RESEARCH ARTICLE
Journal of Biometry Studies (2025) 5(2): 36-44
DOI: 10.61326/jofbs.v5i2.02

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Journal of Biometry Studies

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Identification of *Vibrio parahaemolyticus* found on plastics via matrix assisted laser desorption/ionization time of flight mass spectrometry

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Received: 24/11/2025, Accepted: 16/12/2025

Abstract

Plastic pollution has become a pervasive environmental threat in aquatic ecosystems worldwide, leading to the formation of microplastics that act as substrates for microbial colonization and potential pathogen transmission. This study investigated plastic-associated bacterial communities, with a focus on *Vibrio parahaemolyticus*, along the Western Mediterranean coast of Türkiye, particularly in the Manavgat river and adjacent coastal waters (0-5 m depth). The aim of this study was to characterize plastic-associated bacterial communities, particularly Vibrio parahaemolyticus, in plastic and water samples collected from the Manavgat River and adjacent Western Mediterranean coastal waters using FTIR, PCR, and MALDI-TOF MS. Plastic and water samples were analyzed using a combination of Fourier Transform Infrared Spectroscopy (FTIR), Polymerase Chain Reaction (PCR) and Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS). FTIR results identified polyethylene (PE), polypropylene (PP), polystyrene (PS), polyethylene terephthalate (PET) and high-density polyethylene (HDPE) as the dominant polymers. PCR amplification targeting the *GyrB* gene confirmed the presence of *V. parahaemolyticus* in biofilm-forming bacterial isolates from plastics and seawater. MALDI-TOF MS analyses further supported these findings, yielding genus-level identification scores (1.7-1.9) consistent with established classification thresholds. The results indicate that plastics serve as persistent reservoirs and transport vectors for potentially pathogenic bacteria, facilitating their survival and dissemination in aquatic habitats. This study underscores the significance of the plastisphere as a microbial niche and highlights the public health risks associated with plastic-associated biofilms. Further metagenomic and functional analyses are recommended to elucidate gene exchange dynamics and pathogenic potential within these biofilm communities.

Keywords: Plastic pollution, *Vibrio parahaemolyticus*, Plastisphere, MALDI-TOF MS, PCR, Mediterranean coast, Biofilm

Please cite this article as follows:
Gökdağ, K., & Çağatay, İ. T. (2025). Identification of *Vibrio parahaemolyticus* found on plastics via matrix assisted laser desorption/ionization time of flight mass spectrometry. *Journal of Biometry Studies*, *5*(2), 36-44. https://doi.org/10.61326/jofbs.v5i2.02

1. Introduction

Plastic pollution has emerged as a significant environmental concern to aquatic ecosystems globally (Barnes et al., 2009). Plastics accumulate in rivers, coastal zones and marine habitats due to their durability, low cost, and widespread commercial use (Morritt et al., 2014). Plastics undergo physical, chemical and biological degradation and eventually creating microplastics (<5 mm) (Thompson et al., 2004). After decades of persistence, these particles are now found in sediments, water bodies, biota and even air compartments (O'Brien et al., 2023). Between 1.15 and 2.41 million tons of plastic are thought to enter the ocean annually via rivers (Lebreton et al., 2017). Research indicates that approximately 900 species have been affected by marine litter with plastic constituting 92% of these encounters (Gall & Thompson, 2016). The International union for conservation of nature (IUCN) red list of threatened species including 17% of the species impacted by plastic (Gall & Thompson, 2016). A study by Kühn and Van Franeker (2020) identified 914 species of marine megafauna (including 226 seabird species, 86 marine mammal species, all sea turtle species and 430 species of fish) that are affected by entanglement and/or ingestion. Because microplastics can change water quality, enter food webs, and interact with a variety of chemical and biological pollutants, their ubiquity and endurance generate increasing worries about their effects on the environment and human health (Winiarska et al., 2024).

In addition to their chemical impacts, microplastics serve as carriers of a wide variety of microorganisms, including opportunistic and clinically significant diseases (Zettler et al., 2013). Their hydrophobic surfaces easily absorb contaminants and organic compounds, forming ideal microhabitats that improve microbe adhesion and survival during long distance transport (Bowley et al., 2021). Microplastics can help bacteria like *Vibrio*, *Pseudomonas*, and *Aeromonas* spp. spread throughout riverine and coastal habitats, according to studies, which raises worries about the introduction of dangerous germs into new biological niches (Zettler et al., 2013; Zhang et al., 2020). In areas where anthropogenic pollution and extensive aquaculture activities coexist, microplastics may contribute to the spread of waterborne diseases in addition to posing an ecological danger, according to this vector like activity (Bowley et al., 2021; Odioko & Becer, 2025).

Plastics are rapidly colonized by diverse microbial communities that create resilient biofilms on their surfaces after entering aquatic systems; this phenomenon is known as the "plastisphere" (Amaral-Zettler et al., 2020). These biofilms can create hotspots for gene exchange and microbial survival by enriching pathogenic bacteria and antibiotic-resistant strains at levels higher than those found in the water surrounding those (Amaral-Zettler et al., 2020). Because biofilms offer structural defense, bacteria become more resistant to environmental stress, disinfectants and antibiotics, which may improve the survival of pathogenic species like *Vibrio alginolyticus* and *V. parahaemolyticus* (Kirstein et al., 2016; Oberbeckmann et al., 2018). Because plastics, bacteria and antibiotic residues may coexist and interact in coastal locations, plastic-associated biofilms are a rising health risk for both ecosystems and human populations (Kirstein et al., 2016; Amaral-Zettler et al., 2020).

Studies have shown that *Vibrio* spp. species, isolated from various aquatic organisms and known to cause vibriosis disease, can be accurately identified using MALDI-TOF MS (Yavuzcan et al., 2022; Çağatay, 2024; Gökdağ & Çağatay, 2024). Dieckmann et al. (2010) compared the MALDI-TOF method and reported that a total of 83 *Vibrio* strains were identified. Erler et al. (2015) identified *Vibrio* species in approximately 100 environmental samples by comparing MALDI-TOF MS and some genes.

This study aims to isolate and identify plastic-colonizing bacteria from Türkiye’s Western Mediterranean coast, specifically from the Manavgat River and coastal water at depths of 0-5 m, using genomic PCR proteomics-based matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) techniques together.

2. Material and Method

2.1. Plastic sampling

16 samples were collected in April 2023 at the Manavgat River, discharging into the Western Mediterranean Coast of Türkiye, as well as in adjacent marine areas (0-5 m depth) (Table 1, Figure 1). 14 plastic particles were collected, while water sampling consisted of one seawater sample and two river water samples. Water samples and plastic fragments found along river and coastal zone were collected and placed separately into sterile falcon tubes and 250 ml bottles. During transport to the laboratory, insulated containers with cold chains were used to preserve sample integrity. Upon arrival, water samples were filtered through Whatman filters (Whatman, UK) with a pore size of 0.45 µm and stored at -20°C until further analysis. All collected samples were inoculated into tryptic soy broth (TSB) (Condalab, Spain) and alkaline peptone water (Condalab, Spain) within 24 hours of arrival.

Table 1. Coordinates of the sampling stations

Stations	Lang	Long
MD1	36,805	31,340
MN2	36,747	31,472
MN3	36,744	31,478

2.2. Bacterial growth

Solid and liquid media were used for bacterial isolation from reference bacteria (*V. parahaemolyticus* ATCC 18802), water and plastic samples. To promote the selective growth of *Vibrio* spp., commonly used as selective agars such as chromogenic agar (VCA) (Condalab, Spain) and Thiosulfate-citrate-bile salts-sucrose agar (TCBS) (Condalab, Spain) were applied in parallel with TSA, TSB and nutrient agar (Difco™, France) for the same samples (Di Pinto et al., 2011; Kirstein et al., 2016). Samples were incubated at 25-30°C for 24-48 hours.

2.3. Polymer analyses of plastics with fourier transform infrared spectroscopy (FTIR)

Biofilm structures were examined on eleven different types of plastic samples collected from a total of three stations located at Manavgat River and the coastal area (0-5 m depths). Polymer analyses were carried out using a spectrum 400 fourier transform infrared spectroscopy (FTIR) and FT-NIR spectrometer (Perkin Elmer, USA).

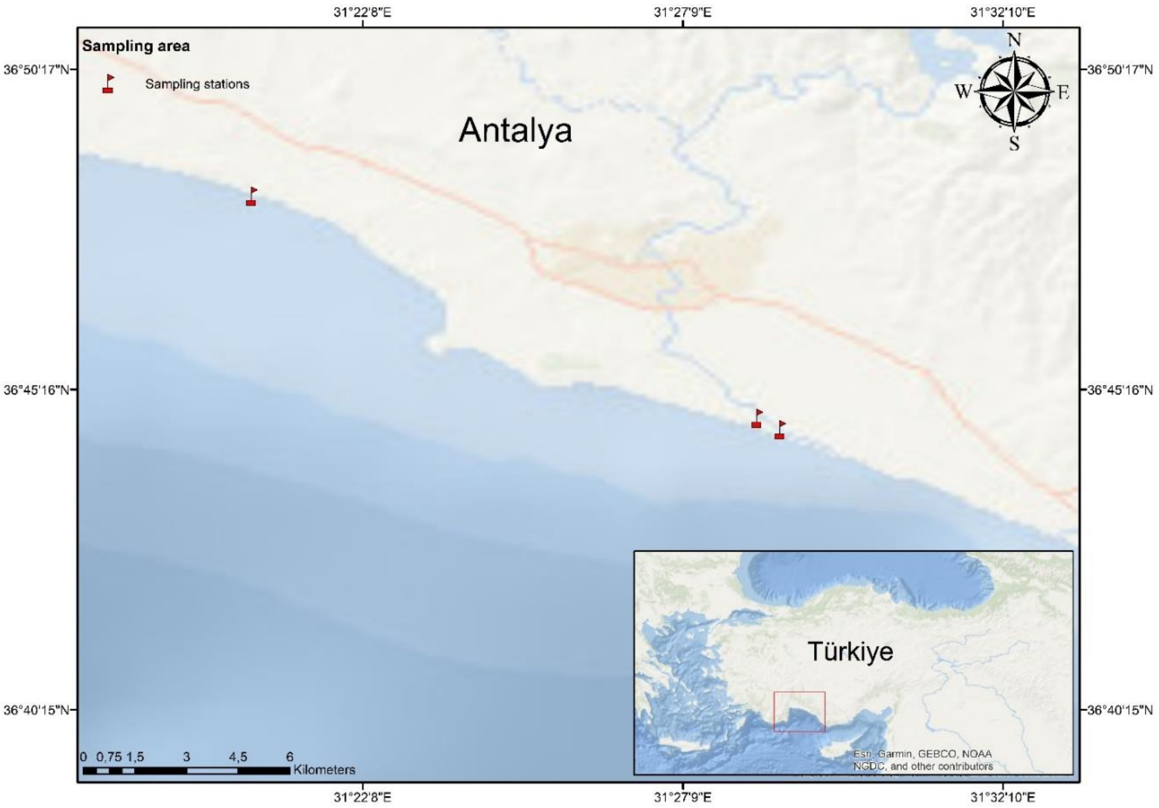


Figure 1. Sampling area

2.4. Molecular detection of *Vibrio* spp. on plastics

DNA isolation method was developed by modifying the protocols described by Comey et al. (1994) and Butler (2011). 1.5 mL of bacterial culture grown in medium for 24-48 hours was transferred into sterile tubes using sterile micropipettes and centrifuged at 5000 rpm for 10 minutes for genomic DNA extraction. The process was repeated until a sufficient pellet was obtained. 500 µL of SET buffer (75 mM NaCl, 25 mM EDTA, and 20 mM Tris-HCl, pH 7.5) (Sigma-Aldrich, Germany) was added to the pellet after removing the supernatant and vortexed until fully resuspended. The tubes were incubated at 95°C for 10 minutes and then allowed to cool to room temperature.

To denature proteins, 50 µL of SDS (sodium dodecyl sulfate) (Sigma-Aldrich, Germany) and 50 µL of Proteinase K (1 mg/mL) (Sigma-Aldrich, Germany) were added. Samples were incubated at 55°C for 2 hours to ensure complete protein degradation. Following incubation, 500 µL of phenol/chloroform/isoamyl alcohol (24:25:1) (Sigma-Aldrich, Germany) was added and the tubes were gently inverted for 5 minutes at room temperature. Samples were centrifuged at 10,000 rpm for 5 minutes, and the upper aqueous phase was transferred to a new sterile tube. An equal volume of chloroform/isoamyl alcohol (24:1) was added, mixed by gentle inversion for 5 minutes, and centrifuged again at 10,000 rpm for 5 minutes. The resulting upper phase was transferred to a new tube, and 1 mL of cold 99% ethanol was added to precipitate the DNA. After centrifugation at 10,000 rpm for 5 minutes, the supernatant was carefully removed. The pellet was washed with 1 mL of 70% ethanol by gentle inversion, followed by centrifugation at 10,000 rpm for 5 minutes. The supernatant was discarded, and the tubes were air-dried at 37°C under frequent monitoring until completely dry. The dried DNA pellets were dissolved in 50-100 µL of 1×TE buffer (10 mM Tris, 1 mM EDTA) depending on pellet size. The purified DNA samples were stored at -20°C for subsequent analyses.

2.5. PCR analyses

Vibrio parahaemolyticus specific primer targeting the *GyrB* gene (DNA Gyrase subunit B) was used to detect bacteria. PCR analysis was conducted by modifying the methods described by Venkateswaran et al. (1998). The primers, targeted gene regions, PCR thermal cycling conditions and references are presented in Table 2.

The PCR reaction mixture consisted of 10 µL PCR buffer (×10), 10 µL $MgCl_2$ (100 mM), 1 µL dNTP mix (10 mM each), 3.1 µL of each forward and reverse primer (50 µM), and 1 µL Taq DNA polymerase (5 U/µL) (Qiagen,USA), adjusted to a final volume of 25 µL with deionized water (Pascual et al., 2010). Amplified PCR products were verified by electrophoresis on 1.5% agarose (Sigma-Aldrich, Germany) gels containing 2 µL ethidium bromide (15 mg/mL) (Merk, Germany). To confirm the accuracy of the amplified gene regions, one randomly selected amplicon from each target gene was submitted for sequencing through a commercial sequencing service.

Table 2. Primers and PCR conditions

Primer name	Primer sequences (5'-3')	Amplicon size (bp)	PCR Conditions	Reference
GyrB	Forward -CGG CGT GGG TGT TTC GGT AGT Reverse-TCC GCT TCG CGC TCA TCA ATA	285	94°C 1min 94°C 1min 58°C 1:30 sec X30 72°C 2:30 sec 72°C 7 min	Venkatesnran et al. (1998)

2.6. MALDI-TOF MS analyses

The formic acid extraction method was applied for bacterial identification using the MALDI-TOF MS system (Bruker Microflex) (Wu et al., 2020). Prior to analysis, bacterial colonies exhibiting distinct morphological characteristics were individually cultured in liquid media to obtain pure isolates. These isolates were sub-cultured as single colonies on TSA, VCA, TCBS, and nutrient agar plates for further bacterial growth. The direct transfer method was employed for MALDI-TOF MS analysis.

MALDI-TOF MS (Bruker Microflex, USA) analyses were performed within 12 hours of bacterial cultivation. For sample preparation, a single bacterial colony from each plate was picked using a sterile toothpick and gently smeared as a thin layer onto the metal 96-well target plate specific to the instrument. Subsequently, 1 µL of formic acid solution was applied to each spot and allowed to dry at room temperature. Within one hour after drying, 1 µL of matrix solution was added to each spot. Once dried at room temperature, the prepared target plate was loaded into the instrument for analysis.

3. Results and Discussion

3.1. Morphologic identification

Isolates were taken from biofilm from plastic surface were inoculated on VCA, TCBS, TSA, TSB and nutrient agar at 25-30°C for 24 hours. Colonies on VCA and TCBS plates were generally green, orange, circular with diameters between 0.2 and 0.4 mm (Figure 2).

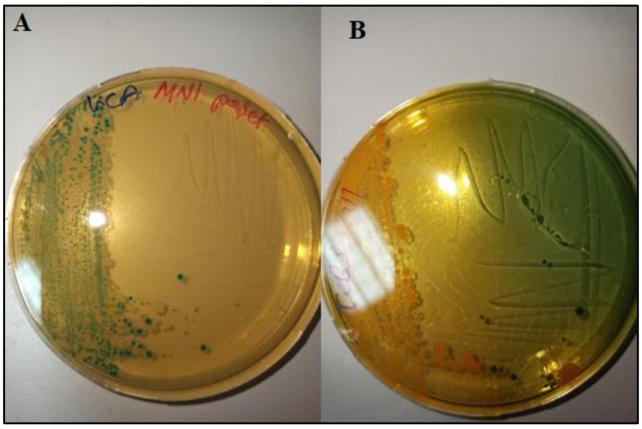


Figure 2. Colony morphology of *Vibrio parahaemolyticus* isolated from (A)Vibrio Chromogenic Agar (VCA) and (B) Thiosulfate-Citrate-Bile Salts-Sucrose Agar (TCBS)

3.2. Identification of Vibrio parahaemolyticus isolates on biofilm-form with PCR

To detect *Vibrio* spp. in the plastisphere (biofilm), species-specific PCR was performed using *GyrB*-Forward and *GyrB*-Reverse specific primers. As a result of this study, *V. parahaemolyticus* species were detected in different samples. The PCR amplicon bands obtained for *V. parahaemolyticus* were visualized on agarose gels. The resulting amplicon sizes were 285 bp for *V. parahaemolyticus*, as shown in Figure 3. For *V. parahaemolyticus*, the *GyrB* primers were used in studies by Vongxay et al. (2006) and Venkateswaran et al. (1998). The PCR bands are presented in Figure 3 and demonstrate consistency between the results of this study and the previous literature.

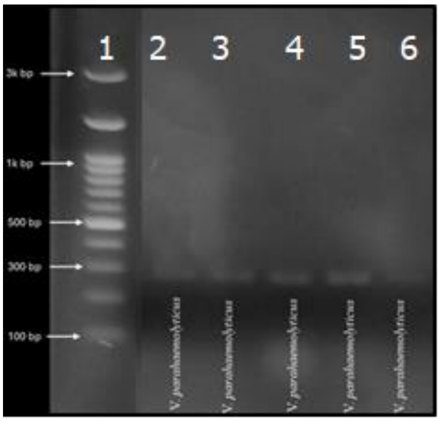


Figure 3. Molecular marker (1), *GyrB* gene amplicons from biofilm of plastics (2-6)

3.3. FTIR analyses of plastic samples

The photographs of plastics (Figure 4) and the FTIR based polymer analysis graphs are shown (Figure 5). The polymer readings were measured in the range of 650-4000 cm^{-1} and the spectra of the identified polymers are presented in the Figure 5.

FTIR analyses revealed the presence of polyethylene (PE), polyethylene terephthalate (PET), polypropylene (PP) (isotactic), polystyrene (PS), propylene-acrylic acid copolymer, and high-density polyethylene (HDPE) polymers. According to Plastics Europe (2023), polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS), polyethylene terephthalate (PET), polyurethane (PUR), thermoplastics and thermoset plastics together accounted for 90% of the global plastic production of 400.3 million tons in 2022. Munari et al.

(2016) reported that plastics along the Adriatic Sea coasts mainly consisted of polyethylene (HD-PE and LD-PE), polypropylene (PP), and polyamide (PA). In Lebanon, polypropylene (PP), polyethylene (PE), polystyrene (PS), polyamide (PA), polyethylene terephthalate (PET), and polyurethane (PUR) were the most frequently detected polymers in seawater, sediment, and fish digestive tract samples (Kazour et al., 2019). Constant et al. (2017) found PE, PS, and PP as the most common polymers in coastal Spain, while Baini et al. (2018) identified PE, PP, PS, ethylene-vinyl acetate (EVA), and styrene-butadiene (SBR) as dominant polymers. Studies from the Turkish Mediterranean coasts have also reported PS, PE, and PP copolymers as dominant in seawater samples (Güven et al., 2017). *V. parahaemolyticus* was detected in four PET plastic fragments and in one seawater sample. However, because all positive plastics belonged to the same polymer category (PET) and the overall sample size was limited, no meaningful comparison could be made regarding polymer-specific detection frequency or potential patterns in MALDI-TOF scores or PCR positivity across different plastic types. The small number of samples represents a methodological limitation, and therefore polymer-dependent inferences should be interpreted with caution

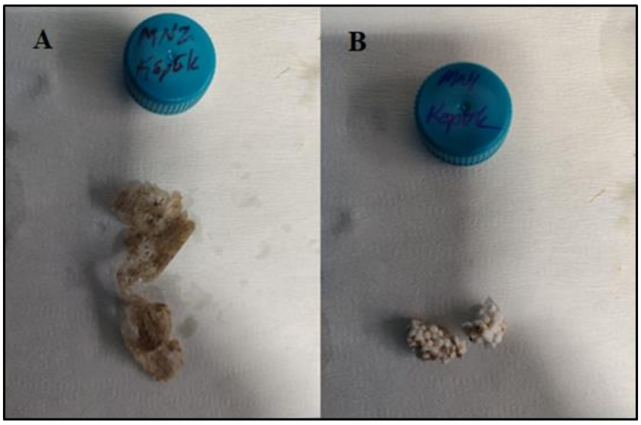
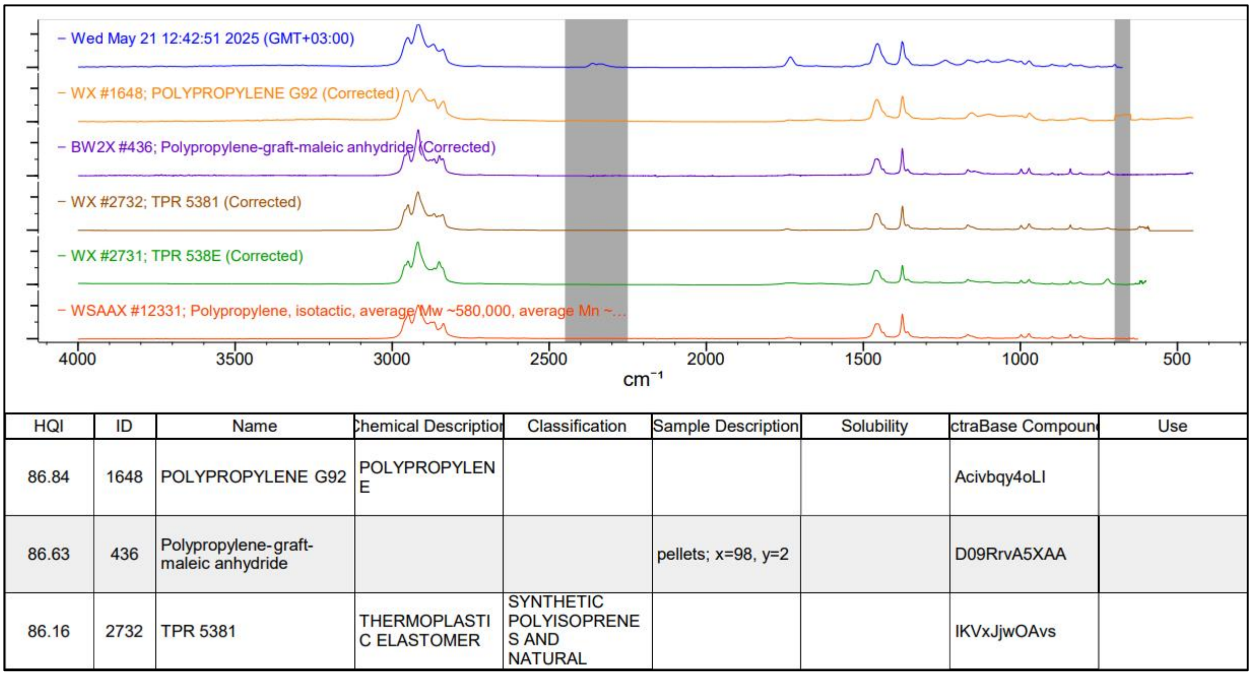


Figure 4. The photographs of plastics



HQI	ID	Name	Chemical Descriptio	Classification	Sample Description	Solubility	ctraBase Compoun	Use
86.84	1648	POLYPROPYLENE G92	POLYPROPYLENE				Acivbqy4oLI	
86.63	436	Polypropylene-graft-maleic anhydride			pellets; x=98, y=2		D09RrvA5XAA	
86.16	2732	TPR 5381	THERMOPLASTIC ELASTOMER	SYNTHETIC POLYISOPRENES AND NATURAL			IKVxJjwOAvs	

Figure 5. Sample of FTIR analyses result

The high abundance of PE and PP polymers in these studies is linked to their low specific gravity (0.89-0.95 g/cm^3 and 0.85-0.92 g/cm^3, respectively), which allows them to float and accumulate along coastal areas due to water currents (Andrady, 2011; Jayasiri et al., 2013; Enders et al., 2015; Zhao et al., 2015; Munari et al., 2016). PE polymers, with an annual production of about 80 million tons, are primarily used in packaging, plastic bags, films, and bottle manufacturing (Thompson et al., 2004). PP polymers, with an annual production of around 50 million tons, are widely used in packaging and in manufacturing reusable containers, stationery, and textiles (Thompson et al., 2004; Jayasiri et al., 2013; Zhao et al., 2015; Munari et al., 2016). Single use PE and PP based items such as plastic bags, ropes, and fishing lines are commonly detected along both Mediterranean and global coastlines (Eriksen et al., 2014; Suaria et al., 2016).

3.4. Identification of Vibrio parahaemolyticus isolates on biofilm form with using MALDI-TOF MS

A bacterial colony of *Vibrio* spp. for identification with using MALDI-TOF MS were placed on a plate and exposed to laser shots. To facilitate efficient absorption of laser energy by the bacterial colony and proton transfer to the molecules, a matrix was added, enabling molecular ionization. Following ionization, gas phase ions were released as free molecules. The signal intensity peaks of the ionized molecules or component proteins were carried to the detector and compared with the reference database for species-level microbial identification. The bacteria identified using the MALDI-TOF MS method and their score values are listed in Table 3.

Table 3. MALDI-TOF scores of *Vibrio parahaemolyticus*

Sample	Species Identification	Score value
Polyethylene terephthalate (PET)	*V. parahaemolyticus*	1.741
Sea water	*V. parahaemolyticus*	1.783
Polyethylene terephthalate (PET)	*V. parahaemolyticus*	1.737
Polyethylene terephthalate (PET)	*V. parahaemolyticus*	1.727
Polyethylene terephthalate (PET)	*V. parahaemolyticus*	1.985

The identification of *Vibrio* spp. is often time-consuming due to their diverse phenotypic traits and long culture durations, which make biochemical identification difficult (Wu et al., 2020). The MALDI-TOF MS method allows rapid and accurate species-level identification using minimal sample volumes (De Bruyne et al., 2011; Malainine et al., 2013; Afanasev et al., 2014; Kirstein et al., 2016; Kapetanović et al., 2023; Bielen et al., 2024; Gökdağ & Çağatay, 2024). It also enables large-scale analysis of isolates from clinical and environmental samples (Dieckmann et al., 2010). This study did not evaluate virulence factors such as the tdh and trh genes or antibiotic resistance profiles; therefore, the virulence potential of the detected *V. parahaemolyticus* isolates remains unknown. Accordingly, statements regarding public health implications should be interpreted cautiously, as the presence of Vibrio on plastics and in seawater does not indicate clinical relevance. Future studies should include virulence-gene screening to better assess pathogenic potential.

Malainine et al. (2013) analyzed seawater, sediment, and mussel samples from Khnifiss Lagoon (Morocco) and identified *V. parahaemolyticus* using MALDI-TOF MS, highlighting its reliability. Afanasev et al. (2014) emphasized the device's high efficiency, ease of use, and low operational cost. Bielen et al. (2024) compared MALDI-TOF MS with 16S rDNA sequencing in mussel isolates and concluded that an expanded database would make it the fastest and most effective tool for culturable bacterial identification. Kirstein et al. (2016) were the first to identify biofilm-forming *Vibrio* spp. (*V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae*) on plastics using MALDI-TOF MS. Kapetanović et al. (2023) also confirmed its high specificity and sensitivity for *Vibrio* detection in plastics, seawater, and sediment samples.

MALDI Biotyper RTC (Bruker®) software assigns score values between 0.00-3.00 (Jansson et al., 2020; Çağatay, 2024). According to the traffic-light classification: green (2.0-3.00) indicates reliable species-level identification, yellow (1.7-1.99) reliable genus-level identification, and red (<1.70) low-confidence or unidentified results. Species (*V. alginolyticus* and *V. parahaemolyticus*) had score values ≥1.7, consistent with previous literature findings, as shown in Table 3.

4. Conclusion

Plastics, as pervasive hydrophobic and hydrophilic pollutants, accumulate in both terrestrial and aquatic environments due to improper disposal and degradation. Their fragmentation into microplastics (<5 mm) enables ingestion by marine organisms, leading to mortality and potential biomagnification through the food chain, posing health risks to humans and other species. Given the continuous rise in global plastic production and consumption, plastic-associated pollution and its ecological impacts are expected to intensify.

This study demonstrated that plastics serve as substrates for microbial colonization of *V. parahaemolyticus*. These biofilms not only promote microbial survival and interaction but also host pathogenic taxa such as Vibrio spp., potentially facilitating pathogen persistence and gene transfer. These findings highlight the need for region-specific monitoring programs and evidence-based policies aimed at reducing plastic inputs and assessing pathogen-bearing biofilms on aquatic plastics. Further research should investigate interactions among resistance-related genes and the impact of single or multiple copies of resistance genes on antibiotic resistance in *V. parahaemolyticus*. Understanding the mechanisms governing biofilm development on microplastics, along with their roles in pollutant adsorption, antibiotic resistance, and bioremediation, requires further investigation, including whole-genome analyses of associated microbial communities.

Acknowledgement

This study, derived from a PhD thesis, was funded by the Scientific Research Projects Coordination Unit of Akdeniz University under project number FDK-2022-5901.

Conflict of interest

The authors declare no conflict of interest.

Ethical Approval

This arcticle does not require ethics committee approval.

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