



REWA

Reduction and assessment of antimicrobial resistance and emerging pollutants in natural-based water treatment systems

D4.2 Guideline on co-selection potentials: Best practice guideline with standardized protocols for evaluation of co-selection potentials in aquatic habitats and report on metal-induced co-selection of antibiotic resistance in aquatic habitats.

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Executive Summary

Antimicrobial resistance (AMR) constitutes an escalating global health crisis, and pollution can increase human health risks via environmental development and dissemination of antibiotic resistance. Apart from antibiotic residues directly selecting for antibiotic resistance, co-selective agents such as metals, biocides, or pharmaceuticals may also expand environmental reservoirs of AMR. Hence, the extensive use of heavy metals associated with various industrial and agricultural activities has raised concerns about the co-selection of antibiotic resistance in aquatic habitats. This report aims to address this issue by presenting some best practice guidelines for the evaluation of co-selection potentials in aquatic microbiomes based on activities from the REWA project. Most of the methods used for studying co-selection are similar to those used for studying AMR in general, and this document should therefore be read in connection with D4.1, where detailed protocols can be found. In addition, we report our evaluations on co-selective potentials in respective case studies. In conclusion, our results suggest a low potential of toxicants to co-select antibiotic resistance in the tested municipal wastewater effluent samples, and we did not obtain any evidence suggesting that vanadium from a Finnish mining site could co-select antibiotic resistance in the studied mining-impacted waters.

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1. Introduction

Antibiotic resistance is an escalating global health crisis, and its impacts reach far beyond clinical settings (World Health Organization, 2015). In recent years, the focus on antibiotic resistance has expanded to encompass a broader ecological perspective, recognizing the significant role that non-clinical environments, such as aquatic habitats, play in the dissemination and persistence of antibiotic resistance genes (ARGs) (Larsson and Flach, 2022). Moreover, as we strive to understand and mitigate this growing threat, it has become increasingly evident that we cannot adequately address the issue without considering the concept of co-selection.

Co-selection is the ability of other pollutants besides antibiotics to select for antibiotic resistance. This can for instance be metals and biocides and can occur through various mechanisms, such as if ARGs and metal or biocide resistance genes are co-located on the same genetic element (co-resistance), or if the same resistance mechanism gives resistance to both metals and antibiotics (cross-resistance) (Baker-Austin *et al.*, 2006). Moreover, the ability of antibiotics to select for antibiotic resistance, especially in environmental settings, is dependent on many factors, including the biodegradation of antibiotics and their sorption to organic particles in the environment (Davies and Davies, 2010; Song *et al.*, 2017). Consequently, co-selection has gained increasing attention as a key player in antibiotic resistance dissemination in the environment.

The extensive use of heavy metals in various industrial, agricultural, and municipal activities has raised concerns about co-selection of antibiotic resistance in aquatic habitats, which serve as reservoirs for diverse microbial communities (Seiler and Berendonk, 2012). The intricate interplay between heavy metals and antibiotics in these environments poses an intricate challenge that calls for a comprehensive understanding and well-defined guidelines for assessment.

This report aims to address this pressing issue by presenting a best practice guideline with standardized protocols for the evaluation of co-selection potentials in aquatic microbiomes as used in the REWA project. Most of the methods used for studying co-selection are the same as those used for studying antibiotic resistance in general, and this document should therefore be read in connection with D4.1. In addition, we report our evaluations on co-selective potentials in respective case studies.

The REWA consortium brings together four Research and Technology Development (RTD) partners and associate partners, providing a multidisciplinary approach with expertise in water engineering, chemistry, materials synthesis, and microbial ecology.

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The project partners are:

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2. Methods for evaluating co-selection potentials in aquatic environments

2.1. Controlled microcosms vs. real-life environmental aquatic samples

As reported in D4.1., it is important to consider experimental setup when studying co-selection as different approaches have different advantages and disadvantages. As discussed previously, either controlled microcosms or real-world samples can be used to study antibiotic resistance, each with pros and cons. Microcosms offer precise control over variables, allowing for focused, replicable studies, but may oversimplify natural ecosystems. Real-world samples provide ecological realism, essential for understanding resistance in complex environments, though they are harder to control and collect. Combining both approaches offers a fuller understanding of resistance in aquatic environments.

2.2. Assessing Co-Selective Pressures by Whole-Cell Bioreporters

Similarly as described in D4.1, it is essential to consider a pollutant's bioavailability when assessing its environmental impact, as only the bioavailable fraction can directly impact microorganisms in a given environment (Song *et al.*, 2017). This is also the case when studying co-selection for antibiotic resistance, by for instance metals. In the REWA project, we are equipped with a range of whole-cell bioreporters useful for studying selective pressures provided by a range of compounds, including Cu, Zn, and general toxicity, and we recommend using these when exploring co-selection potentials. The bioreporter protocols can be found in D.4.1.

2.3. Pollution-Induced Community Tolerance (PICT)

PICT is also a very useful technique to study co-selection, as it can be used to study how a pollutant of interest (e.g. metals) affects antibiotic tolerance in a microbial community. PICT is especially useful for studies of bacterial community tolerance to essential metals such as Cu and Zn, as genetic prediction of resistance to these metals is complicated by the fact that the same gene may be used for cellular homeostasis at low metal concentrations or for metal resistance at high concentrations. In the REWA project, we recommend using the radioactive-labeled leucine incorporation technique, which successfully has been utilized to study co-selection by Zn and Cu previously (Song *et al.*, 2017). The full PICT protocol can be found in D4.1. (**Protocol 4**).

2.4. Co-selection Potential in Bacterial Isolates

2.4.1. Bacterial Isolation and Phenotypic Resistance Screening

Phenotypical resistance (e.g. metal and antibiotic resistance) in isolates is a useful technique for investigating co-selection. As already reported in D.4.1., the isolates can be obtained directly from environments polluted with co-selective agents (e.g. metals) or from controlled microcosm setups exposed to the same compound. The resistance patterns of the isolates can then be compared to a set of isolates obtained from a non-polluted reference site to determine if the pollutant has impacted the level of co-occurring resistances (e.g. metal and antibiotic resistance) in isolates. The procedure for obtaining pure cultures and phenotypical screening can be found in **Protocols 5 and 6** in the appendix of D4.1.

2.4.2. *Aeromonas* as an Indicator Organism for Co-selection in Aquatic Environments

As already described in D4.1., it can be challenging to compare phenotypic resistance patterns in isolates due to variations in resistance patterns among different species. Therefore, in the REWA project, we recommend selecting a specific indicator organism, as this allows for more direct comparisons between polluted and non-polluted environments. As mentioned in D4.1, we recommend using the genus *Aeromonas* as an indicator group in aquatic environments. **Protocol 5** can be found in the appendix of D4.1, and describes how *Aeromonas* species can be isolated from aquatic samples using ADA medium, while the CLSI guidelines should be used for resistance testing in *Aeromonas spp.* strains (Hindler and Richter, 2016; Xu *et al.*, 2025)

2.4.3. Comparative Genomics to Investigate Genetics behind co-resistance

Similar to investigating antibiotic resistance in isolates, it can be useful to use comparative genomics to investigate the genes involved in co-selection. The approach for conducting comparative genomics is similar to that described in D.4.1, and databases such as BacMet (<http://bacmet.biomedicine.gu.se/>) can be used to uncover potential metal or biocide resistance genes that may be connected to the observed resistance traits.

2.5. Co-selection of ARGs in aquatic microbial communities

Co-selection can also be explored in the aquatic microbial community using whole-community DNA-based methods as already described in D4.1. This entails the retrieval of DNA from a sample of interest e.g. water exposed to specific pollutants (e.g. metals) or treatment as already described in D4.1. Subsequently, the impact of the exposure on co-selection for antibiotic resistance genes (ARGs) can be evaluated by quantification of ARGs through methods such as high-throughput quantitative polymerase chain reaction (HT-qPCR) as detailed in D4.1. In the REWA project, we recommend using a high-throughput qPCR SmartChip system as described in D4.1. Recently, there have also been developed HT-qPCR Smartchip systems capable of quantifying metal resistance genes and virulence factor genes (Zhu *et al.*, 2022; Xie *et al.*, 2023). Especially the HT-qPCR system for metal resistance genes can be useful for studying co-selection, as a link between the abundance and diversity of metal and antibiotic resistance genes can be explored (Brinck *et al.*, 2023; Forouzandeh *et al.*, 2023). The protocol for using the HT-qPCR Smartchip system is described in D4.1. Finally, it is also crucial to consider impacts on the microbial community composition when studying co-

selection. In the REWA project, we recommend using amplicon sequencing to study the aquatic microbial community composition as already described in D4.1.

3. Biotoxicity and Co-selection potentials in REWA case studies

3.1. Investigation on Biotoxicity in Case Study 2.

In the original proposal, the intent was to study co-selection by Cu and Zn using samples from case studies #2 and #4. However, upon further evaluation of the source material it was discovered that the wastewater in Case Study #2 already had undergone membrane filtration, and the bacterial density would therefore be too low to use DNA-based or cultivation-based methods. Instead, we quantified chemical wastewater quality using the sensitive *Nitrosomonas europaea* bioreporter assay described in D4.1. Toxicity levels were quantified both before and after treatment with the magnetite-pinebark biosorbent tested in case study #2. The results are reported in D3.2, and a recently published scientific paper (Mohammadzadeh *et al.*, 2025). Our results suggest a low potential of toxicants to co-select antibiotic resistance in the tested wastewater.

3.2. Investigation on Co-selection by Vanadium in Case Study 4.

For case study #4, the source material was changed to vanadium-contaminated wastewater based on decisions taken by the project coordinator. Very little is known about the impact of vanadium on microbial communities and especially on co-selection for antibiotic resistance (Bell *et al.*, 2004; Chen *et al.*, 2021; Zhang *et al.*, 2023). We, therefore, conducted basic experiments to investigate the toxicity and co-selective abilities of vanadium in the microbial community by using a combination of whole-cell bioreporter and phenotypical resistance screening of *Aeromonas spp.* isolates.

As already reported in D3.3, we used a bioluminescence assay with a genetically modified *Nitrosomonas europaea* bioreporter strain to investigate the biotoxicity of vanadium in aqueous solutions. In brief, high concentrations of vanadium (EC₅₀ ~2682 mg/L) inhibited metabolic activity in *N. europaea* (i.e. bioluminescence inhibition), but effective concentrations of vanadium were much higher than typically found in the environment (Zhang *et al.*, 2023). When testing vanadium-contaminated water from the Mustavaara Vanadium mine, we found moderate biotoxicity (25-29% bioluminescence inhibition). However, this toxicity was likely caused by other chemicals than vanadium, as removal of vanadium from the water with *Acacia* tannin coagulants did not reduce biotoxicity.

To further investigate the potential for co-selection of Vanadium and antibiotic resistance, we explored the occurrence of phenotypical vanadium (NaVO₃) and antibiotic resistance in *Aeromonas spp.* Isolates (Figure 1). The isolates were obtained from vanadium-contaminated sediment and non-contaminated reference sediment samples collected at the Muustavaara Vanadium mining site using the protocols referred to in sections 2.4.1. and 2.4.2 (see D4.1 for further details). Results showed that all isolates exhibited a limited level of vanadium resistance with very little difference between isolate origin (i.e. V contaminated or reference sediment). Moreover, the isolates in general exhibited low levels of antibiotic resistance, with only chloramphenicol resistance detected (6 % of the isolates). Overall, environmental

concentrations of vanadium caused negligible toxicity to bacteria and we did not observe a linkage between resistance to vanadium and antibiotics in the present study.

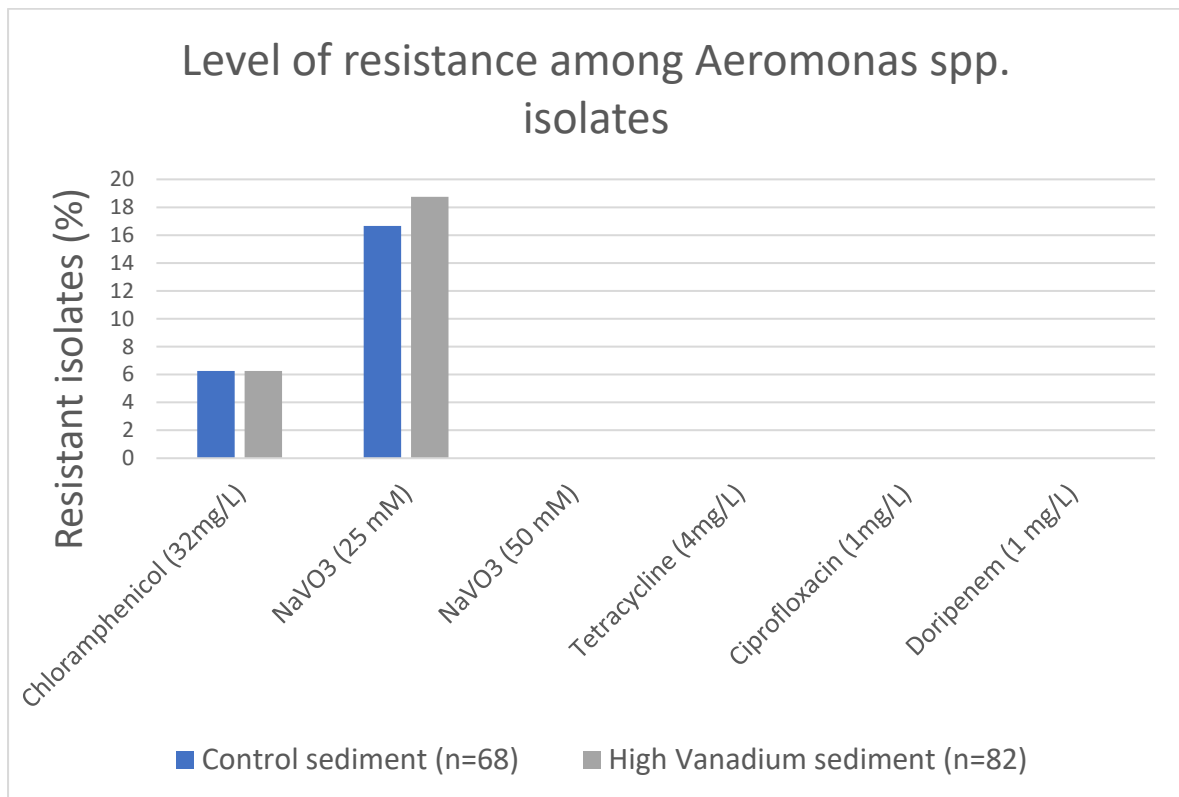


Figure 1: Barplot illustrating the percentage of Aeromonas spp isolates deriving from the Mustaavaara Vanadium mining site resistant to a range of antibiotics and sodium Meta vanadate (NaVO₃). High Vanadium sediment = isolates deriving from sediment samples from a ditch containing ~40 mg/L of Vanadium. Control sediment = isolates deriving from sediment samples from a stream containing low levels of Vanadium (~0.3 mg/L).

4. References

- Baker-Austin, C., Wright, M.S., Stepanauskas, R., and McArthur, J. V. (2006) Co-selection of antibiotic and metal resistance. *Trends Microbiol* **14**: 176–182.
- Bell, J.M.L., Philp, J.C., Kuyukina, M.S., Ivshina, I.B., Dunbar, S.A., Cunningham, C.J., and Anderson, P. (2004) Methods evaluating vanadium tolerance in bacteria isolated from crude oil contaminated land. *J Microbiol Methods* **58**: 87–100.
- Brinck, J.E., Lassen, S.B., Forouzandeh, A., Pan, T., Wang, Y.-Z., Monteiro, A., et al. (2023) Impacts of dietary copper on the swine gut microbiome and antibiotic resistome. *Sci Total Environ* **857**: 159609.
- Chen, L., Liu, J., Hu, W., Gao, J., and Yang, J. (2021) Vanadium in soil-plant system: Source, fate, toxicity, and bioremediation. *J Hazard Mater* **405**: 124200.
- Davies, J. and Davies, D. (2010) Origins and Evolution of Antibiotic Resistance. *Microbiol Mol Biol Rev MMBR* **74**: 417–433.
- Forouzandeh, A., Lassen, S.B., Brinck, J.E., Zhou, Y.-Y., Zhu, J., Solà-Oriol, D., et al. (2023) Limited impacts of high doses of dietary copper on the gut bacterial metal resistome explain negligible co-selection of antibiotic resistance. *Sci Total Environ* **889**: 164183.
- Hindler, J.A. and Richter, S.S. (2016) Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria: M45, 3rd edition. Wayne, PA: Clinical and Laboratory Standards Institute.
- Larsson, D.G.J. and Flach, C.-F. (2022) Antibiotic resistance in the environment. *Nat Rev Microbiol* **20**: 257–269.
- Mohammadzadeh, M., Bello, A., Lassen, S.B., Brandt, K.K., Risteelä, S., and Leiviskä, T. (2025) Pilot-scale adsorption of pharmaceuticals from municipal wastewater effluent using low-cost magnetite-pine bark: Regeneration/enumeration of viable bacteria with a study on their biotoxicity. *Environ Res* **268**: 120774.
- Seiler, C. and Berendonk, T. (2012) Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. *Front Microbiol* **3**.
- Song, J., Rensing, C., Holm, P.E., Virta, M., and Brandt, K.K. (2017) Comparison of Metals and Tetracycline as Selective Agents for Development of Tetracycline Resistant Bacterial Communities in Agricultural Soil. *Environ Sci Technol* **51**: 3040–3047.
- World Health Organization (2015) Global action plan on antimicrobial resistance, Geneva: World Health Organization.
- Xie, S.-T., Ding, L.-J., Huang, F.-Y., Zhao, Y., An, X.-L., Su, J.-Q., et al. (2023) VFG-Chip: A high-throughput qPCR microarray for profiling virulence factor genes from the environment. *Environ Int* **172**: 107761.
- Xu, J., Jensen, M.K.S., Lassen, S.B., Brandt, K.K., Dechesne, A., and Smets, B.F. (2025) *Aeromonas* isolation reveals this genus's contribution to antimicrobial resistance fluxes across the wastewater–treated water–river interface. *J Appl Microbiol* **136**: 1xae302.
- Zhang, B., Zhang, H., He, J., Zhou, S., Dong, H., Rinklebe, J., and Ok, Y.S. (2023) Vanadium in the Environment: Biogeochemistry and Bioremediation. *Environ Sci Technol* **57**: 14770–14786.
- Zhu, J., Huang, Qiong, Peng, X., Zhou, X., Gao, S., Li, Y., et al. (2022) MRG Chip: A High-Throughput qPCR-Based Tool for Assessment of the Heavy Metal(loid) Resistome. *Environ Sci Technol* **56**: 10656–10667.