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**ANTIBIOTIC RESISTANCE AND BIOFILM FORMING
ABILITY OF *PSEUDOMONAS AERUGINOSA* STRAINS
ORIGINATING FROM WASTEWATER TREATMENT
PLANTS**

**Szennyvíztisztító telepekről származó *Pseudomonas aeruginosa* törzsek
antibiotikum rezisztenciájának és biofilmképző képességének vizsgálata**

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Table of Contents

Introduction.....	2
1. Literature review	3
1.1. Water in general.....	3
1.2. Contamination of water.....	3
1.3. Types and characteristics of Wastewater.....	4
1.4. The origin of wastewater	4
1.5. Physical and chemical characteristics of sewage	5
1.6. Biological characteristics	7
1.6.1. The impact of bacterial pathogens on human health.....	7
1.7. Wastewater Treatment processes.....	7
1.7.1. Plastic to microplastic	9
1.7.1.1. Health impacts of MPs	13
1.7.2. Microplastics in WWTPs.....	14
1.7.3. Biofilm formation	15
1.7.4. Antimicrobial Resistance.....	16
1.7.5. <i>Pseudomonas aeruginosa</i>	18
2. Materials and methods	21
2.1. Sampling	21
2.2. Laboratory analysis	22
2.2.1. Isolation and identification of <i>P. aeruginosa</i>	22
2.2.2. DNA isolation.....	23
2.2.3. PA SS PCR: species level identification of <i>P. aeruginosa</i>	24
2.2.4. Gel Electrophoresis.....	25
2.2.5. Antibiotic resistance testing	25
2.2.6. Determination of biofilm forming ability	26
2.2.7. Microtiter plate test	27
2.2.8. Bradford and biofilm viability assays	27
3. Results and Discussion.....	29
3.1.1. Presence and frequency of <i>P. aeruginosa</i> in WWTPs and the affected surface waters ..	29
3.1.2. Antibiotic susceptibility test	30
1.1.1. Biofilm assay on modified microtiter test	31
1.2. Conclusions.....	37
Acknowledgement	38
Summary.....	39
References.....	40
Supplementary Table 1.....	45

Introduction

Since the beginning of the industrial revolution, the generation of waste (as a result of human activities) has been continuously speeding up including solid and liquid fractions, such as wastewater. Wastewater that is usually discharged into surface waters with or without treatment alters the ecological performance and biological diversity of the ecosystem. A major international concern is the environmental impact of contaminants originating from human activity on aquatic ecosystems and their associated long-term effects; thus, wastewater treatment and water quality monitoring are vital issues.

Water pollution occurs when substances that will modify the water in a negative way are discharged. These substances may have human health concerns, endanger animals and their habitat and the environment as a whole.

Wastewater is treated to prevent pollution, therefore to protect the environment, and public health by safeguarding water supplies from waterborne diseases. Safe disposal of human excreta is a prerequisite for the supply of safe drinking water as there are numerous infectious diseases transmitted by excreta. Proper wastewater treatment and disinfection of water supplies can eliminate these waterborne diseases. At the same time, recently a new transmitting factor was identified in wastewater that may play a role in the spread of infectious microorganisms – the microplastics (MPs).

Microplastics are commonly found in wastewater and as a consequence of their low degradation rate, they persist in the environment for many years and interact with chemical compounds and microorganisms. Consequently, they present a new ecological niche for bacteria and eukaryotic colonization, dispersal and biofilm formation after exposure to the environment. MPs also serve as vectors for transportation of antibiotic resistance genes (ARGs) leading to potential human health consequences, since the accumulation and spread of ARGs in bacteria can cause severe issues to the successful prevention and treatment of persistent diseases (Liu et al., 2021). As MPs have been found in all stages of the wastewater treatment process notable attention has been paid to characterize the microbial community of MPs originating from wastewater treatment plants (WWTPs). This thesis work aimed to analyse the potential role of a widely known opportunistic pathogen bacterial species, *Pseudomonas aeruginosa* in the colonization of microplastics.

1. Literature review

1.1. Water in general

Water, in its simplest definition, is a colourless, transparent, odourless liquid that is essential for all forms of life. It's a chemical compound with the formula H_2O , meaning each molecule consists of one oxygen and two hydrogen atoms. Water is the basis of the fluids of living organisms and is found in seas, lakes, rivers, and rain. It's also used in various processes such as cooking, cleaning, and industrial applications (http1). There are four categories of water namely: rainwater, domestic wastewater, agricultural water and industrial water. Industrial water can be subdivided into cooling water, washing effluent and manufacturing or process water; generally, process water poses a greater threat (Crini & Lichtfouse, 2019).

The Action Plan from the United Nations Water Conference held in Mar del Plata in 1977 declared water as a human right and stated that “All peoples, whatever their stage of development and social and economic conditions, have the right to have access to drinking water in quantities and of a quality equal to their basic needs” (Oliveira, 2017). Despite harmonized efforts, factors like the environmental pollution and the climate change make it increasingly hard to provide water in the required quality and quantity to the growing global population.

1.2. Contamination of water

There are multiple sources of water pollution (households, industry, mines and infiltration). As discussed by Ambulkar & Nathanson (2023), pollutants present in the water may originate from point sources or from dispersed sources. A point-source contaminant, like a sewage discharge or outfall pipe, enters the water from a single conduit or channel. Pollutants enter a body of water through dispersed sources. For instance, surface runoff from farms is a scattered source of pollution that enters surrounding waterways with animal waste, fertilizer, pesticides, and sediment. Due to the numerous points at which it enters nearby streams or lakes, urban storm water drainage, which may contain sand and other gritty elements, hydrocarbons, and other chemicals used to melt snow and ice, is also regarded as a distributed source.

Point-source pollutants flow to a single site where treatment procedures may remove them from the water, making them easier to control than dispersed-source contaminants. Pollutants from distant sources, which account for a significant portion of the overall water pollution issue are

typically outside the scope of such regulation. The best way to prevent dispersed-source water pollution is to enforce sensible land-use plans and development requirements.

Pathogenic organisms, oxygen-demanding wastes, plant nutrients, synthetic organic compounds, inorganic chemicals, microplastics, sediments, radioactive materials, oil, and heat are examples of common types of water pollutants. The first three categories' main source is sewage. Some of them also come from farms and industrial settings. Both heat (especially from power plant cooling water) and sediment from degraded topsoil are regarded as pollutants because they can harm aquatic ecosystems and have a negative impact on dissolved oxygen levels and aquatic life in rivers and lakes. Based on the scientific literature and the general experience, non-treated and treated wastewater can be considered as a significant source of various contaminants.

1.3. Types and characteristics of Wastewater

The unavoidable production of liquid waste from human activities mostly ends up as **wastewater** with quality and quantity determined by various factors. Wastewater refers to water that has been utilized in different applications or processes, such as freshwater, raw water, potable water, or saline water. It contains materials like human waste, food remnants, oils, soaps, and chemicals. Sources of wastewater include residential homes, commercial establishments, and industrial facilities (http2). Wastewater can be classified based on several characteristics, such as origin, composition and its hazard.

1.4. The origin of wastewater

According to its **source**, wastewater can be classified into three categories: domestic or municipal sewage, industrial sewage and storm sewage.

Domestic sewage carries used water from households, and it is also called sanitary sewage; it is 99.9% water by weight. The other 0.1% contains a variety of dissolved and suspended impurities. Although these compounds are only present in a smaller fraction, the nature and large volumes of sewage in which they are present make the disposal of domestic wastewater a significant technical and environmental issue. The impurities are usually organic materials and nutrients but can also contain disease causing microbes and various chemicals (pharmaceutical residues, household chemicals). The amount and type of wastewater produced by households (municipal wastewater) is influenced by the behaviour, lifestyle and standard of living of the

residents and the technical and juridical framework of a given area/country. The majority of municipal waste from households will end up as solid and liquid waste fractions and the same considerations apply for the industry. The wastewater composition is significantly affected by the design of the sewer system; most developing countries use a separate sewer system: the rainwater and stormwater are transported in trenches, canals or pipes. Old urban areas have combined sewer system, where different types of wastewaters are mixed (Henze & Comeau, 2008).

Industrial sewage is used water from manufacturing or chemical processes. Industrial wastewater mostly contains specific and easily identifiable chemical compounds, depending on the nature of the industrial process.

Storm sewage is runoff from precipitation that enters the sewer system; it carries organic materials, suspended and dissolved solids, and other substances picked up along the way (Ambulkar & Nathanson, 2023).

1.5. Physical and chemical characteristics of sewage

Physical characteristics are applicable to determine the source, age and composition of sewage, therefore these parameters are useful to plan wastewater treatment. **Turbidity**: the measure of the extent to which suspended matter in water absorbs or scatters radiant light energy that cuts through the suspension. The Jackson turbidimeter is the original measuring device for turbidity. **Colour**: fresh sewage is light brownish grey colour; old sewage is dark grey and black, and it is then called septic. **Odour**: fresh domestic sewage has a slightly soapy or oil odour, septic sewage has a strong odour of hydrogen sulphide. At lower concentrations the odour has no effect, but at higher concentrations it can be an issue for communities living in the vicinity of WWTPs. **Temperature**: normally the average temperature of sewage is in the range of 10-21°C (Jamal, 2008; Sincero & Sincero, 2003).

Chemical characteristics includes any chemicals that can be found in wastewater including several indicator parameters. Chemical composition determines the fate and the effect of wastewater on living organisms. The amount of oxygen needed for the aerobic metabolic process that oxidises the biodegradable organic matter in the water sample is measured by the **biochemical oxygen demand** (BOD). The BOD is a measure of the quantity of oxygen required for oxidation of biodegradable organic matter present in the water (Ramalho, 1977); the greater the BOD, the

more oxygen is needed by microbes to break down the organic materials in sewage. Three types of materials drive wastewater's oxygen demand: (1) carbonaceous organic materials that aerobic organisms can use as a food source; and (2) oxidizable nitrogen that comes from nitrite, ammonia, and (3) organic nitrogen compounds that certain bacteria (like *Nitrosomonas* and *Nitrobacter*) can consume (Ramalho, 1977). It is among the most crucial factors in sewage treatment plant design and operation. BOD levels in industrial sewage can be many times higher than in home sewage. When household sewage and storm sewage are combined in sewerage systems, the BOD of the sewage is of special importance (Ambulkar & Nathanson, 2023).

The quantity of oxygen (mg/l) that is chemically rather than biologically utilized during the oxidation of organic and oxidizable inorganic elements in water is known as **chemical oxygen demand**, or COD. Typically, COD is higher than BOD in water (Kiefer, 2009).

Organic parameters: Organic matter is the main pollutant in wastewater, it is typically measured by the previously mentioned BOD and chemical oxygen demand (COD). COD analysis measures through chemical oxidation by dichromate most of the organic matter present in the sample, these measurements are needed for mass balances in WWT (Henze & Comeau, 2008). Other chemical oxidant used is potassium permanganate, it is a very incomplete oxidation of organic matter, and the analysis is usually only used to estimate the necessary dilutions in the BOD analysis. Dichromate gives a more thorough oxidation including oxidation of inorganic materials. Protein materials make up a large part of the wastewater biosolids particles; proteins and urea are the main sources of nitrogen in wastewater. When proteins are present in large amounts, microorganisms decompose and produce foul odours. Oils and grease are also a major component, they result from wet weather runoff from highways or the improper disposal of motor oil, they are insoluble in water but dissolve in organic substances (e.g., petroleum).

Inorganic parameters: inorganic constituents are usually present in wastewater because of human activities and those include pH, chlorides, alkalinity, nitrogen, phosphorous, sulphur, toxic inorganic compounds, and heavy metals. A lot of vital properties of wastewater are due to the presence of weak acids and bases and their salts (Spellman, 2009).

1.6. Biological characteristics

The presence or absence of microorganisms (bacteria, fungi and viruses) in wastewater is of primary importance. A part of these organisms plays a significant role in the degradation of contaminants and the transformation of nutrients that are present in the wastewater. At the same time, many pathogenic organisms are originating from human excreta that are potentially harmful to the ecosystem or to human health. Pathogens are organisms that are capable of infecting susceptible hosts (humans, animals, or plants). Many of them can be waterborne or can be linked to faecal contamination and wastewater inlet. The major groups of the known pathogen germs are belonging to bacteria, viruses, protozoa, and parasitic worms (Spellman, 2009).

1.6.1. The impact of bacterial pathogens on human health.

Significant waterborne bacterial pathogens that can infect warm-blooded animals and humans' gastrointestinal tracts are released into the environment along with the excreta. All of these infections are primarily responsible for gastrointestinal (diarrhoea) disorders and are spread through the use of faeces-contaminated water. They might all have the capacity to integrate into the microbial communities seen in biofilms (Wingender, 2011).

Obligate pathogens typically originate from faeces and can infect individuals and cause illness, irrespective of their health status. At the same time, **opportunistic pathogens** infect vulnerable groups such as the elderly, youth, immunocompromised individuals, patients with pre-existing medical conditions, and others predisposed to infection (Wingender & Flemming, 2011). Opportunistic pathogens often originate as natural aquatic organisms, adapting to the oligotrophic conditions that are characteristic of numerous drinking water systems (Cloete et al., 2004).

To control and minimize the adverse effects of wastewater on the environment, several treatment options are available.

1.7. Wastewater Treatment processes

According to Sonune and Ghate (2004), wastewater treatment (WWT) consists of a combination of physical, chemical and biological processes and operations to remove solids, organic matter and sometimes nutrients from wastewater. There are different stages of treatment namely: preliminary treatment, primary, secondary and tertiary and/or advanced WWT. The schematic figure of wastewater treatment is summarized in Figure 1 (Birch et al., 2020).

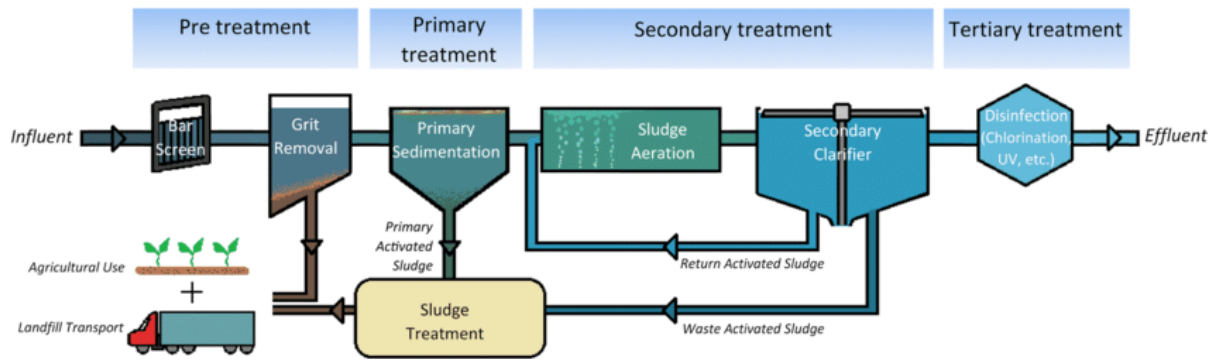


Figure 1: Figure Illustration of the treatment stages of a wastewater treatment plant (Birch et al., 2020).

The preliminary treatment is where the removal of coarse solids and other large materials often found in raw wastewater occurs; it helps to remove or to reduce in size the large, suspended or floating solids. These can be pieces of wood, cloth, paper, plastics, garbage, etc., along with faecal matter. The heavy inorganic solids are removed, for example sand and gravel as well as metal and glass; these objects are called grit and excessive amounts of oils and greases.

Primary treatment is where the removal of organic and inorganic solids by the physical processes of sedimentation and floatation occurs. About 25-50% of the incoming biochemical oxygen demand (BOD), 50-70% of the total suspended solids (SS), and 65% of the oil and grease removed during primary treatment. Some organic nitrogen, organic phosphorus, and heavy metals associated with solids are also removed, however, colloidal and dissolved constituents are not affected.

Secondary treatment is for further treatment of the effluent from primary treatment to remove residual organics and suspended solids. With regards to the size of the solids, the distribution is approximately 30% suspended, 6% colloidal and about 65% dissolved solids (Kalaichelvan & Pandi, 2019). The function of the primary treatment is to remove most of the suspended solids from the wastewater by utilizing clarifiers or settling tanks. Therefore, the effluent from primary treatment contains mainly colloidal and dissolved organic solids. The secondary treatment process involves the biological treatment of wastewater by using various types of microorganisms in a controlled environment; several aerobic biological processes are used. Secondary treatment differs from primary treatment in the way oxygen is supplied to the microorganisms and in the rate at which organisms metabolize the organic matter.

Tertiary treatment is where the residual suspended solids are removed, usually by granular medium filtration or microscreens. Disinfection and nutrient removal also occur in this phase. Advanced treatment involves the removal of dissolved and suspended materials that are remaining after biological treatment when required for different water reuse applications.

Wastewater disinfection is usually accomplished using chemicals, the purpose is to selectively destroy disease-causing organisms. Historically chlorine has been the preferred disinfectant for wastewater, changes in regulations and development of new technologies has affected the use of chlorine. Other chemicals usually used in disinfection include ozone, bromide, iodine, and hydrogen peroxide. There are requirements for low or nondetectable amounts of chlorine residual in treated effluents, alternative disinfection system such as ultraviolet (UV) radiation is used in some cases. There are many factors to consider when choosing the type of chemical to be used for disinfection, such as contact time, intensity and nature of the physical agent, temperature, and type and number of organisms (Tchobanoglous et al., 2003).

The above-mentioned wastewater treatment method can sufficiently decrease the microbial and chemical risk of municipal sewage before its release into the environment. However, there are emerging chemical (e.g., pharmaceutical) and physical contaminants, such as microplastics of which removal means a challenge for the traditional WWTPs.

1.7.1. Plastic to microplastic

Plastics are man-made synthetic/polymers, they are long-chain polymeric materials mainly made from fossil raw materials (Millet et al., 2019); a polymer is a large molecule made up of several identical or similar subunits joined by bonds. Polymers that are found in nature include muscle fibre, DNA, cellulose, silk, and rubber (Geyer, 2020). Plastics are usually created from synthetic polymers by adding different chemical additives to the polymerization of monomers obtained from oil or gas. Currently, there are about 20 distinct categories of plastics, each having a wide range of grades and types (APME, 2006). The common types of plastics are summarized in Figure 2.



Figure 2: Common types of plastics (source: Biorender.com)

In SUP (single use plastic) products, plasticizers like phthalates or phthalic acid esters (henceforth, referred to as "PAEs") are frequently used to increase the plasticity of resin molecules, making the plastic more flexible and easier to process. These plasticizers have been found in food containers, plastic bags, and tableware (Chen et al., 2013; Huo et al., 2017).

Plastic-encased PAEs have the potential to leak into packaged foods or the environment, where they could subsequently find their way into the public food chain (Ustun et al., 2015).

The usual methods for handling plastic waste are landfilling, incineration, recycling, and reuse. The latter three are the most prevalent, with the highest percentage coming from landfilling. According to reports, just 9% of plastic waste was globally recycled and 12% was burned in 2015; the remaining 79% of plastic debris was either landfilled, discarded, or ended up as litter in the environment (Geyer et al., 2017). Only 14% of packaging waste is recycled, according to 2016 World Economic Forum research (<http3>), the remaining 80% is disposed of or discarded as litter, with 40% being landfilled, 14% being burned, and 32% being discharged into the environment.

There are several disposal methods for plastic waste; landfilling has historically been one of the most popular ways to get rid of used plastic, and it still is in many parts of the world (Singh and Sharma, 2016). However, this method wastes finite resources and takes up sometimes valuable and scarce land (Breyer et al., 2017; Canopoli et al., 2020). Another method of waste management

is incineration, which involves burning waste items, including organic materials. Waste products are converted into heat, flue gas, and ash through combustion. Moreover, incineration reduces waste volume by 90–99% (Thanh et al., 2011). However, expensive treatment equipment is needed due to the potential release of toxic chemicals during incineration, such as volatile organic compounds and dioxins. As a result, it is not practical to implement environmental controls on incineration on a large scale, especially in developing nations (Chen et al, 2021).

Waste may be recycled to create valuable new items. Recycling is a globally recognized component of the circular economy concept. However, there are still obstacles to successful recycling, such as the complexity of managing waste streams (USEPA, 2017).

Plastic wastes undergo thermal cracking, which completely breaks down the plastic polymers into a low molar mass state and produces very valuable products. For various types of polymers, the mechanism and results of heat cracking might differ significantly (Kasar and Ahmaruzzaman, 2018).

Given the high carbon content of plastics, carbonisation is becoming a popular method for recycling discarded plastics. This is a heat treatment procedure that turns polymer precursors into carbon materials at high temperatures, such as graphite carbon (carbon nanofibers, graphene, etc.) and amorphous carbon (porous carbon, activated carbon, etc.). The more widely used definition of carbonisation is a two-step process that consists of pyrolysis and carbonisation (Inagaki et al., 2010). Carbonisation offers significant advantages over alternative plastic waste disposal techniques in terms of resource recovery and environmental preservation. Not many intricate pretreatments are needed, and it works with most plastics (Chen et al., 2020).

Due to the long degradation of plastics, these materials can be highly polluting in land, water, and the environment. While certain plastics degrade quickly, others take years or even decades to do so (Derraik, 2002; Ryan & Moloney, 1993). Some plastic sinks to the bottom and remains as litter in the littoral zone; due to their inability to degrade, most plastics last for many years (Browne et al, 2007). Degradable plastic can also endure for a considerable amount of time, depending on several environmental elements such as temperature, duration, and rate of degradation (Barnes et al, 2009).

Plastic deterioration (Figure 3.) has been categorised into photo-oxidative, thermal, ozone, catalytic, mechano-chemical, and biodegradative processes, depending on the physical, chemical, or biological agents responsible (Grassie & Scott, 1985). Plastic breaks down through a process called photooxidative deterioration when exposed to light. UV and visible light both start the deterioration process. Light-induced polymer decomposition produces propyl end groups, esters, and aldehydes (Nagai et al., 2005); Plastic is altered optically, physically, and mechanically by photo degradation, leading to changes in its molecular weight, extensibility, and lifespan (Torikai, 2000).

Both light and thermal deterioration belong to the same category – oxidative degradation. They are nearly identical. Thermal degradation can happen anywhere, but photo degradation starts at the exposed surface (Tyler, 2004). Thermal light and UV cause chain deterioration, which leads to thermal degradation. The parameters influencing thermal deterioration are reactor geometry, reaction media, heating rate, and pressure (Manzoor et al, 2022). Ozone in the air causes polymeric aging, which is the first sign of ozone-induced degradation (Kelefi et al., 1971). The polymer produces a variety of carbonyl compounds based on ketones, lactones, and aliphatic esters when exposed to ozone; over time, this mechanism is followed by the creation of ether, vinyl, and hydroxyl groups. All these products are produced consistently and quickly (Allen et al., 2003).

Mechanochemical polymer degradation is the process by which plastic degrades in response to mechanical stress and intense ultrasonic radiation. Mechano-chemical degradation is the process by which the role of mechanical force causes molecular chains to break and a chemical reaction to occur (Li et al., 2005). Waste polymers (PS, PP, and PE) undergo catalytic degradation, which results in the production of hydrocarbons like polyolefins, which are then catalytically broken down into oils and gases. Catalysts, such as salts, are used to reduce the temperature of decomposition and increase the grade of product generated (Gimouhopoulos et al., 2000). By either decreasing the molecular weight of the polymer or increasing its surface area for the growth of microbial colonies, biodegradation breaks down the polymers. Microorganisms that colonise plastic are the attacking agents in biodegradation, but it is the chemicals (enzymes) that are released from these organisms that break down the polymer (Palmisano & Pettigrew, 1992).

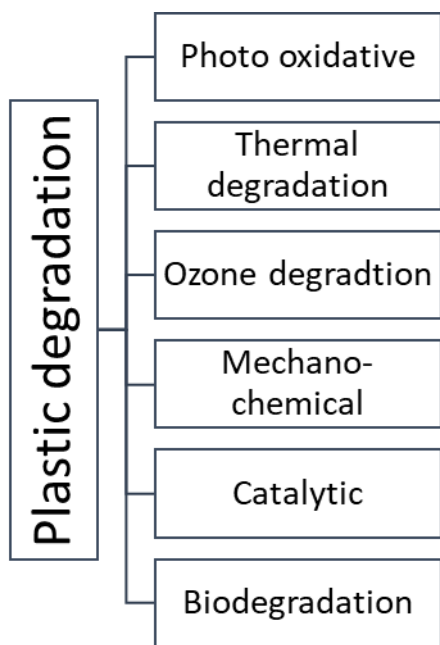


Figure 3: The different processes of plastic degradation. (source: L. Mothoa)

Microplastics (MPs) are plastic fragments or particles that are less than 5 mm in diameter. MPs can be divided into primary MPs which are directly manufactured in small size, and secondary MPs which derive from the breakdown of larger pieces as a result of physical and/ or chemical damage (Hernandez et al., 2019; Galafassi et al., 2021). Shape is an important indicator used for MPs classification, MPs can be categorized into fibre (significantly longer and wider) and fragments/particles (similar width and length). Fibres account for the highest proportion of the observed MPs in wastewater with an average of 52.7%, irregular fragments accounts for an average of 28.8% (Sun et al., 2019). The size of MPs cannot be accurately classified due to the irregularities in shape; the most frequently used size

classification is the dimensions of 25µm, 100µm and 500µm.

Personal care products contain primary MPs, which are processed plastic particles, they are usually in a form of microbeads and can be found in exfoliants in cosmetics, detergents, toothpastes, scrub facial cleansers, and drug carriers (Auta et al., 2017). Secondary MPs result from the degradation of plastic material in the environment (Avio et al., 2017).

1.7.1.1. Health impacts of MPs

MPs are present in various environments; in marine animals especially bivalves (filter feeders), research indicates that they do ingest MPs and they accumulate in their gut then absorbed into their circulatory system (Bouwmeester et al., 2015). It has been determined that the various concentrations of MPs in terrestrial aquatic ecosystems have different degrees of influences on the development, reproduction, behaviour, growth and mortality of aquatic animals (Chae & An, 2017). A study conducted by Deng and his colleagues in 2016, shows that mice exposed to 5 and 20µm PS MPs for a period of 28 days displayed the presence of MPs in the kidneys, liver, and gut; the results related to energy and lipid metabolism indicate the possible detrimental effects after exposure.

In mice, MPs can induce inflammation of the liver and problems with lipid metabolism. Two more studies have demonstrated that mice exposed to PS MPs saw significant alterations in the richness and diversity of their intestinal biota, as well as a decrease in intestinal mucus (Jin et al., 2019; & Lu et al., 2018). According to recent research by Deng et al. (2018), dangerous substances may be adsorbed onto metal nanoparticles (MNPs), where their concentration may be orders of magnitude higher than that of contaminants found in the surrounding environment. Furthermore, recent research raises the likelihood that the compounds that have been adsorbed are more dangerous than the pure substances alone.

Hydrophobic organic chemicals (HOCs) are found in the environment in a variety of forms, and several of them are known to be endocrine-disrupting chemicals (EDCs), including phthalates, BPA (bisphenol-A), PCBs (polychlorinated biphenyls), and PFCs (perfluorinated compounds); it has been demonstrated that HOCs adhere to MPs (Karwacka et al, 2019). Leaching of original plastic additives, such BPA, phthalates, etc., which have been shown to have endocrine-disrupting effects and other toxicities is another important factor to take into account (Gore et al., 2015).

MPs interact with organic materials, inorganic particles, and microbes in aquatic settings (Parrish & Fahrenfeld, 2019). Because of this, a wide variety of microorganisms, such as viruses, bacteria, fungus, algae, and protozoa, can adhere to the surfaces of MPs (Oberbeckmann et al., 2015). These microorganisms have the ability to colonize their surface and produce biofilms, which are intricate ecosystems mostly made up of microbes, cell secretions, and organic and inorganic particles (Flemming, 1997).

1.7.2. Microplastics in WWTPs

Microplastics (MP) were detected in both influent and effluent of WWTPs all around the world; the influent generally contains a higher concentration of MPs. A large part of MPs originates from household discharges. Because of inadequate retention during WWT (Sun et al., 2019), the MPs often get encapsulated in the sewage sludge which is commonly used as organic fertilizer. Over 30 types of MP polymers have been detected in both influent and effluent of WWTPs, the most common are polyester (PES ~28-89%), polyethylene (PE ~4-51%), polyethylene terephthalate (~4-35%) and polyamide (PA ~3-30%). PES, PET and PA are all commonly used in synthetic clothes, while PE is used in personal care products including body and facial scrubs as well as food packaging films and water bottles.

About 80% of MPs concentrations in WWTPs are represented by thermoplastics (PE and PP) and polyesters predominantly with different shapes (fragments and fibres). Due to their small size and low density, MPs from wastewater often seep into treated water. MPs found in wastewater are known to interact with heavy metals, organic pollutants and antibiotics available in wastewater, absorbing these contaminants and therefore, augmenting the ecological risk (Kaur *et al.* 2022).

One of the main concerns of MPs pollution is the MP associated microorganisms (Perveen *et al.*, 2023), MPs can also harbour pathogenic bacteria, antibiotic resistant bacteria, antibiotic resistance genes (ARGs), and metal resistance genes (MRGs) (Galafassi *et al.*, 2021). Pathogenic microbes tend to specialize in opportunistic and surface-associated strategies to exploit their host, heterotrophic bacteria colonizing plastic surfaces survive significantly longer than those in the surrounding seawater; biofilms consisting of complex microbial communities embedded in self-produced extracellular matrix are known to form on MPs in aquatic environments (Lai *et al.*, 2022).

1.7.3. Biofilm formation

The initial attachment of bacteria to a surface or to one another, the formation of microcolonies, the maturation of the biofilm, and the dispersal of the biofilm are thought to constitute the stages of the biofilm developmental cycle (Figure 4.). Planktonic bacteria lack a distinct biofilm biology, as evidenced by the phenotypic responses and bacterial physiology observed in the various biofilm stages (Sauer *et al.*, 2002).

The transformation of the bacteria to produce extracellular matrix components and adhesins, which link them in the biofilm, is necessary for the transition from the solitary planktonic bacterial lifestyle to the community biofilm lifestyle (Whitchurch *et al.*, 2002). The extracellular biofilm matrix acts as a scaffold, contributing to a variety of activities such as cell attachment, cell-to-cell contacts, and antimicrobial tolerance. It also has a crucial structural and cell-to-cell linking role in biofilms, the primary components of the biofilm matrix that the bacteria make include proteins, polysaccharides, and extracellular DNA (Yang *et al.*, 2009).

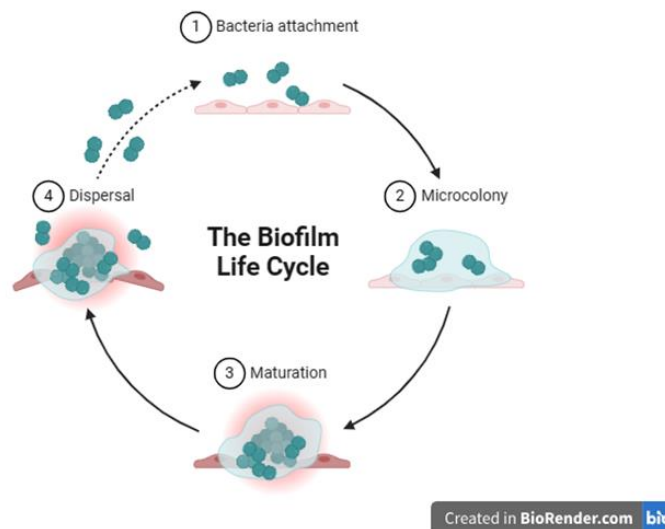


Figure 4. The life cycle of biofilm formation of bacteria. (Source: BioRender.com)

On one hand, microorganisms living in biofilm formations are the key element of MPs degradation. On the other hand, biofilms may compose by human pathogenic bacteria and often considered as hot-spots of antimicrobial resistance.

1.7.4. Antimicrobial Resistance

Bacteria possess an extraordinary genetic adaptability that enables them to counter a broad spectrum of environmental hazards, including the threat of antibiotics that could threaten their survival. Those that inhabit the same ecological niches as antimicrobial-producing organisms have developed ancient survival mechanisms to neutralize the effects of these harmful molecules, allowing them to flourish despite their presence. When bacteria are exposed to antibiotic medications, the same natural phenomenon, known as antibiotic resistance takes place. Antibiotics exert selection pressure on susceptible bacteria, killing or inhibiting them, while bacteria that are intrinsically resistant to the drugs or that have developed resistance to the drugs are more likely to survive and proliferate. Antibiotic resistance is exacerbated not just by misuse of antibiotics but also by their inappropriate use (bad decisions, insufficient dosage, and noncompliance with treatment protocols) (Prestinaci et al., 2015).

Among other mechanisms, evolutionarily, bacteria employ two principal genetic tactics to overcome antibiotic challenges: i) mutations in genes typically related to the antibiotic's mode of action, and ii) the acquisition of external DNA that encodes resistance factors via horizontal gene

transfer (HGT) (Munita & Arias, 2016). The known mechanisms of antimicrobial resistance are summarized in Figure 5.

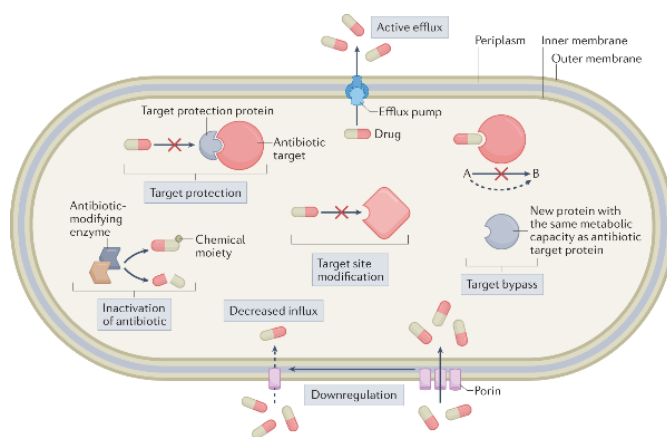


Figure 5: Mechanism of antibiotic resistance. (source: Darby et al., 2023)

According to the US Centre for Disease Control and Prevention (CDC) ([http4](http://www.cdc.gov)), at least 23,000 Americans die due to infections that have become resistant to antibiotics each year, impacting over two million individuals annually.

Numerous studies have shown that certain classes of well-characterized resistance-driving compounds or drivers, such as (i) antimicrobials, such as antibacterial, antifungal, antiviral, and antiparasitic drugs; (ii) heavy metals; and (iii) biocides, primarily surfactants and disinfectants, are responsible for AMR. It has also been observed that resistance to xenobiotics (hexane, toluene, and octanol) and natural chemicals (produced from plants) selects for resistance genes. The co-selection of resistance genes is documented for hazardous substances like solvents, biocides, heavy metals, and antibiotics. There are two distinct mechanisms of co-selection: (i) co-resistance, where the selection of one gene facilitates the selection of another gene that typically does not confer a selective advantage for the compound in question; and (ii) cross-resistance, where a single resistance gene provides protection against various toxic chemicals. The primary transmission routes for these environmental drivers are: (i) industrial and municipal wastewater, (ii) sewage sludge and the application of animal manure, and (iii) aquaculture systems (Samreen et al., 2021).

Antimicrobial resistance is especially a concern, if it interferes with the activity of critically important antibiotics. Therefore, the WHO Expert Committee on Selection and Use of Essential Medicines created the AWaRe Classification of Antibiotics (Figure 6.) in 2017 as a tool to support

local, national, and international efforts to promote antibiotic stewardship (http5). Antibiotics are divided into three groups: Access, Watch, and Reserve. This classification emphasises the importance of using antibiotics appropriately by taking into account the impact that different antibiotics and antibiotic classes have on antimicrobial resistance.

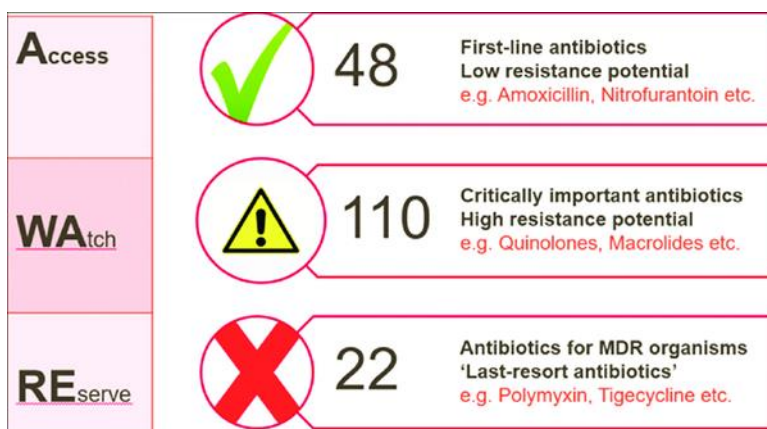


Figure 6: WHO AWaRe Classification of Antibiotics 2019 (Lahiry et al., 2020)

When microorganisms develop mechanisms to simultaneously resist to multiple antibiotic we talk about multidrug resistance (Catalano et al., 2022).

The most common multidrug-resistant bacteria in Europe, *S. aureus*, *Escherichia coli*, *Enterococcus faecium*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, are estimated to cause about 400 000 infections and 25 000 deaths annually, respectively, in 2007 (http6).

1.7.5. *Pseudomonas aeruginosa*

P. aeruginosa is a motile (single polar flagellum), non-spore forming, bacillus rod-shaped Gram-negative aerobic opportunistic pathogenic bacterium that is able to grow in many niches but prefers moist environments. It can be found in low-nutrient or oligotrophic environments and highly nutritious environments, such as sewage and the human body. Multidrug resistant *P. aeruginosa* is thought to emerge from hospitals where large amounts of antibiotics are used, the antibiotic resistance is a result of chromosomal mutations but may also be acquired by horizontal gene transfer. Antibiotic resistant *P. aeruginosa* strains are found in effluent of WWTPs and in the river water downstream of those plants (Slekovec et al., 2012). *P. aeruginosa* exhibits two lifestyle forms: sessile and planktonic. It forms biofilms on surfaces and thus suggests an environment that is resistant to antibiotics and promotes survival in hostile environments (Bruchmann et al., 2013).

P. aeruginosa occurs in both abiotic and biotic environments, from soil and aquatic environments to plant and animal tissues. It can be isolated from a variety of places, such as polluted waters, toilets and sinks, medical equipment like inhalers, dialysis equipment, respirators, and vaporizers, burn wounds, several nosocomial and life-threatening infections in patients with cystic fibrosis (CF), urinary tract infections (UTIs), and pulmonary infections (Botzenhart & Doring, 1993).

The pathogenic microorganism *P. aeruginosa* is a leading contributor to hospital-acquired infections (HAIs), which affect both humans and other animals and plants. In addition to ventilator-associated pneumonia, gastrointestinal infections, dermatitis, UTIs, skin infections like folliculitis and external otitis, bacteraemia, soft tissue infections, respiratory system infections in patients with cystic fibrosis, bone and joint infections, and numerous other infections, it is particularly dangerous for patients with severe burns and for those who are immunocompromised (Azam & Khan, 2019).

P. aeruginosa exhibits various forms of antibiotic resistance. A bacterial species' inherent capacity to reduce an antibiotic's effectiveness through innate structural or functional traits is referred to as **intrinsic** antibiotic resistance (Blair et al., 2015). Due to its reduced outer membrane permeability, capacity to pump antibiotics out of the cell through efflux mechanisms, and synthesis of enzymes such β -lactamases that inactivate antibiotics, *Pseudomonas aeruginosa* has been demonstrated to possess a high level of intrinsic resistance to most antibiotics (Breidenstein et al., 2011).

Restricted outer-membrane permeability, efflux systems that eliminate antibiotics from the cell, and the synthesis of enzymes that inactivate antibiotics are some of the mechanisms of intrinsic antibiotic resistance that *P. aeruginosa* possesses. It was stated that bacteria can obtain resistance genes via horizontal gene transfer (the primary mechanisms of horizontal gene transfer include transformation, transduction, and conjugation) or mutations. Besides the inherent antibiotic resistance of *P. aeruginosa*, **acquired** resistance significantly contributes to the development of multidrug-resistant strains, complicating the pathogen's eradication and leading to a rise in chronic infection cases.

The pattern of biofilm growth in *P. aeruginosa* can be influenced by a number of environmental (Klausen et al, 2003; Singh et al, 2002); the nutritional environment is one of the

important factors. *P. aeruginosa* has been demonstrated to create biofilms that vary from flat and homogenous to highly structured and characterized by widespread aggregates, depending upon the carbon source (Banin et al, 2005). Additionally, micronutrients have a significant impact on biofilm formation patterns. Phosphorus, nitrogen, and iron can all have a significant impact on the biofilm structure of *pseudomonas* sp. (Shrout et al, 2006).

In accordance with the One Health approach (McEwen & Collignon, 2018) and based on the overview of the scientific literature we can conclude wastewater streams may play a significant role in the spread of pathogen and/or antibiotic resistant microorganisms, pharmaceutical residues and microplastics. However, we still don't have detailed information about the role of different types of polymers in the spread of critically important, biofilm forming and antibiotic resistant microorganisms such as *P. aeruginosa*. Therefore, the aim of this research was to:

- Identify and isolate *P. aeruginosa* from surface water and WWTP.
- Determine the antibiotic resistance.
- Determine the biofilm forming ability of *P. aeruginosa* strains from different phases of the WWT.
- Determine which polymer is a preferred surface for *P. aeruginosa* to attach and multiply.

2. Materials and methods

2.1. Sampling

The samples were collected from the following matrix type: influent (untreated) wastewater, treated wastewater (TWW), and from the recipient waterbody upstream, downstream. Simultaneously, six different types of polymers were incubated in the examined WWTPs in plastic colonizers, of which we tried to isolate bacterial strains as well. The samples were collected in nine different cities within Hungary: Table 1. and Figure 7. below indicates the sampling sites and dates.

Table 1. Sampling sites and sampling dates of WWTPs and surface waters receiving treated wastewater in Hungary		
Sampling site	Recipient water body (River)	Sampling date(s)
1. Nagykanizsa	Principális-csatorna	4/12/2023; 6/21/2023
2. Veszprem	Veszprémi-Séd	3/22/2023; 6/29/2023
3. Zalaegerszeg	Zala	5/17/2023
4. Jaszfenyszaru	Zagyva	3/29/2023; 8/30/2023
5. Budapest	Danube	5/31/2023
6. Szeged	Tisza	6/15/2023
7. Miskolc	Sajó	5/10/2023
8. Kecskemet	Csukás-ér-főcsatorna	4/19/2023
9. Szentendre	Danube	4/5/2023; 7/27/2023

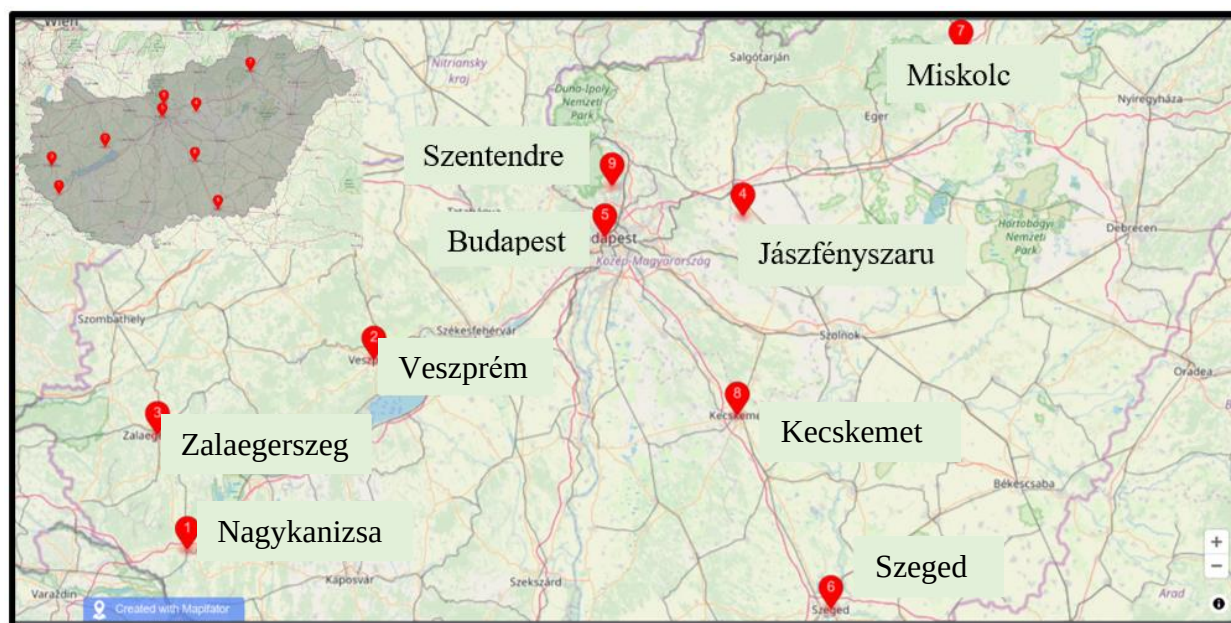


Figure 7: The map above shows the locations where the samples were taken. (Source: Mapify)

The necessary international ISO and Hungarian MSZ standards (MSZ 12750-2:1971), examination of surface waters (Felszíni vizek vizsgálata), MSZ EN ISO 5667-1:2007 sampling surface waters (Mintavétel felszíni vizekből) were followed during the sampling process. Samples were taken aseptically into sterile 1L volume bottles and were transported immediately to the laboratory of the Institute of Aquaculture and Environmental Safety. During transportation and temporary storage, samples were cooled down to 4°C. Samples were processed within 24h.

2.2. Laboratory analysis

The flowchart of the laboratory analysis is summarized in Figure 8. Upon collection of the samples, selective culturing was performed to cultivate the *Pseudomonas* strains, then from the presumably *Pseudomonas* strains DNA was isolated and *P. aeruginosa* identified at species level by a species-specific molecular biology method (PCR). Antibiotic resistance and biofilm forming ability on different polymer types were determined by phenotypic and enzymatic methods.

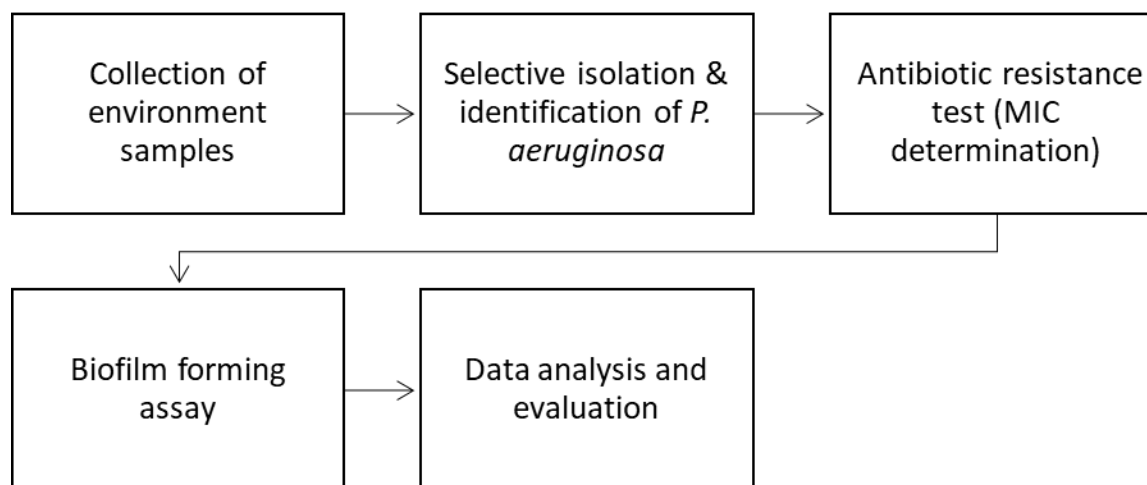


Figure 8: summary of the laboratory steps.

2.2.1. Isolation and identification of *P. aeruginosa*

In order to isolate the strains of *P. aeruginosa*, 1 ml of liquid samples were added to asparagine broth containing a mixture of (g/L): 3 L-asparagine; 1 K₂HPO₄; 0.5 MgSO₄·7H₂O; and 10 mL of glycerol at a pH of 7.0. 48 hours of 42°C incubation in test tubes. The bacterial suspensions were then transferred to cetrimide agar (MERCK 105284). After incubating the plates for a full day at 37°C, the colonies that produced trimethylamine odour and showed signs of

pyocyanin production were further inoculated into acetamide broth that included the following (g/L): pH 6.8, K_2HPO_4 , 2, $MgSO_4 \cdot 7H_2O$, 1, acetamide, 5, and NaCl. Nessler's reagent will confirm that acetamide is decomposing if there is a colour shift from colourless to yellow, brown discolouration, or precipitation (Hungarian Standard MSZ 21470-77:1988) which verifies the presence of *Pseudomonas aeruginosa*. This last step is not mandatory, since the species level identification of *P. aeruginosa* was confirmed with molecular methods as well. Following two to three rounds of solo colony subculturing, the isolated bacterial strains were stored at $-80^{\circ}C$ for future use.

2.2.2. DNA isolation

For DNA extraction, microwave extraction method (Figure 9.) was performed using 1400 μ L overnight bacterial suspension. In the first step, liquid cultures were centrifuged for 2 minutes at 11000 RPM. Then the supernatant was removed and the bacterial pellet was

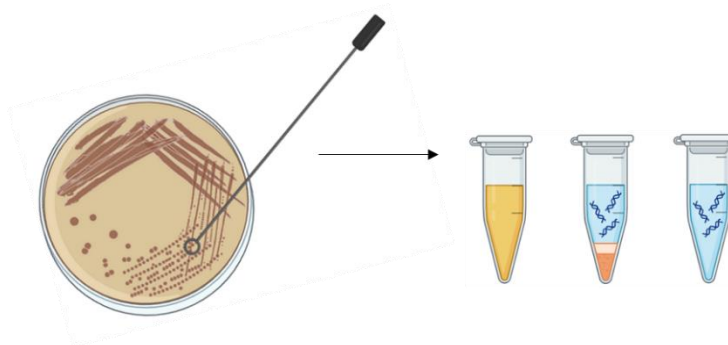


Figure 9: DNA isolation with microwave extraction method.

microwaved for 2 minutes at maximum power. After 2 minutes incubation at room temperature, microwave treatment was repeated for another 1 minute at maximum power. In the last step, bacterial cells were suspended in 50 μ L of MQ water, were vortexed and then centrifuged for 2 minutes at 11 000 RPM. After the centrifugation process, the supernatant (bacterial DNA) was transferred into a new and clean Eppendorf tube. Isolated DNA was stored at $-20^{\circ}C$ until further use.

When microwave extraction failed, the Zymo Quick Miniprep Plus Kit was used for DNA extraction. The reagent was prepared as follows: add 1060 μ L Proteinase K storage buffer to each Proteinase K tube before use. The final concentration is approximately 20mg/ml, and it is stored at $-20^{\circ}C$.

For this DNA isolation protocol fresh liquid culture of bacteria is used; the cells should be pelleted in order to remove the media. This is done by adding 2x700 μ L of liquid culture to a clean Eppendorf tube and centrifuge at a speed of 11000RPM for 2 minutes then remove supernatant. 200 μ L of DNA elution buffer is used to resuspend the cultured pellet, into a microcentrifuge tube

add 200 μ L of the sample, 200 μ L Biofluid and cell buffer, and 20 μ L Proteinase K. Vortex for 10-15 seconds and then incubate the tube for 10 minutes at 55°C, 1 volume of Genomic Binding Buffer is added to the digested sample and vortex for 10-15 seconds. The mixture is then transferred to a Zymo-spin column in a collection tube, centrifuge at 12000 RMP for a minute then dispose the collection tube along with the flow through.

The spin column is washed 3 times. Firstly, add 400 μ L of DNA Pre-Wash Buffer to the spin column in a new collection tube, centrifuge at 12000RMP for a minute and then empty the tube. Secondly, add 700 μ L g-DNA wash buffer to the spin column and thirdly, add 200 μ L of g-DNA wash buffer, centrifuge at 12000RMP for a minute in between each wash and also discard the flow through. The spin column is then transferred to a clean microcentrifuge tube, 50 μ L of DNA Elution Buffer is added directly onto the matrix, then 5 minutes incubation at room temperature and centrifuge for a minute at maximum speed to elute DNA.

2.2.3. PA SS PCR: species level identification of *P. aeruginosa*

The identification of the strain is achieved by using PA-SS PCR using specific primers (PA-SS-F (5'-GGGGGATCTTCGGACCTCA-3'); PA-SS-R (5'TCCTTAGAGTGCCCCACCCG-3') targeting the variable regions V2 and V8 of the 16S rDNA with an amplicon size of 956 bp. Using this protocol, sequence analysis is not required, as the amplification of the relied DNA fragment sufficiently verifies the presence of species *P. aeruginosa* (Atzél et al., 2008; Spilker et al., 2004)

Before running a PCR, a master mix (M/M) was prepared. A M/M that was used in this experiment contained the following:

Table 2. Parameters of PA-SS PCR to identify *P. aeruginosa*

Master mix:		Heat profile:
GreenTaq buffer (10x)	5 μ L	95°C, 2 min
PA-SS forward primer (100pM)	0.5 μ L	94°C, 25 sec
PA-SS reverse primer (100pM)	0.5 μ L	58°C, 40 sec
dNTP (4mM)	10.0 μ L	72°C, 40 sec
Template (DNA)	1.0 μ L	72°C, 1 min
Taq polymerase	0.25 μ L	4°C, ∞
MQ water	32.75 μ L	
Final volume	50 μ L	

} 25x

2.2.4. Gel Electrophoresis

This technique is used for the separation and purification of proteins and nucleic acids. The gel contains 0.7g agarose and 70 mL SB buffer (0.4 g NaOH, 3.044 g H₄BO₄, 1000 mL distilled water). To prepare the gel, agarose and SB buffer were mixed into a glass bottle, heated in microwave until agarose fully dissolved, and the gel mixture was poured into the gel tank with the combs placed at one end to create wells where samples (PCR products) were be loaded. For testing, 7 mL of PA-SS PCR product were loaded into each well. Electrophoresis was set to 110 constant V for 40 min. PCR products were checked with UV transillumination (Figure 10).

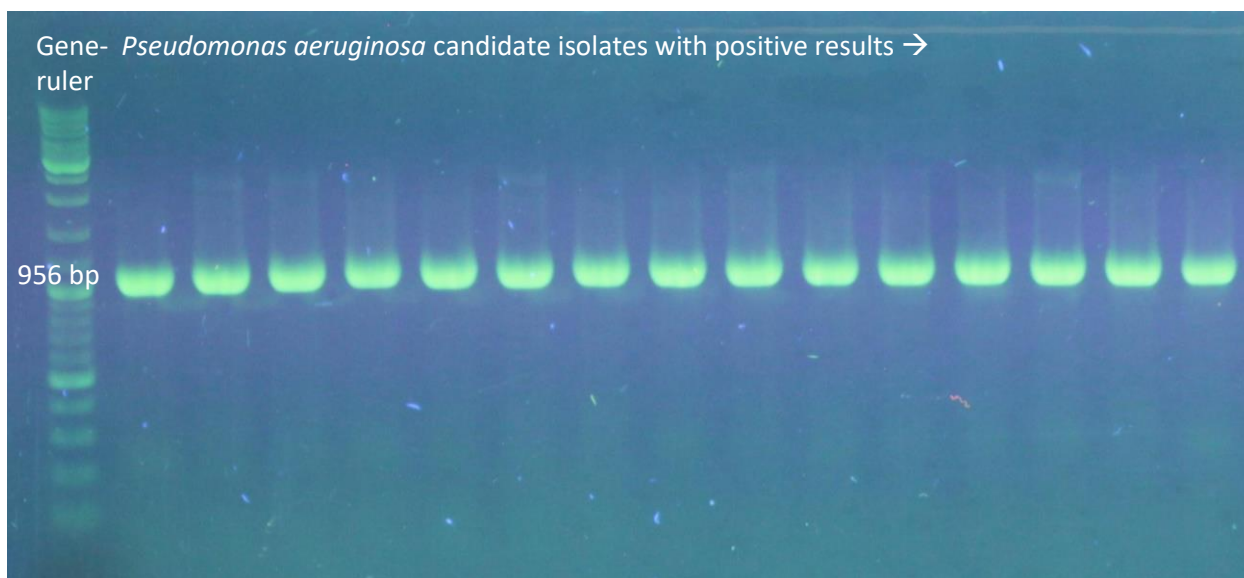


Figure 10: Gel electrophoresis showing PCR products.

2.2.5. Antibiotic resistance testing

After species level identification, verified *P. aeruginosa* strains were selected then cultivated on Mueller-Hinton (MH) agar (MERCK 105435) following the EUCAST (European Committee on Antimicrobial Susceptibility Testing) recommendation. Minimal inhibitory concentrations (MIC) test strips were used to detect the MICs of the strains. The following MICs test strips were used colistin (CS), doripenem (DOR), imipenem (IMI), levofloxacin (LEV), piperacillin (PIP), aztreonam (ATM), ceftazidime (CAZ), cefepime (FEP), ciprofloxacin (CIP), netilmicin (NET).

Table 3: Name and class, AWARE classification of the antibiotics tested, their concentration range and MIC breakpoints					
Name	Class	Concentration range	AWaRe classification (WHO)	MIC breakpoint (mg/L) (EUCAST)	
				Sensitive ≥	Resistant <

Aztreonam	Monobactam	0.016-256 mg/L	Reserve	0.001	16
Cefepime	Cephalosporin	0.016-256 mg/L	Watch	0.001	8
Ceftazidime	Cephalosporin	0.016-256 mg/L	Watch	0.001	8
Ciprofloxacin	Fluoroquinolone	0.002-32 mg/L	Watch	0.001	0.5
Colistin	Polymyxin	0.016-256 mg/L	Reserve	(4) ²	(4) ²
Doripenem	Carbapenem	0.002-32 mg/L	Watch	0.001	2
Imipenem	Carbapenems	0.016-256 mg/L	Watch	0.001	4
Levofloxacin	Fluoroquinolones	0.002-32 mg/L	Watch	0.001	2
Netilmicin	Aminoglycoside	0.016-256 mg/L	Watch	IE	IE
Piperacillin	Penicillin's	0.016-256 mg/L	Watch	0.001	16

In one round, 5 bacterial strains were tested. Therefore, 50 petri dishes were prepared with 20ml of Muller Hinton (MH) agar and set aside to solidify, bacterial suspension was prepared from overnight bacterial cultures in 5ml physiological saline suspension until it reached 0.5 MacFarland density. The bacterial suspensions were spread onto the MH agar plates (10 plates for each strain), and the MICs test strips were placed in the middle of the plate using a sterile forceps and incubated at 35°C. The MICs results are recorded after 24 hours. Figure 11. displays one of the strains that were isolated and tested for antibiotic resistance.

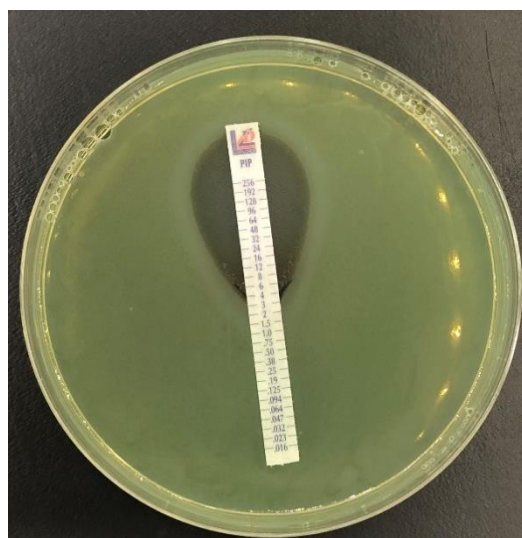


Figure 11: Bacterial culture (strain MP PAE 32) with test strip (MIC) and inhibition zone.

2.2.6. Determination of biofilm forming ability

To determine the biofilm forming ability of the isolated *P. aeruginosa* strains, three different approaches have been used. For initial screening, a rapid **microtiter plate test** was used to evaluate the biofilm forming ability of wastewater originating *P. aeruginosa* strains on PS microplates.

The second and third methods aimed to develop new approach methodologies to determine the biofilm formation on 6 polymer types (LDPE, PP, PVC, PET, PLA, and PS) using only reference strains of *P. aeruginosa* that are publicly available in strain collections (ATCC 10145; ATCC 115442; ATCC 27853) with **biofilm viability** and **protein production assays**.

2.2.7. Microtiter plate test

A modified microtiter plate test was used to assess biofilm-forming ability with parameters and conditions described by Stepanovic et al. (2000) in tryptic-soy broth (TSB) medium, 96 well polystyrene (PS) plates with flat bottom. Into the wells go 230 μ L TSB and 20 μ L bacterial suspension (OD_{600} : 0.6), 250 μ L of sterile TSB medium goes into the wells reserved for abiotic control, incubation was set at 35°C for 48h.

After 48h the wells were poured out and washed 3 times with sterile phosphate buffered saline (PBS) solution ((Phosphate Buffered Saline: 8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4 , 0.24g KH_2PO_4 ; 1 L distilled water), methanol is used to fix the adhered bacterial cells, after 15 minutes the methanol is decanted and the cells were stained with crystal violet staining solution (1%) and set for 20 minutes at room temperature, the wells were washed out 3 times with distilled water, the acetic acid (33%) was used to resolve the adhered stained cells. Measurement of the optical density was done with BioTek ELx800 Absorbance Microplate Reader at OD 550. The results were analysed using recommendations from Stepanovic et al. (2000).

$OD \leq OD_C$	No biofilm production (0)
$OD_C < OD \leq 2x OD_C$	Weak biofilm production (+)
$2x OD_C < OD \leq 4x OD_C$	Moderate biofilm production (++)
$4x OD_C < OD$	Strong biofilm production (+++)

2.2.8. Bradford and biofilm viability assays

The following tests were performed to determine which type of polymer (if any) is a preferred surface for *P. aeruginosa* to attach and multiply. During these experiments, 6 polymer types (LDPE, PP, PVC, PET, PLA, and PS) were used in the form of test objects with a diameter of 4mm. For testing, three reference strains of *P. aeruginosa* that are publicly available in strain collections (ATCC 10145; ATCC 115442; ATCC 27853), because these methods are not commonly used and needed preliminary testing and validation.

To assess biofilm-forming ability, a modified microtiter plate test was used in LB medium with static, flat bottom, 96-well plates in 250 μ l final volumes using 20 μ l bacterial suspension ($OD_{600}=0.6$) and 230 μ l LB. Abiotic control wells contained 250 μ l sterile LB medium and test object. Test was performed in twelve repetitions, in case of every material type object and

evaluated after 48h incubation at 35°C. After incubation, from each wells test objects were removed and washed out three times with sterile PBS solution. Then test objects were placed into Eppendorf tubes (1 ml).

In case of the **(Bradford)** assay, three test objects were placed in one Eppendorf tube and 0.8 ml PBS was added, then until the Bradford assay will be performed, they have been placed in the freezer for further analysis.

The **biofilm viability assay** was used to determine the enzyme activity – in that case, one test object and 200 µL PBS was placed in one Eppendorf. Then 20 µl of 5 mg/mL MTT pour to the tubes (0.5 mg/mL MTT final concentration). Incubate the mixtures at 37°C for 20 min with the tube cap open. Collect the bacteria–formazan crystal complexes by centrifugation (10,000 g, 30 s) and remove the supernatant using a pipette. The volume of residual liquor should be less than 20 µL to reduce its interference with formazan dissolution. Add 800 µL DMSO (Dimethyl sulfoxide) to the tubes. Manually mix the tubes to accelerate the dissolution process, incubate in DMSO for 20 min. From each tube in three replicates pipette 200 µL into flat bottom 96-well plates. Record the absorbance at 550 nm of the mixtures.

3. Results and Discussion

3.1.1. Presence and frequency of *P. aeruginosa* in WWTPs and the affected surface waters

During the examination period 2023 March through to 2023 August, 87 wastewater and surface water samples were analysed to selectively isolate *P. aeruginosa* as it is summarized in Supplementary Table 1.

From these samples, 66 (75.86%) were positive for *P. aeruginosa* with traditional and molecular methods, mainly originating from Zagyva stream in Jászfényszaru. All the samples from Jászfényszaru were positive to *P. aeruginosa* except 2 isolated strains (Supplementary Table 1.). The strain from below stream that was sampled on the 29th of March 2023 showed no presence of *P. aeruginosa*, however, samples taken at the same location on the 30th of August 2023 were all positive for *P. aeruginosa*.

In Veszprém, samples were taken on two occasions, March 22nd, 2023, and June 29th, 2023, on both occasion *P. aeruginosa* was found to be present in most of the samples except for the samples taken from below the sewage inlet in the Veszprémi séd stream. Sampling in Nagykanizsa was also done on two occasions, 12th of April and 21st June, all the samples were positive for *P. aeruginosa*. Sampling in Észak-Pest and Zalaegerszeg was only done on one occasion; 17th of May in Zalaegerszeg, where samples from the influent and below stream showed no presence of *P. aeruginosa*. Sampling in Észak-Pest was done on the 31st of May and only the samples from below and above the sewage inlet showed no presence of *P. aeruginosa*, the other samples were positive.

Sampling in Szeged was done on the 15th of June, all the samples were selectively cultured, there was no presence of *P. aeruginosa* in the samples from below stream and from the influent. Samples taken in Kecskemet from the influent showed a presence of *P. aeruginosa*, but samples from the treated and below stream were negative; samples from Miskolc that were selectively cultured from the influent were all positive to *P. aeruginosa*.

Based on these results we can conclude, that raw (untreated) sewage samples were all positive to *P. aeruginosa*, and sewage treatment (with the option of chlorination) did not successfully eliminate this pathogen in all cases – species *P. aeruginosa* was detectable in the majority of treated sewage samples as well. Surface water receivers above sewage inlet were

various regarding the presence of *P. aeruginosa*, but below sewage inlet, the number of detection have increased. Consequently, the presence of opportunistic *P. aeruginosa* most likely linked to treated sewage inlet in the examined surface water samples.

3.1.2. Antibiotic susceptibility test

In the frame of my thesis work, I had the chance to perform antibiotic resistance tests against 29 *P. aeruginosa* strains isolated from wastewater-related samples. Among these strains, 18 strains were comprehensively characterized against 10 different types of antibiotics (Table 4.). Antibiotics were selected according to the 2019 WHO AWaRe Classification of antibiotics for evaluation and monitoring of use: access group, that includes antibiotics that are more susceptible to a wide variety of pathogens and has a lower resistance potential. Access group antibiotics are first or second choice for treatment options. Second on the list are the watch group antibiotics, antibiotics in this group have a higher resistance. The third class are the reserve group antibiotics, and antibiotics in this group are treated as the last resort as they are highly resistant to multiple drugs.

The results are evaluated in the following way, IE shows that there is insufficient evidence that the organism or group is a good target for the therapy with the agent. S: Susceptible, standard dosing regimen: a microorganism is categorised as susceptible, standard dosing regimen, when there is a high likelihood of therapeutic success using a standard dosing regimen of the agent. I: Susceptible/Intermediate, increased exposure: a microorganism is categorised as Susceptible, increased exposure when there is a high likelihood of therapeutic success because exposure to the agent is increased by adjusting the dosing regimen or by its concentration at the site of infection. R: Resistant: A microorganism is categorised as Resistant when there is a high likelihood of therapeutic failure even when there is increased exposure.

Based on Table 4. we can clearly see that most strains belong to intermediate (I) category; strain MP PAE 75 which is from treated wastewater shows resistance to most antibiotics, MP PAE 70, 66, 29 and 6, also show resistance towards 4 out of 10 of the antibiotics used during this experiment. 70.5% of the strains show resistance towards imipenem, and these strains are from different phases of the WWTP, 47% show resistance towards colistin.

A study done in the Czech Republic by Roulova and colleagues (2022) found that the *P. aeruginosa* strains isolated from hospital effluents show resistance to the following antibiotics, ceftazidime (11.5%), piperacillin (11.5%) and aztreonam (8.5%); in our study resistance to

ceftazidime is at 5.9%, piperacillin is 35.3% and aztreonam is 5.9%, the difference in these results is because in our study we collected and tested samples from various phases of the WWTP, whereas the study done in the Czech Republic only looked at the untreated hospital effluents.

In our study the rate of resistance to imipenem is 70.6% and this correlates with the study done in Brazil where the rate of resistance to imipenem in the *P. aeruginosa* strains is found to be less than 70% (Peixoto de Freitas & Barth, 2002).

Non-susceptibility to at least one agent in three or more antimicrobial categories is known as multidrug resistance (MDR) (Magiorakos et al., 2012); among the comprehensively examined 18 isolates, five strains were considered as MDR (MP PAE 75, 70, 66, 29 and 6). MP PAE 75 is non-susceptible to antibiotics from 5 categories, fluoroquinolones (CIP & LEV), cephalosporins (FEP), penicillin (PIP), carbapenems (IMI) and polymyxins (CS). MP PAE 70 is MDR to antibiotics from 3 categories: cephalosporins (FEP), carbapenems (IMI & DOR) and polymyxin (CS). The strain MP PAE 66 is also non-susceptible to 3 categories: cephalosporins (FEP), fluoroquinolones (LEV) and carbapenems (IMI and DOR). MP PAE 29 shows MDR to antibiotics from 4 categories: cephalosporins (CAZ), fluoroquinolones (LEV), carbapenems (IMI) and polymyxins (CS). The last strain from our research that is MDR to 3 categories of antibiotics is MP PAE 6, it's MDR to monobactams (ATM), penicillins (PIP) and carbapenems (DOR).

1.1.1. Biofilm assay on modified microtiter test

The results of the modified microtiter assay for screening were analysed using the recommendations from Stepanovic et al. (2000). In the frame of my thesis work, I could analyse the biofilm forming ability of 36 wastewater-related *P. aeruginosa* strains (Table 5.). Most of the strains showed a strong ability (+++) to form biofilm, only two strains did not form a biofilm – both of them originated from the upstream section of the surface water receiver, i.e., these non-biofilm forming strains were not directly linked to the wastewater load. The strains with a strong biofilm production are from the treated WW (e.g., MP PAE 81,32, and 43), downstream section of the surface water receiver (e.g., MP PAE 82) and from the influent sewage (e.g., MP PAE 69 and 70). The moderately (++) biofilm-forming were from the upstream section of surface waters (e.g., MP PAE 45 and 63), downstream (MP PAE74), treated WW and from plastic colonizers incubated in the treated WW (MP PAE 75 originated from PLA surface and 76 from PS). The

latest two isolates, even they are considered as moderate biofilm producers were isolated from the surface of MPs, therefore, they are verified contributors in microplastic colonization.

Date of reading:		2023.07.19						2023.07.27						2023.09.01						2023.09.08						2024.12.13						01-02-2024					
Antibiotics	Code	MP-PAGE-75	MP-PAGE-70	MP-PAGE-66	MP-PAGE-74	MP-PAGE-60	MP-PAGE-42	MP-PAGE-29	MP-PAGE-26	MP-PAGE-63	MP-PAGE-25	MP-PAGE-45	MP-PAGE-43	MP-PAGE-32	MP-PAGE-31	MP-PAGE-33	MP-PAGE-6	MP-PAGE-30	MP-PAGE-25	MP-PAGE-24	MP-PAGE-4	MP-PAGE-76	MP-PAGE-88	MP-PAGE-67	MP-PAGE-71	MP-PAGE-57	MP-PAGE-10	MP-PAGE-13	MP-PAGE-18	MP-PAGE-29	MP-PAGE-31						
Nedimicin	MET	IE	IE	IE	IE	IE	IE	IE	IE	IE	IE	IE	IE	IE	IE	IE	IE	IE	IE	IE	IE	IE	IE	IE	IE	IE	IE	IE	IE	IE	IE						
Ciprofloxacin	CIP	R	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I						
Cefepime	CEP	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R						
Ceftazidime	CAZ	I	I	I	I	I	I	R	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	R						
Aceclofenac	ATM	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	R	I	I	I	I	I	I	I	I	I	I	I	I	I	I						
Piperacillin	PIP	R	I	I	I	I	I	I	I	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R						
Levofloxacin	LEV	R	I	R	I	I	I	R	I	I	I	I	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R						
Imipenem	IMI	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R						
Doripenem	DOR	I	R	R	I	I	I	I	I	I	I	I	I	I	I	I	R	I	I	I	I	I	I	I	I	I	I	I	I	I	I						
Colistin	CS	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R						

Table 4. Results from antibiotic resistance test using MICs according to the EUCAST MIC breakpoint tables.

S: Susceptible, is colour coded green
I: Intermediary, colour coded orange
R: resistant, colour coded red
IE: insufficient evidence

Table 5. Biofilm forming ability and origin of the *P. aeruginosa* strains.

Strain ID:	MP-PAE-63	MP-PAE-68	MP-PAE-69	MP-PAE-70	MP-PAE-72	MP-PAE-71	MP-PAE-73	MP-PAE-74	MP-PAE-75
Biofilm production:	++	+++	+++	+++	+++	+++	++	++	++
	upstream	influent	influent	influent	treated ww	treated ww	treated ww	downstream	treated ww from plastic colonizer (PLA)
	MP-PAE-76	MP-PAE-66	MP-PAE-64	MP-PAE-65	MP-PAE-67	MP-PAE-4	MP-PAE-6	MP-PAE-25	MP-PAE-81
	++	++	+++	++	+	++	0	++	+++
	treated ww from plastic colonizer (PS)	treated ww	influent	influent	below stream	treated ww	upstream	treated ww	treated ww
	MP-PAE-82	MP-PAE-83	MP-PAE-24	MP-PAE-32	MP-PAE-43	MP-PAE-45	MP-PAE-84	MP-PAE-88	MP-PAE-92
	+++	0	+++	+++	+++	++	++	+++	+++
	downstream	upstream	treated ww	treated ww	treated ww	upstream	treated ww	upstream	downstream
	MP PAE 10	MP PAE 13	MP PAE 18	MP PAE 29	MP PAE 41	MP PAE 53	MP PAE 60	MP PAE 62	MP PAE 96
	+	++	+	+	+	+	++	++	+

Markings: no biofilm production (0); weak biofilm production (+); moderate biofilm production (++); strong biofilm production (+++) Yellow colour: *P. aeruginosa* strains with a complete antibiotic resistance profile.

Our results about the great biofilm production of *P. aeruginosa* are in accordance with the scientific literature as *P. aeruginosa* is known as a biofilm forming microorganism on various surfaces (de Celis et al., 2022). However, as my results show, there can be significant differences among strains of different origin, because 2 out of 3 upstream (background) strains were non-biofilm producers.

1.1.2. Results of Bradford and biofilm viability assays

The previously shown results on biofilm formation were applicable for preliminary screening, but the modified microtiter plate assay can analyse the biofilm forming ability on PS only. To better understand the role of environmental *P. aeruginosa* on plastic colonization, new approach methodologies were tested. During our experiment, 6 polymer types were used (LDPE, PP, PVC, PET, PLA, and PS) and three reference strains of *P. aeruginosa* that are publicly available in strain collections (ATCC 10145; ATCC 115442; ATCC 27853). The first approach was the Bradford protein production assay, which is still in progress at the time of writing the thesis work, therefore the results are not shown.

The results of the biofilm viability assay, i.e., the MTT enzyme activity linked to biofilm production were repeated three times for the examined reference strains. The results of the repeated measurements are summarized in Figures 12 to 14.

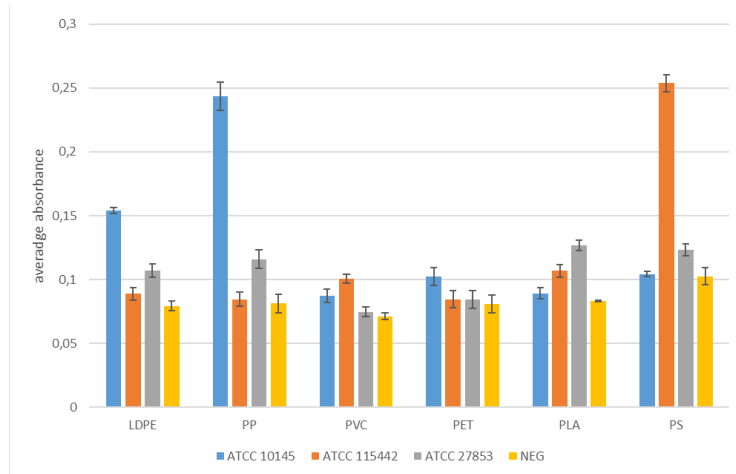


Figure 12: Enzyme activity assay on polymers using three strains.

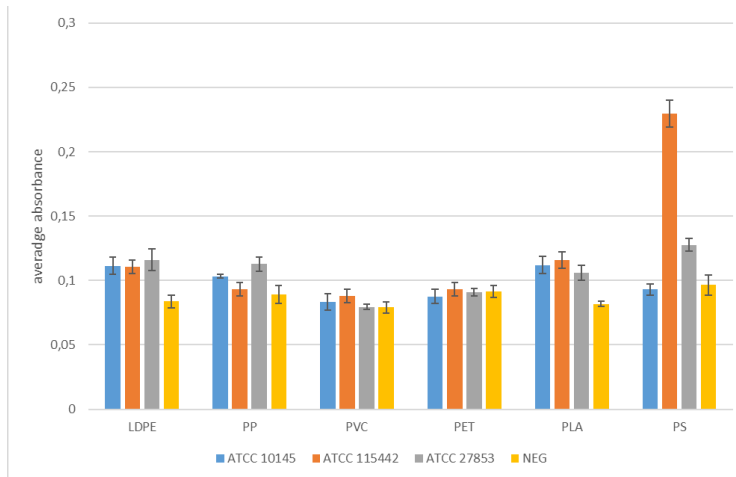


Figure 13: Enzyme activity assay on polymers using three strains.

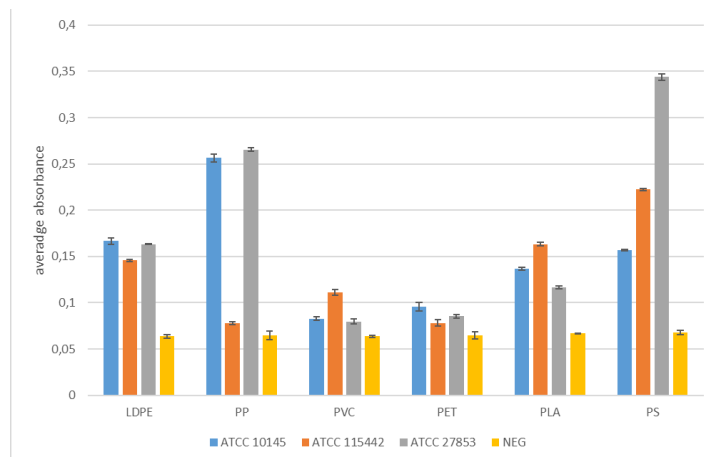


Figure 14: Enzyme activity assay on polymers using three strains.

Based on Figures 12-14., the preference of the examined strains to attach to different polymers were ranked as follows:

1. ATCC10145: **PP**>LDPE>PET/PS>PVC>PLA
2. ATCC10145: **LDPE**/PLA>PP>PS>PET>PVC
3. ATCC10145: **PP**>LDPE>PS>PS>PLA>PET>PVC

1. ATCC 115442: **PS**>PLA>PVC>LDPE>PP/PET
2. ATCC115442: **PS**>PLA>LDPE>PP/PET>PVC
3. ATCC115442: **PS**>PLA>LDPE>PVC>PP

1. ATCC 27853: **PLA**>PS>PP>LDPE>PET>PVC
2. ATCC27853: **PS**>LDPE>PP>PLA>PET>PVC
3. ATCC27853: **PS**>PP>LDPE>PLA>PET>PVC

We can see that polymer type PS shows generally a higher absorbance which means more bacterial cells are attached and formed biofilm to its surface. It shows a higher concentration for the strain ATCC 115442, the rest of the polymers show an average absorbance for this strain. The strain ATCC 10145 shows a higher absorbance on PP and LDPE; the remaining strain ATCC 27853 shows an average absorbance on all the polymer types, however, it has a slightly higher absorbance on PP and PS. Based on this experiment it is evident that PS, PP, PLA and LDPE are the most preferred polymers for *P. aeruginosa* strains to attach, but based on the comparison to the negative control, in most cases, the examined bacterial strains formed a biofilm on other polymers with the exception of PET where biofilm formation of the examined three strains was negligible and, PVC ,where strain ATCC 27853 did not form a biofilm.

In general, the polymer types of PS, PP and LDPE are a more preferred surface for the *P. aeruginosa* strains to attach. PET did not form any biofilm for the strains ATCC 27853 and 115442, and PVC also did not form any biofilm for the strain ATCC 115442; we use the negative (NEG) control as a point of reference.

The polymer types of PS, PP and PET are reported as the most common polymers in different stages of WWTP (Uoginte et al., 2022), this is due to these polymers being widely used for packaging that is utilized daily (Huang et al., 2022). According to my results, WW originating *P.*

aeruginosa strains show different ability to attach to the examined polymer types and grow better on the surface of PP, PS and LDPE.

According to Ayush and colleagues (2022), the density of polymers influences the interaction with *P. aeruginosa*. If the polymer has high density, then during incubation it sinks to the bottom of the well and collides with the bottom and wall of the well. Whereas polymers with a lower density than water float on the liquid media so they interact only with the wall. In their work, the polymer types that have a lower density were PP and PE. In our work PP is one of the polymers that is a preferred surface for *P. aeruginosa* to attach and multiply, therefore, our results using different approaches for biofilm quantification widened our knowledge in this field.

1.2. Conclusions

Our findings show that *P. aeruginosa* is present at different stages of the wastewater treatment and have a higher frequency in sample types with a direct connection to wastewater treatment.

The majority of the isolated *P. aeruginosa* strains did indicate resistance towards certain antibiotics that were used during this experiment; five strains are multidrug resistant (MDR), with resistance against the main categories of antibiotics such as fluoroquinolones, cephalosporins, penicillin, carbapenems and polymyxin. 12 out of the 17 strains were resistant to imipenem (carbapenems), which is a great concern to human health.

The biofilm formation of the strains that were tested during our work were usually moderate to strong; the majority of the strains show a strong (+++) or moderate (++) biofilm formation with a modified microtiter test on PS surface. Only two upstream *P. aeruginosa* strains (originated from the natural surface water above the sewage inlet) were non-biofilm producers.

Generally, polymers provide a suitable surface for *P. aeruginosa* (and other bacteria), however, some may be more suitable than the others. Based on our biofilm viability assay, among the six examined polymer types, *P. aeruginosa* strains showed the highest concentration of MTT enzyme on PS and PP. This is a cause for concern because studies show that these polymers are the most common in the environment. However, there are polymers where the examined opportunistic pathogenic strains did not form any biofilm which means that the polymer type is a determining factor in the biofilm formation of *P. aeruginosa*.

Regarding the link between antibiotic resistance and biofilm formation, strain MP PAE 6 did not form any biofilm; however, it does show resistance towards 3 out of 10 antibiotics (DOR, PIP & ATM) that were used during our experiment. MP PAE 75 was resistant to most of the antibiotics used in this experiment and it showed a moderate biofilm formation. On the contrary the strain MP PAE 69 shows a strong biofilm formation, but it is only resistant to one antibiotic (imipenem, most of the strains show resistance to imipenem). Based on this variety of results in antibiotic resistance and biofilm formation, we can conclude that there is no direct link between these phenotypic features. Further molecular studies (gene expression, whole genome sequencing, determination of acquired antibiotic resistance genes with the PCR) are required to find a correlation between these parameters. Moreover, to validate biofilm viability tests, we plan to conduct the Bradford assay to

determine protein concentrations. Currently there's no other literature available that explains this research.

In the future, the developed methods and our results can be used to choose among different polymer types for specific applications, like drinking water pipelines or medical devices. In the meantime, based on our finding about the frequency of *P. aeruginosa* in wastewater-related samples, it is strongly recommended to continuously monitor its presence in wastewater and in surface water receivers and to properly operate the third stage of wastewater treatment (i.e., disinfection) whenever it is necessary.

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Summary

Plastic has evolved over the previous century from being non-existent to a common and essential component of contemporary life. Although plastic has many benefits over other materials, its widespread manufacture and quick disposal are causing serious problems for the environment, the economy, and ethics (Sun et al., 2019). Six hundred million of the eight hundred million tons of plastic manufactured up until 2015 were thrown away as waste (Schell et al., 2019). As a consequence of their low degradation rate, microplastics (MP) persist in the environment for many years and interact with chemical compounds and microorganisms. Consequently, MPs present a new ecological niche for bacteria and eukaryotic colonization, dispersal and biofilm formation after exposure to the environment (Kruglova et al., 2022), named as 'Plastisphere' (Zettler et al, 2018).

Wastewater originating MPs often reach the environment and present abiotic surface for biofilm formation. This ecological niche called plastisphere is considered as a hotspot of antimicrobial resistance. The aim of this research was to isolate biofilm forming and antibiotic resistant opportunistic pathogenic *P. aeruginosa* from wastewater treatment plants surface water receivers and plastic colonizers, to evaluate their antibiotic resistance profile and to determine their role in MPs colonization.

P. aeruginosa was found to be present in samples from all the stages of wastewater treatment plant (WWTP), we tested 87 samples and from these samples, 66 (75.86%) were positive for *P. aeruginosa*. Antibiotic assay was performed on a set of the isolated strains and most strains showed intermediate or resistant category towards the tested antibiotics. Five strains were evaluated as multidrug resistant (MDR) with resistance against the main categories of antibiotics such as fluoroquinolones, cephalosporins, penicillin, carbapenems and polymyxin. Preliminary biofilm assay of the strains showed a moderate to strong biofilm formation on PS microplates.

To develop novel methods for biofilm detection of *P. aeruginosa*, six types of polymers (PA, PLA, LDPE, PVC, PET and PS) with a diameter of 4mm were used to determine which one is the preferred surface for *P. aeruginosa* to attach and multiply. According to our results, PP, PS and LDPE were the most preferred surface for *P. aeruginosa*. Our results may contribute in polymer testing and application in the future and emphasize the role of wastewater treatment in the removal of opportunistic pathogens such as *P. aeruginosa*.

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Supplementary Table 1. The isolated and identified *P. aeruginosa* strains of wastewater-related sources

WWTP	STREAM	SAMPLING SITE	Sample ID	Date of sampling	PA-SS_PCR	IDENTIFIED AS <i>P. AERUGINOSA</i>	STRAIN ID
Jászfényszaru	Zagyva	TREATED_WW	JUT1	2023.03.29	✓	✓	MP-PAE-4
					✓	X	MP-PAE-5
		ABOVE_STREAM	JUF1		✓	✓	MP-PAE-6
					n.d.	n.d.	MP-PAE-7
		BELLOW_STREAM	JUA1		✓	X	MP-PAE-1
					✓	X	MP-PAE-2
					n.d.	n.d.	MP-PAE-3
		INFLUENT_WW	JUPH-IN1		n.d.	n.d.	MP-PAE-8
					✓	✓	MP-PAE-9
		SLUDGE	JUPH-SL1		✓	✓	MP-PAE-10
					✓	✓	MP-PAE-11
		COLONIZER_TREATED_WW	JFT-PLA	n.d.	✓	✓	MP-PAE-75
			JFT-PS	n.d.	✓	✓	MP-PAE-76
		TREATED_WW	JUT 2	2023.08.30	✓	✓	MP-PAE-84
					✓	✓	MP-PAE-85
					✓	✓	MP-PAE-86
		ABOVE_STREAM	JUF 2		✓	✓	MP-PAE-87
					✓	✓	MP-PAE-88
					✓	✓	MP-PAE-89
		BELLOW_STREAM	JUA 2		✓	✓	MP-PAE-90
					✓	✓	MP-PAE-91
					✓	✓	MP-PAE-92
		INFLUENT_WW	JUPH-IN-2		✓	✓	MP-PAE-93
					✓	✓	MP-PAE-94
					✓	✓	MP-PAE-95

		SLUDGE	JUPH-SL-2		✓	✓	MP-PAE-96
					✓	✓	MP-PAE-97
					✓	✓	MP-PAE-98
Szentendre	Duna	TREATED_WW	SET1	2023.04.05	✓	✓	MP-PAE-25
		ABOVE_STREAM	SEF1		n.d.	n.d.	
		BELLOW_STREAM	SEA1		n.d.	n.d.	
		INFLUENT_WW	SEPH-IN1		✓	✓	MP-PAE-15
					n.d.	n.d.	MP-PAE-16
					n.d.	n.d.	MP-PAE-17
		SLUDGE	SEPH-SL1		✓	✓	MP-PAE-18
		INFLUENT_WW	SEPH-IN_2	2023.07.27	✓	✓	MP-PAE-77
			SEPH-IN_2		✓	✓	MP-PAE-78
		SLUDGE	SEPH-SL-2		✓	✓	MP-PAE-79
			SEPH-SL-2		✓	✓	MP-PAE-80
		TREATED_WW	SET_2		✓	✓	MP-PAE-81
		BELLOW_STREAM	SEA_2		✓	✓	MP-PAE-82
		ABOVE_STREAM	SEF_2		✓	✓	MP-PAE-83
Veszprém	Veszprémi sód	TREATED_WW	VMT1	2023.03.22	n.d.	n.d.	MP-PAE-23
					✓	✓	MP-PAE-24
		ABOVE_STREAM	VMF1		n.d.	n.d.	
		BELLOW_STREAM	VMA1		n.d.	n.d.	MP-PAE-20
					✓	X	MP-PAE-21
		INFLUENT_WW	VMPH-IN-1		✓	✓	MP-PAE-12
		SLUDGE	VMPH-SL-1		✓	✓	MP-PAE-13
					✓	✓	MP-PAE-14
		TREATED_WW	VMT2	2023.06.29	✓	✓	MP-PAE-66
		ABOVE_STREAM	VMF2	2023.06.29	n.d.	n.d.	
		BELLOW_STREAM	VMA-2	2023.06.29	✓	X	

					✓	✓	MP-PAE-67
					✓	✓	MP-PAE-68
		INFLUENT_WW	VMPH-IN-2	2023.06.29	✓	✓	MP-PAE-69
					✓	✓	MP-PAE-70
		SLUDGE	VMPH-SL-2	2023.06.29	n.d.	n.d.	
Nagykanizsa	Principális-cs.	TREATED_WW	NAT1	2023.04.12	n.d.	n.d.	
		ABOVE_STREAM	NAF1		n.d.	n.d.	
		BELLOW_STREAM	NAA1		n.d.	n.d.	
					✓	✓	MP-PAE-26
		INFLUENT_WW	NAPH-IN1		✓	✓	MP-PAE-27
					✓	✓	MP-PAE-28
					✓	✓	MP-PAE-29
		SLUDGE	NAPH-SL1		✓	✓	MP-PAE-30
					✓	✓	MP-PAE-31
		TREATED_WW	NAT2	2023.06.21	✓	✓	MP-PAE-71
					✓	✓	MP-PAE-72
					✓	✓	MP-PAE-73
Észak-Pest	Duna	ABOVE_STREAM	NAF2	2023.06.21	n.d.	n.d.	
		BELLOW_STREAM	NAA2	2023.06.21	✓	✓	MP-PAE-74
		TREATED_WW	ÉPMT	2023.05.31	✓	✓	MP-PAE-32
					✓	✓	MP-PAE-33
					✓	✓	MP-PAE-34
		ABOVE_STREAM	ÉPMF		n.d.	n.d.	
					✓	X	MP-PAE-35
		BELLOW_STREAM	ÉPMA		✓	X	MP-PAE-36
					✓	X	MP-PAE-37
					✓	✓	MP-PAE-38
		INFLUENT_WW	ÉPIN		✓	✓	MP-PAE-39

					✓	✓	MP-PAE-40
		SLUDGE	ÉPSL		✓	✓	MP-PAE-41
					n.d.	n.d.	MP-PAE-42
Zalaegerszeg	Zala	TREATED_WW	ZGMT	2023.05.17	✓	✓	MP-PAE-43
					✓	✓	MP-PAE-44
		ABOVE_STREAM	ZGMF		✓	✓	MP-PAE-45
					n.d.	n.d.	MP-PAE-46
		BELLOW_STREAM	ZGMA		✓	X	MP-PAE-47
					✓	X	MP-PAE-48
					✓	X	MP-PAE-49
		INFLUENT_WW	ZGIN		✓	✓	MP-PAE-50
					✓	X	MP-PAE-51
					✓	X	MP-PAE-52
		SLUDGE	ZGSL		✓	✓	MP-PAE-53
					✓	✓	MP-PAE-54
					✓	✓	MP-PAE-55
Kecskemét	Csukás-éri fcs.	TREATED_WW	KTMT	2023.04.19	✓	X	MP-PAE-56
		ABOVE_STREAM	KTMF		n.d.	n.d.	MP-PAE-57
		BELLOW_STREAM	KTMA		✓	X	MP-PAE-58
		INFLUENT_WW	KTIN		✓	✓	MP-PAE-59
		SLUDGE	KTSL		✓	✓	MP-PAE-60
Szeged	Tisza	TREATED_WW	SDMT	2023.06.15	n.d.	n.d.	
		ABOVE_STREAM	SDMF	2023.06.15	✓	✓	MP-PAE-63
		BELLOW_STREAM	SDMA	2023.06.15	✓	X	
				2023.06.15	✓	X	
		INFLUENT_WW	SDIN	2023.06.15	✓	✓	MP-PAE-64
				2023.06.15	✓	✓	MP-PAE-65
				2023.06.15	✓	X	

		SLUDGE	SDSL	2023.06.15	✓	X	
				2023.06.15	✓	X	
				2023.06.15	✓	X	
Miskolc	Sajó	TREATED_WW	MCMT	2023.05.10	n.d.	n.d.	
		ABOVE_STREAM	MCMF		n.d.	n.d.	
		BELLOW_STREAM	MCMA		n.d.	n.d.	
		INFLUENT_WW	MCIN		✓	✓	MP-PAE-61
		SLUDGE	MCSL		✓	✓	MP-PAE-62