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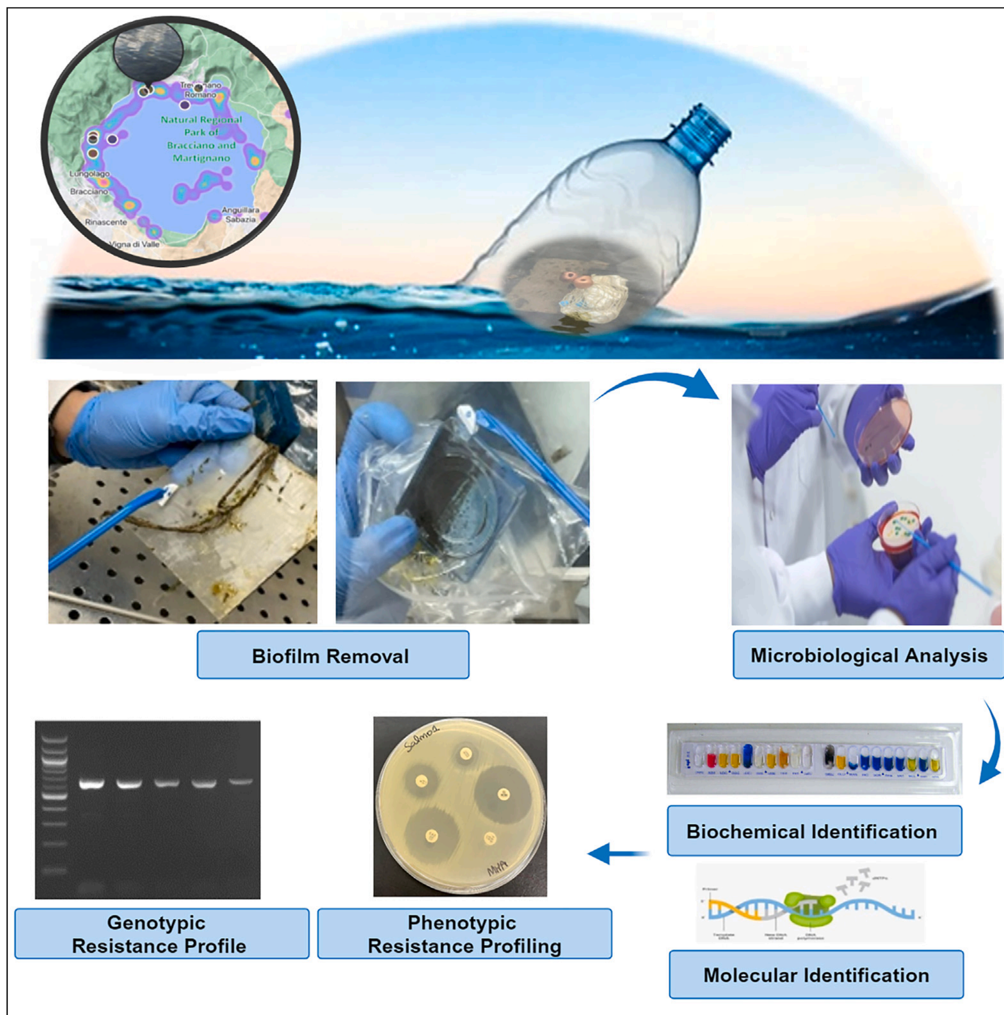

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Highlights

Plastic plays a vital role in disseminating superbugs in aquatic ecosystem

Plastic-associated antibiotic resistant bacteria pose hidden risks for humans

Detection of emerging and re-emerging pathogens in environmental sector is threatening

Improving One-Health prevention by enhancing the detection of AMR in aquatic ecosystem

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Vehicle transmission of antibiotic-resistant pathogens mediated by plastic debris in aquatic ecosystems

Ifra Ferheen,[1] Roberto Spurio,[1] and Stefania Marcheggiani[2,3,*]

SUMMARY

Plastic materials are emerging environmental pollutants acting as potential vehicles for accumulation and spread of multidrug-resistant bacteria. The current study investigates the role of plastics in favoring the dispersal of specific pathogens and their associated antibiotic resistant genes (ARGs). Artificial plastic substrates (APSs) were submerged in seven sampling points of Lake Bracciano (Italy), and after one-month both APSs and raw water (RW) samples were collected. Through the combination of standard microbiological and biochemical techniques, 272 bacterial strains were identified and characterized for antibiotic resistant profiling. Our results revealed a notable difference in terms of diversity and abundance of pathogenic bacteria recovered from APSs, compared to RW. In addition, higher resistance patterns were detected in APSs isolates, with frequent appearance of relevant ARGs and class 1 integrons. These findings reinforce the idea that plastic materials in aquatic ecosystems serve as a reservoir for superbugs, significantly contributing to the dissemination of ARGs.

INTRODUCTION

Plastic waste in aquatic ecosystems has been considered a significant environmental and health problem for decades; the widespread consumption of plastic products has worsened the issue, leading to the emergence of new chemical contaminants. In addition, the plastic debris surface can also serve as new attachment sites and stable habitats for biofilm-forming bacteria and their spread.[1] One of the less explored features of these biofilms, is their potential to act as "hotspots" for horizontal gene transfer (HGT),[2–4] leading to the exchange of genetic material, allowing the spread of traits such as antibiotic resistance.[5] The rise of antibiotic resistance has become a significant concern for global public health.[6] The increase in morbidity and mortality rates due to multidrug-resistant bacteria (MDR), and the associated healthcare costs are all attributed directly to improper use of antibiotics.[7] According to the World Health Organization, antibiotic-resistant bacteria (ARB) and antibiotic-resistant genes (ARGs) are the two major concerns to public health in the twenty-first century.[8,9]

Antibiotic-resistant pathogenic bacteria pose a substantial hazard to human health because they can lead to serious infections that are challenging to treat.[6,10–12] From a One-Health perspective, the widespread distribution of MDR bacteria in aquatic ecosystems is threatening, as these bacteria can spread to new hosts and potentially infect other organisms within the ecosystem.[13,14]

During the last few decades, extended-spectrum β-lactamases (ESBLs) produced by pathogenic bacteria constitute a worldwide concern associated with treatment complications and high mortality rates.[15,16] The rapid dissemination of ESBL-producing strains, and the horizontal gene transfer mediated by plasmids harboring *β-lactamase* genes are regarded as key factors contributing to this global issue for animal, human, and ecosystem health.[16–18]

Pathogens and plastic particles can both cause various health problems[19–21]; for this reason, environmental and medical researchers are increasingly investigating the combined effect of the two factors, namely the ability of polymers to bind and disperse potential pathogenic species to new hosts and in the environment.[2,19,22] Considering the polymers' hydrophobic surface, plastic debris can serve as new habitats, providing sites of attachment for bacteria, leading to biofilm formation and acting as a vehicle for pathogenic microorganisms and their associated genetic determinants. Marine ecosystems have received significant attention, but the impact of plastic debris on freshwater bodies remains inadequately understood. The concentration of MDR bacteria and ARGs mediated by plastic materials in freshwater bodies ultimately leads to their transfer to humans through direct contact, water reuse, and the food chain, posing significant risks to human health.[23,24]

Our research intends to bridge this knowledge gap by investigating the potential role of freshwater environments in facilitating the spread of plastic-associated superbugs. In the present study, through a comparative analysis of multi-drug resistance bacteria residing in raw water (RW) and those colonizing on artificial plastic substrates (APSs), we have investigated the role of plastic particles as potential hotspots for the colonization, amplification, and dispersal of bacteria known to cause diseases in humans and animals.

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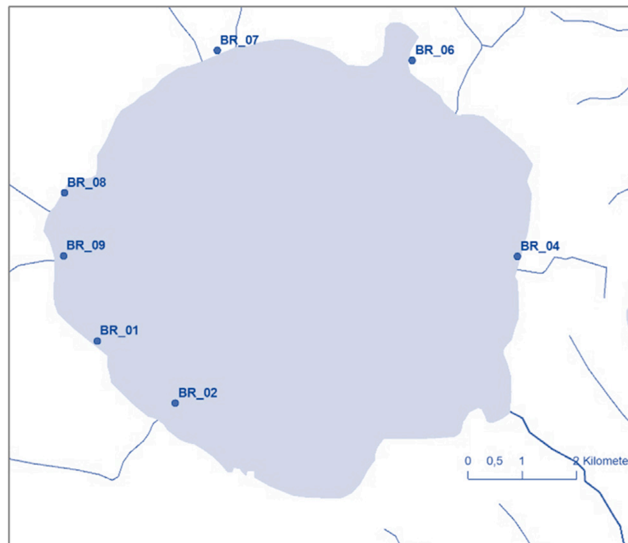

Figure 1. Geographical localization of sampling sites

Geographical coordinates of the seven sites selected for the sampling campaign at Lake Bracciano (Italy): BR-01 E; 42°06′27.06″ N; 12°11′07.35", BR-02 E; 42°05′78.00 "N; 12°12′04.83", BR-04 E; 42°07′08.34 "N; 12°16′47.80", BR-06 E; 42°09′08.60″N; 12°15′29.27″, BR-07 E; 42°09′20.66″N; 12°12′43.68", BR-08 42°07′56.87″N; 12°10′45.57″, BR-09 E; 42°07′18.98″N; 12°10′42.92".

RESULTS

The present study involved seven different geographical sites located along the banks of Lake Bracciano, Latium Region (Italy) Figure 1. The site selection process was based on considering land cover within a 2-kilometer radius of each site, using the "*Geographical Information System* "ArcGIS (Figure S1). Specifically, the BR-01, BR-02, and BR-06 sites were found to be in close proximity to the discontinuous urban fabric, and over 45% of their area comprised complex cultivation patterns. On the other hand, the BR-04, BR-07, BR-08, BR-09 sites exhibited a predominance of broad-leaf forest cover and were located close to farmhouses, beaches, tourist attractions, and recreational activity sites.

Abundance of bacteria isolated from an aquatic ecosystem

Overall, 272 bacterial strains were isolated from both RW samples and artificial plastic substrates (APSs) (Figure S2) in the three sampling campaigns conducted in the summer, winter, and spring seasons Figure 2. The bacterial richness and composition along three sampling campaigns was evaluated by principal-component analysis (PCA) (Figure 3A) and exhibited for APSs samples a descending order (winter > summer > spring), consistent with the trend observed for the RW samples. The Pearson correlation analysis demonstrates a significant correlation in terms of bacterial abundance (referring to the selected panel of cultivable bacteria) in RW winter and RW summer samples ($r = 0.92$), in APSs summer and APSs spring samples ($r = 0.87$). The correlation analysis of RW samples compared to APSs samples revealed a significant correlation for APSs summer and RW summer ($r = 0.84$) and APSs spring and RW spring ($r = 0.87$). Moderate and low levels of correlation were observed in all the other cases, as illustrated in Table S1.

Identification of environmental isolates

Biochemical identification of 272 environmental bacterial isolates (Table 1) depicted a noteworthy diversity and abundance of ESBL producing bacteria between APSs and RW samples. In particular, the majority of ESBL-producing isolates from APSs belonged to *Escherichia coli*, *Vibrio fluvialis, Klebsiella pneumoniae*, *Burkholderia pseudomallei, Proteus vulgaris*, *Aeromonas salmonicida*, *Chryseobacterium indologenes*, and *Serratia odorifera*. Bacteria isolated from RW samples included *Pseudomonas putida*, *Pseudomonas alcaligenes*, *Burkholderia gladioli*, *Aeromonas hydrophila*, and *Serratia liquefaciens*. Among the non-ESBL producing strains, the dominant species in APSs samples were *S. liquefaciens*, *S. marcescens*, *Citrobacter freundii*, *Cronobacter* spp., *Erwinia* spp., *Enterobacter cloacae*, *B. gladioli*, *E. coli*, *V. fluvialis*, *Salmonella enterica*, and a few gram-positive bacteria, including *Staphylococcus cohnii*, *Staphylococcus lantus*, *Staphylococcus intermedius*, *Staphylococcus sciuri*, and *Staphylococcus aureus*, while from RW samples *E. coli.*, *V. fluvialis*, *S. liquefaciens*, *S. odorifera*, and *S. marcescens* were recovered. Cluster analysis was performed to evaluate the similarity and dissimilarity of environmental isolates among APSs and RW samples collected in three seasons. The dendrogram revealed the clustering into two primary groups, RW, and APSs, with three subgroups representing seasonal variations.

16S rDNA sequencing-based identification of 42 isolates derived from RW ($n = 11$) and APSs ($n = 31$) samples revealed the predominance of strains belonging to different species of *Serratia*, *Pseudomonas*, *Aeromonas*, and *Lysinobacillus*. Furthermore, a few bacterial strains exclusively isolated from APSs samples, were identified as *Morganella morganii*, *Citrobacter freundii*, *Klebsiella* spp., *Exiguobacterium* spp.,

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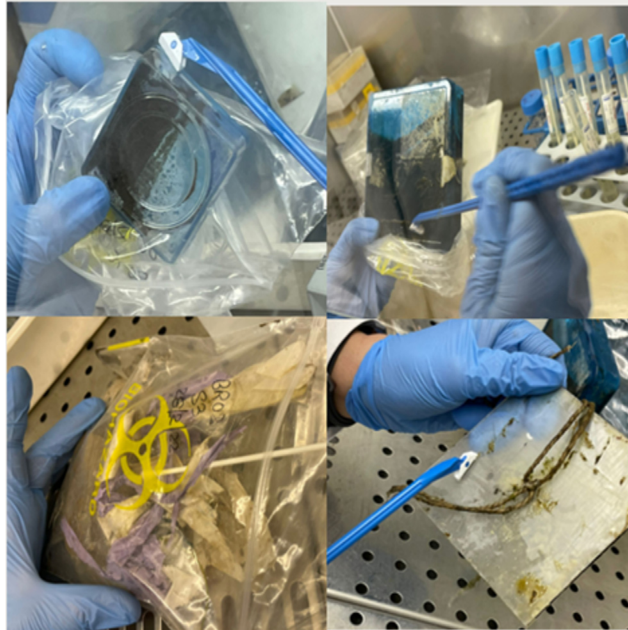

Figure 2. Biofilm removal from artificial plastic substrate

Recovery of surface-attached bacteria from four plastic polymers used as artificial plastic substrates, under sterile conditions.

Enterobacter cloacae, and *Salmonella enterica*. The resulting DNA sequences were submitted to NCBI database with corresponding accession numbers detailed in key resource table.

In some cases, when we tried to superimpose the results of the biochemical identification with the molecular analysis based on the 16S rDNA sequence, we realized that the commercial biochemical kits display low efficiency in the identification of environmental bacterial strains either at the genus level or at the species level. Our results showed that 11 strains biochemically identified as *Vibrio fluvialis* were not confirmed by rDNA sequence analysis, as two of them were *Serratia* spp., three *Aeromonas* spp., three *Morganella morganii*, one *Pseudomonas* spp., one *Dickeya zeae*, and one *Klebsiella* spp. In a similar trend, out of nine strains recognized as *Staphylococcus* spp., six were identified as *Lysinibacillus* spp., and three as *Exiguobacterium* spp., based on molecular identification (Table 2).

Antimicrobial resistance profiling of environmental isolates

The prevalence of antibiotic resistance bacteria was significantly higher among APSs isolates (89.1%), as compared to the rate observed for RW isolates (61.3%) (Figure 4). The antibiotic resistance profile of gram-negative bacteria (APSs = 130 and RW = 101) revealed the following

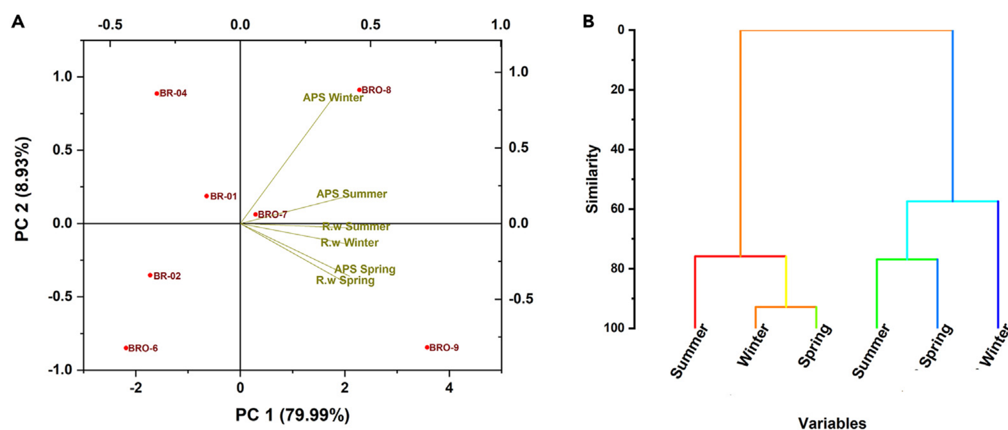

Figure 3. The principal-component and cluster analysis of environmental isolates

(A) The PCA biplot depicts the relationships among samples based on the measured variables, including RW (summer, winter and spring samples), APSs (summer, winter, and spring samples), in relation to the seven selected sampling sites.

(B) Hierarchical cluster analysis based on the similarity of 272 biochemically identified isolates in three seasons.

Table 1. List of isolates recovered from artificial plastic substrate (APS) and raw water (RW), biochemically identified up-to-genus level

Environmental isolates	RW samples (n = 101)	APSs samples (n = 171)	Summer (n = 85)	Winter (n = 118)	Spring (n = 69)	Enterobacteriaceae member	ESBL production	Pathogenicity
Vibrio spp. (n = 19)	0%	11.1%	31.5%	42.1%	26.3%	No	Yes	Cholera, wound infections, gastroenteritis, septicemia[43]
Serratia spp. (n = 41)	20.7%	11.6%	34.4%	39.0%	26.8%	Yes	Yes	Pneumonia, urinary tract infections, wound infections, sepsis[44]
Pseudomonas spp. (n = 44)	22.7%	12.2%	29.5%	40.9%	29.5%	No	Yes	Pneumonia, urinary tract infections, sepsis, wound infections[25]
Burkholderia spp. (n = 35)	13.8%	11.1%	34.2%	42.8%	22.8%	No	Yes	Respiratory infections, skin infections, sepsis[45]
Klebsiella spp. (n = 14)	0%	8.1%	35.7%	42.8%	21.4%	Yes	Yes	Pneumonia, urinary tract infections, sepsis[46]
Chryseobacterium spp. (n = 5)	0%	2.9%	40.0%	60.0%	0.0%	No	Yes	wound infections, sepsis[47]
Aeromonas spp. (n = 41)	23.7%	9.9%	24.3%	43.9%	31.7%	No	Yes	Gastroenteritis, wound infections, sepsis[48]
Enterobacter spp. (n = 11)	0%	6.4%	36.3%	54.5%	9.0%	Yes	No	Pneumonia, urinary tract infections, sepsis[49]
E. coli (n = 28)	18.8%	6.4%	32.1%	25.0%	25.0%	Yes	Yes	Gastroenteritis, urinary tract infections, Shiga toxin-producing *E. coli* (STEC) infections[50]
Salmonella spp. (n = 4)	0%	2.3%	25.0%	50.0%	25.0%	Yes	No	Salmonellosis, typhoid fever[51]
Erwinia spp. (n = 2)	0%	1.1%	0.0%	100%	0.0%	Yes	No	Plant diseases[52]
Cronobacter spp. (n = 4)	0%	2.3%	25.0%	50.0%	25.0%	Yes	No	Neonatal meningitis, sepsis[53]
Proteus spp. (n = 3)	0%	1.7%	33.3%	66.6%	0.0%	Yes	Yes	Urinary tract infections, wound infections[54]
Staphylococcus spp. (n = 21)	0%	12.2%	33.3%	38.0%	28.5%	No	No	Skin infections, respiratory infections, sepsis[55]

Shapiro-wilk analysis illustrates that “RW Summer”, “RW Winter”, “RW Spring” and “APSs Spring” samples showed a p value ≤ 0.05, indicating non-normal distribution. Conversely, for “APSs Winter” and APSs Summer p value ≥ 0.05 suggests normal distribution for the obtained data. The Chi-square test revealed a p value < 0.05 indicating a significant difference between RW and APSs samples.

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Table 2. Experimental methods and conditions adopted for microbiological analysis

Targeted bacteria	Aliquots analyzed (RW Sample)	Methods	Growth medium	Growth conditions	Colony phenotype
Staphylococcus spp.	1mL	Isolation by streak plate method	Rapid Staph Agar	44°C for 48 h	Black
Salmonella spp.[56]	1L	1) Membrane filtration and pre-enrichment 2) Enrichment 3) Isolation by streak plate method	1) Peptone water 2) Rappaport Vassiliadis Broth company 3) Xylose Lysine Deoxycholate Agar (XLD) Chromogenic agar	1) 37°C for 18 h 2) 44°C for 24 h 3) 37°C for 24 h	Turbidity and Black Color
KESC (Klebsiella, Enterobacter, Serratia, Citrobacter) and Proteaceae *(Proteus, Morganella Providencia)*	500 mL	1) Membrane filtration and pre-enrichment 2) Enrichment	1) Muller-Hinton (MH) Broth 2) Chrome Art Extended Spectrum Beta Lactamase agar (ESBL)	1) 37°C for 18–24 h 2) 37°C for 18–24 h	Turbidity Blue/Green Brown White Red
Yersinia spp.	500 mL	1) Membrane filtration 2) Pre-enrichment	1) MH Broth 2) Chrome Art Chromogenic *Yersinia* sp. Agar	30°C 18–24 h	Red/Purple

percentages: chloramphenicol CAM (APSs: 98.2%, RW: 54.7%), tetracycline TET (APSs: 94.5%, RW: 48.7%), sulfamethoxazole SMX (APSs: 93.7%, RW: 45.0%), gentamicin GEN (APSs: 96%, RW: 49.4%), ciprofloxacin CIP (APSs: 87.9%, RW: 42.1%), ceftazidime CAZ. (APSs: 87.9%, RW: 45.7%), kanamycin K (APSs: 84.9%, RW: 68.7%), cefoxitin FOX (APSs: 97.9%, RW: 44.7%), and meropenem MEM (APSs: 82.9%, RW: 34.7%). The pattern of resistance profile obtained for gram-positive bacteria ($n = 41$) retrieved from APSs only, revealed the following percentages: oxacillin OXA (87.7%), gentamycin GEN (75.8%), tetracycline TET (89.6%), sulfamethoxazole SMX (72.9%), ciprofloxacin CIP (97.6%), meropenem MEM (83%), and ceftazidime CAZ (78.3%).

Some isolates obtained from the APSs samples, which include potential human pathogens like *Klebsiella* spp., *Chryseobacterium* spp., *Cronobacter* spp., *Salmonella* spp., and *Morganella morgani* were resistant to all nine tested antibiotics. For other species recovered from both matrices, the percentage of resistant isolates was lower, but still remarkably higher for APS samples: *Serratia* spp. (APSs: 75.2%, RW: 32.1%), *Aeromonas* spp. (APSs: 71.3%, RW: 29.2%), and *Pseudomonas* spp. (APSs: 65.4%, RW: 35.1%).

Moreover, considering the influence of the geographical distribution of sampling points, the results indicate that samples collected in sites BR-07, BR-08, and BR-09 exhibited a substantially higher resistance rate as compared to sites BR-01, BR-02, BR-04, and BR-06. The current study classified strains as MDR strains if they exhibited non-susceptibility to at least three or more antibiotics tested.

Strains displaying a prominent phenotypic antibiotic resistance profile were further analyzed by molecular methods to gain deeper insights into the genetic mechanisms underlying antibiotic resistance. For this purpose, a panel of 18 antibiotic resistance genes (ARGs) was selected for targeted DNA amplification by PCR (Table S2). Among the 42 relevant MDR isolates, 13 of the 18 ARGs were positively detected; specifically, two tetracycline resistance genes (*tetA*, *tetW*), three sulfonamide resistance genes (*sul1*, *sul2*, *sul3*), one chloramphenicol resistance gene (*cmlA1*), four β-lactamase resistance genes (*blaCTX-M*, *blaSHV*, *blaTEM*, *mecA*), and three multi-drug resistance genes (*acrB*, *acrF*, *adeA*) were detected as illustrated in Figure 5. Notably, the most frequently detected ARGs were *sul1*, *blaCTX-M*, and *cmlA1*, while five genes of the panel (*cmx(A)*, *acrA*, *acrR*, *tetB*, *tetM*) were absent in all 42 environmental isolates.

Overall, the detection rate of ARGs in APSs isolates was up to 72.2%, whereas in RW isolates it was considerably lower (approx. 50%) (Figure 5). Among the various antibiotic classes, β-lactamase resistance genes exhibited the highest prevalence in APSs isolates (68.2%), surpassing that in RW isolates (40.9%). A similar trend was observed for the detection of sulfonamide resistance genes (APSs: 61.0%, RW: 41.7%), tetracycline resistance genes (APSs: 60.2%, RW: 38.3%), and chloramphenicol resistance genes (APSs: 51.5%, RW: 35.2%).

Moreover, other types of ARGs contributing to multi-drug resistance (*acrB*, *acrF*, *adeA*) in these environmental isolates were detected in the APSs samples at 64.9% rate, a much higher value compared to 32.7% in RW samples (Figure 6). The highest number of ARGs detected in gram-negative bacteria was found in *Serratia* spp., *Pseudomonas* spp. and *Aeromonas* spp. Interestingly, some potential opportunistic human pathogens like *Morganella morganii*, *Citrobacter freundii*, *Klebsiella* spp., and *Salmonella enterica* detected in this study, also showed prominent antibiotic resistance genotypic profiles, which is a serious health concern.

DISCUSSION

The environmental fate of plastic debris and their role in transmitting potential pathogenic bacteria and antibiotic-resistance genes to humans and animals is still unexplored, as only inadequate scientific evidence is available. These pollutants, act as a new route for the exposure

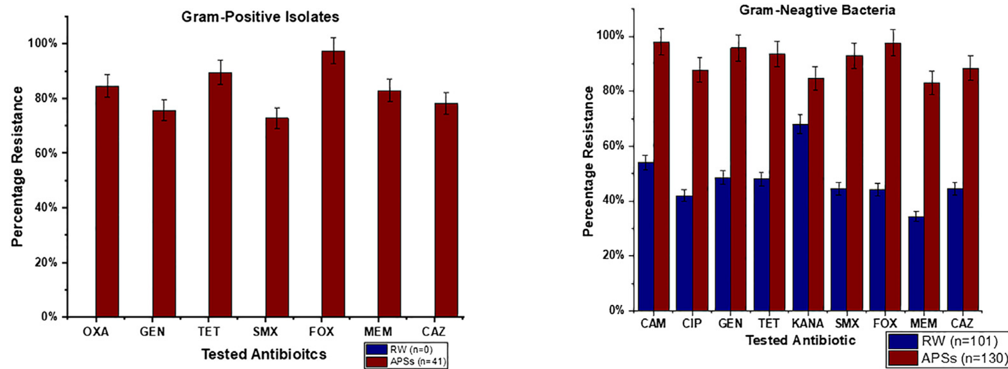

Figure 4. Phenotypic resistance profile of environmental isolates

A different panel of antibiotics was used for gram-positive and gram-negative bacteria of 272 isolates. Results of the paired t test indicate a significant difference between the resistance patterns of isolates obtained from RW and APSs samples in both cases (p value ≤ 0.05). Data are represented as mean $\pm$ 5 SEM.

of humans and animals to bacteria-mediated diseases. In this study, we have investigated the role of plastic debris in harboring MDR bacteria and their associated ARGs in aquatic ecosystems.

The study area of Lake Bracciano is notable, owing to: (i) its classification as a regional natural park, (ii) its paramount significance as the principal reservoir of the drinking water supply of the city of Rome, (iii) its susceptibility to anthropogenic activities. From the seven selected sites, and from both matrices (RWand APSs) 272 environmental strains were isolated and identified using standard microbiological and biochemical methods. Our results revealed, on plastic substrates, the presence of bacterial strains known from previous studies to colonize diverse environmental niches and exhibit varying degrees of resistance patterns. Some of these strains have been reported in the literature for causing various infections, like urinary tract infections (UTIs), and pneumonia, a common acute respiratory infection. Specifically, *Pseudomonas putida*, *Pseudomonas alcaligenes*, *Aeromonas hydrophila*, and *Serratia liquefaciens* are ubiquitous in soil and water[25] and are known to cause opportunistic human infections. On the other hand, *Vibrio* spp. cause different types of infections (e.g., gastrointestinal, wound, and blood), which are responsible in humans for a wide array of symptoms and may cause even death in extreme cases.[26] The most significant finding of this study was the isolation of ESBL-producing strains like *Vibrio* spp., *Aeromonas* spp., *Morganella morganii*, and *Klebsiella* spp., which is alarming evidence in the context of One Health. The association between ESBL-producing bacteria and plastic materials suggests that individuals who are in direct contact with recreational waters are at higher risk of developing certain coinfections.[27] Currently, few reports are available on ESBL-producing Vibrio, specifically,[28] and no such strains from plastic debris have been described. The significantly lower rate of ESBL-producing bacteria in RW samples compared to APSs suggests that plastic may serve as a repository for concentration of multidrug-resistant bacteria, highlighting the negative impacts of this new ecological niche. Despite the application of several infection control strategies, the persistent detection

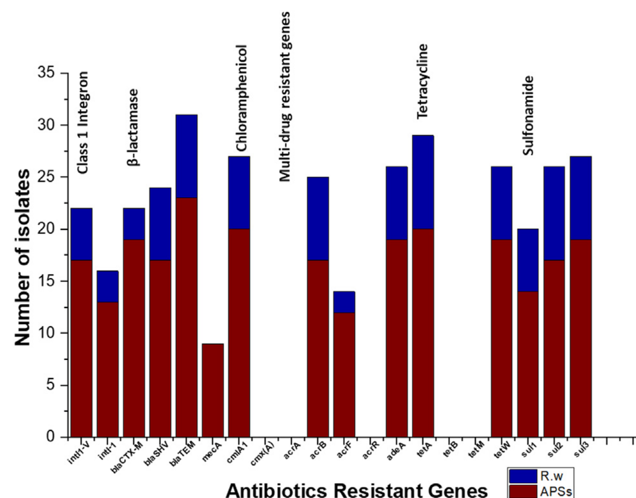

Figure 5. Antibiotic resistant genes detected in raw water and artificial plastic substrates

A significant difference between ARGs detected in RW and APSs isolates were observed, as revealed by Kruskal-Wallis ANOVA test (p value ≤ 0.05).

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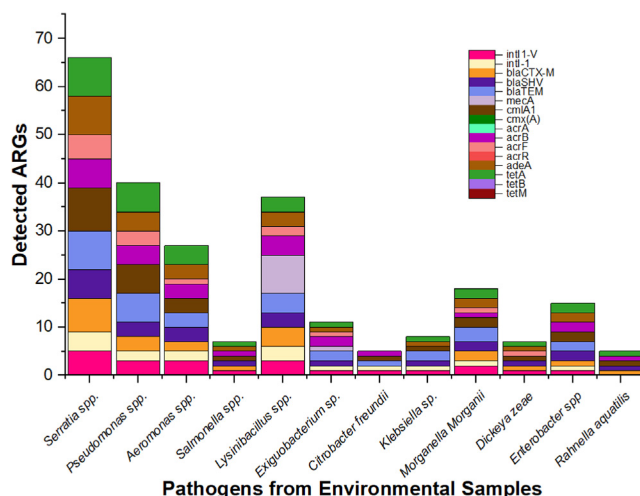

Figure 6. Genotypic resistance profile of environmental isolates

Genotypic profiling of 42 multi drug resistant strains obtained by DNA amplification of ARGs by PCR. Detection of antibiotic resistance genes (ARGs) and two Class 1 integrons across the 42 relevant environmental bacterial species.

of these ESBL-producing strains in hospitals and in the environment, suggests that they might possess strategies that enable them to survive in different habitats for very long time.[27,29] Owing to their acquired resistance to antibiotics, such as penicillin and cephalosporins commonly employed in human therapeutics, ESBL-producing bacteria pose a significant health threat.[15,30] To mitigate the risks posed by plastic-associated ESBL-producing bacteria, it is necessary to implement preventative initiatives such as controlling antibiotic misuse and better managing plastic pollution.[31,32]

As a result of our investigation, it was found a notable discrepancy in the identification of bacterial species obtained through biochemical and molecular identification methods. This inconsistency, associated with environmental bacteria, is of great concern as it may lead to inaccurate identification and to unreliable procedures of both early-warning systems and water treatment process. The existing literature survey revealed that environmental strains of *Vibrio* spp. are often misidentified as *Aeromonas* spp. through the biochemical identification method, as these two genera are closely related.[33,34] Based on previous reports, the human pathogenic bacteria *Burkholderia cepacia* cannot be discriminated from a related genus (e.g., *Pseudomonas*) by conventional phenotypic and biochemical methods, because there is an overlap in the biochemical characteristics.[35] Similar conclusions were drawn by comparing molecular and biochemical methods for detecting environmental strains of *Vibrio parahaemolyticus*.[36,37] The results of our study demonstrated that rapid identification systems, such as API-20E, API-20N, and API-Staph kits, primarily designed for clinical isolates, have a high chance of producing false positive or false negative results when used for identifying environmental strains. This may be due to the prevalence of different species of the same genus within the consortium, continuously evolving by horizontal gene transfer. Therefore, frequent updating of the database of the API test kits may aid in improving its accuracy for environmental strains[37,38]

Antibiotic resistance is an inevitable consequence of selective pressure, and it can rapidly develop in surface-attached bacteria. Biofilms are formed when microorganisms adopt a benthic state, and under this condition, bacteria can be up to 1,000 times more resistant to antimicrobial agents than their planktonic state.[39] Plastic debris can serve as a vehicle for disseminating ARGs by providing a stable substrate for bacteria to proliferate and to exchange genetic information.[40] Antibiotic susceptibility assays performed in this study revealed that bacteria bound to APSs display resistance levels to commonly used antibiotics, significantly higher than those recorded for RW isolates.[14,40]

Furthermore, a close relationship between genotypic and phenotypic resistance analysis was observed for the majority of the strains. Our results demonstrate the higher prevalence of multi-drug resistant bacteria and antibiotic-resistant genes in APSs samples compared to RW samples, strengthening the notion that plastic debris could act as underestimated trafficking agents. The results revealed critical insights into the prevalence of potential human pathogens and antibiotic-resistant bacteria on plastic surfaces in freshwater bodies, emphasizing the urgent need to develop and implement new, efficient, and reliable strategies to mitigate the plastic pollution.

Focusing on the geographical locations of the sampling sites, specifically BR-07, BR-08, and BR-09, we detected a considerably higher bacterial diversity, as well as high resistance patterns, in comparison to the other four sampling sites (BR-01, BR-02, BR-04, and BR-06). These differences may be due to some factors such as population density, industrial zones, agricultural practices, and wastewater treatment plants, which can significantly impact the prevalence of antibiotic-resistant bacteria in aquatic environments.[41,42]

In a nutshell, the main goal achieved in this study was setting a reproducible sampling method by using APSs, as an efficient and easy-to-handle tool for monitoring the presence of MDR bacteria. These results hold significant importance from the One Health perspective, as they

enhance our understanding of the environmental fate of MDR bacteria and their associated genes, revealing a new route of exposure to humans and animals.

Limitation of the study

In the current study, the sampling method was optimized for recovering MDR bacteria colonizing on plastic substrates submerged in lentic water. However, this method may not be suitable for certain marine environments, such as sandy beach coastal ecosystems. A challenge faced while performing this experiment on sites of recreational activities, was the recovery of samples after one month, due to unforeseen circumstances, such as human influence and turbulence in water, which may result in detachment of APSs cages from the anchoring point.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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  - Biochemical and molecular identification of isolates
  - Phenotypic and genotypic profiling of isolates
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.110026.

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AUTHOR CONTRIBUTIONS

S.M. and R.S.: conceptualization; S.M. and I.F.: experimental set-up, formal analysis, and writing original draft; R.S., and S.M.: supervision, reviewing, and editing; S.M.: funding acquisition.

DECLARATION OF INTERESTS

The authors have no conflicts of interest to declare.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides and recombinant proteins		
Muller Hinton Broth	Condalab, Madrid, Spain	Lot: 411213
Muller Hinton Agar	Condalab, Madrid, Spain	Lot: 303169
Rapid Staph Agar	Condalab, Madrid, Spain	Lot: 64316113
Peptone water	Liofilchem srl, Roseto degli Abruzzi (TE), Italy	Lot: 032818503
Rappaport Vassiliadis Broth	Condalab, Madrid, Spain	Lot: 401303
Xylose Lysine Deoxycholate Agar	VWR Chemicals, Milano (MI), Italy.	Lot: 1395
Chrome Art Extended Spectrum Beta Lactamase agar	Biomerieux, Marcy-l'Étoile, France	Lot: 4080252
Tryptone Bile X-glucuronide	Condalab, Madrid, Spain	Lot: 310281
Chrome Art Chromogenic Yersinia spp. Agar	Biomerieux, Marcy-l'Étoile, France	Lot: 765534644
Slanetz and Bartley Agar Base	Condalab, Madrid, Spain	Lot:528449
Chloramphenicol	Condalab, Madrid, Spain	Lot: 210610J
Tetracycline	Condalab, Madrid, Spain	Lot: C06602B
Sulfamethoxazole	Condalab, Madrid, Spain	Lot: C11909A
Gentamicin	Condalab, Madrid, Spain	Lot: C27710A
Ciprofloxacin	Condalab, Madrid, Spain	Lot: 210325A
Ceftazidime	Condalab, Madrid, Spain	Lot: 210604B
Kanamycin	Condalab, Madrid, Spain	Lot: C01301b
Cefoxitin	Condalab, Madrid, Spain	Lot: C10903B
Meropenem	Condalab, Madrid, Spain	Lot: 210205B
Oxacillin	Condalab, Madrid, Spain	Lot: 210604C
Agarose	BBI Life Sciences, Sanghai, China	Lot: EB01BA0023
GRS Taq Polymerase	GRISP, Porto, Portugal	Lot:7E50506B
GRS Taq polymerase buffer	GRISP, Porto, Portugal	Lot:7E50506B
Cryobank	VWR Chemicals, Milano (MI), Italy.	Lot: 147302
API 20E	BioMeriuex, Grassina (FI), Italy	Lot:1010350310
API-20NE	BioMeriuex, Grassina (FI), Italy	Lot: 1010269460
API-Staph kit	BioMeriuex, Grassina (FI), Italy	Lot: 1009857160
DNA extraction kit	GRISP, Porto, Portugal	Lot: 7E50423A
PCR and Gel band Purification kit	GRISP, Porto, Portugal	Lot: 7E60404A
Sanger DNA sequencing	Eurofins Genomics (Ebersberg, Germany)	https://eurofinsgenomics.eu/en/custom-dna-sequencing/eurofins-services/
Software and algorithms		
MEGA11 software	Molecular Evolutionary Genetic Analysis, version 11	https://www.megasoftware.net/
ArcGIS 10.8 software	Geographical Information System ArcGIS based on Corine Land Cover (CLC)	https://desktop.arcgis.com/
Origin Pro 2019	Origin Lab (USA), Origin 2019, Graphing and Analysis	https://www.originlab.com/2019

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Basic Local Alignment Search Tool	NCBI Blast Analysis, Nucleotide Blast	https://blast.ncbi.nlm.nih.gov/Blast.cgi
Deposited data		
BL12–APSs–Winter	This Study	*Pseudomonas putida GenBank:* OQ346170
BL13–APSs–Winter	This Study	*Aeromonas caviae* GenBank: OQ346171
BL36–APSs–Winter	This Study	*Morganella morganii* GenBank: OQ346173
BL37–APSs–Winter	This Study	*Dickeya zeae* GenBank: OQ519845
BL44–APSs–Winter	This Study	*Klebsiella michiganensis* GenBank: OQ346182
BL36a–APSs–Winter	This Study	*Morganella morganii* GenBank: OP420858
BL36b–APSs–Winter	This Study	*Morganella morganii* GenBank: OP420859
BL40–APSs–Winter	This Study	*Serratia nematodiphila* GenBank: OQ346176
BL45–APSs–Winter	This Study	*Serratia marcescens* GenBank: OQ346181
CY4–APSs–Spring	This Study	*Serratia marcescens* GenBank: OQ346119
E13–APSs–Spring	This Study	*Serratia fonticola* GenBank: OQ346121
BL03–APSs–Winter	This Study	*Enterobacter* sp. GenBank: OQ346174
BL51–APSs–Winter	This Study	*Klebsiella oxytoca* GenBank: OQ519846
CY10–APSs–Spring	This Study	*Pseudomonas putida* GenBank: OQ519847
E1–APSs–Spring	This Study	*Pseudomonas tructae* GenBank: OQ346120
BL01–APSs–Winter	This Study	*Serratia marcescens* GenBank: OQ346175
BL42–APSs–Summer	This Study	*Enterobacter cloacae* GenBank: OQ346164
BL39–APSs–Winter	This Study	*Citrobacter freundii* GenBank: OQ346178
BL49–APSs–Summer	This Study	*Exiguobacterium acetylicum* GenBank: OQ346165
BL47–APSs–Summer	This Study	*Exiguobacterium acetylicum* GenBank: OQ346166
BL46–APSs–Summer	This Study	*Lysinibacillus fusiformis* GenBank: OQ346167
BL48–APSs–Summer	This Study	*Enterobacter cloacae* GenBank: OQ519848
RS1–APSs–Summer	This Study	*Lysinibacillus boronitolerans* GenBank: OQ346123

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
RS14–APSs–Spring	This Study	*Lysinibacillus sphaericus* GenBank: OQ346125
RS15APSs –Spring	This Study	*Bacillus thuringiensis* GenBank: OQ519862
RS19 –APSs–Spring	This Study	*Lysinibacillus fusiformis* GenBank: OQ519849
BL50–APSs–Spring	This Study	*Salmonella enterica* GenBank: OQ519863
RS25–APSs–Spring	This Study	*Lysinibacillus macrolides* GenBank: OQ519850
RS2–APSs–Spring	This Study	*Lysinibacillus macroides* GenBank: OQ346124
RS28–APSs–Spring	This Study	*Lysinibacillus fusiformis* GenBank: OQ519851
BL09–RW–Winter	This Study	*Serratia marcescens* GenBank: OQ346168
BL10–RW–Winter	This Study	*Serratia marcescens* GenBank: OQ346169
BL14–RW–Winter	This Study	*Aeromonas hydrophila* GenBank: OQ346172
BL23–RW–Summer	This Study	*Aeromonas hydrophila* GenBank: OQ346163
BL02–RW–Winter	This Study	*Serratia marcescens* GenBank: OQ346177
E30–RW–Winter	This Study	*Rahnella aquatilis* GenBank: OQ346122
BL43–RW–Winter	This Study	*Serratia marcescens* GenBank: OQ346179
BL41–RW–Winter	This Study	*Serratia nematodiphila* GenBank: OQ346180
CY3–RW–Spring	This Study	*Pseudomonas putida* GenBank: OQ346118
BL19–RW–Winter	This Study	*Pseudomonas putida* GenBank: OQ519852
BL20–RW–Winter	This Study	*Pseudomonas putida* GenBank: OQ519853

RESOURCE AVAILABILITY

Lead contact

For further information regarding reagents and resources, contact corresponding author, Stefania Marcheggiani (stefania.marcheggiani@iss.it).

Materials availability

This study did not generate new unique materials.

Data and code availability

- 16S rDNA sequences of 42 bacterial strains have been deposited on the NCBI Genome Bank (publicly available at https://www.ncbi.nlm.nih.gov/gene/) with the accession number mentioned in key resources table.
- All the software and reagents used are mentioned in key resources table, any additional detail that supports the findings of this study are available from the lead contact upon request.

- Comprehensive details on experiment design, execution protocols, and any additional information are readily accessible from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

This study does not incorporate experimental models or human or animal participants.

METHOD DETAILS

Study area and sampling

The current study was performed on a caldera-type volcanic lake located 32 km northwest of Rome, Lazio, Italy (Figure 5). In seven geographical sites of Lake Bracciano, three sampling campaigns were conducted in three different seasons, i.e., summer, winter, and spring of 2021–2022. The land use analysis was performed using ArcGIS 10.8 software (ESRI) based on Corine Land Cover (CLC) from 2000 to the latest update in 2018 (Copernicus Land Monitoring Service 2000–2018).[43] All influential factors like discontinuous urban fabric, broad leaf forest, non-irrigatable land, and industrial and commercial units were considered for each selected site. For the isolation of the bacterial communities, both raw water (RW) samples (3 L) and Artificial Plastic Substrates (APSs) were collected from each sampling point, adequately labeled, transported to the laboratory, stored under dark conditions at $\pm 4°C$, and analyzed within 24 h.

Microbiological analysis of raw water samples

Recovery and concentration of bacteria from raw water (RW) samples collected from seven sites was performed using membrane filtration process. In particular, for isolation of *Escherichia coli* and intestinal enterococci (IE), 100 mL of RW was filtered using cellulose nitrate filter with a 0.45 μm diameter pore size (Sartorius Stadium Biotech, Göttingen, Germany).[44–46] Filters were placed on Tryptone Bile X-glucuronide (TBX) agar at 44°C for 24 h to isolate *E. coli*, while Slanetz and Bartley Agar Base (SB) was used to isolate IE, following incubation at 37°C for 48 h. The recovery of other selected bacteria was performed using methods, media, incubation conditions, and aliquots of RW analyzed, as reported in Table 2. Briefly, under sterile conditions, aliquots of the RW samples were filtered and were inoculated into 100 mL of pre-enrichment broth incubated at 37°C for 18–24 h.[47,48] Pre-enrichment step was performed to substantially improve the limit of detection for specific bacterial species, by allowing the few cells present in the sample to recover in Muller Hinton media (or Peptone water in the case of *Salmonella*) and subsequently to reach detectable levels in chromogenic selective media.[49,50]

Subsequently, 100 μL aliquots of pre-enriched culture were spread onto a chromogenic medium for isolation of *Salmonella* spp., *Yersinia* spp., *Klebsiella* spp., *Pseudomonas* spp., and *Proteus* spp. In contrast, to isolate *Staphylococcus* spp., 1 mL aliquot of RW was spread on the chromogenic medium.

Single colonies from each chromogenic medium were selected and isolated onto MH agar plates incubated at 37°C for 24 h. Results referring to *E. coli* and intestinal enterococci were interpreted based on colony-forming units (cf..u.) per mL; on the other hand, when qualitative methodologies were applied for other potential pathogenic species, results were interpreted on the presence or absence of specific colony phenotypes. All experiments were conducted in triplicates, incorporating both positive and negative controls. To validate the incubation conditions and medium suitability for the targeted bacteria, relevant ATCC strains (*S. aureus* ATCC25923, *P. aeruginosa* ATCC27853, *K. pneumoniae* ATCC13883, and *E. coli* ATCC25922) were employed as positive controls. To detect potential contaminations in the media, sterile broth was used as negative control.

Microbiological analysis of artificial plastic substrate samples

An efficient experimental set-up was designed for reproducible and efficient investigation of plastic fragments, providing a matrix for bacterial colonization and adherence. Plastic pollution was simulated by using four polymers as an artificial plastic substrate (APS), i.e., Polypropylene (PP), Polystyrene (PS), Styrene Acrylonitrile Resin (SAN), and Polyethylene terephthalate (PET). APSs fragments were caged in a net filled with stones for anchoring at the bottom of the lake, and to allow plastisphere adhesion and proliferation they were submerged deep (1.5 m) in the euphotic zone of each site. After one month, APSs samples were collected from all sites and stored under sterile and dark conditions at $\pm$ 4°C in the properly labeled collection bags, and transported to the laboratory. Further analyses were performed within 24 h of collection. The recovery of the bacterial community that colonized the APSs surface was performed using sterile scrapers and swabs under a laminar hood (AURA, Biosafety Cabinet Class 2) (Figure 6).

The collected material was inoculated into 100 mL of pre-enrichment Muller-Hinton broth and incubated at a temperature comparable to the one recorded in the lake at the time of sample collection, i.e., 20°C for 18–24 h. Subsequently, 100 μL aliquots of pre-enriched culture were spread onto a chromogenic or selective agar medium and incubated at the appropriate temperature, as summarized in Table 2.

To secure long-term storage of the retrieved bacteria and to ensure the possibility to perform further analyses, a cryobank was established using single colonies of bacteria isolated from RW and APSs isolates, following the manufacturer's guidelines.

Biochemical and molecular identification of isolates

The environmental isolates were identified up to the genus level using the biochemical identification method. The API 20E biochemical identification kit (BioMeriuex, Grassina (FI), Italy) was used for Gram-negative bacterial identification, while the API-Staph kit (BioMeriuex, Grassina

(FI), Italy) and API-20NE kit (Biomerieux; Grassina (FI), Italy) for *Staphylococcus* spp. and *Pseudomonas* spp. identification, respectively, following the manufacturer's instructions. Each bacterial species was assigned a unique seven-digit identification code based on the positive and negative results following the API color scale. The isolates were then identified using the APIWEB software by entering the generated seven-digit code.

For molecular identification, isolates were subjected to genomic DNA extraction using a DNA extraction kit (GRISP, Porto, Portugal). DNA quantity and quality were determined by 1% agarose gel electrophoresis. The bacterial 16S rDNA gene was amplified by universal primers (forward 5′-AGAGTTTGATCMTGGCTCAG-3′ and reverse 5′- TACGGYTACCTTGTTACGACTT-3′). PCR reaction mixture contained 0.4 μM of each primer, 1 ng/μL genomic DNA template, 0.06 U/μL of Grisp Taq DNA Polymerase, 1x Taq PCR Buffer, and 200 μM dNTPs. Reactions were conducted using the following conditions: 3 min for initial denaturation at 95°C, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 60 s, and a final extension at 72°C for 10 min. PCR products were purified using PCR and Gel band Purification kit (GRISP, Porto, Portugal), and Sanger DNA sequencing was performed by Eurofins Genomics (Ebersberg, Germany). All sequence alignments were conducted by a BLAST search in the NCBI database, and phylogenetic analysis was done using MEGA11 software (Molecular Evolutionary Genetic Analysis, version 11).[51] Kimura's two-parameter model (K2P) was used to determine the model of nucleotide substitution that best fits the data.[52]

Phenotypic and genotypic profiling of isolates

The antibiotic susceptibility test (AST) of isolates was determined using the disk diffusion method following the Clinical and Laboratory Standards Institute (CLSI) guidelines.[53,54] Nine commercial antibiotic disks (Condalab, Madrid, Spain) of different antimicrobial classes were tested, as listed in Table S3. Briefly, each bacterial isolate obtained from RW and APSs was revived from the cryobank and suspended in 5 mL of sterile saline solution (0.85% NaCl) to match 0.5 McFarland standards in turbidity. 100 μL of the inoculum was spread on MH agar plates using sterile swabs, and antibiotic discs (Condalab, Madrid, Spain) were placed on it and incubated at 37°C for 24 h. The inhibition zone diameters were measured to determine the bacterial isolates' antibiotic susceptibility.[54] The *Klebsiella pneumoniae* ATCC13883 strain was used as a positive control to ensure the quality of antibiotic susceptibility testing. The inhibition zones were measured and interpreted according to the Clinical and Laboratory Standards Institute (CLSI) breakpoint criteria.[54] If the isolates exhibited non-susceptibility to at least one antimicrobial agent in three or more antimicrobial classes, they were classified as multidrug-resistant (MDR) strains.

The environmental isolates exhibiting a phenotypic resistance profile against all tested antibiotics, were subjected to resistance genotypic profiling. Specifically, 18 ARGs and two class 1 integrons (*intI1* and *intI1-V*) were examined in these isolates. Briefly, purified genomic DNA of all isolates was used as the template (2 ng/μL) for amplification by PCR using Grisp Taq DNA polymerase with the primers as listed in Table S2.[1,55] For the PCR reaction mix 0.4 μM of forward and reverse primers, 0.06 U/μL of Grisp Taq DNA Polymerase, 1× Taq PCR Buffer, and 200 μM dNTPs were used. PCR reactions were performed using the following conditions: 3 min for initial denaturation at 95°C, 30 cycles of denaturation at 95°C for 30 s, annealing at specific temperatures mentioned in Table S2 for 30 s, elongation at 72°C for 60 s, and a final extension at 72°C for 10 min. Results were analyzed on 1% agarose gel.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was performed by using ORIGIN Pro 2019 Software (OriginLab, United States of America). A Chi-Square test was conducted to determine any statistically significant association between the microbial communities obtained from raw water (RW) and artificial plastic substrate (APS) samples, with a significance level set at $p\text{-value} \leq 0.05$.

To evaluate the seasonal dynamics of microbial community composition, a Principal Component Analysis (PCA) was performed, taking into consideration three seasons, the sampling sites, the number of bacteria recovered, and the two matrices (RW and APS). Additionally, a Hierarchical Cluster Analysis has been applied to bacteria species count obtained from the RW and APSs in three seasons.

Given the present study's sample size (n = 272), the Shapiro-Wilk Test was the more appropriate method to evaluate its normal distribution. If a $p\text{-value} > 0.05$ is the null hypothesis, then the data is normally distributed. For the data normally distributed, parametric test was performed (two samples t-test), while for data with rejected normality non parametric test (Kruskal-Wallis ANOVA) was used. In particular, phenotypic antibiotic profiles of RW and APS isolates were compared using two samples t-test, and for genotypic antibiotic profile of isolates, Kruskal-Wallis ANOVA test was performed.