April 2025

Pilot study on antimicrobial resistance monitoring in European surface waters – Final report of the Eionet Working Group

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European Topic Centre
Biodiversity and ecosystems

EIONET

Cover design: NIVA
Cover image: © Carsten Schwermer, NIVA
Layout: Claudia Neitzel [NIVA]

Version: 1.0

Publication Date: 29 April 2025

ETC-BE coordinator and contributing partner: Norwegian Institute for Water Research (NIVA)

Eionet WG on AMR in surface waters:

The European Environment Information and Observation Network (Eionet) is a partnership network of the European Environment Agency (EEA) and its 38 member and cooperating countries. EEA and Eionet gather and develop data, knowledge, and advice to policy makers about Europe's environment.

The Eionet Working Group on AMR in surface waters was established under the Council Recommendation on Stepping up EU Actions to combat AMR in a One Health Approach (2023/C 220/01).

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doi: 10.5281/zenodo.15296568

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https://www.eionet.europa.eu/etcs/etc-be

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https://www.eionet.europa.eu/

European Environment Agency
https://www.eea.europa.eu/en

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Acknowledgements

We kindly thank the following people for their valuable support and contributions to this pilot:

Austria: Irina Dielacher (sampling, laboratory, data processing), Moritz Pellegrini (laboratory, data processing), all WWTPs for providing samples.

Belgium: Sébastien Adam (sampling), Léa Grimonster and Elisa Delrée (aboratory), and Leslie Crettels (data processing) from ISSEP (Institut Scientifique de Service public /Scientific Institute of Public Service).

Germany: Sebastian Gläsel and Janin Fanta (sampling, laboratory); Corinne Pippig, Lydia Lehniger and Sven Pfeifer for data processing and reporting support to the pilot study.

Ireland: Prof Dearbháile Morris, Dr Zina Alfahl, Niamh Cahill and Aneta Kovarova (University of Galway, Ireland) for providing sampling, analysis and reporting support to the pilot study in Ireland.

Switzerland: Karin Beck (sampling, laboratory), Sasikaladevi Rathinavelu (laboratory, data processing)

The Netherlands: Guusje Jacob and Hetty Blaak (sampling, laboratory, data processing).

Layout: Claudia Neitzel (NIVA, Norway)

1. Summary

This report documents the process and outcomes of a pilot study conducted by the Eionet Working Group (WG) on antimicrobial resistance (AMR) monitoring in European surface waters. Recognizing AMR as a major global public health threat and the environment's potential role in its spread, this pilot was conducted aiming to enable a harmonized approach for data collection and reporting of selected antimicrobial resistance indicators in surface waters at European scale. This initiative is relevant to ongoing revisions of EU water-related directives.

Method development for the AMR pilot study. The Eionet WG, led by the European Environment Agency (EEA[1]) and experts from the European Topic Centre Biodiversity and Ecosystems (ETC-BE[2]) together with experts from EEA member countries and relevant European agencies, developed methodologies for all stages of the pilot study. The goal was developing a reliable methodology that could serve as foundation for a future European surveillance effort for AMR in water, constrained by what could be implemented on a voluntary basis by WG members within a limited project period (June 2023-March 2025). Given the emphasis on the impact of human activities, sampling in this pilot focused on rivers downstream of urban wastewater treatment plants (WWTPs) and their effluents. The WG developed **practical guidelines** that set out details for the method, covering sampling frequency, sample preparation, target indicators, analyses, data reporting and quality control procedures. Six gene targets (16S rRNA, intl1 and the four ARGs addA1, ermB, $bla_{CTX\text{-}M1}$ and vanA) for quantitative Polymerase Chain Reaction (qPCR) analysis, along with culturing of *Escherichia coli* (*E. coli*) and Extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* (ESBL-Ec), were monitored to estimate AMR pollution in surface waters. The indicators selected for the purpose of this pilot should not be seen as a recommendation for others - rather, they represent what was possible and relevant within the tight constraints of this activity.

Ten out of fourteen countries nominated to the Eionet AMR WG participated in the pilot survey by reporting AMR data. For analysing the samples, **qPCR analysis** was used and prioritized over other potential methods, such as metagenomic analysis, due to cost-effectiveness and availability. Recommended primer and probe sets and measures for inhibition reduction were developed. Bespoke standards for qPCR calibration[3] were designed for this study and used to improve the comparability of the results for the specific ARG targets analysed in the different laboratories. For **culturing** and quantifying *E. coli* and ESBL-Ec on selective media[4], WHO's protocol on integrated global surveillance on ESBL-producing *E. coli* (Tricycle protocol) (WHO, 2021) was followed. The Tricycle protocol was used as standardized EU methods are currently only available for food-producing animals and meat, and not for water. An offline **data reporting** template was developed, based on EEA's WISE-6[5] (Water quality) data model. Reporting guidance set out required parameters, units and metadata.

AMR pilot study results. The survey provided data on the concentrations of the selected gene and bacterial targets at WWTP and river sampling locations. The abundance of the detected gene targets was expressed both as the absolute abundance and in relative terms (as a proportion of the 16S rRNA gene). As expected, the general pattern of gene abundance, based on median values, and bacterial indicators was in the order 'WWTP inlet' > 'WWTP outlet' > 'river downstream' > 'river upstream', where WWTP inlet showed the highest concentrations, was evident for 16S rRNA, intl1, aadA1, ermB and vanA, aligning with other research studies. The relative abundance across the entire data set of ARGs could be broadly categorized into three groups: 'high abundance' (including intl1, aadA1, sul1, ermB, tetW), 'intermediate abundance' ($bla_{CTX\text{-}M1}$), and 'low abundance' (vanA). Estimated removal effectiveness owing to wastewater treatment for the most abundant genes (intl1, aadA1, ermB, vanA) ranged between 1.3 and 2.9 log_{10}-units (94.5%-99.9%), which is somewhat lower than findings from other studies. The proportion of ESBL-Ec

[1] https://www.eea.europa.eu/en

[2] https://www.eionet.europa.eu/etcs/etc-be

[3] A commercially-available product composed of synthetic gene fragments was used as positive control in the qPCR

[4] Cefotaxime amended. Cefotaxime is a third-generation cephalosporin β-lactam antibiotic used to treat a variety of bacterial infections

[5] https://cdr.eionet.europa.eu/help/WISE_SoE/wise6

among the total number of *E. coli* exhibited a large variation across countries, ranging between 0 and 14.5%, depending on the sampling location. The reduction effectiveness during wastewater treatment processes was 2.3-2.4 $\log_{10}$ units (99.5%-99.6%) for both *E. coli* and *ESBL-Ec*, amongst all countries. Since the pilot's primary goal was to enable harmonization of AMR monitoring across European countries, rather than to study the actual target concentrations and trends between countries, only limited interpretation of the results has been undertaken at this stage.

Experiences gained and challenges. Developing harmonized AMR monitoring in the framework of this pilot presented challenges and opportunities for shared learning. Participants experienced timetable and funding constraints that limited the scope and scale of sampling and analysis, requiring integration with existing monitoring programs and/or ongoing R&D projects in most countries. The WG adapted the initial scope to accommodate varying capacities and resources amongst the participants. There were significant differences in experience with qPCR and culturing techniques amongst the WG members. Methodological considerations included budget-influenced site selection, preference for cheaper but less specific methods, and debates on the relevance of certain clinical markers in the environment. Quality assurance and control challenges included the lack of readily available certified reference materials for AMR targets and target bacteria.

Priority areas for future EU-wide monitoring. Several priority areas were identified for future Europe-wide AMR monitoring including to:

- Foster agreement on the objectives of environmental AMR monitoring;
- Strengthen harmonization of methods and protocols;
- Develop and implement robust quality assurance/quality control (QA/QC) procedures;
- Establish a centralized and integrated data reporting system and solutions for data sharing.

The WG members evaluated this pilot study as a valuable first step towards harmonized AMR monitoring in European surface waters by fostering collaboration amongst the participants and developing a foundational methodology.

2. Purpose of report

This document is a report on the process and outcomes of a pilot study developed and conducted by the Eionet Working Group (WG) on antimicrobial resistance (AMR) monitoring in European surface waters. It encompasses methodology, results from monitoring AMR indicators in rivers, urban wastewater, and urban wastewater treatment plant discharges, as well as 'lessons learned' by the Eionet WG members to improve future AMR monitoring. This report aims to support future work on AMR monitoring in the aquatic environment by the EEA, Eionet, Commission services and national agencies.

3. Context

The World Health Organization (WHO) declared AMR to be amongst the top ten global public health threats (WHO, 2022). The health burden from AMR infections in the EU/EEA area is comparable to that of influenza, tuberculosis, and HIV/AIDS combined (OECD, 2019; ECDC, 2024[6]). An estimated 1.9 million deaths attributable to AMR and 8.2 million deaths associated with AMR could occur globally in 2050 according to recent forecasts (GBD, 2024). The main entry routes of AMR and antibiotics originate with human and veterinary use of antibiotics and the release of AMR and antibiotics via wastewater and sludge from wastewater treatment plants (WWTPs), via discharges from e.g., pharmaceutical industries, and via veterinary wastes and natural fertilizers such as manure (Krzeminski et al., 2020).

During recent years there have been several actions from the European Union (EU) on antimicrobial resistance[7]. In June 2023, the EU Council adopted the Recommendation on stepping up EU actions to combat antimicrobial resistance in a One Health approach[8], which extends and complements the 2017 EU One Health Action Plan against AMR in all three dimensions of the One Health spectrum to maximize synergies and attain a strong and effective response against AMR across the EU (EC, 2017). The plan outlined over 70 actions covering human health, animal health and the environment, however it was recognised that further action is needed, particularly in the areas of human health and the environment. The need to strengthen One Health national action plans on AMR and to reinforce surveillance and monitoring of AMR and antimicrobial consumption were among the objectives of the EU Council recommendations. Robust surveillance and monitoring on AMR and antimicrobial consumption (AMC) at all levels in human health, but also in the veterinary, plant, and environmental sectors, are crucial to assess the spread of AMR, support the prudent use of antimicrobials and inform infection prevention and control responses. While the environmental dimension of AMR has been comparatively less in focus than AMR in human or animal health, growing evidence shows that the natural environment may be a major reservoir and driver of AMR. In line with the One Health approach, environmental monitoring of AMR in groundwaters and surface waters, including coastal waters and wastewater, is essential to further understand the role played by the presence in the environment of antimicrobial residues in the emergence and spread of AMR, the levels of environmental contamination and the risks posed to human health. Accordingly, the EU Council encouraged Member States to develop integrated systems for the surveillance of AMR and AMC encompassing human health, animal health, plant health, food, wastewater, agricultural soils and the environment (in particular water and soil), taking into account other past and ongoing activities (e.g., QTG-AIS[9], WHO Tricycle protocol[10]). Such integrated and continuous intersectoral monitoring should be designed to detect emerging resistant infections and outbreaks efficiently and rapidly. There is also a need to determine the presence of AMR genes and antimicrobials, trends and toxicity in soil and water bodies. The results of this surveillance should inform effective strategies to tackle AMR across sectors and at appropriate administrative levels.

[6] https://www.ecdc.europa.eu/assets/amr-targets-2024/index.html
[7] https://health.ec.europa.eu/antimicrobial-resistance/eu-action-antimicrobial-resistance_en
[8] https://health.ec.europa.eu/publications/council-recommendation-stepping-eu-actions-combat-antimicrobial-resistance-one-health-approach_en
[9] https://www.who.int/news-room/articles-detail/call-for-experts-qtg-amr-amu-integrated-surveillance
[10] https://www.who.int/publications/i/item/9789240021402

Revisions to the Urban Wastewater Treatment Directive 2024 (UWWTD) include monitoring of AMR at large wastewater treatment plants (WWTPs), while proposed revisions to the Environmental Quality Standards (EQS) and Groundwater Directives would enable monitoring of antibiotic-resistant genes in surface and groundwaters. Reporting under all these legislations will be to the EEA. The EEA therefore aims to understand what should be reported and how, given the current lack of standardized methods for AMR monitoring and reporting in aquatic environments.

The European Environment Agency (EEA) works with the Eionet (European Environment Information and Observation Network) as the partner network which supports implementation of the EEA's work program. Together with experts from the European Topic Centre Biodiversity and Ecosystems (ETC-BE) at the Norwegian Institute for Water Research (NIVA) in Oslo, the Eionet Working Group on AMR in European surface waters prepared a pilot study on AMR in surface waters for 2024 (Figure 1). The work involved significant effort in harmonizing, among others, sampling methods, indicator parameters, sample analysis and data reporting.

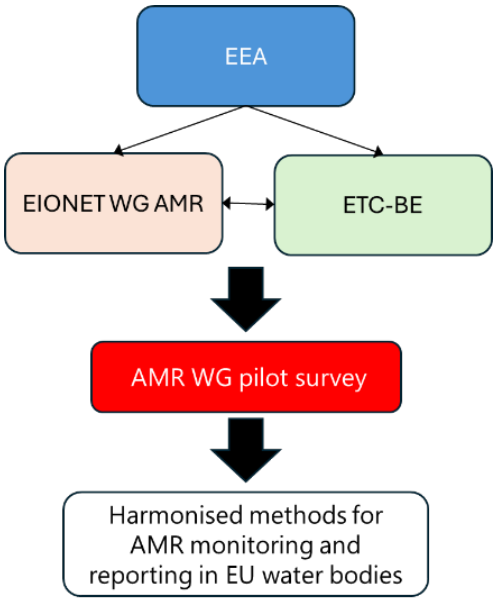

Figure 1. Organizational structure of the Eionet WG task on antimicrobial resistance in European surface waters.

4. Introduction

4.1 Objectives of the AMR pilot study

The overarching aim of the AMR pilot study was to enable a harmonized approach for data collection and reporting of selected resistance indicators surface waters at European scale. While the survey results were intended to be informative, the primary focus was on developing a robust and consistent methodology that can be implemented by members of the WG, building capacity and experience. The outcome should inform the development of a coordinated, harmonized environmental AMR surveillance across Europe. Specific objectives included:

1) **Establish an Eionet collaboration.** The AMR pilot study was led by the ETC-BE in collaboration with the Eionet. Representatives from the Joint Research Centre (JRC) and the European Food Safety Authority (EFSA) were included in the Eionet WG to bring their expertise and familiarity with related EU initiatives into the discussion. This collaborative effort aimed to bring together experts and representatives from various European countries to share knowledge, expertise, and build capacity in AMR area.
2) **Develop a harmonized methodology.** A core objective was to create a standardized methodology enabling surveying AMR in surface waters, encompassing:
    - **Monitoring objective:** Environmental surveillance requires agreement on the objectives, the aim or the final use of the obtained results i.e., collecting data on AMR across different WG members, as well as on the related decisions.
    - **Sampling locations:** The focus was on water bodies downstream of WWTPs, inlet and outlet of WWTPs due to the relevance of anthropogenic impact on water bodies, while optional sampling was done from upstream locations and points of low impact.
    - **Target indicators:** The objective was to select a limited number of common and relevant bacteria and ARGs and use the 16S rRNA gene as a measure of total bacterial abundance.
    - **Analytical methods:** The emphasis was on prioritizing widely applicable techniques already available or implementable in WG member laboratories, such as quantitative PCR (qPCR) and culturing, while the future potential of metagenomics was acknowledged during the WG discussions.
3) **Facilitate data collection and reporting.** The pilot study aimed to gather information on the abundance of selected AMR indicators data from participating WG members at designated sampling locations and report the data in a form suitable for the EEA. It involved developing a pilot dataflow with standardized data reporting procedures using a dedicated Excel template compatible with the existing WISE-6 dataflow.
4) **Build capacity and share knowledge.** The collaborative nature of the pilot study aimed to facilitate knowledge exchange between WG members with varying levels of previous expertise in AMR monitoring. This in turn enabled capacity-building in countries with less developed AMR monitoring programs.
5) **Inform future policy and monitoring strategies.** This objective focused on the possibility to inform the European Commission's proposed revisions to the Water Framework Directive, revisions to the Urban Wastewater Treatment Directive in relation to AMR monitoring and to support the development of standardized AMR dataflows within the WISE-State of Environment reporting (SoE).

4.2 Goal of this report

This report aims to answer the following questions:

1. Which **methodologies** were developed and used by the Eionet AMR WG to (a) run the AMR survey; (b) analyse the samples; and (c) report the results. (section 5)
2. What **experiences** have been gained from developing harmonized AMR monitoring? Which areas presented challenges in relation to the harmonization, and where were compromises made to accommodate pilot objectives, timetable, and funding constraints? (section 6)
3. What **results were obtained during the AMR survey**? (section 0)
4. What **priority areas** should be addressed by others when establishing a European-wide monitoring and reporting scheme? (section 10)

4.3 Focus of this report

The initially proposed methodology for the AMR pilot study and the final applied methodology, detailing the changes and experiences made by the Eionet WG during the process.

4.4 Out of scope for this report

- Designing analytical methods for detection of AMR in water.
- Covering all the potential objectives of an AMR monitoring, such as risk of AMR evolution, risk for ecological effects, population-level resistance prevalence, population-level antibiotic use, or socio-economic impact.
- Method optimization was not a primary aim of the project. Still, in cases of analytical challenges optimization was supported/facilitated by the WG. WG members used existing in-house methods where possible. The developed method(s) were intended for use within the specific task of the Eionet AMR WG. Hence, their applicability may not be suitable for all conditions and purposes beyond this pilot.
- In-depth interpretation of data beyond the provided graphs obtained from the results of the pilot.
- Policy demands (while recognizing that there is a policy need for evidence-gathering in this area).

4.5 Relevance of the task

The ETC-BE AMR task is of relevance to EU legislation recently under revision listed in Table 1.

Table 1: AMR and EU water legislation

Legislation	Content
Water Reuse Regulation 2020 (WRR)	Identifies AMR as a possible risk, requiring monitoring and reporting of AMR in wastewater for reuse.
Water Framework Directive (WFD), Environmental Quality Standards Directive (EQSD) and Groundwater Directive - PROPOSAL	Selected ARGs to be included in the Surface Water (SW) and Groundwater (GW) Watch List and suitable monitoring methods for AMR to be identified and/or developed.
Urban Wastewater Treatment Directive (UWWTD) 2024	Implementing Act setting out methodology for monitoring of AMR in discharges from large WWTPs by June 2026. Member States to start reporting by Dec 2030
Bathing Water Directive (BWD)	The BWD has been evaluated: a decision on whether a review will take place is pending.

This ETC-BE AMR task aimed to inform developments at the European level, focusing on the process in member countries for implementing AMR monitoring in water. Legislative developments reflect growing concern about AMR as a public health threat and the recognition of the need for standardized monitoring and reporting of AMR in water bodies. The Eionet AMR WG played a crucial role in addressing these needs by developing a proposal for harmonized methods and data collection and analysis workflows. The relevance of this activity to EU legislation lies in the process and the development of understanding among the countries – what is needed to establish AMR monitoring in surface water, rather than the specific details of the actions. In addition, the relevance of this activity also lies in the methodology developed and the targets identified, as this is crucial for an AMR monitoring study and an agreement between countries is not still reached.

4.6 Importance and benefits of a Europe-wide environmental AMR monitoring and reporting

Efforts to deliver a One Health[11] response to AMR need the environmental component to support efforts already under way in the food, human and animal health sectors. Environmental AMR monitoring and reporting could play an important role for understanding the occurrence of AMR in waters, protecting public health, supporting policy and regulation, ensuring data comparability, optimizing resources, fostering collaboration, advancing research, and demonstrating global leadership in addressing the AMR challenge.

Public health protection and the role of the environment. AMR is recognized as one of the top global public health threats. It leads to infections that are harder to treat, resulting in prolonged illness, higher medical costs, and increased mortality. A Europe-wide scheme can enable early detection of AMR trends and hotspots, allowing for timely interventions to prevent the spread of resistant bacteria. The environment plays a significant role in the emergence and spread of AMR (BIOHAZ, 2021; Klemm, 2018). Monitoring AMR in surface waters may help to understand how and how many ARB and ARG enter the environment that may help to assess the probability of transmission to humans. WWTPs are critical points through which antibiotic residues and resistant bacteria can enter surface waters. Monitoring these points, considering the abundance in water and sludge phases, helps assess the AMR releases to the environment and the effectiveness of wastewater treatment. Thus, it allows to identify under designed WWTPs and identify difference in AMR mitigation efficiency amongst the WWTPs.

Policy and regulation support, data comparability and standardization. Data from a harmonized monitoring scheme can inform policymakers and support the development of regulations and directives aimed at controlling AMR. Revisions to EU directives, such as the Urban Wastewater Treatment Directive and that proposed for the Environmental Quality Standards and Groundwater Directives, include monitoring for AMR/ARGs. A standardized scheme ensures compliance and effective implementation. Standardized monitoring methods and protocols ensure that data collected across different countries are comparable, reliable, and consistent. A centralized data reporting system, like the WISE-6 template, facilitates the integration and analysis of data, providing a comprehensive overview of AMR in the surface water in Europe.

Resource optimization, support and knowledge sharing. Different countries have varying capabilities (including such as AMR laboratory analysis/infrastructure) and resources for AMR monitoring in the environment. In the EU, the monitoring of AMR in zoonotic and commensal bacteria from food-producing animals and meat thereof is performed yearly by the EU Member States and 3 EFTA countries in a harmonized way as laid down in the legislation (Directive 2003/99/EC[12], Commission Implementing Decision 2013/652/EU and its update (EU) 2020/1729). In line with EFSA, the National Reference Laboratories for Antimicrobial Resistance (NRLs), part of the Network from the European Reference

[11] One Health: One Health is an integrated, unifying approach that aims to sustainably balance and optimize the health of people, animals and ecosystems (WHO; https://www.who.int/health-topics/one-health#tab=tab_1)

[12] Directive 2003/99/EC of the European Parliament and of the Council of 17 November 2003 on the monitoring of zoonoses and zoonotic agents, amending Council Decision 90/424/EEC and repealing Council Directive 92/117/EEC. OJ L 325, 12.12.2003, p.31–40.

Laboratory (EURL-AR)[13], participate in this monitoring and have experience in the subject, so knowledge can be shared. AMR analytical infrastructure is available in each EU member State and EFTA countries; however, whether that is linked to environmental regulation can be a separate matter. For Eionet countries outside of the EU, AMR capabilities could be less developed. Secondly, even though EU countries have experience on AMR monitoring in the clinical sector, monitoring water for AMR is not developed.

Research & Innovation, global leadership and responsibility. Monitoring AMR in the environment provides valuable data for scientific research, helping to understand the mechanisms of resistance development and transmission, as well as support risk assessment. Harmonized monitoring drives science-based policy making. Insights gained from monitoring can drive innovation in wastewater treatment technologies and other interventions to reduce pollution with AMR in the environment. By establishing a comprehensive monitoring scheme, Europe can lead by example in the global fight against AMR, setting standards and best practices for other regions to follow. AMR is a global issue, and efforts to monitor and control it in Europe contribute to global health security and the reduction of AMR worldwide.

[13] https://www.food.dtu.dk/english/topics/antimicrobial-resistance/eurl-ar/participants

5. Methodologies developed and used by the Eionet WG

5.1 Eionet WG formation and collaboration

Invitation and recruitment of participants

Eionet National Focal Points (NFPs) were requested to nominate members for the working group. In many cases, this resulted in nomination of national researchers, particularly where the (water) authorities lacked expertise in the AMR area. The ETC-BE was tasked with creating a proposal for a task flow that outlines what should be done, why it should be done, and when it should be done, essentially outlining a proposed methodology. However, each country would have the autonomy to decide whether the proposed methodology was feasible for them.

Facilitation of information exchange and knowledge sharing

It was expected that discussions between Eionet WG members would help to:

- Identify and understand differences in AMR monitoring status between WG members.
- Comprehend the complexity of the AMR problem.
- Determine what is needed and desired in terms of AMR monitoring.
- Gain insights into the actions taken by WG members thus far and explore how knowledge can be shared among participants.
- Identify knowledge that is relevant for Eionet countries.
- Strike a balance between what is ideal and what is achievable.

The process involved:

- Identifying aspects that might require debate prior to the meetings.
- Using the insights gained from discussions to identify actions that are feasible within the available resources.

5.2 AMR pilot questionnaire methodology

Baseline questionnaire development and distribution

In summer 2023, a questionnaire was conducted among the participating Eionet WG members from 11 countries to determine the status of AMR monitoring in surface waters. This included identifying which countries have already started to do AMR monitoring, and if so to what extent, aiming to better understand current capabilities of the WG members in the field of AMR and environmental monitoring. The information provided in the questionnaire included the following countries: Austria, Belgium, Czechia, Estonia, France, Germany, Ireland, Latvia, Luxembourg, the Netherlands, Spain, Switzerland.

5.3 AMR pilot study design

The AMR pilot study was designed based on agreement between the Eionet AMR WG participants. A detailed protocol was developed and drafted by ETC-BE which then was reviewed by the WG. **The protocol for the pilot** is found in Appendix A2. The protocol set out what was to be measured, when it should be measured, where and how it should be reported. Key information is presented below: however, for details, please consult the protocol.

a) Defining monitoring objectives and scope for the pilot study

The objective for the WG, given by EEA and formulated in the Terms of Reference (Appendix 1 Terms of Reference), was to conduct a pilot study enabling a harmonized approach for data collection and reporting. General objectives of surveillance of AMR in the environment consist of the detection of trends in the prevalence of AMR in specific environmental compartments, in the detection of new forms of AMR as they can emerge, as well as in the production of data contributing to human or animal risk assessments (i.e.,

through generating data on the exposure of humans or animals to AMR through contact with the environment), or in the evaluation of the efficiency of interventions.

b) Selection of water types and sampling locations

The pilot study focused on surface waters, with the Eionet WG on AMR identifying specific environmental settings for sampling:

- **Rivers** downstream of WWTPs: This environment was prioritized as a core sampling location because it is likely to reflect the release of ARB and ARGs from WWTPs into receiving water bodies.
- **WWTP effluents:** Collecting samples from treated wastewater directly is important to understand the abundance and diversity of AMR determinants released from WWTPs.
- **Bathing waters:** While not explicitly included as a core sampling environment, several WG members emphasized the importance of bathing waters due to the potential risk of AMR transmission to bathers. **Seawater**, restricted to transitional waters and coastal waters.

Sampling locations were prioritized. All WG members were asked to collect samples from the receiving water body downstream of the WWTP outlet and - if possible - WWTP effluents (outlet). Additional options (lower priority) were to sample bathing water, receiving water bodies upstream of the WWTP outlet, WWTP inlet, low impacted sites in rivers or seawater. **Groundwater** was also discussed as a potential area of interest but was deemed of low priority here.

The locations where water samples should have been collected in the survey are listed in Table 2. A short explanation for including it into the program is given.

Table 2. Sampling locations discussed and selected in this pilot.

Water type	Sampling collection location	Explanation	Priority in this pilot study
Rivers (freshwater)	River downstream of WWTPs	The location in rivers downstream of WWTPs outlets, where WWTP effluents mix with the river water, was considered crucial for assessing the impact of treated wastewater effluent on the receiving water environment; hence reflecting the human impact on the environment.	high
	Upstream of the WWTPs	Providing baseline data on ARG levels before point source input from the WWTP. However, it was not selected as a core sampling location for this pilot study.	optional
	Control sample	When including a low impacted site in the river ('river control'), this represents areas with minimal human impact, providing background levels of ARGs for comparison with impacted sites.	optional
Rivers, lakes and coastal waters	Bathing water	Bathing water is highly relevant due to risks of exposure on human health associated with recreational activities. This location can reflect the presence and spread of AMR from various sources, including WWTPs, agricultural runoff, and other anthropogenic influences. Samples to be collected according to the Bathing Water Directive.	optional

Water type	Sampling collection location	Explanation	Priority in this pilot study
WWTP effluents	WWTP outlet (treated wastewater)	Sampling directly from the WWTP outlet provides insights into the levels of ARGs and ARB being released from the treatment process allowing to understanding the contribution of WWTPs to AMR dissemination in the environment.	high
	WWTP inlet (untreated wastewater)	When combined with information from the level of ARB/ARG in the inlet of WWTPs, this allows for evaluating the effectiveness of WWTPs in removing AMR.	optional
Seawater transitional waters and coastal waters	Downstream	Can inform on the presence of AMR pollution from rivers, carrying pollution downstream of anthropogenic influence such as WWTPs or cities, to the sea and oceans.	optional

c) Sample types, collection methods and sample processing

The pilot study called for the collection of water samples from the designated sampling sites. The working group agreed on the following points regarding sample collection and processing:

- **Sample volume:** The WG agreed to collect a 10-500 mL of water at each sampling point to allow for sub-sampling for both qPCR and culturing analyses. This was water quality dependent and is detailed in the 'Summary of AMR pilot study' document.
- **River/lake sampling:** For river and lake samples, the WG recommended collecting a minimum of one sample per sampling spot and occasion, with sampling occasions ideally spread across several months (e.g., May, June, July). Collecting time-integrated samples in rivers was suggested to account for potential fluctuations in AMR levels throughout the day.
- **WWTP effluent sampling:** For WWTP effluent at the plant outlet, composite samples, collected routinely by the WWTPs as part of their monitoring obligations, are preferred. This involves taking multiple sub-samples over a specified period and combining them into a single representative sample, providing a more accurate reflection of AMR levels in the effluent over time.
- **Filtration:** After collection, water samples were filtered through 0.22 µm pore size polycarbonate (PC) filters. If PC filters were unavailable, alternative filter types like PES, GSWG, or PVDF could be used.

d) Establishing sampling frequency

The frequency and timing of the sampling collection was up the individual WG members. Decisive was the timeframe given by the duration of the Eionet task and the final report both due in March 2025. For the pilot, WG members were asked to collect samples from each location at least 2-3 times between April – August 2024. Regarding the number of samples per sampling location and sampling date, WG members were asked to provide at least one sample per site and sampling date. It was recommended to avoid sampling during/shortly after heavy rain, if possible, to improve consistency of results.

e) Selection of analytical methods

The AMR pilot study utilized a dual approach to analyse water samples for AMR, employing quantitative PCR (qPCR) and culture-based methods.

i. Quantitative PCR (qPCR)

The qPCR, a DNA-based method enabling the quantification of specific genes, including those associated with AMR, was selected for the pilot study. Key considerations for qPCR analysis included cost effectiveness, availability and ease of implementation.

ii. Culture-based methods

The Tricycle protocol was selected as the preferred method for culturing bacteria and assessing resistance to antibiotics (WHO 2021). This protocol involves culturing bacteria on selective media containing specific antibiotics, allowing for the isolation and enumeration of resistant bacteria.

Reasons for choosing these methods:

The decision to adopt a two-pronged approach using qPCR and culturing reflects a compromise between practicality and comprehensiveness. Culturing, though less precise than qPCR, offers a cost-effective way to assess the abundance of resistant bacteria and to study bacterial population structure in the environment (through phylogenetics). It also enables comparisons to data from human and animal sectors, supporting the One Health perspective. The qPCR, on the other hand, provides specific genetic information about the presence and abundance of AMR genes. This dual approach enabled the pilot study to achieve a balanced and informative assessment of AMR.

5.4 Development of harmonized protocols

The main information related to the protocol for the AMR pilot study is stated below. For the details, please consult the dedicated protocol/guideline developed by ETC-BE to which WG members could give their expert input (see Appendix A2).

5.4.1 DNA extraction

Recognizing the potential variability introduced by using different DNA extraction kits, the WG decided to use commercially available kits, particularly the Power Water kit, whenever possible.

5.4.2 qPCR analysis

Primers and probes. The selection of appropriate primers and probes for the targeted AMR genes was crucial for accurate qPCR analysis. The Eionet WG agreed on using published and validated primer and probe sets, with some WG members already using these sets in their R&D AMR monitoring programs.

Standards in the form of gBlocks. Commercially available synthetic DNA fragments called gBlocks were used as standards for qPCR calibration to ensure consistency across laboratories. A common template sequence composition for the gBlocks was designed and used by all members.

Inhibition reduction. Recognizing that inhibitors present in environmental water samples can interfere with qPCR, the protocol included measures for inhibition mitigation, including dilution, typically 10 or 100 times, and the use of inhibitor-tolerant DNA extraction kits and polymerases.

5.4.3 Culturing methods

The pilot study employed *Escherichia coli* (*E. coli*) and ESBL-producing *E. coli* (*ESBL-Ec*) as bacterial indicators, using culture-based methods alongside. This allowed for assessment of both the genetic potential for resistance and the presence of resistant bacteria in the water samples.

Standardized Protocol. The Tricycle protocol was chosen for culturing and quantifying *E. coli* and ESBL-Ec. The protocol involves filtering water samples, culturing the bacteria on selective agar plates containing the

antibiotic cefotaxime (CTX), checking the presence of ESBL by synergy test, and counting the resulting colonies that produce ESBL.

Reference Strain. The Netherlands offered to provide a reference ESBL-Ec strain to other WG members for quality control purposes, ensuring consistency in culturing and identifying resistant bacteria across laboratories. Several WG members used the offered reference strain.

Cefotaxime concentration. A cefotaxime (CTX) concentration of 4 µg/mL in the ESBL-Ec selective medium was chosen to maintain consistency with the Tricycle protocol's recommendations and ensure comparability with global monitoring data.

5.4.4 Selection of gene targets, variants, primers and culturing targets

a) Criteria for qPCR gene target selection

The Eionet WG selected six gene targets for the pilot study, balancing the selection of commonly occurring, easily detectable genes with less abundant, but clinically relevant targets, to reflect human exposure and efficiency of AMR interventions, such as WWTP sanitation. Besides the relevance, the aspects such as targets abundance and stability were also considered. The choice of the primers and the qPCR conditions were discussed during meetings. Details on the primers, qPCR conditions and references are given in the 'Summary of AMR pilot study approach'. Also, the fact that one indicator may have several variants, which may lead to different results, was discussed amongst the WG. The final selection for the WG pilot study was a compromise between scientific ideal and practical limitations and should not be seen as a recommendation for others.

The chosen targets are listed in Table 3. While all WG members were asked to analyse for 16S rDNA, *intI1*, *addA1*, *ermB*, $bla_{CTX\text{-}M1}$ and *vanA* in their samples collected in the framework of the AMR pilot, the detection of *tetW*, *sul1* and bla_{KPC} were additional options for WG members.

Reasons for choosing these individual gene targets include:

- Relevance to the AMR pilot study objective i.e., the AMR dissemination, the risk of transmission.
- Diversity by targeting genes from different gene families rather than different variants of the same gene.
- Targets with protocols that produce as little amplicon as possible were selected to reduce the total length of the gBlocks, that minimized the costs and while favouring sensitivity of the assay(s).
- Targeted ARGs based on the recommendations from the scientific literature.
- Available SYBR green qPCR protocol

Table 3: Gene targets selected in this pilot. Core targets in bold. Criterions partially adapted from Manaia (2023).

Gene	Description	Criterion
16S rRNA	**A general bacterial marker, providing context for the abundance of other targeted genes**. The 16S rDNA gene is highly conserved among bacteria, making it an excellent marker for identifying and classifying bacterial species. By sequencing this gene, researchers can determine the phylogenetic relationships between different bacterial strains. Measuring 16S rDNA allows to quantify the abundance and diversity of bacterial communities in environmental samples. This helps in understanding the overall bacterial population dynamics and how they might influence the spread of ARGs. By analysing 16S rDNA sequences alongside ARGs, the co-occurrence and	Assessment of - Bacterial abundance - Bacterial removal/increase - ARG relative abundance

Gene	Description	Criterion
	potential horizontal gene transfer of resistance genes within bacterial communities can be investigated. This helps in identifying specific bacterial hosts that carry and spread ARGs.	
intl1	- **Codes for class 1 integrase**, allowing acquisition and reshuffling of ARG cassettes in type 1 integrons. Integrons are genetic elements that can capture and express genes, including ARGs. - Particularly important as intl1 facilitates the horizontal gene transfer of ARGs among bacteria. - Occurs in the chromosome and plasmids - intl1 is related with mobile genetic elements (MGE) that may harbor ARG. - Commonly found in various environments, including wastewater, soil, and clinical settings, making it a reliable marker for monitoring AMR (Gatica, et al. 2016).	- Among the most used AMR indicators - Abundant in wastewater - Related with ARGs - Occurs in multiple wastewater bacterial species
aadA1	- addA1 encodes an enzyme that **inactivates aminoglycoside antibiotics**, such as streptomycin and spectinomycin, by adding a nucleotide group to them. - frequently found on plasmids, transposons, and integrons in a wide range of bacterial species, contributing to its widespread presence in the environment.	- Aminoglycoside resistance genes are among the most abundant in wastewater - Associated with intl1 (variable region) - Occurs in multiple wastewater bacterial species
ermB	- **Confers resistance to macrolides**, lincosamides and streptogramins antibiotics by methylating the 23S rRNA, which prevents antibiotic binding. - Commonly found in both clinical and environmental bacterial isolates, making it a key target for monitoring AMR.	- Macrolide resistance genes are among the most abundant in wastewater - Occur in multiple wastewater bacterial species
$\boldsymbol{bla}_{\text{CTX-M1}}$	- Codes for an extended-spectrum beta-lactamase that hydrolyses **third-generation cephalosporins**. - It was chosen in the list of targets due to its relevance to public health. - Occurs in the chromosome and plasmids	- Occurs in multiple wastewater bacterial species - Include extended-spectrum beta-lactamase producers, which are of high clinical relevance (relevant for risk assessments)
vanA	- **Confers resistance to vancomycin**, a last-resort antibiotic used to treat serious infections caused by Gram-positive bacteria. - Typically located on plasmids and on transposons that integrate into the chromosome - Often associated with mobile genetic elements, including integrons, which facilitate horizontal gene transfer.	- Commonly found in wastewater, particularly in hospital effluents - Found in various bacterial species in wastewater, predominantly in *Enterococcus faecium* and *Enterococcus faecalis*

Gene	Description	Criterion
		- detected in surface waters, especially in areas impacted by wastewater discharge
tetW	- Encodes a ribosomal protection protein that **confers resistance to tetracycline** by preventing the antibiotic from inhibiting protein synthesis in bacteria. - Can be located on both plasmids and chromosomes - Often associated with integrons like intI1.	- Prevalent in wastewater environments. - Found in surface waters, particularly in areas receiving wastewater effluents. - Occurs in multiple wastewater bacterial species, including both Gram-positive and Gram-negative bacteria
sul1	- **Confers resistance to sulfonamindes**. - Encodes a sulfonamide-resistant variant of dihydropteroate synthase, which allows bacteria to bypass the inhibitory effects of sulfonamide antibiotics. - Occurs in the chromosome and plasmid - Can be associated with intI1.	- Among the most examined ARGs in wastewater - Abundant in wastewater - Occurs in multiple wastewater bacterial species
bla_{KPC}	- Encodes a **carbapenemase enzyme**, which hydrolyses carbapenems and other beta-lactam antibiotics. - Typically located on plasmids, but can also be integrate into the chromosome - Often associated with intI1	- Frequently detected in wastewater, especially in hospital settings - Occurs in surface waters, particularly in areas impacted by wastewater discharge - Found in multiple bacterial species in wastewater, including various Enterobacteriaceae.

The selection of targets was an area for discussion and the selection used here, driven by the ability of participants to monitor and analyse at short notice, should not be seen as a recommendation for other surveys. It is to be highlighted that for future routine monitoring stakeholders may also have an opinion about the significance of the selected gene targets for AMR surveillance.

Particularly the relevance and inclusion of bla_{KPC} into the monitoring was debated. The bla_{KPC} gene encodes for *Klebsiella pneumoniae* carbapenemase (KPC), an enzyme that confers resistance to carbapenem antibiotics. This gene is a significant factor in the increasing incidence of carbapenem-resistant *K. pneumoniae* bloodstream infections and the spread of high-risk lineages of carbapenem-resistant *K. pneumoniae* in hospitals. The presence of bla_{KPC} genes contributes to the convergence of virulence and resistance in *K. pneumoniae*, the emergence of new *Enterobacterales* species carrying carbapenemase genes, and the plasmid-mediated spread of these genes causing outbreaks within hospitals and across healthcare networks. Based on the deteriorating epidemiological situation related to carbapenem resistance in Enterobacterales, the probability of further spread of CRE in the EU/EEA has lately been

stated to be high (ECDC, 2025; EFSA BIOHAZ, 2025). This may explain the interest of some WG members including blaKPC amongst the indicator in the survey.

b) Criteria for culturing targets selection

Culture-based methods with using ***E. coli*** and **Extended Spectrum Beta-Lactamase producing *E. coli* (ESBL-Ec)** was applied in the pilot study. This decision was driven by several factors: *E. coli* is commonly shared between humans and animals and is a widely recognized indicator of faecal contamination in water, making it a relevant target for assessing the potential presence of other pathogens, including those carrying antibiotic resistance genes ESBL-Ec are of significant clinical importance due to their resistance to a wide range of antibiotics, including those commonly used to treat human infections. Monitoring ESBL-Ec in surface waters helps assess the potential for transmission of clinically relevant antibiotic-resistant bacteria and resistance genes to humans. These two target bacteria are also included in the AMR EU monitoring of food-producing animals and meat thereof (EU 2020/1729).

Besides these bacterial targets, the possibility of including *Enterococcus* and/or *Klebsiella*, which are relevant indicators of faecal contamination and can carry antibiotic resistance genes, was discussed but these targets were not retained. The decision to focus on *E. coli* and ESBL-Ec reflected the need to prioritize targets based on feasibility and relevance to the study objectives. The choice of *E. coli* and ESBL-Ec provides a balanced approach, considering both general faecal contamination and the presence of clinically relevant antibiotic resistance.

5.5 Data reporting methodology for AMR pilot study

5.5.1 Development of a data reporting template

Adapting the WISE-6 dataflow. WISE-SoE (Water Information System for Europe - State of the Environment) refers to the dataflows used by the EEA to collect and report environmental data related to water. WISE-6 is a specific dataflow within this system that focuses on water quality reporting for inland, coastal, and marine waters. Prior to the pilot reporting, the ETC-BE adopted Excel spreadsheets for data reporting instead of EEA's standard, online WISE-6 reporting. This approach allowed flexibility, accommodating WG members with varying levels of experience in AMR data reporting, and developing experience for EEA and ETC-BE in managing a new data type. However, a long-term goal would be to integrate AMR data into EEA data reporting, such as the WISE-6 dataflow, aligning with the broader goal of streamlining environmental data reporting and establishing a standardized and sustainable system within the EEA's existing infrastructure.

Defining data elements and metadata. The ETC-BE aimed to provide clear guidance to participating WG members on the required data, specifying parameters, units, and metadata requirements. While documenting unusual conditions during sampling, such as heavy rainfall or increased flow rates at WWTPs, was recommended, the WG did not detail specific types of metadata to be collected. There is a balance to be struck in reporting, between detail and what can be expected at European level.

The Excel data reporting template to be used by reporters, utilized existing elements of the WISE-6, such as reporting elements and code lists, to the greatest extent possible, and further adjusted to AMR reporting needs where necessary. The WISE-SoE Water Quality (WISE-6) for disaggregated data was used as the starting point for template design, since considered as most suitable for AMR reporting. It was decided that the template should contain two sheets – one for sampling site information, and one for analysis data. Elements were selected or excluded based on relevance and adaptability to AMR reporting. Allowed cell entries and methodology for obtaining data were also retrieved from WISE-6, and when necessary new cell entries were made. For example, new allowed cell entries (extended code list) had to be made for unit of measure element and determinant code element to allow for selected genes and bacteria and allow for adequate units of measure.

Ensuring clarity and simplicity. Based on WG members varied experience with EEA's reporting infrastructure and use of adjusted reporting template from WISE-6, a need was identified to design an AMR data reporting guidance. The offline excel guidance file was designed based on the structure of WISE-6 websites for reporting guidance, containing the three relevant levels of reporting – level 2 datasets, level 3 elements and level 4 vocabulary. Microsoft TEAMS was used to communicate reporting template, reporting guidance file, submitted AMR reporting files from members and general reporting help. Each country had a separate folder to work in and submit their AMR reports. After members had submitted their AMR report, the ETC-BE would quality control (QC) the file. QC was performed using Microsoft Access (Version 2501), where coherence between reporting guidance and the report were ensured. If necessary, the file was sent back with feedback to the reporter for alterations. The QC process ensured identical determinant (genes and bacteria names), coherence between the sampling site information and analysis records, correct units, reported limit of quantifications (LOQ) and that unique records were reported.

5.5.2 Data analysis, presentation and storage

Adapting visualization based on data quality. The participants considered various visualization designs, including heatmaps and boxplots, while recognizing the need to tailor the presentation based on the data's nature and the intended message.

Some challenges related to data handling were identified related to data comparability, where ensuring comparability of data across different laboratories using various qPCR machines was identified as a key consideration. This concern underscores the importance of robust quality control measures, standardized protocols, and potentially the use of a common quality assurance sample, a standard, with known gene concentrations. Data analyses and visualization were performed in R studio[14]with all AMR data reports compiled using Access. Data of absolute abundance of ARGs contained censored data (records reported as under limit of quantification (LOQ)), where each record had one LOQ value and the total data report had several LOQ values. It was therefore considered the best approach to utilize the "NADA2" package (Helsel, 2024), that specializes in handling censored data. This method also visually demonstrates the proportion of data below LOQ and enables comparison between group differences. Censored data were given estimated values below the respective LOQ value, where the whole dataset was used as basis for calculating the estimates. If a censored records had a value below LOQ reported, the original value was retained. Data of relative abundance to 16S rRNA and cultivation data did not contain censored data. Records of relative abundance to the 16S rRNA gene, where the basis of gene target copy number/dL was below LOQ, were excluded. The basis of calculation has two different calibration curves (gene target and 16S rRNA gene) which define separate LOQ values that define above or below LOQ. Whereas to define a combined LOQ value is not possible and would be necessary to have if values below LOQ for relative abundance were to be included in the dataset.

The reported data was categorized in four sampling locations 'WWTP inlet', 'WWTP outlet', 'river upstream' 'WWTP discharge' point and 'river downstream' WWTP discharge point. To enable a descriptive overview of the robustness of the data, number of collected samples and proportion of samples below level of quantification for each determinant, country and sampling location was made (Table 4). Further on, to present a visual overview of the data, heatmaps presenting a relative abundance of the data for each determinant, country, and sampling location, were made for the relative abundance to 16S rRNA gene data, absolute abundance and cultivation data. A more detailed presentation of the data was presented in boxplots, providing additional statistics such as range, median, outliers and distribution of data. Due to boxplots nature compromising visualization when several variables are needed, WG members were grouped together and the total abundance amongst all WG members were presented for each determinant and sampling location. To also include country as a variable, bar plots were made as well, visualizing the mean concentrations with standard deviation (SD) for each determinant, country and sampling location (Appendix A4).

[14] R studio version (v. 2024.12-0+467)

Selection of reporting units. From the quantitative analysis by membrane filter methods, it was decided to use and report the following information and units:

For qPCR, results were reported as '*gene copy number relative to gene copy number of 16S rRNA gene*', i.e., prevalence. In addition, the concentration '*gene copy per 100 mL*' (i.e., abundance) of original water sample, considering gene copy number, reaction volume, DNA sample dilution, and filtered volume in the respective filtration was used.

For culturing, concentrations of *E. coli* and *ESBL-Ec* the unit was '*colony forming units (CFU) per dL' i.e. per 100 mL* of filtered sample. In addition, the proportions of *E. coli* producing *ESBL* should be calculated by dividing *ESBL-Ec* CFU by total *E. coli* CFU. Calculating this proportion helps to determine the prevalence of antibiotic resistance within the bacterial population. This ratio provides insight into the extent of resistance and helps in monitoring trends over time. Importantly, while relative abundance is indicative for selection processes, especially across WWTPs, the absolute abundance allows discussion of mass transport, exposure risk, and elimination. Both perspectives are essential for an evaluation of environmental AMR.

Using these units allows for standardized comparisons across different samples and studies. They are widely accepted in microbiology for water quality testing and are often stated in AMR surveillance-related literature and recommendations, both in human and animal sectors.

Data storage. Data reports were delivered in EEA ETC-BE teams for WG Antimicrobial resistance with each WG as ownership of their data report. Compiled datasets were also uploaded to the workspace of Eionet WG on AMR and are stored there for access for all members of WG AMR.

6. General experiences encountered during establishing and implementing the pilot study of AMR in surface waters

In this section, the input of the WG members to the general experiences encountered during establishing and implementing the pilot study regarding different topics is stated. The WG members were asked to give their opinions, which was collected and summarized. The conclusive summary of all input from the seven WG members is given below.

6.1 Timetable and funding constraints

Eionet is a network of experts in EEA member countries and Working Group activities are voluntary, with members of the WG being nominated by their National Focal Point. Financial resources were not available for supporting WG members in undertaking the pilot study, although EEA and ETC-BE supported the activities of the group in preparation of documents, meeting organization, etc.

- Several WG members highlight the lack of specific funding for the project, which led to reliance on internal grants or funding from other projects. This limitation affected the scope and scale of sampling, the number of samples and analysis activities. Some had to integrate their sampling campaigns with R&D projects and/or existing chemical monitoring schedules to reduce costs and simplify workloads.
- Over time, various sources of funding were secured, including support from national authorities, environmental agencies, and networks. These funds helped offset some upfront costs and facilitated broader and better-planned sampling and analysis campaigns.
- The process of securing funding and approvals caused delays in the initiation of the pilot study in several WG members. For example, the request for tender process and approval for sample access took several weeks, impacting the overall timeline.
- Collaboration with authorities responsible for chemical monitoring and other stakeholders was crucial in facilitating sampling and reducing costs. This integration helped streamline the workload and ensure the successful implementation of the pilot study.

Oving to these constraints the pilot scope was adapted to the practical limitations and resources available. The fact that many WG members contributed to the realization of the work and contributed with data, shows that this was a feasible and successful approach.

6.2 Variability in national capabilities and resources

The questionnaire performed at the start of the AMR pilot study showed the status of AMR monitoring in surface waters in Europe, as then understood by WG members (autumn 2023). A clear barrier to AMR monitoring was the lack of harmonization and legislative drivers to do so. When establishing and implementing a pilot study of AMR in surface water, a wide range in national capabilities and resources, as well as differences in AMR monitoring experience, were evident. The level of experience with qPCR varied significantly among WG members, as did their familiarity with culturing techniques. Some had to involve experienced colleagues to address resource limitations and constraints, while others had no difficulties due to prior experience with similar projects. In some cases, detailed cost estimations for lab work were provided, highlighting the expenses associated with reagents, labour, and specific sampling campaigns. However, in countries with a smaller base for AMR studies, the work was often carried out by a limited number of individuals who were responsible for both cultivation and PCR techniques. Additionally, budget constraints influenced how extensive the sampling scheme was for some participants, emphasizing the need to balance costs with the benefits of monitoring.

6.3 Methodological considerations and debates related to selection of sampling locations, choice of analytical methods, selection of target indicators and harmonization

Selection of sampling locations. Budget constraints significantly influenced the choice of sampling locations, often limiting samples to sites closest to workplaces or integrating sampling with existing chemical monitoring schedules to reduce costs. This approach ensured feasibility but restricted the

diversity of sampling sites. For example, one country managed to sample the two largest WWTPs in the country due to their proximity to workplaces. Another country highlighted the need for protocols to clearly indicate sampling methods for different water bodies, including freshwater and seawater/coastal water.

Choice of analytical methods. There was a preference for SYBR green methods due to simplicity and cost-effectiveness, although some participants favoured probe-based approaches such as TaqMan for their lower error rates. Cultivation methods posed challenges, such as overgrowth on TBX-CTX for ESBL isolation. One participant mentioned the need for specific incubation temperatures to manage accompanying flora, suggesting a 4-hour incubation at 36°C followed by incubation at 44°C. One country used MALDI-TOF to confirm species identity and the combination disk test to confirm ESBL production.

Selection of target indicators. Extensive discussions were held to identify relevant ARGs, with some participants questioning the relevance of certain clinical markers in environmental settings. Markers such as vanB, blaCTX, and blaKPC, which are crucial in clinical settings but very rare in the environment were discussed. Difficulties with quantification were noted for specific qPCR targets, such as 16S rDNA and vanA.

Harmonization of protocols. Standardization of protocols was crucial but challenging. Participants noted deviations from ideal standardized approaches and emphasized the need for detailed protocols and transparency. Issues with qPCR assay protocols, filtration volumes, and the impact of weather conditions on results underscored the importance of harmonizing specific protocol aspects and adapting them to different instruments and sample types. One country highlighted the need for standardized concentrations for setting up standard curves and standard operating procedure (SOP) for each specific instrument, as well as clarity on which mastermixes to use. One country reported that heavy rainfall preceding sampling dates had a notable impact on results, leading to increased ESBL-Ec counts and quantification of ARGs, highlighting the influence of weather conditions on environmental microbial populations.

Collaborative efforts: Collaboration with authorities and stakeholders was essential for facilitating sampling and reducing costs. This integration helped streamline the workload and ensure successful implementation despite methodological challenges. It was noted that a lot of "lab wisdom" needed to be spelled out in considerable detail and turned into protocol for things to work for everyone, emphasizing the need for attention to detail for full-scale implementation at the European level.

6.4 Quality assurance and control challenges

6.4.1 Establishing QA/QC procedures

Quality assurance (QA) and quality control (QC) measures were implemented for both culturing and qPCR analyses to ensure reliability and comparability. This proved to be a challenging part of the pilot for qPCR, as there was a need to ensure high quality data collection but with a low impact on the resources required during the pilot study implementation. For example, there are no established reference materials and there is a lack of common calibration standards while it is known that use of different qPCR instruments, reagents, DNA extraction kits, operating conditions of the qPCR reactions may have an impact on the detection and quantification.

6.4.2 Use of controls and interlaboratory comparisons

a) qPCR: Several measures were outlined to ensure reliability and comparability:

- **Standardized DNA extraction:** The decision to use commercially available DNA extraction kits, specifically the Power Water kit, was made to minimize variability.
- **gBlocks for calibration:** The use of commercially available gBlocks as standards in qPCR was agreed upon.
- gBlocks are double-stranded, sequence-verified DNA fragments that act as synthetic templates for qPCR. The use of a common gBlock aimed to improve comparability between WG members by providing an identical positive control for all six qPCR assays.
- The WG discussed different approaches to gBlock concentration determination, including using Nanodrop and Qubit instruments. While obtaining an identical starting concentration for the gBlock across labs was deemed important, it was considered less critical than using a common

gBlock, as quantities would also be normalized against 16S rDNA results. Details on the chosen gBlock are found in Appendix A2.

- **Dilution to reduce inhibition:** Dilution prior qPCR, usually ending up with 1/10 for quantification, was decided to be the preferred method to assess and reduce inhibition risk and was carried out by WG members. A dilution of 1/100 was used if 1/10 dilution was negative for 16S rDNA analysis. This is further explained in the guidance AMR pilot method Appendix A2.
- **Negative controls:** Using negative qPCR controls for sampling, DNA extraction, and the mastermix was done to identify potential contamination issues.
- **Efficiency of extraction:** To maintain some quality control and use high quality qPCR data, the threshold for reporting was set so that only records where reporting extraction efficiency was between >90% and <120% should be reported.

b) Culturing: Quality assurance for culturing methods was viewed important due to the potential variability in culturing practices. While standardized control samples could be created and shipped for quality control, this was not implemented during the pilot phase. It was commented by WG members that fresh preparation of TBX and TBX-CTX agar plates can sometimes be challenging.

6.4.3 Difficulties with implementing interlaboratory comparisons

Interlaboratory comparisons, or "ring tests" were considered as a means of assessing comparability between laboratories. However, although this was deemed a valuable approach, implementing them during the pilot was challenging due to logistical constraints e.g. limited duration of the project, budget limitations, and concerns about adding complexity. Use of standardized protocols, reagents, and quality control measures was identified as critical and sufficient to ensure the reliability and comparability of qPCR data in the AMR pilot study. If the WG would have had more time and resources, the interlaboratory calibration would have been highly prioritized.

6.4.4 What could/should have been done?

The lack of readily available certified reference materials (or similar) for AMR targets (for qPCR) represents a significant issue in relation to providing confidence in absolute concentrations reported by multiple reporters, and a harmonized reporting at European level. To some extent, this is mitigated by normalization to the 16S rRNA gene. In addition, there are no commercial quantitative reference materials for resistant bacteria on the market. Homemade reference materials are laborious to prepare. A certified reference material, similar to the one developed by the JRC for COVID-19, would increase the comparability of the qPCR quantifications.

7. Baseline questionnaire on AMR monitoring in the environment

Summary

In summary, the online questionnaire on AMR monitoring activities amongst Eionet WG members revealed that none of the responding members had established a national monitoring program for AMR in surface waters at the time of the survey (June 2023). This highlights the nascent stage of AMR monitoring in the European water environment. While some WG members indicated that research-based activities related to AMR in surface waters were underway, the lack of formal monitoring programs underscored the need for a harmonized approach across Europe.

Key findings and insights from the questionnaire were:

i. **Large range in expertise in monitoring of AMR in the environment.** Overall, there was limited knowledge about the status of AMR monitoring in surface waters amongst WG members.
ii. **No formal monitoring programs:** None of the WG members that responded to the questionnaire had a formal program in place for routinely monitoring AMR in surface waters. This finding highlight confirmed the need for capacity building and knowledge sharing within the Eionet working group. Specific findings were that routine monitoring is expected in three countries within five years while four WG members have no set timeframe, and the prime purpose of monitoring is to gain insights into AMR dissemination and transmission. Furthermore, that monitoring is currently primarily in the research stage, with findings published in scientific papers and reports.
iii. **Research-based activities:** Despite the absence of formal monitoring, most responding WG members indicated that research activities related to AMR in surface waters were being conducted. This suggests an existing, albeit fragmented, knowledge base upon which to build a standardized monitoring approach.
iv. Specific findings show that AMR monitoring is mostly done at WWTPs & hospitals and in some rivers. WG members use PCR- or culture-based methods to measure antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs).
v. **Need for standardization:** The questionnaire results highlighted the absence of standardized methods and targets for AMR monitoring in surface waters across Europe. This lack of harmonization poses a significant challenge for data comparability and necessitates the development of a common methodological framework.

The current barriers to implementation:

- **Lack of legislative drivers:** The absence of legislative drivers, such as EU legislation or the implementation of the UWWTD proposal, hinders the establishment of formal monitoring programs.
- **Lack of standards and harmonized targets:** The absence of standardized methods and harmonized targets makes data comparison and interpretation difficult.
- **Limited connection to other surveillance programs:** Except for Belgium, environmental AMR monitoring is not connected to other surveillance programs.
- **Data availability without standardized handling:** While data is available in the form of scientific papers, there is no standardized approach to data handling, storage, interpretation, and publication.

Limitations of the questionnaire findings:

Insights are based on 11 responses while the Eionet working group comprised 14 WG members and three institutions, not all of which responded to the initial questionnaire.

8. Results of pilot study of AMR in European surface waters

After collection of the samples and analysis, the data was reported by the countries, which then was quality controlled, compiled, and visualized by ETC-BE (the detailed procedure described in section 0). This section addresses the data's variability and limitations and presents the total dataset from all participating countries using overview heatmaps and box plots to compare ARG and bacterial target abundance across different countries and sampling locations. More country-specific data is found in Appendix A4.

8.1 qPCR analysis & culturing results

Variability and data representation

In total, 1.164 samples were collected at all sampling locations by all countries in the period of sampling collection between April and December 2024 (Table 4). Due to the **differing number of samples collected at various locations**, the amount of data accumulated varied between them: while most data were obtained from the 'river downstream' and 'WWTP outlet' locations. Although the same sampling location names are used in the graphs of this section, the locations actually differed in terms of distance from the WWTP discharge spots (with different conditions), water quality (freshwater and brackish water), and the recipient type (river, reservoir, canal, estuary). In addition, the number of samples taken at the different sampling locations varied between countries (Appendix A4), and samples were collected at different time points. Related to the targets, most samples were analysed for the six core gene targets (16S rRNA, intI1, aadA1, $bla_{CTX\text{-}M1}$, ermB, and vanA), and both bacterial targets. However, sul1 and tetW, which were optional to measure, were measured in fewer samples (Table 4), therefore the reliability of those genes is considered low due to the significantly fewer data points and should be taken into account when interpreting the results. Despite these heterogeneities, the data is presented cumulatively for all countries and simplified for easier visualization and comparison between member countries. Thus, drawing comparisons between disparate elements (i.e., sampling locations, countries and gene targets) is limited.

Table 4 also shows the **proportion of samples below the LOQ**. Regarding gene targets, for four of the six core gene targets (16S rRNA, int1, sul1, and aadA1), and both bacterial indicators a higher percentage of data is above LOQ relative to the total target abundance in the data set. In contrast, vanA, blaCTX-M, tetW and ermB have a higher percentage (11%-79%) of data below LOQ. This indicates a general higher concentration of the four core gene targets and bacterial indicators compared to the other gene targets. The higher proportion of data below LOQ for the rare genes $bla_{CTX\text{-}M1}$ and vanA poses challenges for estimating the proportion of data below LOQ and skews visualization, which should be considered when interpreting the results. Table 4 shows a varied number of samples among the four sampling locations, with ‘river downstream’ and ‘WWTP outlet’ containing most samples (as they were the core locations to be sampled). Regarding the proportion of data below LOQ, the sampling locations can be ranked as ‘river upstream’ > ‘river downstream’ > ‘WWTP outlet’ > ‘WWTP inlet’, with ‘river upstream presenting the highest number of samples below LOQ.

Table 4. Number of samples (n) collected by the WG members during the AMR pilot study and proportion (%) of samples below level of quantification (LOQ) for all gene targets and sampling locations.

Target	WWTP inlet		WWTP outlet		River upstream		River downstream	
	n(samples)	% below LOQ	n(samples)	% below LOQ	n(samples)	% below LOQ	n(samples)	% below LOQ
E.coli	18	11	31	3	17	0	35	3
ESBL-Ec	18	11	31	3	17	6	35	9
16S rRNA	25	4	44	5	31	0	51	0
IntI1	25	0	44	0	31	7	51	2
aadA1	25	4	44	0	31	29	51	6
blaCTX-M1	25	8	44	46	31	74	51	63
ermB	25	0	44	7	31	23	51	14
vanA	25	36	44	80	31	100	51	86
sul1	0		13	0	6	17	13	8
tetW	0		9	0	6	67	9	11
blaKPC	0		0		0		0	

Abundance of monitored gene targets

The **relative abundances of detected gene targets in proportion to the 16S rRNA gene** were quantified. Figure 2 illustrates the gene target data reported for each country and, and for four sampling locations. The relative abundance of gene targets at 'WWTP inlet' is generally higher compared to the other sampling locations. Samples from 'WWTP inlet' and 'WWTP outlet' show higher gene target levels compared to the sampling locations in the river, which is expected considering the dilution effect in the receiving waterbodies.

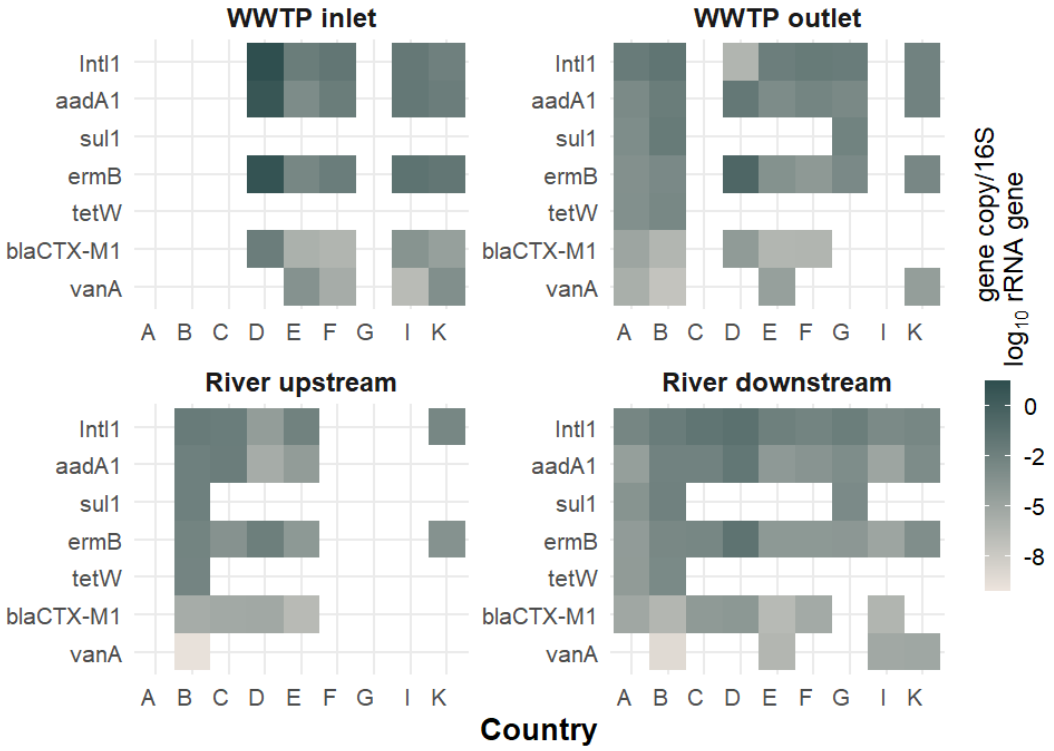

Figure 2. Abundance of gene targets relative to the 16S rRNA gene in samples collected at WWTP and river sampling locations in each country.

Notes to Figure 2: Numbers are expressed in log10 gene copy number/16S rRNA gene, with a colour scale where dark grey and beige indicate high and low abundance, respectively, relative to the gene abundance in the entire dataset received from all WG members. No colour indicates no records reported for the given gene target. Only data where the basis of calculation had an absolute gene target abundance (gene copies/dL) above LOQ are included.

Figure 3 shows the **absolute abundance of gene targets** for all WG members at the four sampling locations. Concentrations are expressed in $\log_{10}$ gene copy number/dL (i.e. 100 mL). The colour scale is indicative for the gene concentration, with dark grey indicating high abundance and beige indicating low abundance relative to the gene concentration in the entire dataset. No colour represents no available data for the given target gene, country and sampling location. Proportion of data below LOQ are estimated using Regression on Order Statistics (ROS) estimation. The trend in Figure 3 indicates that compared to relative abundance the pattern across sample locations and genes have less variations, implying that the AMR prevalence is also important to show relative to the general bacterial abundance. The heatmap indicates a higher abundance of ARGs and bacteria (detected as 16s rRNA) in WWTP inlet, and also for intl1, aadA1, sul1, ermB at all sample locations. In addition, the heatmap implies that 16S rRNA genes show higher concentrations than the other gene targets.

As compared to the relative expression of gene abundance per 16S rRNA gene shown in Figure 2, the expression of absolute number of genes per volume allows visualization of the 16S rRNA gene concentration and comparison with the other targets (also ARB, although this is not shown in Figure 3) and for the calculation of differences between sampling locations, i.e. removal rates across WWTPs and the impact of WWTPs on the receiving rivers.

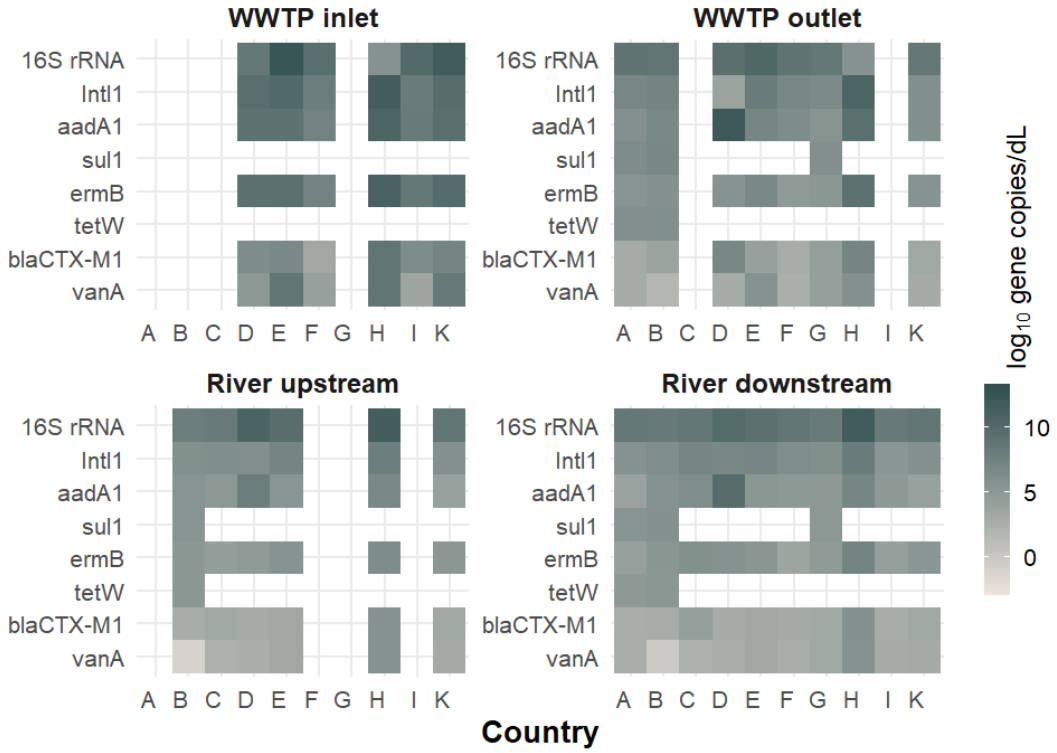

Figure 3. Concentrations of gene targets in samples collected at WWTP and river sampling locations in each country.

Notes to Figure 3: Gene target concentrations are expressed in $\log_{10}$ gene copy numbers/dL. The colours are indicative for the gene target concentration, where dark grey and beige indicates high and low abundance, respectively. No colour indicates no records reported for the given gene target. Note that sampling location 'river downstream' for country H is brackish water.

Figure 4 illustrates the median of the measured proportion for the data of the **relative abundances of detected gene targets in proportion to the 16S rRNA gene** from all countries and are grouped by sampling locations and presented as box plots. The abundance of gene targets could be distinguished in three discrete groups of 'high', 'intermediate' and 'low abundance' genes, where intl1, aadA1, sul1, ermB, tetW form the group with relatively high concentrations, $bla_{CTX\text{-}M1}$ with an intermediate concentration and vanA with the lowest concentration, relative to the other genes in the total data set.

Spatial abundance. When analysing the abundance of genes across sampling locations (i.e. WWTP and rivers sampling locations), no clear differentiation was possible due to similar abundances for most detected genes at the four locations. This is evident from the overlapping boxes in Figure 4. The only discernible trends are: (i) among the high-abundance genes, 'WWTP inlet' showed the highest concentrations for ermB, aadA1 and intl1; (ii) concentrations of intl1, sul1, and tetW were higher at the 'WWTP outlet' compared to both river sampling locations, while concentrations for addA1 and ermB at 'WWTP outlet' and were not clearly different to river sampling locations; (iii) no clear differences between the river sampling locations are seen independent of the gene target.

The expected pattern of gene abundance, i.e., 'WWTP inlet' > 'WWTP outlet' > 'river downstream' > 'river upstream', with 'WWTP inlet' having the highest concentrations, is not clearly visible when the entire data range is considered indicated by the boxes in Figure 4. This is due to grouping all countries as a variable resulting in a large range of data (A4.2, Figure 10), lacking data for specific sampling locations and gene targets, or a combination of these factors.

However, when only considering the **median concentrations** in Figure 4, the expected pattern of gene abundance at these locations can be observed for aadA1, ermB, and vanA. For the high-abundance genes sul1 and tetW, 'WWTP outlet' values were higher than both river locations, with higher median values in 'river downstream' than 'river upstream'. For all genes, except $bla_{CTX\text{-}M1}$, median concentrations at both WWTP sampling locations were higher than at both river locations.

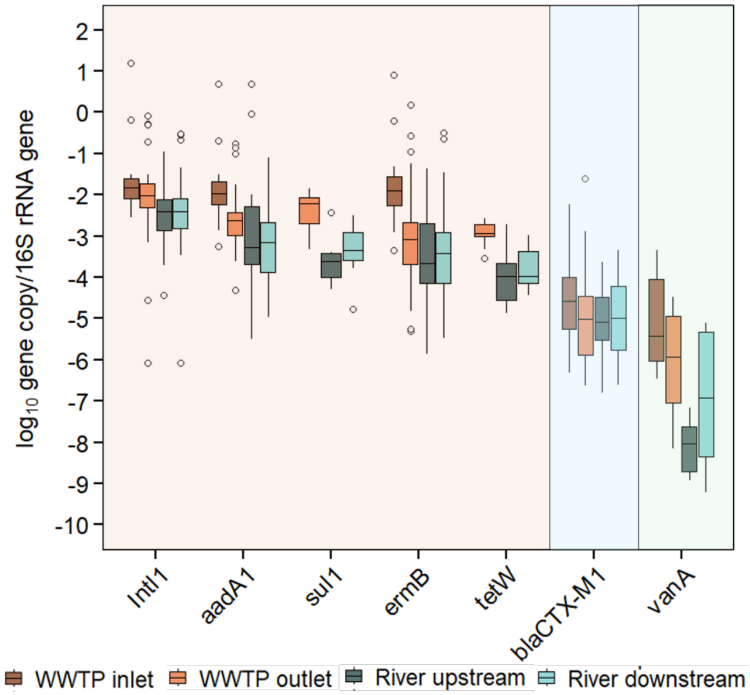

Figure 4. Abundance of gene targets relative to 16S rRNA genes in samples from all WG members collected at WWTP and river sampling locations.

Notes to Figure 4: The abundance is categorized in three main groups of gene abundance with high (orange), intermediate (blue) and low abundance (green), relative to the other genes in the total data set. Boxes indicate the median (horizontal line) and quartiles. Points represent outliers that are defined as data points being < 1.5 times the interquartile range from the box. Whiskers extend to the most extreme data points not being classified as outliers.

Figure 5 presents the **absolute abundance of gene targets** for each sampling location in samples from all countries. The concentration of total bacteria, measured as the 16S rRNA gene, ranged between 8-11 log_{10}-units/dL with highest concentration in the 'WWTP inlets', but also relatively high levels in the 'river upstream'. The trend of the total abundance was ranked into targets with high, intermediate, and low abundance adapted from Figure 4. However, as the concentrations of $bla_{CTX\text{-}M1}$ and vanA in the intermediate and low concentration groups overlapped, this trend was not as clear as when data was expressed as the relative gene abundance per 16S rRNA gene.

Spatial abundance. Similar to the statements made for the gene copy number relative to the 16S rRNA gene above, when analysing the **abundance of genes across the four sampling locations** based on the concentrations presented in Figure 5, no clear differentiation was possible due to similar concentrations for most of the detected genes at all locations. The only discernible trends are:

(i) amongst the detected high-abundance genes, 16S rRNA, intl1, addA1, and ermB showed highest concentrations in the ‚WWTP inlet';

(ii) (ii) when considering both WWTP sampling locations, none of the genes clearly showed higher concentrations than in river sampling locations;

(iii) (iii) clear differences between the river sampling locations is seen only for sul1 and tetW,, with 'river downstream' higher than 'river upstream'.

Thus, the expected abundance pattern ('WWTP inlet' > 'WWTP outlet' > 'river downstream' > 'river upstream') is also not clearly visible from the 'total target abundance' scheme. However, when considering only the **median concentrations** the expected abundance pattern could be observed for 16S rRNA, intl1, aadA1, and ermB. For the two high abundance genes sul1 and tetW, 'WWTP outlet' values were higher than both river locations, with higher median values in river downstream than upstream. For all genes, except $bla_{CTX\text{-}M1}$ and vanA, both WWTP sampling locations had higher median concentrations than in both river locations.

The **WWTP removal effectiveness** (based on median values) for the four core abundant genes intl 1, addA1, ermB, vanA ranged between 1.3 and 2.9 log_{10}-units (equal to 94.5% and 99.9% removal), which is somewhat lower than was reported for Dutch WWTPs (3.3-3.5 log-units) (Pallares-Vega et al. 2019) (Note: details on removal rates are presented in Appendix 4, Table 5). The difference between WWTP inlet and outlet concentrations for the three most abundant genes intl 1, addA1, ermB, when expressed as the total abundance, is more pronounced than when expressed as the gene target to 16S rRNA gene ratio. The concentrations for intl1, aadA1 and sul1 in wastewater detected in the pilot were comparable to those reported by Manaia (2023), where a comparative meta-analysis of several published datasets of WWTPs was done. A recent study at the Danube River also consistently detected intl1 and sul1 at high concentrations (with 4.2 – 6.7 log_{10} and 3.6 to 6.5 log_{10}, respectively) while the ESBL gene $blaC_{TX\text{-}M1}$ was only slightly above the threshold of detection and only sporadically detected (Schachner-Groehs et al., 2024).

Compared to the relative expression of gene abundance per 16S rRNA gene shown in Figure 4, the 'absolute number of genes per volume', in Figure 5 allows visualization of 16S rRNA gene concentration alongside other targets. This facilitates the calculation of differences in bacterial abundance by means of the 16S rRNA genes between sampling locations, such as removal rates across WWTPs and the impact of WWTPs on receiving water bodies.

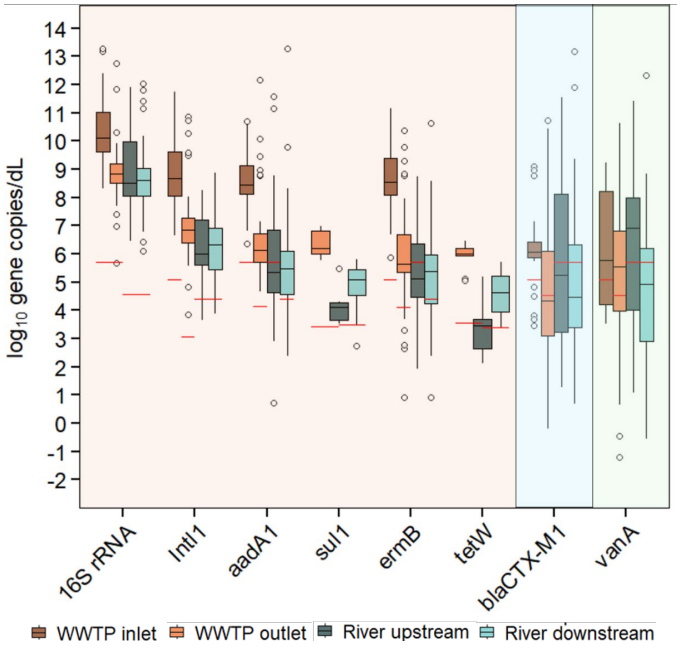

Figure 5. Concentrations of gene targets in samples from all WG members collected at WWTP and river sampling locations.

Notes to Figure 5: The abundance is categorized in three main groups of gene abundance, i.e. high (orange), intermediate (blue) and low (green) abundance relative to the other genes in the total data set, where the colour shading has been adapted from Figure 4. Boxes indicate the median and quartiles. Points represent outliers, the latter defined as data points >1.5 times the interquartile range from the box. Whiskers extend to the most extreme data points not classified as outliers. Red lines show maximum LOQ values, and proportions under LOQ are estimated using ROS estimation. Note that sampling location 'river downstream' for country H is brackish water.

Abundance of E. coli and ESBL-Ec

The concentrations of *E. coli* and *ESBL-Ec* were quantified (in colony forming units, CFU, per 100 mL), and the ratio (percentage) *ESBL-Ec* of *E. coli* for each sample was calculated. Figure 6 illustrates the overview of the abundance of bacterial targets and Figure 6 shows their ratio for each sampling location and country. As expected, *E. coli* concentrations are higher than those of *ESBL-Ec*. Also, both bacterial targets have highest abundance at WWTP inlets, minor in WWTP outlet and lowest abundance in river upstream and river downstream (challenging to distinguish in Figure 6).

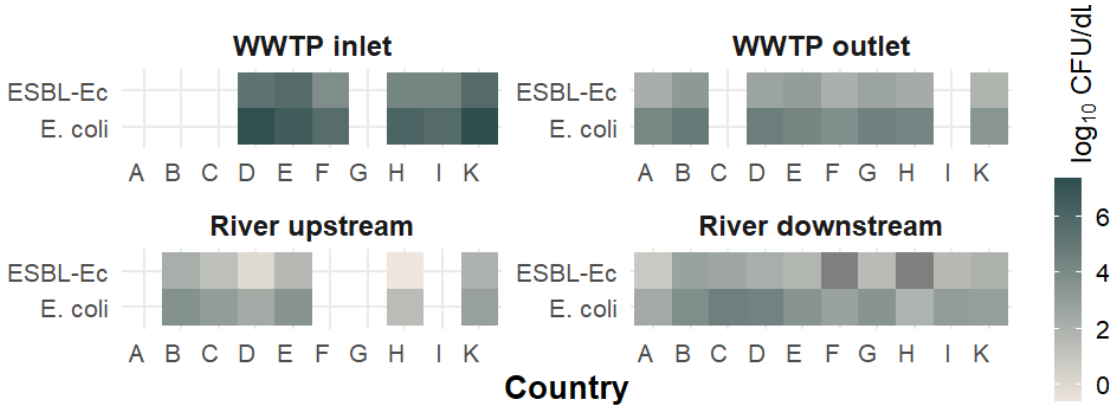

Figure 6. Concentration of E. coli and ESBL-Ec in samples collected at WWTP and river sampling locations in each country.

Notes to Figure 6: Concentrations are expressed in log10 CFU/dL, with a colour scale where dark grey indicates high abundance (6 log10 CFU/dL) and beige colour indicates low abundance (0 log10 CFU/dL). No colour indicates no records reported for the bacterial target. Note that sampling location 'river downstream' for country H is brackish water.

The ratio of ESBL-Ec and *E. coli* are in the lower range of the scale, but some values, such as country E for both of the WWTP locations and country K for both of the river locations, stand out as higher compared to other (Figure 7). In general, the pilot study data for ratio are considered as high abundance, and higher compared to other studies, for instance done by Jørgensen et al. (2017).

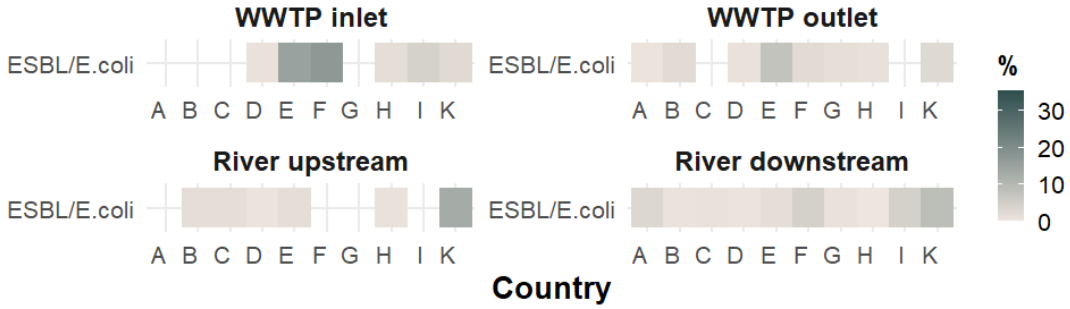

Figure 7. Ratio of ESBL-Ec to E. coli in samples collected at WWTP and river sampling locations in each country**.**

Notes to Figure 7: Ratios are expressed in percentage, with a colour scale where dark grey indicates high abundance (over 30%) and beige colour indicates low abundance (0%). No colour indicates no records reported for the bacterial target. Note that sampling location 'river downstream' for country H is brackish water.

Figure 8 shows *E. coli* concentrations per dL compared with *ESBL-Ec* per dL of samples from all WG members and collected at different sampling locations in form of a box plot. *E. coli* concentrations were 1.5 – 2 $\log_{10}$ units larger than those of *ESBL-Ec*. The general trend of abundance across sampling locations based on the median values has the following order: 'WWTP inlet' > 'WWTP outlet' > 'river downstream' and 'river upstream', with 'WWTP inlet' showing the highest abundance and river upstream and downstream the lowest abundance. The same pattern is observed for *E. coli* and *ESBL-Ec.* However, when considering the range of dataset visualized in the box plots, not such clear difference between the river sampling locations is seen owing to overlapping boxes.

The reduction efficiency during wastewater treatment processes based on median values were ca. 2.4 and 2.3 log10 units (equal to 99.6% and 99.5%), for E. coli and ESBL-Ec, respectively (Appendix 4, Table 5). There is a clear difference in concentrations between 'WWTP inlet' and 'WWTP outlet' (Δlog10 = 2.4 and 2.3 for E. coli and ESBL-Ec). In addition, there is a clear difference between WWTP sampling locations (Δlog10 ranging between 4-7 and 2.2-5 for E. coli and ESBL-Ec) compared to both river sampling locations (Δlog10 ranging between 3-3.5 and 1.5-2 for E. coli and ESBL-Ec). However, this difference in concentrations is not equally clear between 'river upstream' and 'river downstream' sampling locations owing to overlapping error bars. This despite that lower concentrations of both indicators were measured upstream than downstream in some WG members. In addition, the samples downstream of WWTPs in the WG members were collected in different distance from the WWTP discharge point, making comparability difficult. Thus, the current dataset limits the possibility for interpretations on the quantitative contribution of WWTP effluent input on the receiving rivers.

The proportion of *ESBL-Ec* among the total number of *E. coli* in water samples from all WG members collected at different sampling locations is shown in Figure 9. A large variation, with median concentrations ranging between 0.5% and 14.5%, is observed across the countries, depending on the sampling locations.

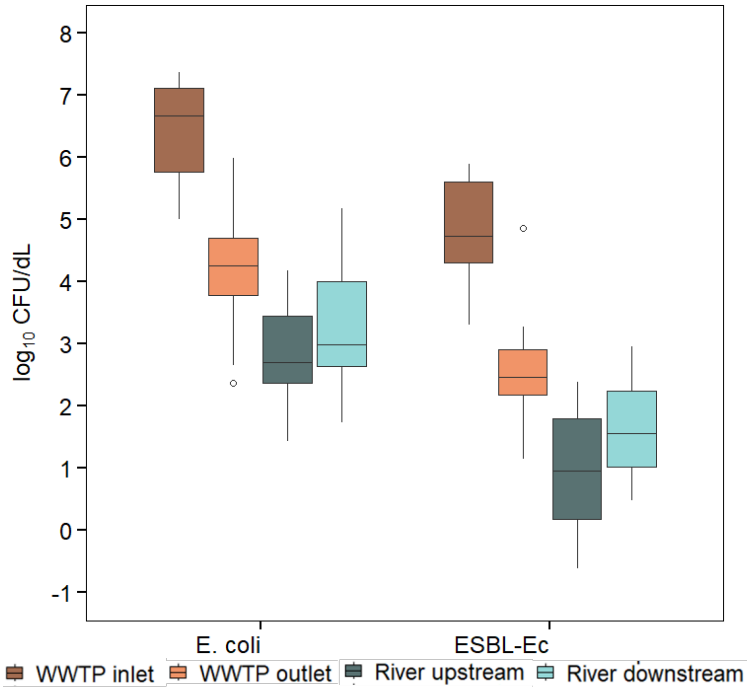

Figure 8. Concentrations of E. coli and ESBL-Ec in samples collected from all WG members at WWTP and river sampling locations.

Notes to Figure 8: E. coli = Escherichia. coli, ESBL-Ec = Extended-Spectrum Beta-Lactamase producing E. coli; CFU = colony forming units. Note that sampling location 'river downstream' for country H is brackish water.

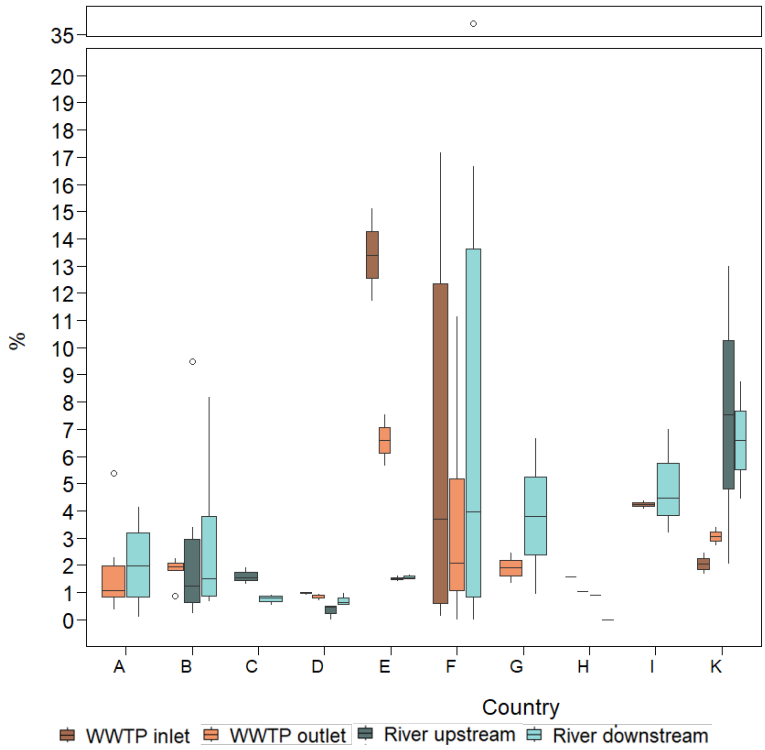

Figure 9. Proportion (in %) of ESBL-Ec to total E. coli in samples from all WG members collected at WWTP and river sampling locations.

Notes to Figure 9: The data point at 35% for country F is an outlier. E. coli = Escherichia. coli, ESBL-Ec = Extended-Spectrum Beta-Lactamase producing E. coli. Note that sampling location 'river downstream' for country H is samples is brackish water.

8.2 Summary of AMR pilot results and potential uses of data

8.2.1 Summary

Quality and robustness of the dataset. In total, 1.164 samples were collected from WWTP and water bodies between April and December 2024, with most data from 'river downstream' and 'WWTP outlet.' Locations differed in distance from WWTP discharge spots, water quality, and recipient type. Most samples were analysed for six core gene targets and bacterial targets, while sul1 and tetW were measured less frequently, making their reliability lower. Despite heterogeneity, data is presented cumulatively for easier visualization and comparison between the countries. Therefore, only limited comparison has been made between disparate elements such as sampling locations, countries and gene targets. Four core gene targets (16S rRNA, int1, sul1, and aadA1) and both bacterial indicators had higher percentages above LOQ, while vanA, blaCTX-M, tetW, and ermB had higher percentages below LOQ, indicating higher concentrations of core gene targets and bacterial indicators as expected. Sampling locations ranked by proportion of data below LOQ are 'river upstream' > 'river downstream' > 'WWTP outlet' > 'WWTP inlet.'

qPCR results. The pilot study quantified the relative and absolute abundance of selected gene targets across WWTP inlet, WWTP outlet, river upstream, and river downstream sampling locations. The relative abundance of the monitored ARGs has been broadly categorized into three groups: high abundance (including intl1, aadA1, sul1, ermB, tetW), intermediate abundance ($bla_{CTX\text{-}M1}$), and low abundance (vanA) across the entire dataset. The bla_{KPC} gene, despite being included into the monitoring plan by several countries was not reported by any of them. Despite the use of standards for qPCR calibration (i.e., gBlocks), the comparability of the specific ARG targets levels between the WG members could not be fully validated since no interlaboratory calibration was carried out.

Spatial differentiation. When integrating the data from all countries, the general trend with regard to spatial differentiation is that 'WWTP inlet' has the highest concentrations for 16S rRNA, intl1, addA1 and ermB among the high-abundance genes. Concentrations of intl1, sul1, and tetW were higher at the 'WWTP outlet' compared to both river sampling locations, while concentrations for addA1 and ermB at 'WWTP outlet' and were not clearly different to river sampling locations. There were no clear differences between the 'river downstream' and 'river upstream' locations for most genes. The pattern of gene abundance, i.e., 'WWTP inlet' > 'WWTP outlet' > 'river downstream' > 'river upstream', with 'WWTP inlet,' that would be expected is only visible when considering median concentrations but is not clearly obvious when considering the entire data range at a location. The latter is due to grouping of all countries as a variable resulting in a large range of data, lacking data for specific sampling locations and gene targets, or a combination of these factors. These trends might vary among countries, but interpreting the available data was beyond the scope of this pilot.

The metric of absolute gene target abundance allowed for a comparison with total bacterial abundance (16S rRNA gene) and facilitates the calculation of removal rates across WWTPs and the impact of WWTPs on receiving waterbodies. Estimated removal rates in WWTPs for the most abundant genes (intl1, aadA1, ermB, vanA) ranged between 1.3 and 2.9 log_{10}-units (equal to 94.5% and 99.9%), which was noted to be somewhat lower than findings from other studies. When expressed as total abundance, the difference between WWTP inlet and outlet concentrations for these genes was more pronounced.

Culturing results. The general trend of abundance of *E. coli* and ESBL-Ec across sampling locations based on the median values has the following order: 'WWTP inlet' > 'WWTP outlet' > 'river downstream' and 'river upstream', with 'WWTP inlet' showing the highest and river upstream and downstream the lowest abundance, respectively. However, when considering the entire range of dataset for one sampling point, not such clear difference between the river sampling locations is seen owing to overlapping ranges. This follows the pattern observed for the detected gene targets. The reduction efficiency during wastewater treatment processes based on the available numbers was estimated with ca. 2.4 and 2.3 log_{10} units (equal to 99.6% and 99.5%), for *E. coli* and *ESBL-Ec*, respectively. While a clear difference in concentrations was evident between WWTP sampling locations and river sampling points, the distinction between river upstream and river downstream was less clear based on the overall dataset. The proportion of ESBL-Ec among the total number of *E. coli* in water samples exhibited a large variation across countries, ranging between 0% and 14.5%, depending on the sampling location.

8.2.2 Potential use of data

The pilot study data provides a basis for understanding the abundance of the selected gene targets in surface waters but can also be utilized for other purposes. This kind of dataset enables assessment of the effectiveness of WWTPs, the input of WWTPs into surface waters, and the potential impact of WWTP size and treatment type on the presence of genes and bacteria in receiving waters. This includes the possibility of calculating removal effectiveness of WWTPs and comparison of ARG loads from WWTPs with different wastewater inputs. In addition, assessing environmental impact by comparing upstream and downstream concentrations of WWTP discharge in rivers may be possible. Since the main goal of the pilot was to enable AMR monitoring amongst European countries rather than to study the actual concentrations and trends, only limited interpretation was made of the results in section 8. Depending on which question should be answered, AMR in water data could in general be used in several areas, such as:

- **Assessing dissemination of AMR and risk for transmission**

Monitoring of AMR dissemination in the water environment enables tracking of changes in the spatial and temporal patterns of AMR in the environment. It also allows identification of background levels for the ARG targets. AMR data are also needed for the risk assessment of environmental exposure to, and transmission of, ARB to humans and/or animals via environmental routes.

- **Assessing wastewater treatment plant effectiveness**

Understanding the removal efficiency of bacteria and resistance genes helps in evaluating the performance of WWTPs and identifying areas for improvement. From the measured bacterial and gene

concentrations at the inlet and outlet of the treatment plant, the reduction in concentrations can be calculated to assess the removal effectiveness of the treatment process. This information is crucial for determining the current capacity of WWTPs to reduce ARG emissions and for identifying potential areas for improvement.

The current lack of routine monitoring and discharge regulations for AMR is noted as a reason for their uncontrolled release. Data on ARG loads can provide the evidence needed to support the development and implementation of discharge regulations, treatment targets, and best management practices aimed at reducing the environmental spread of AMR.

Comparing ARG loads from WWTPs receiving different types of wastewater, such as those with and without hospital input, can provide insights into the sources and pathways of ARGs entering the environment.

- **Assessing the environmental impact**

Assessing the impact of discharges from WWTPs or other AMR sources on receiving water bodies can inform and drive implementation of strategies to mitigate the spread of AMR in the environment. Comparison of concentrations upstream and downstream of the treatment plant discharge may be assessed to evaluate the impact of the WWTPs effluent on the river's (or recipient's) microbial and resistance gene load. Monitoring ARG loads in receiving rivers under different conditions, such as varying flow rates, allows for the evaluation of how hydrological characteristics influence the fate and transport of ARGs (Schwermer & Uhl, 2021). Example for how these assessments inclusive details on the calculations can be applied is stated for instance in Schwermer & Uhl, 2021 and Cacace et al. 2019.

- **Correlation analysis**

A statistical analysis can be done to identify correlations between bacterial concentrations and resistance gene concentrations, which can help understand the relationship between bacterial presence and resistance gene prevalence. Analysing trends over regions involves comparing data from different geographical areas to identify patterns and differences in wastewater treatment effectiveness and the prevalence of ARB. This can help in understanding local/regional differences and tailoring interventions accordingly.

- **Trends over time**

Initial assessments of ARG loads in WWTPs and receiving waters can serve as a baseline for future monitoring efforts. This allows for tracking changes in ARG prevalence over time and evaluation of the effectiveness of interventions to mitigate AMR. Analysing data over time to identify trends in bacterial and resistance gene concentrations can indicate seasonal variations or the impact of specific events (e.g., heavy rainfall). The benefits of longitudinal data analysis include:

- Temporal trends, where seasonal variations and long-term trends in bacterial and resistance gene concentrations can be identified
- Impact assessments, where the effectiveness of interventions and changes in wastewater treatment processes over time can be evaluated.
- Predictive modelling, where models to predict future trends and potential outbreaks based on historical data can be developed.
- Policy development, which aims to inform policy decisions and regulations based on observed trends and impacts.

- **Public Health protection, policy, and regulation**

The presence of ARGs in receiving waters can pose risks to human health through direct exposure to ARB and ARGs in water used for various purposes like irrigation, recreation, reuse, and potentially drinking water. Monitoring concentrations of AMR, in combination with other pathogens and compounds of emerging concern, may help in assessing the risk to public health and developing measures to prevent the spread of resistant pathogens. Surveillance monitoring of influent water at WWTPs reflects the community

and/or AMR sources. Therefore, it can also support the public health monitoring through a wastewater-based epidemiology concept such as done during the COVID-19 pandemic and even function as an early warning system. Data from these analyses can support the development of policies and regulations aimed at controlling antibiotic resistance in the environment.

- **Research and development**

Insights gained from such analyses can guide research on AMR dissemination, AMR sources, new treatment technologies and methods to effectively remove ARB and ARGs from wastewater.

9. End of activity survey – Feedback questionnaire

At the end of the project period, the WG participants, including member country experts and the ETC-BE team, responded to an online questionnaire collecting feedback on the lessons learned and experience from working of the group. It consisted of 11 questions. A summary of the feedback of all participants is given below.

Summary

All 16 responses said they would recommend the Eionet Working Group on AMR in surface waters activity to others. Most WG members (15/16) understood their role in the Working Group and were able to get their points across in meetings and discussions. The process for developing the sampling methodology and analytical methodologies for the AMR pilot study worked well. Most participants (11/13) found the process of reporting data to be easy or straightforward. Resourcing the work for the working group was challenging for some members. The process for drafting the report of the WG worked well for those who contributed. The WG progress and Eionet collaboration were considered worthwhile and positively influenced future AMR monitoring. All respondents thought the outcome of this Eionet WG activity would be communicated and addressed. Most (13/16) thought that there should be further activities on AMR in water with the Eionet.

The following comments and suggestions were made:

- Some participants noted the need for more time to devote to precise objectives and better formal guidance.
- There were suggestions to rethink the panel of targets and types of sites to be monitored.
- The complexity of data reporting was a challenge for some, but technical assistance was appreciated.
- The process of resourcing work was challenging, with some needing external support and permissions.

10. Priority areas for establishing a Europe-wide AMR monitoring scheme in the future

Based on the experiences and outcomes of the Eionet WG AMR pilot study in surface waters, the following suggestions are made to facilitate the establishment of a robust and effective Europe-wide AMR monitoring scheme:

1. Foster agreement on the objectives of environmental AMR monitoring

- Clearly define the main objective of the monitoring program, since this purpose drives the selection of other parameters. Examples of objectives include: the identification of key sources and transmission routes; assessment of human exposure risks; tracking of AMR trends; evaluation of the effectiveness of interventions.
- Align the selection of target indicators, sampling locations, and sampling frequency with the defined monitoring objectives to ensure the collected data is relevant and informative.

2. Strengthen harmonization of methods and protocols

- Continue and expand the harmonization of sampling methods, target indicators, analytical techniques (both culture-based and molecular), and data reporting procedures. Develop detailed and standardized protocols that are adaptable to different water types (freshwater, seawater, wastewater).
- The choice of analytical method(s) to be used for measuring the AMR pollution targets needs to be based on and targeted to the objective of the monitoring program. This should be flexible to support new developments in this field.
- If PCR is chosen as one of the available options, then prioritize the development and validation of standardized qPCR assays, including consensus on primer and probe sets, standard curve preparation and cycling conditions for different instrument types. Limitations on the harmonization that is realistically achievable (e.g. different instruments or access to materials) emphasize the need for robust QA/QC procedures).
- Establish standardized filtration volumes for different water matrices to ensure adequate recovery of microbial DNA and comparable results.
- Recognize the importance of balancing standardization with flexibility to accommodate the diverse environmental conditions and monitoring objectives across Europe.
- Future decisions around frequency of sampling need to carefully consider extreme weather events.
- Harmonize international protocols for culture (WHO Tricycle) with existing European methodology from other sectors (cefotaxime selective concentrations)

3. Develop and implement robust quality assurance/quality control (QA/QC) procedures

- Prioritize the establishment of comprehensive QA/QC procedures for all stages of the monitoring process, from sampling to data analysis and reporting.
- Establish mechanisms for regular interlaboratory comparisons (ring tests) using common reference materials to assess variability between laboratories and improve data comparability.
- Promote the development and use of (certified) reference materials (or similar) for key AMR targets to enhance confidence in absolute quantification and ensure usage of proper control samples.
- Develop clear guidelines for data validation, including, for instance, acceptance criteria for qPCR efficiency and the handling of data below the limit of quantification.

4. Establish a centralized and integrated data reporting system & solutions for data sharing

- Further develop and implement a centralized data reporting system, potentially utilizing and expanding the WISE-6 platform, to ensure efficient data collection, storage, and sharing.
- Define clear data elements, metadata requirements (including relevant environmental conditions like rainfall events), and reporting guidelines to ensure data quality, consistency, and interpretability.

- Consider the audience for the reported data (e.g., policymakers, researchers, the public) and tailor data presentation formats accordingly to maximize its utility.
- A reference list of harmonized and unambiguous nomenclature for targets/indicators is required (cf CAS codes or similar for chemicals).
- Ensure data availability and sharing amongst experts, stakeholders and the public.
- Consider the integration of AMR surface water surveillance into existing national and international surveillances programs.

5. Advance towards the establishment of environmental quality criteria for AMR

- Initiate a process for making progress towards defining environmental quality criteria (EQC) for AMR abundance in different water uses, acknowledging the current scientific complexities and controversies related to risk assessment. This may involve further research on exposure and transmission pathways through the environment (human and animal health), dose-response relationships, and the effectiveness of mitigation measures.

6. Enhance communication, knowledge sharing, and capacity building

- Promote dialogue among relevant stakeholders on the priorities and objectives of AMR surveillance in water.
- Foster effective communication and knowledge sharing among researchers, policymakers, and the public regarding environmental AMR. Utilize platforms like the Eionet WG for continued collaboration and exchange of best practices.
- Develop and implement capacity-building initiatives, including training programs and workshops, to enhance the expertise and capabilities of countries with less experience in environmental AMR monitoring.

11. Conclusions

There is an urgent need to better understand the abundance, extent and risk presented by AMR dissemination and transmission in the environment. The Eionet WG on AMR developed and carried out a pilot study to enable a harmonized approach for data collection and reporting of selected resistance indicators in surface waters at European scale. The initiative aimed to develop a methodology that could serve as foundation for a future European surveillance effort for AMR in water, constrained by what could be implemented on a voluntary basis by members of the WG within the given project time period (June 2023-March 2025).

The activity established an Eionet collaboration, bringing together experts from European Environment Agency (EEA) member countries, the EEA, the European Topic Centre Biodiversity and Ecosystems (ETC-BE), the Joint Research Centre (JRC), and the European Food Safety Authority (EFSA). This collaborative effort facilitated knowledge exchange, adapted to differences in national capabilities, and fostered a shared understanding of the complexities of environmental AMR monitoring. The willingness of country experts to collaborate, compromise and share knowledge was crucial for the project's progress.

The **AMR pilot study** developed a harmonized **methodology** encompassing monitoring objectives, sampling locations, target indicators, analytical methods, and data reporting procedures, which was successfully applied by the WG. The methodology was summarized in a short protocol for the AMR pilot that WG members endorsed and followed (Appendix A2). A dual **analysis approach** using qPCR and culturing was adopted as a practical compromise between cost-effectiveness and comprehensiveness. The qPCR gene targets selected for the purpose of the pilot, including six ARG core targets and three optional ARG additions, are not intended as a recommendation to others, as the list owed largely to the analytical resources available to WG members in the pilot study. Within these restrictions, selection aimed for diversity across gene families and considered abundance, health relevance, and detectability. The selection of the culturing targets E. coli and ESBL-Ec considered their relevance as faecal contamination indicators and their clinical significance. Culture methods were based on existing protocols used to analyse water (WHO Tricycle) and therefore differed from some used for EU AMR monitoring in food-producing animals and meat (Commission Implementing Decision (EU) 2020/1729)). The development of a **data reporting template** adapted from EEA's WISE-6[15] dataflow, and a dedicated guidance file facilitated data submission and quality control.

The WG encountered several **challenges during pilot study**. Timetable and funding constraints limited the scope and scale of sampling and analysis activities in many participating countries, often necessitating reliance on core budgets or integration with existing monitoring programs. The variability in national capabilities and resources, including experience with qPCR and culturing techniques, posed a challenge to complete harmonization. Methodological considerations and fruitful debates arose regarding the selection of sampling locations, analytical methods, and target indicators, often requiring compromises between scientific ideals and practical limitations. Full harmonization of protocols, especially for details on sample collection and the qPCR assays, demonstrated potential for further enhancement. Quality assurance and control challenges were also encountered and discussed, notably the difficulties in implementing an interlaboratory comparison (ring test) due to logistical and budgetary limitations. The lack of readily available certified reference materials for AMR targets and ARBs represents a significant issue for ensuring confidence in reported absolute concentrations. Lack of a harmonized nomenclature for targets/indicators (cf CAS codes for chemicals[16]) makes harmonized reporting for a wider range of AMR indicators challenging.

The **results of the AMR pilot study** provided an overview of the relative and absolute abundance of monitored gene targets and bacterial indicators across the participating countries and their sampling locations. The relative abundance of the monitored ARGs has been broadly categorized into three groups:

[15] https://cdr.eionet.europa.eu/help/WISE_SoE/wise6
[16] https://www.cas.org/cas-data/cas-registry

high abundance (including intl1, aadA1, sul1, ermB, tetW), intermediate abundance (blaCTX-M1), and low abundance (vanA) across the entire dataset.

Regarding the spatial differentiation of target abundance, the general trend is that both WWTP sampling locations exhibit higher gene concentrations for all genes, except $bla_{CTX\text{-}M1}$ and vanA, compared to the river locations. The pattern of gene abundance that would be expected, i.e., 'WWTP inlet' > 'WWTP outlet' > 'river downstream' > 'river upstream', with 'WWTP inlet having highest concentrations, is evident for 16S rRNA, intl1, aadA1, ermB and vanA, as well as for both bacterial indicators targets. These statements are however only true when considering the median concentrations for the targets but is not equally clear when considering the entire data range from all countries at a sampling location. The latter is due to grouping of the data from all countries and/or lack of data for specific sampling locations and gene targets. These trends might vary among countries as compared to the cumulative country data, but interpreting the available individual country data was beyond the scope of this pilot.

Reporting of data with respect to types of target genes and sampling locations varied amongst WG members, which should act as a caution when interpreting the results. Since the pilot's primary goal was to enable AMR monitoring across European countries rather than to study the actual target concentrations and trends between countries, only very limited interpretation was made here. However, further potential in the data sets is set to be explored by members of the WG following this Eionet action.

In conclusion, the Eionet AMR pilot study represents the first step towards establishing a harmonized Europe-wide monitoring scheme for AMR in surface waters. It successfully fostered collaboration, developed a foundational methodology, and provided valuable insights into the challenges and complexities of environmental AMR surveillance among the participating members and countries. The lessons learned during the Eionet WG task regarding funding, harmonization, quality control, and the variation in national capabilities are useful for informing future Europe-wide AMR monitoring schemes.

12. List of Abbreviations

Abbreviation	Name	Reference
AMC	antimicrobial consumption	
AMR	antimicrobial resistance	
ARB	antibiotic-resistant bacteria	
ARG	antibiotic resistance genes	
BWD	Bathing Water Directive	
CAS	Chemical Abstracts Service	
CTX	cefotaxime	
DNA	deoxyribonucleic acid	
ECHA	European Chemicals Agency	https://echa.europa.eu
Eionet	European Environment Information and Observation Network	https://www.eionet.europa.eu/
EEA	European Environment Agency	www.eea.europa.eu
EFSA	European Food Safety Authority	https://www.efsa.europa.eu/en
EFTA	European Free Trade Association	https://www.efta.int/
ESBL	Extended-spectrum beta-lactamase	
ETC-BE	European Topic Centre Biodiversity and Ecosystems	https://www.eionet.europa.eu/etcs/etc-be
EQC	Environmental Quality Criteria	
EQSD	Environmental Quality Standards Directive	
EU	European Union	https://european-union.europa.eu
GW	Groundwater	
JRC	Joint Research Centre	https://joint-research-centre.ec.europa.eu
KPC	Klebsiella pneumoniae carbapenemase	
LOQ	limit of quantification	
NFP	National Focal Point	
NIVA	Norwegian Institute for Water Research	https://www.niva.no
NRLs	National Reference Laboratories	
PCR	Polymerase chain reaction	
PES	Polyethersulfone	
PVDF	Polyvinylidene Fluoride	
qPCR	quantitative Polymerase Chain Reaction	
R&D	Research and development	
QA	Quality assurance	
QC	Quality control	
SOP	standard operating procedure	
rDNA	Recombinant DNA	
SD	Standard deviation	
SoE	State of Environment	
SW	Surface water	
UWWTD	Urban Wastewater Treatment Directive	
WFD	Water Framework Directive	
WG	Eionet Working Group	
WHO	World Health Organization	https://www.who.int
WISE	Water Information System for Europe	
WRR	Water Reuse Regulation	
WWTPs	wastewater treatment plants	

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Appendix 1 Terms of Reference

Eionet Working Group – Terms of Reference

Eionet Working Group	**Antimicrobial resistance in surface waters**
Rationale	European collaboration on the food and health aspects of antimicrobial resistance (AMR) is well-established. The third part of the One Health approach – environment – is behind this effort and the current activity aims to lay the foundation for future development. The aims of the Eionet Working Group are: • to conduct a pilot survey of AMR in surface waters during 2024, enabling the first harmonised data collection and reporting of the selected resistance indicators at European scale, • to build capacity and experience for more quantitative monitoring in future. Given the wide range in capability and knowledge between countries, the focus is expected to be on gaining experience in what can be measured and reported in relation to AMR in surface waters, as developed and agreed by the group itself. ETC-BE will support the work, and we will liaise with key national/EU experts as appropriate. Outputs of the work, expected in 2024-25, would be used for example: • to inform the European Commission's Water Framework Directive Common Implementation Strategy (Working Group Chemicals) and developments under the revised Urban Wastewater Treatment Directive in relation to monitoring of AMR. • to prepare WISE SoE dataflows for AMR reporting. • to inform a briefing or WISE FW page on AMR in European rivers and lakes, possibly also transitional and coastal waters. This group would cease after the pilot survey results have been reported and summarised, i.e. Q4/2024 – Q2/2025, to avoid potential overlaps with the Commission's proposed legislation on AMR in the water area.
Expertise and expectations	EEA and ETC-BE will work with countries and experts to agree on sampling, analytical and reporting methodologies, building on existing knowledge, capabilities and resources available in participating countries. The derived approach needs to be applied to a pilot survey in 2024. The focus of the pilot is likely to be on surface waters influenced by discharges of urban wastewater, to be confirmed at the first meeting. Three online meetings are expected in 2023 (June, September and October/ November). A pilot survey undertaken by country participants in 2024 would be supported with 3-4 meetings to detail planning, reporting and to review the results. One meeting could be physical, subject to resources being available. Participants would need to allocate resources time to support activities outside of meetings, related to carrying out the pilot survey, analysis and reporting, and in supporting ETC-BE AMR team in their preparation of a report on the pilot.

Policy process to be supported	The EU water legislation is going through a review procedure. AMR features as a possible risk under the Water Reuse Regulation 2020 (WRR). Meanwhile, recently published proposals from the European Commission envisage reporting of AMR under the revised directives. Large urban wastewater treatment plants would be required to monitor AMR under the proposed revision to the Urban Wastewater Treatment Directive. Monitoring of antimicrobial resistant genes (ARGs) could be required under the revised Environmental Quality Standards Directive (EQSD) and Groundwater watch lists, subject to suitable monitoring and analysis methods being identified.
Links to other Eionet groups	Biodiversity 1 and Thematic Group Water Environment and Health Group
Relevant organisations and networks	ETC-BE, WISE-SoE European Commission (DGs ENV + SANTE; JRC) EU agencies e.g. EFSA, ECDC, ECHA Water regulators, managers and utilities

European Environment Agency
European Topic Centre
Biodiversity and ecosystems