UNIVERSITY OF GOTHENBURG

Unveiling pesticide effects and antibiotic resistance in freshwater microorganisms through genomics

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Abstract

A large proportion of the rivers worldwide are impacted by human activities and pollution. Freshwater microorganisms, including bacteria, algae, and fungi are crucial for nutrient cycling and primary production. Chemical pollution can alter aquatic microorganisms at both cellular and community-levels, sometimes leading to acclimation or adaptation responses.

Antibiotic resistance is the mechanism by which bacteria increase their tolerance towards antibiotic exposure. The rapid increase of antibiotic resistance in pathogenic bacteria during the last decades has become a major global health problem. River sediments, can act as reservoirs of ARGs that persist and spread in the environment.

The four papers in this thesis investigate how freshwater microorganisms respond and acclimate to pesticides at both cellular and community levels and explore the presence and characteristics of ARGs in a high-flow Scandinavian river. Altogether, this thesis combines single-organism and community approaches in laboratory and field settings, integrating metagenomics, transcriptomic, phenotypic, and chemical profiling tools.

Paper I showed that the green alga *Raphidocelis subcapitata* acclimated within four weeks to toxic but environmental levels of the herbicide diflufenican. This acclimation was accompanied by fitness trade-offs. Acclimated cells were smaller and overexpressed stress-related genes, such as genes related to DNA repair and replication, and cellular division. **Paper II** demonstrated that aquatic biofilms exposed to environmental levels of the fungicide tebuconazole exhibited a reduction in fungal biomass, while algae and bacteria were unaffected. As a response to tebuconazole, the fungal community composition shifted. Moreover, biofilms also partly degraded tebuconazole, releasing unknown transformation products, suggesting a potential acclimation or adaptation mechanism. In **paper III**, a field ecotoxicological assessment was performed using periphyton. A reduction in algal species richness was observed, accompanied by changes in community composition in periphyton of agricultural streams, compared to non-agricultural streams. Agricultural streams had a higher pesticide mixture toxic pressure, and green algae (*Chlorophyta*), decreased in relative abundance in the agricultural streams, while opportunistic cyanobacteria (*Cyanobacteriota*) increased, likely replacing them. **Paper IV** characterized the sediment resistome of the Göta Älv river and examined the potential influence of WWTP effluents as sources of antimicrobials and ARGs. ARGs were found to accumulate downstream, with distinct resistome composition and taxonomic diversity compared to the upstream sites.

Overall, this thesis investigates how human activities influence freshwater microorganisms using high-throughput molecular methods. The studies reveal molecular mechanisms, cellular responses, community shifts, and ecologically or clinically relevant genes in freshwater microbial communities resulting from pesticide exposure as well as agricultural and urban activities.

Keywords: Algae, fungi, bacteria, biofilms, sediments, ARGs, resistome, transcriptomics, shotgun metagenomics, amplicon sequencing, pesticides, pesticide toxicity, acclimation, adaptation

Svensk sammanfattning

En stor andel av världens floder påverkas av mänskliga aktiviteter och föroreningar. Sötvattensmikroorganismer, däribland bakterier, alger och svampar, spelar en viktig roll i floders näringsämnesflöden och primärproduktion. Kemiska föroreningar kan påverka akvatiska mikroorganismer både på cellulär nivå och på organismsamhällesnivå, vilket kan leda till acklimatisering och adaption.

Antibiotikaresistens är den mekanism genom vilken bakterier ökar sin tolerans mot antibiotika. Den snabba ökningen av antibiotikaresistens hos sjukdomsalstrande bakterier under de senaste decennierna är ett stort globalt hälsoproblem. Flodsediment kan fungera som reservoarer för antibiotikaresistensgener som då förblir, och sprids vidare, i miljön.

I de fyra artiklarna i denna avhandling undersöks hur sötvattensmikroorganismer svarar på och acklimatiserar sig till bekämpningsmedel på både cellulär och organismsamhällesnivå. Vidare utforskas förekomsten och egenskaperna hos resistensgener i en snabbflytande svensk flod. Avhandlingen kombinerar studier av enskilda organismer med studier på organismsamhällesnivå under laboratorie- och fältförhållanden. Integration av metagenomik, transkriptomik, fenotypisk och kemisk profilering har varit ett viktigt tillvägagångssätt under avhandlingsarbetet.

Artikel I visar att den gröna algen Raphidocelis subcapitata acklimatiserade sig inom fyra veckor till toxiska men likväl miljö-autentiska nivåer av herbiciden diflufenikan. Denna acklimatisering åtföljdes av fitness-avvägningar. De acklimatiserade cellerna var mindre och överuttryckte stressrelaterade gener, såsom gener kopplade till DNA-reparation och replikation samt celldelning. **Artikel II** visar att biofilmssvampar exponerade för miljönivåer av fungiciden tebukonazol uppvisade en minskning i biomassa, medan alger och bakterier påverkades i mer begränsad utsträckning. Som svar på tebukonazol förändrades det akvatiska svampsamhällets sammansättning. Dessutom degraderade biofilmerna delvis tebukonazol, vilket frigjorde okända transformationsprodukter. Detta antyder en potentiell acklimatiserings- eller adaptationsmekanism.

I **artikel III** befanns algartsrikedomen vara lägre - och sammansättningen av organismsamhällen annorlunda - i perifyton i jordbruksnära vattendrag jämfört med vattendrag som inte låg i omedelbar anslutning till jordbruk. De jordbruksnära vattendragen hade en högre toxisk belastning från blandade bekämpningsmedel, och gröna alger (*Chlorophyta*) minskade i relativ förekomst, medan opportunistiska cyanobakterier (*Cyanobacteriota*) ökade, till synes på de förras bekostnad. I **artikel IV** karakteriserades resistensgener i sedimentet från Göta Älv. Den potentiella påverkan av avloppsvatten från reningsverk som källor till antimikrobiella ämnen och resistensgener undersöktes. Resistensgener befanns ackumulera nedströms, och dessa nedströms belägna lokaler uppvisade annorlunda resistensgenessammansättning och taxonomisk mångfald jämfört med uppströmslokalerna.

Sammanfattningsvis använder denna avhandling högeffektiva molekylära metoder för att undersöka hur mänskliga aktiviteter påverkar sötvattensmikroorganismer. Studierna avslöjar molekylära mekanismer, cellulära svar, förändringar i organismsamhällens sammansättning och ekologiskt samt kliniskt relevanta gener i sötvattensmikrobiella organismsamhällen som ett resultat av exponering mot bekämpningsmedel och jordbruks- och stadslivsrelaterade aktiviteter.

Nyckelord: Alger, svamp, bakterier, biofilm, sediment, restistensgener, resistom, transkriptomik, shotgun metagenomics, amplicon sequencing, bekämpnigsmedel, bekämpningsmedelstoxicitet, acklimatisering, adaption

Resumen en español

Una fracción considerable de los ríos a nivel global está afectada por actividades antropogénicas y procesos de contaminación. Los microorganismos de agua dulce, incluyendo bacterias, algas y hongos, desempeñan un papel fundamental en los ciclos biogeoquímicos y en la producción primaria de las redes tróficas. Sin embargo, la contaminación química puede alterar el funcionamiento normal de los microorganismos acuáticos, lo que, en ocasiones, induce respuestas de aclimatación o adaptación tanto a nivel celular como a nivel comunitario.

La resistencia a los antibióticos constituye el mecanismo mediante el cual las bacterias incrementan su tolerancia frente a la exposición a antibióticos. Durante las últimas décadas, el rápido incremento de la resistencia a antimicrobianos en bacterias patógenas se ha consolidado como un problema sanitario de escala global. Los sedimentos fluviales pueden actuar como reservorios de genes de resistencia a antibióticos (ARGs por sus siglas en inglés), que persisten y se dispersan en el medio ambiente.

Los cuatro artículos que integran esta tesis examinan cómo los microorganismos de agua dulce responden y se aclimatan a la presencia de varios pesticidas, a nivel celular y de comunidad. Asimismo, analiza la presencia y características de ARGs en un río escandinavo de alto caudal. En general, la tesis combina trabajo de campo y laboratorio, integrando herramientas de metagenómica, transcriptómica, caracterización fenotípica y análisis químico para evaluar distintos niveles de organización biológica.

En el **artículo I**, se demostró que el alga verde *Raphidocelis subcapitata* logró aclimatarse a concentraciones tóxicas, pero frecuentemente detectadas en el medio ambiente, del herbicida diflufenican en el transcurso de cuatro semanas. Sin embargo, la aclimatación comprometió evolutivamente a las algas, puesto que las células aclimatadas redujeron su tamaño. Además, sobreexpresaron genes asociados al estrés, relacionados con la reparación y replicación del ADN, y la división celular. El **artículo II** evidenció que los hongos asociados a biopelículas, expuestos a concentraciones ambientales del fungicida tebuconazol, disminuyeron su biomasa. Además, la composición de la comunidad fúngica se modificó, mientras que las algas

y bacterias no resultaron afectadas. A su vez, las biopelículas degradaron parcialmente el tebuconazol, liberando productos de transformación desconocidos, lo cual sugiere un posible mecanismo de aclimatación o adaptación. En el **artículo III** se observó una disminución en la riqueza de especies algales, así como cambios en la composición de la comunidad algal en arroyos agrícolas en comparación con arroyos no agrícolas. Los arroyos agrícolas presentaron una mayor presión tóxica derivada de la contaminación por mezclas de pesticidas, y las algas verdes (*Chlorophyta*) disminuyeron en abundancia relativa, mientras que cianobacterias oportunistas (*Cyanobacteriota*) aumentaron, probablemente reemplazándolas. El **artículo IV** caracterizó el resistoma sedimentario del río Göta Älv y evaluó la posible influencia de los efluentes de plantas de tratamiento de aguas residuales como fuentes de antibióticos y ARGs. Los resultados mostraron una acumulación de ARGs aguas abajo, acompañada de una composición del resistoma y una diversidad taxonómica claramente diferenciadas respecto a los puntos de muestreo aguas arriba.

En resumen, esta tesis analiza la influencia de las actividades antropogénicas sobre los microorganismos de agua dulce mediante el uso de tecnologías de secuenciación de alto rendimiento. Los estudios realizados describen mecanismos moleculares, respuestas celulares, cambios en la estructura comunitaria y la presencia de genes de relevancia ecológica o clínica en comunidades microbianas de agua dulce, como consecuencia de la exposición a pesticidas, así como de actividades agrícolas y urbanas.

Palabras clave: Algas, hongos, bacterias, biopelículas, sedimentos, ARGs, resistoma, transcriptómica, shotgun metagenomics, amplicon sequencing, pesticidas, toxicidad de pesticidas, aclimatación, adaptación

List of papers:

I. **Gómez-Martínez, D.**, Bengtson, J., Nilsson, A. K., Clarke, A. K., Nilsson, R. H., Kristiansson, E., & Corcoll, N. (2023). Phenotypic and transcriptomic acclimation of the green microalga *Raphidocelis subcapitata* to high environmental levels of the herbicide diflufenican. *Science of The Total Environment*, *875*, 162604.
doi: 10.1016/j.scitotenv.2023.162604

II. **Gómez-Martínez, D.**, Selvin, M. A., Nilsson, A. K., Carmona, E., Ngou, J. S, Kristiansson, E., Nilsson, R. H., Corcoll, N. (2024). Environmental concentrations of the fungicide tebuconazole alter microbial biodiversity and trigger biofilm-released transformation products. *Chemosphere*, *369*, 143854.
doi: 10.1016/j.chemosphere.2024.143854

III. **Gómez-Martínez, D.**, Gustavsson, M., Nilsson, R. H., Kristiansson, E., Corcoll, N. Field-based ecotoxicological assessment of the effects of pesticide pollution on algal biodiversity in southern Swedish agricultural streams. *Manuscript.*

IV. **Gómez-Martínez, D.**, Ngou, J. S., Ugolini, V., Lai, F. Y., Nilsson, R. H., Kristiansson, E., Corcoll, N. Antibiotic resistance gradient along a large Scandinavian river influenced by wastewater treatment plants. *Under review in FEMS Microbiology Ecology.*

Other papers published during the PhD but not included in the thesis:

- Vidal-Verdú, À., **Gómez-Martínez, D.**, Latorre-Pérez, A., Peretó, J., & Porcar, M. (2022) The car tank lid bacteriome: a reservoir of bacteria with potential in bioremediation of fuel. *npj Biofilms Microbiomes* 8, 32. doi: 10.1038/s41522-022-00299-8
- Abarenkov, K., Kristiansson, E., Ryberg, M., Nogal-Prata, S., **Gómez-Martínez, D.**, Stüer-Patowsky, K., Jansson, T., Põlme, S., Ghobad-Nejhad, M., Corcoll, N., Scharn, R., Sánchez-García, M., Khomich, M., Wurzbacher, C., Nilsson, R. H. (2022) The curse of the uncultured fungus. *MycoKeys* 86: 177-194. doi: 10.3897/mycokeys.86.76053
- Nilsson, R. H., Ryberg, M., Wurzbacher, C., Tedersoo, L., Anslan, S., Põlme, S., Spirin, V., Mikryukov, V., Svantesson, S., Hartmann, M., Lennartsdotter, C., Belford, P., Khomich, M., Retter, A., Corcoll, N., **Gómez-Martínez, D.**, Jansson, T., Ghobad-Nejhad, M., Vu, D., Sanchez-Garcia, M., Kristiansson, E., Abarenkov, K. (2023) How, not if, is the question mycologists should be asking about DNA-based typification. *MycoKeys* 96: 143-157. doi: 10.3897/mycokeys.96.102669
- Nilsson, R. H., Jansson, A. T., Wurzbacher, C., Anslan, S., Belford, P., Corcoll, N., Dombrowski, A., Ghobad-Nejhad, M., Gustavsson, M., **Gómez-Martínez, D.**, Kalsoom, Khan F., Khomich, M., Lennartsdotter, C., Lund, D., Van Der Merwe, B., Mikryukov, V., Peterson, M., Porter, T. M., Põlme, S., Retter, A., Sanchez-Garcia, M., Svantesson, S., Svedberg, P., Vu, D., Ryberg, M., Abarenkov, K., Kristiansson, E. (2024) 20 years of bibliometric data illustrates a lack of concordance between journal impact factor and fungal species discovery in systematic mycology. *MycoKeys* 110: 273-285. doi: 10.3897/mycokeys.110.136048
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Author contributions

Paper I: Participated in the study design. Performed the laboratory experiments and measured all phenotypic endpoints. Extracted the RNA and analyzed the transcriptomic data form raw sequences to final results. Performed all statistical analysis and generated all plots. Drafted and edited the manuscript.

Paper II: Participated in the study design. Participated in the laboratory experiments and measured all phenotypic endpoints. Participated in the chemical analysis of tebuconazole. Analyzed the amplicon sequencing data from raw sequences to final results. Performed all statistical analysis and generated all plots. Drafted and edited the manuscript.

Paper III: Participated in the study design. Participated in the field work, led one sampling campaign. Performed DNA extractions and analyzed the amplicon sequencing data from raw sequences to final results. Participated in the chemical mixture toxicity assessment part of the project. Performed all statistical analysis and generated plots. Drafted and edited the manuscript.

Paper IV: Participated in the study design. Participated in the field work. Performed DNA extractions and analyzed the shotgun metagenomics data from raw sequences to final results. Performed all statistical analysis and generated all plots. Drafted and edited the manuscript.

Background

River ecosystems

> *"The rivers in this land are the reason we are here."*
> Lauren Abdel-Razek

River ecosystems are flowing waters that drain the continents and link terrestrial and marine ecosystems through water, energy, and matter exchanges (Valentim et al., 2024). River ecosystems are classic examples of lotic ecosystems. The word 'lotic' is derived from the Latin *lotus*, which means washed, referring to flowing waters (Marsh & Fairbridge, 1999). Lotic systems range in size from narrow streams to major rivers that span several kilometers in width (Jana, 2025). Together, they form part of larger watershed networks or catchments, where small headwater streams drain into mid-size streams, which then flow into larger river systems(Allan & Castillo, 2007).

River ecosystems comprise both biotic interactions among plants, animals and microorganisms, and abiotic interactions, involving the physical and chemical components of the environment (Allan et al., 2021). Due to the high occurrence of these complex interactions, rivers harbor a remarkable diversity of lifeforms, while simultaneously providing essential services to billions of people, including drinking water, energy production, climate regulation, navigation and nutrient cycling (Valentim et al. 2024).

Currently, a large proportion of the rivers worldwide are moderately to severely impacted by human activities at some point along their course (Battin et al., 2023; Best, 2019). Although rivers occupy less than 1% of the Earth's surface, they provide about 80% of the renewable fresh water used by human society (Vörösmarty et al., 2015). Therefore, conserving this vital resource is one of the greatest challenges for ensuring a sustainable future (Allan et al. 2021).

Freshwater microorganisms

Microorganisms are defined as organisms that are not visible to the naked eye, typically measuring less than one millimeter in size (Weisse, 2006). They comprise both prokaryotic and eukaryotic lifeforms and are distributed across all three domains of life plus viruses. These include bacteria, archaea, microalgae, fungi, and protozoa, representing an extraordinarily diverse group in terms of both taxonomy and function. Moreover, they are highly abundant and ubiquitous (Sigee, 2005; Zinger et al., 2012).

This thesis focuses on riverine microalgae, fungi, and bacteria, which play a key role in maintaining the health, resilience, and functioning of freshwater ecosystems.

Microalgae are single-celled photosynthetic organisms that can be found individually, in chains, or in clusters. Their size ranges widely, from just a few micrometers to up to 100 µm. These microorganisms play a vital role as primary producers due to their high abundance and photosynthetic activity. As a result, they are the base of the food web and contribute to nearly 50% of global photosynthesis, supplying energy and organic matter to zooplankton and fish (Daneshvar et al., 2021; Shen et al., 2025).

Microalgae represent a phylogenetically diverse group of organisms, exhibiting a wide variation in both morphological and ecological adaptations (Kezlya et al., 2023). In freshwater ecosystems, the three most abundant and ecologically significant microalgal groups are green algae (*Chlorophyta*), diatoms (*Bacillariophyta*) and blue-green algae or cyanobacteria (*Cyanobacteriota*). Although most microalgae are eukaryotic, cyanobacteria are the only prokaryotes classified as algae, due to their photosynthetic capability, presence of chlorophyll-a, and accessory pigments that resemble those of eukaryotic algae (Khan et al., 2009).

Aquatic fungi are fungi that depend on aquatic environments for all or part of their life cycle. Like algae, they are eukaryotes and form a highly diverse, non-monophyletic group in terms of morphology, phylogeny, and ecology (Grossart et al., 2019). Their inherent wide metabolic diversity makes them essential players in the remineralization and decomposition of organic matter, as well as important predators, endophytes, symbionts and even pathogens, that are crucial to maintain the flow of energy and equilibrium within aquatic food webs (Grossart & Rojas-Jimenez, 2016).

Fungal species that are commonly found in freshwater environments include members of the phyla *Ascomycota*, *Basidiomycota*, and *Chytridiomycota* (Shearer et al., 2007). Yet, aquatic fungi are still poorly characterized in terms of their diversity and metabolic capabilities, making them one of the major "unknowns" in the field of freshwater microbiology (Grossart & Rojas-Jimenez, 2016).

Bacteria are prokaryotic organisms. Together with fungi, bacteria lead organic matter decomposition and play a key role in the nitrogen, phosphorus, and carbon cycles, as well as in the degradation and transformation of pollutants (Zeglin, 2015).

The most prominent bacterial groups in rivers are *Proteobacteria* and *Bacteroidetes*. They are able to break down humic substances and process complex macromolecules (Besemer, 2015).

Forms of living

Freshwater microorganisms live as complex communities, either as free-living plankton suspended in the water column (Figure 1), or in the benthos (bottom of the river), as biofilms on cobbles or stones (ephilitic), or in hyporheic sediments (epipsammic) (Li et al., 2021). While freshwater microorganisms are often classified as either planktonic or benthic, many species transition between both phases during their life cycle (Sigee, 2005). This thesis is focused on benthic living forms of freshwater microorganisms, although planktonic algae are also studied.

In streams and rivers, biofilms, are the dominant form of microbial growth (Figure 1). Biofilms are structured communities of bacteria, algae, cyanobacteria, fungi, and protozoa embedded in a self-produced matrix of extracellular polymeric substances (EPS), which is composed of exo-polysaccharides, proteins, glycoproteins, and glycolipids, together with extracellular DNA (Bonnineau et al., 2021). When biofilms are predominantly autotrophic, or when the focus of the study is on the algal compartment of the biofilm, they are called periphyton. Their inherent high diversity and physical closeness allow them to play a key role in the uptake and retention of inorganic and organic nutrients, as well as in oxygen production in river ecosystems (Fernandes et al., 2020; Sabater et al., 2007). Their attachment increases their residence time compared to the faster-moving flow of the water, giving them a higher opportunity to metabolize the available nutrients and enhancing their ability to protect themselves, making them more metabolically efficient than free-floating (planktonic) cells (Battin et al., 2016). As a result, biofilms form complex networks with multicellular-like behavior and three-dimensional structures, making them a remarkable hotspot of riverine taxonomic and functional diversity.

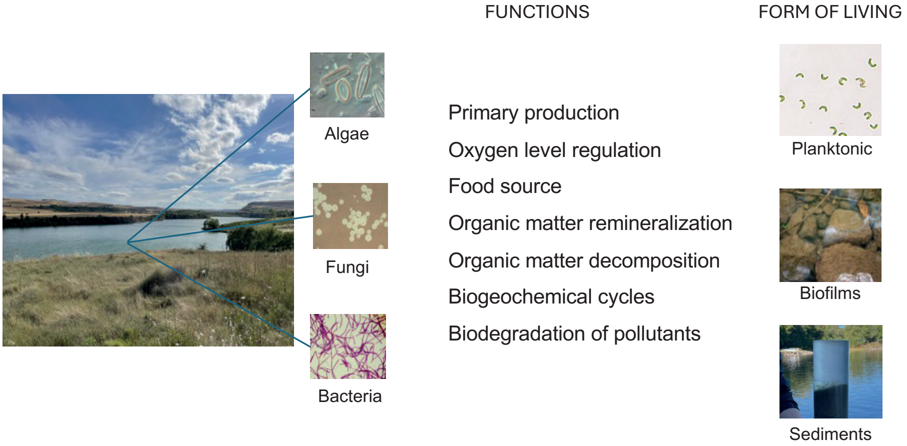


Figure 1: Schematic overview of the key ecological roles and life forms of riverine algae, fungi, and bacteria. From left to right: representative microscopic images of each group of organisms, major ecological functions (e.g., primary production, organic matter decomposition), and predominant life forms, from planktonic to biofilms, and sediments.

Sensitivity and adaptation

River ecosystems are influenced by multiple stressors that co-occur simultaneously. Most of them are caused by human activities. A stressor is any external abiotic or biotic factor that disrupts the normal functioning of a biological system. Such stressors can have either negative or positive impacts on individual organisms, entire communities, or on the overall ecosystem processes (Sabater et al., 2019).

In riverine environments, biofilms, and freshwater microorganisms in general, are among the first components to interact with such stressors, such as dissolved substances and pollutants. As a result, biofilms and freshwater microorganisms in general are highly responsive to changes in water quality and serve as effective indicators for detecting the early impacts of environmental disturbances on the ecosystem (Sabater et al., 2007).

Freshwater microbial communities, when exposed to toxic chemicals in the water column exhibit primary effects, also called short-term responses. A primary effect is a defined biological change in the organism, caused by a chemical reaction between the toxicant and a certain biochemical target. Under prolonged exposures, organisms may exhibit two distinct long-term responses that differ in their temporal scale: acclimation and adaptation. Acclimation responses involve physiological changes driven by alterations in gene expression. Acclimation is typically temporary, although it can sometimes lead to irreversible effects. Moreover, when a species acclimates to toxic concentrations of a substance it might be at the expense of downgrading other metabolic processes. These responses are called fitness trade-offs. Adaptation responses include more permanent genomic modifications and changes in community composition, and therefore they take longer time to appear. Adaptation may lead to the replacement of sensitive taxa with more tolerant ones, potentially resulting in the loss of key species and subsequent disruption of ecosystem functions. The rise of new community assemblages could also lead to new biological responses, such as the capacity of biodegrade and even mineralize the toxicant.

The rapid occurrence of short- and long-term responses in freshwater microbial communities proves their value as sensitive early warning indicators of toxicant exposure in aquatic ecosystems. Therefore, the understanding of such effects is crucial to preserve the ecosystem's health (Muñoz et al., 2015; Sabater et al., 2007).

Pesticide pollution and effects on non-target microorganisms

As previously mentioned, lotic ecosystems are globally subjected to a wide range of environmental stressors, especially contaminants introduced through both point and non-point sources (Berríos-Rolón et al., 2025; Feckler et al., 2015; Parlak, 2024).

Pesticides are chemical substances that prevent, destroy or control a harmful organism (pest) or disease (Brock, 2024). They are typically classified based on their target organisms, such as insecticides (for insects), herbicides (for weeds), and fungicides (for fungi), or based on their molecular structure properties. The rapid growth of the human population has intensified the demand for food production, prompting the agricultural sector to increase their use of pesticides to enhance crop yield. Agriculture accounts for approximately 85% of global pesticide usage (Carvalho, 2017). Sales of pesticides in the EU were approximately 350,000 tones between the years 2011 and 2020, but less than 0.1% of the applied pesticides effectively reach their intended targets (Dhananjayan et al., 2020). The residual amounts often enter and pollute surface waters via different pathways, such as spray drift, leaching, runoff, volatilization, and atmospheric deposition (Zhang, Luo, et al., 2018).

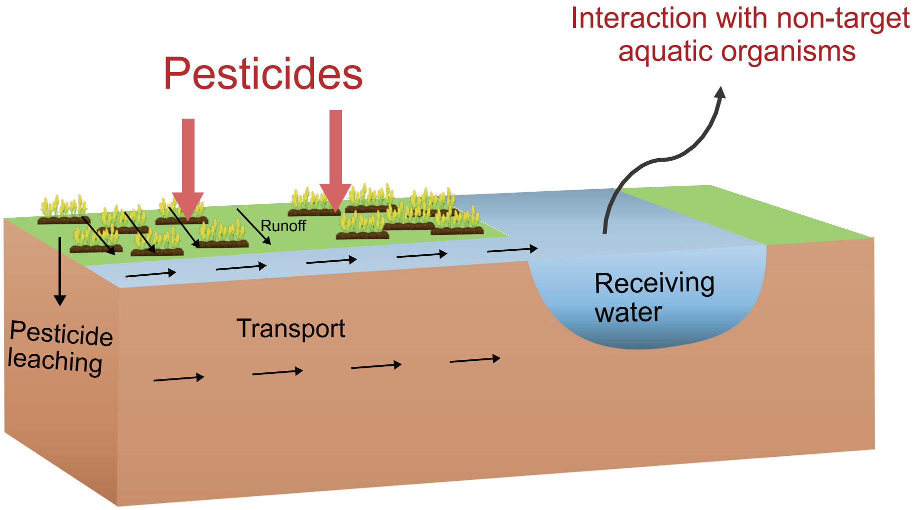


Figure 2: Main pathways of surface water pesticide pollution. Pesticides are applied in crops and end up in recipient water through different pathways, such as pesticide runoff and leaching. Adapted from Xia et al. (2020).

These pathways become notably active during heavy rainfall, leading to sudden spikes in pesticide concentrations in agricultural streams, often surpassing ecologically safe thresholds (Halbach et al., 2021). Due to the close phylogenetic relationships between target and non-target organisms (*e.g.* weeds and algae), pesticides can exert toxic effects on non-target aquatic microorganisms, including algae, bacteria and fungi (Staley et al., 2015).

Ecotoxicity testing and environmental realism

The effects of pesticides on microorganisms can be investigated through toxicity testing. Toxicity testing aims to evaluate the potential environmental hazards posed by chemicals (McCarty et al., 2018). The sensitivity of organisms is typically assessed with standardized laboratory protocols, which provide detailed information about the test design, including the test organism, toxicant concentration and duration. The estimation of selected endpoints is also specified, as is the calculation of the standard toxicity thresholds like the half-maximal effect concentration (EC50), and the no effect concentration (NOEC). These standard laboratory tests involve the exposure of a single species to a single chemical, under controlled laboratory conditions (Brock et al., 2024). Internationally recognized protocols for such tests are mainly established by the Organization for Economic Co-operation and Development (OECD), among others. In Europe, the European Food Safety Authority (EFSA) guidance documents for the risk assessment of pesticides advocate the use of these standardized methods to generate baseline toxicity data (EFSA, 2013).

For instance, the green microalga *Raphidocelis subcapitata* is used as a freshwater algal representative species for standardized laboratory testing (OECD, Test No. 201, http://www.oecd.org/). Endpoints such as the percentage of growth inhibition compared to the control, or yield after 72-96 h based on cell counts or biomass (i.e., chlorophyll-a concentration) are used to calculate the EC50 and NOEC, giving the first indications of how toxic a substance is for a group of organisms. However, such simplified laboratory setups involving one specie and one chemical do not fully reflect the ecological complexity of natural environments, where species live in communities and different stressors co-occur.

More environmentally realistic approaches have been developed to assess chemical effects on community and ecosystem levels. Ecotoxicological methods at higher levels of environmental realism include the use of microcosms and field experiments (Morin & Artigas, 2023; Wijewardene et al., 2021). This thesis covers a gradient of methodological complexity, beginning with standardized single-species laboratory assays and progressing to microcosm and field studies, allowing a more comprehensive assessment of ecological responses across multiple levels of biological organization.

Microcosms studies allow the integration of more realistic exposure scenarios, with long-term assessment of endpoints at the community level (Polazzo et al., 2022). Diversity and composition are commonly used community-level endpoints to characterize structural responses. Typical community responses to chemical exposure include an increase or decrease in the abundance of certain species or groups of species. However, some species might not show any changes. This variability is caused by differences in species sensitivity and indirect effects resulting from the interactions between species (Preston, 2002). In general, microcosm experiments still allow for control of the experimental conditions and the inclusion of replicates, while also recreating more environmentally realistic conditions. This facilitates a more robust determination of casual relationships between chemical exposure and community-level effects in comparison to field studies, where it is often difficult to clearly link observed changes to chemical toxicity (Romero et al., 2018).

In the environment, pesticides occur in complex multi-component mixtures and often co-occur with other stressors that shape the structure and functioning of the aquatic ecosystems (Backhaus & Faust, 2012). Microorganisms exist within communities rather than as isolated cells, which adds further complexity.

Field studies provide the highest degree of environmental realism. They allow the assessment of integrative effects under realistic exposure scenarios of pesticide mixtures in natural microbial communities.

Moreover, they allow us to assess the impacts of chemical pollution together with non-chemical stressors, providing a better explanation for the community changes rather than the effect of pesticides alone. However, this also makes it difficult to clearly establish causality between pesticide presence and observed biological effects (Bighiu et al., 2020). Assessing pesticide effects on microorganisms at the individual and community level is still challenging as it is difficult to balance environmental realism with clear causal relationships. Moreover, data on the impact of pesticides on aquatic microbial communities remains relatively scarce (Feckler et al., 2015).

Antibiotic resistance in freshwater environments

Antibiotic resistance is defined as the mechanism by which bacteria increase their tolerance against antibiotic exposure (Larsson & Flach, 2022). This poses an obvious threat to human health and has become one of the most significant public health issues of the 21st century (Ferri et al., 2017). Genes encoding biochemical mechanisms for this trait are known as antibiotic resistance genes (ARGs) and are commonly transferred horizontally between bacterial cells. The high usage of antibiotics has created a strong selection pressure, allowing resistance genes to evolve, move, and spread between bacteria much faster than they would in the absence of prolonged antibiotic exposures (Ebmeyer et al., 2021). This entire set of known resistance genes is known as the resistome.

Mobile ARGs can be transferred between different environments (Bengtsson-Palme et al., 2018). This transfer can occur, for instance, when wastewater treatment plant (WWTP) effluents containing resistant bacteria enter rivers that are used as sources of drinking water (Figure 3). Studies have shown that resistant bacteria can be reintroduced into the human microbiome through exposure to water contaminated with resistant bacteria and ARGs. This may happen through activities such as ingesting sewage-polluted water during recreation, consuming crops irrigated with contaminated surface water, or through other situations involving poor sanitation (Bengtsson-Palme et al., 2023; O'Flaherty et al., 2018).

Many studies have focused on characterizing the resistome, especially in the human gut and WWTP sludge microbiomes, since they are considered ARGs hotspots due to the frequent co-occurrence of pathogens and strong antibiotic selection pressure (Berglund et al., 2023; Guo et al., 2017; Salazar et al., 2022; Zhang et al., 2022). However, the freshwater environmental resistome has received far less attention, even though they may constitute a link between these two ARGs hotspots (Shin et al., 2023; Spänig et al., 2021). Therefore, to fully understand how ARGs move from non-pathogens to pathogens, it is as important to study the genetic reservoirs in freshwater environments.

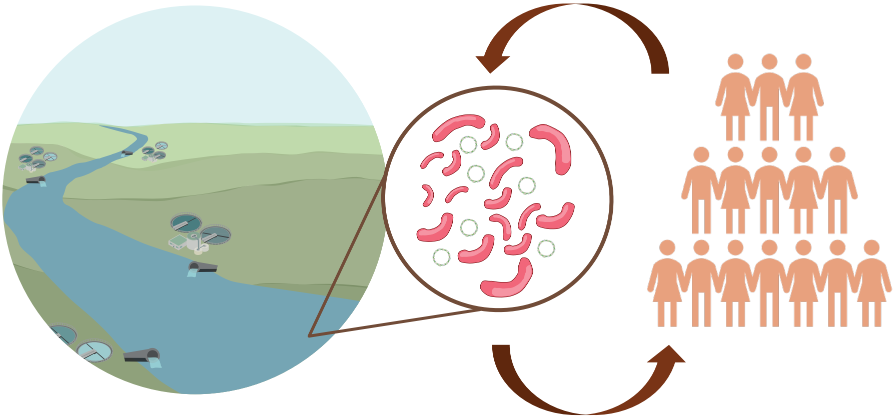


Figure 3: Conceptual illustration of ARG exchanges between humans and freshwater environments. WWTP effluents can potentially release ARGs that enrich the environmental resistome, which can re-enter the human gut via water, food, or recreation. Conversely, human-derived ARGs can enter WWTPs and contribute to their spread in aquatic systems.

Sediments can serve as an interface that promotes the transfer, persistence, and dissemination of ARGs among the bacterial communities that inhabit them (Marti et al., 2014). It is reasonable to infer that a wide range of resistant bacteria may be present in these habitats, especially in river sediments exposed to effluent from WWTPs. Additionally, ARGs can persist in sediments over extended periods and may become more accessible during high-flow events, when sediments are resuspended into the water column.

Thus, the characterization of the resistome in sediments is important for understanding which ARGs are present in environmental communities and how they may spread between environmental and host-associated bacteria, potentially becoming mobilized and posing clinical risks.

Environmental genomics and transcriptomics

Genetic information is encoded in the linear sequence of nucleotides within DNA. Each DNA molecule consists of a double helix formed by two complementary nucleotide strands, joined together to form units known as base pairs (bp): adenine (A) pairs with thymine (T), and cytosine (C) with guanine (G). The observable traits of each organism, known as the phenotype, are mainly determined by the exact sequence of nucleic acids in its genome. The genome is divided in specific sequence units known as genes, most of which are transcribed into RNA and subsequently translated into proteins (Alberts, 2003; Crick, 1970). DNA (and RNA) sequencing is the method that involves the identification and determination of the order of these nucleotide bases.

Transcriptomics

The transcriptome encompasses the full range of RNA molecules present in a cell, including messenger RNA (mRNA), which plays a central role in translating genetic information, carrying the protein-coding parts. Analyzing the transcriptome of a cell or a population offers a snapshot of genes actively expressed at a particular moment.

Transcriptomics refers to the study of the transcriptome. It reveals patterns of gene expression and the regulatory mechanisms that control them, and it is studied through high-throughput sequencing technologies that are capable of sequencing large amounts of RNA (converted to cDNA) or DNA sequences simultaneously (Schirmer et al., 2010).

Metagenomics: amplicon sequencing and shotgun metagenomics

Metagenomics refers to the culture-independent genomic analysis of microbial communities. As most microorganisms in nature are unculturable by traditional methods, environmental genomics has emerged as an essential and routine approach for characterizing microbial diversity in natural settings (Kumar et al., 2021). This method enables the study of the full spectrum of microbial life, including many previously inaccessible taxa. Metagenomic sequencing is commonly employed to assess the taxonomic composition of microbial communities and/or to quantify the relative abundance of functional genes within these communities. There are two main types of metagenomic analysis: amplicon sequencing and shotgun metagenomics.

Amplicon sequencing is a commonly used method for analyzing the taxonomic composition of microbial communities. This approach targets specific genetic markers that generally contain "hypervariable regions" that show sequence diversity among different species and can be used for species identification, but are also flanked by conserved sequences, making them easy to amplify through PCR using universal primers (Chakravorty et al., 2007). Some examples are the 16S rRNA gene for bacteria, the 18S and 23S rRNA gene for eukaryotes, and the ITS region for fungi, which are used in this thesis. In contrast, shotgun sequencing involves the extraction and sequencing of the total DNA from an environmental sample, enabling a more comprehensive analysis of the microbial genome. The obtained sequencing reads can be either assembled into contigs or directly mapped to a reference gene database. This approach provides detailed insights into the functional, genetic and taxonomic composition of the microbiome (Azli et al., 2022).

One of the greatest challenges in ecotoxicology is the reliable assessment of the effects of chemical pollutants at the community and ecosystem levels. Conventionally, effect assessment is focused on toxicological phenotypic endpoints, biased by regulatory requirements rather than ecological relevance.

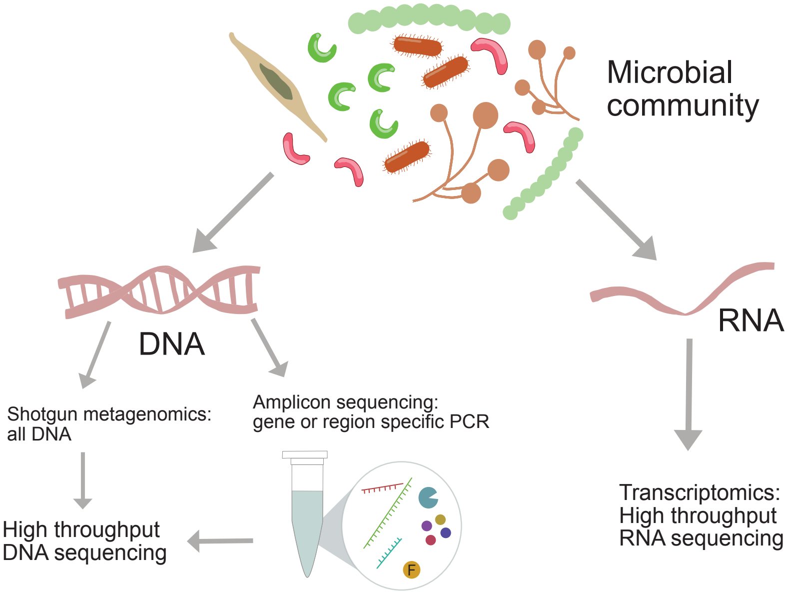


Figure 4: Schematic overview of the genomic and transcriptomic approaches used in this thesis. Starting from cells of a single species or a community, DNA can be directly subjected to high-throughput sequencing after library preparation or amplified at target regions by PCR prior to sequencing. RNA is first reverse-transcribed into cDNA and then sequenced using high-throughput platforms.

In the context of microbial ecotoxicology, high throughput sequencing methods offer a significant potential to detect and quantify biological changes across multiple levels of organization, from molecular and individual responses to population and community dynamics. These tools provide a comprehensive view of cellular processes within organisms, as well as shifts in the composition of natural microbial communities in response to environmental stressors. Therefore, the use of high throughput sequencing methods represents a transformative opportunity to deepen our understanding of the mechanisms driving ecological responses to toxic substances and environmental degradation (Zhang, et al., 2018).

Aims of the thesis

This thesis aims to provide new knowledge on the individual and community-level effects and acclimation mechanisms towards pesticides in freshwater microorganisms, and on the presence and nature of antibiotic resistance genes (ARGs) in a large Scandinavian river using a combination of transcriptomics and metagenomics. This thesis includes both single-organism and community level approaches, and both laboratory and field studies, using a combination of phenotypic endpoints, metagenomics, transcriptomics, and chemical profiling tools. The specific aims of the four research papers that are included in the thesis are as follows:

Paper I: Assess both phenotypic and transcriptomic changes associated with the acclimation mechanisms of the green microalga *Raphidocelis subcapitata* towards the herbicide diflufenican at environmental concentrations.

Paper II: Investigate the short- and long-term effects of the fungicide tebuconazole on biofilm fungal and bacterial biomass, as well as potential shifts in community composition associated with these exposures. Additionally, assess the role of biofilms in the biotransformation of tebuconazole from the water column.

Paper III: Characterize algal diversity in the periphyton of three streams with differing levels of pesticide pollution, and explore the relationships between algal diversity patterns, the toxic pressure of pesticide mixtures, and other agricultural inputs.

Paper IV: Characterize the presence of ARGs within sediment bacterial communities along the Göta Älv, a large boreal river in Sweden. This study also aims to assess if WWTP discharges serve as sources of antimicrobials and ARGs into the Göta Älv, as well as to characterize and link the bacterial diversity of river sediment with its resistome.

Main methods used in the thesis

This section describes the key methodologies used in the studies presented in this thesis. Detailed descriptions of the methods, as well as additional ones that are not covered in this section, can be found in the respective papers.

Types of study: from low to high environmental realism

Laboratory studies

As previously stated, ecotoxicological studies range from simplified laboratory experiments to more complex laboratory and field studies. While this progression better reflects the ecological complexity of natural environments, it also introduces more variables that are difficult to control.

Single-species bioassays

The algal model species *Raphidocelis subcapitata* was exposed for 12 weeks in axenic conditions to two different concentrations of diflufenican, one close to its half-maximal effect concentration (EC50) and one close to its 10% effect concentration (EC10) (i.e., 10 and 310 ng/L). This duration enabled the algae to acclimate to diflufenican exposures and maintain the acclimation status over time. Diflufenican was chosen due to its high toxicity to algae (Book et al., 2022; Weyman et al., 2012) and its high presence in Swedish rivers, as well as in other European rivers near agricultural areas.

To culture the algae, a serial batch culture approach was followed (LaPanse et al., 2021), harvesting cells during the late exponential growth phase and re-inoculating them weekly into fresh medium containing diflufenican. Cultures were grown in 50 mL flasks with medium C (Ichimura, 1971), a nutrient- and vitamin-rich medium that prevents nutrient limitation so that the herbicide was the only stressor. Light and temperature conditions were along the lines of those standardized in the OECD Guideline 201.

Microcosms

Natural biofilm communities from a Swedish river were exposed in microcosms to two environmentally relevant concentrations, in both chronic (24 days at 10 or 100 µg/L) and acute manners (96 h at 100 µg/L). Each microcosm consisted of a rectangular glass vessel with a holding volume of 1.5 L covered with a glass lid. Each vessel contained a final volume of 1.2 L of river water amended with nutrients, and the biofilms were attached to submerged glass slides, leaning semi-vertically on a tubular glass rod fixed to the bottom (Figure 5). The experiment comprised 18 independent microcosms in total. Water in each microcosm was renewed every 3 to 4 days, and constant agitation was maintained with horizontal shakers set at 47 rpm to simulate river flow.

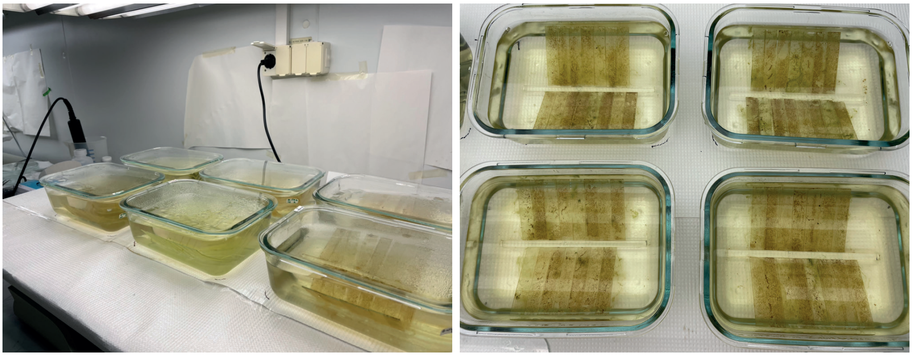


Figure 5: Microcosms in shakers (left) and detail of biofilms grown on the glass slides of microcosms (right).

In **paper II**, it is important to clarify the distinction between acute and chronic exposures. Generally, the term acute is used when referring to short-term exposures, when one single dose of toxicant produces observed effects over no more than one life cycle. The term chronic traditionally involves longer-term exposures, covering multiple generations. However, due to the short life cycle of microorganisms, what would traditionally be considered chronic exposures (24–96 hours) are treated as acute in this thesis and generally in microbiological research, whereas exposures longer than 96h are considered chronic.

Tebuconazole was selected due to its high toxicity to fungi and its widespread presence in European rivers at harmful concentrations (Finckh et al., 2024; Lefrancq et al., 2017).

Field studies

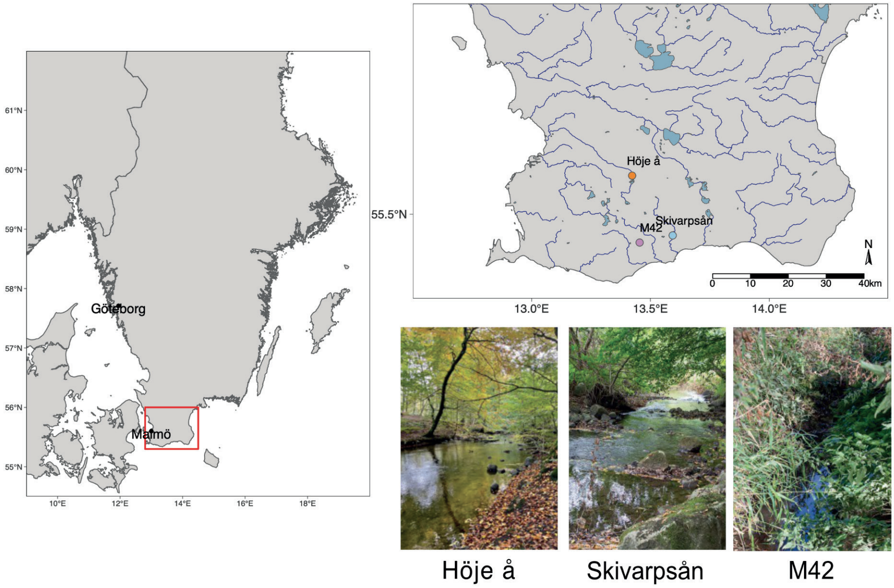
Paper III and **paper IV** are field studies, achieving the highest environmental realism. These field studies were performed in different Swedish rivers and streams, each characterized by different land uses in their catchment areas. The following text describes the characteristics and importance of the selected sampling sites and sampling strategies to fulfill the specific aim of each paper, as well as the type of biological sampling according to the aims of each study.

Pesticide pollution in Skåne

Paper III investigates whether expected differences in biofilm-algal biodiversity among three streams are linked to expected different pesticide pollution levels. The study took place in three flowing water systems in southern Sweden's Skåne region: the rivers Höje å and Skivarpsån, and the stream M42 (Figure 6), all of which drain into the Baltic Sea.

As part of Sweden's national pesticide monitoring program, the Swedish University of Agricultural Sciences (SLU) reports annual pesticide concentrations for four streams and two rivers, to which Skivarpsån and M42 belong (Boye et al., 2019). This long-term pesticide monitoring has shown that herbicides are the most prevalent pesticide group in the Swedish surface waters included in the monitoring, particularly in agricultural regions, which is why this area was selected for the study. Both Skivarpsån and M42 sampling sites are located in typical cereal-growing regions dominated by autumn- and spring-sown crops (Bighiu et al., 2020).

Höje å flows mainly through agricultural land, but the sampling site studied in **paper III** is at its headwaters, at the Häckeberga nature reserve. This area is surrounded by deciduous forest, with dense canopy cover. Because of these natural and semi-undisturbed conditions, we selected this site as our reference location.



Höje å Skivarpsån M42

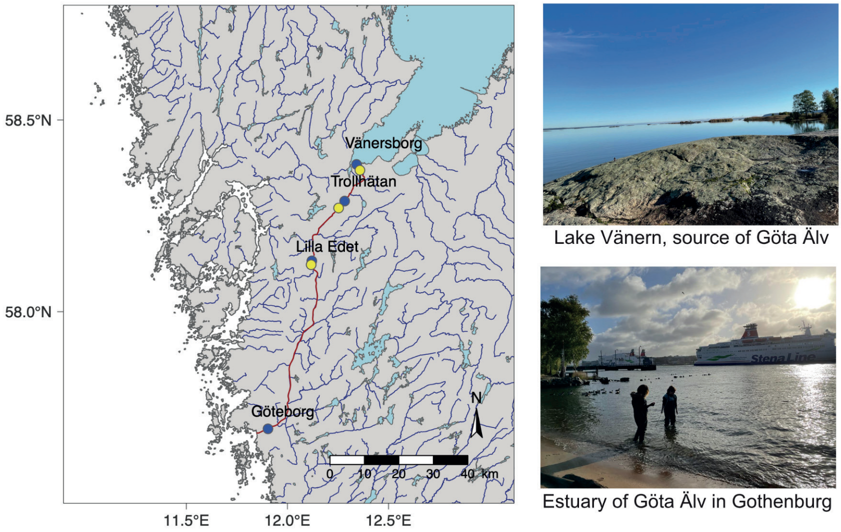
Figure 6: Study area in southern Sweden (left) and close-up map with pictures of each site (right). From Gómez-Martínez et al. 2025 (*Manuscript*).

Göta Älv

Paper IV focuses on the study of the resistome in bacterial sediments from a high-flow river impacted by the effluent of several wastewater treatment plants (WWTPs) along its course. The description of the resistome in bacterial sediments from large, high-discharge rivers in countries where antibiotic levels are reported to be generally low has been little studied. The Göta Älv is a good example of such rivers. This 96-kilometer boreal river originates in Lake Vänern, the largest lake in the European Union, and flows into the Kattegat Sea by the city of Gothenburg on the Atlantic Swedish west coast.

The Göta Älv supplies drinking water to over one million people in Gothenburg (the second largest city in Sweden) and nearby towns. Its estuary hosts the port of Gothenburg, the largest in Scandinavia. The river and its catchment have a long history of human activity and receive wastewater discharges from four municipalities, including Gothenburg's WWTP.

Used as a water source since the late 1800s, the Göta Älv meets drinking water standards for most pollutants, but fecal contamination remains a significant problem mainly due to floods and storms. When heavy rainfall occurs, the untreated influents arriving to WWTP are bypassed and directly discharged to the river, which makes it a potential threat for an increase of ARGs levels in the water and sediment.



Lake Vänern, source of Göta Älv

Estuary of Göta Älv in Gothenburg

Figure 7: On the left, map of the Göta Älv course (red), from lake Vänern (top right) to the Kattegat Sea, passing by the city of Gothenburg (bottom right). Blue dots in the map indicate river sediment sampling sites upstream of a WWTP, and yellow dots indicate river sediment sampling sites downstream of a WWTP. From Gómez-Martínez et al., 2025 (*Manuscript under review in FEMS Microbiology Ecology*).

Phenotypic endpoints

Phenotypic (sometimes called apical) endpoints refer to traditional whole-organism responses that can be directly observed and measured in *in vivo* tests (OECD, 2012). An important aspect of this thesis (**Papers I and II**) is the integration of phenotypic endpoints with genomic tools to better understand the effects of chemicals on freshwater microorganisms. While phenotypic endpoints are more commonly used in ecotoxicology, they sometimes fail to detect subtle or underlying adverse or acclimation effects. On the other side, phenotypic endpoints can also clarify and give context to genomic responses. Used together, these approaches complement each other and provide a more integrative understanding of a toxicant's overall impact.

In **paper I**, the physiological status of the algae was assessed through several measurements. Algal growth rate was estimated fluorometrically by measuring chlorophyll-a content at excitation/emission wavelengths of 425/680 nm. Cell size was determined by flow cytometry, using chlorophyll-a autofluorescence plotted against the forward scatter. The relative abundance of 13 photosynthetic pigments, including chlorophyll-a and carotenoids, which are the primary targets of diflufenican, was quantified using high-performance liquid chromatography (HPLC). Finally, photosynthetic performance was evaluated as the quantum yield of photochemical energy conversion in the photosystem II (PSII) by measuring chlorophyll-a fluorescence using a Pulse Amplitude Modulated (PAM) fluorometer.

In **paper II**, phenotypic endpoints were used to estimate the total biomass of the three main microbial groups in biofilms: fungi, bacteria, and algae. Fungal biomass was assessed using ergosterol, a mycosterol found in fungal cell membranes and commonly used as a biomass proxy. Ergosterol was extracted from biofilm samples and quantified by HPLC–MS/MS. Algal biomass was measured as chlorophyll-a content via HPLC. Bacterial abundance was estimated by quantifying the *rpoB* gene through quantitative polymerase chain reaction (qPCR), with the number of *rpoB* copies serving as a proxy for bacterial cell counts and expressed as copies of *rpoB* per cm^2. Although bacterial abundance was determined by qPCR (which is technically not an apical endpoint) in the present thesis, it is included here because it serves as an estimate of total number of bacterial cells.

Environmental genomics and transcriptomics

A significant part of this thesis involves transcriptomic and metagenomic approaches. Therefore, this methodology is central to grasp the scope of this work. In this section, I describe the common procedures applied across the transcriptomics, amplicon sequencing and shotgun metagenomics analyses presented in this thesis. These include DNA and RNA extraction, library preparation, sequencing using Illumina technologies, and general bioinformatics processing.

DNA or RNA extraction

All genomics and transcriptomics methods start with DNA or RNA extraction from the cells, which are generally performed using readily available kits. The working principles and main steps of these kits, from whole cells to purified DNA or RNA, are mostly similar. They typically begin with cell lysis using a lysis buffer containing chaotropic salts and enzymes that destabilize cell structures, digest proteins, and disrupt the association of nucleic acids with water. This facilitates the binding of DNA or RNA to a selective, silica-based membrane. Once bound to the silica column, a purification step is performed in which (usually) ethanol is used to wash away salts and other unwanted lysate components while retaining the nucleic acids. A subsequent wash step removes remaining impurities such as polysaccharides and pigments. Finally, the clean DNA or RNA is released from the column by elution in water after a brief drying spin.

Library preparation

Before sequencing a biological sample, a library of the sample's DNA or RNA needs to be prepared. In the case of amplicon sequencing, a previous step is involved, were the gene of interest (for example, 16S) is amplified before library preparation. This is typically achieved through PCR using primers that flank hypervariable regions of the genetic marker. These primers generate amplicons that contain both conserved and variable regions, enabling later taxonomic differentiation of the sequences.

Moreover, in the case of RNA sequencing, the RNA is reverse transcribed to cDNA prior to library preparation, in order to allow amplification and the use of sequencing technologies.

The library preparation procedure involves labeling (tagging) the DNA fragments of interest. These tags, known as adapters, are short DNA sequences that attach to the fragments and later enable binding for amplification and sequencing in the Illumina flow cell. The final library, ready for sequencing, is a collection of DNA fragments that together represent the genomic content of a given sample in the case of shotgun metagenomics (**paper IV**), the transcriptome (**paper I**), or the collection of DNA amplicons of interest (i.e., 16S, ITS2, and 23S in **papers II and III**).

Sequencing using Illumina technologies

High-throughput sequencing (HTS) is a method for analyzing genetic material, enabling rapid sequencing of large quantities of DNA or RNA. Unlike traditional approaches, HTS can sequence millions of short DNA fragments simultaneously. In this thesis, Illumina sequencing was employed, which uses sequencing-by-synthesis chemistry to detect individual bases as they are incorporated into the complementary DNA strands. The output consists of large FASTQ files containing both the nucleotide sequences and quality scores for each base call.

Quality control check and trimming

Before aligning the sequences to reference databases, a quality control check needs to be performed, in order to only keep the sequences with high quality and remove primers and adapters. In **papers I and IV**, the quality was evaluated using FastQC, which is a software tool that allows to observe the quality of each base along the length of the reads, adapter contamination, and other warnings that might be of concern for downstream analysis. Based on these observations, the sequences were filtered, and adapters were removed using the software tool BBDuk.

In **papers II and III**, a different approach was taken, as amplicon sequencing was performed. The DADA2 algorithm was used, which models sequencing errors introduced during amplicon sequencing and applies this model to infer the true composition of the sample (Callahan et al., 2017). This workflow includes quality filtering, denoising, merging paired-end reads, and removing PCR artifacts such as chimeras. The result is a set of inferred unique DNA sequences, referred to as amplicon sequence variants (ASVs), where each of them represents a species.

In **paper II**, fungal taxonomy was described. However, sequences were not clustered into ASVs but into operational taxonomic units (OTUs) using the VSEARCH algorithm. This choice is due to the high level of intraspecific variation in the ITS2 marker (Kauserud, 2023). In community studies, OTUs group different ITS alleles into broader sequence clusters, providing a closer approximation to species-level resolution, while this is not possible when using ASVs.

When performing shotgun metagenomics, short-read alignments can be assembled into longer sequences known as contigs, a process that is computationally demanding. In **paper IV**, we bypassed assembly and instead aligned reads directly to the Comprehensive Antibiotic Resistance Database (CARD) reference database using the DIAMOND software tool, applying strict alignment criteria to minimize false positives.

Alignments to databases or reference genomes

Once the raw reads are quality filtered, and clustered, if necessary, then they are aligned based on their similarity to the reference sequences collected in specific databases or reference genomes. Subsequently, the number of hits to each gene are counted, producing a count matrix.

In the case of **paper I**, the reads were aligned using the Rsubread package v.2.10.4 (Liao et al., 2019) to the reference genome of *Raphidocelis subcapitata*, since the goal is to analyze different gene expression patterns in one single species. The analysis was followed by the estimation of read counts for each gene using the function featureCounts in Rsubread.

In **papers II and III**, the ASVs or OTUs were aligned to databases containing amplicon sequences that correspond to different species. In the case of bacteria (16S), they were aligned to the silva classifier reference database (Quast et al., 2013), in the case of fungi to UNITE database (Nilsson et al., 2019) and in the case of algae to the microgreen database (Djemiel et al., 2020).

For the identification of resistance genes in bacterial sediment metagenomes (**paper IV**), the shotgun reads were aligned to the database CARD, which contains clinically relevant ARG sequences.

Statistical analysis

Differentially expressed genes and ASVs.

Once the count matrix is obtained, statistical methods need to be applied to assess whether there are significant differences between samples (**papers III and IV**) or treatments (**papers I and II**) in terms of genes, genetic marker or species relative abundances.

To estimate differentially expressed, differentially abundant genes (**papers I and IV**) or differentially abundant ASVs and OTUs (**paper II**), the DESeq2 R package was used. DESeq2 applies a statistical model to compare gene counts expression between two groups (Wald test) or among more than two groups (Likelihood Ratio Test, LRT). The analysis begins by estimating the variance in expression levels and then fitting a negative binomial distribution to each gene. This distribution accounts for overdispersion in sequencing data, producing p-values adjusted using the Benjamini-Hochberg false discovery rate (FDR) algorithm. A p-value threshold is then applied to identify differentially expressed genes/genetic markers, with smaller values providing stronger evidence for differential expression. The calculated p-value for each gene indicates the likelihood that the observed difference occurred by chance, and the FDR is the expected proportion of false positive among genes that are classified as differentially expressed or abundant.

In **paper IV**, a slightly different approach was required because there was only one metagenomic sample per site. Differentially abundant ARGs among sediment samples were identified using DESeq2 with a LRT instead of a Wald test, allowing the evaluation of changes across more than two levels (each kilometer of the river was treated as a level).

Microbial diversity measurements and statistics.

When assessing the microbiome of a sample from sequencing data, certain key concepts are frequently used, including alpha and beta diversity. In this thesis, both measures were applied to compare diversity across sites and treatments. In brief, alpha diversity summarizes the diversity within a single sample (within-sample diversity), whereas beta diversity quantifies the similarity or dissimilarity between communities (between-sample diversity). Alpha diversity is commonly calculated using indices such as

Shannon diversity or species richness, while beta diversity can be derived from dissimilarity metrics such as Bray–Curtis distances.

Chemical profiling and mixture toxicity assessment

Although it is not a major focus of this thesis, chemical profiling has been important, since we used it to estimate the levels of toxicants present in our experimental conditions and at our sampling sites.

In all papers, identification of pesticides and antibiotics was mainly performed using high-performance liquid chromatography (HPLC) coupled or in tandem with a mass spectrometer (MS or MS/MS). In this method, the chemical mixture is first passed through a column that separates compounds based on their polarity and the changing composition of the solvent. As the compounds leave the column, they pass through a UV detector connected to an electrospray ionization source, which turns them into charged particles. These particles then enter the mass spectrometer, where they are sorted by their mass-to-charge (m/z) ratio. The ions (precursor ions) are detected and fragmented into product ions, which are also detected. The detector records the number and strength of the signals, producing a pattern of peaks that shows which compounds are present and in what amounts. These patterns are compared with known standards or database entries to confirm and quantify each compound.

This method was used to detect diflufenican in **paper I**, tebuconazole and its metabolites in **paper II**, and several targeted pesticides and antibiotic mixtures in **paper III** and **paper IV**, respectively.

Pesticide mixture toxicity assessment

In **paper III**, the overall toxicity of pesticide mixtures detected at each site was estimated using the sum of toxic units (STU) for each location. Each toxic unit (TU) was calculated by dividing the measured pesticide concentration at a site by the median EC50 value for that compound, which was derived from the EC50 values of all tested algal species. The EC50 values were obtained from the US EPA ECOTOX database and EFSA pesticide reports. This method of assessing mixture toxicity, known as the concentration addition (CA) approach, is the one recommended by the Water Framework Directive (European Commission, 2011) for evaluating chemical mixtures.

Following the CA approach, we defined the sum of toxic units (STU_{median}) at each site and sampling occasion as follows:

$$STU_{median} = \sum_{i=1}^{n} \left(\frac{MEC_i}{EC50_i median}\right)$$

Finally, as pesticides can be highly specific to certain groups of algae, we also determined the STU for the individual phyla *Chlorophyta*, *Bacillariophyta*, and *Cyanobacteriota*.

Main results and discussion

Paper I

Algal acclimation towards environmental concentrations of diflufenican

The algal model species *Raphidocelis subcapitata* was exposed for 12 weeks to diflufenican at the concentrations of 10 and 310 ng/L. The exposed algal cells were able to reach an acclimation phase after four weeks from the start of the experiment, and these acclimation changes were characterized in terms of phenotype and transcriptome. During the first week, growth rate, pigment composition and photosynthetic quantum yield decreased in a dose-dependent manner (Figure 8). This phase was referred to as the stress phase. During the next two weeks the exposed algal populations recovered in terms of growth and photosynthetic yield (recovery phase). After four weeks of exposure, the exposed algae were able to acclimate by maintaining a growth rate at levels similar to those of the non-exposed populations. Moreover, algae acclimated to diflufenican were increasingly more tolerant to diflufenican (1.3 and 2.4 times, respectively) than non-exposed algae (Figure 9).

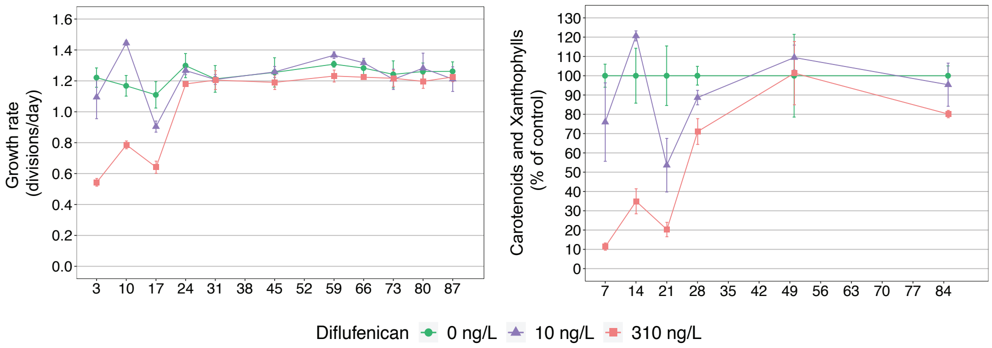


Figure 8: Changes in *R. subcapitata* during the 12 weeks of the experiment in terms of growth rate after 3 days of re-inoculation of new medium (left), and relative carotenoids and xanthophyll content in relation to the control (right), when exposed to 0 ng/L (●) 10 ng/L (▲), and 310 ng/L (■) of diflufenican. The figure shows mean and standard deviation of the three replicates per treatment. From Gómez-Martínez et al. 2023 (*Science of the Total Environment.* 2023 Jun 1; 875,162604).

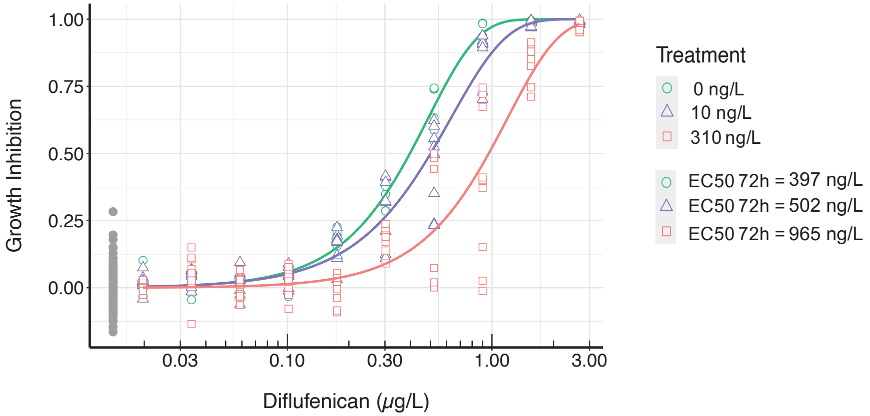


Figure 9: Diflufenican 72 h dose-response curves for R. subcapitata unexposed (green circles), acclimated to 10 ng/L (violet triangles), and acclimated to 310 ng/L of diflufenican (red squares) at the end of the experiment. The effect is calculated as relative inhibition of cell growth on a scale from 0 to 1 (0 to 100 % inhibition). Upper and lower EC50 values from 95 % confidence intervals are plotted and presented in brackets, next to the EC50 values. From Gómez-Martínez et al. 2023 (*Science of the Total Environment*. 2023 Jun 1; 875,162604).

Diflufenican inhibits the synthesis of carotenoids, which are accessory pigments involved in absorbing and dissipating the excess light captured during photosynthesis (Böger, 1996; Dang et al., 2019; Feckler et al., 2018; Miras-Moreno et al., 2019). At week 7, the algae exposed to the highest dose of diflufenican recovered their carotenoid content up to 80% compared to the controls and maintained those values over time. Sequencing of mRNA revealed that the transcription of the genes involved in the carotenoid biosynthesis pathway downstream of diflufenican's target enzyme, phytoene desaturase, were upregulated (genes are more expressed than in the controls). Moreover, the transcription of the genes involved in this pathway upstream phytoene desaturase were downregulated. This pattern matches the classical negative/positive feedback regulation, enabling active maintenance of the carotenoid biosynthetic pathway and demonstrating the algae's remarkable phenotypic plasticity (Figure 10).

One of the observed fitness costs to acclimation was that acclimated algae were smaller in size. Coping with a stressor such as diflufenican can increase the demand for resources (O2, CO2, and nutrients), and it is known that microalgae can reduce their cell size to surface area in order to enhance uptake of nutrients and reduce metabolic costs under situations of stress (Staehr & Birkeland, 2006). Moreover, changing environmental conditions can result in a loss of equilibrium between the energy flux provided by photosynthesis and the synthesis of macromolecules, leading to changes in cell cycle activity (Wagner et al., 2017; Wilhelm & Jakob, 2011). Gene sets associated with cellular structure, cellular division, DNA repair and replication, and protein folding were upregulated. This general response may reflect shifts in carbon allocation with the purpose of maintaining a stable cellular growth.

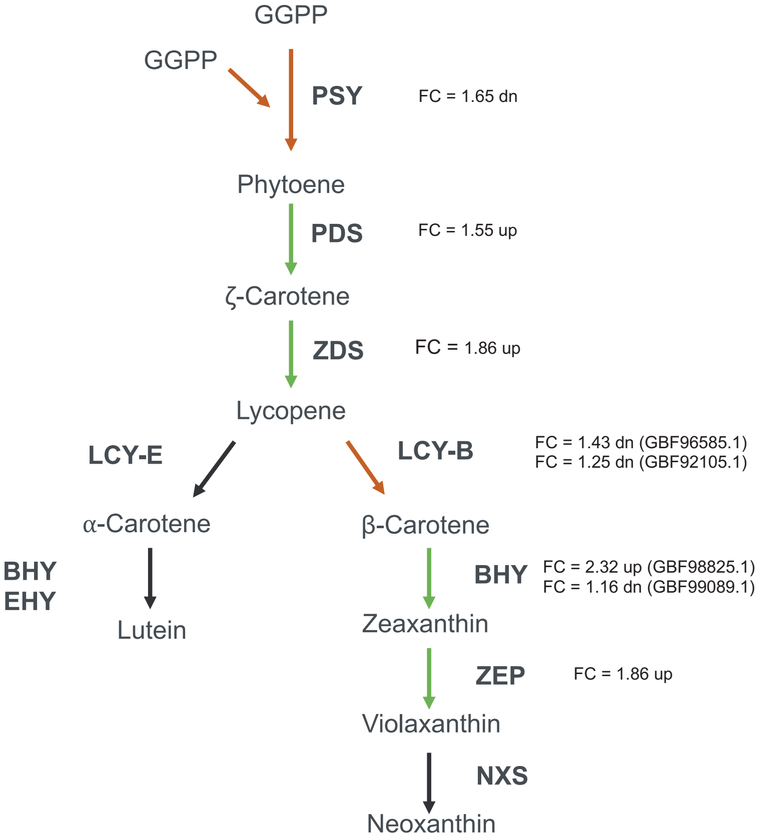


Figure 10: Scheme of the carotenoid biosynthesis pathway and main enzymes up- or down-regulated in *R. subcapitata* after 80 d of exposure to 310 ng/L of diflufenican in comparison to non-exposed algal individuals (0 ng/L diflufenican). Up-regulated genes coding for enzymes involved in the reaction steps according to the differential expression analysis performed are marked in green arrows, and down-regulated genes are marked in red arrows. The stated fold-change (FC) indicated for each enzyme is obtained from DeSeq2 analysis (Wald test). From Gómez-Martínez et al. 2023 (*Science of the Total Environment*. 2023 Jun 1; 875,162604).

Finally, acclimated algae showed a trend towards the upregulation of the enzyme zeaxanthin epoxidase, which is involved in the synthesis of abscisic acid, a phytohormone involved in the regulation of the cell cycle by inhibiting the G1/S phase progression. A drop in carotenoids during the stress phase could trigger faster cell division and upregulation of carotenoid pathway genes during acclimation.

Paper II

Biotransformation of tebuconazole and changes in fungal diversity

In **paper II**, natural biofilm communities from a Swedish river were exposed in microcosms to two environmental concentrations of the fungicide tebuconazole in both chronic (24 days at 10 or 100 µg/L) and acute manners (96 h at 100 µg/L). We observed that biofilms chronically exposed to 100 µg/L of tebuconazole can dissipate around 20% of the compound and release tebuconazole transformation products at concentrations that are 10-fold higher compared to the controls. Although studies that specifically target the biodegradation capacity of aquatic biofilms towards tebuconazole are lacking, our results are similar to those of Bertrans-Tubau et al. (2023), who reported that aquatic biofilms in microcosms were able to partially dissipate a pesticide mixture where tebuconazole was present. Studies performed in soil microbiomes have previously reported up to 22 transformation products originating from tebuconazole (El Azhari et al., 2018; Storck et al., 2016). We were not able to identify the molecular structures of the detected transformation products. However, our study revealed that the release of transformation products occurs not only in soil, but also in aquatic environments.

In terms of fungal biomass, biofilms exposed chronically to tebuconazole were more affected than those exposed acutely (Figure 11), which is in line with findings from previous studies (Artigas et al., 2012; Donnadieu et al., 2016). Even though changes in bacterial and algal biomass were expected due to their antagonistic and synergistic interactions with fungi (Dimitrov et al., 2014; Gulis and Suberkropp, 2003), our results showed that tebuconazole does not influence either bacterial or algal biomass (Figure

11). However, both fungal and bacterial community composition were influenced by tebuconazole exposures.

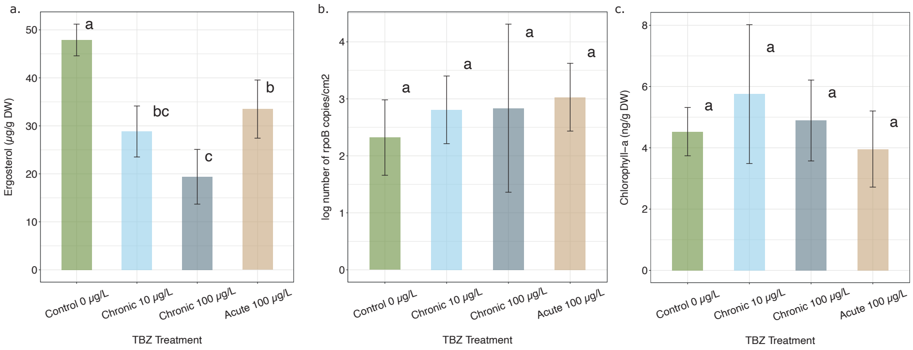


Figure 11: Biofilm responses upon exposure to tebuconazole acute (96 h) and chronic (24 days) in terms of fungal biomass – ergosterol concentration (a), bacterial biomass – number of rpoB copies (b), and algal biomass – chlorophyll-a concentration (c). From Gómez-Martínez et al. 2024 (*Chemosphere*. 2024 Dec; 369,143854).

Although biofilms exposed chronically to tebuconazole showed a 60% decrease in fungal biomass, their diversity values were higher than those exposed in an acute manner, suggesting that some taxa thrived at high tebuconazole concentrations and may be more tolerant (Figure 12).

Two species proved to be significantly more abundant in biofilms exposed chronically to 100 µg/L of tebuconazole, one belonging to the fungal class *Malasseziomycetes* and the other to *Eurotiomycetes*. These two species could potentially have different levels of ergosterol compared to the others. Previous research has documented considerable variation in ergosterol content among fungal species. For example, Gessner & Chauvet (1993) observed pronounced differences in ergosterol concentrations between different strains. Similarly, Charcosset & Chauvet (2001) found up to a 14-fold difference in ergosterol levels among four aquatic hyphomycete species under different culture conditions. These findings suggest that species with lower ergosterol content might be less sensitive to tebuconazole exposure than those with higher ergosterol levels, or vice versa.

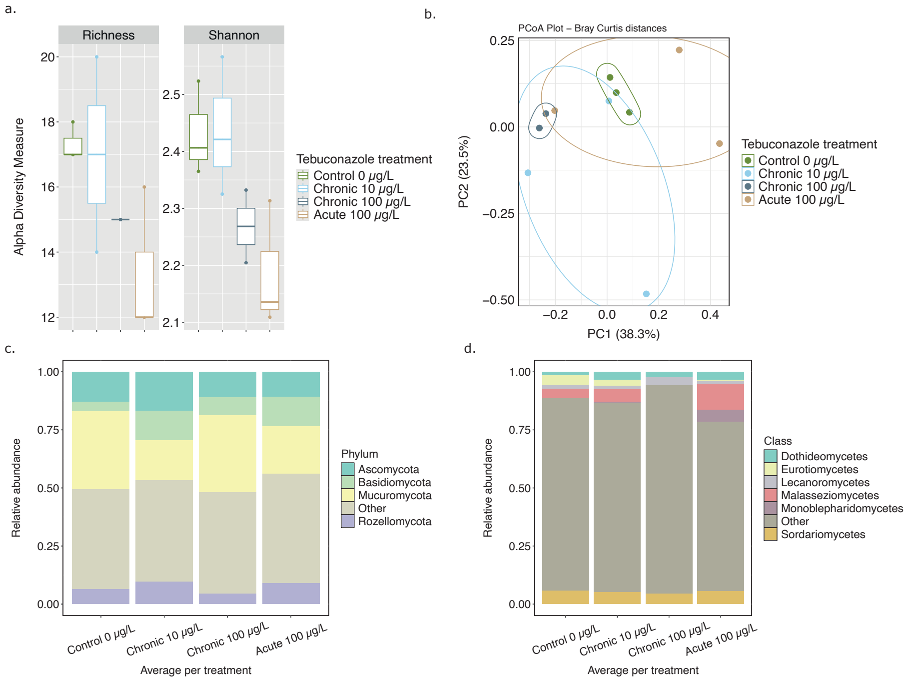


Figure 12: Fungal richness and Shannon alpha diversity indexes (a), PCoA plot based on Bray-Curtis distances (b), and fungal taxonomic profiles at the phylum (c) and class (d) levels, resulting from the RNA metabarcoding (ITS2) from the control biofilms, biofilms exposed for 24 days chronically to 10 and 100 µg/L of tebuconazole, and biofilms exposed in a 96 h acute pulse to 100 µg/L of tebuconazole, respectively. From Gómez-Martínez et al. 2024 (*Chemosphere*. 2024 Dec; 369,143854).

Bacterial community composition (beta-diversity) was different between treatments. Members of the families *Phycispheraceae* (phylum *Planctomycetota*) and *Saprosphyraceae* (phylum *Bacteroidota*) were significantly more present in biofilms exposed to tebuconazole. Both families are widespread in freshwater systems and possess a diverse set of carbohydrate-active enzymes (CAZy), that enable the degradation of a broad range of complex polysaccharides (Chiriac et al., 2023). Therefore, these bacterial groups may partially compensate for the fungal role in organic matter degradation during prolonged tebuconazole exposures.

Paper III
Community composition changes linked to pesticide pollution

Paper III is an ecotoxicological field study that compares the algal biodiversity of two agricultural streams (Skivarpsån and M42) with a less-impacted stream (Höje å), all located in southern Sweden. Sampling was conducted six times over an eight-year period (2016, 2017, and 2024).

Biodiversity changes in the agricultural streams were partly linked to higher pesticide toxic pressure and differences in nutrient composition between sites.

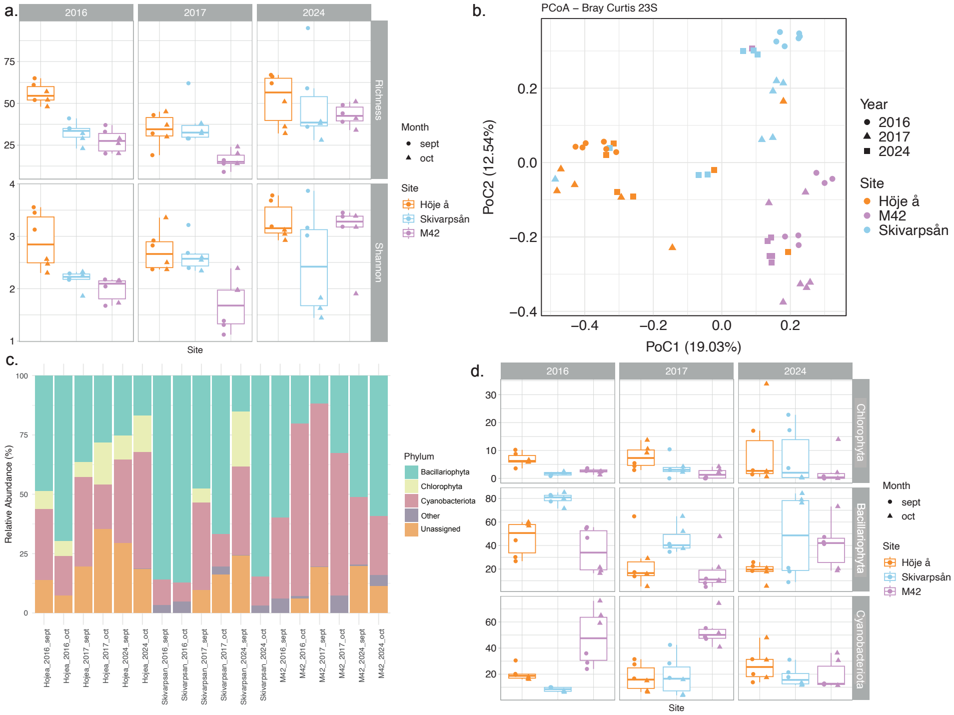


Figure 13: Algal community composition based on 23S amplicon sequencing. Algal richness and Shannon diversity indices by site and year (a); PCoA of Bray-Curtis distances (b); Phylum-level relative abundances (c); Relative abundances of *Chlorophyta*, *Bacillariophyta*, and *Cyanobacteriota* by site and year (d). Sites are represented in different colors: Höje å (orange), Skivarpsån (blue), and M42 (purple). In (a) and (d), months are denoted by shape: circles (September), and triangles (October). In (b), shapes indicate year: circles (2016), triangles (2017), and squares (2024). Each boxplot contains six replicates. From Gómez-Martínez et al. 2025 (*Manuscript*).

Algal species richness was significantly lower in M42 than in Höje å. In 2016 and 2017, alpha diversity values (species richness and Shannon Index) were lower in the agricultural streams compared to the non-agricultural stream. By 2024, however, diversity levels had converged across all sites (Figure 13.a).

Community composition consistently differed between Höje å and the two agricultural streams across all years (Figure 13.b). Moreover, the relative abundance of *Chlorophyta* showed a lower trend in the agricultural streams, while *Cyanobacteriota* showed a higher trend (Figure 13).

The environmental risk posed by pesticide mixtures in Swedish freshwater ecosystems has been well described up to 2018 (Bighiu et al., 2020; Bundschuh et al., 2014; Gustavsson et al., 2017; Rydh Stenström et al., 2021). Given that our sampling sites are located in agricultural regions where high levels of herbicides have been previously reported, we investigated whether changes in algal biodiversity could be linked to pesticide toxic pressure.

To do so, we estimated the sum of toxic units (STU). Consistent with previous studies, our results indicate a relatively low toxic pressure on algal species, with STU values remaining below the critical threshold for algal toxicity (STU < 0.1). Yet, STU values were consistently higher in Skivarpsån and M42 than in Höje å, suggesting a greater ecotoxicological stress from pesticide mixtures in the agricultural streams (Figure 14.a).

Algal species richness was significantly lower in M42 than in Höje å, with M42 also exhibiting the highest toxic pressure. In 2016 and 2017, the agricultural streams had lower species richness and Shannon diversity than the non-agricultural stream. However, by 2024, diversity levels had converged across all sites. This pattern reflects the increased resilience in pesticide-impacted streams rather than a reduction in pesticide use, as sales data indicate little change over time (Reusch et al., 2005).

Community composition also differed consistently between Höje å and the agricultural streams, supporting previous findings that pesticides can alter periphyton structure (Lips et al., 2022; Lu et al., 2020; Malbezin et al., 2024).

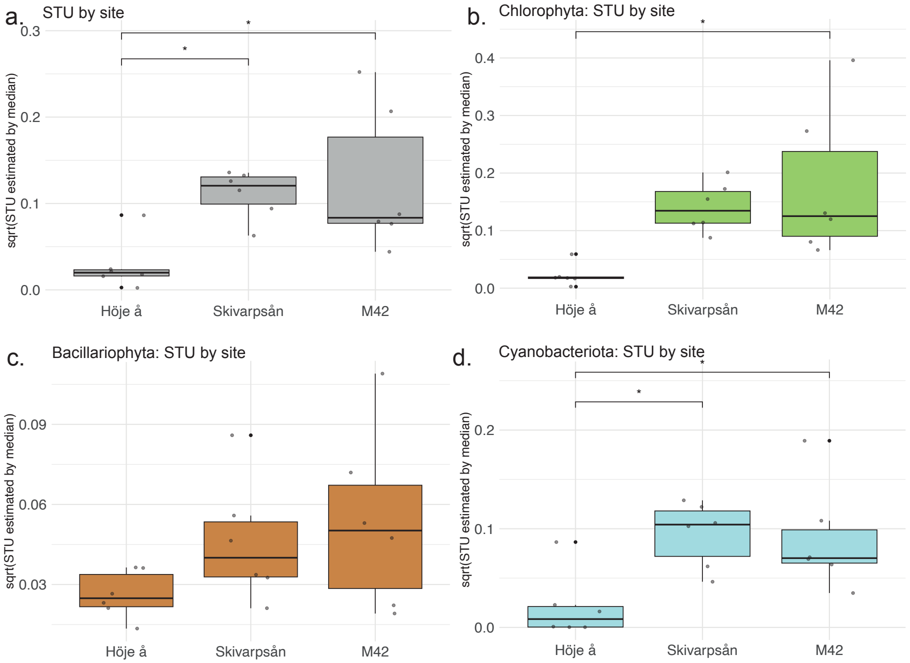


Figure 14: Cumulative toxic units (STU) estimated by the EC50 median approach, including all phyla, for all sites (a), STU for toxic pressure for *Chlorophyta* (b), *Bacillariophyta* (c), and *Cyanobacteriota* (d). In each box, the central line indicates the median (the 50th percentile), and the box itself spans from the 25th to the 75th percentile of the data. Stars denote significant differences between sites after Tukey HSD postHoc test. For all boxes, the number of replicates was 6. All STU values were below 0.1, which is the critical threshold value for algal toxicity according to EFSA (2013). From Gómez-Martínez et al. 2025 (*Manuscript*).

In **paper III**, the toxic pressure was individually estimated for the three most relevant algal phyla, which are *Chlorophyta*, *Cyanobacteriota* and *Bacillariophyta*. For both *Chlorophyta* and *Cyanobacteriota*, individual values of STU were significantly higher in Skivarpsån and M42 (Figure 14.b and c). However, only *Chlorophyta* showed lower relative abundance in the agricultural streams. Previous studies suggest that this algal group is more sensitive to pesticides than *Bacillariophyta* and *Cyanobacteriota* (e.g., Malbezin et al., 2024; Peng et al., 2019). In contrast, *Cyanobacteriota* is known to adapt quickly to rising herbicide levels in freshwater through rapid evolutionary processes (Melero-Jiménez et al., 2021), which may explain why they outcompeted *Chlorophyta*. For example, Karier et al. (2017) reported that while *Chlorophyta* are usually dominant, exposure to metazachlor can create conditions favoring *Cyanobacteriota*. Similarly, Lin et al. (2023) found that the addition of glyphosate can strongly boost the competitiveness of *Cyanobacteriota*, emphasizing their adaptive advantage.

Paper IV

Resistome profiling of bacterial sediments in the Göta Älv

In **paper IV**, we characterized the resistome of sediment bacterial communities in the Göta Älv, the largest river in Sweden, through two culture-independent approaches: qPCR and shotgun metagenomics. The analysis of six clinically relevant mobile genes through qPCR (i.e., *sul1*, *ermB*, *bla*$_{CTX\text{-}M}$, *qnrS, tetX* and *tetA*) not only in sediments, but also in sludge and effluents revealed that the genes that were most prevalent in the sludge and the effluents, were also the most prevalent in the river sediments. These were *sul1* and *ermB*. Resistance to sulfonamides (e.g., *sul1*) and macrolides (e.g., *ermB*) has been previously revealed in river sediments (Adeniji et al., 2020; Brown et al., 2019; Harnisz et al., 2020; Li & Zhang, 2020; Ohore et al., 2019; Proia et al., 2013; Rieke et al., 2018; Stoll et al., 2012; Su et al., 2014). In addition, *sul1* has been reported as the gene with highest average relative abundance compared to the rest of the studied genes in surface water from the rivers Eskilstunaån and Fyrisån, located on the Swedish east coast (Lai et al., 2021). In this study, the different abundances of *sul1* gene copies in the effluents did not relate to changes in abundances of *sul1* in river sediment, even though some studies have found the opposite, particularly for *sul1* (Koczura et al., 2016). However, evidence also indicates rapid decreases in ARGs from the effluent to the river within short distances (2–2.5 km), downstream of WWTP discharge points (Lee et al., 2021).

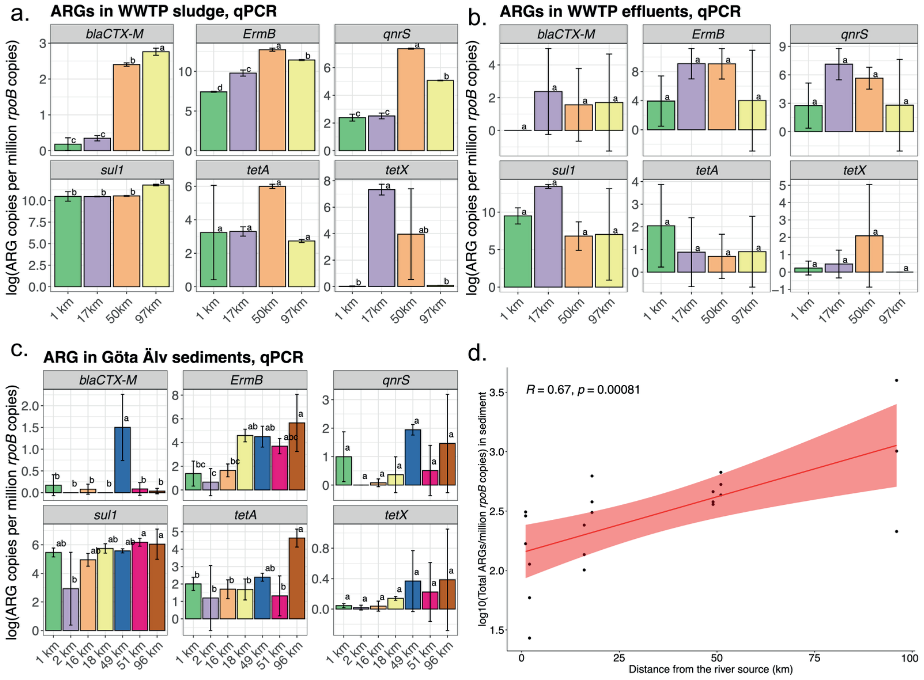


Figure 15: Abundance of the six qPCR-targeted ARGs (*blaCTX-M, ErmB, qnrS, sul1, tetA,* and *tetX*) in the WWTP sludge (a), WWTP effluents (b), and Göta Älv's sediments (c), expressed in number of ARG copies per million *rpoB* gene copies. The different sampling sites along the Göta Älv river from the source (0 km) to the estuary (96 km) are represented in the x-axis (Figures 2.a, 2.b and 2.c). Coloured bars represent average values, error bars represent the standard deviation (n=3), and the displayed letters on top of the bars indicate a significant result between treatments in Tukey's multiple comparisons post hoc test (p<0.05). The results of Pearson correlation analysis between distance from the river source and total ARG copies in sediment samples (d). From Gómez-Martínez et al. 2025 (*Manuscript under review in FEMS Microbiology Ecology*).

The analysis of ARGs via qPCR also revealed a positive correlation between the total abundance of the six targeted genes and the distance from the river source. Our findings align with previous studies. For instance, Lai et al. (2021) reported a higher amount of ARGs relative abundances in urban recipient water bodies compared to their upstream sites.

These results indicate that the transport of ARB and free DNA along the river contributes to higher ARG abundances in downstream sections of human-impacted rivers. Furthermore, co-selection of antibiotic-resistant bacteria driven by chemical pollutants, such as heavy metals, should not be overlooked, especially since the port of Gothenburg, located at the estuary of the Göta Älv, is known to experience substantial contamination of water and sediments from boat-associated pollutants such as heavy metals (Gupta et al., 2022; Norén et al., 2020).

Shotgun metagenomics revealed that *sul1* and *ermB* were among the 15 most abundant genes in sludge samples. Although undetected in sediment through metagenomics, qPCR showed that their copy numbers correlated with metagenomic counts in the sludge, suggesting that thousands of copies per million bacterial cells are required for detection in metagenomics, highlighting one of the limitations of shotgun metagenomics.

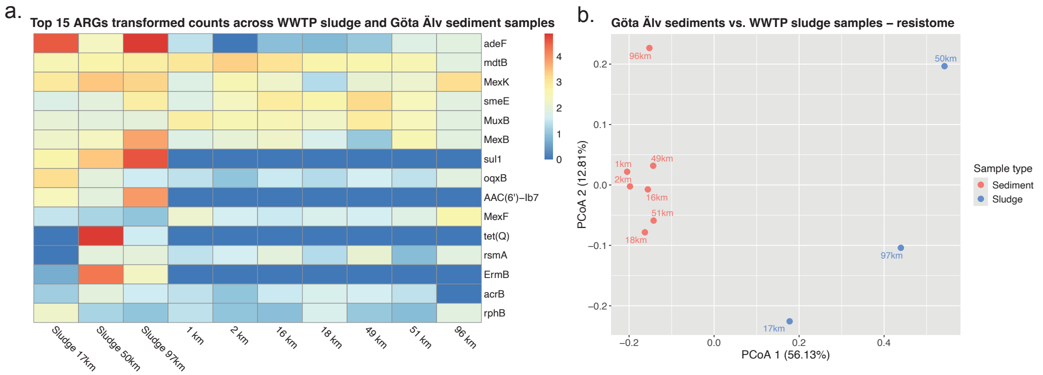


Figure 16: Resistome profile obtained from shotgun metagenomics analysis. Heatmap containing top 15 ARGs log-transformed counts across WWTP sludge and sediment samples (a), and PCoA plot based on Bray-Curtis distances, including sludge and sediment samples from the river source at 1 km to the river mouth at 96 km (b). From Gómez-Martínez et al. 2025 (*Manuscript under review in FEMS Microbiology Ecology*).

Moreover, shotgun metagenomics revealed a high presence of multidrug resistance efflux pumps both in sludge and river sediments (i.e., Mex-type multidrug resistance genes) and indicated that the resistome and taxonomic profiles at the river mouth (Gothenburg) differ from the rest of the upstream samples. The Mex-type multidrug resistance efflux pumps are the Resistance Nodulaltion Division (RND) efflux pumps, which are responsible for antibiotic efflux and resistance in *Pseudomonas aeruginosa* (Langendonk et al., 2021). In our study, Mex-type genes were among the most abundant genes found in the sediment. These genes form part of antibiotic specific efflux pumps (i.e., *MexAB-OprM* and *MexEF-OprN*) that are of clinical relevance but also comprise an intrinsic form of resistance (Alcalde-Rico et al., 2018; Puzari & Chetia, 2017).

Genus-level beta-diversity differed between sludge and sediments, and the river mouth sediment was also separated from the rest. Such shifts in microbial diversity often coincide with changes in the resistome (Chen et al., 2019; Rubin-Blum et al., 2023; Samson et al., 2023). Subirats et al. (2023) further suggested that increased ARG efflux genes may confer a selective advantage in chemically polluted environments, altering microbiome diversity.

Among the dominant genera in sediment were *Streptomyces*, *Pseudomonas*, and *Nocardioides*. As previously said, *Pseudomonas* carries Mex-type genes, while *Nocardioides* is known for carrying sulfonamide and tetracycline resistance genes (Chen et al., 2019; Guo et al., 2019). We observed a higher abundance of *Nocardioides* in the estuarine region (river mouth). This genus can use a wide range of organic substances as carbon sources, including petroleum hydrocarbons (Ma et al., 2023). Its prevalence may therefore indicate environmental pollution potentially linked to boat traffic and other anthropogenic activities.

Conclusions and future perspectives

In this thesis I aimed to provide new insights on the individual and community-level effects, acclimation and adaptation mechanisms towards pesticide exposures in freshwater microorganisms. Moreover, I also aimed to describe the sediment resistome of the Göta Älv, and the influence of WWTP effluents along its course. Some questions have been answered, while others remain only partially resolved, and new ones have emerged.

In **paper I**, the phenotypic plasticity of *Raphidocelis subcapitata* was highlighted. This green algal model species was able to acclimate to environmental concentrations of diflufenican that were initially toxic, in a time frame of four weeks. However, this acclimation came with trade-off expenses, on both phenotype and transcriptome levels. The acclimated algal cells were reduced in size and genes related to protein damage and translational dysfunction, protein unfolding, DNA repair and malfunctioning of the cell cycle were overexpressed, indicating that a residual stress state remains in the acclimated algae. Moreover, this study has shown that phenotypic endpoints are sometimes not enough to unveil all the effects a herbicide has on algae. The major limitation of this study is also one of its advantages. We exposed one single algal species to one single herbicide, which allowed us to describe in high detail the acclimation effects, not only at on a phenotypic but also on a transcriptomic level, leaving us with no doubt that the observed effects were solely due to the diflufenican exposures.

This acclimation study is only the first step towards a better assessment of not only effects, but also acclimation processes that occur in microalgal species when exposed to herbicides. Further studies including other environmental variables such as temperature, light intensity, pesticide mixtures and algal communities would be of great relevance in the assessment of acclimation effects under more realistic environmental conditions.

In **paper II**, we moved one step forward in terms of biological complexity, by exposing environmental biofilms to the fungicide tebuconazole in a microcosm approach. Fungi exposed to tebuconazole, both in an acute and a chronic matter, showed a decrease in biomass. Meanwhile algae and bacteria remained comparable to the controls. Moreover, the biofilms were able to partly degrade tebuconazole and release unknown transformation products into the water column. This could be one acclimation or adaptation mechanism towards tebuconazole. One new question arising from these results is the toxicity of such transformation products. We could not characterize these compounds in terms of chemical structure or toxicity, and therefore further toxicity and chemical analytical tests would be needed to do so.

Tebuconazole also induced changes in the fungal community composition, and two species were differentially abundant in tebuconazole-exposed biofilms. These emerging species could potentially be more efficient at coping with tebuconazole, potentially by degrading it, however, this efficiency could also involve fitness trade-offs that could imply a lower efficiency at other functions, as observed in **paper I**. A complementary or follow-up study to **paper II** could involve a better characterization of the environmental functions of the exposed fungi to test if there are any fitness trade-offs, for example, the quantification of genes related to organic matter decomposition, as well as a better characterization of the released tebuconazole transformation products and their degradation pathway, possibly by q-PCR-targeting only specific genes that are known to be involved in general fungal degradation pathways. Overall, our results and the suggested further studies would provide a better assessment of the effects of environmental concentrations of this fungicide. **Paper III** uses concepts introduced and explored in **paper I** and **paper II**, such as the effect of herbicides in algae and the study of community composition changes through metabarcoding and brings them together in a field-based study. In **paper III,** we attempted to answer whether the observed differences in periphytic algal biodiversity between two agricultural and one non-agricultural impacted river were associated with different levels of pesticide mixture toxicity, as well as other factors that are consequences of agricultural land use, such as high levels of nutrients or conductivity. The results of **paper III** are much more inconclusive compared to **papers I**

and II, because of its high environmental realism. Moreover, **paper III** contains measurements from three different years, spread across an eight-year span, and two different sampling occasions per year, which makes the variability much higher. Overall, we observed that the streams impacted by agricultural land use had lower species richness and different algal community composition. Also, the algal phylum *Chlorophyta* showed a lower relative abundance trend in the agricultural streams, while *Cyanobacteria* showed a higher relative abundance trend. The overall pesticide toxic pressure was higher in the agricultural streams. More specifically, the toxic pressure for both *Chlorophyta* and *Cyanobacteria* was higher in agricultural streams. However, only *Chlorophyta* decreased in the agricultural streams, suggesting that this phylum might be more sensitive to herbicides and/or high discharge of nutrients, and opportunistic species belonging to the phylum *Cyanobacteria* might have replaced them. In turn, the ecosystem's food web of agricultural streams might be disrupted, since this algal group is considered a low-quality food source due to its low content in polyunsaturated fatty acids (PUFAs) and sterols. Further studies assessing predator abundance are required to verify this hypothesis. At the algal trophic level, however, potential acclimation mechanisms and related fitness trade-offs remain unknown. Given that diflufenican has long been one of the dominant herbicides driving the toxicity of these mixtures, it could be expected, based on findings from **paper I**, that acclimation changes may have occurred, potentially leading to fitness trade-offs as well. Moreover, as in **paper II**, a characterization of bacterial and fungal diversity would also provide answers on how the entire biofilm dynamics is affected by the pesticide mixtures.

Antibiotic resistance is one of the most significant public health issues of the 21st century. However, the freshwater environmental resistome remains understudied compared to the human and animal gut resistomes, making this topic worth exploring. **Paper IV** aimed to characterize the resistome of the sediments in the Göta Älv and to investigate if WWTP effluents serve as sources of antimicrobials and ARGs into the river, and if they influence the resistome in the sediments. We found that ARGs accumulate along the Göta Älv and show a distinct diversity compared to upstream locations, not only in resistome composition but also at a taxonomic level. **Paper IV** also offered the chance to evaluate the two most widely applied DNA-based approaches for ARG detection, illustrating how they complement one another to provide a more comprehensive view. As a future direction, it could be suggested that an initial survey using shotgun metagenomics would have identified the most prevalent genes, which could then have been selectively quantified by qPCR to achieve a more refined profile, and then profiled over time. In summary, **paper IV** contributes to the comprehension of the distribution and features of ARGs in a high-flow urban river and delivers valuable site-specific insights that are particularly relevant since the Göta Älv ultimately supplies raw water for humans and domestic animals that depend on effective antibiotics.

In this thesis, each chapter examines how human activities influence freshwater microorganisms from different perspectives. This outlook section encourages the reader to think beyond classical ecotoxicological approaches. The first three papers collectively highlight that fitness trade-offs, often overlooked in traditional ecotoxicological bioassays, can be as important and ecologically impactful as conventional phenotypic endpoints. A key conclusion is that, microbial communities acclimate, adapt and change in response to pesticides through different mechanisms, and although it is well established that microorganisms live in communities, there is still no standardized framework for assessing toxicity endpoints at the community level. Moreover, even less progress has been made in evaluating the toxicity of pesticide mixtures in such contexts. This gap makes it particularly challenging to associate the toxicity of pesticide mixtures to changes in community structure and function. Finally, this thesis stresses the importance of characterizing rivers that serve as drinking water sources in terms of antibiotic resistance genes (ARGs), which remain underrepresented compared to other ARG reservoirs. While studying all these processes is complex, this thesis demonstrates that high-throughput sequencing technologies can effectively reveal molecular mechanisms, cell-level responses, community-level dynamics, and genes associated with traits relevant to both ecological function and human health.

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