



Surveillance of Emerging Pathogens and Antibiotic Resistances in Aquatic Ecosystems

KEY MESSAGES

- Wastewater surveillance is a sensitive method to detect spill over of zoonotic infections and early pandemic emergence.
- Technological advances offer new possibilities for microbial monitoring by inclusion of parameters such as viruses, antibiotic resistance genes, antibiotic resistant bacteria and metagenomes. These parameters and methods should be taken into account in future regulations.
- Microbial source tracking methods are sensitive tools for identifying human contamination of water resources and helping to develop specific protection measures.
- Extreme events, such as rainfall after prolonged dry periods, can lead to increased microbiological contamination of surface water.
- Monitoring methods have been successfully harmonised and implemented and can be recommended for future application.

Context

The analysis of the microbiological quality of water is an essential step for identifying the sanitary and environmental risks caused by faecal contamination. Assessment of the entire range of pathogen microorganisms is difficult and expensive, so microbial indicators are typically used for water quality management. However, a better understanding of the fate of pathogenic viruses and antibiotic resistant bacteria from the sources to river basins and estuaries are urgently required.

The AquaticPollutant project SARA “Surveillance of Emerging Pathogens and Antibiotic Resistances in Aquatic Ecosystems” determined the prevalence of pathogenic viruses (including SARS-CoV-2), microbial indicators, antibiotic resistance, and microbial source tracking markers in wastewater, surface water, coastal sea waters, sediment and bivalve molluscan shellfish in catchments located in different climate areas (Sweden, Germany, France, Spain, Portugal, Israel, Uganda, and Mozambique).

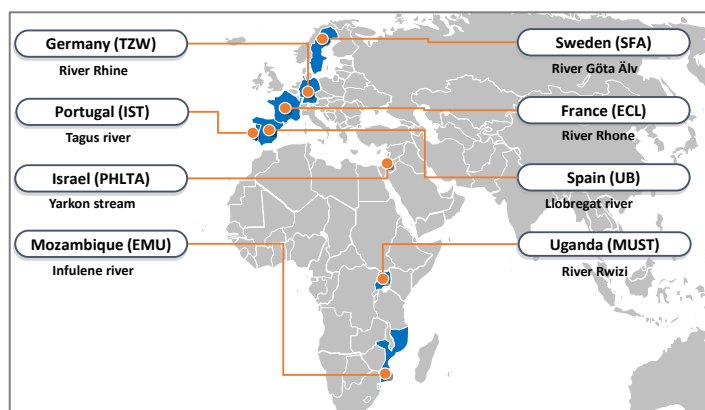


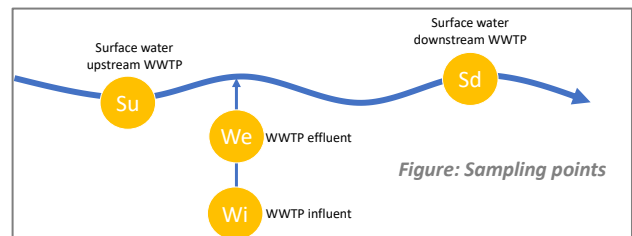
Figure: Project consortium and model areas

Findings

Harmonisation of methods: The project used both culture and molecular methods to monitor different model areas. In the first phase, the culture-based analyses and the sample preparation for the molecular biological analyses were harmonised (booklet available on the SARA website www.sara-project.info). The use of harmonised methods provided the basis for the comparison of monitoring data and can be recommended for future monitoring programmes.

SARS-CoV-2 monitoring: The results of wastewater-based surveillance from the partners demonstrate that this approach is effective in tracking COVID-19 trends and providing sentinel warning of variant emergence, regardless of the differences in detection method. This was confirmed by analysis of wastewater samples from Spain, Germany, Portugal, and Uganda. Samples collected during a period of high reported cases showed the highest biomarker concentrations. The application of wastewater-based surveillance to public health protection in low-and-middle income countries like Uganda is of particular interest since it provides the opportunity to overcome several barriers to effective surveillance. However, the implementation of wastewater monitoring poses challenges in terms of obtaining representative and meaningful samples. In particular, it is difficult to sample small catchments, critical facilities (e.g. hospitals) or low-income countries where the use of automatic samplers is not possible or the samplers are not available. To overcome these problems, a low-cost and easy-to-use passive sampler that also is suitable for antibiotic resistance genes has been developed. In summary, wastewater data provide a less biased alternative to reported cases from individual testing. Our studies highlight the importance of using wastewater data as a complementary tool to improve understanding and estimation of infection prevalence.

Aquatic catchments: To monitor the model sites, F-specific and somatic **bacteriophages** were used as viral indicators in addition to the classical faecal indicator *E. coli*. Overall, the concentration of somatic phages was higher than that of F-specific phages, making somatic phages more suitable for routine monitoring.



There were clear differences in the presence of **human pathogenic viruses** between the different catchments and wastewater treatment plants. Wastewater treatment plants using activated sludge and tickling filter showed a decline by 1-2 log levels in viral load between influent and effluent. The stabilisation ponds in Uganda showed a reduction in some samples, while in others there was almost no virus removal, suggesting that the process used in this treatment is not reliable and stable.

Norovirus levels in wastewater appear to increase to high levels during the colder months and the reduction of norovirus and other viruses in wastewater treatment plants could be limited depending on the treatment processes used. Genotyping identified the dominant norovirus genotypes, with no major differences between the European samples. However, genotype GII.13 was dominant in the Ugandan samples, whereas it was rarely or not observed in any of the European samples. Understanding the genotypic diversity of norovirus at the population level is essential due to the high burden of norovirus outbreaks and the lack of vaccines and antivirals. In this context, surveillance of wastewater using nucleic acid-based methods in combination with genotyping can provide complementary data to clinical samples and represent an effective strategy for monitoring the circulation and emergence of epidemic genotypes of noroviruses.

A key objective was to study the dynamics of **antibiotic resistance genes and antibiotic resistant bacteria** in Europe and Africa. Extended-spectrum β -lactamase-producing *E. coli* have been selected as indicators of

antibiotic resistant bacteria. The ratio of extended-spectrum β -lactamase-producing *E. coli* to *E. coli* shows that there is a high variance depending on the model site and sampling location, with higher abundance of extended-spectrum β -lactamase-producing *E. coli* in Uganda and low abundance in Sweden.

In the **quantitative polymerase chain reaction-based analysis for antibiotic resistance genes**, the sulfonamide resistance gene *sul1* was the antibiotic resistance gene detected with the highest prevalence in all model areas (see Figure below). The colistin resistance gene *mcr-1* gene was detected only in influent and effluent samples from the wastewater treatment plants in Germany, Israel and Uganda. It was not detected in the corresponding samples from Sweden and France. In surface waters, the tetracycline resistance gene *tetC* and the β -lactamase genes *bla*_{TEM}, *bla*_{CMY-2} and *bla*_{OXA-48} and were detected at higher gene copy numbers especially in Germany, Spain, Israel, Uganda and Mozambique. Overall, the relative abundance of antibiotic resistance genes was higher in Uganda. In contrast, significantly fewer antibiotic resistance genes were detected in samples from the Swedish model area. Levels of antibiotic use and resistance in Sweden are among the lowest in Europe, both in the human and animal sectors. This is due to early awareness and long-term interdisciplinary and intersectoral action. For example, Sweden was the first country in the world to ban all use of antibiotics as growth promoters in food animal production, and the Swedish regulation of antimicrobial use in farm animals is strict from a European perspective.

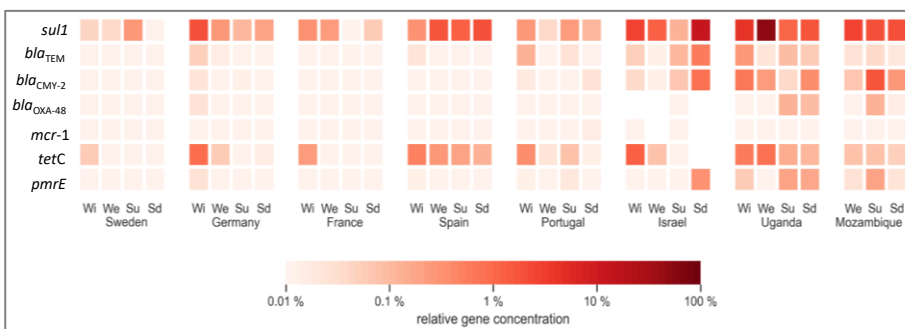


Figure: Heatmap of the relative abundance (normalised to 16S rRNA gene) of key antibiotic resistance genes in the different catchment areas monitoring (Wi: wastewater treatment plant influent, We: wastewater treatment plant effluent, Su: Surface water upstream wastewater treatment plant, Sd: Surface water downstream wastewater treatment plant).

The **metagenomic approach** was used on over 300 samples to determine the type, relative abundance and distribution of antibiotic resistance genes in the different partner countries. The majority of antibiotic resistance genes were classified as efflux pumps. In addition to evaluating the sequence reads, we assembled the reads into contigs and binned the contigs into metagenome-assembled genomes. These studies showed considerable variation in the relative abundance of metagenome-assembled genomes with and without antibiotic resistance genes. Downstream samples from Portugal contributed significantly to metagenome-assembled genomes containing tetracycline resistance genes, and overall the abundance of both antibiotic resistance genes-containing metagenome-assembled genomes and antibiotic resistance genes is relatively low in the model area in Sweden compared to the other catchments. Antibiotic resistance genes quantification by polymerase chain reaction is characterised by higher accuracy and sensitivity. However, metagenomics provides a higher coverage of antibiotic resistance genes, so the analysis methods complement each other well.

Studies on the **stability of antibiotic resistance** in the environment and the **role of the non-bacterial fraction** in its transmission: Microcosm experiments were conducted to determine the persistence of human-associated and oligotrophic environmental antibiotic resistance bacteria and genes in anthropogenically polluted river waters. The study evaluated the die-off of human-associated antibiotic resistant bacteria and antibiotic resistance genes in the aquatic environment. Environmental antibiotic resistant bacteria (oligotrophic extended-spectrum β -lactamase-producing bacteria) and antibiotic resistance genes (e.g. *sul1*) persisted in the microcosms. The isolated oligotrophic extended-spectrum β -lactamase-producing bacteria

contained β -lactam resistance genes found in viral contigs, suggesting that bacteriophages may play an important role in the spread of antibiotic resistance genes in aquatic ecosystems. Antibiotic resistance genes were also detected in the non-bacterial fraction of the water samples, specifically within phage particles, in rivers and wastewater treatment plants. The substantial concentrations found within these phages suggest that they play a significant role in the transmission of antibiotic resistance genes in these environments.

Human MST markers (crAssphage, HMBif and HF183) were detected in all model areas, including the African areas. crAssphage was the most abundant across all samples and sites. HMBif and HF183 showed similar results but were generally detected less frequently and at lower concentrations. Marker levels varied significantly between catchment areas, particularly in wastewater treatment plant effluent and river water samples. Our study indicated that long periods of drought correlated with slightly lower levels of MST markers in river water samples, whereas heavy rainfall increased marker concentration. Strong correlations were observed among all MST markers and with cultivable bacterial and bacteriophage faecal indicators, supporting their use as indicators of human faecal pollution.

In addition to the sampling campaigns conducted under standard conditions, samples were also taken during specific events, including dry periods, rainfalls after a long dry season, and flooding. The monitoring of these **extreme events** has demonstrated that especially rainfall events occurring after extended periods of drought can result in a significant increase in the microbiological contamination of surface waters, with *E. coli*, coliform bacteria, somatic phages and ESBL-producing *E. coli* numbers exhibiting a 100-1000 fold increase compared to standard conditions. This has been demonstrated in model areas in Germany and Spain. Potential causes for the higher pollutions include the remobilisation of river sediments, the inflow of surface runoff, the discharge of untreated wastewater from combined sewer overflow basins, and underperformance of wastewater treatment plants.

Bivalve molluscan shellfish and sediment as time integrating parameters: When sediment and water phases were analysed, the sediment samples showed a higher abundance of selected antibiotic resistance genes. Therefore, sediment analysis can provide additional information to water quality. Studies have shown the presence of antibiotic resistance genes in bivalve molluscs. Shells from the German study area have also been found to contain extended-spectrum beta-lactamase producing *Enterobacteriaceae* and *Pseudomonas*.

Monitoring Recommendations

Water management is affected by a wide range of water quality and quantity issues. These include the presence of microbial contaminants entering the water cycle from point sources such as industrial discharges, urban wastewater, littering and urban runoff. The increasing frequency of extreme weather events (e.g. floods, droughts) due to climate change and discharges from combined sewer overflows or urban storm water runoff also exacerbate water management challenges. The following recommendations for surface water monitoring were derived from the project results.



Figure: Sampling campaign in Uganda

- Wastewater-based surveillance is a successful tool for monitoring the circulation of SARS-CoV-2, including variants and sub-lineages and antibiotic resistance genes in the population and should be used internationally in the future as a sentinel system for the early detection, prevention and real-time monitoring of disease threats and outbreaks.

- Wastewater treatment plant effluents are an important source of antibiotic resistant bacteria, antibiotic resistance genes and viruses in surface waters and should therefore be monitored in accordance with the one health approach. More efficient removal of antibiotic resistance from wastewater can be achieved by upgrading the treatment process with advanced treatment processes such as ozonation, UV irradiation or chlorination.
- Somatic bacteriophages are more suitable for faecal pollution monitoring than F-specific phages due to their higher concentration and environmental persistence.
- Extended-spectrum β -lactamase-producing *E. coli* is a possible and good indicator for clinically relevant antibiotic resistant bacteria.
- Antibiotic-resistant environmental bacteria, such as extended-spectrum β -lactamase-producing oligotrophic bacteria, should be considered for environmental antimicrobial surveillance to provide a complete picture.
- Extreme events can result in an increase of faecal contamination (factor 100-1000) and should be included in monitoring programmes.
- When selecting antibiotic resistance for screening, representative genes from each of the categories (1) frequently detected at high levels, 2) frequently detected at intermediate levels and 3) not detected or detected at low levels) and genes conferring resistance to different classes of antibiotics should be considered.
- Antibiotic resistance genes analysis by polymerase chain reaction is more sensitive and metagenomics provides a higher coverage and a more comprehensive picture of antibiotic resistance genes. The data obtained are complementary and therefore both methods should be used for monitoring.
- Sweden has a relatively low per capita use of antibiotics and a comparatively favourable situation with regard to antibiotic resistance. This situation is influenced by factors such as a long-standing adherence to evidence-based guidelines, strong local commitment and strategic efforts at both regional and national level, in both human and animal health. The successful Swedish strategic programme against antimicrobial resistance could be implemented by countries in their own national action plans against antimicrobial resistance.
- The monitoring of antimicrobial resistance in the environment should also include the bacteriophage fraction and the sediments.
- Passive sampling is a good tool for monitoring pathogenic viruses and antibiotic resistance genes, especially in places where automatic sampling systems cannot be installed or are not available.
- Using crAssphage in water monitoring programs could significantly improve the ability to track faecal contamination, particularly in high-risk areas. Its effectiveness in diverse climates and during extreme weather events makes it an important tool for microbial source tracking.

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