



TUDOMÁNYOS DIÁKKÖRI KONFERENCIA

2021. november 24.

**OXITOP MIKROKOZMOSZ MODELL, MINT AZ
ANTIBIOTIKUM REZISZTENCIA MIKROBIOÁLIS
KÖZÖSSÉGRE GYAKOROLT HATÁSÁNAK
VIZSGÁLATÁRA ALKALMAS MÓDSZER**

**OxiTop microcosm model as a possible tool to study the effects of
antibiotic exposure on the microbial community**

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**Gödöllő
2021.**

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List of abbreviations

AMR	Antimicrobial resistance
ARB	Antibiotic resistance bacterial
ARGs	Antibiotic resistance genes
ARMs	Antibiotic resistance microorganisms
ATC	Anatomical therapeutic
BOD	Biochemical oxygen demand
CFUs	Colony forming units
Eps	Emerging pollutants
FCA	Fluoroquinolone, quinolone, chloramphenicol, florfenicol and amphenicol
LB	Luria-Bertani
MDR	Multidrug resistance
MGE	Mobile genetic element
MLSB	Macrolide-lincosamide-streptogramin B
PCR	Polymerase chain reaction
PDR	Pan drug resistance
STP	Sewage treatment plant
WWTPs	Wastewater treatment plants
XDR	Extensively drug resistant

1. Introduction

The era of antimicrobial resistance is now upon us. With the rapid growth of the population and the gradual improvement of human living standards all over the world, bacterial antibiotic resistance is widespread in the environment and in healthcare institutes, which has become a major threat to environmental safety and human health. In general, antibiotic resistance problems are especially visible in hospital settings, as the overuse of antibiotics or infection due to the failure during medical treatment occurs there. The World Health Organization has listed antibiotic resistance as one of the three major threats to human health (Pruden et al., 2006).

Due to the selective pressure of antibiotics, large numbers of drug-resistant pathogens have arisen widespread which is a huge concern and often related to transferable antibiotic resistance genes. The presence of antibiotic resistance genes in the environment and their spread among the members of the microbial ecosystem are often more harmful to the environment than antibiotics themselves. Sewage is a verified source of antibiotic load, resistant microorganisms, and resistance genes, but its effect on Hungarian surface water's microbiome has not been examined in detail.

Based on this background, we decided to examine a chosen surface water body with and without sewage load to evaluate the effect of antibiotic pressure on the activity and composition of the microbial communities. To reach this goal, we validated a microcosm method (WTW OxiTop system) and set different treatments of some chosen antibiotics (colistin, florfenicol, imipenem, ceftazidime).

The initial hypothesis of this work was that samples affected by sewage inlets show a higher number of antibiotic resistant representatives of the cultivable, and non-cultivable bacterial community. To check this hypothesis the main questions that should have been answered were as follows:

- What is the composition of microbial community with and without sewage load?
- What is the effect of antibiotic pressure on the composition, activity, and diversity of the microbial communities in the examined sample types

2. Literature Review

2.1. What is the environment?

The environment is closely related to human life. It is the total of all conditions and influences that affect the development and life of all organisms on earth ([http 1](#)). Environment includes all physical, chemical, and biological factors external to the human host, and all related behaviors (Prüss-Üstün et al., 2007). It includes everything around the organism: abiotic (non-living) and biotic (living) environment. Abiotic environment consists of soil, water, and air while the biotic environment includes all other organisms with which the organism comes into regular contact (Chauhan, 2008).

Natural environment includes the atmosphere, hydrosphere, soil, lithosphere, and biosphere. The artificial environment is a subset of the natural environment and refers to the secondary environment of human processing and transformation that emerges from it (Li, 2011).

Human activities for various purposes will inevitably affect the surrounding environment and may destroy it. However, human beings are one of the internal elements of the environment, and the environment is the carrier of human social life and other activities. Humans and the environment need to interact and develop together (Wang, 2019). Surface water bodies are under a continuous and intensive effect of human activities and emissions. Since water quality and analysis is a focus in my thesis work, in the upcoming section I will discuss water quality and protection in details.

2.1.1. Water

Water covers about 71% of the surface of the earth. Water types collected on the surface of earth are considered surface waters including streams, rivers, lakes, wetlands, reservoirs, creeks and saltwater (seas and oceans). The hydrologic cycle, often known as the water cycle, involves the transfer of water from and to the Earth's surface. Surface water bodies are fed by precipitation and runoff. Water bodies, on the other hand, lose water due to evaporation and infiltration into the soil (Khan, 2019). Groundwater is hydrologically connected to the surface

water. It is present in permeable geologic material, usually small rocks, and gravel (http 2).

Water is an essential and basic resource required for food security, human health, natural ecosystems, sustaining life and livelihoods. Safe water is required for drinking, hygiene, and providing food; adequate water to produce energy and support economic activities such as industry and transportation. Water in the natural environment ensures the provision of a multitude of ecosystem services to meet basic human needs and support economic and cultural activities (UNESCO, 2012). Achieving sufficient freshwater is a fundamental right and requirement for many biological species including humans (Makate et al., 2016). Water also provides a habitat for millions of aquatic animals and plants. Water is one of the most important components of any ecosystem. All living organisms need water to grow and survive. In an ecosystem, water cycles through the atmosphere, soil, rivers, lakes, and oceans, as is shown in Figure 1.

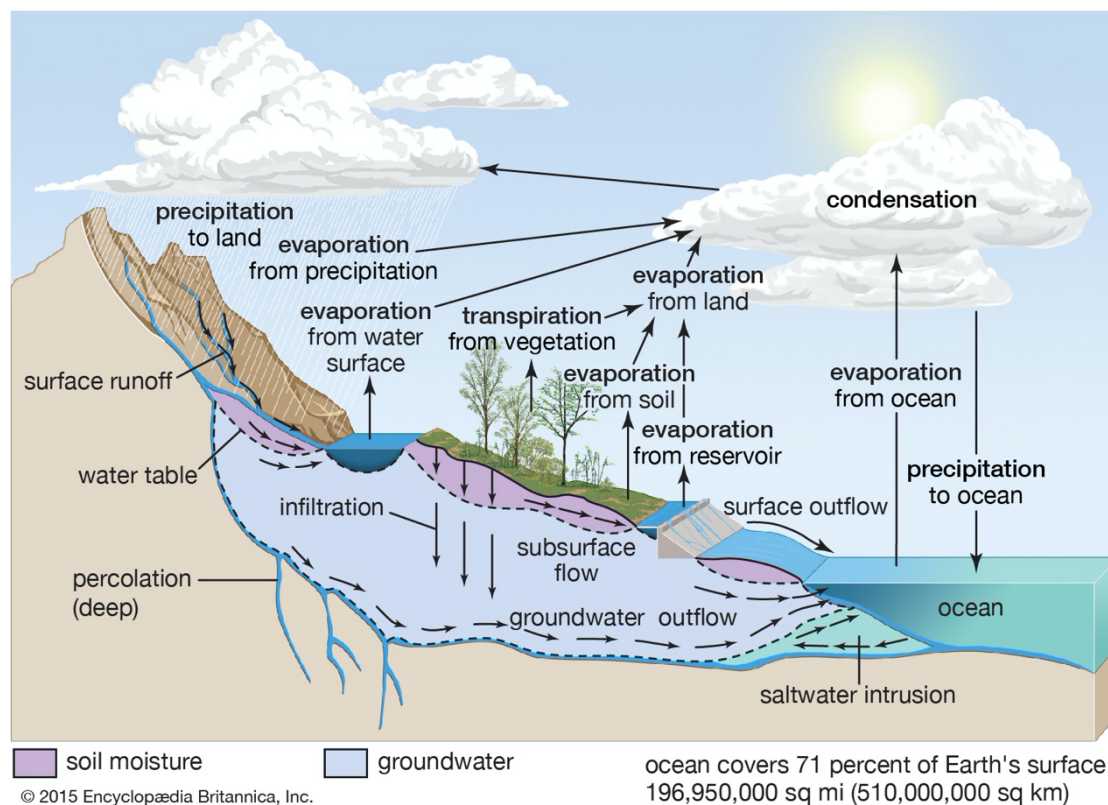


Figure 1. Hydrologic Cycle (Rafferty, 2015)

2.1.2. Microbial ecosystem

The most prolific source of biodiversity on the planet is microorganisms. Microbes are an

important group of decomposing metabolic groups in living things, without which the biosphere's physical energy cycle will be interrupted and life on Earth will be difficult to reproduce. Climate change, geographic shifts, geochemical cycles, and biological evolution are all influenced by microbes in the biosphere (Lan et al., 2008). However, due to the scale of microorganisms and the limitations of research methodologies, many microbe populations are unable to be separated and cultivated, and the percentage of known species to estimated species remains low. The statistics Table 1. below demonstrates this.

Table 1. The number of known species of microorganisms and the estimated total number of species (Science Cloud Platform of Chinese Academy of Sciences, 2004)

Group	The number of known species	Estimate the total number of species	Percentage of known species
Virus	5000	130000	4%
Bacteria	4760	40000	12%
Fungus	72000	1500000	5%

Microorganisms are an important group of organisms with catabolic and anabolic potential. Microorganisms are necessary for life on Earth to exist. They are the first life forms on the planet, and their biodiversity is necessary to regulating the biosphere and providing humans with vast and unexplored resources. In addition to species diversity, microorganism variety covers a wide range of physiological categories, ecological types, and genetic diversity (Jun et al., 2014). Microorganisms can adapt to various environmental condition, and they play a key role in biodegradation and biotransformation, namely the decomposition of various environmental contaminants.

2.2. Environmental pollution

The introduction of dangerous substances into the environment is referred to as pollution. A pollutant is any substance that could, in a high enough concentration, harm human beings, animals, vegetation or material. Contaminants can be natural, such as volcanic ash produced by volcanic eruptions, various salt particles brought into the air when seawater evaporates, liquid

particles produced by splashing waves on the ocean, and dust raised by strong winds (Gang et al., 2012). Human activities, such as wastewater from factories being discharged into runoff, can also cause contamination. Pollutants have the potential to deteriorate the quality of the air, water, and soil and biosphere.

Pollution is a worldwide issue. Even though metropolitan regions are typically more polluted than rural areas, pollution can migrate to isolated areas where no one lives. For example, pesticides and other persistent chemicals have been found in the Antarctic ice sheet. In the middle of the northern Pacific Ocean, a massive concentration of microscopic plastic particles forms what is known as the Great Pacific Garbage Patch ([http 3](#)).

Because of the many interconnected factors between the environment and humans, it is a complex issue. Population increase, the nature of modern technology (produce plastics, chemical products, human-made detergents, and so on), deforestation, agricultural development, industrial development, and urbanization are all examples of these activities (Appannagari, 2018).

Surface water is of a great concern because we use it as a drinking water source. It is a habitat for aquatic organisms, used for recreation and irrigation. Surface waters has a lot of environmental loads from various human activities. One of these activities is sewage load, which has a significant effect on the quality of surface water.

2.2.1. Water pollution

With the growth of the economy, the water contamination problem is becoming progressively more serious, consequently, influencing the quality of drinking water. According to research, 50% of global diseases are caused by contaminated drinking water (Scott, 2013). Nowadays, with population growth, the increasing demand for water resources from agriculture and industry, and the worsening impact of climate change, water resources are facing extremely serious threats. Water pollution can be divided as physical (e.g., plastic), biological (e.g., infectious microorganisms), chemical (e.g., organic, and inorganic pollutants) and radiological contamination ([http 4](#)). Pathogens (mostly from human and animal waste), organic debris (including plant nutrients from agricultural run-off such as nitrogen or phosphorus), chemical

pollution, and salt can come from irrigation, domestic wastewater, and runoff of mines into rivers. These contaminants may be naturally occurring or man-made. They can be organic or inorganic. Examples of chemical contaminants include nitrogen, bleach, salts, pesticides, metals, toxins produced by bacteria, and human or animal drugs. The harm of some new pollutants to public health and the ecological environment is gradually emerging. Emerging pollutants (EPs) are defined as synthetic or naturally occurring chemicals that are not commonly monitored in the environment, but which have the potential to enter the environment and cause known or suspected adverse ecological and/or human health effects. In some cases, the release of emerging pollutants to the environment has likely occurred for a long time but may not have been recognized until new detection methods were developed (Geissen, et al., 2015). Emerging pollutants include new chemicals such as environmental hormones and antibiotics, and fine particles such as microplastics. Emerging pollutants like medicines, are putting our world's waterways in jeopardy, yet the amount and consequences of their presence in our freshwater are mostly unknown (http 5). Once water is contaminated, it is difficult, costly, and often impossible to remove the pollutants. Still today, 80 percent of global wastewater enters surface water recipients without pre-treatment (http 6). Freshwater's fitness for various human activities, such as drinking, bathing, and agriculture, is determined by the nature and number of contaminants present. Furthermore, contamination of freshwater ecosystems can have an influence on fish and other wildlife's habitat and quality of life.

After the general introduction of the environment and water, from now I will talk about antibiotics because it is relevant to my work.

2.3. Antibiotics

Antibiotics are antimicrobial chemicals that are either natural, semi-synthetic, or synthetic. They're commonly used to treat infectious diseases in humans and animals, and they're also added to animal feed as growth promoters (Martinez, 2009).

Humans began to utilize antibiotics extensively since penicillin was discovered and used in clinical practice. Antibiotics were then used in animal husbandry production, where they played a critical role in preventing and treating animal infections as well as stimulating animal

growth, hence fostering large-scale breeding, and improving animal husbandry's economic benefits (Gao et al., 2013). However, 'REGULATION (EC) No 1831/2003 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 22 September 2003, on additives for use in animal nutrition, other than coccidiostats or histomonostats' forbid the authorization of any further antibiotics for use as feed additives. Within the framework of the phasing out of antibiotics used as growth promoters and to ensure a high level of protection of animal health, the European Food Safety Authority will be asked to review the progress achieved in the development of alternative substances and alternative methods of management, feeding, hygiene, etc., before 2005. as from 1 January 2006, antibiotics shall be deleted from the Register.

Antibiotics are the main solutions to treat bacterial infections since the discovery of the first agent in 1930. A lot of antimicrobial drugs have been found already, that could focus on weak points of bacteria. Through the mechanism of action, they can be divided into 5 classes: Cell Wall Synthesis Inhibitors; Protein Synthesis Inhibitors; Nucleic-acid (DNA or RNA) Synthesis Inhibitors; Membrane Integrity Disruptors; Metabolic Pathway Inhibitors. Some antibiotics have a broad-spectrum, others can affect only a special group of microorganisms. The main types of antibiotics and their mechanisms are summarized in Figure 2 (Sauvage, et al., 2008).

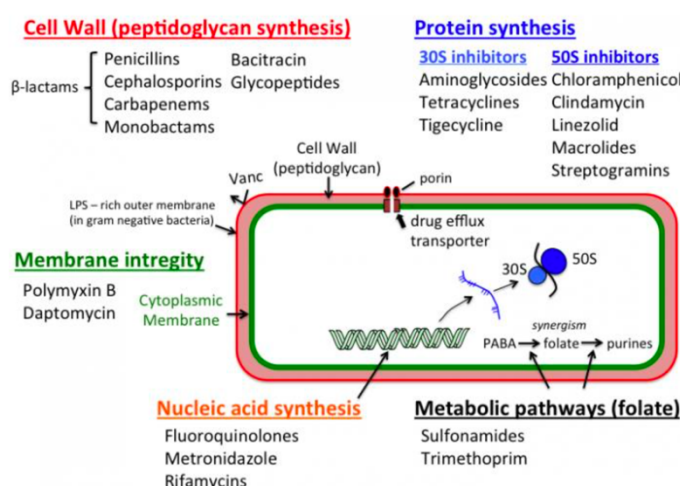


Figure 2. Bacterial structure & mechanisms of action of antibacterial drugs (http 7)

Vanc - Vancomycin

Antibiotics have made significant contributions to the cure of diseases and the promotion of livestock production, but their widespread usage and even abuse has resulted in a slew of environmental issues. Most antibiotics consumed by humans and animals are not fully absorbed

and utilized, and they end up in the sewage treatment plant with feces or in the environment. Even though many antibiotics have a short half-life, their widespread use and ease of entrance into the environment has resulted in the establishment of a “pseudo-persistence” phenomenon (Gao, 2013), making them a new sort of environmental pollutant. Antibiotics have induced the arise of drug-resistant bacteria in the environment, posing a threat to human health and the ecosystem.

2.3.1. Categories of antibiotics

According to their chemical structure, antibiotics are classified as β -lactams, sulfonamides, quinolones, macrolides, tetracyclines, aminoglycosides, and polypeptides (Etebu et al., 2016).

Another type of classification is according to the role and importance of antibiotics in therapy (Lahiry et al., 2020):

Antibiotics can be classified as Access, Watch or Reserve, based on their WHO Essential Medicines List status. Access Group of Antibiotics includes 48 antibiotics, 19 of which are included individually on the WHO Model List of Essential Medicines as first- or second -choice empiric treatment options for specified infectious syndromes. Watch Group Antibiotics includes 110 antibiotics that have higher resistance potential and includes most of the highest priority agents among the Critically Important Antimicrobials for Human Medicine and/or antibiotics that are at relatively high risk of selection of bacterial resistance. Reserve Group Antibiotics: This group includes 22 antibiotics and antibiotic classes that should be reserved for treatment of confirmed or suspected infections due to multi-drug-resistant organisms. Antibiotics in Reserve group should be treated as “last resort” options ([http 8](http://8)).

Access		48	First-line antibiotics Low resistance potential e.g. Amoxicillin, Nitrofurantoin etc.
WA _{tch}		110	Critically important antibiotics High resistance potential e.g. Quinolones, Macrolides etc.
RE _{serve}		22	Antibiotics for MDR organisms 'Last-resort antibiotics' e.g. Polymyxin, Tigecycline etc.

Figure 3. WHO AWaRe Classification of Antibiotics 2019 (Lahirv et al., 2020)

2.3.2. Status of antibiotic use

Antibiotic consumption estimates in global agriculture range from roughly 63,000 tons per year to over 240,000 tons per year, owing to poor surveillance and data collecting in many countries (Figure 4). 70-80% of antibiotics given to fish are excreted in the water, where they spread quickly across water systems. Antibiotics are excreted unmetabolized by 75-90% of studied animals and end up in sewage systems and water sources (http 9).

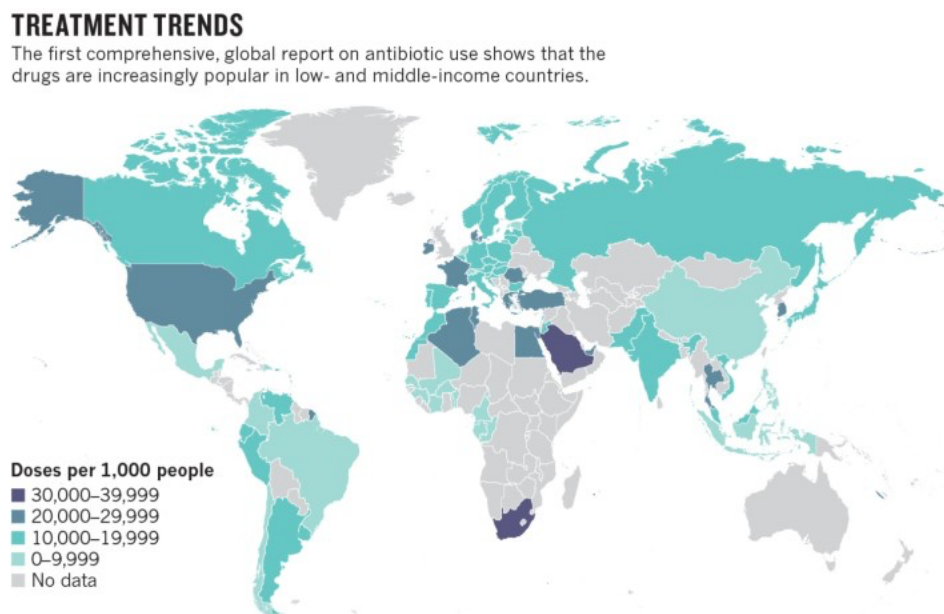


Figure 4. The use of antibiotics worldwide (Reardon, 2015)

Antibiotics are used differently in different countries and locations. Streptomycin, for example, is commonly used in fruit farming in the United States, although it is prohibited in Germany. In 1999, 65% of the 1320,000 tons of antibiotics used in the European Union and Switzerland were for the treatment and prevention of human diseases, 29% for veterinary medications, and 6% for growth boosters (Moreno-Bondi et al., 2009).

2.3.3. The fate of antibiotics in the environment

Medical antibiotics, animal husbandry, aquaculture, and manufacturing discharge are the main sources of antibiotics in the environment.

Antibiotics cannot be entirely absorbed by the human body, and a significant portion of

them is expelled in the form of original medications or metabolites through urine and feces, where they eventually end up in the municipal sewage treatment plant. According to scientific studies, most antibiotics cannot be entirely eliminated in a sewage treatment facility (Huang, et al., 2021). A considerable proportion of antibiotics enters surface water with the effluent, polluting groundwater and even drinking water.

Antibiotics in surface water can accumulate in sediments and in aquatic organisms such as fish and shrimps, entering their bodies, and then the food chain. Through sewage irrigation and sludge fertilization, antibiotics in sewage and sludge can infiltrate the soil environment. Then, through surface runoff and subsurface infiltration, it contaminates surface and groundwater.

Antibiotics discharged through animal urine and feces can reach the surface water and soil environment, polluting groundwater and drinking water in animal husbandry and aquaculture. A huge number of antibiotics are used directly in aquaculture. It directly pollutes surface water, and can be accumulated in the sediment, making degradation difficult. Antibiotics secreted by animals in the urine and feces can reach the surface water and soil environment, polluting groundwater and drinking water in animal husbandry and aquaculture. A lot of antibiotics are given straight to the water in aquaculture and can persist in the water and the sediment (Kummerer, 2008).

The specific origins and migration pathways of antibiotics are shown in Figure 5.

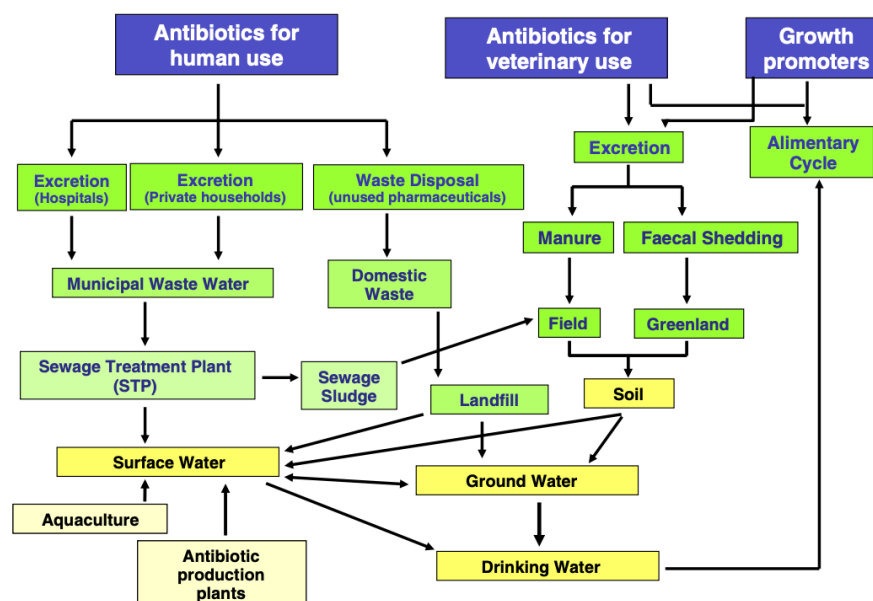


Figure 5. Sources and pathways of antibiotics in the environment (Moreno-Bondi et al., 2009)

As can be seen from Figure 5, wastewater and waste discharged from the production of antibiotics can enter the water and soil environment, and then migrate and transform in the environment through a variety of ways. Furthermore, one of the sources of antibiotics in the environment is direct dumping of antibiotics. Through sewage discharge and rainwater washing, buried antibiotics can contaminate surface and groundwater, eventually reaching drinking water.

2.4. The risk profile of antibiotics

Antibiotics are special compounds have a unique risk profile that isn't only toxic. They are still active compounds when they enter the environment, and they can kill sensitive organisms, but the resistant microorganisms will survive. Therefore, antibiotics differ cause a selective pressure on the microorganisms, and force the microbial ecosystem to evolve to antibiotics resistance. In the following sections I will discuss the main terms of antibiotic resistance.

2.5. Antibiotic resistance

Antibiotics are used like general prescription and administration, for veterinary purposes, but their efficiency is under threat. The reason is that targets develop antibiotic resistance when the antibiotic is misused. It means the microorganisms which can be treated by specific antibiotics will form the ability to resist the action of these antibiotics. In these cases, the old antibiotics may not work to target microorganisms anymore ([http 10](#)). When a bacterium that was previously susceptible to an antibiotic evolves resistance, we are talking about acquired resistance.

Antimicrobial resistance (AMR) has different levels like multidrug resistance (MDR), extensively drug resistance (XDR), and pan drug resistance (PDR). MDR was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories, XDR was defined as non-susceptibility one or two agents of different antimicrobial categories. PDR was defined as nonsusceptibility to all agents in all antimicrobial categories (Magiorakos et al., 2011).

Antibiotic resistance is driven by various factors including environmental drivers. Castro-Sánchez and his team published a report showing that the major driving force is the misuse of

antibiotics in humans and animals. Suboptimal dosing, availability of diagnostics, and environmental contamination are a minimum force on developing antibiotic resistance (Castro-Sánchez et al., 2016).

2.5.1. Mechanisms

Bacteria resist to antibiotic's action in two ways: one, by preventing the antibiotic from reaching its target at a high enough concentration, and the other, by modifying or bypassing the antibiotic's target. How bacteria prevent antibiotics from reaching their goals and modify or bypass the goal of antibiotics are as follows ([http 11](http://11)):

- Pump the antibiotic out from the bacterial cell with the over-expression of efflux pumps.
- Decrease permeability of the membrane that surrounds the bacterial cell. Certain changes in the bacterial membrane make it more difficult to pass through. In this way, antibiotic cannot enter bacterial cytoplasm in an effective concentration.
- Destroy the antibiotic. There are bacterial enzymes that can inactivate antibiotics.
- Modify the antibiotic. Bacteria can sometimes produce enzymes that can add different chemical groups to antibiotics.
- Disguise the target. Changes in the composition or structure of the target caused by mutations in bacterial DNA can prevent the antibiotic from interacting with the target.
- Express replacement proteins. Some bacteria can produce substitute proteins, which can be used to replace proteins that are inhibited by antibiotics.
- Reprogram the target. Sometimes bacteria can produce different variants of the structure it needs.

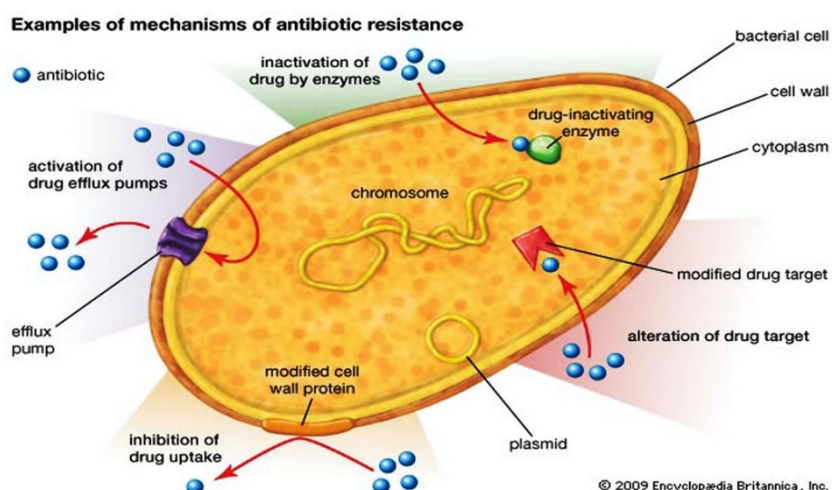


Figure 6. Antibiotic resistance strategies in bacteria (Bbosa et al., 2014)

2.5.2. The spread of antibiotic resistance genes in the environment

The resistance of bacteria to antibiotics is generally manifested as intrinsic resistance and acquired resistance. Intrinsic resistance means that bacteria are naturally insensitive to certain antibiotics. Acquired resistance involves changes in the genetic background of bacteria. Bacteria can acquire resistance through random mutation, or express potential resistance genes, or through horizontal transfer of resistance genes (Xu, 2010). The resistant strains can transfer the resistance genes to the soil indigenous microorganisms (Che-Sanford, 2001)

The sewage treatment system is an important way for resistance genes to enter the water environment. Antibiotic resistance genes enter the sewage treatment system along with medical wastewater, pharmaceutical wastewater, and animal farm wastewater. Since the existing water treatment technology has no obvious removal effect on many antibiotics, there is still a considerable concentration of antibiotics in the sewage effluent (Auerbach et al., 2007).

Wastewater treatment plants (WWTPs) are believed to be probable hotspots for antibiotic resistance dissemination in the environment as they offer convenient conditions for Antibiotic resistance bacteria (ARB) proliferation as well as for the horizontal transfer of Antibiotic resistance genes (ARGs) among different microorganisms. Based on the culture-dependent methods, it was observed that antibiotic-resistant bacterial species most frequently detected in WWTPs samples belong to the common indicators of fecal contamination: *E. coli*, total coliforms, and enterococci (Pazda et al., 2019). Some studies available in the scientific literature

show that biological processes may positively affect ARB spread and selection as well as ARGs transfer (Auerbach et al., 2007; Ferreira da Silva et al., 2006; Luczkiewicz et al., 2010). The persistent selective pressure from antibiotic residues at sub-inhibitory concentrations, as well as high density and diversity of microorganisms (including pathogenic, commensal, environmental and indigenous) sustained by a nutrient rich environment create favorable conditions for antibiotic resistance dissemination (Rizzo et al., 2013). WWTPs sludge was recognized as the main source of tetracycline- and sulfonamide-resistant bacteria and genes discharged into the water environment (Munir et al., 2011).

Based on the overview of scientific literature, sewage is an important factor in the antibiotic and ARGs load of surface water recipients. Some recent studies tried to find connection between the abundance of antibiotic resistance microorganisms (ARMs)/ARGs and the antibiotic load of surface water bodies, but in Hungary our knowledge in this field is still limited.

Therefore, my aim was to examine the microbial community of sewage affected and non-affected water samples and to validate OxiTop system, as a possible tool to evaluate the effect of antibiotic load on the composition of microbial community. To reach this goal the following steps were performed:

1. Choose a surface water body in Hungary with the same location with and without sewage load.
2. Set a microcosm experiment using non-affected (clean) and sewage affected water samples with and without antibiotic treatment to track the changes in the microbial community
3. Monitor the biological activity of the microbial community under antibiotic pressure in OxiTop system
4. Identify the changes in microbial community with community profiling (amplicon sequencing)
5. Isolate cultivable microorganisms from the different settings for further analysis

In the following section I will introduce the major steps, materials, and methods of our experiment.

3. Materials and methods

The main steps of our experiment are summarized in Figure 7.

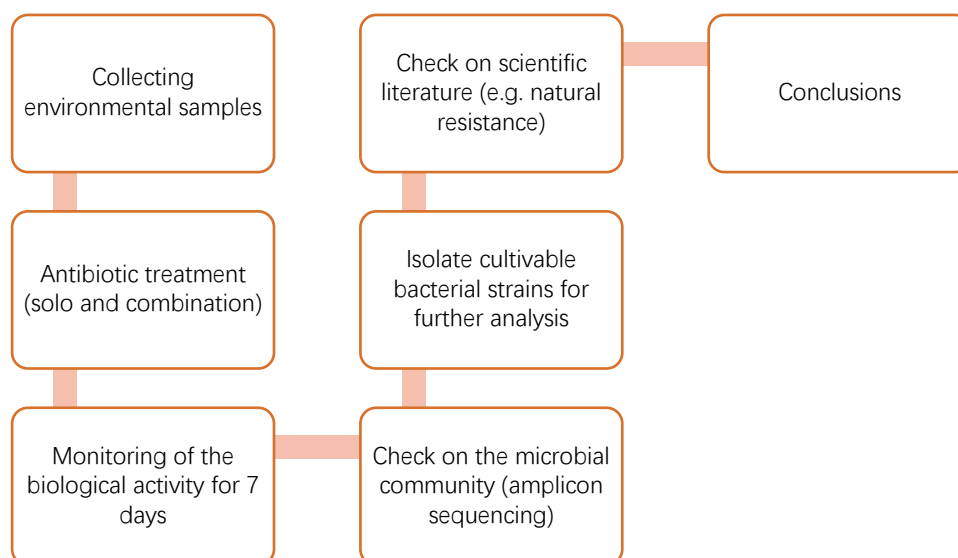


Figure 7. The summary of experimental processes

3.1. Sampling

Our strategy was to find a sampling site with a surface water body with and without sewage load. To reach this goal, our choice was the section of the Rákospatak stream localized between Gödöllő and Isaszeg (see Figure 8.). Samples were taken on the 30th of March 2021.

Samples were taken aseptically into sterile 1L volume bottles. Samples were immediately

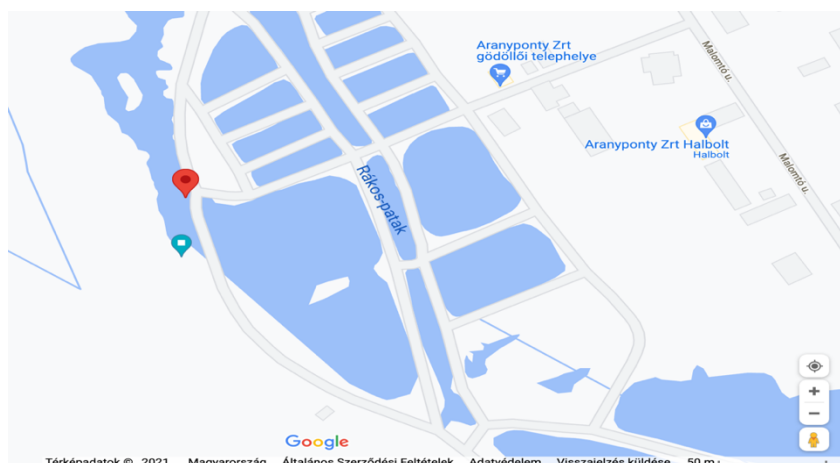


Figure 8. Map of the sampling site (credit: Google Map)

Sewage load (green tag): 47°33'53.6"N 19°22'02.9"E (47.564900, 19.367463)

Clean water sample (red tag): 47°33'54.8"N 19°22'03.0"E (47.565225, 19.367489)

transported to the laboratory of the Institute of Aquaculture and Environmental Safety and were processed for traditional cultivation and for OxiTop microcosm experiment.

3.2. OxiTop System

The method used in my experiment was a manometric method. The principle of this method is based on measuring pressure differences via piezoresistive electronic pressure sensors (Ijoma, 2010). Measurements are taken automatically for a 7-day period. The sample is kept in a sealed container fitted with a pressure sensor. A substance absorbing carbon dioxide is added in the container above the sample level. During incubation, oxygen is consumed, and carbon dioxide is released by the biologically active microorganisms. The total amount of gas, thus the pressure, decreases because carbon dioxide is absorbed. From the drop of pressure, the electronic device computes and displays the consumed quantity of oxygen.

For determining the biochemical oxygen demand (BOD) of the microbial community, i.e., the microbiological activity, we used a WTW OxiTop® respirometer system (Figure 9.).

200 ml environmental samples were added aseptically into the 0.5 L volume sterile bottles,



Figure 9. OxiTop System

in three technical replicates. At the same time, three different types of antibiotic treatment were set to measure the effect of solo and combined antibiotic pressure. All samples were treated with 300 µg/L final antibiotic concentration with the application of disks containing the relevant antibiotics. Sample types and treatment were marked as follows:

- Native (non-treated) samples: CW (Clean water) and SL (sewage load)
- Colistin-treated samples: CW-COL (Clean water with colistin) and SL-COL (sewage load with colistin)

- Florfenicol-treated samples: CW-FLOR (clean water with florfenicol) and SL-FLOR (sewage load with florfenicol)
- Colistin, imipenem, ceftazidime-treated samples (100-100-100 µg/L of each antibiotics): CW-MIX (clean water with antibiotic mixture), SL-MIX (sewage load with antibiotic mixture)

According to the previously mentioned AWaRe classification of WHO, two of the chosen antibiotics (imipenem and ceftazidime) are Watch class, and colistin is a Reserve class antibiotic in human therapy, while florfenicol is a commonly used veterinary type of antibiotic.

As CO₂ absorber, sodium hydroxide pellets were added to a separate container. Incubation temperature was 15°C, incubation time was 7 days with continuous stirring using the platforms of the OxiTop® system ([http 12](http://12)). Negative pressure (that is correlating with the microbial activity of the sample) were measured in every 30 minutes during the incubation period. At the end of our experiment, outlier replicates (with more than ±10 Pa deviation) were excluded from further data analysis. Pressure curves of the remaining technical replicates were averaged.

3.2.1. Cultivation methods

Cultivation aims to culture cell lines of bacteria from the environmental samples for further analysis including antibiotic resistance. Figure 10. shows the steps of culturing bacteria.

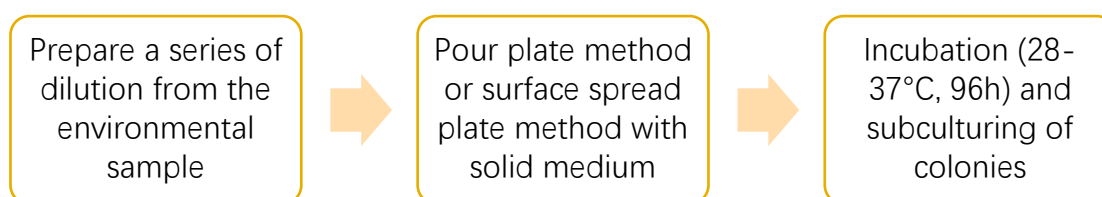


Figure 10. Steps of plating method

3.2.2. Medium for cultivation

To evaluate the available media for the cultivation and isolation of antibiotic resistant microorganisms originating from environmental samples, we set a preliminary experiment using environmental samples of the same site (Rákos stream). Samples were preincubated in 5

ml Mueller-Hinton broth containing 2 µg/ml colistin at 35°C for 24h. Preincubated samples were transferred to Luria-Bertani (LB) medium, MacConkey Medium, R2A, Asparagine broth (for the isolation of *P. aeruginosa*), and CHROMagar® *Acinetobacter* medium. The exact composition of these media can be found in Supplementary Material 1. According to our preliminary results on the diversity and colony forming units growing on the applied media (data not shown) our choice was R2A and LB medium for further use. During antibiotic resistant tests, we used the standardized Mueller-Hinton medium (Sigma-Aldrich).

3.2.3. Subculturing

After incubation at 28°C for 120h, colony forming units (CFUs) of the tenfold dilutions were counted and averaged to calculate the CFU/ml values of the original (100%) samples. Colonies with typical, or unique morphology were subcultured with spreading onto the surface of a fresh nutritive (LB) plate. After subculturing, clean bacterial cultures were inoculated into liquid broth medium and were incubated at 28°C for 48 hours at 150 RPM (round per minute). The liquid culture was used for extracting DNA and for storage at -80°C. To store microbial cultures, 700 µL glycerol (30 v/v%) was mixed with 700 µL bacterial suspension in cryogenic tubes. After this step we can do the PCR starting with DNA extraction from liquid cultures.

3.3. Identification

3.3.1. DNA extraction

For DNA extraction, we use the Zymo Quick DNA Miniprep Plus Kit following the manufacturer's instruction. The following steps are how to use this kit to extract DNA from the obtained strain.

First, we use fresh liquid culture of a bacterial strain. Liquid medium should be removed before processing by pelleting cells. In this step, I've added 2x700 µL liquid culture to a clean Eppendorf tube and centrifugated it at approximately 500 x g (11000 RPM) for 2 minutes depending on volume and cell type and removed the supernatant.

Then I resuspended the cultured cell pellet using 100 µL DNA elution buffer. First, I added

up to 200 μ L sample to a microcentrifuge tube and added 200 μ L Biofluid and cell buffer (Red) and 20 μ L Proteinase K (to prepare Proteinase K, I added 1060 μ L storage buffer to each Proteinase K tube prior to use to reach a final concentration of 20 mg/ml). Second, I mixed the sample and the ingredients thoroughly with vortex for 10-15 seconds and then incubated the tube at 55°C for 10 minutes. Third, I added 1 volume Genomic Binding Buffer to the digested sample then vortex for 10-15 seconds. Fourth, I transferred the mixture to a Zymo-Spin column in a collection tube and centrifugated at ≥ 12000 RPM for 1 minute. Then I discarded the collection tube with the flow through.

After these steps, I prepared new collection tubes for next operation. I added 400 μ L DNA Pre-Wash Buffer, 700 μ L g-DNA Wash Buffer, and 200 μ L g-DNA Wash Buffer to the spin column in a new collection tube separately, and Centrifuge at ≥ 12000 RPM for 1 minute to empty the collection tube after each addition. Then I discarded the collection tube with the flow through.

Finally, I transferred the spin column to a clean microcentrifuge tube and added ≤ 50 μ L DNA Elution Buffer directly on the matrix. I left tubes for 5 minutes at room temperature then I centrifuged the samples at a maximum speed for 1 minute to elute the DNA. The eluted DNA can be used immediately for molecular based applications.

The species level identification of sub-cultured isolates using the eluted DNA is performed by 16S rDNA sequencing as it is summarized in Figure 11.

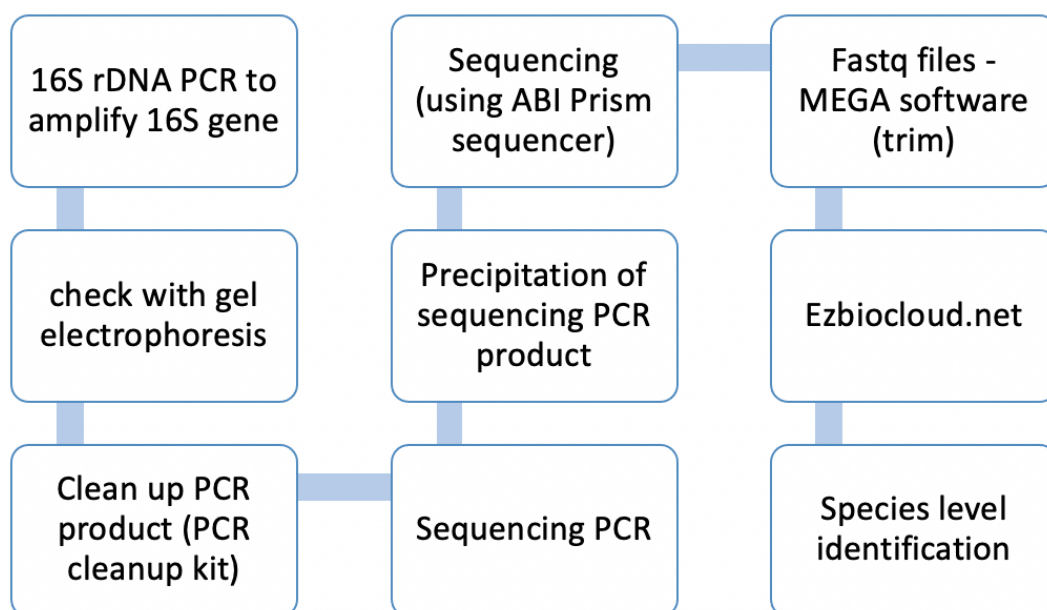


Figure 11. The protocol for PCR-based identification of bacterial cultures

3.3.2. 16S rDNA PCR

16S ribosomal DNA (rDNA) gene sequencing is not only faster and more accurate than conventional methods, but also allows identification of strains that are difficult to grow in laboratory conditions. Furthermore, differentiation of strains at the molecular level enables discrimination between phenotypically identical bacteria (Woo, et al., 2008). 16S rDNA contains variable regions, which can be used as identifying features for specific species. These characteristics make the 16S rDNA gene an ideal genetic fragment for bacterial identification, comparison, and phylogenetic classification ([http 13](http://13)).

16S rRNA gene sequencing is based on the polymerase chain reaction (PCR) followed by DNA sequencing. PCR is a molecular biology method used to amplify specific fragments of DNA through a series of cycles that include Denaturation of a double stranded DNA template, annealing of primers (short oligonucleotides) that are complementary to the template and extension of primers by the DNA polymerase enzyme, which synthesizes a new DNA strand (Shendure, 2017).

PCR reaction mixture contained 0.8 μ L of DNA, 0.5 μ L of 27f (5'-AGAGTTTGATCMTGGCTCAG - 3') and 1492r (5'-GGTTACCTTGTTACGACTT- 3') primers, 5 μ L Taq buffer, 10 μ L dNTP, 32.5 μ L Taq polymerase, and it was supplemented with molecular-grade water to the final volume (50 μ L). The heat profile was the following: initial denaturation for 3 min at 95 °C, 32 cycles of amplification (30 sec 94°C, 30 sec 52°C, 1 min 72°C). The last step was a final extension for 10 min at 72 °C. All amplifications were carried out in an Eppendorf Mastercycler PCR System (Germany).

3.3.3. PCR clean-up

The purpose of this step is to purify DNA molecules from PCR from enzymes, dNTPs, salts, and primers with using NucleoSpin Extract II DNA Clean-up set.

I added 90 μ L NT(I) buffer to PCR product, washing in and transfer the mixture to a membrane tube. After centrifugation with 11000 RPM for 1 min, I decanted filtrate and replaced the column. I added 650 μ L NT3 buffer and centrifugated with 11000 RPM for 1 min then

decanted filtrate. Then re-centrifugate with 11000 RPM for 2 min to make sure that there is no excess liquid in the tube.

Then I prepared a clean Eppendorf tube, placed the silica membrane column and added 35-50 μ L NE buffer. I incubated the tube for 1 min in room temperature then centrifugated with 11000 RPM for 2 min.

3.3.4. Agarose gel electrophoresis

Agarose gel electrophoresis is the method for the visualization of PCR results. In the first step I was mixing 70 ml SB Buffer (0.4 g NaOH; 3.044 g H₃BO₄; 1000 cm³ distilled water) and 0.7 g Agarose and homogenized it with heating up. Then I added 3.5 μ L Eco Safe nucleic acid staining solution (Pacific Image Electronics) and poured the gel. After the gel solidified, the comb was removed leaving wells for samples. I loaded 4 μ L PCR product with 1 μ L DNA loading dye (Thermo Scientific, USA). As a reference, I added 3 μ L Gene Ruler DNA Ladder Mix (Fermentas) to each line, to check on the approximate size of PCR products.

To separate the DNA fragments with different sizes, I ran gel electrophoresis with 100V for 40 min, then I checked and evaluated the result under UV transillumination.

3.3.5. 16S rDNA Sequencing

Sequencing is a reaction when we can check on the order of the bases of given genes. Using broad range 16S rDNA PCR, followed by DNA sequencing, the amplification and identification of any bacterial DNA present in a clinical or environmental sample will be possible (Patel et al., 2017). After sequencing, the sequences are compared with known nucleotide sequences of databases to identify the bacteria (Woo, et al., 2008).

In sequencing we are using the Sanger Dideoxy termination method. The first step is to run a sequencing reaction using the following parameters (Table 2.):

Table 2. Parameters of sequencing

Master mix		Heat profile:	
Big Dye	1 μ L	96°C	1 min
Big Dye buffer	0.5 μ L	96°C	10 sec
Primer	0.5 μ L (only forward)	51°C	5 sec
DNA template	3.5 μ L	60°C	4 min
MQ water	3.5 μ L	4°C	∞
Final volume	10 μ L		

} 28x

To recover the fluorescently labelled fragments, I added acetate mix to the product of the sequencing PCR and vortex it. The composition of Acetate mixture is: 3 μ L NaAc (3M), 62.5 μ L EtOH (95%) and 14.5 μ L MQ water. (NaAc is sodium acetate made of 80 mL distilled water and 40.0824 g $\text{CH}_3\text{CO}_2\text{Na} \cdot 3\text{H}_2\text{O}$. We adjusted pH to 5.2 with glacial acetic acid and volume to 100 mL with distilled water, sterilized by autoclaving and stored at room temperature).

The mixture was incubated for 10 minutes in room temperature, and centrifugated with 3220 g at 4°C for 30 minutes. After this step, I decanted supernatant, added 180 μ L EtOH (70%), and centrifugated the mixture with 3220 g at 4°C for 20 minutes. I decanted the residual supernatant completely and incubated at 50°C for 15-20 minutes to evaporate the residual liquid. Then I added 20 μ L Hi-Di formamide (Thermo Fischer, USA) and incubated for at least 24h at 4°C.

After these steps, we used capillary gel electrophoresis (sequencing) in an ABI Prism 3130 Genetic Analyzer (Applied Biosystems, USA) and did sequence analysis.

Sequences were manually processed in MEGA7 (version 7.0.26) and were exported in *.txt and *. fasta format. Partial 16S rDNA sequences were compared to the EzBioCloud database ([http 14](http://14)), a public data and analytics platform focuses on Bacteria and Archaea taxonomy, ecology, genomics, metagenomics, and microbiome.

3.4. Amplicon sequencing

To determine the genus level composition of the whole bacterial community of the examined samples, we performed a next-generation method called amplicon sequencing. Amplicon sequencing is a highly targeted method for analyzing genetic variation in specific genomic areas. Variant identification and characterization are made easier using ultra-deep

sequencing of PCR products (amplicons). Amplicon sequencing of the bacterial community's 16S rRNA genes across various species is a widely utilized approach for phylogeny and taxonomy investigations, particularly in diverse metagenomics samples ([http 15](#)).

3.4.1. Sample processing

For amplicon sequencing, we aseptically filtered 200-300 ml volume of each setting using 0.22 μ m MCR membrane filter at the end of the 7-day OxiTop experiment (Millipore). I isolated community DNA from the filtrate using the DNeasy Powersoil DNA extraction kit (Qiagen, Germany) according to the manufacturer's instructions.

During my experiment, we amplified bacteria domain-specific genetic elements from the community DNA. For paired-end 16S rDNA amplicon sequencing, the variable V3 and V4 regions of the 16S rDNA gene were amplified using forward (5'-AGA GTT TGA TCC TGG CTC AG-3') and reverse (5'-ACC TTG TTA CGA CTT-3') primers. Amplification products were sequenced in the Illumina MiSeq platform (2x250 bp read length) by Biomi Ltd.

3.4.2. Data analysis

Raw reads (fastq files) of amplicon sequencing were processed and analyzed by the mothur software (Schloss et al., 2009) using the high-performance computer of the Institute of Aquaculture and Environmental Safety under Linux operation system. Mothur is an open-source, platform-independent, community-supported software for describing and comparing microbial communities. With mothur software we can analyze data using command lines. The major steps of data analysis were as follows:

- Remove the too short or too long sequences and sequences with any ambiguous bases,
- Deal with the number of homopolymers, which are the repetitive sequences of the DNA
- Choose the unique sequences found in a fasta-formatted sequence file
- Count the number of sequences represented by the representative sequence
- Obtain an alignment database. In our data analysis, we used Silva database as a reference ([http 16](#)).
- Implement a pseudo-single linkage algorithm with the goal of removing sequences that are

likely due to sequencing errors.

After data processing, mothur software summarize taxonomy and diversity results of the examined samples in data files. Data can be exported to excel for further analysis.

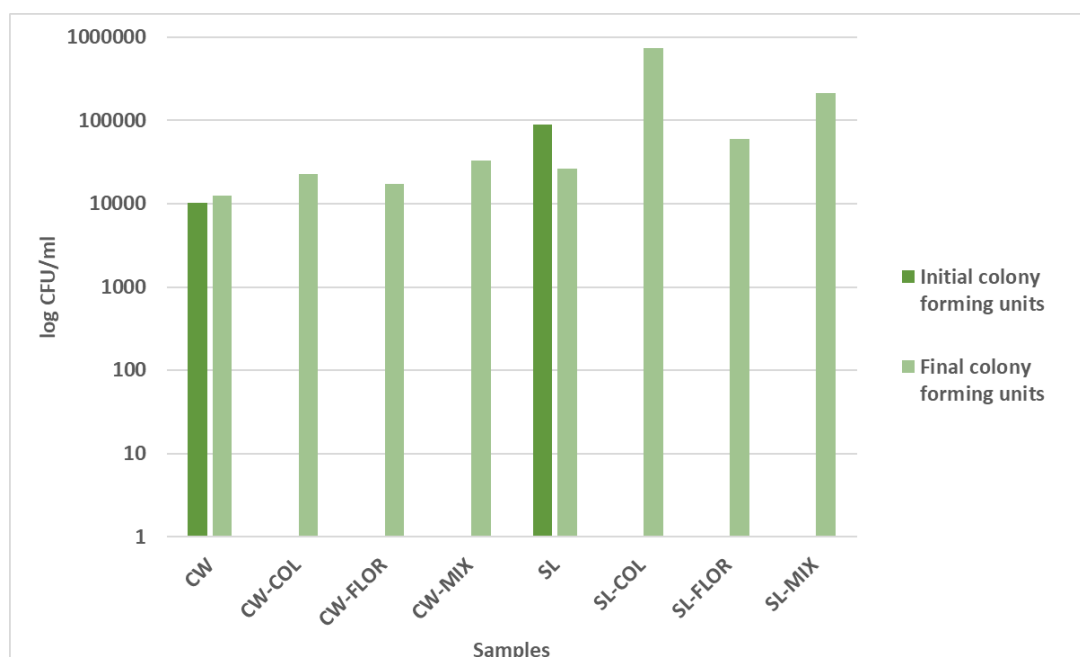
4. Result and Discussion

Based on the examination of environmental samples with and without sewage load of the same geographical location (CW and SL samples) and their treatment with different types of antibiotics (see section 3.2.), we gained information about the composition and activity of the microbial community. Hereby I present my results in the following order:

- Cell counts (colony forming units)
- BOD values
- Microbial community (amplicon sequencing)
- Isolation and identification

4.1. Cell counts (colony forming units)

With traditional cultivation, colony forming units were determined on R2A medium at the beginning and at the end of the 7 days long experiment. CFU values are summarized in Graph 1.



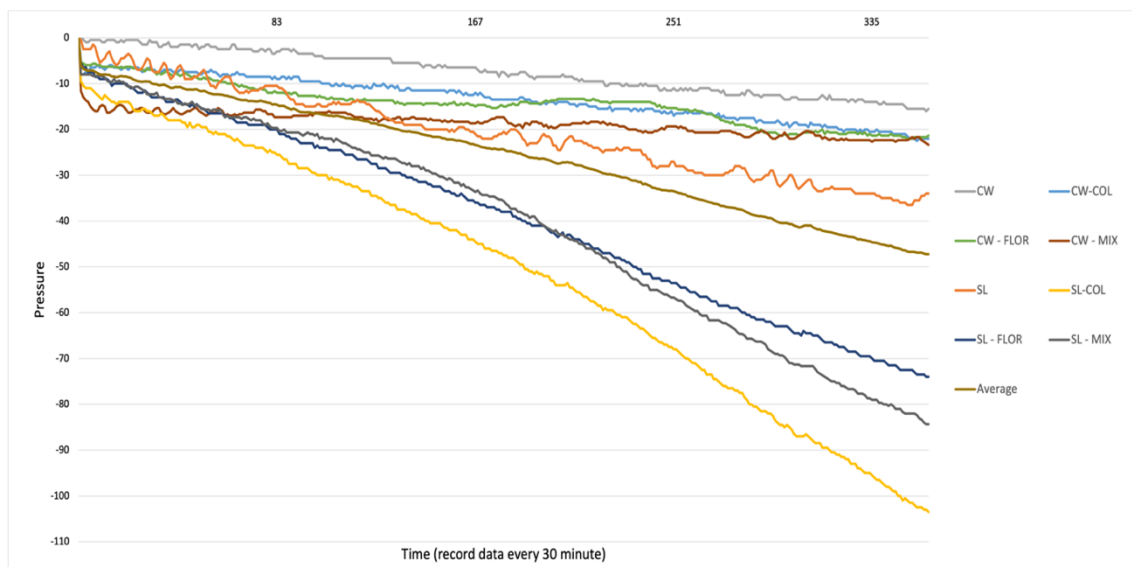
Graph 1. Colony Forming Unit

In Graph 1 the cell count of CW sample was stable, there were no significant increase, or

decrease in microbial counts. At the same time, SW samples especially with colistin treatment, increased their microbial numbers, which was an unexpected outcome of antibiotic treatment. The difference was higher in the case of sewage samples means that in the sewage load microbial community, the adaptability to antibiotics is higher. However, the stable or increasing cell counts were unexpected: We have expected that antibiotic treatment will kill or reduce the number of microorganisms. A possible explanation is that with the effect of antibiotics, sensitive microorganisms were eradicated and without the competition, resistant bacteria could grow faster, so their cell count is increased.

4.2. The effect of microbiological activity with different antibiotics treatment

During the 7 days long experiment, we have continuously detected the changes of negative pressure in each bottle and replicates which correlates with microbial activity. The result of biological oxygen demands of the eight different settings (CW and SL samples, the other 6 samples treated separately by antibiotics and their combinations (CW / SL-COL, CW / SL-FLOR, CW / SL-MIX)) are summarized in Graph 2. supplemented with the average value of all settings.



Graph 2. Microbiological activity of the microbial community expressed in negative pressure measured by OxiTop system
(Average values of three replicate bottles)

In Graph 2. the pressure is going down when the microbial community is consuming a significant amount of oxygen, which means that they were biologically active. If the curve is flat, it means there was no real biological activity because the microbe community was not using so much oxygen.

In this graph we can see that the lowest level of biological activity was in the original clean water (CW) sample (without sewage load). A similar level of biological activity was in clean water with colistin, clean water with florfenicol, clean water with the mixture of colistin, imipenem and ceftazidime sample. According to the BOD values of CW samples, we can conclude that the antibiotic treatment did not cause a significant change in the biological activity of the microbial community.

In the sewage group, the original sewage sample had the lowest degree of biological activity. The biological activities of the organisms in the sewage-affected sample with florfenicol and sewage with colistin, imipenem, ceftazidime samples were similar. However, comparing to florfenicol treatment, the microbial community in the SL-MIX sample treated with colistin, imipenem, ceftazidime was less active in the first half of incubation period, but was more active in the second half of our experiment. In the SL-COL sample, biological oxygen demand was the highest suggesting that it was the most active microbial community of all examined settings, that it can be a result of the slightly higher microbial cell counts.

In the case of the same initial sample (SL), the results are different depending on the antibiotics added to the system.

In the clean water group, samples without antibiotic treatment had the lowest microbiological activities. When a combination or the sole application of antibiotic are added to the samples, the activity of the microorganisms is increased. The three combined antibiotics of colistin, imipenem, ceftazidime had the greatest impact on microbial activity in the sample.

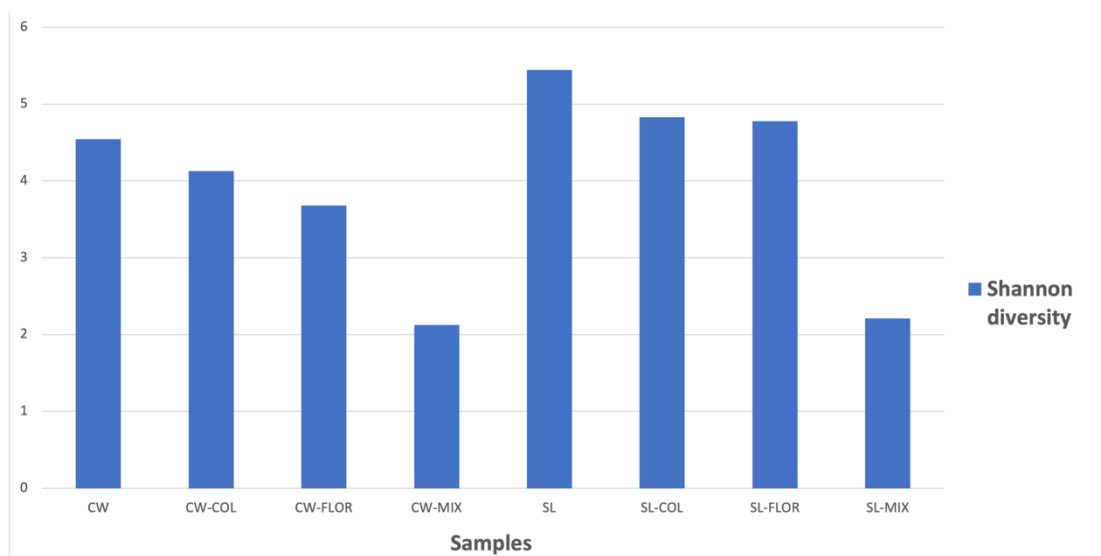
It is obvious that antibiotics have a significant impact on microbial activity, particularly in the case of colistin, but the determined effect was not inhibition. BOD is the measure of the amount of oxygen taken up by aerobic microorganisms that decompose organic waste matter in water. It is therefore used as a measure of the amount of certain types of organic pollutants in water. A high BOD indicates the presence of a large number of microorganisms, which suggests a high level of pollution (Ijoma, 2010). Therefore, we can conclude that the applied

concentrations of antibiotics are rather carbon sources for the microorganisms and slightly increase their biological activity and there is no measurable inhibition of bacterial growth.

Comparing to scientific literature, I couldn't find any similar studies regarding the comparison of BOD values of microbial communities under different antibiotic treatments. Panta and Doerrler described colistin resistance as a complex and multifactorial phenomenon (Panta and Doerrler, 2021). They have found that colistin resistance can be linked to pH and oxygen level of the medium as well. Li and her co-authors mentioned that the relative abundances of ARGs were negatively correlated with temperature and BOD (Li, et al., 2020) which is also a possibility in our case.

4.3. Diversity and taxonomy

The bacterial diversity of the samples was determined by the mothur algorithm and is expressed with Shannon diversity index as it is summarized in Graph 3.

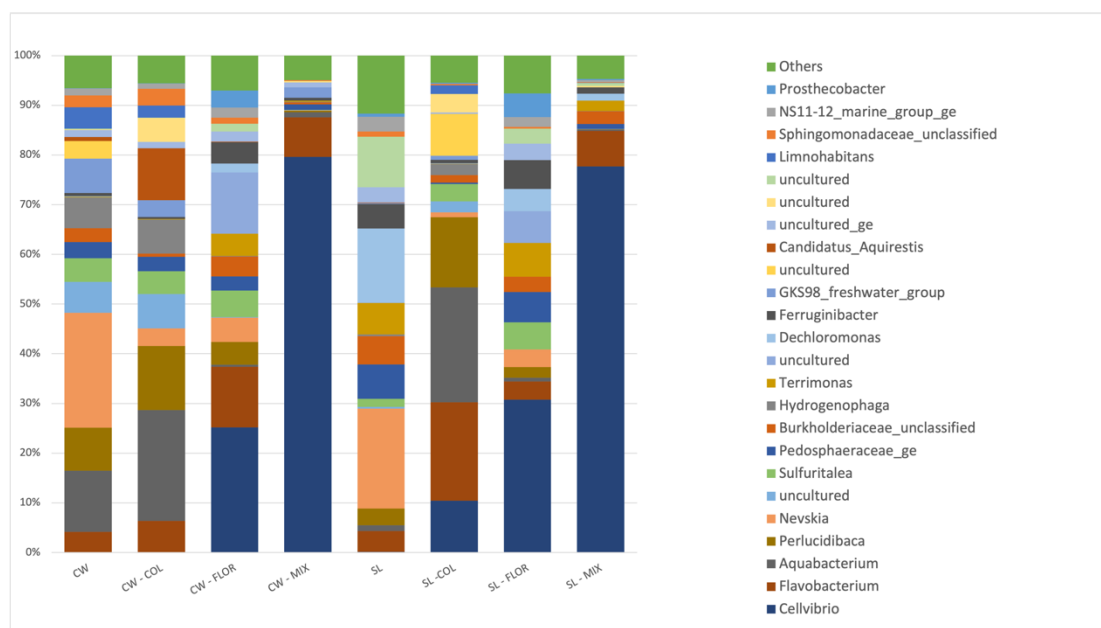


Graph 3. Shannon diversity index of the examined samples

As it can be seen, CW samples had a lower level of microbial diversity, while the sample with sewage load had a higher Shannon index. Comparing to native (CW and SL) samples, all antibiotic treatment led to a lower level of bacterial diversity. According to our results, SL sample has the highest diversity, CW-MIX sample has the lowest diversity of all settings. Comparing to the determined CFU values of each sample (Graph 1.), there is a reverse correlation between diversity and cell count. According to this information, we can state the

antibiotic treatment caused a significant decrease in the microbial diversity of the examined sample, while the microbial counts remained the same or slightly increased. A possible explanation is that antibiotic treatment eradicated the sensitive microorganisms and opened a niche to the fast growing, antibiotic resistant species.

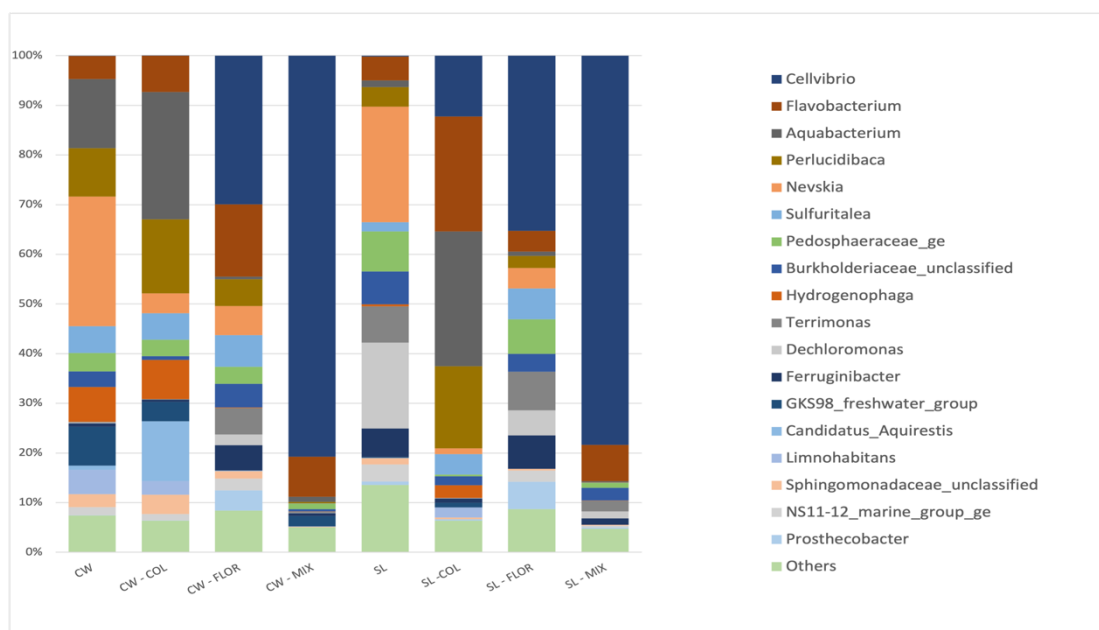
Graph 4. below shows the composition of the bacterial community under different treatments for some samples at the end of the experiment (CW/SL, CW/SL-COL, CW/SL-FLOR, CW/SL-MIX) determined by amplicon sequencing method.



Graph 4. The composition of the bacterial community

Graph 4. shows that in the CW/SL-FLOR, SL-COL, and CW/SL-MIX *Cellvibrio* species had the highest abundance and *Flavobacterium* species had at least 5% abundance in all examined settings. *Aquabacterium* were dominant in CW, CW-COL and SL-COL samples. *Perlucidibaca* and *Nevskia* were important members of the native (CW and SL) microbial communities but did not appear in CW/SL-MIX samples. As it can be seen, after treatment, the dominance of the significant *Nevskia* genus in untreated samples was suppressed, while the proportions of the *Flavobacterium*, *Aquabacterium*, and *Perlucidibaca* communities did not change significantly with colistin and florfenicol.

There were a lot of “uncultured” hits, these are bacteria whose 16S sequence was not found at the genus level by the mothur program when compared to the SILVA database. To find some relevant information about the known species of the identified microbial communities, I’ve excluded the “uncultured” genera and reset the graph (see Graph 5.).



Graph 5. The composition of the bacterial community (remove "uncultured")

After this data processing step, we could identify the main, known representatives of each sample. From now on, I have listed a table about the top six identified taxa of each sample (Table 4.). I choose 6 most interesting species among them and will give a brief description of these members of the microbial communities.

Table 2. Identified taxa of the examined samples

CW	Percentage	CW-COL	Percentage	CW-FLOR	Percentage	CW-MIX	Percentage
Nevskia	15.19%	Aquabacterium	16.86%	Aquabacterium	17.87%	Cellvibrio	66.03%
Aquabacterium	8.09%	Perlucibaca	9.76%	Flavobacterium	15.28%	Flavobacterium	6.60%
Perlucibaca	5.65%	Candidatus_Aquirestis	7.91%	Perlucibaca	10.90%	Algoriphagus	3.12%
GKS98_freshwater_group	4.53%	Hydrogenophaga	5.21%	Cellvibrio	8.06%	GKS98_freshwater_group	1.72%
Hydrogenophaga	4.07%	Flavobacterium	4.81%	Hydrogenophaga	1.69%	Aquabacterium	0.87%
Flavobacterium	2.69%	Nevskia	2.66%	Nevskia	0.77%	Perlucibaca	0.24%
SL	Percentage	SL-COL	Percentage	SL-FLOR	Percentage	SL-MIX	Percentage
Nevskia	7.40%	Cellvibrio	15.00%	Cellvibrio	17.02%	Cellvibrio	67.49%
Dechloromonas	5.49%	Flavobacterium	7.28%	Terrimonas	3.76%	Flavobacterium	6.24%
Pedosphaeraceae_ge	2.56%	Sulfuritalea	3.20%	Pedosphaeraceae_ge	3.36%	Burkholderiaceae_unclassified	2.23%
Flavobacterium	1.54%	Nevskia	2.92%	Flavobacterium	2.02%	Terrimonas	1.85%
Perlucibaca	1.24%	Perlucibaca	2.72%	Nevskia	1.97%	Dechloromonas	1.20%
Aquabacterium	0.42%	Terrimonas	2.68%	Perlucibaca	1.19%	Aquabacterium	0.22%

4.3.1. Characterization of the most important identified taxa

The jump in, the dominance of the genus *Cellvibrio* in the treated samples (except CW-COL) is outstanding. This genus involved in the breakdown of cellulose. It has been described that its presence (and that of *Flavobacterium* species) can be closely related to the frequency of antibiotic resistance genes (Han, et al., 2018). According to the results of Han and his co-workers, *Cellvibrio* species are possible hosts of ARGs in agricultural soils treated with different sources of animal manures (Han et al., 2018). *Flavobacterium* was significantly correlated with 10 aminoglycoside resistance genes, 5 tetracycline resistance genes, 3 Macrolide-lincosamide-streptogramin B (MLSB) resistance genes, 2 FCA (fluoroquinolone, quinolone, chloramphenicol, florfenicol and amphenicol) resistance genes, 2 sulfonamide resistance genes, 1 β -lactam resistance gene, 1 multidrug resistance gene and 1 MGE (mobile genetic element). *Cellvibrio* also had significant relationships with diverse ARGs especially those conferring resistance to aminoglycoside, tetracycline and MLSB (Han et al., 2018).

Species of the genus *Nevskia* have a wide nutritional spectrum and are able to utilize sugars as growth substrates (Gutierrez et al., 2012). *Nevskia ramosa* is a widely distributed epineustonic bacterium (Pladdies et al., 2004). Based on the scientific literature, *Nevskia* species are not discussed in context with antibiotic resistance, which is supported with our results: in the native samples, *Nevskia* species were relatively abundant, but their abundance significantly decreased under antibiotic treatment.

The genera *Aquabacterium* and *Perlucidibaca* were abundant in the native CW sample, and under antibiotic treatment (COL and FLOR) *Aquabacterium* became dominant. Similarly, *Perlucidibaca* became more abundant with colistin treatment. In case of SL samples, there were no significant difference in their abundance with and without antibiotic treatment. The genus *Aquabacterium* belongs to the family *Comamonadaceae*, order *Burkholderiales*, in the class *Betaproteobacteria* (Pham et al., 2015). Five species of the genus *Aquabacterium* have been identified to date. *Aquabacterium citratiphilum*, *A. commune* and *A. parvum* were isolated from biofilm from the Berlin drinking water system (Pham et al., 2015), while *A. fontiphilum* and *A. limnoticum* were found in freshwater springs in China (Lin et al., 2009; Chen et al., 2012). Some potential pathogens for example *Aquabacterium* had the highest abundances in biofilms

samples and were potential hosts harboring many resistance genes (Zhao, et al., 2021). The genus *Perlucidibaca* was described in 2008 (Song et al., 2008). *Perlucidibaca* are Gram-negative freshwater bacteria belonging to the family *Moraxellaceae*. *Aquabacterium* and *Perlucidibaca* can be considered as the “core microbiome” of microplastics traveling in wastewater, i.e., they presumably play a role in the early colonization of microplastics traveling in wastewater (Martinez-Campos, et al., 2021). The preponderance of these genera in CW-COL samples is understandable, given that members of the *Comamonadaceae* and *Moraxellaceae* families are innately resistant to colistin. At the same time, I couldn't find any information about *Aquabacterium* species' florfenicol resistance, which should be investigated further in the future.

Hydrogenophaga species are the predominant bacteria cultured from granular activated carbon filters in water treatment (Magic-Knezev, et al., 2009). Descriptions of the nutritional versatility of the predominating genera confirm that *Hydrogenophaga* prefer carboxylic acids and/or amino acids as growth substrates but are limited in the utilization of carbohydrates and generally do not hydrolyze proteins, carbohydrates, or fats (Garrrity et al., 2005; Magic-Knezev and Van der Kooij 2006). *Hydrogenophaga* was a new host of ARGs in distinct setting samples after ozone disinfection. Following disinfection with O_3/Cl_2 , *Hydrogenophaga* became a bacterial host for beta-lactam ARGs (Zhang, et al., 2019). *Hydrogenophaga* and *Aquabacterium* species are verified to harbor Class 1 integrons that are playing a significant role in the worldwide problem of antibiotic resistance (Gillings et al., 2008). In our case, *Hydrogenophaga* was found in the CW, CW-COL and CW-FLOR samples and was the most abundant in the CW-COL setting.

4.4. Traditional cultivation

After incubation at the required temperature (28 and 37°C), representative colonies were sub-cultured. The number of cultures from the preliminary experiment were 53 bacterial cultures, and 16 bacterial cultures were isolated from Rákos stream. Bacterial DNA was isolated using the Zymo DNA purification kit and species-level identification of the isolated strains is in progress using 16S rDNA sequencing method. Due to the unfortunate problems with sequencing method, the isolated strains were not comprehensively identified, described, and

characterized, but in the future the missing experiments will be finished to find a link between the amplicon sequencing data and the cultivable species. I listed the strains, their colony morphology, and the results of the successful identifications in the Supplementary material 2. According to the available list of the cultivable species, we could not find the representatives of the dominant genera identified by amplicon sequencing method. With cultivation, mainly naturally colistin resistant bacterial species (e.g., *Proteus faecis*, *Serratia marcescens*, *Morganella morganii*) were identified – almost all of them from the preliminary experiments. We will try to cultivate the principal bacterial species from the discovered microbial communities in the experimental conditions in the future.

5. Conclusions and suggestions

In our experiment using samples taken from the Rákos stream, the diversity of microbial community was correlated with the origin of the sample (clean water, sewage load) and the different antibiotic treatments (COL, FLOR, MIX). It was verified that antibiotic resistant bacteria are present in both sample types, but sewage affected microbial community showed a higher level of tolerance. This result is a verification of our hypothesis and an expected outcome of sewage load effect the presence and frequency of antibiotic resistant, cultivable, and non-cultivable bacterial community.

The OxiTop system has been adapted and optimized for the examination of antibiotic treatment in natural freshwater samples with and without sewage load. We have verified that a freshwater sample with sewage load have a higher number of microorganisms with the ability to adapt to antibiotic treatment. We established that antibiotic load reduces the diversity of environmental samples while marginally increasing the cultivable number of bacteria, this is the answer of the second question we mentioned in the hypothesis.

Based on our results, there is no significant difference in the categories of species in the original composition of microbial community with and without sewage load, but there is a significant variation in the proportion of the total number. Several of them have vastly different abundance: *Nevskia*, *Aquabacterium*, *Flavobacterium*, *Perlucidibaca*, *Hydrogenophaga*.

After comparing our results to scientific literature, we discovered that some of the identified bacterial genera are verified to be antibiotic-resistant or to harbor antibiotic resistance genes. For example, *Flavobacterium* interacted with 10 aminoglycoside resistance genes, while *Cellvibrio* and *Aquabacterium* could be possible hosts containing numerous resistance genes. Since the sensitive bacteria have been eradicated by antibiotics, the growth rate of the resistant bacteria has accelerated due to reduced competition, resulting in an increase in the number of potentially antibiotic resistance harboring microorganisms.

In the future, we will check on the presence or absence and the quantity of different antibiotic resistance genes with molecular methods and finish the identification and antibiotic resistance profiling of the already cultivated microorganisms. According to scientific sources, some physical properties, such as pH can be important factors in antibiotic (colistin) resistance

(Panta and Doerrler, 2021). Based on this information, I suggest checking on the differences in pH during OxiTop measurements as well. Temperature is another factor effecting the relative abundance of ARGs (Li, et al., 2020), therefore, in the future it is recommended to repeat the experiment with different temperature settings.

6. Summary

Almost all antibiotics used in clinical practice now contain resistant bacteria, and multi-drug resistant strains are on the rise, with super germs capable of surviving most conventional treatments appearing. According to the World Health Organization (WHO), antibiotic resistance is one of the three most serious threats to human health.

Antibiotic resistance develops because of several genetically linked biological mechanisms. Antibiotic resistance genes, which are the source of bacterial resistance, have been classed as a new form of pollution. Human activities can transmit antibiotic resistance genes (ARGs) throughout the surface water ecosystem.

The purpose of my thesis is to see how sewage inlets affect the presence and frequency of antibiotic-resistant, cultivable, and non-cultivable bacterial communities, as well as to look for antibiotic resistance genes (ARGs) in environmental samples. The objective is to figure out how common they are in the environment.

The research methods are using the OxiTop system to check BOD, traditional cultivation, molecular biology (PCR), and amplicon sequencing. According to changes in pressure, BOD can show how the biological activity was. The research method of antibiotic resistance is mainly based on the culture of microorganisms, and the resistance will be evaluated by the resistance phenotype. Amplicon sequencing is a highly targeted method for analyzing genetic variation in specific genomic areas. In my laboratory work, PCR and sequencing is used for the species-level identification of bacterial strains isolated from environmental samples.

We estimated the biochemical oxygen requirement of the microbial community through tests and successfully isolated 16 bacterial strains from the surface water samples investigated. We extracted DNA and used PCR to find the species-specific 16S rDNA gene. We discovered that some species can be the host of multiple antibiotic resistance genes after additional investigation and comparison with scientific sources. Finally, we will obtain information on sample diversity and cell count, as well as the predominance of taxa in the specified surface water environment.

7. Acknowledgement

First and foremost, I must express my gratitude to my supervisor, Dr. Kaszab Edit, who works in the Department of Environmental Safety and has taught me a variety of skills and guided me through laboratory work. She assisted me in correcting errors in my thesis work. I appreciate everything she has done for me. I'd also like to express my sincere thanks to Jiang Dongze, a PhD student in the Environmental Safety Department. He advised and assisted me throughout the experiment.

Then, I have to express my thankful feeling to the Stipendium Hungaricum program, the Hungarian Government, and China Scholarship Council, thank them to supported me study in Hungary to complete the Bachelor's degree. I'd want to convey my gratefulness to Hungarian University of Agriculture and Life Sciences and Department of Environmental Safety, where I completed my thesis. In the meantime, this project was supported by the 2020-1.1.2-PIACI-KFI-2021-00239 and the Thematic Excellence Programme 2020, and National Challenges Subprogramme (TKP2020-NKA-16). My supervisor Dr. Edit Kaszab was supported by ÚNKP-21-5. I am very grateful for these supports.

Here I also want to thank Dr. Árpád Hegyi for assisting us in collecting environmental samples, Dr. Mátyás Cserhádi for helping me to collecting data in the OxiTop system, Dr. Milán Farkas for teaching me how to use the mothur software, and Brigitta Edőcs for demonstrating the filtration procedure.

I am appreciative to the professors who taught me and the friends who supported me over the years; they all promoted me to grow and improve.

Furthermore, I want to thank my families for everything they have done for me. They always motivate me and provide me with suggestion and strength so that I may finish my thesis effectively. Heartfelt thanks for everything.

Supplementary material 1

- **Mueller-Hinton** broth (Sigma-Aldrich).

Reagent	Amount to add (g / Liter)
Agar	17.0
Beef infusion solids	2.0
Casein hydrolysate	17.5
Starch	1.5

- **Luria-Bertani** (LB) medium

Reagent	Amount to add
Tryptone	10.0 g
Yeast extract	5.0 g
NaCl	9.0 g
Distill Water	1000 ml

- **MacConkey** Medium

Reagent	Amount to add (g / Liter)
Peptone from gelatin	17.0
Peptone from casein	1.5
Peptone from meat	1.5
Sodium chloride	5.0
Lactose	10.0
Bile salt mixture	1.5
Neutralred	0.03
Crystal violet	0.001
Agar-Agar	13.5

- **R2A**

Reagent	Amount to add (g / Liter)
Agar	15
Casein acid hydrolysate	0.5
Dextrose	0.5
Dipotassium phosphate	0.3
Magnesium sulfate	0.024
Proteose peptone	0.5
Sodium pyruvate	0.3
Starch (soluble)	0.5
Yeast extract	0.5

- **Asparagine** broth (for the isolation of *P. aeruginosa*)

Reagent	Amount to add (g / Liter)
DL-asparagine	2
Dipotassium phosphate (anhydrous)	1
Magnesium sulfate	0.5
Potassium sulfate	10
L-proline	1

- **CHROMagar® Acinetobacter** medium (Chebio, Ltd., Budapest)

Supplementary material 2

List of the strains and colony morphology

Sample ID	Sampling site (location)	Time of sampling	Designation of strain	Colony morphology	Species (16S rDNA)
2 MCR3 (Sample 2, colistin concentration: 3 ug/ml)	Isaszeg lakes	12-10-2020	C1	5-6 mm, round, entire, raised, smooth, brown-yellow	<i>Serratia marcescens</i>
			C2	4 mm, round, entire, raised, smooth, brown-yellow	<i>Proteus faecis</i>
			C3	6-7 mm, round, entire, raised, smooth, brown-yellow	<i>Proteus faecis</i>
			C4	2 mm, round, entire, raised, smooth, brown-yellow	<i>Serratia marcescens</i>
			C5	1-2 mm, round, entire, raised, smooth, brown-yellow	<i>Morganella morganii</i>
			C6	1-2 mm, round, entire, raised, smooth, brown-yellow	<i>Chromobacterium aquaticum</i>
			C7	2 mm, round, entire, raised, smooth, brown-yellow	<i>Chryseobacterium rhizoplanæ</i>
			C8	1-2 mm, round, entire, raised, smooth, brown-yellow	<i>Chryseobacterium rhizoplanæ</i>
			C9	1 mm, round, entire, raised, smooth, brown-yellow	In process

Sample ID	Sampling site (location)	Time of sampling	Designation of strain	Colony morphology	Species (16S rDNA)
2 MCR2 (Sample 2, colistin concentration: 2 ug/ml)	Isaszeg lakes	12-10-2020	C10	0.5 mm, round, entire, raised, smooth, brown-yellow	<i>Enterococcus gallinarum</i>
			C11	1 mm, round, entire, raised, smooth, brown-yellow	In process
			C12	0.5 mm, round, entire, raised, smooth, brown-yellow	<i>Serratia surfactantfaciens</i>
			C13	1 mm, round, entire, raised, smooth, brown-yellow	<i>Morganella morganii</i> subsp. <i>Morganii</i>
			C14	1-2 mm, round, entire, raised, smooth, brown-yellow	In process
			C15	1 mm, round, entire, raised, smooth, brown-yellow	In process
			C16	0.3-0.5 mm, round, entire, raised, smooth, brown-yellow	In process
			C17	1 mm, round, entire, raised, smooth, brown-yellow	In process

Sample ID	Sampling site (location)	Time of sampling	Designation of strain	Colony morphology	Species (16S rDNA)
1 MCR2 (Sample 1, colistin concentration: 2 ug/ml)	Isaszeg lakes	12-10-2020	C18	1-2 mm, round, entire, raised, smooth, brown-yellow	In process
			C19	1-2 mm, round, entire, raised, smooth, brown-yellow	In process
			C20	1 mm, round, entire, raised, smooth, brown-yellow	In process
			C21	1-2 mm, irregular, undulate, raised, smooth, brown-yellow	In process
			C22	0.1 mm, round, entire, raised, smooth, brown-yellow	<i>Serratia marcescens</i>

Sample ID	Sampling site (location)	Time of sampling	Designation of strain	Colony morphology	Species (16S rDNA)
PLA- MCR2 (Sample in the plastic, colistin concentration: 2 ug/ml)	Isaszeg lakes	12-10-2020	C23	0.1 mm, round, entire, raised, smooth, brown-yellow	In process
			C24	5 mm, round, entire, raised, smooth, brown-yellow	<i>Morganella morganii</i>
			C25	1 mm, round, entire, raised, smooth, brown-yellow	<i>Morganella morganii</i>
			C26	1 mm, round, entire, raised, smooth, brown-yellow	In process
			C27	<0.1 mm, round, entire, raised, smooth, brown-yellow	Mixed sequence
			C28	2-3 mm, round, entire, raised, smooth, brown-yellow	In process
			C29	1 mm, round, entire, raised, smooth, brown-yellow	In process
			C30	0.5 mm, round, entire, raised, smooth, brown-yellow	In process

Sample ID	Sampling site (location)	Time of sampling	Designation of strain	Colony morphology	Species (16S rDNA)
PLA- MCR3 (Sample in the plastic, colistin concentration: 3 ug/ml)	Isaszeg lakes	12-10-2020	C31	2-3 mm, round, entire, raised, smooth, brown-yellow	<i>Morganella morganii</i> ssp. <i>Morganii</i>
			C32	1-2 mm, round, entire, raised, smooth, brown-yellow	In process
			C33	1 mm, round, entire, raised, smooth, brown-yellow	In process
			C34	1-2 mm, round, entire, raised, smooth, brown-yellow	In process
			C35	<1 mm, round, entire, raised, smooth, brown-yellow	In process
			C36	1 mm, round, entire, raised, smooth, brown-yellow	In process
			C37	1 mm, round, entire, raised, smooth, brown-yellow	In process
			C38	0.5 mm, round, entire, raised, smooth, brown-yellow	In process

Sample ID	Sampling site (location)	Time of sampling	Designation of strain	Colony morphology	Species (16S rDNA)
PLA (Sample in the plastic)	Isaszeg lakes	12-10-2020	C39	1 mm, round, entire, raised, smooth, brown-yellow	In process
			C40	0.5 mm, round, entire, raised, smooth, brown-yellow	In process
			C41	1-2 mm, round, entire, raised, smooth, brown-yellow	In process
			C42	0.5 mm, round, entire, raised, smooth, brown-yellow	In process
			C43	<0.5 mm, round, entire, raised, smooth, brown-yellow	In process
			C44	0.5 mm, round, entire, raised, smooth, brown-yellow	In process
			C45	1 mm, round, entire, raised, smooth, brown-yellow	In process
			C46	1-2 mm, round, entire, raised, smooth, brown-yellow	In process
			C47	0.5-1 mm, irregular, undulate, flat, smooth, brown-yellow	In process
			C48	1 mm, round, entire, raised, smooth, brown-yellow	In process
			C49	0.5 mm, round, entire, raised, smooth, brown-yellow	In process
			C50	0.5 mm, round, entire, raised, smooth, brown-yellow	In process
			C51	1-2 mm, round, entire, raised, smooth, brown-yellow	In process
			C52	1 mm, round, entire, raised, smooth, brown-yellow	In process
			C53	1 mm, round, entire, raised, smooth, brown-yellow	In process

Sample ID	Sampling site (location)	Time of sampling	Designation of strain	Colony morphology	Species (16S rDNA)
CW	Rákös stream	30-03-2021	1	1-2 mm, circular, entire, rough, flat, yellow	Paucibacter aquatilis
			2	<1 mm, circular, entire, rough, flat, yellow	In process

Sample ID	Sampling site (location)	Time of sampling	Designation of strain	Colony morphology	Species (16S rDNA)
CW-COL	Rákös stream	30-03-2021	3	3-4 mm, circular, entire, smooth, flat, yellow	In process
			4	1-2 mm, circular, entire, smooth, flat, yellow	In process

Sample ID	Sampling site (location)	Time of sampling	Designation of strain	Colony morphology	Species (16S rDNA)
CW-FLOR	Rákös stream	30-03-2021	5	2-3 mm, circular, entire, smooth, raised, purple	Dyadobacter hamtensis
			6	3-4 mm, irregular, entire, rough, flat, yellow	In process

Sample ID	Sampling site (location)	Time of sampling	Designation of strain	Colony morphology	Species (16S rDNA)
CW-MIX	Rákös stream	30-03-2021	7	1 mm, circular, entire, smooth, flat, yellow	In process
			8	2-3 mm, circular, entire, smooth, flat, yellow	Pseudomonas kitaguniensis

Sample ID	Sampling site (location)	Time of sampling	Designation of strain	Colony morphology	Species (16S rDNA)
SL	Rákos stream	30-03-2021	9	1-2 mm, irregular, undulate, smooth, flat, yellow	In process
			10	1 mm, circular, entire, smooth, flat, yellow	In process

Sample ID	Sampling site (location)	Time of sampling	Designation of strain	Colony morphology	Species (16S rDNA)
SL-COL	Rákos stream	30-03-2021	11	<1 mm, circular, entire, smooth, flat, yellow	In process
			12	1-2 mm, circular, entire, smooth, flat, yellow	In process

Sample ID	Sampling site (location)	Time of sampling	Designation of strain	Colony morphology	Species (16S rDNA)
SL-FLOR	Rákos stream	30-03-2021	13	<1 mm, circular, entire, smooth, flat, yellow	In process
			14	1 mm, circular, entire, smooth, flat, yellow	In process

Sample ID	Sampling site (location)	Time of sampling	Designation of strain	Colony morphology	Species (16S rDNA)
SL-MIX	Rákos stream	30-03-2021	15	2-3 mm, circular, entire, smooth, flat, yellow	In process
			16	1-2 mm, irregular, undulate, smooth, flat, yellow	In process

Supplementary material 3 - Resume

OXITOP MIKROKOZMOSZ MODELL, MINT AZ ANTIBIOTIKUM REZISZTENCIA MIKROBIOÁLIS KÖZÖSSÉGRE GYAKOROLT HATÁSÁNAK VIZSGÁLATÁRA ALKALMAS MÓDSZER

OxiTop microcosm model as a possible tool to study the effects of antibiotic exposure on the microbial community.

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A pályamunka nyelve: angol.

Az antibiotikum rezisztencia kialakulása és terjedése a Világegészségügyi Szervezet (WHO) álláspontja szerint napjaink egyik legnagyobb környezetegészségügyi kihívása. Az antibiotikum rezisztencia számos biotikus és abiotikus tényező hatására kialakulhat, melyek feltárására számos tudományos kutatás zajlik. Munkám során célul tűztem ki annak vizsgálatát, hogy hogyan befolyásolja antibiotikumokkal végzett kezelés egy adott felszíni víztest szennyvízterhelés alatt álló, vagy szennyvízterhelés nélküli mikrobaközösségét. Hipotézisem alapján a szennyvízterhelés alatt álló mikrobaközösség tagjai jobban tolerálják az antibiotikumok jelentette szelekciós nyomást. A vizsgálatok végrehajtásához egy kiválasztott mintavételi pont szennyvízzel terhelt és háttér mintáinak felhasználásával OxiTop mikrokozmosz vizsgálati rendszerben követtem nyomon a mikrobaközösség változását három különböző antibiotikum kezelés (colistin, florfenicol, valamint colistin/imipenem/ceftazidime) hatására. A 7 napos vizsgálat során rögzítésre kerültek a mikrobaközösség biológiai oxigénigényében bekövetkező változások, a kiindulási és végpontban tenyészthető mikrobaszám, valamint összehasonlítottam a kezelt és kezeletlen minták mikrobaközösségét újgenerációs szekvenálási módszerrel (amplikonszekvenálás). Emellett kísérletet tettem a tenyészthető mikrobaközösség reprezentatív tagjainak kimutatására és faj szintű azonosítására. Eredményeim alapján a 300 µg/L koncentrációban végzett antibiotikum kezelés egyik minta esetében sem okozott a sejtszám, vagy biológiai aktivitás tekintetében csökkenést, ugyanakkor az antibiotikum kezelés hatására (különösen a kombinációban alkalmazott készítményeknél) jelentősen csökkent a vizsgált minták biológiai diverzitása. A szennyvízbevezetés hatása alatt álló mikrobaközösség mind sejtszám, mind biológiai aktivitás tekintetében jobban tolerálta az antibiotikumokkal végzett kezelést, mint a háttér minta közössége. Munkánk során több olyan, antibiotikum rezisztencia szempontjából eddig kevésbé vizsgált, bár potenciálisan jelentős nemzetség dominanciáját igazoltam a kezelésekre hatására, mint a *Cellvibrio*, *Flavobacterium* és a *Perlucidibaca*. A kísérlet további eredménye, hogy sikeresen adaptáltuk és optimalizáltuk az OxiTop vizsgálati rendszert antibiotikum-kezelések végrehajtásához, mely a jövőben hasznos eszköz lehet a vizsgálatok folytatásához.

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