“UNDER PRESSURE”

INTERACTION BETWEEN PHARMACEUTICALS AND RIVER BACTERIA

Aleksandra Hagberg
To my Mother

Thank you for always being there for me.
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Abstract

Pharmaceuticals are often entering the environment without being completely decomposed. Once released in the environment they continue to carry on their main function but instead targeting the inhabitants of the aquatic ecosystem. Our interest was drawn towards the bacteria, that are often present in the environment in the form of biofilms. Despite their small size, they are carrying on important functions for the ecosystem. Any disruption in their work can potentially result a disturbance in the whole ecosystem. Thus, knowing the possible effect of the pharmaceuticals on bacterial biofilms can give us more understanding about the mechanisms that lie beneath pharmaceutical pollution.

Natural degradation processes like photolysis, hydrolysis, and biodegradation can reduce pollutant concentrations. Bacterial biofilms, common in aquatic ecosystems, play a crucial role in pharmaceutical degradation process. The extracellular polymeric substances (EPS) produced by biofilms enhance their tolerance to environmental stressors.

This study focuses on bacterial biofilms chronically exposed to low levels of pharmaceuticals remaining in the treated waste water released from a STP into the Knivsta River, Sweden. Using the sequencing we mapped the species that inhabited the sampling location.
Model biofilm consortia were constructed and characterized using various analysis techniques. Experiments investigated bacterial motility, biofilm formation, and interactions between isolates. The bacterial isolates exhibited diverse motility patterns. Cross-cultivation assays indicated coexistence without negative interactions among isolates. Chemical analysis using ATR-FTIR spectroscopy and cryo-XPS revealed differences in macromolecular composition among isolates.

The impact of pharmaceuticals, such as Trimethoprim and Diclofenac, on bacterial growth was studied.

The findings contribute to understanding the complex interactions between pharmaceuticals and bacterial biofilms, crucial for assessing environmental risks and designing possible wastewater treatment strategies.
Abbreviations

WWTP – Waste water treatment plant
STP – Sewage treatment plant
SP – sampling point
CV – crystal violet
ATR FTIR - Attenuated total reflectance Fourier transform infrared
XPS – X-ray photoelectron spectroscopy
EPS – extracellular polymeric substance
STP - Sewage treatment plant
OD – Optical density
OPLS -DA -Orthogonal Partial Squares Discriminant Analysis
PCA – Principal component analysis
LC-MC – liquid chromatography mass spectrometry
List of publications

This thesis is based on the following paper, which are provided as appendices.

I. Aleksandra Hagberg, Shashank Gupta, Olena Rzhepishevska, Jerker Fick, Mette Burmølle, Madeleine Ramstedt.


II. Aleksandra Hagberg, Olena Rzhepishevska, Anastasiia Semenets, David A.Cisneros, Madeleine Ramstedt

Surface analysis of bacterial systems using cryo-X-ray photoelectron spectroscopy. *Surface and Interface Analysis* 52(1) (June 2020)

III. Elena Yunda, Aleksandra Hagberg, Thibault Duteil, Fabienne Quilès, Madeleine Ramstedt

Biochemical composition and biofilm formation by isolates of river bacteria in presence and absence of environmental stressors.

IV. Aleksandra Hagberg, Olena Rzhepishevska, Christine Gallampois, Fabienne Quilès, Madeleine Ramsted

Metabolic alterations in a Swedish *Pseudomonas sp.* river isolate in presence of diclofenac
Author’s Contribution

Paper I

The author participated in the last part of the sampling performed the experimental work and was involved in drafting the text.

Paper II

The author performed part of the experiments and wrote a section about microscopy in the paper.

Paper III

The author planned and executed large parts of the experimental work, as well contributed to manuscript preparation and writing.

Paper IV

The author was involved in planning of the study, performed some of the experiments and analytical work, as well as contributed to manuscript writing.
Introduction

The environmental impact of pharmaceutical pollutants was not considered as significant until the late 90s when the first data regarding the surface water contamination arrived. Richardson and Bowron made an early prediction regarding the growing amount of pharmaceutical residues in the environment in the 80s, and it took over a decade until their theory was proven. Today pharmaceutical residues are found worldwide: in freshwater, ground water, marine water and even in the ice of the Antarctic. Pharmaceuticals are compounds picked from other substances due to their ability to affect a specific biological target better than the other chemicals. Once released in the environment, they continue to be biologically active and the impact of pharmaceuticals on the environment is not entirely known yet. For example, 80% of antimicrobials that are released in environment stay active and intact.

The pharmaceutical consumption is growing every day resulting to a bigger number of pharmaceutical residues in the environment. The majority of the pharmaceuticals are entering the environment through three main pathways: via patients with further excretion to wastewater, from industrial wastewater and from improper disposal of expired pharmaceuticals. For instance, between 30 to 90% of antibiotics that enter the aquatic environment are excreted via urine as active substances. There are the also a minor pathway such as landfills and livestock activities. Pharmaceuticals, as well as other contaminants, undergo a series
of changes during wastewater treatment in the STP. Part of them breaks down to smaller fragments potentially less harmful than the original substances. But some of them remain unchanged, and those contaminants are potentially posing danger to aquatic biota.

In the environment, the concentration of pollutants could slowly be fading away without human intervention. Due to photolysis, hydrolysis and biodegradation, pharmaceuticals potentially can be decomposed into less hazardous substances. Biodegradation differs from the other environmental reactions since it involves living organisms - bacteria, fungus, and yeasts. For this reason, bacteria are widely used in wastewater cleanup. All environmental degradation relies on the chemical and physical properties of the pollutants, and therefore may differ between substances. Some groups of pharmaceuticals that are not degradable under environmental conditions are prone to bind to suspended matter in the aquatic environment or may deposit in sediment for a long time.

Despite that the chemical and physical properties of each pharmaceutical component are well studied, the fate of pollutants in the environment is not known. One reason may be that each location where the sewage water is released has its unique properties. Thereby it becomes hard to predict the future fate of pharmaceuticals once they are released in the environment.
In the environment, bacteria are often present in the form of biofilms, sessile microbial communities, often in cooperation with different species such as fungi and algae. Biofilm play an essential role in fundamental ecological processes, for instance biogeochemical cycling of nutrients. They are able to degrade pollutants and other xenobiotic compounds, both organic and inorganic. This way they may be controlling the water quality. However, if the pollutant hampers microbiological activity, for example by compromising the bacterial structure and its activity, this can potentially lead to disruption of the whole ecosystem.

One of the most distinctive features of bacteria inside a biofilm is their ability to create a protective layer, matrix, which consists of a complex mixture of polysaccharides, proteins, extracellular DNA, and water (over 97% water). For example, soil bacteria continuously experience water stress. By producing extracellular polymer substances (EPS), bacterial biofilms prevent water loss. This puts them in a better position than free-living bacteria that do not benefit from the presence of a matrix of EPS. The composition of EPS in the matrix can vary depending on the environment as well as species that inhabit the biofilm. The EPS increase bacterial biofilm tolerance against wide variety of environmental stressors. In addition, the protective ability of the biofilms is enhanced when multiple species are present.
Bacterial biofilms use sorption through the matrix as an effective way to provide resources for themselves\textsuperscript{43}. The ability of the biofilms to capture substances from the environment is not compound specific\textsuperscript{35}. Thus, toxic substances, as well as nutrients, can be trapped inside the biofilm \textsuperscript{44}. Thus, making the biofilms a potential reservoir for pollutants. Biofilms play an essential role in the aquatic ecosystem as a food source for grazing organisms \textsuperscript{45}, for instance protozoa\textsuperscript{46}. Therefore, bacteria biofilms are located at the very beginning of the food chain in aquatic ecosystem. Consequently, contributing to the contamination of the whole food chain up to the higher predators, where contaminants tend to accumulate at higher levels (biomagnification)\textsuperscript{44}. Importantly biofilms interact with both biotic and abiotic matter\textsuperscript{37}. By biodegradation, biofilms decompose the organic components into smaller constitutes that continue their life cycle in the ecosystem\textsuperscript{47}. Any change in this process could result in an undesirable effect on the whole ecosystem. Knowing how pharmaceuticals interact with aquatic habitants therefore provides an understanding of processes that are a result of pharmaceutical pollution and water quality.

The aim of this thesis work was to investigate the effect of environmental stressors, in particularly pharmaceutical residues, on bacterial biofilms. We were interested in the influence of contaminants both on macro and micro levels. The first represented by a field study and the second by experimental work done in the lab. On the macro level we investigated the influence of pharmaceutical contaminants on bacterial communities in a small...
fresh-water stream. We also studied how exposed community composition differed from the communities that had not experienced continuous exposure. On the micro level we studied the influence of contaminants on bacteria isolates separately and made a first attempt to model biofilm. It this way we were hoping to build knowledge about possible bacterial responses to the pharmaceutical exposure.
Materials and methods

Microbial field sampling

Four sampling points were chosen. Two were located upstream the Knivsta sewage treatment plant that releases the effluent water into the stream, Knivstaån (Location №1: 59°43'37.6"N17°47'05.0"E) (Location №2: 59°43'25.6"N17°47'13.7"E). Two were downstream the contamination (Location №3: 59°43'04.1"N17°47'29.0"E) (Location №4: 59°42'16.9"N17°47'05.0"E). Sampling were made three times in one year (May, June, and October, 2018). The water level was differed depending on season, in June the lowest water level was registered, in comparison to May and October. It was an important fact, considering that Knivsta river is small in size and a lower water level could influence the concentration of the contaminants from the treated sewage water.

Since biofilms more likely would grow on a solid surface, we picked small stones from the bottom of the river in an area of 10 x 10 cm, using forceps previously sterilized in ethanol and in lighter flame. Stones were collected in two 50 ml Falcon tubes: A and B. Tube A contained stones for further isolation on R2A media (medium for freshwater samples). Tube B was used for DNA extraction directly from the substrate. By using specific media and terms of cultivation we expect to have lost certain species that require different growing conditions. By comparing results obtained from tube A and B we would get information of which
species were not cultivated on the selected media and that therefore were not present on the plates.

Some sampling points did not contain any stones and the bottom of the river was covered with mud. In order to still sample in these points, we picked parts of plants which were under water. We also "planted" new stones that were previously collected in another place, washed and autoclaved. They were supposed to be an attractive alternative surface for biofilm growth in those areas where there was a lack of natural stones. From this point, all samples were labelled according to on which substrate they were growing: natural stones, planted stones and plant material. It was important to distinguish samples in this way to enable investigations concerning if there were any difference in bacterial communities and their diversity on various substrates.

To separate biofilms from the substrate, stones from Tube A were vortexed with 1 ml PBS for 5 min. Thereafter 100 µl were separated and diluted 1:10 and plated on 1/10 R2A agar (media designed for freshwater samples) with cyclohexadiene (50mg/mL added before pouring the media.) to prevent fungal growth. On each plate 100 µL of obtained suspension was spread. After four days of cultivation, the surface of the Petri dish was covered with various bacterial colonies. Approximately 15 colonies were picked from each plate and individual freezer stocks were made out of them. The rest of the colonies were pooled and stored in plastic 15 ml tubes with 50 % glycerol at -80-degree C to enable future
continued isolation if needed (more details can be found in paper I).

Purification of the collection of individual isolates
Bacteria were cultivated from individual freezer stocks on Petri dishes with R2A agar for three to four days at room temperature. All plates were kept under a large glass beaker with a glass of water to keep the air moist. Most of the isolates grew as a homogeneous culture and appeared to contain one type of bacteria. To make sure that further, we would be dealing with single isolates, each isolate was recultivated at least two times. Once the strains were considered as single isolates, they were taken from the plate and resuspended in 1 ml of R2A 100% media. These liquid samples were placed in 15 ml Falcon tubes in a shaker at slow mixing for 24 hours. Thereafter they were frozen with Glycerol (50%) in individual tubes, and stored at -80 degrees C.

Creating a library of isolates
Dealing with a large number of isolates, it was essential to create an accessible description of the library that would allow us to navigate through and help select and us narrow down the number of bacteria for future experiments. By limiting the number of isolates for further stages of our research, we wished to move towards experiments with controlled laboratory conditions. This approach means that the conditions that we created for model biofilms do not recreate the full complexity of biofilms in the environment.
For this purpose, all bacterial isolates were photographed on an agar plate as a first step. After that, I created criteria to describe the morphology of the colonies on the plate considering their size, shape, color, and any specific visible characteristics, such as high viscosity or an abundance of water in the EPS of the colonies. All those details were noted down in order to allow bacterial isolates to be distinguished according to their appearance during subsequent work. The whole initial library contained 192 isolates.

Sequencing of consortia
During the sampling we collected the material into two tubes (A and B) for sequencing (Figure 1). Tube A samples underwent several stages of isolations and recultivations, as mentioned above, before sequencing.

To facilitate the experimental work, separate isolates were merged together according to sampling location and the time of the year when they were collected. As a result, we ended up having 15 tubes (4 tubes from sampling points 1-4 in May; 4 tubes from points 1-4 from June; 4 tubes from points 1-4 from October; and 3 tubes obtained from the planted stones collected in October in points 1, 2 and 4, since these stones were lost in point 3). Thereafter DNA was extracted from the pooled samples for DNA sequencing. For the tubes marked with B, DNA extraction was done directly from the substrate, skipping the isolation steps.
Figure 1. Two strategies for sequencing the samples picked from the river Knivsta.

The DNA extraction for both tubes was performed the same way using the Qiagen Powerbiofilm Kit protocol, except that the stone samples were sonicated with metal beads for 15 min before the extraction. This was done to separate biofilms from the substrate. Further PCR was made using a High-fidelity PCR kit and bacterial primers: 341F: CCT AYG GGR BGC AG and 806R: GGA CTA CHV GGG TWT CTA AT. To assure that the extracted samples contained DNA, we used agarose gel electrophoresis to visualize the DNA in the sample.
Shortly after the first PCR, a second PCR was performed in order to have more material. The same procedure was used with the exception that no primers were present in the second PCR. The second PCR was also followed with agarose gel electrophoresis (Figure 2).

Sequencing of colonies from sampling point 4 from May. The majority of the strains that grew on plates had the same phenotype: small white colonies. We assumed that some of them might belong to the same species. To ensure a varied collection, we decided to select colonies with different morphologies. Thus, we chose 16 isolates to sequence using 16S rRNA, from the 26 initially collected isolates. The isolates were sent for sequencing to a commercial lab (Eurofin) on agar plates with R2A media.
Limiting the number of the bacteria samples
We aimed to design the model biofilm based on a few isolates for further experiments (Figure 2). For that, we needed to select a smaller number of isolates from the larger collection. This approach was chosen to allow us to perform controlled experiments and study the processes occurring between the pharmaceutical contaminants and the biofilms in more detail.

At first, we were choosing one certain media and cultivating conditions to benefit the growth of slow growing river strains. That would mean that many species that were in the river would no longer participate in the model. Then, we specifically choose to use isolates from locations that had already been exposed to contaminants remaining in the treated sewage water; i.e. Sampling points 3 and 4. Since we experienced problems sampling in location number 3, we decided to focus on Sampling point 4. From this sampling point we had isolated of 26 isolates and from them 16 were sent for 16S rRNA sequencing. From the sequenced strains four isolates were selected for in-depth studies (Paper 3 and 4).
Figure 3. The graphical representation of process of selecting the isolates for future experiments.
Characterization of bacterial strains

Before performing any experiment where we would expose the biofilm to pharmaceutical contaminants, we needed to look closer at our bacteria. Therefore, we performed several experiments to study the bacteria from different points of view, thus learning their morphology, as well as physical and chemical properties.

Crystal violet
One of the first experiments that we performed was to characterize the bacteria isolates was a biofilm assay using Crystal Violet (CV) as a quantitative measure. It’s a classical assay to determine biofilm formation. We grew bacteria separately and together in a group of four over 24 h in 96 well plate, and subsequently colored with CV. The OD were measured on Perkin Elmer Wallac 1420 Victor 2 Microplate Reader (Paper 3).

Growth curves
We performed two types of growth- curve experiments, a manual version and an automated version. Early in the project manual growth curves were done to learn the growth dynamic of bacteria. Results from manual curves were also compared to results from automatic curves in Paper 3.
For the second type of growth curve experiment we used an automated protocol and 96-well plates. These experiments also
included adding the pharmaceutical in the various concentrations and monitoring bacterial growth (Paper 3 and 4).

To establish whether the bacteria samples were able to coexist we performed a series of cross-cultivation experiment on R2A plates (10% R2A). Two bacteria strains from the frozen stock were streaked across each other on a plate (Paper 3). The cultivation of bacteria took between 3 and 4 days at 25 C.

Motility
To examine what types of motility the bacteria were able to do we performed a series of experiments using agar plates of different density. Two biological replicas and four repeats were done per isolate for each type of movement (twitching, swarming, and swimming). Since our bacteria grow very slowly, we decided to prolong the experiment to 72 h and perform measurement every 24 h. We measured the diameter of the bacterial colony; if the shape was not circular, the measurement was taken at the longest distance (Paper 3).

AFM analyses
For AFM, we prepared samples in two different ways. The first set was cultivated on a culture plate. One colony was taken and mixed with 100 µL of MQ water. After that, 10 µL of solution was placed on mica on top of a sample holder and left to dry, in air, in a laminar flow cabinet. The second set was prepared in the same
way except that suspended samples were centrifuged at low speed for 2 to 5 minutes and only the pellet was placed on the mica before drying. Thus, separating the bacteria from EPS (Paper 2).

SEM analyses
To get a closer look at the morphology studied bacteria we also performed SEM analyses (Paper 2 & 3). The preparation stage required the samples to be dried in a series of ethanol baths with a concentration going from 70% to 98%. Thereafter, samples were critical point dried and coated with 5 mm of iridium to avoid charge build-up on the surface of the sample during analysis. Samples were analysed with Carl Zeiss Merlin Microscope. For cryo-SEM, bacteria were grown on an agar plate with R2A media. A piece of agar with a colony on top was placed on carbon tape. The sample was immersed into liquid nitrogen and subsequently analysed with Carl Zeiss Merlin Microscope at low temperature. However, *Pseudomonas* and *Rhizobium* isolates easily detached from the agar. As a consequence, we scraped colonies from agar and put them directly in the fixative solution and thereafter onto the sample holder for Paper 3.

Exposure to pharmaceuticals
In our investigation particular attention was directed towards Trimethoprim due to its antibacterial properties. Based on results from the growth curves we picked concentration of 25 mg/L. Utilizing this concentration as the upper limit, we further explored two additional concentrations (12 mg/L and 6 mg/L). The bacteria
were cultivated under static conditions, and at the end of the growth period, they were pipetted into cuvettes for OD 600 measurements. By scraping the bottom of the well plate with sterile pipet tip, I aimed to collect both planktonic cells and biofilm.

Despite being water-soluble, when added to sterile MQ water, Trimethoprim resulted in the formation of white sediment. To address this issue, acetic acid was introduced; however, this led to a significant decrease in the solution's pH, creating an acidic environment. Given that our studied bacteria thrive in a neutral pH range, it became crucial to determine whether growth inhibition was due to trimethoprim itself or the acidic conditions. Consequently, additional controls were implemented, consisting of bacteria and acid alone, and bacteria with neutralized acid (presence of acetate), to isolate and understand the impact of acidity on bacterial growth. The cultivation period lasted up to 24 hours, and metal foil was used to cover the plates, preventing exposure to light.
Results and discussion

The bacterial consortia present in the Knivsta River have been exposed to purified wastewater for a long time. Presumably, this means that the locations downstream from the STP have experienced selective pressure from the presence of the pharmaceuticals and may have adapted to these environmental conditions. In this thesis project, we are interested in seeing what processes lie beneath the biofilm - pharmaceutical interactions.

We approached this task from the macro and micro scale. Where on a macro scale we studied the bacterial composition of the four location (two of them upstream and two downstream from the STP). That would allow us to compare the contaminated area to the one that was not contaminated.

Bacterial composition in the Knivsta river

To get an overview of bacterial species variability in the river as well as investigate if it correlated with the pharmaceutical concentration, we sequenced the collected isolates as well as direct extracts from substrates taken in the river (Paper 1). The data were then analysed using multivariate analysis. The PCA plot (Figure 4a) describes the factors that have contributed to the differences observed between the samples. It showed the largest separation between the sampling points upstream and downstream, which is showed in the first principal component (x-axis) in the PCA plot (Figure 4b,c).
This large separation between samples in the plot reflects the presence of pharmaceuticals. In addition, there is a difference between which bacterial species were identified upstream and downstream. This suggests an effect of the pharmaceuticals on the bacterial composition. However, it is quite possible that, together with pharmaceuticals, wastewater brings nutrients into the river that affect the river biota.

The second-largest separation (Figure 4) is visualized as the second component (in the y-axis). It describes the differences in bacterial composition from the various substrates and the cultivated isolates. As expected, a larger variability was observed from the species composition in samples sequenced directly from the substrate that in those samples that had been isolated.
Figure 4. a) Loading plot for the score plots in b) and c). The pharmaceuticals are highlighted in a red circle pointing that their presence brings a large contribution to the separation in the score plot. B) The PCA score plot color coded by sampling point and showing separation between upstream and
downstream sampling locations, from the left to the right. C) The same score plot color coded according to if sequencing was performed directly from the substrate or from a collection of isolated bacteria.

Certainly, the presence of the pharmaceuticals from the effluent water, as well all other compounds that were present in waste water were creating a selective pressure on bacterial communities. In fact, they presence appears to have benefitted bacterial species such as *Pseudomonas, Serratia, Aeromonas, Acinetobacter, Rahnella, Acidovorax, Pedobacter, Flavobacterium, Enterobacter*. At the same time creating possible pressure against other genera such as: *Crenothrix, Hyphomicrobium*, family *Methylomonaceae, Ferruginibacter*, family *Burkholder*, *Rhodoferax, Novosphingobium*, family *Rhodobacte*, family *Sphingomon*, and *Rhodobacter* (Figure 5a).

**Sampling method development – Planted substrates**
Due to the lack of stones in some sampling points, we used cleaned and autoclaved stones that we placed into the river. For three months, those «planted» stones were at the bottom of the river, thereby becoming an attractive surface for bacterial growth. To evaluate this type of approach, we picked up the planted stones and natural stones from the same location at the last sampling occasion (October 2018). After 16s RNA sequencing, we were able to compare the bacterial composition between the existing stones and the “planted” stones.
As a result, we observed some differences between the bacterial consortia, but a large diversity of species was observed even on the introduced stones. Thus, this approach can serve as a good replacement for biofilm sampling in locations lacking suitable substrates for sampling.
Figure 5. The relative abundance of genera in the samples taken from different sampling points and different time periods. Genera which abundance was higher than 1% are shown. The first bar in each point and time represent data was obtained from samples that were cultivated on media, the second one represents genus sequenced directly from the substrate. Bars that are missing represent data that has been excluded due to low quality.
Figure 6. Relative abundance of bacterial genera from the different substrates: “planted” stones and natural ones.

Creating a collection of bacterial isolates from the Knivsta river
As a first step, I performed isolation of bacterial strains to form a library of isolates that was frozen. In this way, the collected biofilm samples from the Knivsta river were separated into individual strains for further work. To make sure each isolate was one bacterial species, each isolate was cultivated and re-streaked
several times to get a pure culture. Through all cultivation steps, I used R2A media that designed for fresh water samples, to keep the same cultivation conditions.

Some isolates did not grow on plates after the second cultivation from freezer stocks. After several attempts, those samples were excluded from the collection. Around 17% samples from the initial library didn't show any growth. Isolates that could be cultivated were summarized into a library and described using the visual characteristics of their colonies (colony morphology) (Fig. 7). Four groups of colony types were observed:

a. Small white colonies

b. White colonies with an excessive amount of EPS

c. Small yellow colonies

d. Yellow colonies with an excessive amount of EPS
**Figure 7.** Examples of the different colony morphologies in the library.

- **A:** Small white colonies;
- **B:** White colonies with an excessive amount of EPS;
- **C:** Small yellow colonies;
- **D:** Yellow colonies with an excessive amount of EPS.

The table describing this library of isolates can be found in Appendix 1. It contains information about sampling location, when samples were collected, from which substrate they were isolated,
the optical density in cultures grown from freezer stock, and a brief description of how the colony looks like.

Sequencing of individual isolates from SP4
The sequence identity of individual strains from sampling point 4 in May (AH 066-AH089) are shown in Table 1. In addition, sequencing was also done to obtain the identity of one strain from sampling point 3 in May (AH123) that was used in the microscopy experiments for Paper 2 (described further below).

Table 1. Bacterial isolates sequenced as individual isolates from Sampling point 4 in May, and one isolate from Sampling point 3 (AH123).

<table>
<thead>
<tr>
<th>Number</th>
<th>Bacteria species</th>
</tr>
</thead>
<tbody>
<tr>
<td>AU066 (1)</td>
<td>Acinetobacter bohemicus</td>
</tr>
<tr>
<td>AU066</td>
<td>Flavobacterium spec.</td>
</tr>
<tr>
<td>AU067</td>
<td>Flavobacterium spec.</td>
</tr>
<tr>
<td>AU069</td>
<td>Sphingomonas spec</td>
</tr>
<tr>
<td>AU072</td>
<td>Flavobacterium spec</td>
</tr>
<tr>
<td>AU074</td>
<td>Arthrobacter spec</td>
</tr>
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<td>AU075</td>
<td>Flavobacterium spec</td>
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<td>Flavobacterium spec</td>
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<tr>
<td>AU080</td>
<td>Pseudomonas spec</td>
</tr>
<tr>
<td>AU081</td>
<td>Rhizobium giardinii</td>
</tr>
<tr>
<td>AU082</td>
<td>Pseudomonas spec.</td>
</tr>
<tr>
<td>AU083</td>
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</tr>
<tr>
<td>AU085</td>
<td>Arthrobacter spec</td>
</tr>
<tr>
<td>AU087</td>
<td>Flavobacterium spec.</td>
</tr>
<tr>
<td>AU089</td>
<td>Arthrobacter spec</td>
</tr>
</tbody>
</table>
Characterization of individual bacterial strains

The microscale of the research included more detailed approach for studying the bacteria including the characterisation and the exposing the isolates individually to the pharmaceutical contaminants,

In order to obtain more information about individual isolates that we wanted to use to form model biofilms, initial characterization was done. The initial characterization started by looking for methods to image the strains with microscopy using the strain *Rhanella aquatilis* (strain AH123, Paper 2). This particular isolate was selected due to its ability to produce an excessive amount of EPS. Therefore, it became a model for initial testing of different techniques for analyzing the individual isolates.

In the later stages of the project, we moved our research focus to the isolates from Sampling point 4, as mentioned above. Two out of our four selected isolates were able to produce a large amount of the EPS, in the same way as *Rhanella aquatilis*, thus the methods and approaches that we developed at the start of the project worked efficiently also with the other isolates.

We had to find an approach to visualize bacteria that produced an extensive amount of EPS on the agar plate and in liquid culture (Paper 2). The isolate (AH 0123) *Rahnella aqualitis* from sampling point 3 was a perfect candidate, as it produced a large amount of EPS.
In initial SEM analyses (Figure 8a), the images showed different odd-shaped structures that suggested that this isolate might contain different types of bacteria and was not a pure culture. However, Cryo SEM (Figure 8b) helped us to clarify this issue. The images from Cryo SEM showed rod shaped bacteria covered in a layer of EPS, suggesting that the initial result from classical SEM was showing artifacts originating from the large amounts of EPS. The EPS presumably collapses due to the sample drying, creating the peculiar shapes and structures observed initially.
To further investigate this, we performed AFM imaging (Figure 8c,d) to visualize bacteria in the absence and presence EPS. Bacterial isolate imaging was done using two approaches: one involving washing with PBS and centrifugation at low speed, and the other without washing. The AFM images show that the

**Figure 8.** a, b Scanning electron microscopy (SEM) and c, d Atomic force microscopy (AFM)
washing procedure effectively removed EPS, enabling clear visualization of the rod-shaped bacteria.

The preparation technique for the different types of microscopy influenced the end result, bringing at first some confusion, but later giving improved methods that could be used for the other strains.

Bacterial growth
The studied isolates were all slowly growing bacteria. It could take up to 72 hours for colonies to appear on the R2A 10% agar plates. This correlated with what I observed for growth in liquid media (R2A 100%) as discussed in Paper 3. For *Pseudarthrobacter*, *Sphingomonas*, and *Rhizobium*, the growth patterns were quite similar. Growth started after 16 hours for all three bacterial isolates, reaching its peak between 30 and 35 hours, followed by the stationary phase. However, the growth rate of the *Pseudomonas* isolate was almost twice as fast as the other three isolates. Growth started after 7 hours of cultivation, with the first peak emerging at 14 hours, followed by a slight decline in OD at 15 hours. Subsequently, a second peak appeared at 20 hours, followed again by a decline until 25 hours, where the isolate seemed to have reached some form of stationary phase. This oscillating growth pattern could be explained by the sequential usage of different nutrient sources in the media, as many different species, including *Pseudomonas*, have been previously described to exhibit a similar
growth pattern. Catabolite repression, a regulatory mechanism, could stand behind this odd-shaped growth pattern of *Pseudomonas*, preventing the bacteria from utilizing secondary carbon sources until the preferred carbon source is consumed.

Another possible reason for the oscillating growth could be the formation of a biofilm that scatters light during the measurement. The results for this experiment were obtained from an instrument that automatically conducted measurements every 30 minutes with 10 seconds of shaking beforehand. Thus, the measured data comes from both biofilm and planktonic cells.

In contrast, the classical growth curve assay showed no oscillating growth pattern for *Pseudomonas*. One contributing factor could be that, before each measurement, the bacterial suspension was vigorously shaken on a vortex to make the suspension homogeneous. The studied isolates had previously shown the ability to form aggregates in overnight liquid media (as well as biofilm). *Pseudarthrobacter*, *Sphingomonas*, and *Rhizobium* can grow in liquid media in the form of semitransparent oddly shaped aggregates that are easily broken once the suspension is shaken on a vortex for a couple of seconds. *Pseudomonas* creates a white dense pellet at the bottom of the tube that is also easily homogenized. Therefore, growing bacterial isolates for the classical growth curve assay required thorough mixing, which may have influenced the results.
Figure 9. Growth curve for bacteria river isolates over 48 h. Where Blue is for Sphingomonas, Grey – Pseudomonas, Orange – Rhizobium, Yellow – Pseudarthrobacter.

Biofilm formation
All four species were able to form a biofilm (Figure 9). *Sphingomonas* showed the lowest ability to form biofilm according to the CV assay. However, the data from ATR-FTIR spectroscopy showed a different perspective. We were able to grow the bacteria isolates over a longer period of time (3 days compared to CV that lasted 24 hours) and record the growth dynamics every 9 hours. However, the intensity of the spectra from *Rhizobium* and
*Sphingomonas* did not show abundance of the growth either, compared to the other isolates. This can indicate a lower biomass production of these strains, in general. However, it can be that these bacterial isolates are able to form aggregates that are not attached to any surface. Since the infrared beam penetrates into the sample not deeper than ~2 µm (from the bottom of the biofilm), these formations would be beyond the instrument's detection.

Data from confocal microscopy provided another look at the bacterial isolates and their own unique biofilm architecture. *Pseudomonas* formed island structures distanced from each other with a few µm, their high reached ~40 µm. Significant number of cells were unattached to the surface and showed high motility.

The rest of the biofilms showed to be more sessile. Both *Rhizobium* and *Pseudarthrobacter* biofilms were up to 60 µm thick. *Pseudarthrobacter* biofilms had a three-dimensional structure that was dense and elastic. *Sphingomonas* formed biofilm with thickness up to 150 µm. Beneath the biofilm structures formed by *Sphingomonas* were located voids. That can explain the low intensity of the signal from the ATR-FTIR measurements. The biofilm that is lightly attached to the surface would also be easily washed away during CV sample preparation, which would explain the low biofilm formation results for CV.
Synergy or antagonism between isolates
In order to study how bacteria interacted with each other, I performed a Cross Cultivation assay. All combinations of isolates showed an excellent ability to coexist, and there were no signs of
negative interactions. In the case of *Pseudomonas* and *Sphingomonas*, we could see that tiny white colonies of *Pseudomonas* are embedded inside the watery EPS created by *Sphingomonas*.

One possible reason that different bacteria species can coexist is that they were all isolates from the same location. Therefore, they may have been used to exist side-by-side.

Bacterial Motility
Data from the previous experiments showed that Pseudomonas has an ability to move. Therefore, I decided to perform a series of motility experiments to find out what type of bacterial motility is performed by Pseudomonas, and whether other isolates are also capable of movement. Since the studied isolates were slow-growing, I performed several measurements creating a curve showing the evolution of motility for 72h. However, it is important to point out that as bacteria multiply,
the colony expands in size, even when not showing any movement. Thus, in the cases where the motility was very low, we can assume that what is observed is only growth and that this type of motility does not occur.

All four bacterial isolates demonstrated the capability to execute various types of motilities. Specifically, Pseudomonas exhibited the highest swimming proficiency compared to the other bacteria. SEM images revealed that the Pseudomonas isolate possesses multiple polar flagella, providing an explanation for its swimming ability. In contrast, Sphingomonas displayed the ability to perform swarming, even though SEM images did not indicate the presence of flagella associated with the swarming movement. Therefore, these results likely indicate gliding movement. Gliding and swarming, as previously explained, can be easily confused. Additionally, Sphingomonas species have been previously documented being capable of gliding. Consequently, we can suggest that the obtained results correspond to gliding, facilitated by a large amount of EPS secreted by the bacteria. None of the isolates demonstrated the ability to perform twitching
Figure 12. Three motility types for four river isolates. Where Red color represents *Pseudarthrobacter*; Orange – *Rhizobium*; Green – *Pseudomonas*; Blue – *Sphingomonas*. 
Chemical Characterization

We used several different spectroscopy methods to study bacteria isolates from a chemical perspective. These methods allowed us to probe the macromolecular composition of cells (Paper 3).
Figure 13. Spectroscopic characterisation of river isolates after 72 hours growth on 10% R2A plates: a) ATR-FTIR spectra illustrating biochemical composition in the fingerprint region; b) relative content of peptides, lipids, and polysaccharides on bacterial surface estimated from the C signal in cryo-XPS analysis of Pseudomonas, Rhizobium, Pseudarthrobacter and Sphingomonas.
Analysis was made on hydrated cells to avoid the appearance of artefacts as a result of drying of EPS and the risk of cell rupture.

To study difference in chemical composition and surface chemistry of the isolates we used ATR-FTIR and cryo-XPS. ATR-FTIR spectra showed presence of proteins, nucleic acids, polysaccharides, and phospholipids. The composition of each of the bacterial isolates differed from each other. *Pseudomonas* spectra showed a lower contribution from polysaccharides compared to the other isolates in FTIR. The same results came from the cryo-XPS. This data aligned with fact that among all four isolates *Pseudomonas* produces less EPS when growing on agar plates. This can be compared to *Pseudarthrobacter* and *Sphingomonas* that showed a high content of polysaccharides both in ATR-FTIR and cryo-XPS. Both isolates produce extensive amount of EPS.

Spectra collected from *Sphingomonas* did not show any amide bands from proteins, and since proteins are abundant in bacterial cells it is possible that material that was collected from agar plates and analysed in FTIR was mainly extracellular. *Sphingomonas* spectra included bands that corresponds to polyhydroxyalkanoates (PHA), bacterial polymers typically present in the intracellular compartment and serve as energy storage compounds. Thus, results that we obtained from ATR-FTIR on *Sphingomonas* suggest that these polymers were present in the extracellular matrix of bacterial colonies.
Bands relating to polysaccharides were also present in FTIR spectra of all the bacteria. This could be originating from the energy-storage molecule glycogen. Notably, the R2A media that was used to grow bacteria for all experiments contained starch that has a molecular structure similar to glycogen. To eliminate the possibility that component from the media was responsible for peaks, we performed a control experiment on 10 % R2A plates without starch. As result, we saw that carbohydrate bands in the control experiment were similar to the experiments with starch in the medium, suggesting that a large part of the vibrations comes from the glycogen or other polysaccharides produced by cells.

Raman spectroscopy was used to complement the existing results from ATR-FTIR and cryo-XPS. *Pseudomonas, Pseudarthrobacter* and *Rhizobium* showed spectra corresponding to cytochromes. *Sphingomonas* displayed carotenoids bands in the spectra, which is correlated with the slight orange color of the colonies when grown on R2A media agar plates. The intense resonance that came from both cytochromes and carotenoids made the bands so strong that other signals could not easily be detected. This was limiting the information that could be obtained from Raman spectroscopy. Nevertheless, the strong difference between *Sphingomonas* and the other three river bacteria will make it easier to track the isolate in future experiment when all bacteria are co-cultured.

Overall, the experiments showed that all four bacteria have different chemical compositions, that may result in different
interaction with pharmaceutical pollutants. This could also potentially lead to synergetic effects where all bacteria species are benefiting from each other presence since they differ from each other.

Exposing bacteria to pharmaceuticals

Bacterial growth after 24 hours
Trimethoprim is a synthetic antimicrobial pharmaceutical that binds to the enzyme dihydrofolate reductase inhibiting the folic acid synthesis pathway. It is usually used to treat uncomplicated urinary tract infections. Trimethoprim is rarely used alone in the treatment, but is often combined with sulfamethoxazole for synergetic effects. Trimethoprim alone has a bacteriostatic effect. However, when combined with sulphonamides it can be bactericidal. The sulfamethoxazole was not detected in the Knivsta river.

Trimethoprim is considered a water-soluble pharmaceutical. But, to increase solubility, we decided to add small amounts of acetic acid to increase solubility by protonating it. We decided to study the effect of trimethoprim at both neutral pH and at pH 5, to investigate if we could see any effect of protonation (Paper 3). We wanted to investigate whether the growth changed as a result of the antibacterial effect of trimethoprim, presence of acetic acid or an acidic environment. Thus, we included additional controls containing acid. Two groups of experiments were created with
identical protocols except for pH; one was at pH 5, and the other was at pH 7. Compared to the control, we did not observe significant growth inhibition of *Rhizobium, Pseudarthrobacter, Sphingomonas*, and *Pseudomonas*, with acid alone. We can, therefore, conclude that acidic environment did not affect the bacterial growth of any studied isolate.

However, an effect of trimethoprim was clearly seen (Figure 13). The lower the concentration of the pharmaceutical was, the higher the optical density of the bacterial culture was for *Rhizobium, Pseudarthrobacter and Sphingomonas*. On the other hand, the *Pseudomonas* showed no sensitivity to the different concentrations of Trimethoprim at pH 5 or pH 7. All three concentrations plus the experiment with the acid remained at the same level as the control.
Figure 13. Growth of four bacterial isolates after 24 hours exposed to various concentrations of Trimethoprim (25 mg/L, 12 mg/L, 6 mg/L).
Growth in presence of Trimethoprim
To more closely see what is happening during the cultivation of bacteria in the presence of the pharmaceutical, we made growth curves at different concentrations between 50 mg/L – 1.5 mg/L.

**Pseudomonas with Trimethoprim**

**Rizobium with Trimethoprim**
**Figure 14.** Growth curves over 48 hours with different concentrations of Trimethoprim (from 50 mg/L to 1.5 mg/L)
The results from experiments with *Pseudomonas* corresponded to what was observed in other experiments after 24 hours. Growth curves at all concentrations grew to similar values of optical density and had similar pattern as the growth control. The presence of the pharmaceutical also did not delay the exponential phase of the bacteria.

*Rhizobium* and *Pseudarthrobacter* showed a change in the growth curves depending on the concentrations of Trimethoprim. The lower the concentration of pharmaceutical was the closer the growth was to that of the control.

In case of *Sphingomonas* the growth of the bacterial culture was delayed by approximately 8-10 hours. The growth in the control sample started around 15 hours, while in the samples that contained Trimethoprim the exponential phase started around 25 h. This shows that *Sphingomonas* was the most affected by the presence of the Trimethoprim.

Growth with Diclofenac

The growth of *Pseudomonas* showed no signs of suppression or slowdown when exposed to trimethoprim. Consequently, we were interested to explore whether any other pharmaceutical previously detected in the Knivsta river might potentially influence *Pseudomonas* growth. Our focus turned to diclofenac, as previous studies have demonstrated that certain *Pseudomonas* species can degrade diclofenac to some extent. Therefore, we performed a series of experiments where we exposed the *Pseudomonas* to Diclofenac at various concentrations (Paper 4).
Diclofenac is one of the most frequently used non-steroid anti-inflammatory drugs for treating fever or pain. It often can be bought without a prescription\textsuperscript{66}. Its presence has been detected in wastewater due to human consumption\textsuperscript{67,68}. In 2012 together with 17 alpha and beta estradiol diclofenac was placed on list of Priority Substances under Water Framework Directive by European commission. It is on a special watch list for drugs that should be monitored but that do not yet have Environmental Quality standard designed for them\textsuperscript{69}.

As the first step we exposed *Pseudomonas* to six different concentration of diclofenac (from 10g/L to 0,6 g/L) in the same experimental setup as described before with Trimethoprim. This time we used a slightly different approach by studying the growth dynamics with R2A media diluted in 10 times. This way we could monitor possible effects relating to reduced nutrient availability. The maximum OD of the bacteria in the experiment with diluted media appeared to be 4 times lower compared to 100 % media. In the control solution with diclofenac and media only, we observed light scattering presumably caused by small particles or micelles formed by the pharmaceutical. The aggregation of diclofenac does not seem to be linked to nutrient composition of the R2A media. However, the aggregation could be caused by the gradual change in pH, since at the start of the experiment the pH of the solution with 5 g/L of diclofenac was 7,3, but after 24 h cultivation on a tilting-table it was decreased to 6.6. This slight acidification of the medium was likely due to CO2 dissolution and could have led to the increase of the diclofenac scatter over time. This effect was
most pronounced at high concentration of the pharmaceutical (10 g/L) where the obtained result therefore was a representation of a combination of several factors together i.e. planktonic growth, biofilm formation and scattering from the diclofenac aggregates. The growth of *Pseudomonas* was limited in the presence of 10g/L and 5 g/L diclofenac regardless of the concentration of R2A media. Concentration-dependent changes in bacteria growth were observed primarily in 100% R2A media, affecting both the start of the exponential growth phase and bacterial density at the stationary phase. The smaller relative effect at lower nutrient content led us to the hypothesis that the river isolate alters its metabolism in the presence of diclofenac, potentially utilizing it as a carbon source, as observed in other *Pseudomonas* species.
Figure 15. a) Pseudomonas growth curve at different diclofenac concentrations in 100% R2A media displaying the concentration dependent decrease of OD; b) Growth curves at 10% concentration of R2A media. Legend for panels a & b: light blue - R2A medium control, orange - control growth in R2A medium, light grey - diclofenac in medium, yellow - bacterial growth in 10 g/L diclofenac, blue - growth in 5 g/L of diclofenac, green - growth in 2.5 g/L of diclofenac, dark blue - growth in 1.2 g/L diclofenac, brown - growth in 0.6 g/L diclofenac and dark grey - growth in 0.3 g/L diclofenac; c) Two replicate control growth curves for Pseudomonas in 100% R2A medium (squares in black and blue) and scatter from control solutions with 10 g/L of diclofenac (crosses in black and blue). d) Scattering of diclofenac in 12 replicate experiments. (Standard deviation not shown in panel d for clarity). Triangles
represent scattering in 100 % R2A and circles in 10 % R2A. All data in panels a-d were collected from 96-well plates where each measurement point represents an average of 6 wells. Error bars show standard deviation between these 6 wells.

As second step, we used gas chromatography together with mass spectrometry (GC-MS) to learn what types of the metabolites are consumed by *Pseudomonas*. The findings obtained from these experiments originate from a small pilot study that is part of the larger project that is ongoing at the time of these thesis printing.

We used unsupervised multivariate analysis to identity the differences in metabolite patterns between the media before bacterium growth and media after bacterial growth (referred as “spend medium”) (Figure 15).

The PCA plot indicated that the first component reflects the differences observed between the R2A media without bacteria and media after bacterial growth. The second component showed the differences between the highest concentration of diclofenac and the supernatants obtained from the lower concentrations, i.e. pure media and 0.3 g/L diclofenac. According to the data, bacterial growth led to the depletion of various substances such as amino acids, lipid metabolites, organic acids, and nucleotides in the media. Some metabolites showed an increase of the concentration in the “spent” media compared to the initial media. An increased level of palmitoleic acid (lp4), putrescine (am8), and 1,4-
butanediamide (am7), were associated with samples from 5 g/L, suggesting that these metabolites were released by the bacteria into the medium.

Similarities between the “spent” media with diclofenac at concentration 0.3 mg/L and without it, led us think that bacteria had not significantly altered their secreted metabolite profile in the presence of diclofenac at 0.3 g/L. This separation between “spent media” and pure media is most likely connected to bacteria growth and consumption of nutrients from the media. The difference becomes more visible for samples with diclofenac at 5 g/L that separates clearly from the other samples in the PCA score plot, indicating both consumption of the nutrients from the R2A media and release of metabolites into the media.

When studying metabolite patterns in cells we discovered changes in a variety of metabolites. This indicated that several metabolic pathways in *Pseudomonas* could be changed as result of its interaction with diclofenac. *Pseudomonas* were able to continue their growth in the 10 % medium despite the increase in concentration of diclofenac and did not show large signs of reduced metabolic activity (since they still took up metabolites from the medium). Nevertheless, the results obtained from the cells and surrounding supernatant indicated that the presence of diclofenac triggered an alteration in metabolic processes, and that it was concentration dependent. The difference in metabolite patterns we observed in the pilot study, are suggesting that potential alterations happened in the lipid metabolism,
presumably rigidifying the membrane, as described in previous research \(^6\). The reason behind this alteration should be studied in future projects.

The increased presence of organic acids in the supernatant in exposed cells suggested that their production could be a result of defense mechanisms against toxic aromatic substances. Organic acids can change the surrounding pH and previous studies have shown that this can reduce the activity of some toxins \(^6\). This mechanism would also be quite efficient against diclofenac, considering the fact that diclofenac has a solubility that is decreased in acidic environment. However, future work is required to prove this hypothesis.

The metabolite dataset provided additional data suggesting that possibly the regulatory pathway (Crc) may be activated as a response to the presence of diclofenac. This regulatory protein has previously been recognized for its involvement in the degradation of aromatic compounds \(^7\). Additionally, a possible transformation product, called tropone, was observed inside the cells that had been exposed to diclofenac at 0.3 g/L. This observation led us to consider the possibility that bacteria might utilize diclofenac as a carbon source. To delve deeper in this hypothesis, we continued our search for additional degradation products in the supernatant using LC-MS. Several transformation products were found. Future work will identify their exact identity.
Conclusions

This thesis aimed to study the influence of pharmaceutical contaminants both on a macro and a micro level. On the macro level, we observed the changes in species composition in bacterial communities in a river. These changes may have been due to the presence of the xenobiotic compounds that were brought into the stream by the STP. To test this hypothesis, we went to the micro level. The micro level involved isolation and characterization of bacterial strains, in order to develop a biofilm model and expose it to selected pharmaceuticals.

The results, on the macro level, showed a clear influence of effluent water on the bacterial composition in the river, evident in difference of taxa upstream and downstream from the STP. Although, the richness of taxa in contaminated areas was not influenced, we observed a clear difference in bacterial composition downstream compared to the areas that were located upstream. This suggested that the presence of the waste water was creating a negative pressure against bacterial species such as: *Crenothrix*, *Hyphomicrobium*, family *Methylomonaceae*, *Ferruginibacter*, family *Burkholder*, *Rhodoferax*, *Novosphingobium*, family *Rhodobacte*, family *Sphingomon*, and *Rhodobacter*, and benefiting the species like: *Pseudomonas*, *Serratia*, *Aeromonas*, *Acinetobacter*, *Rahnella*, *Acidovorax*, *Pedobacter*, *Flavobacterium*, *Enterobacter*. 
To learn about the interaction between bacterial isolates and pharmaceuticals we moved to the micro scale of the project. We selected isolates from a contaminated site (Sampling point 4, collected in May), that had been continuously polluted with effluent water. In this way we were studying bacteria that may have created adaptation mechanisms against pharmaceutical pollutants. We detected a number of pharmaceutical pollutants in two locations (3 and 4) downstream from the STP. These pharmaceuticals were likely to have been introduced into the water through the STP plant. For the further experiments we picked Trimethoprim for its antimicrobial properties and Diclofenac as it is a very commonly used pharmaceutical. The experiments demonstrated varying bacterial responses to these compounds, indicating different sensitivity among the isolates.

*Sphingomonas* appeared to be the most sensitive strain to Trimethoprim displaying the largest reduction in growth, 20% compared to the control with R2A media. The morphology of the cells was significantly affected in the presence on Trimethoprim resulting in cell elongation. However, when co-cultivated in a biofilm containing several other isolates, *Sphingomonas* seemed more resilient to stress factors. The second largest growth reduction were observed in *Pseudarthrobacter* with 40% growth compared to control. Thickness of the biofilm in the presence of Trimethoprim was not compromised and remained the same compared to control experiments, suggesting that the ability of the bacteria to produce matrix was not affected. *Rhizobium* was the
third most sensitive strain with 60 % growth compared to the control.

*Pseudomonas*, in particular, was resilient to trimethoprim and exhibited interesting dynamics in the presence of diclofenac. Preliminary data investigating interactions between *Pseudomonas* and diclofenac suggested that it can metabolise this pharmaceutical.

Overall the results in this thesis illustrate that some microbial isolates appear to exhibit a resilience against pharmaceutical pollutants, and respond to changes relatively quickly and adapt to environmental changes.
Future perspectives

The findings of this study open for many possibilities for future research aimed at deeper exploration and understanding of microbial responses to pharmaceutical exposure in aquatic ecosystems.

Further research is planned that will be focused on studying EPS produced by the 4 river isolates described in this thesis work. It will use methods like FTIR, Raman and colorimetric assays to study chemical and physical properties of EPS extracted from the bacteria. This work also plans to investigate exposure of Trimethoprim and learn how it affects the EPS, since it is a first barrier between the contaminants and bacteria. CryoSEM will be used to study the EPS structure. In Paper 2 we determined that this microscopy method can successfully capture the bacteria with EPS without compromising its structure and creating artefacts. Therefore cryo-SEM is suitable to study how and if the EPS structure is compromised in the presence of the contaminant.

Furthermore, it would be interesting to study the effect of *Pseudomonas* on Diclofenac over a longer period of time, to see if further degradation occurs in the presence of these bacteria. Testing the effect of the Diclofenac on other bacterial isolates could also show interesting interaction processes. Moving towards growing all species together and exposing the system to diclofenac could shed a light on the question about their synergetic effects with other drugs than trimetoprim. Additional metabolomics
analysis is planned, and required, in order to have more replicas that can give reliable statistic and strengthen the models.
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Appendix