points or inflow junctions. Ensure that the sampling point is accessible and safe to approach.
4. **Preservation Methods:** Consider using non-toxic preservatives such as sodium thiosulfate to neutralise chlorine or other reactive substances.
5. **Rapid and Safe Sample Transport:** Establish a clear protocol for the secure and efficient transportation of samples from the confined location to the laboratory. Use pre-cooled, insulated containers with ice packs to keep samples at a stable temperature of 4°C. Ensure that the transport route is planned to minimise transit time.
6. **Detailed Sampling Documentation:** Document all aspects of the sampling process with precision, including the specific confined space location, the method of entry and exit, the exact point of sample collection, the time of collection, and the volume of the sample taken.
7. **Ventilation and Environmental Monitoring:** Continuously monitor the atmosphere within the confined space for hazardous gases and ensure adequate ventilation to protect the health of the sampling personnel.
8. **Communication and Coordination:** Maintain clear lines of communication between the sampling team and support personnel. Coordinate with facility managers or local authorities to ensure uninterrupted access and to inform them of the sampling activities.

6.2.4.5 SAMPLING IN EMERGENCIES FOR INSTANCES AFTER NATURAL DISASTERS

MASTER CHEF: **AHMET METE SAATCI / BILGE ALPASLAN KOCAMEMI**

In the aftermath of natural disasters such as floods, hurricanes, or earthquakes, wastewater sampling must be conducted with urgency and adaptability to assess public health risks. Here's how the process can be improved:

1. **Rapid Response Location Assessment:** Prioritise sampling locations based on the severity of the disaster's impact and the vulnerability of the population. Coordinate with emergency response teams to identify areas where wastewater infrastructure has been compromised and where sampling can provide critical public health information.
2. **Emergency-Ready Equipment:** Use robust, sterilised containers that are easy to transport and can be securely sealed. Equipment should be chosen for its durability and ability to function in potentially harsh and unpredictable environments.
3. **Adaptive Sampling Approach:** In disaster scenarios, traditional sampling points may not be accessible. Adapt your strategy to collect samples from makeshift drainage points or standing water that may have mixed with sewage. Be prepared to make on-the-spot judgments about the most

appropriate locations for sampling, given the altered landscape.

4. **Safe Preservation Under Adverse Conditions:** Given the potential for compromised safety protocols during disasters, use preservatives that are safe to handle, such as sodium thiosulfate. Ensure that preservation methods are compatible with the likely presence of varied contaminants due to the disaster.

5. **Expedited Sample Transport and Analysis:** Due to the potential for rapid changes in the contamination profile post-disaster, organise for immediate transport of samples to the laboratory. Keep samples refrigerated with portable cooling elements, and prioritise their analysis upon arrival to inform timely public health decisions.

6. **Comprehensive Documentation with Context:** Document the sample collection meticulously, noting the disaster context, including the specific emergency, the exact sampling location, and environmental conditions. Include any deviations from standard protocols necessitated by the emergency circumstances.

7. **Safety Protocols for Emergency Conditions:** Ensure that all personnel involved in sampling are equipped with appropriate safety gear, including personal flotation devices in flood conditions, helmets in areas with aftershocks, or other relevant protective equipment.

8. **Collaboration with Relief Agencies:** Work in conjunction with relief agencies and local authorities for coordinated efforts. Share information about sampling plans and findings to aid in the broader disaster response and recovery efforts.

6.2.4.6 SAMPLING WITHOUT SEWAGE SYSTEM IN INFORMAL SETTLEMENTS

MASTER CHEF: **REHNUMA HAQUE**

The term "without sewage system" typically refers to areas that lack the infrastructure for collecting, treating, and disposing of sewage or wastewater. In such contexts, human waste management often relies on alternatives like septic tanks, pit latrines, or other forms of decentralised sanitation.

Unfortunately, in some scenarios, these systems may discharge untreated faecal matter into open drains or ditches, leading to environmental contamination. According to the World Health Organization, approximately 3.5 billion people, or 43% of the global population, do not have access to safely managed sanitation. This encompasses a lack of proper facilities that ensure the treatment or safe disposal of excreta (*Progress on WASH in Health Care Facilities 2000-2021*, 2023).

Conducting wastewater surveillance in areas with limited or no sewage infrastructure poses significant challenges. The absence of a centralised sewage network complicates the identification of appropriate sampling points and the estimation of the catchment population size—key parameters for WBS.
In response to these challenges, we suggest combined targeted field strategies with technological and community-based approaches:

1. **Hydraulic Flow Identification and Targeted Location Mapping:** Determining the direction of hydrologic flow is crucial for understanding how sewage travels from the community to open environments. Identifying a single, well-placed collection point downstream can represent the entire community. Advancements in remote sensing and Geographic Information System (GIS) technology can help map sewage flow in areas where traditional infrastructure is lacking. Collaborate with community leaders to understand local practices and identify sites such as communal latrines, open drains, or dumpsites that serve as informal wastewater collection points.

2. **Defined Catchment Size and Community-Engaged Sampling Strategy:** After establishing a downstream sampling point, it's vital to map areas contributing to the main flow. User-friendly mapping applications like STRAVA and Map My Ride can assist with area mapping. However, field visits by an expert team are essential for accurately assessing environmental conditions and selecting optimal sampling sites that avoid issues of dilution or sample contamination.

3. **Appropriate Equipment for Field Conditions:** Use robust, sterile containers that can be tightly sealed to prevent spillage and contamination during transport. Containers should be lightweight and portable, allowing for ease of use in potentially dense and difficult-to-navigate settlements.

4. **Community-Engaged Sampling Strategy:** Engage with residents to determine the most appropriate times and locations for sample collection, such as early morning or late evening when communal areas are less crowded. Consider

using discreet sampling methods to respect privacy and cultural norms.

5. **Culturally Sensitive Preservation:** Opt for safer, less toxic preservatives when in close proximity to living areas, such as sodium thiosulfate for neutralising chlorine. Preservation methods should be chosen for their effectiveness as well as their minimal risk to both the sampler and the local population.

6. **Sludge Sampling as a Concentrated Source:** Recognising that enveloped viruses like SARS-CoV-2 largely partition to the solid phase of wastewater, sludge sampling offers a direct approach without the need for additional concentration steps. We collect primary sludge samples—rich in organic matter, including faecal content—through grab or composite methods while considering the heterogeneity of the matrix.

7. **Rapid and Secure Transport:** Organise for swift transportation of the samples to a laboratory, utilising portable coolers with ice packs to maintain a consistent refrigerated temperature. Given the informal nature of these settlements, ensure that the transport method is practical and can navigate potentially unconventional routes.

8. **Detailed and Contextual Documentation:** Document each sample with care, noting the specifics of the informal settlement, including its location, the exact point of sample collection, any observed environmental factors, and the community dynamics that may influence the waste profile.

9. **Health and Safety Adherence:** Equip sampling teams with appropriate personal protective equipment (PPE) and sanitation supplies, as they may be exposed to higher levels of contaminants in these settings. Ensure that all team members are trained in health and safety protocols relevant to working in informal settlements.

10. **Capacity Building and Education:** Where possible, involve and train local residents in the sampling process, building local capacity for ongoing monitoring and fostering a sense of ownership and understanding of the importance of wastewater surveillance.

Chapter 7

Methods and Methodologies- Transportation protocols

7 METHODS AND METHODOLOGIES – TRANSPORTATION PROTOCOLS

7.1 CHAIN OF CUSTODY

MASTER CHEF: MÁRTA VARGHA

Correct interpretation of wastewater surveillance data requires consistent data management. Samples should be traceable along the entire sample processing chain: conditions of sampling, sample transport, sample storage and analysis should be documented in the custody records (Association of Public Health Laboratories, 2022; US EPA, 2023). Electronic laboratory information management systems are robust and reliable means of record keeping, but well-structured paper-based custody forms also provide a viable alternative.

7.1.1 KEY INGREDIENTS

- **Labelling material:** For unambiguous sample identification, including unique sample identifiers, collection data, and sampling points.
- **Custody Forms:** Well-structured paper-based or electronic records to document sample details, such as sampling method, site information, and on-site measurements.
- **Coolants and Insulated Containers:** To maintain sample temperature during transport.
- **Temperature Monitors:** Devices to record temperature during shipping, ensuring sample preservation.
- **Protective Packaging:** To comply with regulations for transporting potentially infectious substances and to safeguard sample integrity.
- **Laboratory Information Management Systems (LIMS):** Electronic systems for reliable record-keeping and data management.
- **Quality Assurance Standards:** Accreditation documents or compliance certificates to ascertain laboratory adherence to recognised standards.

7.1.2 RECIPE

1. **Sampling and labelling**

a. Primary sample receptacles should be properly labelled in order to ensure unambiguous sample identification. Labels can be written or bar-coded, but in either case they should contain at least the following information: unique sample identifier, date and time of the sample collection, sampling point.

b. Additional data to record during sampling: sampling method, sampling site and location, sample container type (e.g. glass or plastic), sample volume, preservatives (if applied), sampler ID, any other relevant or unusual circumstances.

c. Sampling method should be recorded in detail: grab, composite or passive sample. For composite samples, whether time or flow-weighted, frequency of sampling. For passive samples, length of passive sampling.

d. The following sampling site information should be provided: exact location of the site (geoinformation), name of the sampled facility, type of the sampled facility (wastewater treatment plant (WWTP), in the case of WWTPs, the exact sampling point (for example technology step) within the WWTP, other institution, such hospitals, long-term care facilities, correctional institutions, educational facilities, residential buildings, or transportation vehicles, such as ships or aeroplanes etc.), type of sampling location (e.g. influent wastewater, upstream sample in the collection system, retention pond, septic tank etc.), sample type (e.g. raw sewage, sedimented wastewater, wastewater solids, etc.).

e. Results of on-site measurements, such as temperature, pH, conductivity, etc. should be recorded.

2. **Sample transport**

a. Special labelling requirements apply to packages transported by fast delivery services. See international regulations on shipping Category B infectious substances (further details are provided under Section 7.3).

b. For all means of transportation, time (beginning and end) of shipping, method of cooling, and temperature during transport (if measured) should be recorded.

c. Other information or problems during the shipment (for example accidental freezing of samples) should be also recorded.

3. Receipt of the sample in the laboratory

a. Upon arrival at the laboratory, sample(s) should be checked for integrity. Any visible damage or anomalous condition should be recorded. Temperature of the sample on arrival should also be noted.

b. If samples are stored before beginning sample processing, storage conditions (temperature, length of storage) should be recorded. Note if there was a matrix recovery control added before storage. Samples are often divided into subsamples when received in the laboratory (e.g. subsamples for immediate analysis, storage and chemical analysis), these should be labelled with a unique identifier as subsamples of the original sample. All directions of analysis should be listed.

4. Sample processing

a. All steps of sample processing in the laboratory should be traceable. It is recommended to have forms (electronic or paper-based) with pre-set answer choices, such as tick-boxes, rather than free text, to facilitate data analysis.

b. Pre-treatment steps to be recorded include pasteurisation (time and temperature), centrifugation (velocity, time and temperature), pre-filtration (pore size), and addition of matrix recovery control (volume, concentration, type, lot number).

c. Data on sample concentration should include date and time, specification of the method (e.g. membrane filtration, ultrafiltration, precipitation, specific branding of filters etc.), processed volume, final volume of the concentrate and any deviations from the normal procedure. For filtration-based methods, the time required to filter a sample can vary, in this case time needed for sample processing should also be noted. For some methods, cross-contamination is possible between samples processed in parallel or sequentially, in this case order of the samples should also be recorded.

d. For nucleic acid extraction, date and time, specification of the method (name of the commercial nucleic acid extraction kit or reference to the laboratory method SOP), the starting and the end volume should be recorded.

e. Records of SARS-CoV-2 detection should include date and time of the analysis, method specification (e.g. qPCR, digital droplet PCR), information about the detection kit (name, batch number), target gene, use of inhibition control (type, batch number), standards used for quantification (type, lot number), volume of nucleic acid used, results of the analysis (Ct value, copy numbers with confidence interval, limit of detection), ID of the PCR run (with instrument ID, if relevant). Information on observed inhibition during analysis or quality assurance issues should also be noted.

5. Indicators for normalisation

a. Dilution of wastewater by precipitation or non-communal (e.g. industrial) sewage can bias detected SARS-CoV-2 concentration. It is necessary to correct for this bias by normalising results to the human faecal fraction of wastewater.

b. Most commonly used viral faecal indicators are pepper mild mottle virus (PMMoV) and CrAssphage quantified by qPCR (see also Section 4.4.4.5). Same data should be recorded as for SARS-CoV-2 detection.

c. Chemical human biomarkers, such as caffeine or creatinine can also be used for normalisation. Specifications of the method and determined concentration of the biomarker should be recorded.

d. Wastewater flow volumes are also indicative of dilution by precipitation in combined sewers. This variable is listed under point 5. of the Metadata.

6. Metadata

a. Additional information that may influence data interpretation can be obtained from external sources.

b. If WWTPs are sampled, data on the served population, wastewater flow volumes and sewage travel time should be recorded. Some WWTPs have online monitoring for parameters such as temperature, pH, conductivity, dissolved oxygen, that can be useful for data analysis. Preferably, mapping files of the WWTP catchment will also be provided to aid population targeting and reporting.

c. If individual institutions or transport vehicles are sampled, the number of residents/users should be noted.

d. Weather conditions, especially precipitation volume may be relevant.

e. Other wastewater characteristics, such as total dissolved solids and biological oxygen demand are also informative.

7. **Quality assurance**

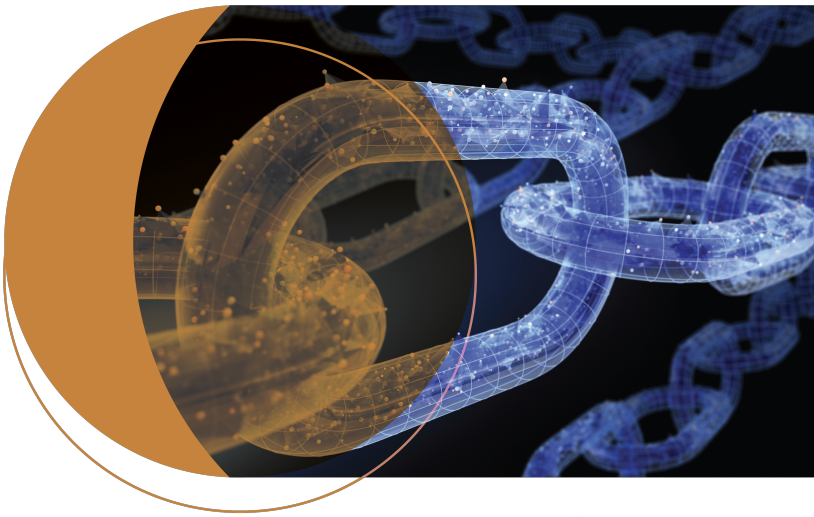
a. In the initial phases of setting up a wastewater surveillance system, method development is often carried out by research or university laboratories, which might not be accredited. However, for established regional or national surveillance schemes it is recommended that all participating laboratories should operate a formal quality assurance system (e.g. accreditation by a national authority, compliance with ISO 17025 or GLP standards).

b. Chain of custody protocols should be in line with the requirements of the quality assurance system of the laboratory.

c. Since various protocols are used by the different laboratories, it is good practice to organise national inter-laboratory proficiency testing (PT) or participate in international PT scheme, where available.

7.1.3 TIPS

- **Use barcodes for efficient sample tracking** and to minimise errors in data entry
- **Maintain a detailed log** of every individual who handles the samples at each stage.
- **Use lockable coolers** during transport to prevent unauthorised access.
- **Regularly calibrate temperature monitors** to ensure accuracy.
- **Have a contingency plan** for unexpected events, such as delays in transportation.
- **Conduct periodic audits** of your chain of custody process to identify and correct any potential weaknesses.
- **Engage in continuous training of personnel** to ensure adherence to the protocols and the latest standards.



7.1.4 THE SCIENCE BEHIND

Within the technical framework of wastewater surveillance, the foundational elements of transportation protocols and chain of custody are anchored in safeguarding sample integrity and ensuring meticulous traceability. The comprehensive procedure, which spans from the initial collection of the sample to the final analytical evaluation in the laboratory, is meticulously structured to maintain the sample's pristine state and ascertain the precision of the subsequent data obtained.

SAMPLE INTEGRITY

Maintaining the integrity of wastewater samples is crucial for reliable surveillance. Any changes in the sample between collection and analysis—whether due to chemical reactions, biological degradation, or physical alterations—can compromise the data. To mitigate these risks, transportation protocols dictate the use of preservatives, temperature-controlled containers, and swift delivery to the laboratory. The chain of custody supports sample integrity by documenting each transition and handling of the sample, creating a timeline that can be reviewed for potential sources of error or contamination.

TRACEABILITY

The ability to trace a sample back to its origin is essential for interpreting data accurately. This traceability allows researchers and public health officials to associate the data with specific locations, times, and conditions under which the sample was collected. Detailed labelling, which includes unique identifiers and metadata such as the date, time, and sampling point, enables this level of traceability. The use of electronic management systems or structured paper-based forms enhances the reliability of this tracking process.

QUALITY ASSURANCE

Quality assurance practices are embedded in the transportation protocols and chain of custody to ensure that wastewater surveillance data meets specific standards. By adhering to international shipping regulations, employing standard operating procedures, and participating in proficiency testing, laboratories demonstrate their commitment to producing valid and defensible data. These practices are not only important for individual sample analysis but also for the comparability of data across different sampling sites and laboratories.

NORMALISATION

Transportation and chain of custody protocols also play a role in the normalisation of surveillance data. The normalisation process corrects for variables that could skew the concentration of target analytes, such as the dilution of wastewater from rainfall or variations in wastewater flow. By carefully documenting sampling conditions and integrating external metadata, researchers can adjust their findings to more accurately reflect the true levels of target substances or pathogens within the community.

7.2 HYGIENE AND SAFETY

MASTER CHEF: MÁRTA VARGHA

Biosafety considerations should be addressed at every step of the wastewater sampling and analysis workflow. Although according to currently available scientific evidence the risk of SARS-CoV-2 transmission via wastewater is low, wastewater samples should be handled as substances potentially containing infectious SARS-CoV-2 viruses. Biosafety procedures should be developed for the surveillance system, including measures on vaccination, use of personal protective equipment (PPE), protocols for sample handling, requirements for laboratory space, disinfection, waste management and training for the samplers and laboratory personnel (Association of Public Health Laboratories, 2022; Iwen et al., 2020; World Health Organization, 2020b).

7.2.1 KEY INGREDIENTS



- **Personal Protective Equipment (PPE):** Essential for safeguarding personnel from exposure to pathogens and hazardous chemicals. PPE includes gloves, face shields, goggles, lab coats, and appropriate footwear.
- **Disinfectants:** Solutions like 0.1% hypochlorite or 70% ethanol are critical for surface decontamination and inactivation of viruses.
- **Plastic Containers:** Used for the safe collection and transport of samples, these containers must be sturdy and leak-proof.
- **Biosafety Cabinets:** Enclosed, ventilated laboratory workspaces offering protection to users from aerosolised pathogens during sample handling
- **Autoclaves:** Sterilisation equipment necessary for the decontamination of waste materials potentially containing infectious agents.
- **Biosafety Level 2 (BSL-2) Facility:** A laboratory environment equipped with the necessary containment to safely handle wastewater samples.
- **Training Programmes:** Structured courses to educate personnel on proper sample management, safety protocols, and emergency procedures.

7.2.2 RECIPE



1. **Sampling**

a. Safety of samplers is critical during the collection of wastewater samples. Untreated wastewater contains various enteric and respiratory pathogens and even chemicals that pose a hazard for the workers of wastewater treatment plants (WWTPs). The risk for samplers is similar to that of the workers.

b. Since there is no evidence of SARS-CoV-2 transmission by wastewater, standard procedures used for the protection of workers are sufficient, no additional SARS-CoV-2 specific measures are necessary for samples. Adequate PPE and safe work practices should be used during sampling.

c. Other safety risks – unrelated to SARS-CoV-2 – such as physical injury from slipping, tripping, head strikes or entrapment should also be addressed, but they are not discussed further in this document.

2. **Handling wastewater samples**

a. Wastewater samples should be collected in plastic containers. Outside of the containers should be disinfected by approved disinfectants (see point 5.). Samples should be packaged and shipped as Category B infectious substance (UN 3373, see also Section 7.3).

b. Upon arrival at the laboratory, samples should be opened in a designated area. Outside of the containers they should be disinfected upon opening. Within the laboratory, samples should be transferred in high-rimmed trays to avoid spilling.

3. Laboratory analysis

a. For adequate level of protection, wastewater samples should be processed in a biosafety level 2 (BSL-2) laboratory with unidirectional airflow. Separate areas should be designated for donning and doffing personal protective equipment to avoid cross-contamination.

b. Work procedures should be designed to be unidirectional within the laboratory. All equipment and consumables should be in the place of use so laboratory personnel do not have to leave and re-enter the lab during sample processing.

c. Samples are recommended to be opened and handled in a biosafety cabinet (BSC). The required level of protection during sample processing depends on the applied methods. Concentration by membrane filtration, for instance, can be carried out in a BSL-2 space. For potentially aerosol generating procedures, such as vortexing, mixing, pipetting or decanting wastewater samples, use of a BSC cabined is recommended. Centrifugation is also an aerosol generating process, therefore it should be carried out in closed centrifuge tubes or a closed rotor.

4. Pasteurisation

a. Pasteurisation of wastewater samples is practised by many laboratories to reduce the risk of infection through contact or aerosol generating procedures. Heating wastewater samples to 56°C for 30-90 min was shown to inactivate enveloped viruses.

b. Data on the effect of heat treatment on viral RNA concentration is controversial. Some publications report considerable loss of detectable SARS-CoV-2, while according to others, it is negligible. One report even observed an increase in SARS-CoV-2 signal after pasteurisation at 60°C.

c. Due to this controversy, pasteurisation is not a universally recommended treatment. Adequate matrix recovery controls should be used to detect any loss of viral RNA, if pasteurisation is applied.

5. Decontamination

a. Work surfaces, equipment and other surfaces that were potentially contaminated by aerosols generated during sample processing should be disinfected by a disinfectant with confirm efficiency against enveloped viruses.

b. REACH approved disinfectants should be used. Hypochlorite solution 0.1% or alcohol-based products with at least 70% ethanol are suitable for the elimination of SARS-CoV-2.

6. Waste management

a. Leftover samples and all laboratory waste that may contain SARS-CoV-2 should be decontaminated by autoclaving. Waste should be managed and disposed of in accordance with BSL-2 guidelines.

b. Waste management practices should comply with local or national waste regulations.

c. During disposal of waste containing SARS-CoV-2 PCR products (e.g. PCR plates) care should be taken to avoid cross-contamination of rooms used for sample processing and PCR-mix preparation.

7. Personnel

a. Samplers and laboratory personnel should be trained in proper management of potentially infectious samples and the necessary safety measures including.

b. All personnel should have an up-to-date vaccination record.

c. Adequate personal protective equipment should be used for all sampling and sampling processing steps, based on the biosafety risk assessment.

8. Risk assessment

a. Site-specific and activity-specific risk assessment should be carried out for each laboratory participating in wastewater surveillance.

b. Risk assessment takes into account the specific conditions of the laboratory. Considerations include sample volume, laboratory procedures, risk of aerosol generation, existing facility controls, and the training and competence of the staff.

c. Risk mitigation measures should address the hazards identified during risk assessment.

7.2.3 TIPS

- **Reassess and update safety protocols regularly** to align with emerging research and changes in regulatory standards.
- **Engage in open communication** and regular training refreshers to reinforce the importance of hygiene and safety protocols among all personnel.
- **Ensure that all materials and equipment are readily available** and well-maintained to prevent any lapses in safety measures.
- **Develop clear and concise emergency response** plans to address any accidental exposures or spills promptly and effectively.
- **Foster a culture of safety** where all team members feel empowered to report potential risks and contribute to the continuous improvement of biosafety practices.



7.2.4 THE SCIENCE BEHIND

Wastewater surveillance serves as a critical tool in monitoring public health, particularly in the detection and tracking of pathogenic microbes such as the SARS-CoV-2 virus. The science behind the established protocols for transportation, hygiene, and safety in wastewater surveillance is grounded in understanding the behaviour of pathogens within the wastewater matrix, the routes of potential exposure to humans, and the methods to mitigate these risks.

THE BEHAVIOUR OF PATHOGENS IN WASTEWATER

Wastewater contains a complex mixture of biological and chemical substances, including a variety of pathogens like bacteria, viruses, and protozoa. These pathogens can persist in wastewater for varying durations and may retain the potential to cause infection. The SARS-CoV-2 virus, although primarily a respiratory pathogen, can be shed in the faeces of infected individuals and thus be present in wastewater. Given the right conditions, these pathogens can pose a risk to humans, particularly those involved in the collection and analysis of wastewater samples.

ROUTES OF POTENTIAL EXPOSURE

The primary routes of exposure to infectious agents in wastewater include direct contact, ingestion, and inhalation of aerosols. During sampling and laboratory analysis, personnel may come into contact with contaminated water or surfaces, or they may inadvertently inhale aerosols generated during the handling and processing of samples. Therefore, the established protocols focus on preventing such exposures through the use of personal protective equipment (PPE), engineering controls like biosafety cabinets, and safe handling practices.

RISK MITIGATION STRATEGIES

The cornerstone of risk mitigation in wastewater surveillance is the implementation of biosafety protocols that align with the level of risk associated with handling wastewater samples. The use of PPE such as gloves, face shields, and lab coats is mandatory to create a barrier between the individual and the potentially infectious material. Disinfectants play a crucial role in inactivating pathogens on surfaces and equipment, thereby reducing the risk of indirect transmission.

Biosafety cabinets are employed to contain and exhaust potentially infectious aerosols, protecting the laboratory personnel and the environment. High-rimmed trays and closed containers are used to prevent spills and leaks during transportation within the laboratory, while autoclaves are utilised to decontaminate waste effectively.

Pasteurisation is a method employed by some labs to reduce the viability of enveloped viruses in wastewater samples, though its impact on viral RNA detection varies. Matrix recovery controls are essential to ensure that the analytical methods remain effective following any treatment of the samples.

Training and education of personnel are vital components of a comprehensive safety protocol, ensuring that all individuals involved are aware of the risks and are proficient in the necessary preventative measures. Regular risk assessments tailored to the specific activities and conditions of each laboratory are

fundamental to identifying potential hazards and implementing appropriate controls to mitigate them.

Wastewater surveillance is a dynamic field, and the science behind hygiene and safety protocols is continually evolving. As new research emerges and our understanding of the behaviour of pathogens in wastewater deepens, protocols are updated to reflect the current state of knowledge. This ensures the continued protection of public health while enabling the valuable insights that wastewater surveillance provides.

7.3 SHIPMENT

MASTER CHEF: **MÁRTA VARGHA**

Shipping arrangements are critical for timely and safe delivery of wastewater samples to the analysing laboratory. Transportation procedures should be optimised individually for each surveillance system, taking into account the number and location of laboratories participating in surveillance, sample number and volume, sampling frequency, time demand of shipping, cost efficiency, and biosecurity considerations (International Air Transport Association (IATA), 2021; World Health Organization, 2022).

7.3.1 KEY INGREDIENTS



- **Sample Containers:** Durable plastic containers to prevent breakage.
- **Disinfectants:** Hypochlorite, hydrogen-peroxide, or alcohol-based disinfectant for surface sterilisation.
- **Secondary Packaging:** Waterproof and sift-proof bags or containers for additional security.
- **Absorbent Material:** Cushioning to prevent leakage and absorb any potential spill.
- **Cooling Equipment:** Icepacks, melting ice, or cooling vans to maintain sample temperature.
- **Temperature Data Logger:** For monitoring sample temperature during transport.
- **Labelling Materials:** To ensure clear and unambiguous sample identification.
- **Documentation:** To maintain chain of custody and provide essential sample details.
- **Trained Personnel:** Individuals skilled in handling and packaging infectious substances.

7.3.2 RECIPE



1. **Means of sample transportation**

a. In research focused or pilot studies it might be feasible for the laboratory staff to collect and transport the samples to the laboratory. Large scale surveillance systems, however, require organised means of transport. The following arrangements are examples that are being used in practice.

b. Shipping samples by fast delivery service is practical if wastewater samples are shipped for large distances. It does not require extra staff for the laboratory and it is comparatively easy to organise. Wastewater samples should be shipped as Category B infectious substances (UN 3373). Shipping hazardous substances, such as wastewater, especially larger volumes is expensive, long-term financial sustainability should be considered.

c. For smaller distances, a designated delivery vehicle can be employed to pick up samples from various sampling locations and transport them to the laboratory. It is more cost efficient, but requires substantial human resources, depending on the number of sampling sites. It also requires careful planning to establish optimal collection routes and continuous coordination with the sampling team or staff.

d. The most cost-efficient option is to utilise already established routes of transport, e.g. for the transport of clinical samples or routine environmental monitoring systems. This arrangement also requires extensive coordination of the involved partners.

2. **Time of transportation.** Ideally, sample processing should start on the day of sampling, both to avoid degradation of the viral RNA and to minimise turn-around time of results for public health decision-making. If that is not feasible, samples should be transported to the laboratory within 24 hours. If fast delivery services are employed, same-day or overnight shipping should be ordered.

3. **Packaging**

a. Wastewater samples should be shipped in plastic containers to avoid breakage. Outside of the containers should be disinfected by disinfectants approved for the elimination of SARS-CoV-2 and other pathogens (e.g. hypochlorite, hydrogen-peroxide or alcohol-based disinfectant).

b. For shipping services, packaging should comply with the regulation for infectious substances (Category B, UN 3373). This means that sample receptacles should be cushioned with absorbing material, and placed into a secondary waterproof and sift-proof container, e.g. a bag, that can hold the entire volume of the packed samples. Outside of the secondary packaging should also be disinfected.

c. For other means of transport, similar basic principles apply: containers should be leak-proof, and positioned securely within the storage box to avoid shifting during shipping. Secondary packaging (i.e. placing sample containers in a plastic bag) is recommended.

d. The personnel responsible for packaging samples or opening sample packages should be trained in handling and packaging infectious substances. Optimally, these personnel should have this training and competency formally certified.

4. **Cooling**

a. Wastewater samples should be transported cold, but not frozen. Ideally, samples should be cooled immediately after sampling to 4°C and maintain that temperature until sample processing. If that is not possible, samples should be protected from warming up.

b. If shipping services are employed, cooled shipping at 4°C should be ordered.

c. Using other means of transport, samples can be shipped in a cooling van, or in a cooler box on melting ice or with icepacks. Melting ice can cool down the sample, but care should be taken that it does not freeze. Obtaining melting ice might not be feasible in all settings. Ice packs can keep the sample from warming up, but can only reduce the temperature of small sample volumes or if several of them are used per sample. Ice packs are easier to obtain and handle than melting ice.

d. A temperature data logger in the cooler box is useful for tracking the temperature of the samples.

e. Outside temperature should also be considered when selecting the method of cooling.

5. **Documentation and labelling**

a. Samples for transport should be labelled to ensure unambiguous identification. Documentation – either on paper or in electronic format – should include sample ID, sampling date and time, sample location, sampling method, the name of the sampler and any other relevant information on the sampling (see also Section 7.1).

b. For delivery by shipping services, package labelling should comply with the regulation for shipping infectious substances, including a biohazard sign.

7.3.3 TIPS

- **Prioritise the choice of transportation** based on the sample's stability, the urgency of analysis, and the geographical layout of the sampling sites.
- **Ensure all personnel involved in the transportation process are adequately trained** in biosecurity and sample handling protocols.
- **Establish a contingency plan** for unexpected delays or issues during transport to mitigate any potential impact on sample integrity.
- **Coordinate closely with the sampling teams and laboratories** to streamline the collection, transport, and analysis workflow.
- **Regularly review and update the transportation protocols** to adapt to new regulations, technological advancements, or changes in the surveillance system's scope.

7.4 STABILITY TESTS AND ASSESSMENTS

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The process of wastewater analysis for viral nucleic acid detection and quantification includes several phases, each exposed to physical, chemical, or biological factors that risk affecting the stability of target nucleic acids. These factors impact the entire pathway from viral

shedding, through passage via the sewerage network, to sampling, transport, analysis, and the short- and long-term storage of sample aliquots prior to further analysis. Enveloped viruses have, on average, higher decay rates than non-enveloped viruses, although there are clear differences between the decay rate constants of different virus families, as shown by a recent systematic review and meta-analysis of the persistence of viruses in environmental waters and wastewater in the absence of disinfectants (Silverman & Boehm, 2021).

7.4.1 KEY INGREDIENTS

- **Temperature Control Devices:** Essential for maintaining specific temperature conditions during sample collection, transport, and storage.
- **Dark, Opaque Containers:** To protect samples from light, particularly ultraviolet (UV), which can degrade nucleic acids.
- **RNase Inhibitors:** Chemicals that inhibit the activity of RNases, safeguarding RNA integrity.
- **pH Buffers:** To maintain a stable pH in samples, as fluctuations can impact viral stability.
- **Protective Gear:** Necessary for handling samples safely, especially when dealing with potential pathogens.
- **Inactivation Agents:** Such as heat blocks or UV lamps for sample inactivation while preserving nucleic acid integrity for analysis.

7.4.2 RECIPE

1. **Sample Collection:** Collect samples in dark, opaque containers to mitigate light exposure. Add pH buffers to stabilise the sample environment.
2. **Transportation:** Keep samples at 4°C using ice packs or refrigerated containers to prevent RNA degradation.
3. **Short-Term Storage:** Store samples at 4°C if analysis will occur within seven days. If analysis is delayed, freeze samples at −20°C or −80°C, but be mindful of the effects of freeze-thaw cycles.
4. **Inactivation Procedures:** If necessary, inactivate samples using heat treatment or UV light, ensuring that the chosen method does not overly compromise RNA integrity.
5. **Long-Term Storage:** For storage beyond a week, freeze samples at −80°C, preferably in single-use aliquots to minimise freeze-thaw cycles.

7.4.3 TIPS

- To minimise degradation, **begin laboratory analysis on the day of collection** or as soon as possible.
- **If using inactivation procedures, validate that they do not significantly degrade RNA**, as this would compromise the analysis.
- When freezing samples, **avoid multiple freeze-thaw cycles** by storing in single-use aliquots.
- **Implement an isochronous study design** for a systematic approach to evaluating stability under various conditions. This will help determine the shelf-life of your samples with a quantifiable margin of uncertainty.



7.4.4 THE SCIENCE BEHIND

7.4.4.1 FACTORS THAT IMPACT THE VIRAL STABILITY

Due to the multiplicity and complexity of factors and processes affecting viral stability, evaluating stability in field conditions from sampling to detection can be challenging. Isochronous study design can be of great support in elucidating degradation mechanisms during both transport and storage (Gawlik et al., 2012). Factors affecting the stability of wastewater samples are shown in Figure 24, along with suggestions for surveillance programme operators to minimise decay

and/or loss of SARS CoV-2 in wastewater. The main factors impacting viral stability in wastewater include:

PHYSICAL FACTORS

Sunlight is one of the most important environmental stressors leading to microorganism inactivation with a significant effect on the decay rates of both enveloped and non-enveloped viruses (Nelson et al., 2018). However, due to the underground nature of sewerage systems and to the turbidity of wastewater, the effect of sunlight on viral decay in wastewater may be less profound than in other water environments. Temperature has a significant effect on virus decay, due to protein denaturation and an enhanced activity of enzymes, impacting the integrity of both the viral envelope and the capsid, as well as of viral RNA in water. According to experimental and field studies, with the increase of temperature, SARS-CoV-2 genome concentration decays with a first order kinetics (Ahmed et al., 2020; Hart & Halden, 2020). In freezing temperatures, at -20°C or lower, SARS-CoV-2 genome seems to persist for months and more (Hokajärvi et al., 2021), although prolonged storage of the sample at −20 °C can lead to a reduction in viral loads of SAR-CoV-2 or surrogates (Markt et al., 2021). Sample freezing and thawing can lead to the degradation of SARS-CoV-2 genetic material by the RNAses present in wastewater. Aggregation / Adsorption onto solid matter also protect viruses from inactivation.

CHEMICAL FACTORS

Water composition in terms of pH, salinity, organic substance, and chemicals influences viral persistence in water and RNA integrity in the sample. Indeed, SARS-CoV-2 RNA persists longer in clean water, as demonstrated for other viruses as well (Ahmed et al., 2020). Wastewater varies in terms of composition and strength, according to the type of sewerage (combined or separate), the origin of discharges (urban, industrial, agricultural), and the distance travelled from source to treatment plant. A high concentration of organic matter in wastewater usually increases the persistence of the viral population.

BIOLOGICAL FACTORS

The presence of antagonist microorganisms can decrease viral persistence in water. Several microorganisms can reduce viral concentrations in wastewater - live bacteria may do so by producing antiviral substances, while protozoa through adsorption or predation, and in fact, viral persistence has been shown to be longer in autoclaved wastewater than in untreated wastewater (W. Ahmed et al., 2020).

7.4.4.2 SHORT TERM STABILITY

Temperature and time delay are the most critical factors known to cause decay in SARS-CoV-2 RNA copy numbers in wastewater. The time for 90% reduction of viable SARS-CoV-2 at room temperature is less than two days, and only minutes at 50°C and 70°C (Bivins, Greaves, et al., 2020). However, wastewater-based monitoring of SARS-CoV-2 is based on the detection and quantification of virus RNA, which is generally more persistent than viable virus.

Good practices during wastewater sampling and transport of samples from wastewater treatment plant to analysing laboratory can prevent the deterioration of viral targets (see specific chapters for further details). Laboratory analysis of SARS-CoV-2 from wastewater should ideally start on the day of sample collection or, in the case of composite sampling, on the day sampling is completed. However, logistics issues, such as long distances between wastewater sampling points and the laboratory, might cause delays in the initiation of sample processing. The delay prior to initiation of sample processing should be kept to a minimum, however. On some occasions, sample processing is therefore initiated already at the sampling site (at the wastewater treatment plant). Temperatures of about 4°C are often the most favourable and practical temperatures both for sample collection and for sample transportation. While no decay was observed at freezing temperatures (-20°C and -80°C), at 4°C, Hokajärvi et al. (2021) reported a linear decay of SARS-CoV-2 RNA over 21 days. SARS-CoV-2 RNA exhibited little decay in primary influent stored for over 7 days at both 4 and 25°C (W. Ahmed, Simpson, et al., 2022; Bivins et al., 2021). Other authors reported even longer stability, for up to 9 and 14 days at 4°C (Islam et al., 2022; Markt et al., 2021).

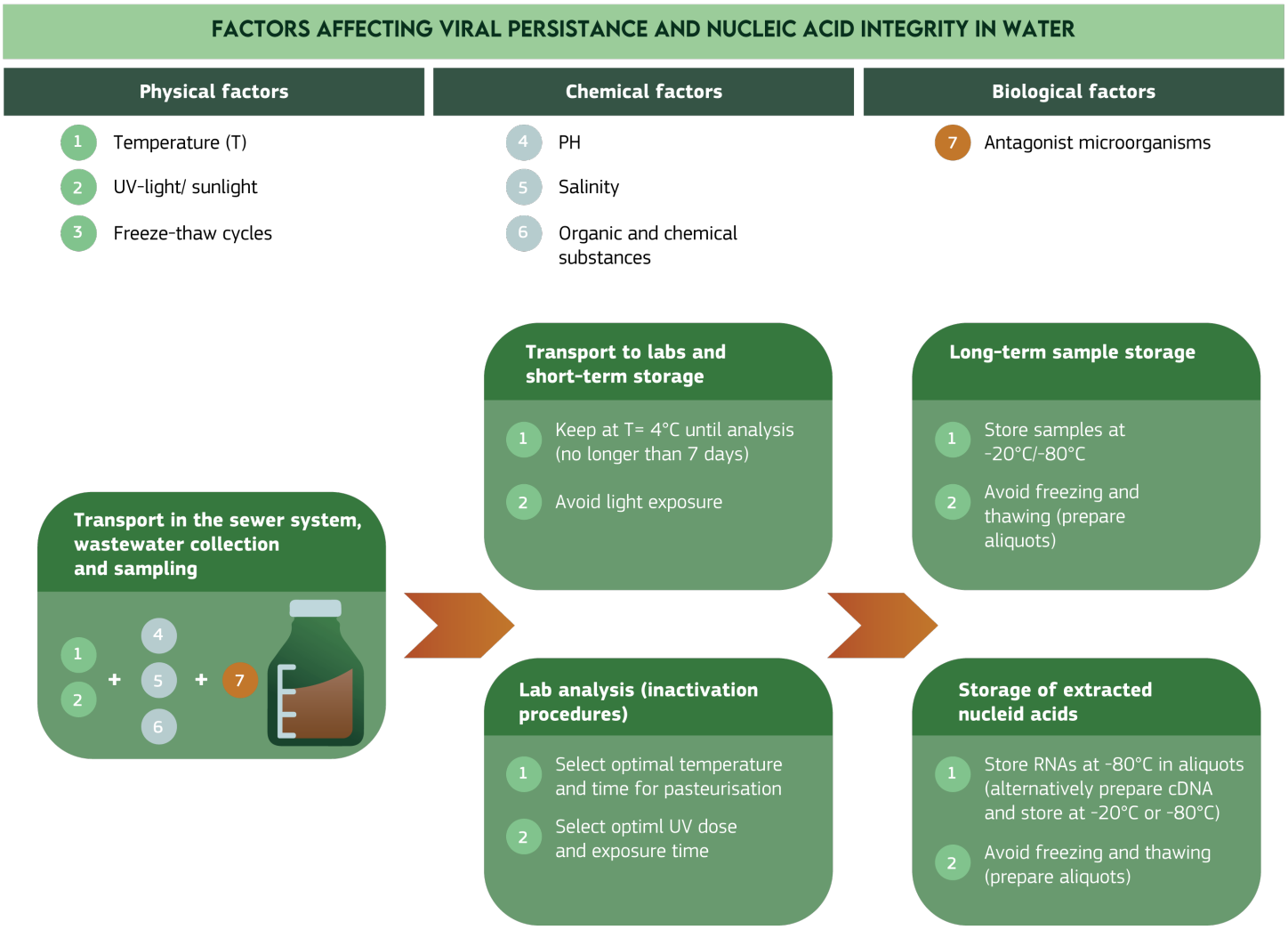
Thus, for a sufficient conservative recommendation, when immediate initiation of the analysis is not possible, wastewater samples should be stored at 4°C for delays up to seven days, and at freezing temperatures of −20°C or −80°C in cases of longer delays.

7.4.4.3 EFFECT OF INACTIVATION PROCEDURES

In Europe, SARS-CoV-2 is currently classified as a hazard group 3 pathogen (Health and Safety Authority, n.d.). Inactivation or reduction of its infectivity is of utmost importance to avoid laboratory-acquired infections. For this reason, samples are sometimes subjected to pre-treatment, prior to viral concentration. In general, physical inactivation procedures such as heat, UV light and gamma-irradiation of the virus contribute to risk mitigation (Darnell et al., 2004; Patterson et al., 2020) and have the advantage of not requiring that the lids of the bottles containing sewage samples be opened. Nevertheless, nucleic acids should be preserved for a valid subsequent molecular analysis. Several in vitro studies have explored the effect of inactivation treatments on SARS-CoV-2 infectivity and genome persistence, as described below.

HEAT TREATMENT: Heat treatment is the most frequently used viral inactivation procedure to reduce the risk of occupational infection. Most studies on this procedure have been conducted on infected cell cultures and on clinical specimens containing SARS-CoV-2. Heat treatment at 56°C for 30-mins and 60°C for 60-mins resulted in complete SARS-CoV-2 inactivation (starting infectious titre of 3.5 x 105 TCID50/ml), with little impact on RNA detection on laboratory-spiked clinical samples (Chin et al., 2020; Kim et al., 2020; Pastorino et al., 2020). Similar results were obtained on SARS-CoV-2 viral supernatant samples: complete inactivation was achieved after 15-mins at 65°C, and RNA loss was

Figure 24: Factors affecting viral persistence and nucleic acid integrity in water



Source: G. La Rosa unpublished (redesigned)

negligible even after 30-mins of treatment (Bivins, Greaves, et al., 2020; Chin et al., 2020, 2020; Loveday et al., 2021). When the temperature was increased to 70°C, time for virus inactivation was reduced to 5 minutes (Chin et al., 2020). Nevertheless, SARS-CoV-2 RNA disappeared when temperature was increased to 92°C for 15 minutes (Burton et al., 2021; Kim et al., 2020; Pastorino et al., 2020). These results suggest that 56°C for 30 minutes is a good compromise between laboratory safety and molecular analysis, and that temperature should be strictly controlled during sewage inactivation.

UV LIGHT TREATMENT: Ultraviolet (UV) irradiation is an extensively tested, widely used and effective no-contact method for inactivating viral pathogens. UV-C (100–280 nm) is commonly employed in germicidal applications (Storm et al., 2020). Nevertheless, viral inactivation at 254 nm results from direct UV-C light absorption and photochemical damage to nucleic acid, leading to the disruption of viral replication.
For this reason, this inactivation approach is not widely used in molecular assays designed to detect viral nucleic acid. Loveday et al., report that a 1-min exposure to UV-C (254 nm) with a 0.07 J/cm2 UV dose was responsible for a 7-Log^{10} reduction in infectious SARS-CoV-2 titres, corresponding to the complete inactivation of the tested SARS-CoV-2 suspension. At the same experimental con-ditions, RNA levels decreased by 2-Log^{10}, and up to a 3-Log^{10} reduction was observed after 10-mins of treatment following a linear decay curve (Loveday et al., 2021).

GAMMA-IRRADIATION: One Mrad of radiation was able to completely inactivate SARS-CoV-2 in a suspension of 106.5 TCID50/ml with a slight decay of RNA (< 0.5 Log^{10}). RNA loss remained stable after increa-sing the radiation dose up to 5 Mrads (Leung et al., 2020). The limited impact of 5 Mrads of radiation on SARS-CoV-2 RNA has been confirmed by Ahmed et al. on influent sewage waters, although the authors did not evaluate its effect on virus infectivity (W. Ahmed et al., 2020).

7.4.4.4 LONG-TERM STABILITY

A wastewater surveillance programme should provide rapid testing for sample measurements, ideally within 24-48 hours, but the storage of samples for short or long periods of time may be necessary for various reasons. For example, analyses may need to be repeated if laboratory instruments fail or results do not pass quality controls. Moreover, archived wastewater samples could become valuable at a later date for retrospective epidemiological studies either on SARS-CoV-2 (La Rosa et al., 2021), or on other viruses (W. J. Lodder et al., 2013; Suffredini et al., 2018) or chemical agents. Therefore, both sewage samples and the extracted nucleic acids need to be stored under conditions that would preserve their integrity even for long periods (months/years). In future, the establishment of long-term storage solutions for wastewater and RNA samples, modelled on clinical biobanks, may prove useful (Thaler & Sakmar, 2021).

As with short-term storage, temperature is a key factor in long-term storage. Hokajärvi et al. found no decay of SARS-CoV-2 RNA in wastewater within 58 days at the freezing temperatures of −20°C and −80°C (Hokajärvi et al., 2021). In contrast, Fernandez-Cassi et al. reported an extensive reduction in SARS-CoV-2 RNA concen-trations measured in raw influent wastewater stored at −20°C after one month (Fernandez-Cassi et al., 2021). Therefore, where possible, frozen wastewater samples should be stored at -80°C, possibly in ready aliquots to avoid multiple thawing cycles, as specified above. Other options for long-term storage (months or years) are solid phase samples, membranes or other concentrates rather than wastewater (Beattie et al., 2022).

The preservation of extracted nucleic acids is also crucial. After completion of the analyses, the spare extract can be stored, so as to be available for any additional or repeat testing. Maintaining the integrity of RNA molecules during storage is also a challenge. Indeed, RNA is highly susceptible to degradation by RNase, which is a ubiquitous enzyme (M. R. Green & Sambrook, 2019). The addition of commercially available RNase inhibitors before freezing is advisable, to prevent degradation without affecting analyses. In general, the most common approach to increasing the longevity of nucleic acid extracts is to freeze the samples at -20°C (DNA samples) or -80°C (RNA samples) in single-use aliquots to avoid multiple freeze-thaw cycles. RNA samples may also be converted into cDNA using commercially available kits; cDNA is more stable than RNA, and is therefore suitable for long-term storage at -20°C.

7.4.4.5 EFFECT OF FREEZE-THAW

Wastewater samples collected for viral testing are usually analysed within a short time, but in most cases, storage at the laboratory is needed prior to further processing and analysis. Storage at lower temperatures (-20°C or−80°C) is necessary to maintain sample integrity for longer periods, but data on the effect of freezing and thawing on virus viability are scarce (Michael-Kordatou et al., 2020). A study on MS2 bacteriophages in wastewater (Olson et al., 2004) revealed increased viral degradation at -20°C compared to −80°C, due to the formation of large ice crystals. Similar data were also shown on coliphages: each cycle

of freezing/thawing caused further viral reduction (Gunnarsdóttir et al., 2012). As for SARS-CoV-2, multiple studies have shown freeze-thaw cycles to significantly reduce the quantifiable viral signal (Bivins et al., 2021; Markt et al., 2021). This may be due to the cycle disrupting cells, with the release of contents possibly including proteases and RNases, which may impair SARS-CoV-2 detection. Bivins and colleagues investigated the effect of freeze–thaw cycles on SARS-CoV-2 RNA with lab scale experiments by freezing wastewater aliquots at −80°C for 48 h, thawing at 4°C and refreezing at −80°C for up to three cycles (Bivins et al., 2021). After three freeze–thaw cycles, RNA copy numbers decreased considerably. Recently, Huge and co-workers observed a sevenfold decrease in viral load in a wastewater sample that had been frozen at 80°C for 92 days compared to results obtained without freezing, concluding that freezing samples before analysis should be discouraged (Huge et al., 2022).

After analysis, it is advisable to freeze the samples in individual aliquots ready for use, to avoid repeated freeze-thawing cycles.

7.4.4.6 TESTING SCHEMES

While studies on the influence of temperature on other enveloped viruses in wastewater have identified 4°C as appropriate for short-term storage (1-5 days) (W. Ahmed et al., 2020), SARS-CoV-2 wastewater samples have been kept at 4°C, -20°C and -80° before analysis (Alygizakis et al., 2021) and transported at 4°C (Navarro et al., 2021) with little information on their stability. The results of a survey on the methodologies applied to wastewater samples for quantification of SARS-CoV-2 by participants of the Pan-European Sewage Sentinel System confirmed variability in storing conditions (4°C, <10°C, -20°C) (European Commission. Joint Research Centre., 2021). The stability of wastewater samples collected for SARS-CoV-2 viral analyses should be tested under the relevant conditions to ascertain possible degradation during both transport and storage.

The isochronous stability study design and statistical analysis proposed by Gawlik et al. in 2012 (Gawlik et al., 2012) would be helpful in this context. In an isochronous stability study, samples are exposed to varying storage conditions for specified intervals of time (days, weeks or months) and stored back at the chosen reference temperature before being analysed under repeatability conditions (i.e.: one single analytical run). The study of the stability and related uncertainty of quantification are hence based on a regression analysis of concentration measurements over time intervals and on significance testing of the regression line (Linsinger et al., 2001). The stability of the measured gene concentration is evaluated through a linear equation, with the assumption of a linear degradation over time. Isochronous stability data must first be analysed to exclude any evident significant trend of degradation. The significance of the degradation trend can be determined using either the t-test or the F-test. Likewise, the shelf-life or expiry date for a sample can be identified with an acceptable range of uncertainty (i.e., 20%). The shelf-life, defined generally as “the length of time a product may be stored without becoming unsuitable for use” (Tanner, 2016), can indeed be interpreted as an expression of the acceptable uncertainty attributable to degradation of SARS-CoV-2 measured gene in wastewater samples. Obviously, if the slope value is significantly different from 0, indicating degradation, the shelf-life is not computed.

7.4.4.7 CONCLUSIONS

Several physical, chemical, or biological factors can affect the stability of wastewater samples, during different phases from collection to analysis and storage. The viral signal can be affected by any or a combination of these factors, with the overall effect also depending on the extent and length of virus exposure. This chapter provides practical information and good practices for minimising decay and/or loss of SARS CoV-2 in laboratory workflows from sampling to storage of wastewater influent samples.

7.5 STORAGE

MASTER CHEF: MÁRTA VARGHA

Wastewater or wastewater solid samples can be stored at different stages of sample processing. Adequate storage conditions (especially temperature and storage duration) are critical for the stability of analytes, and the reliability and reproducibility of results. SARS-CoV-2 viral particles cannot replicate and have limited persistence in wastewater, however its genetic material (RNA) is relatively stable.

As a rule of thumb, untreated samples should be processed as soon as possible. Extended storage may lead to the deterioration of the genetic material of SARS-CoV-2, thus leading to underestimation of the actual viral concentration. It can also compromise the ability of WBS to provide real-time information on the circulation of the virus in the population or to predict the number of symptomatic infections or hospitalised patients. On the other hand, stored (sub)-samples are very useful if re-analysis or more in-depth analysis (e.g. variant detection) is necessary.

Optimal storage conditions at various steps of sample processing are outlined below. More information on

testing and assessing the stability of SARS-CoV-2 RNA in wastewater samples is included in Section 7.4.

7.5.1 KEY INGREDIENTS

- **Refrigerators and Freezers:** Essential for maintaining specific temperatures to ensure the stability of your samples.
- **High-Quality Plastic Containers:** These are crucial for storing and freezing samples. They must be durable, leak-proof, and suitable for low temperatures to prevent breakage and sample loss.
- **Pipettes and Cryogenic Vials:** Used for dividing samples into smaller portions. This practice is important to minimise freeze-thaw cycles that can degrade sample quality.
- **Protective Packaging:** Double-bagging your samples in sturdy plastic bags can help prevent cross-contamination in the event of a container failure.
- **Labelling Materials:** Waterproof labels and markers ensure that samples can be accurately identified, tracked, and correlated with their corresponding data.
- **Temperature and Time Monitoring Devices:** These devices are imperative for recording and monitoring the storage conditions. They help in maintaining the integrity of the samples by providing a detailed storage history
- **Space Management:** Shelving and racking systems allow for optimal use of space and helps prevent accidental sample mishandling or misplacement.

7.5.2 RECIPE

1. **Process samples as soon as possible** to maintain analyte stability.
2. **Store unprocessed wastewater samples at 4°C** and viral concentrates and nucleic acids at ultralow temperatures for optimal results.

a. Besides storage time and temperature, other factors, such as wastewater composition, pH, applied chemicals, etc. can also influence the stability of the viral genetic material and thus viral recovery. The applied storage methods should therefore be validated by each laboratory. Natural samples of high viral concentration or spiked samples are both suitable for testing stability under the applied conditions

3. **Ensure consistent storage conditions** and thorough data recording to achieve reliable and reproducible results in WBS studies.

7.5.3 TIPS

- **Always prioritise processing samples** as quickly as possible to maintain the most accurate representation of analyte concentrations.
- When freezing is unavoidable, **consider the evidence – avoid -20°C if possible** and go for the colder option.
- **Use plastic containers for freezing** and double-bag to prevent contamination from any potential breakage.
- **Be mindful of the space required for storing** large volumes of unprocessed wastewater. Plan your laboratory's capacity accordingly.
- When in doubt, **err on the side of colder and quicker.** The stability of your analytes depends on it.



7.5.4 VARIANTS OF THE RECIPE

7.5.4.1 STORAGE OF UNPROCESSED WASTEWATER SAMPLES

1. Extended storage of untreated sewage samples should be avoided, whenever possible.

2. Samples should be cooled immediately after sampling and kept cool until processing (see also section 7.3 on shipping). Currently available evidence indicates that SARS-CoV-2 RNA concentration is stable for up to two weeks at 4°C.

3. In settings where cooling is not feasible, samples should be processed within one day. Some evidence exists that wastewater samples can be stored at room temperature for up to 3 days.

4. Data on freezing wastewater samples is controversial. Most studies indicate that significant loss of genetic material is experienced at -20°C, but not below -75°C. Samples should be aliquoted into volumes necessary for further processing and stored, in order to avoid repeated thawing. Freeze-thaw cycles have been shown to considerably reduce the concentration of detectable viral RNA.

5. Plastic containers should be used for freezing wastewater samples to avoid breakage. It is recommended to place sample containers for freezing into plastic bags to prevent contamination if breakage occurs.

6. Storing large volumes (in general, 50-1,000 ml) of multiple unprocessed wastewater samples requires a lot of storage space. Availability of sufficient refrigerator or freezer capacity can be a limiting factor in long-term storage of samples, and should be taken into account when designing laboratory procedures.

7.5.4.2 STORAGE OF UNPROCESSED WASTEWATER SOLIDS

Similar principles apply as for unprocessed wastewater. Due to the lower water content, usually smaller volumes are sufficient for viral RNA detection, hence storage typically requires less space.

7.5.4.3 STORAGE OF PRE-TREATED SAMPLES

1. Various pre-treatment steps (including pre-filtration, centrifugation and/or pasteurisation) are often applied during sample processing of wastewater.

2. Pre-filtration and centrifugation remove suspended solids that would interfere with downstream steps of concentrating viral RNA (e.g. ultrafiltration). Enveloped viruses are likely to attach to particulate matter, as it was shown for SARS-CoV-2. Thus, this step leads to the loss of detectable viruses. However, if applied consistently, it does not compromise the value of wastewater monitoring and was not shown to influence storage.

3. Pasteurisation may be applied primarily as a safety measure to reduce infectivity of wastewater samples. According to the current scientific consensus, the risk of SARS-CoV-2 transmission via wastewater is very low, therefore this step is dispensable. Reports on the impact of heat treatment on viral RNA recovery are ambiguous: pasteurisation of wastewater samples at 60°C for 30-90 min was reported both to increase and to decrease the concentration of recovered viral RNA. However, pasteurisation did not affect the stability of viral RNA during cold storage.

7.5.4.4 STORAGE OF VIRAL CONCENTRATES

1. Different methods are used to concentrate wastewater samples, including precipitation, (ultra)filtration and ultracentrifugation methods. Viral concentrates are either recovered in a considerably smaller volume (<1 mL), or absorbed on filters.

2. Concentrates should be stored frozen at -20°C, or -75-80°C. Viral RNA was shown stable for multiple months under these conditions. In case of filters, recovery was reported to improve after freezing.

3. Concentrates should be frozen in aliquots corresponding to the volume used for nucleic acid extraction to avoid repeated thawing.

4. Due to their smaller volume, frozen storage of concentrates for future analysis requires less freezer space.

7.5.4.5 STORAGE OF NUCLEIC ACIDS

Extracted viral RNA can be stored at -20°C prior to analysis. For long-term storage, ultra-low temperature freezing (-75°C or lower) is recommended.



Chapter 8

Conclusions

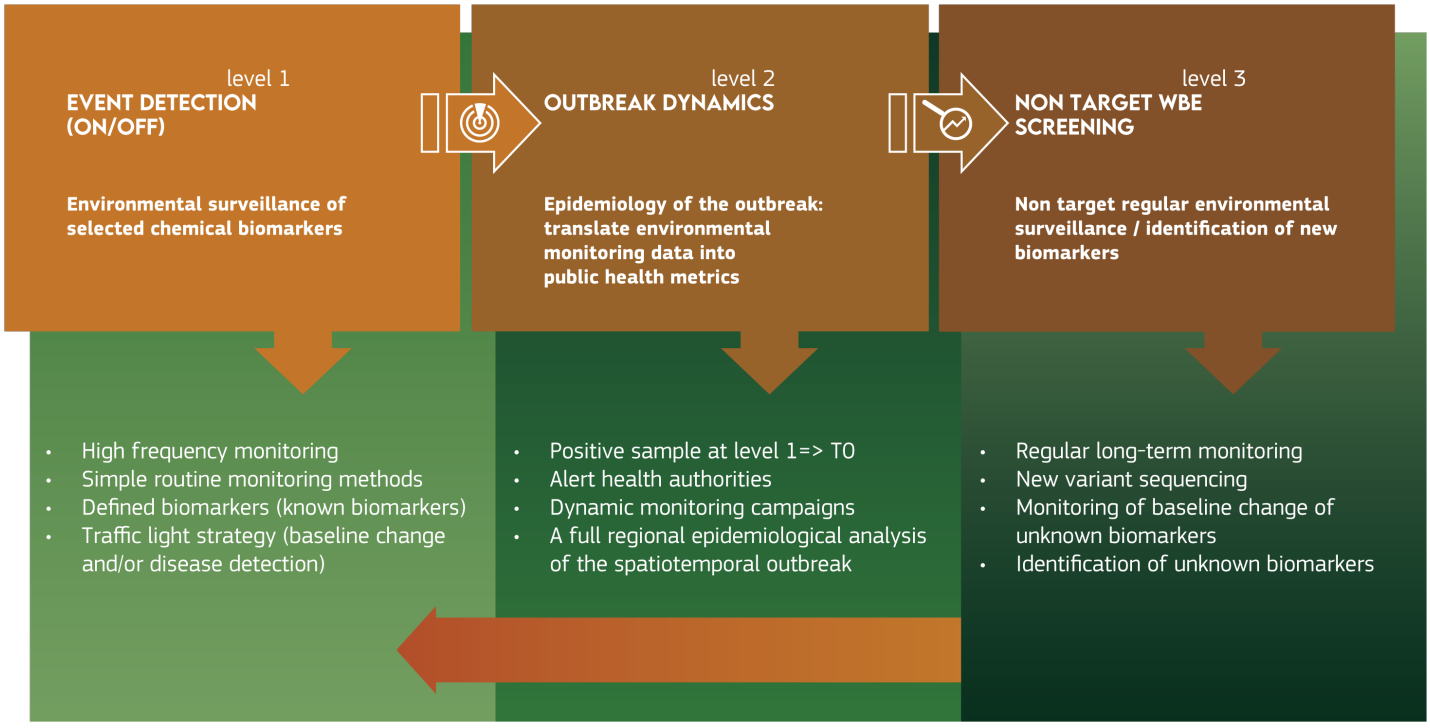
8 CONCLUSIONS

The thorough investigation and application of Wastewater-Based Surveillance (WBS) and its progression into Wastewater Epidemiology Surveillance (WES) during the COVID-19 pandemic have emphasised its vital role as a comprehensive public health instrument. The strategic deployment of WBS/WES, as elaborated in the preceding chapters, has established a structured approach for detecting events, analysing outbreak dynamics, and conducting broad-spectrum screening. This has provided valuable insights into the presence and proliferation of SARS-CoV-2 and within populations and is a concept that can be widened to other pathogens and compounds detectable in wastewater.

The strategic implementation of WBS/WES, as detailed in the preceding chapters, has provided a framework for event detection (Level 1), outbreak dynamics (Level 2), and non-targeted screening (Level 3), offering insights into the presence and spread of SARS-CoV-2 and other pathogens within communities. This tiered approach (see Figure 25), encompassing levels 1 through 3, has facilitated the early detection of viral events, the understanding of outbreak progression, and the exploration of promising parameters that combine chemical and biological markers.

Incorporating WBS/WES into a One Health strategy accentuates the interdependence of human, animal, and environmental health, endorsing a comprehensive approach to public health surveillance. The discovery of innovative parameters and the formation of a global network of partnerships, such as GLOWACON, are crucial in propelling the field forward. These international collaborations among nations, organisations, academics, industries, and the public-private sectors have cultivated a new standard of shared knowledge and a framework for data exchange, which stands as one of the pivotal teachings from the pandemic.

Figure 25: Comprehensive strategy of WBE in a One Health approach



Source: Adopted from Remonnay, personal communication (redesigned)

Likewise, the economic significance of water systems has also come into the spotlight, with the value of water extending beyond its conventional roles. The notion of the water system as a "Resource Recovery Facility" or “Ecofactory” has surfaced, reimagining a system that not only provides water but also generates energy, supports climate and biodiversity initiatives, recovers resources such as phosphorus, and acts as a sentinel within the One Water – One Health framework.

This innovative concept applies to all water systems, including rivers and local water systems, underscoring the importance of WES as an all-encompassing surveillance instrument. The chapters of this Cookbook have offered practical, hands-on guidance in the form of "recipes," as well as a thorough overview of the various facets of WBS/WES used for SARS-CoV-2, from conceptual frameworks to methodologies, transport protocols, and storage considerations. The integration of these components into the wider context of public health surveillance, governance, communication, and financial evaluation has been explored, along with the ethical, anthropological, and social underpinnings of the practice.

In summary, the advancements in WBS and the advent of WES signify a revolutionary change in environmental surveillance, with the potential to profoundly influence public health monitoring and policymaking. As we continue to refine these systems and adopt the Resource Recovery Facility concept, we must strike a balance between innovation and caution, ensuring equitable data use and adherence to ethical standards. The future of WBS/WES is filled with promise, and the lessons learned during the COVID-19 pandemic will undoubtedly serve as a foundation for future public health initiatives.

Resource Recovery Facilities, as a modern interpretation of wastewater treatment plants, are at the forefront of this sustainable approach. They are not only pivotal in managing waste but also serve as hubs of innovation that:

1. **Reclaim and repurpose water**, contributing to water security and conservation efforts.
2. **Extract and recycle nutrients**, turning waste into valuable fertilisers and contributing to sustainable agriculture.
3. **Generate renewable energy**, reducing the carbon footprint and contributing to energy independence.
4. **Provide real-time public health data**, enabling proactive responses to emerging health threats.
5. **Support the One Health approach**, recognising the intricate connections between the health of people, animals, and our shared environment.

As we look to the future, the expansion of WBS/WES could include:

- **Monitoring a wider array of pathogens,** to not only track existing diseases but also to be vigilant against new threats.
- **Integrating antimicrobial resistance tracking,** to combat the growing challenge of drug-resistant organisms.
- **Incorporating biomarker analysis,** to detect a range of health indicators that can inform public health strategies.
- **Strengthening global partnerships,** to share knowledge, standardise methodologies, and foster innovation across borders.

The Cookbook's recommendations, such as establishing clear communication, ensuring data quality, and promoting transparency, are not just guidelines for current practice but also principles that will underpin the evolution of WBS/WES. By adhering to these recommendations, we can ensure that WBS/WES remains a robust, responsive, and respected tool in the arsenal of public health surveillance, ready to adapt to the challenges of tomorrow.

The final recipe



Based on the information provided here, the final recipe that arises from the WBS/WES 'Cookbook':

- **Establish Clear Communication.**
  Ensure that all stakeholders understand what WBS is and what it isn't, and communicate the significance of the data collected.
- **Frequent Data Updates**
  Update results frequently and make all data available to ensure timely and informed decision-making.
- **Contextualize Results**
  Put WBS results into context to provide meaningful insights for public health implications.
- **Focus on Trends**
  Highlight trends and alerting results to identify and respond to public health threats promptly.
- **Engage Stakeholders**
  Engage with various users of the data, including wastewater plant personnel, epidemiologists, and

statisticians, but also medical staff and the general public, to foster collaboration and improve outcomes.

- **Ensure Data Quality**
  Implement rigorous data documentation, quality assurance, and control measures to maintain the integrity of WBS data
- **Data Accessibility**
  Share data in a way that is accessible and understandable, respecting privacy and ethical standards.
- **Integrate Data**
  Combine wastewater data with other health surveillance data to enhance insights into public health trends.
- **Adapt and Innovate**
  Remain adaptable in data documentation and quality assurance practices to accommodate new methodologies and emerging threats.
- **Promote Transparency and Trust**
  Maintain detailed records of data provenance and encourage feedback on the data model development process.

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List of abbreviations and definitions

Abbreviations	Definitions
ACIC	Australian Criminal Intelligence Commission
AI	Artificial intelligence
AIC	Akaike information criteria
AMR	Antimicrobial resistance
API	Application programming interface
B2M	β-2 Microglobulin
BIC	Bayesian information criteria
BOD	Biochemical oxygen demand
BSC	Biosafety cabinet
CDC	Centers for Disease Control and Prevention
cDNA	Complementary deoxyribonucleic acid
COD	Chemical oxygen demand
crAssphage	Cross-Assembly phage
CRM	Certified reference material
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DOI	Digital object identifier
dPCR	Digital polymerase chain reaction
EC	European Commission
ECDC	European Centre for Disease Prevention and Control
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
EPHO	Essential Public Health Operations
EU4S-DEEP	European Union Digital European Exchange Platform
FAIR	Findability, Accessibility, Interoperability, and Reusability
FAO	Food and Agriculture Organization

Abbreviations	Definitions
GAM	Generalised additive models
gc	Gene copies
GIS	Geographic information system
GLASS	Global antimicrobial resistance surveillance system
GPS	Global positioning system
GSAID	Global Initiative on Sharing All Influenza Data
GWPP	Global water pathogens project
GWRC	Global Water Research Coalition
HERA	Health Emergency Preparedness and Response Authority
HIC	High-income country
IATA	International Air Transport Association
ICU	Intensive care unit
IfSG	German Protection against Infection Act
IPC	Infection prevention and control
IWA	International Water Association
JRC	Joint Research Centre
LDGV	Lineage-determining genetic variations
LDM	Lineage-defining mutations
LIMS	Laboratory Information Management System
LMIC	Low- and middle-income country
LoD	Limit of Detection
LOESS	Locally estimated scatterplot smoothing
LoQ	Limit of Quantification
LOWESS	Locally weighted scatterplot smoothing
MK	Mann-Kendall
MOH	Ministry of Health
NASBA	Nucleic acid sequence-based amplification
NH_3	Ammonia
NH_4-N	Ammonium
NGO	Non-Governmental Organisation

Abbreviations	Definitions
NGS	Next-Generation Sequencing
NIST	National Institute of Standards and Technology
NSAID	Nonsteroidal anti-inflammatory medication
NWSS	National Wastewater Surveillance System
ODM	Open Data Model
OECD	Organisation for Economic Co-operation and Development
OSHA	Occupational Safety and Health Administration
PBM	Polymorphism-based markers
PCR	Polymerase chain reaction
PE	Population Equivalents
PMMoV	Pepper Mild Mottle Virus
PPE	Personal Protective Equipment
PT	Proficiency testing
QA	Quality assurance
QC	Quality control
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative Reverse Transcription PCR
RAG	Risk Assessment Group
RIVM	Netherlands National Institute for Public Health and the Environment
RM	Reference material
RMG	Risk Management Group
RMSE	Root mean square error
RNA	Ribonucleic acid
RSI	Relative Strength Index
RT-PCR	Reverse Transcription-PCR
SMA	Simple Moving Average
SNP	Single Nucleotide Polymorphism
SOP	Standard Operating Procedure
STP	Sewage treatment plant
TN	Total Nitrogen

Abbreviations	Definitions
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
VOC	Variants of Concern
VOI	Variants of Interest
VUM	Variants Under Monitoring
WBE	Wastewater-Based Epidemiology
WBS	Wastewater-Based Surveillance
WC	Wastewater signal to clinical cases
WEF	Water Environment Federation
WHO	World Health Organization
WRF	Water Research Foundation
WWTP	Wastewater treatment plants

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Annex

ANNEX 1. OVERVIEW OF THE PUBLIC HEALTH ENVIRONMENTAL SURVEILLANCE OPEN DATA MODEL (PHES-ODM)

MODEL OVERVIEW

The ODM has fourteen tables that represent WBS's real-world entities.

DAY-TO-DAY DATA COLLECTION

Samples table - a sample is a representative amount of wastewater, air, or surface substance. Each sample contains up to 23 attributes. Examples of sample attributes include the sample material (e.g. raw sewage, holding tank sewage) and collection (e.g. grab sample, Moore swab).

Measures table - the location to store all measurements and observations of any substance, including a biological, physical or chemical substance. Measures can be taken at sites or on samples. Each measure contains up to 24 attributes, including the measured value, unit, aggregation, quality descriptor and measurement method.

SUPPORT FOR SAMPLES AND MEASURES TABLES

Measure sets - If needed, measures are grouped into measure sets. Applications of measure sets include measure replicates and measures that must be interpreted as a group (e.g., proportions of various variants detected in a single assay).

Quality reports - Each sample, measure or measure set can have one or more quality reports or flags. For example, a sample may have leaked or been stored outside the recommended temperature range.

PROTOCOL REPORTING

Reporting methods are performed as a series of steps that can be grouped into a protocol. Protocols can be versioned and updated. You can start with existing published protocols and revise sections.

Protocol steps - an entry or 'step' within the protocol steps table can be a method or a measure. An analogy is making a cake: you mix the dry ingredients (a method), but first, you measure the amount of flour and sugar.

Protocols - where to store a group of method steps such as sample concentration, extraction, inhibition, and measurement.

Protocol relationships – How to describe the order of protocol steps, including revisions to existing protocols.

Instruments - Each protocol step can include details about the instrument(s) used to perform the method. Instruments may also be linked to measures.

SITES AND ORGANISATIONS

Information about 'who' collects data and 'where' sampling and measurements are made is stored in five tables: sites, polygons, organisations, contacts, and addresses. The site table records where wastewater samples are taken. Sites can have measures such as wastewater flow rates and temperature. The polygon table stores the geometry or shape of a geographic area. Polygons can be associated with two entities: wastewater samples, such as a sewershed (the area served by a wastewater collection system); and populations, such as a municipal boundary or health region. In turn, population measures can be about people, such as Covid-19 hospitalisation, or the geographic area, such as rainfall.

Each data entry can have identifiers, including the organisation and contact name of the person who generated the data. The data identifiers support data provenance and lineage.

Datasets - holds details about the dataset and includes data custodians and funders responsible for data contained in the ODM. Dataset information enables the attribution of credit to data collectors and enhanced traceability. Datasets can include a dataset's digital object identifier (DOI). A sharing and access schema provides information regarding who can access data.

LOOK-UP TABLES

There are four look-up tables to support reporting. Within the ODM, everything is a part and recorded in the part table. There are 20 part types, including tables, measures, methods, attributions, units, aggregations, categories, sets, domains, groups, etc. There are over 800 parts within the ODM, which can be expanded as needed. The additional look-up tables are sets, translations and languages. Any part can be translated into any language.

PART ATTRIBUTES

LABEL	DESCRIPTION
Aggregation scale	The scale of an aggregation set. Aggregation scales include quantitative and qualitative.
Aggregation sets	Sets of aggregations that can be used for measures. Examples of aggregation sets logarithm, linear and Boolean aggregations.
Aggregations	Statistical measures used to report a measure. Each measure reported as a number should be reported with an aggregation.
Attributes	An attribute of a domain, specimen, compartment, or reportTable.
Categories	A discrete list of values that can be reported for a measure, method or attribute.
Category sets	A set of categories. For example, site type has a list of different sites, such as a wastewater treatment plant, a septic tank, or a pumping station.
Classes	A class is a collection of one or more related measures or methods within a group. A group can have one more classes to describe different parts or the class.
Compartments	A substance from which a sample was taken. Compartments include air, water, surface, and humans.
Compartment Sets	Sets of compartments. Compartment sets are used to identify when a measure can be recorded for more than one compartment. For example, SARS-CoV-2 can be measured in people (humans), water, surface, or air.
Data types	The data type for a part.
Description of change	A description of change in a part from the previous version.
Domains	Domain is the highest level of describing a measure. There are three domain types: biologic (i.e. Covid-19, chemical (i.e. nitrogen), physical measure (temperature).
First released version	The version in which a part was first released.
Groups	A collection of related measures.
Label	A human readable label of a part.
Last updated date	The date that the part was last updated.
Latex expression	Latex expression used to generate mathematical formulas for units.
Maximum length	The minimum length of the value of a part or measure.
Maximum value	The maximum length of the value of a part or measure.
Measure	An observation recorded from a specimen (i.e. a site, sample, person or population).
Method	A procedure or step that is performed to make a measure.
Minimum value	The minimum value of measure.
Maximum length	The maximum value of measure.
Missingness	The part type for missingness values.
Missingness set	The part type for missingness sets.

LABEL	DESCRIPTION
Nomenclature	A classification system to report the measure class.
Ontology reference	Ontology reference for a part.
Part Description	The description of the part.
Part ID	The unique identify of any entity within the dictionary.
Part Instruction	Additional notes and instructions on how a part is used and/or defined.
Part types	Part types describe the purpose or use of the part.
Quality indicators	A measure of the quality of a reported value or sample.
Quality Sets	Sets of quality measures. For example, PCR have a quality measure set.
Set type	The type of set. i.e. quality set, aggregation set, unit set
Short Names	Shortened names for tables and other important parts for use in wide names.
Specimen sets	Sets of specimens. Specimen sets are used to identify when a measure can be recorded for more than one specimen. For example, SARS-CoV-2 can be measured in people (humans) or samples. Flow rates are measures from sites.
Specimens	Measures or observations are taken from three types of substances: populations (humans or geographic areas), samples, sites.
Status	Whether the part is still active and can be used in the most current ODM version. Values are 'active' or 'inactive'.
Tables	Tables are where measures, methods and attributes are recorded. Tables represent the main entities of the environmental surveillance.
Unit sets	Sets of units. Measurement classes can use a set of related units that are recorded using unit sets.
Units	The unit of the measurement.

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