IDENTIFICATION OF MICROPLASTIC-ASSOCIATED MICROBIAL COMMUNITIES FROM VARIOUS STAGES OF WASTEWATER TREATMENT AND RECIPIENT SURFACE WATERS USING MALDI-TOF MASS SPECTROMETRY

MIKROMŰANYAGOKHOZ TÁRSULÓ MIKROBIÁLIS KÖZÖSSÉGEK AZONOSÍTÁSA A SZENNYVÍZTISZTÍTÁS KÜLÖNBÖZŐ FÁZISAIBAN ÉS A BEFOGADÓ FELSZÍNI VIZEKBEN MALDI-TOF MS MÓDSZERREL

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Abstract

Recent studies have identified microplastics (MPs) in wastewater as surfaces that facilitate microbial colonization and act as vectors for the dissemination of antibiotic resistance genes (ARGs) and antibiotic-resistant bacteria (ARBs). Their presence throughout the wastewater treatment process highlights the importance of investigating the microbial communities associated with MPs in wastewater treatment plants (WWTPs) and their potential implications for environmental and human health.

This study investigates microbial colonization on MPs in wastewater by sampling six types of MPs deployed at various phases of wastewater treatment in three different WWTPs across Hungary, between June 2024 and March 2025. MPs were incubated in plastic colonizers submerged in treated and untreated wastewater. Post-retrieval, MPs were processed using selective media to ARBs, using Chromatic agar plates and selective medium for Pseudomonas aeruginosa. Strains were identified by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) at the genus level. Based on our results, Stenotrophomonas, Aeromonas genera, and members of the Enterobacteriaceae family were the dominant antibiotic-resistant bacterial taxa associated with MPs.

Keywords: *microplastic, wastewater, Aeromonas, Stenotrophomonas, MALDI-TOF MS*
JEL Code: *F64, Q53, O13*

Összefoglaló

A legújabb kutatások alapján a szennyvizekben található mikroműanyagok elősegíthetik a mikroorganizmusok kolonizációját, és vektorként szolgálhatnak az antibiotikum-rezisztencia gének (ARG-k) és az antibiotikum-rezisztens baktériumok (ARB-k) terjedésében. A mikroműanyagok jelenléte a szennyvíztisztítás teljes folyamatában igazolt, mely indokolja a szennyvíztisztító telepeken előforduló, mikroműanyagokhoz kötődő baktériumközösség vizsgálatát, különös tekintettel a környezeti és humán egészségre gyakorolt hatásokra.

Jelen tanulmány hatféle mikroműanyag mikrobiális kolonizációját vizsgálta szennyvízben, amelyek 2024 júniusa és 2025 márciusa között, három magyarországi szennyvíztisztító telep

különböző kezelési fázisaiban (nyers és tisztított szennyvízben) kerültek kihelyezésre. Az inkubációt követően visszagyűjtött mikroműanyagokról szelektív Chromatic agar táptalajon tenyésztettünk antibiotikum-rezisztens baktériumokat, illetve szintén szelektív táptalajon dúsítottuk másik célszervezetünket, a Pseudomonas aeruginosa fajt. Az izolátumok azonosítása nemzetség szinten történt, mátrix-asszisztált lézerdeszorpciós/ionizációs repülési idő tömegspektrometriával (MALDI-TOF MS). Eredményeink alapján a Stenotrophomonas és Aeromonas nemzetségek, valamint az Enterobacteriaceae család tagjai voltak a mikroműanyagokhoz társuló domináns antibiotikum-rezisztens bakteriális taxonok.
Kulcsszavak: *mikroműanyag, szennyvíz, Aeromonas, Stenotrophomonas, MALDI-TOF MS*

Introduction

Since the Industrial Revolution, human activity has led to a continuous and accelerating increase in waste production, including both solid and liquid forms such as wastewater. When released into surface waters with or without adequate treatment, wastewater can negatively affect ecological functions and reduce biodiversity. Globally, there is growing concern about the environmental impact of pollutants derived from anthropogenic sources, particularly regarding their long-term effects on aquatic ecosystems. As a result, effective wastewater treatment and regular monitoring of water quality are considered essential measures (EINSCHLAG and CARLOS, 2013).

Water pollution occurs when harmful substances are introduced into water bodies, potentially threatening human health, wildlife, and the broader environment. Treating wastewater is critical to minimizing pollution and protecting public health by preventing the spread of waterborne illnesses. Safe management of human waste is fundamental to maintaining clean drinking water, given the risk of numerous diseases transmitted through faecal contamination. Proper treatment and disinfection can significantly reduce the incidence of such illnesses (GRAY, 2004).

Recently, microplastics (MPs) have emerged as a growing concern associated with wastewater. MPs are plastic fragments or particles that are less than 5 mm in diameter (HERNANDEZ et al., 2019). These synthetic particles are prevalent in wastewater systems and persist in the environment due to their extended degradation. MPs can interact with chemical pollutants and microorganisms, providing a novel habitat for microbial colonization, biofilm development, and dispersal (KARKANORACHAKI et al., 2021). MPs enter wastewater treatment plants (WWTPs) through various pathways, primarily originating from domestic, industrial, and urban sources. A significant contributor is household wastewater, where synthetic textiles release microfibers during laundry processes (ACARER, 2023). Despite the treatment processes in WWTPs, studies have shown that a considerable number of microplastics can bypass filtration systems and be released into aquatic environments through effluents (IYARE et al., 2020).

Moreover, MPs can act as carriers of antibiotic-resistant bacteria (ARBs), which harbour antibiotic resistance genes (ARGs), raising public health concerns, as the proliferation of ARGs among bacteria poses a challenge to the effective treatment of infectious diseases (LIU et al., 2021). Given that MPs have been detected throughout all stages of wastewater treatment (KARKANORACHAKI et al., 2021), there is increasing interest in characterizing the microbial communities colonizing MPs in WWTPs.

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) is a powerful analytical technique not only used for the characterization of biomolecules, including proteins, peptides, but also for the identification of microorganisms. Its rapid, accurate, and high-throughput capabilities have made it indispensable in clinical diagnostics, proteomics, and microbiology (FISSEL, 2024).

This study aimed to identify and analyse the cultivable, antibiotic-resistant bacteria (ARBs) and the opportunistic pathogen species *Pseudomonas aeruginosa* associated with different types of MPs across the technological stages of wastewater treatment and in the surface water bodies receiving treated sewage. The applied methods were traditional cultivation combined with MALDI-TOF MS identification. The types of polymers used in this study were low-density polyethylene (LDPE), polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS), Polylactic Acid (PLA), and polyethylene terephthalate (PET), as they are currently the most commonly used synthetic polymers. When combined, these plastics account for almost 90% of global manufacturing (ANDRADY and NEAL, 2009), and it is generally acknowledged that these materials make up the majority of marine and coastal pollution (ANDRADY, 2011; ENGLER, 2012; MENA and GERBA, 2009). Therefore, revealing their role in the transmission of opportunistic pathogens and ARBs is critically important.

Materials and Methods

Sampling

To determine the plastic-associated, cultivable ARBs and *P. aeruginosa*, in 2024-2025, MPs were incubated throughout the different stages of wastewater treatment and the surface water receivers of three WWTPs located in Hungary. The sampling regime is summarized in Table 1.

Table 1: Characteristics of the WWTPs involved in MPs incubation and the dates of sampling

Sampling sites	Influent wastewater quantity (m^3/day)	Population equivalent (PE)	Sampling dates
WWTP 1	10,617	71,000	10/06/2024
WWTP 2	12,894	123,862	17/07/2024
WWTP 3	830	7,442	25/09/2024 26/09/2024 09/12/2024 01/03/2025

To enable the bacterial colonization of MPs, plastic particles were cut to <5 mm pieces and were incubated in sterile stainless-steel colonizers (Figure 1.A) tied to a metal cage with zip ties, which was submerged into various phases of the examined WWTPs. Influent (untreated) wastewater, effluent (treated) wastewater, upstream and downstream sections of the recipient waterbody were used for the colonization experiment (Figure 1.B). Six types of polymers (LDPE, PS, PLA, PVC, PET and PP) were placed separately into plastic colonizers (20 MP particles/colonizer).

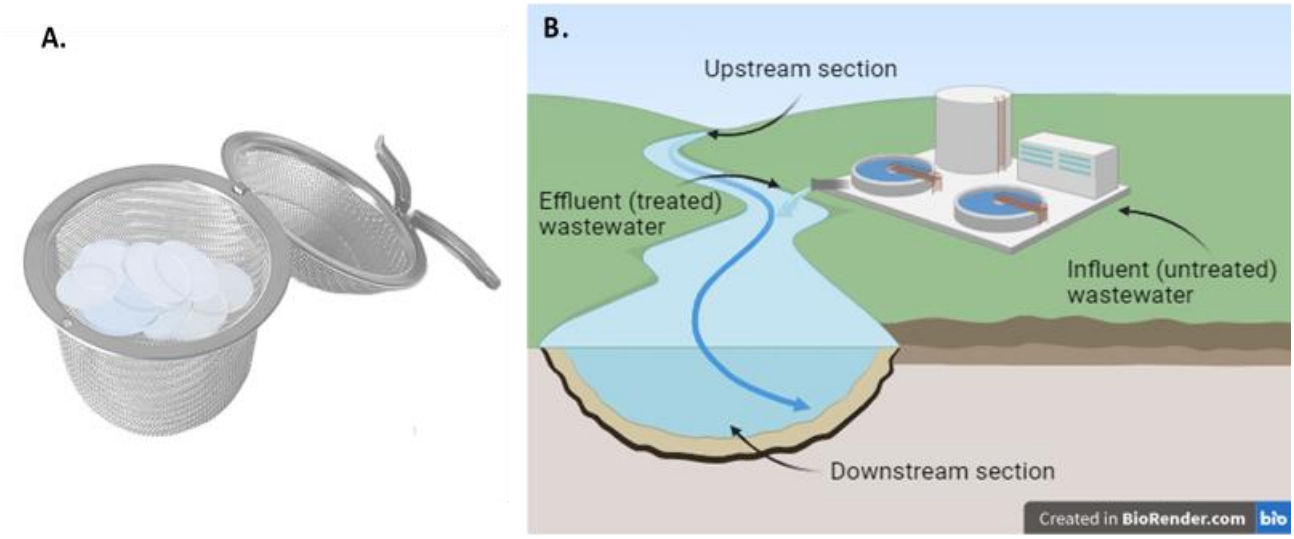

Figure 1: Plastic colonizers used for the incubation of 6 types of polymers (A.) and the stages of wastewater treatment where the colonizers were incubated (B.)

After 3 months of incubation, samples were collected and transferred immediately to the laboratories of the MATE - Institute of Aquaculture and Environmental Safety. Plastic particles were removed from the colonizers using sterile forceps under aseptic conditions and rinsed three times with sterile distilled water to remove debris and planktonic microorganisms originating from the wastewater/water body, but kept the plastic-associated biofilm (SZABÓ et al., 2021).

Isolation of cultivable antibiotic-resistant bacteria and P. aeruginosa

From each type of MPs, one piece was placed into a test tube with 5 mL asparagine broth (3 g L-asparagine; 1 g K_2HPO_4; 0.5 g MgSO4·7H2O; 10 mL glycerol, 1000 mL distilled water, pH 7.0) and incubated at 42°C for 48 hours to selectively enrich opportunistic pathogen species *P. aeruginosa*. After incubation, 100 µL from asparagine broth was transferred and spread onto cetrimide agar plates (Merck 105284), then incubated for another 24 hours at 37°C. Plates with colonies producing fluorescent pigments were considered positive and were subcultured again for further analysis.

To determine the cultivable number of ARBs, three MPs with biofilm were placed into Eppendorf tubes containing 900 µL PBS and vortexed for 1 min. After removing the biofilm, 100 µL of the suspension was spread onto Chromatic colistin (COL) and carbapenem-resistant Enterobacteriaceae (CRE) agar plates (Liofilchem); the plates were incubated at 35°C for 48 hours to selectively enrich microorganisms resistant to these clinically relevant classes of antibiotics (colistin and carbapenems) (ŠAMANIĆ et al., 2021; SILVA et al., 2024). From the Chromatic COL and CRE agar plates, positive colonies (with dark purple and pink pigmentation, as can be seen in Figure 2) were selected, subcultured onto Tryptic Soy Agar (TSA) plates (Biolab, Hungary) and incubated for 48 hours at 35°C. After incubation, the plates underwent another purification step with the streak plate method to reach isolated colonies on the agar plate.

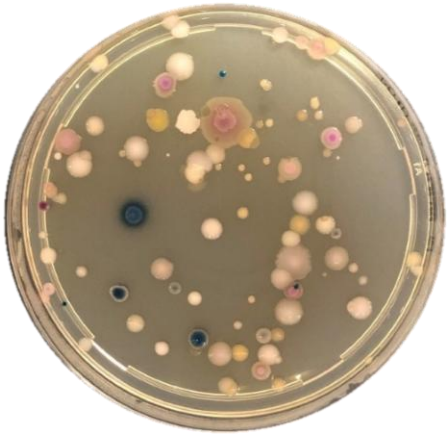

Figure 2: Chromatic carbapenem-resistant Enterobacteriaceae (CRE) agar plate with colonies of antibiotic-resistant bacteria originating from polyethylene terephthalate **(PET) microplastics (MPs). Sample ID: JUF-ET 24.2, Date: 09.13.2024**

After isolation, strains got a unique identifier, and all strains were cryopreserved for later use in 2 mL cryotubes containing 700 µL bacterial suspension and 700 µL 30 v/v% sterile glycerol solution.

MALDI-TOF MS for genus-level identification

Genus-level identification of the MP-associated strains was performed using a MALDI Biotyper 3.0 instrument (Bruker Daltonics, Bremen, Germany) in the Eurofins Food and Feed Testing Ltd., Hungary. For whole cell MALDI-TOF MS identification, the cryopreserved strains were cultured onto Luria Bertani agar plates (10 g tryptone, 5 g yeast extract, 9 g NaCl; 18 g agar, 1000 mL distilled water) and were incubated at 35°C overnight. A pure colony of the fresh bacterial cultures were picked up using sterile toothpicks, and a small amount of biomass was spread onto the spots of a conductive MALDI target plate in two replicates. Protein extraction was performed with acetonitrile/formic acid, then the target was overlayed with 1 µL HCCA (α-cyano-4-hydroxycinnamic acid). This matrix absorbs the ionization laser's energy and facilitates the desorption and ionization of the analyte molecules (LI et al., 2022), enabling the vaporization and transport of the analyte into the gas phase. Ionization of analyte molecules usually results in singly charged ions. These ions travel through a time-of-flight tube where they are separated based on mass-to-charge ratio. The resulting mass spectrum is matched against a database to identify the microorganism at the genus level (TARFEEN et al., 2022; HOU et al., 2019). The quality of the identification is evaluated by automatically generated score values (2.00-3.00 reliable species level identification, 1.70-1.99 reliable genus level identification, 0.00-1.699 not reliable identification).

Results

During the experimental period, a total of 112 cultivable ARBs, originating from the MPs surface, were determined at the genus level by MALDI-TOF MS. Only 14% of the bacterial isolates failed to be identified by the chosen method and reached a non-sufficient score value. Based on our results, there was a notable difference in the cultivable, antibiotic-resistant microbial community of the six different polymers (LDPE, PS, PLA, PVC, PET and PP) that were deployed. Figure 3 below illustrates the differences in microbial diversity and composition of antibiotic-resistant bacterial genera cultured from the biofilms of the chosen polymers. The asparagine enrichment aiming at the isolation of human pathogen *P. aeruginosa* produced

positive results in 90% of the samples; however, when transferred to cetrimide agar, the number of positive strains with typical colony morphologies (green fluorescent pigment production and trimethylamine odour) decreased to 16. The results were categorized based on taxonomic classification at the genus level. Identified taxa included non-Enterobacteriaceae, primarily comprising *Aeromonas* spp., *Stenotrophomonas* spp., *Pseudomonas* spp. and a minor fraction of other non-Enterobacteriaceae, alongside members of the Enterobacteriaceae family.

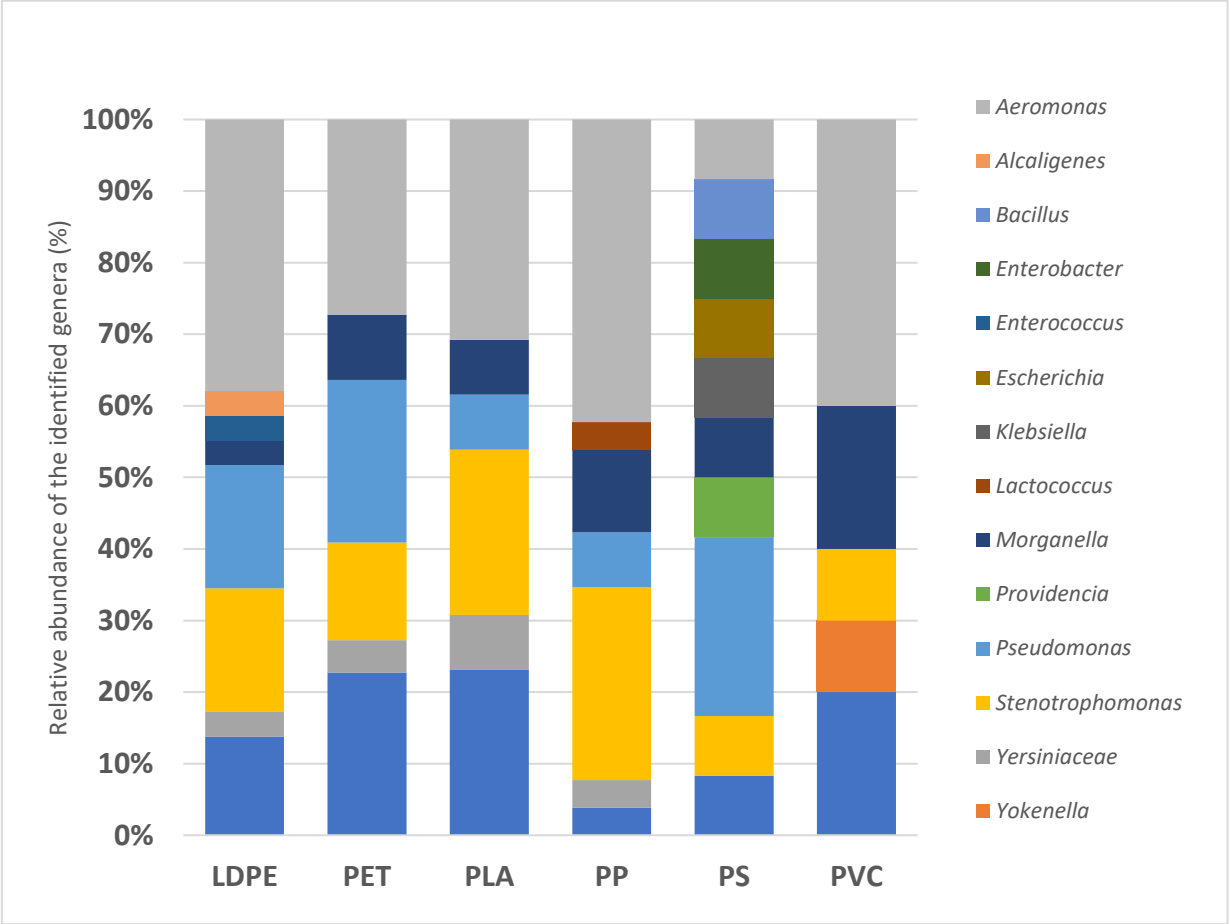

Figure 3. The relative abundance of antibiotic-resistant, cultivable bacteria isolated from six types of microplastics incubated in different phases of wastewater treatment

Note: LDPE - Low-density polyethylene, PET - polyethylene terephthalate, PLA - polylactic acid, PP - polypropylene, PS - polystyrene, PVC –polyvinyl chloride

Discussion

Overall, in this study, it is evident that MPs' material, besides other physicochemical and biological factors, influences the biofilm formation and growth of antibiotic resistant bacteria. Bacterial genera with a strong biofilm forming ability and bacterial taxa that normally inhabit aquatic environments were abundant in the plastisphere, as shown in our results. Some examples of the well-known biofilm-producing microbes from our results are *Pseudomonas* spp. (GHAFOOR et al., 2011), *Aeromonas* spp. (TALAGRAND-REBOUL et al., 2017) and *Stenotrophomonas* spp. (BROOKE, 2012).

PLA, PET and PVC supported the least formation of a diverse microbial community of ARBs primarily due to their physicochemical properties and biodegradation behaviour, which are less conducive to supporting a wide range of microbial colonizers. PLA has low degradability in

nature, and the bioavailable nutrients are also limited (EMADIAN et al., 2017; NARANCIC and O'CONNOR, 2019); it also has a high surface hydrophobicity, which is less favourable for microbial attachment and the formation of biofilms (DUSSUD et al., 2018). According to our results, PS had a more diverse microbial community due to its persistent nature, low hydrophobicity, and surface properties, which create a stable but selective niche that a wide variety of microbes can colonize over time (AMARAL-ZETTLER, 2020; DUSSUD et al., 2018; KIESSLING et al., 2015).

The previously mentioned factors influence which microbes attach to each polymer; some genera appeared only on one type (e.g., *Lactococcus, Alcaligenes* genera were only found on specific polymers like LDPE and PP) because it's uniquely suitable or tolerable for them. Studies have shown that *Escherichia* tends to colonize PS more readily than other MPs, due to its surface characteristics that favour bacterial adhesion and biofilm formation (STEVENSON et al., 2024), which is in accordance with our results.

The *Stenotrophomonas* genus has a high biofilm-forming ability in water and wastewater (MAHTO, 2022; SHEKHAWAT et al., 2020). Consequently, they were the most abundant in the biofilm communities of all polymer types. The presence of *Enterobacter, Escherichia, Klebsiella,* and *Yokenella* genera in wastewater-affected samples can be due to the fact that they are part of the normal gut microbiome of humans, reptiles and mammals (BANDY, 2020). These factors explain their abundance in the sewage-affected microbial communities of MPs' biofilms.

Aeromonas also reached a significant rate in the ARBs' composition of MPs. This genus naturally inhabits aquatic environments (BARGER et al., 2021) and can attach to biotic and abiotic surfaces to form biofilms (CHUNG et al., 2009). Several *Aeromonas* species are fish pathogens and are considered emerging human pathogen organisms (FERNÁNDEZ-BRAVO et al., 2020), which raises the necessity to evaluate their antibiotic resistance and preference in MPs colonization in the future.

Pseudomonas spp. were also dominant in the MPs microbial community, as these species can live in biofilms and are considered emerging waterborne pathogens (BERT et al., 1998). This genus also contains emerging human pathogens besides the well-known opportunistic pathogen species *P. aeruginosa*. Antibiotic-resistant *Pseudomonas* using Chromatic cultivation were detectable on four types of MPs (LDPE, PET, PP, PS); only PVC was negative for testing. The fact that PVC did not contain members of the *Pseudomonas* genus highlights the role of polymer types in the composition of the forming microbial community.

The rest of the cultivated antibiotic-resistant microorganisms were mainly identified as members of the *Providencia, Morganella,* and *Bacillus* genera and had a specific preference to colonize PS; all isolates of antibiotic-resistant *Enterobacter*, *Escherichia*, *Klebsiella*, *Providencia*, *Bacillus*, and *Yokenella* were recovered from this polymer type. These species were previously proven to be present in aquatic ecosystems with or without faecal pollution. According to LECLERC et al. (2001), Enterobacteriaceae that are naturally existing and those that are originating from animal faeces are expected to coexist, at least in certain areas of the urban water cycle (raw water, drinking water, and wastewater). In sewage, for example, strains of the genera *Enterobacter* or *Klebsiella* might multiply and become predominant members of the culturable microbiota. These bacteria also serve as representatives of the bacterial community found in drinking water (BRENNER, 1992; LECLERC et al., 2001; BLANCH et al., 2007). Widespread Enterobacteriaceae are capable of readily migrating throughout various urban water cycle compartments, such as from sewage and wastewater to surface water or, in some cases, enter the drinking water distribution system (FIGUEIRA et al., 2012). Based on our results, microplastics, especially PS, can act as vectors in their transmission.

Conclusions

Our findings indicate that the antibiotic-resistant microbial taxa identified on the incubated MPs' surface are commonly found in aquatic environments and wastewater; therefore, their origin is justified. The biofilm formation of these bacterial species is described in the scientific literature, which also influences their colonization on MPs. Although 14% of the culturable strains were not successfully identified by MALDI-TOF MS, it is still considered a reliable, easy and cheap tool for genus- or even species-level identification of plastic-associated, cultivable microorganisms. Many *Pseudomonas*, *Aeromonas*, *Stenotrophomonas* genus, and some members of the Enterobacteriaceae family (*Escherichia*, *Klebsiella, Enterobacter, Providencia)* are known human pathogens or considered as emerging pathogens according to the scientific literature. Based on our results, the detected differences in the diversity and composition of the microbial community are mainly related to polymer types, but the effect of the specific characteristics of WWTPs or the applied media used for cultivation are worth further investigation. Similarly, to verify the effect of the different polymer types on the non-cultivable microbial composition, next-generation sequencing techniques should be used in the future.

In the frame of our project, a notable collection of plastic-associated ARBs and *P. aeruginosa* was established, which can be deeply investigated and characterized in the future to determine phenotypic antimicrobial susceptibility, the presence of ARGs and mobile genetic elements. The characterization of the strain collection may clarify the role of different types of microplastics in the dissemination of antimicrobial resistance and facilitate the planning of future interventions to improve environmental safety.

Acknowledgments

This research was supported by 2020-1.1.2-PIACI-KFI-2021-00239 provided by the Ministry of Innovation and Technology of Hungary from the National Research, Development and Innovation Fund, financed under the PIACI KFI funding scheme. The scientific work of E. Kaszab was supported by the János Bolyai Research Grant of the Hungarian Academy of Sciences (BO/00236/20/4), and L. E. Mothoa was supported by the Stipendium Hungaricum scholarship. Project No. C2270802 has been implemented with the support provided by the Ministry of Culture and Innovation of Hungary from the National Research, Development and Innovation Fund, financed under the KDP-2023.

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