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

D4.1 Optimized protocols for assessment of antimicrobial resistance (AMR) in aquatic microbiomes.

January 2025

Prepared by: Simon Bo Lassen and Kristian Koefoed Brandt

Section for Microbial Ecology and Biotechnology, Department of Plant and Environmental Sciences, University of Copenhagen, Denmark.

Executive Summary

Antibiotic resistance is an escalating global health threat that extends beyond clinical settings. Wastewater treatment plants represent clinically relevant intervention points for the mitigation of environmental AMR dissemination and transmission to humans. Hence, there is a growing interest in the development and assessment of novel wastewater treatment processes that can reduce the release of antimicrobials and AMR to aquatic recipients. The REWA project aims to develop new wastewater treatment processes for the removal of contaminants of emerging concern (CECs) including both pharmaceuticals and microbial risk factors such as pathogenic or antibiotic-resistant bacteria and antibiotic resistance genes (ARGs). However, there is a lack of standardized methods and guidelines for studying AMR during bench- and pilot-scale development of novel wastewater treatment processes. This report addresses this knowledge gap by presenting protocols used in the REWA project for quantifying antibiotic resistance already during bench- and pilot-scale development of novel wastewater treatment technologies. There is no single optimal method or technique for studying AMR determinants during wastewater treatment processes. We therefore recommend a polyphasic approach using contrasting methodological approaches in parallel. Approaches used in the REWA project include the following: (1) cultivation and subsequent phenotypic and/or genotypic characterization of bacterial isolates belonging to specific indicator groups (e.g. *Aeromonas spp.*), (2) cultivation-independent molecular community analysis to assess the abundance of diverse ARGs and bacterial community shifts, (3) ecophysiological methods for profiling bacterial growth and pollution-induced community tolerance (PICT), and (4) use of whole-cell bacterial bioreporters to assess wastewater toxicity to known indicator organisms and bioavailability of potential selecting agents driving the development of AMR. Detailed protocols are presented in the appendices.

Table of contents

1. Introduction

Antibiotic resistance is an escalating global health threat that extends beyond clinical settings, with far-reaching consequences for ecosystems and human health. In recent years, attention has expanded to include non-clinical environments, particularly aquatic ecosystems, which have been recognized as important reservoirs and pathways for the environmental development and transmission of antibiotic-resistance genes (ARGs) (Larsson and Flach, 2022). Aquatic environments, such as rivers, lakes, and wastewater systems, can harbor antibiotic-resistant bacteria and mobile genetic elements (MGEs), which facilitate the transfer of ARGs between different bacterial species. These environments play a critical role in the development and spread of antimicrobial resistance (AMR), driven by human activities such as wastewater discharge, agricultural runoff, and pharmaceutical pollution.

Wastewater treatment plants represent an important intervention point for the mitigation of environmental AMR dissemination. Hence, there is a growing interest in the development and assessment of novel wastewater treatment processes that can reduce the release of pharmaceuticals and AMR to aquatic recipients. The REWA project aims to develop new wastewater treatment processes for the removal of contaminants of emerging concern (CECs) including both pharmaceuticals and microbial risk factors such as pathogenic or antibiotic-resistant bacteria and ARGs.

There is a lack of standardized methods and guidelines for studying AMR during bench- and pilot-scale development of novel wastewater treatment processes. This report addresses this knowledge gap by presenting protocols used in the REWA project for quantifying antibiotic resistance already during bench- and pilot-scale development of novel wastewater treatment technologies.

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.

This project is funded by the European Commission, the Academy of Finland (Finland), the Ministry of Health (Israel), Innovationsfonden Denmark (Denmark), and the Water Research Commission (South Africa) under the 2020 Aquatic Pollutants Joint call of the Aquatic Pollutants ERA-NET Cofund (GA No 869178). This ERA-NET initiative is an integral part of the activities developed by the Water, Oceans, and Antimicrobial Resistance (AMR) Joint Programming Initiatives (JPIs).

The project partners are:

- Tiina Leiviskä, University of Oulu, Finland (Coordinator)
- Kristian Koefoed Brandt, University of Copenhagen, Denmark
- Giora Rytwo, MIGAL - Galilee Research Institute, Israel
- Bice Martincigh, University of KwaZulu-Natal, South Africa

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

Selection for antibiotic resistance in aquatic environments can be studied in either controlled microcosms or real-world environmental samples, each offering distinct advantages and drawbacks. Microcosms provide precise control over variables like temperature, pH, and nutrients, making it easier to study cause-and-effect relationships and replicate experiments. They are ideal for isolating specific factors, such as resistance gene transfer or antibiotic impact, and can simulate environments that are difficult or unethical to manipulate in real life (Berglund *et al.*, 2014; Mahaney and Franklin, 2022). However, microcosms may oversimplify real-world ecosystems, limiting the study of large-scale processes and sometimes producing artificial results. In contrast, real-world samples reflect the full complexity of natural environments, capturing interactions between species and environmental factors (Manaia *et al.*, 2024). This realism is crucial for understanding how resistance develops and spreads in natural settings. However, the variability of natural environments makes controlling confounding factors difficult, and collecting samples can be logistically challenging and resource-intensive. Both approaches have value: microcosms excel at mechanistic studies at the bench-scale, while field studies are key to understanding fully implemented water treatment technologies. Combining both methods can provide a more complete picture of antibiotic resistance in aquatic environments. In the REWA project, we use a combination of both setups. For instance, in one project we study the impact of cyanobacterial biochar-mediated catalysis of peroxydisulfate to remove antibiotic-resistant bacteria (ARB) and ARGs during wastewater treatment, while also establishing microcosms to study the fate of ARGs in surviving bacteria following the waste water treatment.

3. Assessing water quality by whole-cell bacterial bioreporters

When assessing the environmental impact of a pollutant on antibiotic resistance selection, it is essential to consider the bioavailability of potential selection agents, as only the bioavailable fraction can directly impact microorganisms in a given environment (Song *et al.*, 2017). Whole-cell bioreporter organisms are valuable tools for quantifying pollutant bioavailability and microbial impact, as they specifically react to the bioavailable fraction of the analyte of interest. Bioreporters have been widely utilized to investigate various organic and inorganic compounds in both terrestrial and aquatic environments. For example, highly sensitive and robust bioreporters targeting specific metals and tetracycline have been developed and used to study environmental pollution (Korpela *et al.*, 1998; Tom-Petersen *et al.*, 2001; Virolainen *et al.*, 2008; Hynninen *et al.*, 2010). Bioreporters can most easily be applied in aquatic samples, but it is also possible to assess pollutant toxicity and bioavailability in solid matrices such as sediments or soils. However, when studying sediment or soil, it is essential to consider matrix effects, which may impact the bioluminescence emitted by the biosensor (Brandt *et al.*, 2006, 2008). Batch-to-batch variations are also possible when using living organisms as bioreporters, and can potentially affect the assay's sensitivity (Hansen *et al.*, 2019). Additionally, other compounds present in the samples may be toxic to the bioreporter, potentially limiting the quantification of the compound of interest.

In the REWA project, a whole-cell bioreporter *Nitrosomonas europeae* strain is employed to study selective pressures/toxicity present in water samples before and after water treatment:
- *Nitrosomonas europeae* for overall toxicity/nitrification inhibition (**Protocol 1**)
- *Pseudomonas fluorescens* DF57-Cu15 for Copper bioavailability (**Protocol 2**)
- *Pseudomonas putida* KT2440 for Zinc/lead/Cadmium bioavailability (**Protocol 3**)

4. Pollution-Induced Community Tolerance (PICT)

4.1. [^{3}H]Leucine Incorporation

Selection for antibiotic resistance in aquatic samples can be assessed using a PICT assay. One effective and standardized PICT approach involves tritium-labeled leucine incorporation. The PICT protocol consists of two phases. In the first phase (the PICT selection phase), the bacterial community is exposed to a selecting agent (e.g. antibiotics), either in field-based studies (wastewater samples) or experimental setups such as microcosms. The exposure should be of sufficient duration to allow selection to occur. In the second phase (the PICT detection phase), the bacterial community is extracted from the studied environment and incubated for a short time (e.g., 3 hours) with increasing concentrations of the toxicant of interest (e.g. various antibiotics), along with tritium labeled leucine. Subsequently, the radioactively labeled leucine is quantified to determine short-term protein synthesis in the bacterial community, providing insight into bacterial productivity (i.e. growth). The dose-response relationship can then be modeled to establish an EC50 value, which corresponds to the effective concentration of a toxicant resulting in a half-maximal response of the effect indicator (e.g. leucine incorporation rate). It is essential to keep the PICT detection phase short (<3 hours) and to control pH using a buffer to prevent PICT detection artifacts (Lekfeldt *et al.*, 2014). The PICT method is useful for evaluating the impact of selection pressures on microbial community tolerance. However, PICT only provides a phenotypic measure for tolerance and does not offer direct insight into the mechanisms, resistance genes, or microbes involved. Combining PICT with genotypic investigations is recommended either to link PICT data to changes in the microbial community composition or to link PICT to changes in the relative abundance of resistance genes. Detailed PICT protocol can be found in the appendix (**Protocol 4**).

5. Antibiotic resistance in Bacterial Isolates

5.1. Bacterial Isolation and Phenotypic Resistance Screening

Antibiotic resistance can be studied directly by exploring phenotypical resistance in bacterial isolates. The isolates can be obtained directly from an environment of interest (e.g. wastewater) or from controlled microcosm setups exposed to a compound of interest (e.g. antibiotics). The resistance patterns of the isolates can then be compared to a set of isolates obtained from a reference source (e.g. upstream water or non- polluted microcosm). To study co-selection potentials such as the linkage between metal resistance and antibiotic resistance, it is further recommended to test if antibiotic resistance to different classes of antibiotics is more prevalent among metal-resistant isolates than among metal-susceptible

isolates from a given environment. For aquatic environments, the isolates can be obtained from water samples or corresponding sediments. The procedure for obtaining pure cultures can be found in **Protocol 5** in the appendix. Once pure cultures are obtained, they can be screened for phenotypical resistance against various compounds as described in **Protocol 6** of the appendix.

5.2. *Aeromonas* as an Indicator Organism in Aquatic Environments

Comparing phenotypic resistance patterns in isolates can be challenging due to variations in resistance patterns among different species. Therefore, selecting a specific indicator organism is recommended, as this allows for more direct comparisons between polluted and non-polluted environments. In aquatic environments, *Aeromonas spp*. has been proposed as an ideal indicator organism, as they are ubiquitous in aquatic environments, include pathogenic strains, possess mobile genetic elements (MGEs), exhibit high permissiveness for plasmid uptake, and commonly acquire antibiotic resistance (Grilo *et al.*, 2020). Several commercial selective media are available for cultivating *Aeromonas spp*. from aquatic environments. We have validated some of these media and recommend the use of Ampicillin Dextrin Agar (ADA) and Ampicillin Sheep Blood Agar (ASBA) for this purpose (Xu *et al.*, 2025). ADA showed slightly higher selectivity and specificity, while ASBA recovered a broader diversity of *Aeromona*s spp. **Protocol 5** in the appendix 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).

5.3. Comparative Genomics to Investigate Genetics behind Phenotypical Resistance

Comparative genomics is a valuable tool for exploring the genetics behind the observed phenotypical traits in tested bacterial isolates. This approach involves comparing DNA sequences from different isolates to identify potential genes responsible for the phenotypical trait. In the context of antimicrobial resistance and co-selection, comparative genomics involves scrutinizing the genomes of bacterial isolates, both resistant and susceptible to antibiotics, to identify the ARGs that may be responsible for the observed resistance. The primary objective is to identify genetic elements responsible for resistance and understand how these elements evolve and spread. To conduct comparative genomics in the REWA project, we follow these steps:

1. Obtain high-molecular-weight DNA from bacterial isolates using the Genomic Mini AX Bacteria kit (A&A Biotechnology, Gdynia, Poland) and Qiagen Genomic-tip (20/G) (Hilden, Germany).
2. Remove impurities from the DNA using the Genomic DNA Clean & Concentrator kit (Zymo Research, Irvine, CA, USA).
3. Measure DNA concentrations and quality using a Qubit 2.0 Fluorometer (Invitrogen, USA) and NanoDrop Spectrophotometer ND-1000 (Thermo Fisher Scientific, USA).
4. Prepare libraries using the rapid barcoding sequencing kit (SQK-RBK004) according to the manufacturer's instructions.

5. Perform whole-genome sequencing using a MinION sequencer (Oxford Nanopore Technologies, Oxford, UK) with a FLO-MIN106 flow cell, controlled by MinKNOW (version 19.10.1).
6. Basecall the raw Nanopore reads using GPU-Guppy (version 5.1.12).
7. Trim adaptor sequences from the reads using Porechop (version 0.2.4).
8. Assemble reads into contigs using Flye (version 2.9-b1774).
9. Conduct two rounds of consecutive polishing using Medaka (version 1.4.4).
10. Annotate polished genomes using Prokka (version 1.14.6).
11. Perform whole-genome-based phylogenetic analysis using the Genome Taxonomy Database (GTDB).
12. Identify ARGs and metal/biocide resistance genes using the CARD (https://card.mcmaster.ca/) or BacMet (http://bacmet.biomedicine.gu.se/) databases, respectively.

5.5. Limitations of studying antibiotic resistance in isolates

When focusing on isolates, the primary limitation is the restricted scope of analysis. This method examines individual bacterial strains, which may not adequately represent the full diversity of resistance mechanisms present within a microbial community. Consequently, this approach can overlook the broader context of how resistance genes are distributed across different species and strains. Additionally, isolates may not exhibit the same resistance phenotype in laboratory conditions as they would in their natural environment. This discrepancy arises due to changes in gene expression or interactions with other microorganisms, leading to potential differences between laboratory findings and real-world scenarios. Furthermore, the process of culturing and isolating bacteria is both time-consuming and labor-intensive, requiring significant resources. This limitation can restrict the number of samples that can be processed and analyzed. Lastly, there is a potential for bias in the selection of isolates, as not all bacteria in a community are equally easy to culture. This can result in an overrepresentation of certain species or strains that are more amenable to laboratory conditions.

6. Antibiotic resistance in aquatic microbial communities

6.1. Whole-community DNA extraction from aquatic samples

Selection for antibiotic resistance can also be explored in the aquatic microbial community using whole-community DNA-based methods. This entails the retrieval of DNA from a sample of interest e.g. water exposed to specific pollutants (e.g. antibiotics) or treatments. Subsequently, the impact of the exposure on selection can be evaluated by quantification of ARGs through methods such as high-throughput quantitative polymerase chain reaction (HT-qPCR) as detailed below. For optimal results in obtaining high-quality DNA from aquatic samples, we advise adhering to the standardized protocol outlined by the Water Research Foundation (Liguori *et al.*, 2023).

6.2. Quantification of ARGs using HT-qPCR

Selection for ARGs in the microbial community can be investigated using quantitative PCR (qPCR). A high-throughput qPCR SmartChip system was initially developed in 2012, containing 141 primer sets targeting 18 different groups of ARGs (Looft *et al.*, 2012). The primer set was subsequently improved in 2018 to contain 384 primer sets targeting 315 ARGs and 57 mobile genetic elements (Stedtfeld *et al.*, 2018). The HT-qPCR ARGs chip is a rapid, cost-effective method that enables comprehensive gene quantification in DNA from environmental samples (Bengtsson-Palme, 2018). In the HT-qPCR SmartChip system, more than 5,000 nanoliter-scale qPCR reactions can be conducted simultaneously, targeting various genes. The analysis is carried out by robot pipetting, ensuring reproducibility and precision. The primers designed for the ARG chip have undergone repeated rounds of experimental validation (Stedtfeld *et al.*, 2018). A typical analysis involves three analytical replicates and a negative control for each gene. PCR conditions include an initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 30 seconds at 95°C and 30 seconds at 60°C. The results are analyzed using the Wafergen SmartChip qPCR Software, which excludes amplifications with multiple melting peaks or amplification efficiency beyond the 90%-110% range. After the initial quality check, a threshold cycle (CT) is set, typically at 31 or 27, and all amplifications with values below this threshold are used for further analysis. HT-qPCR can provide the absolute quantity (e.g., genes per L of water), relative quantity (genes per bacterium or the 16S rRNA gene), or comparative quantities (fold-change) of targeted genes, depending on the research question (Zhu *et al.*, 2017; Zhao *et al.*, 2018; Lassen *et al.*, 2022). For operating the Wafergen Smarchip software, we recommend adhering to the user manuals which can be found on the takarabio webpage (https://www.takarabio.com/). Moreover, recommendations and notes for setting up the qPCR reactions can be found in **protocol 7** of the appendix.

6.3. Amplicon Sequencing to Study Microbial Community Composition

When investigating antibiotic resistance it is crucial to consider impacts on the microbial community in response to environmental stressors. Amplicon sequencing is a widely used high-throughput method to identify microbes in different environments (Caporaso *et al.*, 2012). This technique relies on PCR amplification of short hypervariable regions of conserved genes, which are then analyzed using high-throughput sequencing technology, such as Illumina sequencing. Raw reads are processed using various bioinformatics software, including QIIME2, Mothur, or DADA2 (Schloss *et al.*, 2009; Callahan *et al.*, 2016; Bolyen *et al.*, 2019). During data processing, amplicon sequences are clustered into operational taxonomic units (OTUs) or amplicon sequence variants (ASVs). OTUs are generated by clustering sequences based on a 97% sequence identity threshold to minimize the impact of sequencing errors. ASVs, on the other hand, use exact sequences and have statistical confidence values. Once sequences are processed into OTUs or ASVs, databases such as RDP classifier, Greengenes, or SILVA can be used to assign taxonomy to the sequences based on the 16S rRNA gene. Comprehensive guidelines for processing amplicon-sequencing data using the above-mentioned pipelines are available online:

DADA2: https://benjjneb.github.io/dada2/tutorial.html

QIIME2: https://docs.qiime2.org/2023.9/
MOTHUR: https://mothur.org/wiki/miseq_sop/

6.4. Limitations of whole-community DNA-based methods for studying antibiotic resistance

One major limitation of using whole-community DNA-based approaches for studying antibiotic resistance is the complexity of the data generated. These approaches produce vast amounts of information that can be challenging to analyze, given the complexity of microbial communities and the presence of numerous resistance genes. This necessitates the use of sophisticated bioinformatics tools and expertise. Additionally, while HT-qPCR can identify the presence of certain resistance genes, it often lacks the ability to determine their genomic context. This makes it difficult to understand how resistance genes are organized within genomes and how they might be transferred between organisms. Sensitivity and specificity issues also arise, as the approaches may struggle to detect low-abundance resistance genes, potentially leading to an underestimation of resistance levels. Conversely, they may detect genes that are not expressed or functional, resulting in overestimation. Finally, the resource-intensive nature of high-throughput sequencing and the computational demands of data analysis can be prohibitive, limiting the widespread adoption of whole-community DNA approaches.

7. Concluding remarks

All of the above methodologies have their place in antibiotic resistance research, but also come with limitations. Therefore we recommend using a combination of methods to provide a more comprehensive understanding of resistance dynamics in aquatic environments. Researchers must carefully weigh the limitations of each approach and select the most suitable method for their specific research questions.

8. References

Bengtsson-Palme, J. (2018) The diversity of uncharacterized antibiotic resistance genes can be predicted from known gene variants—but not always. *Microbiome* **6**: 125.

Berglund, B., Khan, G.A., Lindberg, R., Fick, J., and Lindgren, P.-E. (2014) Abundance and Dynamics of Antibiotic Resistance Genes and Integrons in Lake Sediment Microcosms. *PLOS ONE* **9**: e108151.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., et al. (2019) Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol* **37**: 852–857.

Brandt, K.K., Holm, P.E., and Nybroe, O. (2006) Bioavailability and toxicity of soil particle-associated copper as determined by two bioluminescent Pseudomonas fluorescens biosensor strains. *Environ Toxicol Chem* **25**: 1738–1741.

Brandt, K.K., Holm, P.E., and Nybroe, O. (2008) Evidence for Bioavailable Copper−Dissolved Organic Matter Complexes and Transiently Increased Copper Bioavailability in Manure-Amended Soils as Determined by Bioluminescent Bacterial Biosensors. *Environ Sci Technol* **42**: 3102–3108.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., and Holmes, S.P. (2016) DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods* **13**: 581–583.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., et al. (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J* **6**: 1621–1624.

Grilo, M.L., Sousa-Santos, C., Robalo, J., and Oliveira, M. (2020) The potential of Aeromonas spp. from wildlife as antimicrobial resistance indicators in aquatic environments. *Ecol Indic* **115**: 106396.

Hansen, M.G., Marcussen, H., Holm, P.E., and Brandt, K.K. (2019) Evaluation of pH-Dependent Metal Speciation Artifacts in Whole-Cell Bioreporter Analysis. *J Environ Qual* **48**: 199–204.

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.

Hynninen, A., Tönismann, K., and Virta, M. (2010) Improving the sensitivity of bacterial bioreporters for heavy metals. *Bioeng Bugs* **1**: 132–138.

Iizumi, T., Mizumoto, M., and Nakamura, K. (1998) A bioluminescence assay using Nitrosomonas europaea for rapid and sensitive detection of nitrification inhibitors. *Appl Environ Microbiol* **64**: 3656–3662.

Korpela, M.T., Kurittu, J.S., Karvinen, J.T., and Karp, M.T. (1998) A Recombinant *Escherichia coli* Sensor Strain for the Detection of Tetracyclines. *Anal Chem* **70**: 4457–4462.

Lassen, S.B., Ahsan, Md.E., Islam, S.R., Zhou, X.-Y., Razzak, M.A., Su, J.-Q., and Brandt, K.K. (2022) Prevalence of antibiotic resistance genes in Pangasianodon hypophthalmus and Oreochromis niloticus aquaculture production systems in Bangladesh. *Sci Total Environ* **813**: 151915.

Lekfeldt, J.D.S., Magid, J., Holm, P.E., Nybroe, O., and Brandt, K.K. (2014) Evaluation of the leucine incorporation technique for detection of pollution-induced community tolerance to copper in a long-term agricultural field trial with urban waste fertilizers. *Environ Pollut* **194**: 78–85.

Liguori, K., Keenum, I., Davis, B., Milligan, E., Heath, L.S., Pruden, A., et al. (2023) Standardizing Methods with QA/QC Standards for Investigating the Occurrence and Removal of Antibiotic Resistant Bacteria/Antibiotic Resistance Genes (ARB/ARGs) in Surface Water, Wastewater, and Recycled Water. *The Water Research Foundation*.

Looft, T., Johnson, T.A., Allen, H.K., Bayles, D.O., Alt, D.P., Stedtfeld, R.D., et al. (2012) In-feed antibiotic effects on the swine intestinal microbiome. *Proc Natl Acad Sci U S A* **109**: 1691–1696.

Mahaney, A.P. and Franklin, R.B. (2022) Persistence of wastewater-associated antibiotic resistant bacteria in river microcosms. *Sci Total Environ* **819**: 153099.

Manaia, C.M., Aga, D.S., Cytryn, E., Gaze, W.H., Graham, D.W., Guo, J., et al. (2024) The Complex Interplay Between Antibiotic Resistance and Pharmaceutical and Personal Care Products in the Environment. *Environ Toxicol Chem* **43**: 637–652.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., et al. (2009) Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. *Appl Environ Microbiol* **75**: 7537–7541.

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.

Stedtfeld, R.D., Guo, X., Stedtfeld, T.M., Sheng, H., Williams, M.R., Hauschild, K., et al. (2018) Primer set 2.0 for highly parallel qPCR array targeting antibiotic resistance genes and mobile genetic elements. *FEMS Microbiol Ecol* **94**: fiy130.

Tom-Petersen, A., Hosbond, C., and Nybroe, O. (2001) Identification of copper-induced genes in Pseudomonas fluorescens and use of a reporter strain to monitor bioavailable copper in soil. *FEMS Microbiol Ecol* **38**: 59–67.

Virolainen, N.E., Pikkemaat, M.G., Elferink, J.W.A., and Karp, M.T. (2008) Rapid detection of tetracyclines and their 4-epimer derivatives from poultry meat with bioluminescent biosensor bacteria. *J Agric Food Chem* **56**: 11065–11070.

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**: lxae302.

Zhao, Y., Su, J.-Q., An, X.-L., Huang, F.-Y., Rensing, C., Brandt, K.K., and Zhu, Y.-G. (2018) Feed additives shift gut microbiota and enrich antibiotic resistance in swine gut. *Sci Total Environ* **621**: 1224–1232.

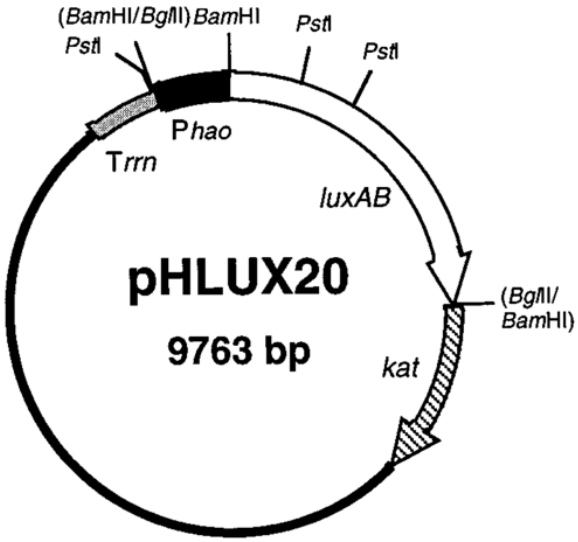
Zhu, Y.-G., Zhao, Y., Li, B., Huang, C.-L., Zhang, S.-Y., Yu, S., et al. (2017) Continental-scale pollution of estuaries with antibiotic resistance genes. *Nat Microbiol* **2**: 1–7.

9. Appendices

Protocol 1: *Nitrosomonas europaea* bioluminescence inhibition assay

Introduction

The bioluminescence assay is based on a transformant of *Nitrosomonas europaea* harboring a plasmid, pHLUX20, with *luxAB* genes (expression controlled by *hao* promotor) and kanamycin resistance. The luxAB-encoded protein (luciferase) catalyzes a biochemical reaction associated with light emission (bioluminescence), and the intensity of this light can be measured quantitively in a plate reader. The luminescent intensity correlates to the energy level of the cell (NAD(P)H and $FMNH_2$) and hence to the respiration rate of the bioreporter cells (Iizumi et al., 1998). A long-chain aliphatic aldehyde (n-decanal) is used in the assay as substrate for the light generating reaction and must be added by the experimenter.
Thus, this assay can be used to assess the inhibitory effect of nitrification inhibitors (biological or synthetic) on ammonia oxidizing bacteria using *N. europaea* as model organisms.



Physical map of pHLUX20
Promoter less luciferase-encoding genes (*luxAB*) from *V. harveyi* and the Tn903-derived kanamycin acetyltransferase encoding gene (kat) are shown as open and striped arrows, respectively, indicating the gene orientations. The *E. coli* 5S rRNA rho-independent terminator (T_{rrn}) and the promoter region of the *N. europaea* HAO-encoding gene (P*hao*) are represented by shaded and solid bars, respectively. The solid line is the region derived from the IncQ plasmid, which is essential for replication. From Iizumi et al. (1998)

Reagents

N-free Ås-medium (Table 1)
10 mL Ammonium solution (Table 2)
Milli-Q water
96% Ethanol solution
50% Ethanol solution
10 mL decanal (aldehyde) solution (0.35%) (Table 3). The solution can only be used during the day it is made.

Recipes

Table 1. N-free Ås-medium

Compound & Molarity	per Liter	per 5 Liters
0.5 M KH_2PO_4	2.94 mL	14.7 mL
0.5 M $CaCl_2 . 2H_2O$	0.28 mL	1.4 mL
0.5 M $MgSO_4 . 7H_2O$	0.32 mL	1.6 mL
Phenol red	1 mg (i.e., 2 mL of a 0.05% solution)	10 mL
Trace metal solution	1.0 mL	5.0 mL
(188) HEPES (powder)	4.77 g	23.85 g
FeNaEDTA (3.8%)	0.1 mL	0.5 mL
1 M $NaHCO_3$ (filter sterilized, add after autoclaving)	1.48 mL	7.4 mL

This solution can be prepared beforehand and stored at room temp. after autoclaving.

Table 2. 75.0 mM Ammonium solution (18.75 mM in final assay volume)

Compound & Molarity	per 10 ml	per liter
N-free As-medium	9.25 mL	925 mL
0.5 M $(NH_4)_2SO_4$	0.75 mL	75 mL

The solution can be used for 2-3 days (keep in fridge)

Table 3. 0.35 % v/V decanal solution.

Before preparing the solution the decanal stock (solid at 5°C) must be thawed first.

Compound & Molarity	per 10 ml	per 15 ml
1. 96% ethanol solution (use fresh i.e., NOT from squeeze bottles)	5 ml	7.5 mL
2. n-decanal solution (in fridge)	35 µL	52.5 µL
3. Milli-Q water	5 ml	7.5 mL

Following the order of addition in Table 3.

Please operate in a fume hood and cover the container with plastic film (smelly)

The decanal solution can only be used on the day of preparation.

Assay protocol

a. Preparation of cell suspension

Turn on the centrifuge and set the temperature to 5 ℃ (allowing it to cool down).

1. Measure NO_2^- concentration of luxAB *N. europaea* cell culture. If above 6000 µM, dilute with Ås-medium to reach 6000 µM NO_2^-.
2. Distribute cell culture into several 50 mL falcon tubes. 100 mL culture is enough to run a full 96-well plate assay.
3. Put tubes in centrifuge, balance the weight and cap the rotor block.
4. Centrifuge culture tubes for 10 mins at 8000g, 5 °C.
5. After centrifuging, carefully take out the tubes and observe a small pink pellet.
6. Pour out the supernatant (GMO waste).
7. Add N-free Ås-medium to each centrifuge tube to concentrate cells 20x, resuspend the cells (vortex at half speed), and combine cells suspensions in one tube.
   E.g., if 50 mL cell culture is centrifuged, the remaining pellet is resuspended in 2.5 mL N-free Ås-medium to reach a 20x up-concentration of cells.
8. Leave cell solution at room temperature for the **starvation phase** for minimum 30 min. The cell solution can be left for an extended period (30-120 min.) without affecting the assay.

b. Preparation of plate reader

1. Turn on the plate reader and computer.
2. Open SMART CONTROL
3. Adjust the temperature to 28 degrees.
4. Open the plate reader (open the reagent chamber)
5. Ensure the syringe holder is in the **park position.**
6. Prepare a bottle for waste liquid and place it under the syringe holder.
7. Go back to the computer and click the prime bottom on the top control panel.
8. Adjust the volume for two syringes to 1000 uL and click prime syringe bottoms:
   a) Prime pump 1 with 1) Milli-Q water and 2) ammonia solution (Table 2)
   b) Prime pump 2 with 1) 50% Ethanol and 2) decanal solution (Table 3)
9. Move the syringe holder to the **work position.**
10. Close the plate reader (close the reagent chamber)

c. Preparation of 96-well plate and bioluminescent measurement

The assay contains the following components in each well:

LuxAB *N. europaea* cells resuspended in N-free Ås-medium	50 µL
Ammonium solution (final conc. NH_4 in 200 µL assay is 18.75 mM)	50 µL
Sample (inhibitor/test chemical/control)	100 µL

1. Set the plate incubator at 250 rpm and 28 °C.

After end of starvation phase, the plate can be set up with the assay. Follow plate setup below if assay is used for BNI screening (when samples/experiment allows)

2. To decrease variability a normal transparent 96-well plate **containing all samples (inhibitor/test chemical/control)** can be prepared prior to the actual assay plate setup. 120 µL of each sample is added to each well, giving a surplus volume for better transfer to the assay plate later. Prepare this "pre-plate" according to the desired plate set up. Keep the "pre-plate" covered with a lid at room temp. until transfer of samples to assay plate.

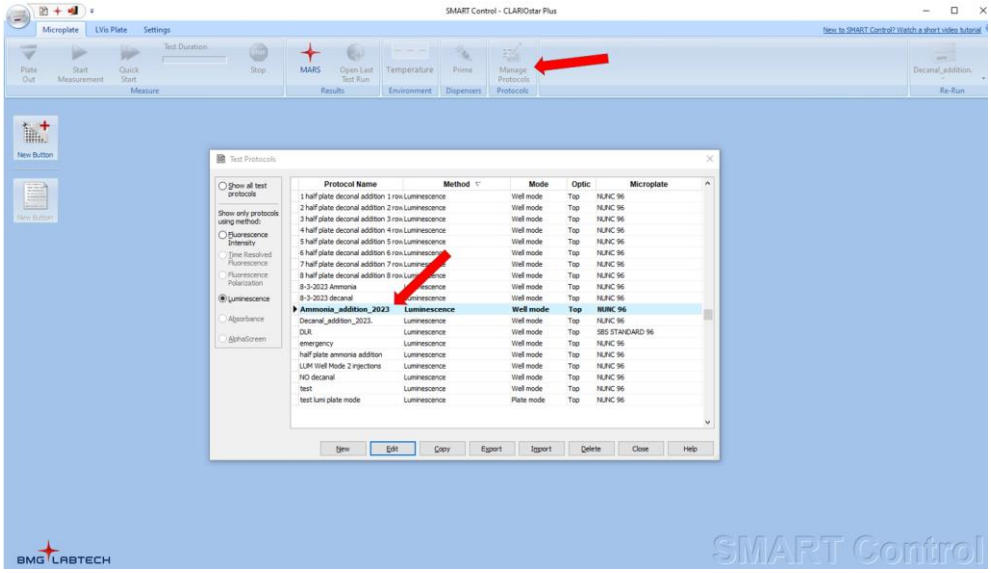
3. The assay is set up in a **white** NUNC 96-well plate.
4. Add 50 µL N-free Ås-medium solution to the "blank" wells.
5. Add 50 µL cell solution to "control" and "inhibitor" wells **using a 8-channel multichannel pipette**.
   Place cell suspension in a clean reagent reservoir. Make sure to gently mix suspension before pipetting into wells.
6. Incubate the microplate in the plate incubator for 10 mins 28 °C.
7. Move the plate to the plate reader.
8. In the SMART CONTROL software:
   Click on "Manage Protocols" in upper panel -> choose protocol "Ammonia_addition_2023". **Make sure that all assay wells have the correct injection volume (50 µL), and that Pump 1 is selected**. Choose "top to bottom" vertical well addition mode (Reading direction). *See guide below.* Run the protocol.
9. Move the plate back to the plate incubator for 5 mins (28 °C) if a full plate (96 wells) is run. Otherwise, the incubation time with ammonium is 20 min i.e. start a 20 min timer when ammonium is added to first well.
10. Add 100 µL inhibitor/test chemical/control to sample wells from the "pre-plate" using a 8-channel pipette. Add samples one row (12 wells) at a time with 1 min. 36 sec. between each row.
11. Move the plate back to the plate incubator for 15 mins (28 °C) if full plate is run (96 wells). If you do not run a full plate, start a **30 min** timer when adding samples to the first well/row.
12. Place the plate in the plate reader. Click on "Manage Protocols" in upper panel -> choose protocol "Decanal_addition_2023". **Make sure that all assay wells have the correct injection volume (25 µL), that Pump 2 is selected, and that the read direction is set correctly (same direction as ammonium and samples were added).** *See guide below.*
13. Run the protocol to add decanal and obtain the bioluminescence readings.
14. Cleaning of the plate reader
    a) Prime pump 1 with Milli-Q water
    b) Prime pump 2 with 50% ethanol and Milli-Q water

SMART CONTROL software guide

Ammonia addition (step 8)

a) Open “Ammonia_addition_2023” protocol

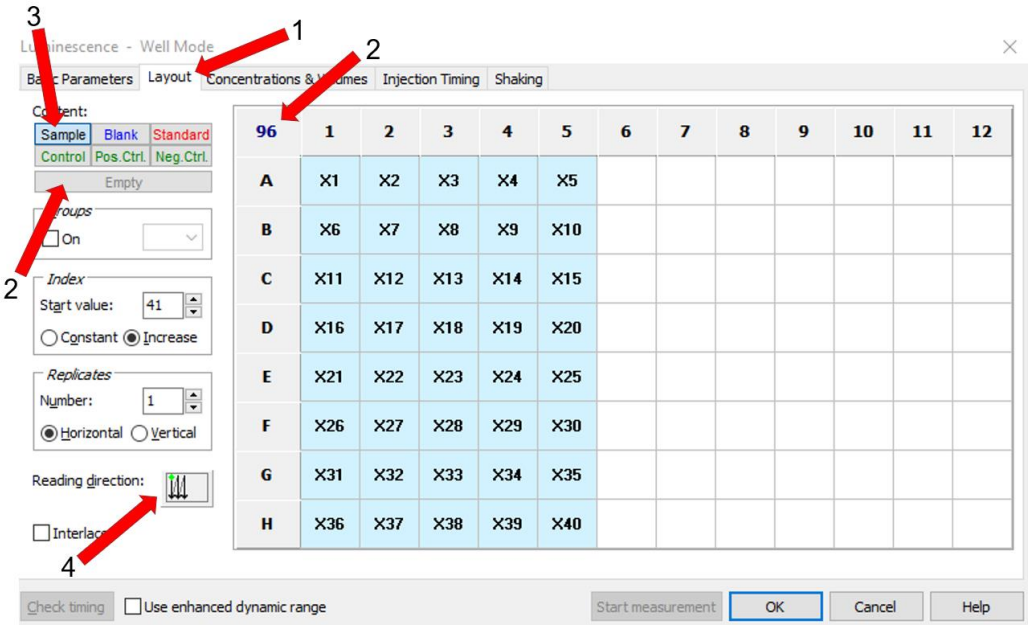
Click on “Manage Protocols” in upper panel -> choose protocol “Ammonia_addition_2023”.



b) Plate setup

Do not change anything in the “Basic Parameters” tab.

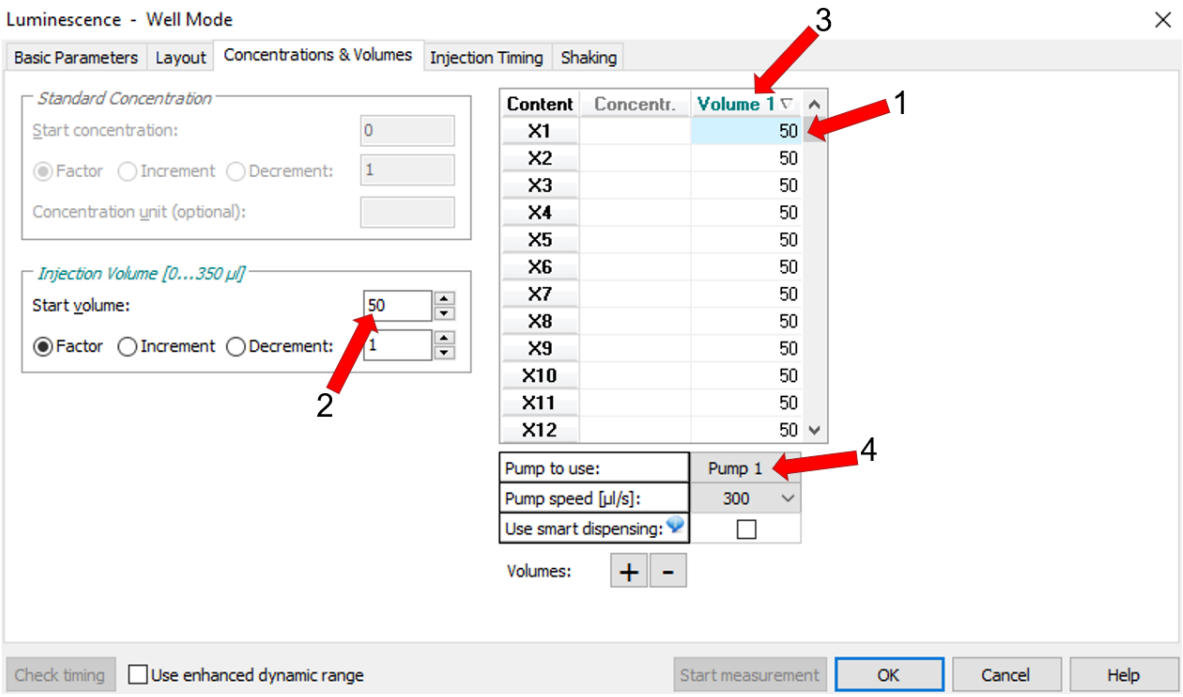
1) Click on the “**Layout**” tab.
2) Clear the plate layout by clicking the “**Empty**” button followed by the “**96**” button in the upper left corner of plate.
3) Mark all wells that should receive ammonium solution as “**Sample**”.
4) Check that the “**Reading direction**” is set correct. This is the sequence/direction of addition that the plate reader will perform.



c) Injection volume and pump selection

1) Check that the injection volume is set to **50 µL** for all wells: Scroll down to **check that all wells** ("Content") you have selected in the "Layout" tab **will receive 50 µL of ammonia solution**.
2) If all wells do not have correct volume, specify the volume by typing "50" in "Start volume".
3) Press the "**Volume 1**" button to mark all wells to receive 50 µL. Check again.
4) Make sure that **Pump 1** is selected.

Initiate protocol by clicking "Start measurement".



Decanal addition (step 12)

a) Open "Decanal_addition_2023" protocol.

Click on "Manage Protocols" in upper panel -> choose protocol "Decanal_addition_2023".

b) Plate setup

Make the plate setup identical to the ammonia addition protocol (see above for screenshots):

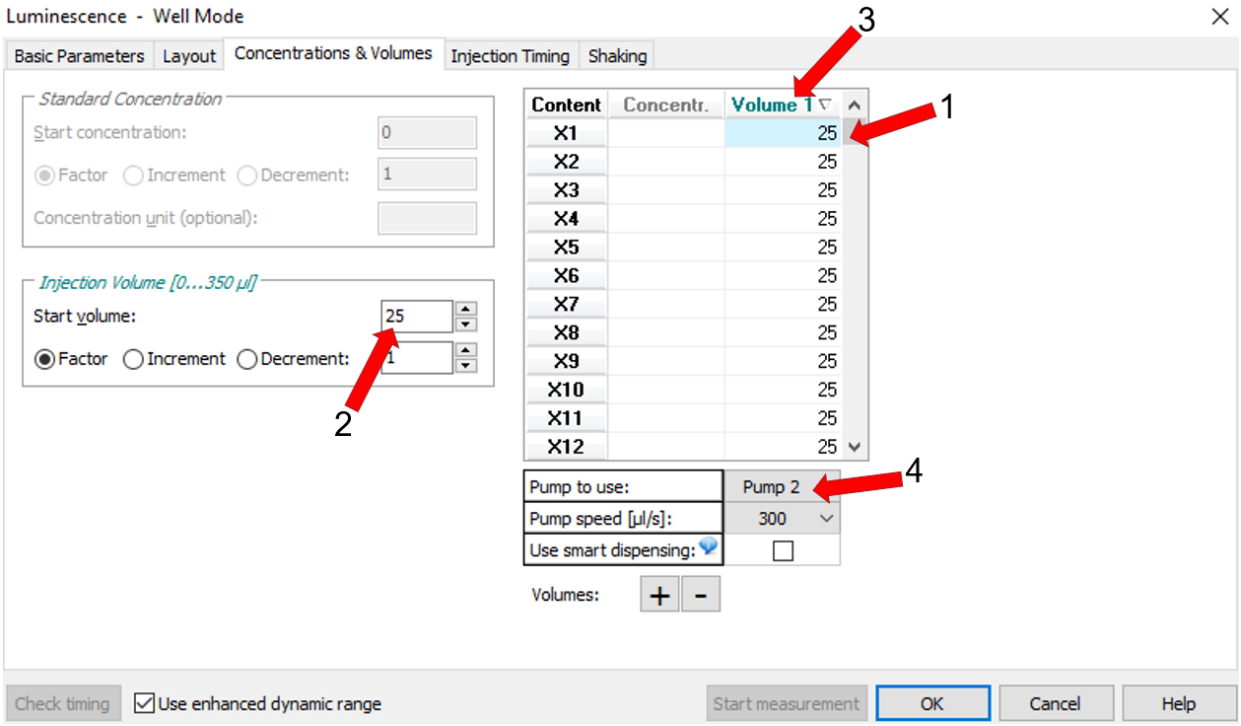
5) Click on the "**Layout**" tab.
6) Clear the plate layout by clicking the "**Empty**" button followed by the "**96**" button in the upper left corner of plate.
7) Mark all wells that should receive ammonium solution as "**Sample**".
8) Check that the "**Reading direction**" is set correct. This is the sequence/direction of addition that the plate reader will perform.

c) Injection volume and pump selection. Same procedure as for ammonia, but injection volume is now **25 µL**.

5) Check that the injection volume is set to **25 µL** for all wells: Scroll down to **check that all wells** ("Content") you have selected in the "Layout" tab **will receive 25 µL of decanal solution**.

6) If all wells do not have the correct volume, specify the volume by typing “25” in “Start volume”.
7) Press the “**Volume 1**” button to mark all wells to receive 50 µL. Check again.
8) Make sure that **Pump 2** is selected.

Initiate the protocol by clicking “Start measurement”.



Literature

Bengtsson-Palme, J. (2018) The diversity of uncharacterized antibiotic resistance genes can be predicted from known gene variants—but not always. *Microbiome* **6**: 125.

Berglund, B., Khan, G.A., Lindberg, R., Fick, J., and Lindgren, P.-E. (2014) Abundance and Dynamics of Antibiotic Resistance Genes and Integrons in Lake Sediment Microcosms. *PLOS ONE* **9**: e108151.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., et al. (2019) Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol* **37**: 852–857.

Brandt, K.K., Holm, P.E., and Nybroe, O. (2006) Bioavailability and toxicity of soil particle-associated copper as determined by two bioluminescent Pseudomonas fluorescens biosensor strains. *Environ Toxicol Chem* **25**: 1738–1741.

Brandt, K.K., Holm, P.E., and Nybroe, O. (2008) Evidence for Bioavailable Copper−Dissolved Organic Matter Complexes and Transiently Increased Copper Bioavailability in Manure-Amended Soils as Determined by Bioluminescent Bacterial Biosensors. *Environ Sci Technol* **42**: 3102–3108.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., and Holmes, S.P. (2016) DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods* **13**: 581–583.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., et al. (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J* **6**: 1621–1624.

Grilo, M.L., Sousa-Santos, C., Robalo, J., and Oliveira, M. (2020) The potential of Aeromonas spp. from wildlife as antimicrobial resistance indicators in aquatic environments. *Ecol Indic* **115**: 106396.

Hansen, M.G., Marcussen, H., Holm, P.E., and Brandt, K.K. (2019) Evaluation of pH-Dependent Metal Speciation Artifacts in Whole-Cell Bioreporter Analysis. *J Environ Qual* **48**: 199–204.

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.

Hynninen, A., Tönismann, K., and Virta, M. (2010) Improving the sensitivity of bacterial bioreporters for heavy metals. *Bioeng Bugs* **1**: 132–138.

Iizumi, T., Mizumoto, M., and Nakamura, K. (1998) A bioluminescence assay using Nitrosomonas europaea for rapid and sensitive detection of nitrification inhibitors. *Appl Environ Microbiol* **64**: 3656–3662.

Korpela, M.T., Kurittu, J.S., Karvinen, J.T., and Karp, M.T. (1998) A Recombinant *Escherichia coli* Sensor Strain for the Detection of Tetracyclines. *Anal Chem* **70**: 4457–4462.

Lassen, S.B., Ahsan, Md.E., Islam, S.R., Zhou, X.-Y., Razzak, M.A., Su, J.-Q., and Brandt, K.K. (2022) Prevalence of antibiotic resistance genes in Pangasianodon hypophthalmus and Oreochromis niloticus aquaculture production systems in Bangladesh. *Sci Total Environ* **813**: 151915.

Lekfeldt, J.D.S., Magid, J., Holm, P.E., Nybroe, O., and Brandt, K.K. (2014) Evaluation of the leucine incorporation technique for detection of pollution-induced community tolerance to copper in a long-term agricultural field trial with urban waste fertilizers. *Environ Pollut* **194**: 78–85.

Liguori, K., Keenum, I., Davis, B., Milligan, E., Heath, L.S., Pruden, A., et al. (2023) Standardizing Methods with QA/QC Standards for Investigating the Occurrence and Removal of Antibiotic Resistant Bacteria/Antibiotic Resistance Genes (ARB/ARGs) in Surface Water, Wastewater, and Recycled Water. *The Water Research Foundation*.

Looft, T., Johnson, T.A., Allen, H.K., Bayles, D.O., Alt, D.P., Stedtfeld, R.D., et al. (2012) In-feed antibiotic effects on the swine intestinal microbiome. *Proc Natl Acad Sci U S A* **109**: 1691–1696.

Mahaney, A.P. and Franklin, R.B. (2022) Persistence of wastewater-associated antibiotic resistant bacteria in river microcosms. *Sci Total Environ* **819**: 153099.

Manaia, C.M., Aga, D.S., Cytryn, E., Gaze, W.H., Graham, D.W., Guo, J., et al. (2024) The Complex Interplay Between Antibiotic Resistance and Pharmaceutical and Personal Care Products in the Environment. *Environ Toxicol Chem* **43**: 637–652.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., et al. (2009) Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. *Appl Environ Microbiol* **75**: 7537–7541.

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.

Stedtfeld, R.D., Guo, X., Stedtfeld, T.M., Sheng, H., Williams, M.R., Hauschild, K., et al. (2018) Primer set 2.0 for highly parallel qPCR array targeting antibiotic resistance genes and mobile genetic elements. *FEMS Microbiol Ecol* **94**: fiy130.

Tom-Petersen, A., Hosbond, C., and Nybroe, O. (2001) Identification of copper-induced genes in Pseudomonas fluorescens and use of a reporter strain to monitor bioavailable copper in soil. *FEMS Microbiol Ecol* **38**: 59–67.

Virolainen, N.E., Pikkemaat, M.G., Elferink, J.W.A., and Karp, M.T. (2008) Rapid detection of tetracyclines and their 4-epimer derivatives from poultry meat with bioluminescent biosensor bacteria. *J Agric Food Chem* **56**: 11065–11070.

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**: lxae302.

Zhao, Y., Su, J.-Q., An, X.-L., Huang, F.-Y., Rensing, C., Brandt, K.K., and Zhu, Y.-G. (2018) Feed additives shift gut microbiota and enrich antibiotic resistance in swine gut. *Sci Total Environ* **621**: 1224–1232.

Zhu, Y.-G., Zhao, Y., Li, B., Huang, C.-L., Zhang, S.-Y., Yu, S., et al. (2017) Continental-scale pollution of estuaries with antibiotic resistance genes. *Nat Microbiol* **2**: 1–7.

Protocol 2: *Pseudomonas fluorescens* DF57-Cu15 assay for determination of bioavailable copper

OVERVIEW

This instruction is designated for the work with the copper reporter strain **DF57-Cu15** but can also be utilized for the general toxicity reporter **DF57-40E7**, which is often used as a control strain to allow for evaluation of sample matrix effects (Brandt et al. 2008, EST).

OBSERVATIONS

1. If strain **DF57-40E7** is to be used solely as a toxicity biosensor, higher concentrations of Cu must be used for the Cu standards.
2. Copper adheres to glass. For growing bacteria and handling samples and standards use sterile plastic tubes.
3. Check lists of materials per step and solutions and chemicals at the end.

Sediment/soil sample preparation:

- For each sample, **1 g** of sediment/soil is mixed with 5 mL of mili-Q water in 15 ml falcon tubes, followed by 2 hours of shaking on a horizontal shaker (250 rpm, 22 °C).
- The supernatant is then collected after centrifugation (10000g, 22°C) for 10 minutes and stored at -20 °C until further use.

Reactivation of the DF57-Cu15 strain and DF57-40E7 (also to be used for Zn)

1. Get the P. fluorescens DF57-Cu15 and **DF57-40E7** glycerol stock from -80°C freezer and place in an ice bucket to avoid thawing (be quick)
2. Using a sterile loop, scrape some of the culture from the frozen tube and streak it on LB agar with kanamycin (25 µg/ml)
3. Place plate in a plastic bag, label and allow to incubate for 2-3 days at 28-30˚C
4. Afterwards, the plates can be stored in a refrigerator for up to one week

Cell cultivation prior to biosensor analysis

Day 1

1. Add 25ml of DMM-Cu to a 50 ml Falcon tube
2. Add 250µl glucose 40% (final concentration 0,4%)
3. Add 25µl kanamycin (25 µg/ml) and swirl to mix
4. Using a sterile loop, pick a single colony of DF57-Cu15 and **DF57-40E7** from the agar plates, transfer to the liquid media and mix
5. Place tube in a plastic bag, label and leave overnight in horizontal position on a shaker at 150-200 rpm, at room temperature

Day 2

1. Add 25ml of DMM-Cu to a 50 ml Falcon tube
2. Add 250µl glucose 40% (final concentration 0,4%)

3. Add 25µl kanamycin (25 µg/ml) and swirl to mix
4. Add 25µl culture from the day before as inoculum to the liquid media and mix
5. Place tube in a plastic bag, label and leave overnight in horizontal position on a shaker at 150-200 rpm, at 28-30˚C incubator (room temperature if leaving more time)

Copper standards

1. Make a dilution series of $CuSO_4$ in Milli-Q water to get the following final Cu concentrations:

1) 1.250 µM	**4)** 0.156 µM	**7)** 0.026 µM	**10)** 0.010 µM
2) 0.625 µM	**5)** 0.078 µM	**8)** 0.020 µM	**11)** 0.005 µM
3) 0.313 µM	**6)** 0.039 µM	**9)** 0.013 µM	**12)** 0 µM

* For $CuSo_4$ pentahydrate (249,68 g/mol), 1 µM = 249,68 µg/L and 1,25 µM = 312,1 µg/L
* use figure at the end as a guide

Testing the DF57-Cu15 culture

6. Measure OD_{600} of the overnight culture and make sure it is between 0.2 - 0.4
    a. Add about 1ml water (or pure DMM-Cu medium) to a plastic cuvette
    b. Use this to calibrate the machine
    c. Add about 1ml culture in liquid media
    d. Insert in the machine and measure value
7. Dilute a sample of culture down to OD_{600}=0,167 in a plastic cuvette
    a. Calculate proportional volume corresponding to OD_{600} = 0,167
    b. Pipette this volume of culture into a new plastic cuvette
    c. Add media completing 1ml
    d. Measure OD_{600} again to check
8. Transfer 500µl of this dilution into a 2ml Eppendorf tube
9. Add 500µl Milli-Q water to the Eppendorf
10. Measure bioluminescence as described in the protocol for the old luminometer (Bio-Orbit 1253)
11. If bioluminescence is higher than 5 relative light units (RLU), stop the experiment (only for strain DF57-Cu15); proceed if bioluminescence is lower than 5 RLU

Preparation of cells

1. Centrifuge the DF57-Cu15 culture Falcon tube at 5000g for 10min at room temperature
2. Pour/suck off the supernatant carefully, making sure to remove all the liquid
3. Resuspend cells in fresh Medium 4 to obtain an OD_{600} = 0,167 (Requirements are approximately 10 ml per plate, and therefore 20 ml for **DF57-40E7 since it is used as matrix control for both Zn and Cu.**
4. Now the cell suspension can be left on the workbench for up to an hour prior to biosensor incubation in samples or standards

Plate preparation

1. Use white microtiter plates to prevent light contamination between wells

2. Make sure you are using the plate type specified in the luminometer method file
3. First, pour 100µl samples or 100µl standards in separate wells of a microtiter plate
4. Note the time before starting to add the cells
5. Add 100µl cell suspension to either the samples or the standards in the microtiter plate, in the same order as they will be analyzed in the luminometer (A1A2A3… B1B2B3…)
6. Incubate the microtiter plate at room temperature without shaking for 90min
7. Measuring bioluminescence should not take more than 1h, since bioluminescence is stable within a timeframe within 2½h after onset of incubation

Procedure for measuring bioluminescence

1. Have fresh aldehyde solution ready in a small glass bottle covered with parafilm
2. Turn on the plate reader
3. Turn on the computer and open the program FLUOstar
4. Turn on the air suction device and place it above the plate reader
5. Set **Reader configuration** to **Luminescence mode**
6. Position the blue labelled cable L (luminometry) on top (check the guide label on the plate reader)
7. The second cabling (red, yellow and green) is not used
8. Set the temperature to 25°C
9. Place a bottle with MilliQ water in the pump chamber and insert the needle in it
10. Place a small empty bottle in the chamber and insert the other tube in it to collect the outflow
11. Rinse pump and tubing with 4ml of water
12. Replace the water bottle with a 50% ethanol bottle and insert the needle in it
13. Rinse pump and tubing with 4ml 50% ethanol to avoid air in tubing
14. Replace the ethanol bottle with the aldehyde one and insert the needle into the parafilm
15. Right before use, rinse the tubing with 2ml aldehyde solution
16. Collect flow through and move the waste bottle to the fume hood (the aldehyde stinks!)
17. Insert the injection needle so that the aldehyde solution will be injected in the microtiter plate to be analyzed
18. Close the cover of the pump chamber
19. Insert the plate with the samples + biosensors with position A1 in the top left corner
20. Choose the correct method under the menu **Test setup**, called **DF57-Cu15/40E7**
21. If running solid phase-contact biosensor assays, choose the version with shaking, **DF57-Cu15/40E7 shake**
22. Click the tab **Concentration/Volume/Shaking** to check if there is 25µl substrate added to all wells on the plate
23. Check whether the reading direction of the wells is correct (Well A1-A12, B1-B12, C1-C12, etc.)
24. Optionally, adjust configurations for filled/empty wells, shaking, number of cycles and interval between them, reading start delay, etc
25. Start measuring

26. **IMPORTANT: Do NOT touch the cover of the pump chamber during measurement!** As long as the cover is opened no individual measurements will be recorded
27. After the last measurement, repeat the rinsing steps with 4ml ethanol first and then 4ml MilliQ water
28. Collect flow through and move the waste bottle to the fume hood
29. All data is automatically saved in an Excel file which can be opened by clicking on the Excel icon in FLUOstar
30. Shut down the computer
31. Shut down the plate reader

MATERIALS

Have everything at hand before starting the procedure.

Step	**Day**	**Qt.**	**Item**
Reactivation of the DF57-Cu15 strain	-	1	Agar plate + kanamycin (25 µg/ml)
		4	Sterile loops
		1	Ice bucket
		1	Frozen bacterial stock
		1	Plastic bag
Cell cultivation prior to biosensor analysis	1	1	Bacterial strain on agar plate
		1	50ml Falcon tube
		25ml	DMM-Cu
			Glucose
			Kanamycin
		1	Sterile loop
		1	Plastic bag
	2	1	Falcon tube with bacterial culture on liquid media from day before
		1	50ml Falcon tube

		-	DMM-Cu
		-	Glucose
		-	Kanamycin
		1	Plastic bag
		1	pipette + tip for 25µl
Copper standards	-		$CuSo_4$
			MilliQ water
		-	Tubes
Testing the DF57-Cu15 culture	-	1	Falcon tube with bacterial culture on liquid media
		-	Pure DMM-Cu media
		3	Plastic cuvette
		1	Pipette + tip for 500µl - 1ml
		-	2ml Eppendorf tube
		-	MilliQ water
		1	Glass beaker
Preparation of cells	-	1	Falcon tube with bacterial culture on liquid media
		-	Pipette + tip
		1	50ml Falcon tube
		-	Medium 4
Plate preparation	-		Black microtiter plate
			Samples
			Cu standards

			Biosensor culture
			100 µl pipette + tips
Procedure for measuring bioluminescence	-		Prepared microtiter plate
			Aldehyde solution
			MilliQ water
			50% ethanol
			Small glass bottle for waste

MEDIA AND SOLUTIONS

Aldehyde Solution

1. Add 5ml 96% ethanol to a 25ml glass bottle
2. Dissolve 35µl n-decanal in the ethanol
3. Add 5 ml Milli-Q water up to a total volume of 10,035ml
   * the solution may be used for one day only
   * keep protected from light

DMM – Cu (i.e. DMM without Cu)

1. Dissolve premixed DMM (Difco) in Milli-Q or double distilled water
2. Add 1ml of trace element solution without Cu per 1L media
3. Autoclave for 20min in Redcap flasks
4. After autoclaving (preferably at the day of use), add glucose to get a final concentration of 0,4% (250µl 40% glucose for 25ml medium)
5. Add kanamycin to a final concentration of 25 µg/ml (25µl kanamycin (25mg/ml) per 25ml medium)

Medium 4

1. For 1 liter of Medium 4 use:

- 7.46g KCl
- 4.19g MOPS
- 1.0g $(NH_4)_2SO_4$
- 1.22g β-glycerofosfat-di natrium salt × 5 H2O (Cat number 157241 [819-83-0])

2. Fill up with Milli-Q water to 1000ml
3. Adjust pH to 7,2 with NaOH
4. Autoclave for 20min in Redcap flasks
5. After autoclaving (preferably at the day of use), add glucose to a final concentration of 0.8% (500µl 40% glucose per 25ml medium)
   * No kanamycin added to this medium

Specifications of Medium 4

- 100mM KCl
- 20mM MOPS (pH 7,2)
- 7.6mM $(NH_4)_2SO_4$
- 4 mM Glycerophosphate
- 0.8% (wt/vol) glucose

CHEMICALS

Chemical	Name	CAS	#	Room	Obs.
Kanamycin	Kanamycin monosulfate	25389-94-0	233	219	Stock solution 25mg/ml in the freezer in R224
DMM (Difco)	Davi`s Minimal Broth without dextrose	-	294	219	
Trace element solution					Stock solution in freezer
Glucose	D+ Glucose	50-99-7	169	219	Stock solution 40% in freezer in R224
KCl	Potassium chloride	7447-40-7	217	219	
MOPS	4-Morpholine propane sulfonic acid	1132-61-2	295,1	219	
$(NH_4)_2SO_4$	Ammonium sulfate	7783-20-2	44	219	
Glycerol diphosphate	Disodium β-glycerophosphate	819-83-0	-	219	Fridge
NaOH	Sodium hydroxide	1310-73-2	322	219	
CuSO4	Copper (II) sulfate pentahydrate	7758-99-8	237	219	
n-decanal	n-Decyl aldehyde	112-31-2	126,5	219	Fridge

Protocol 3: *Pseudomonas putida* KT2440 (pDNP...lux) assay for determination of bioavailable zinc/lead/cadmium

OVERVIEW

This instruction is designated for the work with the following zinc reporter strains (which should I use?):

- **A KT2440 (pDNPczc1lux):** more sensitive
- **B KT2440.2431 (pDNPczc1lux)**: more sensitive, transporter deficient
- **C KT2440 (pDNPcadA1lux):** Zn specific
- **D KT2440.2431 (pDNPcadA1lux):** Zn specific, transporter deficient

The *Pseudomonas fluorescens* **DF57-40E7** can be used as a control strain to allow for evaluation of sample matrix effects, using the same media and replacing the antibiotic tetracycline for kanamycin.

OBSERVATIONS

1. The zinc biosensor can be measured every half hour
2. HMM media should only be prepared at the day of use
3. Lists of materials and solutions and chemicals available at the end

PROTOCOLS

Reactivation of the KT2440 strain, include DF57-40E7 as matrix control (see Cu protocol)

1. Get the *P. putida* KT2440 glycerol stock from -80°C freezer and place in an ice bucket to avoid thawing (be quick)
2. Using a sterile loop, scrape some of the culture from the frozen tube and streak it on LB agar with tetracycline (12µg/ml)
3. Place plate in a plastic bag, label and allow to incubate for 2-3 days at 28-30˚C
4. After growth, the plate can be stored in a refrigerator for up to one week

Cell cultivation prior to biosensor analysis

Day 1

1. Add 25ml of HMM medium to a 50ml Falcon tube
2. Add 30µl tetracycline (12µg/ml) and swirl to mix
3. Using a sterile loop, pick a single colony of KT2440 from the agar plate, transfer to the liquid media and mix
1. Place tube in a plastic bag, label and place in incubator overnight, in horizontal position on a shaker at 150-200 rpm, at 28°C

Day 2

2. Add 25ml of HMM medium to a 50ml Falcon tube
3. Add 30µl tetracycline (12µg/ml) and swirl to mix
4. Add 250µl culture from the day before as inoculum to the liquid media and mix

5. Place tube in a plastic bag, label and place in incubator overnight, in horizontal position on a shaker at 150-200 rpm, at 28°C
4. The zinc-starved cultures are now sensitized to subsequent zinc exposure and thus ready for use as biosensors

Zinc standards

1. Make a dilution series of $ZnSO_4$ in Milli-Q water to get the following final Zn concentrations:

1) 100 µM	**4)** 32 µM	**7)** 8 µM	**10)** 1 µM
2) 64 µM	**5)** 24 µM	**8)** 4 µM	**11)** 0,5 µM
3) 50 µM	**6)** 16 µM	**9)** 2 µM	**12)** 0 µM

* For $ZnSO_4$ heptahydrate (287,56 g/mol), 1 µM = 287,56 µg/L and 0,72 µM = 207,04 µg/L

Sample preparation for Cu and Zn bioavailability:

- For each of the 24 collected samples, **1 g** of soil is mixed with 5 mL of mili-Q water in 15 ml falcon tubes, followed by 2 hours of shaking on a horizontal shaker (250 rpm, 22 °C).
- The supernatant is then collected after centrifugation (10000g, 22°C) for 10 minutes and stored at -20 °C until further use. (a total of approximately 300ul is needed for each sample suspension, which amounts a total of 1200 ul for all assays)

Preparation of cells

1. Centrifuge the KT2440 culture Falcon tube at 5000g for 10min at 20°C
2. Pour/suck off the supernatant carefully, making sure to remove all the liquid
3. Resuspend cells in ~10ml fresh HMM medium (without tetracycline)
4. Measure OD_{600}
    a. Add about 1ml pure HMM medium to a plastic cuvette
    b. Use this to calibrate the machine
    c. Add about 1ml culture in liquid media
    d. Insert in the machine and measure value
5. Dilute a sample of culture down to OD_{600}= 0,015-0,020
    a. Divide measured OD_{600} by desired OD_{600} to find dilution factor
    b. Divide new media volume by dilution factor to find volume of culture to be transferred
    c. Measure OD_{600} again to check

Plate preparation

1. Use white microtiter plates to prevent light contamination between wells
2. Make sure you are using the plate type specified in the luminometer method file
3. First, pour 100µl samples or 100µl standards in separate wells of the plate (2 wells for each, 1 on top of the other (A1B1, A2B2 etc)
4. Note the time before starting to add the cells
5. Add 100µl cell suspension to either the samples or the standards in the microtiter plate, in the same order as they will be analyzed in the luminometer A1-A12, B1-B12, C1-C12, etc.)

6. Incubate the microtiter plate with lid at 28°C for 1,5h
7. Proceed to the luminescence measuring

Procedure for measuring bioluminescence

1. Turn on the plate reader
2. Turn on the computer and open the programme FLUOstar
3. Set **Reader configuration** to **Luminescence mode**
4. Position the blue labelled cable **L** (luminometry) on top (check the guide label on the plate reader)
5. The second cabling (red, yellow and green) and the needle are not used
6. Close the cover of the pump chamber
7. Set the temperature to 28°C
8. Insert the plate without lid with the samples + biosensors with position A1 in the top left corner
9. Choose the correct method under the menu **Test setup**, called **?????**
10. Check whether the reading direction of the wells is correct (A1-A12, B1-B12, C1-C12, etc.)
11. Set number of cycles to 5 or 6 (~30min each)
12. Optionally, adjust configurations for filled/empty wells, shaking, interval between cycles, reading start delay, etc
13. Start measuring
14. **IMPORTANT: Do NOT touch the cover of the pump chamber during measurement!** As long as the cover is opened no individual measurements will be recorded
15. All data is automatically saved in an Excel file which can be opened by clicking on the Excel icon in FLUOstar
16. Leave the machine measuring through the night
17. Take the plate out the following morning
18. Shut down the computer
19. Shut down the plate reader

CALCULATIONS

Induction coefficient (IC) = L_M / L_W

L_M = Luminescence in metal solution

L_W = Background luminescence in MQ water

MEDIA AND SOLUTIONS

HMM (heavy metal MOPS) medium

The HMM medium contains the following chemicals and concentrations. Each color in the table indicates compounds to me mixed in a separate stock solution (7 different solutions in total); the specified amount should be dissolved in the specified volume of MilliQ water. Then, the specified volume of each solution should be mixed to make the media, preferably at the day of use. The remaining volume should be filled with sterile MilliQ water. All the solutions

should be filter sterilized, except for $FeCl_3$, which can be autoclaved. Tetracycline should only be added right before use.

1. 40mM MOPS (pH 7,4)
2. 50mM KCl
3. 10mM NH_4Cl
4. 0,5mM $MgSO_4$
5. 0,4% glucose
6. 1 mM glycerol-2-PO_4
7. 1 µM $FeCl_3$
8. 0,2 µg/ml Thiamine
9. 0,05% Casein hydrolysate
10. 12 µg/ml Tetracycline

Conc/ conc. HMM	Chemical	Amount	Volume H_2O	Concentration	Volume to make 100ml HMM	Obs.	Storage
10x	**MOPs**	8,3704g	100ml	83.704 g/L	10ml	Adjust pH to 7,2 with KOH	Dark, 4°C
	KCl	3,7275g		37,275 g/L			
	NH4Cl	0,535g		5,35 g/L			
	MgSO4	0,06018g		0,6018 g/L			
	Casein hydrolysate	0,5g	100ml	0,5%	10ml		
100X	**Glycerol-2-PO4**	1,09	50ml	21,807 g/l	1ml		Dark, -20°C
	Glucose	20g	50ml	40%	1ml		
1000x	**Tetracycline**	0,6g	50ml	12mg/ml	100µl		
	Thiamine	0,01g	50ml	200 mg/l	100ul		
10000x	**FeCl3**	0,01622g	100ml	0,1622g/l	10µl		Dark, room temperature

CHEMICALS

Chemical	Name	CAS	#	Room	Mol. weight	Obs
Tetracycline	Tetracycline hydrochloride	64-75-5		212		Freezer; stock solution 10mg/ml in freezer in R224
MOPS	4-Morpholine propane sulfonic acid	1132-61-2	295,1	219	209,26 g/mol	
KCl	Potassium chloride	7447-40-7	217	219	74,55 g/mol	
NH_4Cl	Ammonium chloride	12125-02-9	38	219	53,50 g/mol	
$MgSO_4$	Magnesium sulfate	7487-88-9	261	219	120,36 g/mol	
Glucose	D+ Glucose	50-99-7	170	219		
Glycerol-2-PO_4	Disodium β-glycerophosphate	819-83-0	-	219	218,07 g/mol	Fridge
$FeCl_3$	Iron (III) chloride	7705-08-0	213,1	219	162,20 g/mol	
Thiamine	Thiamine hydrochloride	67-03-8	443	219	337,30 g/mol	
Casein hydrolysate	Casein hydrolysate	65072-00-6	95,1	219		
$ZnSO_4$	Zinc sulfate heptahydrate	7446-20-0	481	219	287,54g/mol	
$ZnCl_2$	Zinc chloride	7646-85-7	480	219	136,27g/mol	

MATERIALS

Have everything at hand before starting the procedure.

Step	Day	Qt.	Item
Reactivation of the KT2440 strain	-	1	Agar plate + tetracycline (12 µg/ml)
		4	Sterile loops
		1	Ice bucket
		1	Frozen bacterial stock
		1	Plastic bag
Cell cultivation prior to biosensor analysis	1	1	Bacterial strain on agar plate
		1	50ml Falcon tube
		25ml	HMM medium
			Tetracycline
		1	Sterile loop
		1	Plastic bag
	2	1	Falcon tube with bacterial culture on liquid media from day before
		1	50ml Falcon tube
		25ml	HMM medium
			Tetracycline
		1	Plastic bag
		1	pipette + tip for ~20µl

Zinc standards	-		$ZnSO_4$
			MilliQ water
			Tubes
Testing the DF57-Cu15 culture	-	1	Falcon tube with bacterial culture on liquid media
			HMM medium
		3	Plastic cuvette
		1	Pipette + tip for 500µl - 1ml
Preparation of cells	5	1	Falcon tube with bacterial culture on liquid media
			Pipette + tip
		1	50ml Falcon tube
			HMM medium
Plate preparation	-		White microtiter plate
			Samples
			Cu standards
			Biosensor culture
			100 µl pipette + tips
Procedure for measuring bioluminescence			Prepared microtiter plates

Protocol 4: Bacterials productivity (growth) measured by the [^{3}H]Leucine incorporation technique

OBSERVATIONS

- ➔ This protocol contains instructions to both the Leucine incorporation and the pollution induced community tolerance (PICT) assays, which are very similar with the exception of a few extra steps for the second. The steps for the first are numbered and in black. The additional steps necessary for the PICT assay are included between the lines, in red text marked with stars
- ➔ There are 2 different possibilities when doing the PICT:
  - I. Full dose-response curve: requires ~10 concentrations for each toxicant tested; recommended when there is little information available on the sample and/or at initial steps of experiments; results in a high number of test tubes, which might limit the number of replicates (both biological and technical) that can be tested at a time
  - II. Brief version: only ~3 concentrations used for each toxicant; can be used with systems that are better known, or after an initial full dose-response; because it requires less time/work, it allows for the testing of more replicates, thus increasing reliability of data
- ➔ For the PICT assay, the amount of sample/solution necessary will vary with the experiment; the number of test vials needed for each microcosm/sample tested is equal to:

(# of toxicants X # of concentrations X # of technical replicates)

+ at least 2 no toxicant blanks (concentration 0)

- ➔ The incubation time after the Leu is added depends on the bacterial activity in the sample; more activity requires less incubation time and vice-versa
- ➔ Steps marked with an **F** should be done in the fume hood
- ➔ The procedure involves the analytes, dead controls and blank controls. Steps are the same for them all unless stated otherwise

★ Prepare the toxicant solutions in MilliQ water or MES buffer

1. Add 1g soil from each microcosm to a 15ml Falcon or round bottom tube
   ★ or enough soil for PICT
2. Add 10ml MilliQ water and mix manually (for other soil amounts, add 10x water)
   ★ or use MES buffer with the desired pH instead of MilliQ water for PICT
3. Shake the tubes on a multi shaker at maximum speed for 3min at room temperature
4. Move them to a centrifuge and centrifuge at 1000×g for 10 min at room temperature
5. Carefully pipette 1,5ml supernatant (extracted bacteria) to 2ml tubes, making 2 small tubes (replicates) for each microcosm
   ★ or the required number calculated for PICT
6. In addition to the analytes, pipette 1,5ml supernatant to at least 4 tubes, to make dead controls (negative controls)
   ★ add 50µl toxicant solution to each 2ml tube, a different toxicant and concentration for each tube or tube replicates
   ★ Incubate for 30min
7. **F** Move to the fume hood, covering the surface with spilling-protection paper
8. **F** Add 160µl ice-cold **50%** TCA to each dead control tube
9. **F** Add 50µl [^{3}H]Leu (6,4µM) to each tube, including controls, and vortex
10. **F** Add 50µl [^{3}H]Leu (6,4µM) to 2 extra empty tubes (blank controls, skip to step 24)
11. Incubate for 1 - 4h in the dark at room temperature
12. **F** Add 160µl of ice-cold **50%** TCA to the analytes and vortex to stop the incubation and precipitate
13. Place in the fridge (4°C) for at least 30min (up to 2 weeks)
14. Move the tubes to a micro-centrifuge
15. Orient each tube in the rotor with the hinge up, so the pellet will settle in the same position in all tubes
16. Centrifuge the samples at 20.000×g for 10 min at 4°C
17. **F** Following centrifugation, remove the supernatant (radioactive chemical waste) very carefully, with the aid of a needle connected to an air pump, and discard it
    * do not get the needle too close to the pellet (^{3}H-leucine incorporated into protein)
18. **F** Add 1,5 ml ice-cold **5%** TCA to each tube (including controls) and vortex-mix
19. Repeat the centrifugation step
20. **F** Remove and discard supernatant using the air pump
21. **F** Add 1,5 ml ice-cold 80% ethanol to each tube (including controls) and vortex-mix
22. Repeat the centrifugation step again
23. **F** Remove and discard supernatant using the air pump
24. **F** Add 200µl 1M NaOH to each tube (including controls) and vortex brief and softly

25. **F** Add 200µl 1M NaOH to 4 additional tubes to make controls
26. Leave 1h in the oven at 90°C with a heavy metal plate on top of the tubes to prevent the lids from opening
27. Allow the samples to cool down
28. **F** Add 1ml of scintillation liquid to each tube (including controls and blanks) and vortex-mix
29. Take the samples to the scintillation reader
30. Place the tubes into the reader's individual scintillation vials, in the reading tray
31. Add your protocol flag to the reading tray and set it to the 'not-read' position
32. Start the scintillation counting by 5min protocol

CHEMICALS

Chemical	Name	CAS	#	Room	Obs
TCA	Trichloroacetic acid	76-03-9	449	219	Stock solutions (5 and 50%) in fridge in D226
[^{3}H]Leu	L-Leucine	61-90-5			Stock solution in fridge in D226
80% ethanol	Ethanol				Stock solution in fridge in D226
NaOH	Sodium hydroxide	1310-73-2	322	219	Stock solution in fridge in D226
Scintillation liquid	Optiphase 'hisafe				
MES buffer	2-Morpholinoethanesulfonic acid	4432-31-9	277	219	

Preparation of radiolabeled [^{3}H]Leucine solution

Dissolve 167,94mg L-Leucine (CAS 61-90-5) in 100ml MilliQ water in a 100ml glass bottle (concentration 12,8mM)

In a 15ml Falcon tube, add 0,5ml of the 1st solution to 9,5ml MilliQ water (20x dilution) (concentration 0,64mM)

In a 50ml Falcon tube, add 0,5ml of the 2nd solution to 49,5ml MilliQ water (100x dilution) (concentration 6,4µM)

Distribute 12ml in each of 4 15ml Falcon tubes

Add 39µl radiolabeled [3H] Leucine to each tube

- 12ml 6,4µM L-Leucine
- 39µl radiolabeled [3H] Leucine (185MBq/5ml)

* 1443kBq per 12ml, 6,01kBq per 50µl

Store in freezer

MATERIALS AND EQUIPMENT

Have everything at hand before starting the procedure.

Item	
15ml Falcon or round bottom tubes (1 per soil microcosm)	Multi-pipette + TCA tip
MilliQ water	[^{3}H]Leu (6.4µM)
10ml pipette + tip	Micro centrifuge
Multi shaker	Air pump + radioactive disposal flask
Centrifuge	Multi-pipette + ethanol tip
1,5ml pipette + tip	200µl pipette + tip
2ml tubes (2 per soil microcosm + controls + blanks)	Incubation oven
Spilling-protection paper	Scintillation reader
50% TCA	Plastic bag

Protocol 5: Isolation of bacteria from aquatic environments.

Plate preparation:

- Prepare Reasoner's 2A (R2A) agar plates for heterotrophic bacteria.
- Prepare Ampicilin Dextrin Agar (ADA) plates for cultivation of Aeromonas species.

Bacterial isolation:

1. If sediment samples, bacteria can be recovered by shaking 1 g of sediment sample in 9 mL of 0.9% NaCl solution for one hours at 150 rpm at room temperature. Bacteria in water samples can be recovered directly from the water.
2. Remove 0.1 mL of the suspension (either sediment mix from step 1 or the water sample) with a sterile pipette and transfer it to a 0.9-mL deionized water. Vortex thoroughly, and label as "B".
3. Repeat this dilution step three times, each time with 0.1 mL of the previous suspension and a 0.9-mL deionized water blank. Label these sequentially as tubes C, D, and E. This results in serial dilutions of 10^{-1} through 10^{-5} grams of sediment per mL
4. Spread plate the suspensions (B, C, D and E) on R2A plates and/or ADA plates in triplicates by transferring 100 µL of the suspensions.
5. Incubate for 3-7 days at room temperature, or until colonies are formed. **Note:** If the goal is to isolate *Aeromonas* spp. On ADA medium, plates should be incubated at 37°C for 24h.
6. Count and record the number of bacterial colonies. Only count plates with 30-200 well-separated colonies per plate.

Isolation of Pure Cultures

7. Randomly select individual bacterial colonies from selected plates. Use a high dilution plate, as it tends to have pure colonies that are well-separated. Choose only colonies that are well-separated from neighboring colonies and look morphologically distinct from each other. **Note:** If the goal is to isolate *Aeromonas* spp., ADA medium should be used and only yellow-colored colonies should be selected.
8. Choose desired number of colonies per plate replicate and re-streak (twice) on the same media to obtain pure colonies.
9. After final plating, transfer the isolates to 30% (v/v)glycerol and store at -80 °C for further analysis

Protocol 6: Phenotypical resistance typing of bacterial isolates

Overview

The instructions describe how to prepare microtiter plates containing several pure culture isolates and how to use this plate setup to screen the isolates for phenotypical resistance.

Protocol

Transfer of isolates to Master microtiter plates (see note on Aeromonas spp. isolates below)

Materials: Frozen isolates in cryotubes, R2A plates, microtiter plates, R2B media, toothpicks, and glycerol.

- All isolates should be streaked onto R2A plates using a toothpick to check for viability and purity. It is possible to fit ten isolates per agar plate.
- After, they can be transferred to liquid R2A directly in a microtiter plate (200ul per well); remember to note the exact position of each isolate and make room for blanks without any growth.
- Incubate the microtiter plate at 28C, 200 rpm for 1-2 days, check and note visible growth in each well.
- After incubation, glycerol can be added to each well in the microtiter plate (final concentration 30%) or the isolates can be directly tested for resistance (see "Testing isolates for resistance" below).
- Store master plates at -80 C.

Make a copy of the Master microtiter plate (this is the plates you will use for screening your isolates to minimize contamination of the Master plate).

Materials: microtiter plates containing fresh R2A media (100-150 ul per well), glycerol, the 96 pin replicator, 70 % EtOH, 96% EtOH , glass tray + lid, gas burner and omnitray plate containing R2A (optional).

- Sterilize the 96 pin replicator by first washing it in 70 % EtOH, then emerge it into the glass tray with 96% EtOH – light it on fire/burn it using the gas burner (watch out for the flame), repeat 3 times and leave it in a sideways position in the LAF bench for cooling down.
- Once it is cooled down, dip it into the frozen Master microtiter plate and place it into a new microtiter plate containing fresh R2A media (copy plate). Optional: At the same time, you can transfer your isolates to an omnitray plate containing R2A to check for viability/growth on solid media.
- Incubate the copy microtiter plates at 28C, 200 rpm for 1-2 days, check and note visible growth in each well.

- After incubation, the isolates are ready for resistance testing.

Testing isolates for resistance (see note on Aeromonas spp. isolates below):

Materials: R2A omnitray plates containing antibiotics or metals of interest, non-selective R2A omnitray plates, the 96 pin replicator, 96% and 70 % EtOH, glass tray + lid and gas burner

- Sterilize the 96 pin replicator by first washing it in 70 % EtOH, then emerge it into the glass tray with 96% EtOH – light it on fire/burn it using the gas burner (watch out for the flame), repeat 3 times and leave it in a sideways position in the LAF bench for cooling down.
- Once it is cooled down, dip it into the frozen copy of your master microtiter plate and move it a bit around – stamp the 96 pin replicator onto selective omnitray plates (I usually do two plates per dip, but it might be possible to do more).
- Sterilize the 96-pin replicator by burning 3 times with 96 % EtOH each time you dip it into your "copy master plate", and continue "stamping" onto selective omnitray plates. Remember to include a plate without selective agents to check general viability/growth of each isolates.
- Incubate the omnitray plates at 28C (or room temperature if more than two days).
- Note the growth of each isolates on the plates.

Table 1: Example of test concentrations used for resistance typing of bacterial isolates.

Toxicants	**Stock**	**Solvent**	**Test Concentrations**		
Tetracycline Hydrochloride	16 mg/ml	EtOH	4 mg/l	8 mg/l	16 mg/l
Ampicillin Sodium Salt	64 mg/ml	ddH_2O	32 mg/l	64 mg/l	128 mg/l
Streptomycin Sulphate	128 mg/ml	ddH_2O	16 mg/l	32 mg/l	64 mg/l
Chloramphenicol	32 mg/ml	EtOH	8 mg/l	16 mg/l	32 mg/l
Nalidixic Acid	32 mg/ml	0.3M NaOH	16 mg/l	32 mg/l	64 mg/l
Colistin	0.32 mg/ml	ddH_2O	32 mg/l	64 mg/l	128 mg/l

Note on Resistance screening of Aeromonas spp isolates:

- If only *Aeromonas spp.* strains are to be screened for resistance, the assays should be conducted using Mueller-Hinton agar plates, adhering to the CLSI guidelines. In brief, *Aeromonas* strains should be grown overnight in microwell plates in Mueller-Hinton broth before inoculation onto Mueller Hinton agar plates using a sterilized 96-replicator pin followed by incubation for 24 hours at 35 °C.

Protocol 7: High-throughput qPCR (HT-qPCR) – general recommendations and notes

Overview

The protocol provides general tips and recommendations for conducting HT-qPCR smart chip assays. Please adhere to the Wafergen Smartchip user manual when setting up the system (https://www.takarabio.com/)

Protocol

General steps:

	Step-by-step	**Experimental content**	**Purpose and noted point**
1	DNA extraction	• It can be extracted according to the existing method in the group; • Qubit assay concentration (NanoDrop assay is not recommended, may be overestimated)	1. High DNA yield is the basis for all subsequent experiments 2. Good DNA quality is the key to quantitative success
2	DNA dilution	• Dilute to 10-30 ng/uL. • Try to keep the same batch of samples diluted to the same concentration to facilitate subsequent comparison of results;	1. Too high or too low concentration may lead to quantification failure 2. In general, dilution is strongly recommended when the concentration is too high
3	Conventional qPCR (16S 60bp)	• When the Ct of the sample is higher than the negative control, or 0, it is regarded as no amplification, and the sample can be discarded; • According to current experience, Ct between 10-15 is good.	1. Eliminate samples without 16S amplification, 2. This part of the data will be used in the final data analysis
4	Sample layout	• 384Assay*12Sample as an example: one chip only does 4 samples*3 replicates. • Mix the same concentration and volume of DNA from the same batch of samples. **• It is recommended to include all samples that are to be compared on the same chip in order to avoid chip to chip variability affecting the results. Hence, the number of assay per chip may need to be reduced (see sample/assay volume option further down)**	

5	HT-qPCR and data processing	• Combine the data of 5 with "3 common quantification (16S 61bp)" to calculate relative abundance and absolute abundance	Generate the final result

Notes on instrument adaptation:

Precautions	**Specific description**
Check before use	Check the gas cylinder before use + check the instrument use record to ensure that the instrument is running stably in the near future
Empty liquid waste	The waste liquid tank is cleaned "before use + after use", keeping the machine in a low-load state without waste liquid for a long time, which can reduce the probability of water spraying.
NaClO (0.2 %)	Sodium hypochlorite is easy to decompose. If it cannot be guaranteed to be prepared within three days, it must be remixed. **500 mL H_20 2 mL NaClO**
Aeriation bottle	Replenish the water in the aeration bottle in time. When the liquid level is too low, the spraying will fail. Generally, when the liquid level is less than 10cm, it is necessary to **replenish water and re-aerate**.
Water droplets	If there are water droplets on the SmartChip, the experiment cannot continue. Do not discard the chip, it can be washed after reverse centrifugation and then used again, it will not affect the personal test.

Chips commonly used in the group

Chip	**Gene Number Chip**	**Arrangement A, assay; S, sample**	**Enzyme**	**References for Quantification**
Carbon, nitrogen, phosphorus, sulfur (CNPS)	72	72A × 72S	LightCycler 480 SYBR Green I Master	Zheng, Bangxiao, et al. "QMEC: a tool for high-throughput quantitative assessment of microbial functional potential in C, N, P, and S biogeochemical cycling." Science China Life Sciences 61.12 (2018): 1451-1462.
Arsenic As	80	80A × 64S	LightCycler 480 SYBR Green I Master	Zhao, Yi, et al. "AsChip: a high-throughput qPCR chip for comprehensive profiling of genes linked to microbial cycling of arsenic." Environmental

				science & technology 53.2 (2018): 798-807.
ARGs and MGEs	384	384A × 12S	LightCycler 480 SYBR Green I Master	Stedtfeld, Robert D., et al. "Primer set 2.0 for highly parallel qPCR array targeting antibiotic resistance genes and mobile genetic elements." FEMS microbiology ecology 94.9 (2018): fiy130.
Pathogens	70	72A × 72S	TaqMan™ Gene Expression Master Mix(货号：4369016)	An, Xin-Li, et al. "High-throughput diagnosis of human pathogens and fecal contamination in marine recreational water." Environmental Research 190 (2020): 109982.
Feces contamination	24	24A × 216S	TaqMan™ Gene Expression Master Mix(货号：4369016)	An, Xin-Li, et al. "High-throughput diagnosis of human pathogens and fecal contamination in marine recreational water." Environmental Research 190 (2020): 109982.

The sample volume of different chip arrangement combinations (unit uL)

Assay plate preparation (1 chip)					
Assays	**Samples**	**Enzyme**	**Sterile water**	**Amount of mix to each 384 well**	**Primer amount**
12	384	523	314	14.3	3.6
24	216	523	314	14.3	3.6
36	144	458	275	16.2	4.1
48	108	523	314	14.3	3.6
54	96	556	334	13.7	3.4
72	72	652	391	12.4	3.1
80	64	699	419	12.1	3
96	54	784	470	11.5	2.9
120	42	911	547	10.9	2.7
144	36	1039	623	10.5	2.6
216	24	1430	858	9.9	2.5
248	20	1613	968	9.8	2.4
296	16	1870	1122	9.6	2.4
384	12	2350	1410	9.4	2.3

Sample plate preparation (1 chip)					
Assays	**Samples**	**Enzyme**	**Sterile water**	**Amount of mix to each 384 well**	**DNA**
12	384	2350	1410	9.4	2.3
24	216	1430	858	9.9	2.5
36	144	1039	623	10.5	2.6
48	108	850	510	11.2	2.8
54	96	784	470	11.5	2.9
72	72	652	391	12.4	3.1
80	64	610	366	12.9	3.2
96	54	556	333	13.7	3.4
120	42	493	296	15.2	3.8
144	36	458	275	16.2	4.1
216	24	523	313	14.3	3.6
248	20	481	289	15.5	3.9
296	16	610	366	12.9	3.2
384	12	523	313	14.3	3.6

Assays	**Samples**	**Assay + Sample total volume**
12	384	2873
24	216	1953
36	144	1497
48	108	1373
54	96	1340
72	72	1304
80	64	1309
96	54	1340
120	42	1404
144	36	1497
216	24	1953
248	20	2094
296	16	2480
384	12	2873

Specification for operation of micro-high-throughput work station

Before start:

1) Helium aeriation for **30 minutes**
2) Perform **daily warm-up** three times to ensure system stabilization of the automatic pipette dispenser. Run **daily clean** and **tip clean**.
3) **Turn on the cooler**

Centrifugation steps:

1) Centrifuge primer plates: **2000 rpm, 1 min, 25° C**
2) Mix and centrifuge assay plate after addition of sterile water, enzyme, and primers: **3200 rcf, 2 min, 12° C.**
   After centrifugation, put the plate in the fridge for a few minutes to remove bubbles. Alternatively remove bubbles in the wells using a pipette tip.

3) Centrifuge the chip after assay loading: **3200 rcf, 5 min, 12° C**. Seal the chip gently with a white film.
4) Mix and centrifuge sample/source plate after addition of sterile water, enzyme, and DNA: **3200 rcf, 2 min, 12° C**.
5) Centrifuge the chip after assay+sample loading: **3200 rcf, 5 min, 12° C**. Seal the chip tightly with the blue film.
6) After the chip has been loaded with both assay and sample it is ready for HT-qPCR cycle.