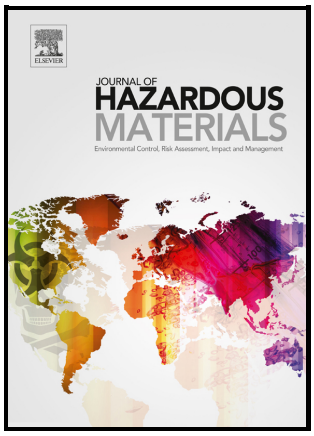

PET particles raise microbiological concerns for human health while tyre wear microplastic particles potentially affect ecosystem services in waters

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TITLE

PET particles raise microbiological concerns for human health while tyre wear microplastic particles potentially affect ecosystem services in waters

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HIGHLIGHTS:

1. Microplastic particles of different origin select for a specific pathobiome
2. PET particles offer a refuge for allochthonous/rare potential pathogens in water
3. Tyre wear particles are an additional source of nutrients for fast growing bacteria

KEYWORDS: microplastic particles, PET, tyre wear particles, pathobiome, anthropogenic pollution

ABSTRACT

Although abundant and chemically peculiar, tyre wear microplastic particles (TWP) and their impact on the microbial communities in water are largely understudied. We tested in laboratory based semi-continuous cultures the impact of TWP and of polyethylene terephthalate (PET) derived particles (following a gradient of relative abundance) on the pathobiome (the group of potential human pathogenic bacteria) of a freshwater microbial community exposed to contamination by the effluent of a urban wastewater treatment plant, for a period of 28 days. We could define the modulated impact of the two types of microplastic particles: while PET does not favour bacterial growth, it offers a refuge to several potential pathogens of allochthonous origin (from the treated sewage effluent), TWP act as an additional carbon source, promoting the development and the massive growth of a biofilm composed by fast-growing bacterial genera including species potentially harmful and competitive in abating biodiversity in surface waters. Our results demonstrate the different ecological role and impact on freshwater environments of TWP and PET particles, and the need to approach the study of this pollutant not as a whole, but considering the origin and the chemical composition of the different particles.

1. INTRODUCTION

Microplastic particles (MPs) are present in almost every aquatic and terrestrial environment on earth including the poles [1]. They are not only emerging pollutants but, in more general terms, a huge group of different polymers, each one with specific chemical and physical properties. Polyethylene (PE), polyamides (PA, e.g. nylon), polypropylene (PP), polystyrene (PS) and expanded polystyrene (EPS), polyethylene terephthalate (PET) and polyvinyl-chloride (PVC) are the most studied MPs [2] both in terms of concentration in various environments and in terms of their impact on the biosphere (MPs microbiome; MPs toxicity and other physiological effects on animals and plants, etc.) [3].

A peculiar group of MPs has been targeted by a very limited number of studies, notwithstanding their ubiquitous presence: particles released from car tyres abrasion (tyre wear particles, TWP). These are complex composite particles, composed by natural rubber (20-35%), synthetic polymers (10-25%), steel (10-22%), textile (up to 4%), different fillers (25%, e.g. carbon black, silica), antioxidants, antiozonants and different curing systems (10-15%) [4]. Several differences in microcomponents of tyres are brand-specific and the original formulas are patented and classified [5]. Further, the origin of the TWP should be considered: while the gross mixtures of car tyres from different producers are rather comparable, they differ between car (where natural rubber accounts for about 30-40% of the total elastomeric part and synthetic rubber accounts for 60-70%) and truck tyres (60-80% and 20-40%, respectively) [6]. Finally, the chemical composition and the aspect of TWP is additionally modified by the presence of attached road wear particles (e.g. asphalt, gravel, oils, and plasticisers) due to the use of the original tyre [7]. The complex and not classifiable chemical composition of these particles, together with their tendency towards sinking or getting dispersed in the water column instead of floating in the surface water layers (thus escaping most of the traditional MPs water column sampling techniques) might be the reason of their underestimation. Still, several studies and technical reports demonstrated their importance as MPs released into the environment in terms of both overall plastic quantity and proportion of MPs [8, 9]. Studies on MPs in rivers near to their estuaries in the sea demonstrated that TWP may represent up to 30%, or even 50% of the whole MP load in Northern Europe [5, 10], and estimations suggest TWP as the most common microplastic pollutant on Earth [11, 12]. Further, differently than other MPs, for which specific actions towards reduction/zeroing release are applied or planned, it is pretty unlike that pollution by particles originated from tyre abrasion will decrease in the near future [7].

In fact, TWP were recognized as pollutants for air, soil, and water already in the 1970s [13], thus much earlier than MPs of different chemical composition and origin. Being produced by abrasion of car tyres against the surface of roads [14], TWP are generally rod shaped, dimensionally ranging from micro- to nano particles, with an uneven and compositionally complex surface [15].

Although TWP are recognized as a prominent water pollutant for a long time, their microbiome in waters (i.e. attached microbes), as well as their impact on the natural microbial communities, is almost unexplored. The microbial communities on MPs of other chemical composition (e.g. PE, PET, PS, PP) is now largely known and have even been generalized with the specific term of *plastisphere* [16]. The risk posed to natural communities and to human health by the plastisphere is targeted by a growing number of studies [17-19]; yet, studies on TWP focused only on bacterial degradation of rubber [18, 19], or on toxicity due to complex molecules or heavy metals in their leachate [15, 22]. Recently, a research focused on the potential bacterial colonization of TWP from new tyres in aquatic environment [23]. In

any case, the peculiar composition of MPs from used tyres and their abundance call for a better understanding of the microbial communities they can host in waters, and of their ecological role in environment.

We approached the problem with a fully empirical and experimental study, to define differences and similarities of TWP and other MPs (i.e. PET particles) impact on natural aquatic microbial communities in an anthropogenic disturbed environment. In this article we focus on the impact of two MPs of different origin in promoting the spread and the persistence of allochthonous potentially pathogenic bacteria of anthropogenic origin (the effluent of a municipal wastewater treatment plant, WWTP) and in the dynamics of resistance genes, in a disturbed aquatic community.

In a semi-continuous culture system of vessels hosting a freshwater bacterial community mixed with the effluent of a WWTP and its load of allochthonous bacteria, we introduced MPs of different origin (TWP and PET particles) in a gradient of relative abundance (from 100% PET to 100% TWP). We assessed the bacterial abundances, phenotypic distribution, and community composition, the abundances of selected antibiotic and metal resistance genes (ARGs, MRGs) and of the integrase (*int*I1) gene of the class 1 integrons (a well-studied marker of anthropogenic impact and ARGs pollution in waters, [24, 25]) in the different vessels. Measurements were performed in water, on PET, and on TWP, focussing on the dynamics of those bacteria that could potentially cause a threat to human health (i.e. the potential pathogens, the so-called *pathobiome* [26]).

Our study represents an early experimental attempt to assess the impact of TWP in waters, and our results are calling for the importance of these neglected MPs in determining the fate of potential pathogens of allochthonous origin and the dynamics of ARGs, MRGs and of the class 1 integrons in the environment.

2. EXPERIMENTAL METHODS

2.1 Experimental set up and sampling

The experiment was set-up in semi-continuous cultures in chemostat vessels. A natural bacterial community from coastal surface waters of oligotrophic subalpine Lake Maggiore (Northern Italy) was used as basal community and added with water from the effluent of the WWTP of Verbania (collected before the final disinfection treatment) to a final concentration of 80% lake water to 20% WWTP water. For additional information on Lake Maggiore and on the WWTP of Verbania see former articles by the MEG Verbania [27-29]. The initial mix was pre-filtered through a 126 µm net and then through a 25 µm net to remove large zooplankton keeping the bacterial communities unaltered and smaller predators. The plastic particles used in the experiment were obtained in the laboratory by shredding a soft drink bottle (for PET particles) and the surface of a used car tyre (Continental Conti Premium Contact 2), further identified as TWP. The diameter of the particles ranged between 0.5 and 3 mm. The particles were then carefully washed with bleach and rinsed several times with sterile *MilliQ* water (Millipore).

The semicontinuous culturing system consisted of ten vessels, previously cleaned with bleach and rinsed three times with *MilliQ* water (Millipore) and then autoclaved. Three of these vessels (V1-V3) were used as replicated Control Treatments and filled only with the mixed waters (750 ml), while the other seven vessels (V4-V10) were filled with mixed water (750 ml) and 250 MPs following a gradient of relative

abundance between PET and TWP (V4: 100% PET, V5: 80% PET + 20% TWP, V6: 60% PET + 40% TWP, V7: 50% PET + 50% TWP, V8: 40% PET + 60% TWP, V9: 20% PET + 80% TWP, V10: 100% TWP). Total surface, volume and mass of the two MPs added in each vessels have been measured: the dry weight of MPs per type and per vessel was directly measured while the overall surface and volume were calculated by approximating the shape of TWP to a cylinder and of PET to a rhomboid. Microaeration was operating in each vessel to prevent anoxia, but also to favour flotation of the MPs thanks to the turbulence produced by the fine bubbling. The system ran for 30 days at 19 °C in the dark to avoid the potential blooming of primary producers. About 20 litres of the original water mix was filtered on 0.22 µm filters (Millex GS, Millipore) and then stored in a cool chamber (-4 °C). Every 48 hours, 15% of the water in each vessel was replaced with this medium in order to prevent nutrients limitation and to favour the stabilization of the original communities. 250 ml water samples from the original sampling sites (Lake water; WWTP effluent and mix) were used to determine the original bacterial community composition, 100 ml of water for chemical analyses of nutrients and other elements of potential interests (e.g. heavy metals), and 1.5 ml for the assessment of bacterial abundances and morphological distribution. Every 48 hours, together with the refilling, 1.5 ml of water from each vessel were collected and fixed (final concentration 2% formaldehyde) for flow cytometry and microscopy counting.

At the end of the experiment (Day 28), 100 ml of water from each vessel was collected and used to determine the microbial community composition and to quantify resistance genes and 50 ml were collected for the chemical analyses. At Day 28, all MPs were manually separated with sterile tweezers on the base of their origin in PET and TWP. They were then gently washed with autoclaved *MilliQ* water (Millipore) and 25 pieces of each MPs type were then used for DNA extraction.

Bacteria from all the water samples used for the molecular analyses were immediately collected on 0.22 µm filters (Isopore GTTP, Millipore) and each filter placed at -20°C until further process. Samples for chemical analyses were collected at T0 and at Day 28 (end of the experiment).

2.2 Bacterial abundances

The bacterial abundance and the morphological distribution in water were measured by flow cytometry (BD Accuri C6) on single sample aliquots of 1.5 ml stained with SYBR Green I (final concentration 1%, Life Technologies). Counts were set to a minimum of 2 $x10^6$ events within the gates designed for single and doubling cells, and 5 $x10^2$ events in the gates for small and large aggregates, the first composed by 3-9 cells and the second by at least 10 cells [30]. Flow cytometry counts and morphologies were confirmed by further epifluorescence microscopy (Axioplan, Zeiss) analysis (DAPI stained samples collected on 0.22 µm black polycarbonate filters, Millipore). The number of cells per aggregate for 600 small and large aggregates were then counted separately in epifluorescence microscopy, in order to assess the average number of cells per event in the small (f) and large aggregate gates (F). These factors were then used to obtain the overall number of bacteria per sample in water (Tot Nr= single cells + nr. of small aggregates x f + nr. of large aggregates x F; [31]). To evaluated stability within the experimental vessels we evaluated whether or not there was a temporal trend in cell numbers using a Mann-Kendall test using the package *trend* [32]. No temporal trend was taken as indication for stability. We therefore tested the data in all Vessels between Day 10 and 28, for Vessel 9, we tested consecutive days since there was a temporal trend until Day 14. Differences in cell numbers, both total and aggregates, at different concentrations of TWP between Day 10 and 28 were evaluated using linear model of log transformed cell number data with the day specified as a random effect using the *lmertest* package [33].

The estimation of overall number of bacterial cells in a vessel was done using the 16SrRNA quantified data from quantitative Real Time PCR (qPCR) [34]. In this way the number of gene copies for each amplification was then brought to number of gene copies per unit (i.e. 1ml of water, 1 medium sized plastic particle), and finally to the overall number of gene copies per substrate per sample in each vessel. This indirect and not ideal way to measure the overall abundances was chosen because of the impossibility to assess cells number on MPs in other ways, and because it is here used simply to give a relative view of the different colonization rates on the two MPs. These measurements were anyway confirmed by observations in epifluorescence microscopy of the surface of the different MPs with enumeration of the bacterial cells attached to them and of the different cell morphologies. Finally, to compare the proportion of potential pathogens among water, PET and TWP, we multiplied the abundances of their processed reads (from the 16SrRNA gene sequencing) for the corresponding 16S rRNA gene values calculated per sample in each vessel. The same was done for the total reads, in order to obtain the proportion of the pathogens, with respect to the total bacterial community, in water, on PET and TWP per vessel. First we evaluated differences in total 16S counts per Vessel between the controls and the treatment vessels using a linear model of log transformed data. Then a linear model of log transformed 16S rRNA gene abundance was made to evaluate the effect of the TWP gradient on its abundance. Differences in 16S rRNA gene abundance between the different substrates (water, TWP and PET) were evaluated through linear models of log transformed data, the output was transformed into an ANOVA table using the *car* package [35], and pair-wise differences were evaluated using the *emmeans* function of the *emmeans* package [36].

2.3 Chemical analysis

Chemical analyses were performed on all water samples (including feeding water at T0). The following variables were analysed: nitrate ($N\text{-}NO_3$), ammonium ($N\text{-}NH_4$), reactive phosphorus (RP), total phosphorus (TP), total nitrogen (TN), total organic carbon (TOC), trace metals (Al, As, B, Ba, Cd, Co, Cr, Cu, Fe, Li, Mn, Ni, Pt, Se, Sr, Tl, V, Zn). $N\text{-}NO_3$ was determined by ionic chromatography, RP, TP, TN and $N\text{-}NH_4$ by UV-VIS spectrophotometry, while TOC by high temperature catalytic combustion. Trace metals were analysed by inductively coupled plasma - optical emission spectrometer (ICP-OES) with concentric nebulizer. All the analyses were performed according to Standard Methods for freshwater samples [37]. Organic nitrogen (ON) was assessed by difference between the measured TN and the inorganic forms of N (nitrate and ammonium). A linear model was applied to evaluate the effect of the TWP gradient on the chemical parameters. The output was transformed into an ANOVA table using the *car* package [35].

2.4 DNA extraction

Filters were cut into two sections, one half was used for DNA extraction the second half was kept as backup. The DNA from water samples was extracted with a commercial kit (PowerSoil Qiagen) according to manufacturer instructions. The DNA from MPs was extracted following the protocol by Debeljak and colleagues [38] using PowerSoil Extraction Kits with some modifications for higher DNA yields. In detail: MPs were transferred in the provided tubes and 10 µl of Lysozyme solution (1000U/µL final concentration) and 5 mg of zirconia beads were added to each tube. The tubes were further incubated at 37° for 30 minutes and then an homogenized (by a PreCellys 24 homogenizer, Bertin Technologies: three times at 6000 rpm for 30 s with 1 min break in between each homogenization step). Thereafter, the extraction continued as detailed in the manufacturer's instructions.

Extracted DNA was quantified fluorometrically using a Qubit (ThermoFisher) and divided in two aliquots: the first was diluted and then used for qPCR analysis; the other aliquot was used for 16SrRNA gene amplicon sequencing, and shipped under controlled condition to an external company for sequencing.

2.5 16SrRNA gene sequencing and bacterial OTUs organization

The V3-V4 regions were used for sequencing of the 16SrRNA gene with the universal bacterial primer pair S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21 [39]. Sequencing was conducted on an Illumina MiSeq platform at IGA technologies (Udine, Italy). Sequences were cleaned and merged using the Usearch pipeline and unique high quality reads were identified and used as sequence variants to cluster the data into so called zero-radius operational taxonomic units (zOTUs) with the unoise2 algorithm [40-42]. Taxonomic assignment was done using the syntax algorithm with the Silva database v. 138 to a genus level with a threshold of minimum 80% similarity [43, 44]. Raw sequence reads are available at NCBI SRA with Bioproject Accession number PRJNA704794.

2.6 qPCR analysis of ARGs, MRG and *int*I1 gene

The following genes, bla_{CTXM} and bla_{TEM} (against β-lactam), *erm*B (against macrolide), *sul*2 (against sulfonamide), *qnr*S (against quinolone), *tet*A (against tetracycline), and *czc*A (against Co, Zn, and Cd), were selected as resistance genes because of their wide distribution in aquatic environments and in WWTP effluents [27, 45, 46]. In addition, *intI*1 gene, proposed as a proxy of anthropogenic pollution in aquatic environments [24], was also chosen. ARGs, MRG, and *intI*1 gene were quantified by qPCR, as previously described [27], using the RT-thermocycler CFX Connect (Bio-Rad). Primer pairs sequences and annealing temperatures are reported in Supplementary Table S1. The standard curves for each tested gene were prepared following the protocol suggested in Di Cesare et al. [47]. The mean value ± standard deviation of the reaction efficiency was 95.4% ± 11.0% and the R^2 for the assays was 0.98 ± 0.02. The limits of quantification (LOQ) per each gene was determined according to Bustin et al. [48] and were 1548, 35, 49, 55, 217, 357, 12, 24, and 11 copy μL^{-1} for 16S rRNA, bla_{CTXM}, bla_{TEM}, *erm*B, *sul*2, *qnr*S, *tet*A, *czc*A, and *int*I1 genes respectively. Gene abundances were normalized by dividing the copy number of the tested gene by the corresponding 16S rRNA gene copy number.

2.7 Pathogenic bacteria identification and statistical analysis

The pathobiome was obtained by subsetting the OTU table to those OTUs which genera were found on the pathogen list of the Bode Science Center (https://www.bode-science- center.com/center/relevant-pathogens-from-a-z.html). According to their presence/absence in the original waters used for the setup of the experiment, it was possible to assign the different pathogenic OTUs to the lake (autochthonous bacteria), to the WWTP effluent used as disturbance (allochthonous bacteria), to both environments (ubiquitous bacteria) or, for those OTUs quantified during the experiment but not detectable in the original media, to the rare biosphere (rare bacteria) [49].

Richness of genera of potential pathogens was compared between the different substrates (control, water, TWP and PET) through linear models of log transformed data, the output was transformed into an ANOVA table using the *car* package [35], and pair-wise differences were evaluated using the *emmeans* function of the *emmeans* package [36]. Percentage of pathogenic reads was evaluated for Day 28 by linear model of the percentage in relation to the TWP gradient.

3. RESULTS

Given that TWP, according to our measurements and approximations, had higher surface, volume and mass than PET, the MPs related colonizable surface per vessel constantly rose (from 4229.9 to 5022.8 mm^3) with increasing the proportion of TWP (Supplementary Table S2). Similar trends were followed also by MPs volume and mass (Supplementary Table S2).The bacterial abundance in each vessel at Day 0 was of 2.3 x 10^6 cells ml^{-1} and faced large fluctuations in the first days, when the communities were adapting to the experimental environment. All vessels reached stability after 10 days of growth in the lab in terms of total bacterial numbers, except for vessel 9 that reached stability at Day 14 (no temporal trend detected with Mann-Kendall between Day 10/14 and 28, statistical output; Supplementary file 1, data; Supplementary Figure 1). Considering all cell number data between Day 10 to the end of the experiment (Day 28) there was a significant increase of total cells with increased proportion of TWP (Figure 1, left panel; linear model (lm): t=6.1, p=1.22x10^{-6}, complete statistical output of the lm; Supplementary file 2). During the experiment, the number of bacterial aggregates composed by more than 10 cells rose from less than 1 x 10^3 aggregates ml^{-1} detected at the beginning of the experiment to an average value of 34.4 x 10^3 aggregates ml^{-1} (Figure 1, right panel), with a clear gradient in increasing aggregation with increasing TWP in the vessel (lm: t=12.5, p=3.4x10^{-13}).

Bacterial marker gene abundances on MPs were quantified by qPCR of the 16S rRNA gene on Day 28, and confirmed by epifluorescence microscopy. Total marker gene abundance, i.e. sum of the abundance in the whole vessel considering MPs and water, was higher in all vessels with MPs compared to the control (lm: t=5.1, p=0.0008), with a tendency, albeit not significant, towards higher number of 16SrRNA copies with higher proportions of TWP (lm: t=2.2, p=0.0779). In any case, and keeping into account all the limitations of the measurements, 16S rRNA marker gene was always more prominent in water with respect to the abundances on both types MPs and its numbers on TWP was then much higher than on PET (Figure 2A, linear model with post-hoc in Supplementary file 2), as confirmed by microphotos showing large biofilms covering the whole surface of the tyre derived MP (Figure 2B and C). Despite on PET the abundances were much lower, the phenotypes were more diverse in comparison to the TWP biofilm (Figure 2B and C) where small rods largely dominated among morphologies. On PET particles a number of different bacterial morphologies with relatively similar abundances (e.g. short, medium and long rods, filaments, cell chains, cocci of different dimensions, others) could be observed (Figure 2 B and C).

The chemical analysis of the water in the vessels (at Day 28) highlighted a significant increment of organic carbon (lm: F= 8.73, p=0.03174; Supplementary File 3 for the complete lm output also for other chemicals), organic nitrogen (lm: F= 13.55, p= 0.01428), Mn (lm: F= 13.29, p= 0.01482), Ni (lm: F= 10.83, p=0.02161), and Zn (lm: F= 86.19, p= 0.00024), with increasing TWP concentrations (Figure 3). Conversely a decrement was observed for $N\text{-}NO_3$ (lm: F= 180.27, p=0.00004), and RP (lm: F= 39.73, p= 0.00148), readily available for bacteria as substrate (Figure 3 and Supplementary Figure 2), while total nitrogen and phosphorus concentrations remained constant along the gradient (lm: F= 2.9065, p= 0.1489; lm: F= 0.68, p=0.448, respectively). The other measured nutrients and heavy metals kept similar concentrations to those measured at the beginning of the experiment for all conditions (Supplementary Table S3).

The overall experimental pathobiome, i.e. Operational Taxonomic Units (OTUs) with genera that were identified as potential pathogens, was composed by 299 OTUs, affiliated with 35 genera with an average

richness of 10 genera in the Controls, 9 in the water of the experimental vessels (V4-V10), 21 on PET and 12 on TWP (Figure 4). The diversity of the pathobiome in the biofilm on PET was higher than in any other group, whereas water and TWP did not differ from the controls (lm followed by post-hoc; Supplementary file 2).

Analysing only data at the end of the experiment (Day 28), the overall proportion of potential pathogens in each vessel (counted as reads assigned to the pathobiome) was higher with higher proportions of TWP (lm: t= 6.7, p= 0.0011, Supplementary file 2), reaching more than 8% of the reads in V9 and V10 (Figure 5, left panel). At the same time, the contribution of biofilms on PET to the overall number of potential pathogens was maximum of 0.2% (in V4, with 100% PET), while the proportion of potential pathogenic bacteria in water was highest fluctuated between 0.4 and 5.8% without any apparent trend (lm: t=1.8, p= 0.14). A comparable distribution was observed also in terms of absolute read numbers (Figure 5, right panel).

The analysis of the composition of the pathobiome (Figure 6) has been organized considering three genera (Acinetobacter, Pseudomonas, and Sphingomonas) separately, as described in the Discussion. These three genera, dominated the pathobiome of MPs biofilm, while their proportion in water kept constantly low during the whole experiment and in every treatment (Figure 6). In particular Acinetobacter belonging reads accounted for about 90% of the overall potential pathogenic reads on MPs in vessels V8, V9 and V10, when TWP was the only microplastic or it was highly predominant. At the end of the experiment, in water, it was possible to detect a selection in favour of Legionella, independently by the presence and the origin of the MPs. Interestingly, when PET were predominant (V4, V5 and V6), a large number of potential pathogenic genera were detected in their biofilm, with a significant number of reads belonging to the genera Bacillus, Brevundimonas, Escherichia and Morganella. At higher concentrations of TWP the relative abundances of potential pathogens excluding the three selected genera dropped (about 1-2% of the reads).

About 14% of the genera of potential pathogens detected along the experiment could be thus considered as autochthonous in Lake Maggiore, 20% as allochthonous, 40% ubiquitous (including the genera Acinetobacter, Pseudomonas and Sphingomonas), and 26% belonging to the rare biosphere (Figure 7). Several genera of potential pathogens of allochthonous origin or belonging to the rare biosphere could be detected only on PET in vessels V4, V5 and V6, while other MPs biofilm and the water favoured ubiquitous genera.

The quantification of six ARGs against the most commonly used class of antibiotics, of a multi-metal resistance gene, and of *int*I1 gene (Supplementary Figure 3) did not evidence particular variations along the gradient neither between the different substrates (bla_{TEM} was never detectable).

4. DISCUSSION

The impact of MPs on aquatic microbial communities is well known: once abundant (e.g. in areas with direct impact of anthropogenic activities, or where water streams and winds cause their accumulation) they act as additional hard substrates favouring the attachment of autochthonous (and sometimes allochthonous) biofilm forming bacteria. This can modify the interactions regulating the microbial communities (e.g. predation and competition) promoting the establishment of different communities in

waters and on the MPs (the plastisphere) thus driving an overall change in the aquatic microbial community composition and physiology [50].

Our results demonstrate that in a given environment the impact of MPs cannot be tested as a whole, as the chemical composition of a MP (and possibly also its shape, structure, and the physical parameters of its surface) can play a fundamental role in determining the attachment of specific aquatic bacteria. For our experiment we compared the composition of potentially pathogenic bacteria in the plastisphere of two very different types of MPs, among the most abundant in open waters: the well-studied PET particles and the rather obscure TWP. The two plastics differ drastically in terms of chemical composition, structure of the surface, presence of metals, buoyancy and compactness of the material. While PET can be compared to an inorganic hard and smooth surface, TWPs are softer, wrinkled, and easy to disrupt losing nano and microparticles together with a variety of chemical compounds [51].

In our experiment we defined a gradient where different proportions of PET and TWP were introduced in a replicated simplified freshwater ecosystem (where organisms larger than 126 µm were removed) disturbed by the effluent of an urban WWTP. This allowed us to assign the potentially pathogenic bacteria detected either to the natural system (autochthonous), to the effluent (allochthonous, of anthropogenic origin), to both (ubiquitous) or to the "rare biosphere", when their numbers became detectable only with the disturbances experimentally imposed. This step has a fundamental importance as the definition of "potentially pathogenic bacteria" is rather ambiguous, and its application as a "black box" can bring to incorrect conclusions [52]. In fact, several studies reported the presence of potentially pathogenic bacteria on MPs [18, 53, 54] (including TWP originated from new tyres of different brands [23]) and a concomitant enrichment in antibiotic resistance genes [53, 55], pointing out the risk for human and environmental health posed by the presence of MPs in waters and their role as hot spot for the selection of potential pathogens. Still, most of the potential pathogens detected on MPs are generally already present in waters (e.g. *Vibrio* spp.), and are known to produce biofilms on aquatic organisms as well as on organic particles of natural origin [56]. Further, many species within genera containing pathogenic bacteria are in fact harmless for humans (e.g. many species belonging to *Vibrio*, *Pseudomonas*, *Acinetobacter*, etc.) and naturally present in open waters, where some of them can also be extremely competitive in disturbed systems. In these environments, nutrients (e.g. organic carbon, phosphorous) can be in excess and the presence of organic particles enhance their competitiveness [57]. In these conditions the fast growth of such species can cause the reduction or even the local extinction of many less competitive bacteria, abating biodiversity, water quality, and the environmental services ensured by the undisturbed system [57, 58].

Our results clearly demonstrate that the presence of TWP is promoting bacterial growth, and that TWP abundance is directly linked to the growth. The same assumption is not valid for PET. The reasons are surely lying on the different characteristics of the two MPs. TWP releases a number of chemicals, either as nanoparticles or molecules, as we could detect from the chemical analysis of the waters [59]. The increment we detected in the bacterial abundance was not limited to the overall cells number but was also detected in bacterial aggregates, suggesting the shift towards a more disturbed but productive system [60].

The amount of specific chemical compounds (organic carbon and some metals, possibly used in manufacturing the tyre, or collected from the roads) was growing with TWP number but, while the potential toxic effects of the metals was not obvious, the spin given by this additional source of

nutrients to the bacterial community, and to some bacterial species, was clear. Our experiment was performed by deriving the TWP from a used car tyre, thus testing a specific material with a peculiar composition that can influence bacterial growth. Further research on the potential effect of tyres with a different composition in macro- (natural and synthetic rubber) and microelements (either in TWP from tyres of different brands but especially from car and truck tyres) could lead to a more precise definition of the importance of leaching and non-dissolvable organic matter proportions in determining the microbial community dynamics [61].

The two MPs also differ in the degree of colonization: as it was possible to define from microphotographs of the plastic surfaces, on PET we could detected only spatially restricted small biofilms, generally composed by cells of very different morphology, while on TWP a huge biofilm of morphologically similar cells covered the whole plastic surface. Considering the exposure time (less than a month), it can be speculated that on PET (hard surface) we still faced an early colonization phase, where several different strains are approaching the newly available surface [62] while on TWP (soft, C-rich surface) the colonization proceeded faster and there was already a selection towards small-medium cocci-shaped cells belonging to opportunistic fast growing bacterial strains, outcompeting less competitive colonizers [63]. This observation is confirmed by enhanced aggregation in water, attributable to strains able to use carbon to grow fast but limited by phosphorous, similarly to those colonizing the TWP surface [31, 64]. The limited increment in available MP surface detected with increasing the proportion of TWP does not seem to be a driver for the observed trends but, the larger shift in MPs mass (and thus, indirectly, in nutrients introduced to the system) could explain the described dynamics. In fact, not only reactive P decreases and TOC increases while higher TWP proportions are in the vessels, but also N presents interesting trends: the decrement in $N\text{-}NO_3$ is fully compensated by an increment in organic N. According to literature data, the ON leached from the TWP [61, 65] can account for about 10% of those actually calculated for our system, while the predominant fraction is possibly derived from bacterial activity, specific for the different communities [66], and possibly concentrated within the biofilm on TWP.

Higher morphological diversity on PET was confirmed by higher taxonomic diversity of the pathobiome. In fact, PET hosted a larger number of genera than water and TWP, including a number of potential pathogenic genera of allochthonous origin (here from a treated WWTP effluent), absent in the other compartments of the system. Still, the overall proportion of potential pathogens on the overall microbial community was extremely low, independently by the number of PET particles in the system.

At the same time, the low morphological diversity on TWP corresponded to a low genotypic diversity with a few genera of fast growing bacteria dominating the pathobiome: *Pseudomonas* spp., *Acinetobacter* spp., and *Sphingomonas* spp. represented the largest majority of the potential pathogenic bacteria present on TWP, and formed large and thick biofilms on the surface of the particles. The same groups were present also on PET, but on TWP their relative abundance became so high to prevent the establishment of other potential pathogens, and thus strongly limited the availability of TWP as a spatial refuge. These three genera are also well known for their ability to increase in number and in relative importance in aquatic microbial communities exposed to disturbances, when nutrients (e.g. organic carbon) and particulate matter are increased [57, 67, 68]. Their fast and exponential growth can cause the collapse of the original aquatic communities, causing losses in biodiversity and modifications in the underlying biogeochemical cycles and in other degradation processes [57]. Even in absence of human pathogenic strains belonging to these groups (e.g. *Pseudomonas aeruginosa*, *Acinetobacter baumannii*),

their impact on the environment can be very strong, reducing its quality according to the existing legislations [69] and thus the environmental services provided.

In our study, the drastic changes detected in the pathobiome when the different MPs were introduced, were not accompanied by changes in abundances of the antibiotic or metal resistance genes tested, nor of the *int*I1 gene. This result, quite surprising, is in conflict with other observations where MPs had a significant impact in promoting antibiotic resistance in the plastisphere [19, 70]. We can speculate that the limited exposure time, as well as the limited number of genes tested and the relatively high quality of the effluent water used, is in fact reducing the depth of our results, still, they call for deeper analyses in the ecological role of MPs in selecting antibiotic resistances. The stability of *czc*A and *int*I1 genes in the communities along the gradient are also contraposing the limited but significant increment we detected for zinc, probably leached from the TWP. The dynamics of both these genes are used as proxy of adaptation of bacteria to specific stressors, the first selected under pressure by metals (cobalt, zinc, or cadmium [46]), the second by generic factors of anthropogenic origin (antibiotics, pharmaceuticals, metals [24]). Their missing increment in our system is an additional evidence of the striking role played by nutrients, and more specifically by C and P and their ratio, in selecting the different communities adhese to the MPs.

This study is filling a current gap in the literature, by presenting more than one line of evidence supporting our conclusions; still a number of limits in our setting should be taken into account: a single used TWP and PET source was tested, and the outcome generalized. Specific studies on the differences between TWP and PET of different origin and age could strengthen the observed trends. Further, although not observed in our study, the leachate from TWP of brand specific metals and toxic substances could impact bacterial dynamics and strain selection to a certain extent, and thus influence the response of the aquatic microbial communities to the pollutant. The results were achieved by testing two MPs representing a large part of the particles present in water, but can be speculated that other important MPs (e.g. PE, PVC, PS, others) can even affect in different, nowadays obscure ways, the fate of pathogens in waters. Our experiment followed a classical reductionistic approach, testing under artificial conditions a simplified system with a controlled number of variables; only the test of our findings in different environmental settings will allow for a generalization of our observations (considering natural conditions for the aeration of MPs, which might sink after the formation of thick biofilms [71, 72], the influence of different concomitant contaminations, the effect of limiting factors other than those considered, etc.).

Finally, we could only speculate on the role of MPs as (point) source of nutrients and of toxic substances, both impacting on the aquatic microbial community composition. Specific research on the role of different MPs not only as refuge for human pathogens, but also in selecting and supporting the growth of bacterial strains causing the reduction of diversity and stability of an aquatic community, is needed to define the magnitude of their impact on the underneath ecosystem services.

All in all, our results demonstrate, under controlled experimental conditions, that chemical composition and origin of MPs can drastically change the impact on the pathobiome of an aquatic microbial community, offering either a refuge or a substrate (an additional niche) where, otherwise limited, bacterial groups can grow. The largely understudied TWP had thus a very strong impact on the pathobiome, selecting fast growing species that can pose a threat for human health but, more directly,

can deplete the environmental services characterizing a freshwater microbial community. PET particles confirmed their role as ecological refuge for allochthonous and rare potential pathogens, without a strong support of their growth but with a clear role in their maintenance in aquatic systems, posing a direct threat for human health.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

MB Sathicq: Methodology, Validation, Formal analysis, Investigation, Writing - review & editing, Funding acquisition. R Sabatino: Methodology, Validation, Formal analysis, Data curation, Writing - review & editing. A Di Cesare: Conceptualization, Methodology, Writing - review & editing. EM Eckert: Methodology, Validation, Writing - review & editing. D Fontaneto: Formal analysis, Writing - review & editing. M Rogora: Formal analysis, Writing - review & editing. G Corno: Conceptualization, Formal analysis, Investigation, Data curation, Writing - original draft, Funding acquisition.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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FIGURE LEGENDS

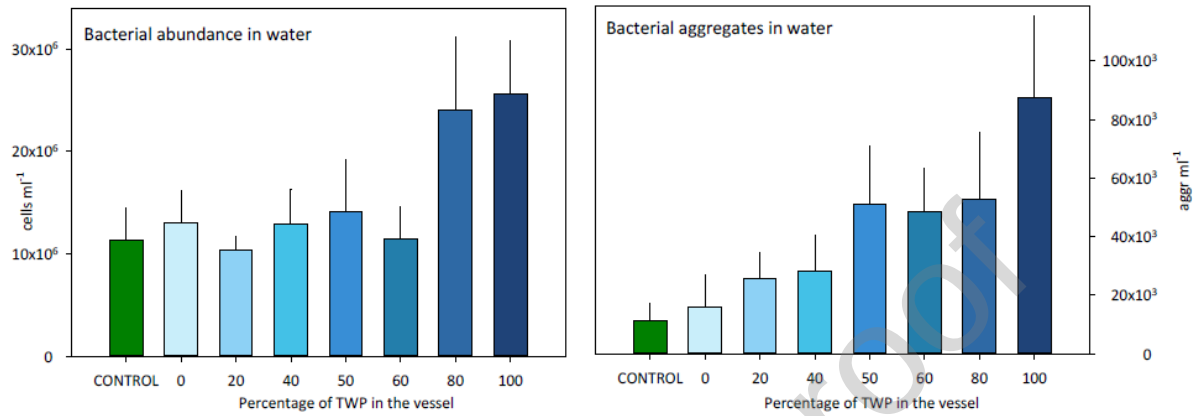


Figure 1. Overall bacterial cells number and bacterial aggregates (>10 cells) in water, in the different treatments (mean ± s.d.) from Day 10 to the end of the experiment. Control values are mean of three replicated vessels (V1-V3). Colour gradient from V4 to V10 represents the proportion TWP/PET.

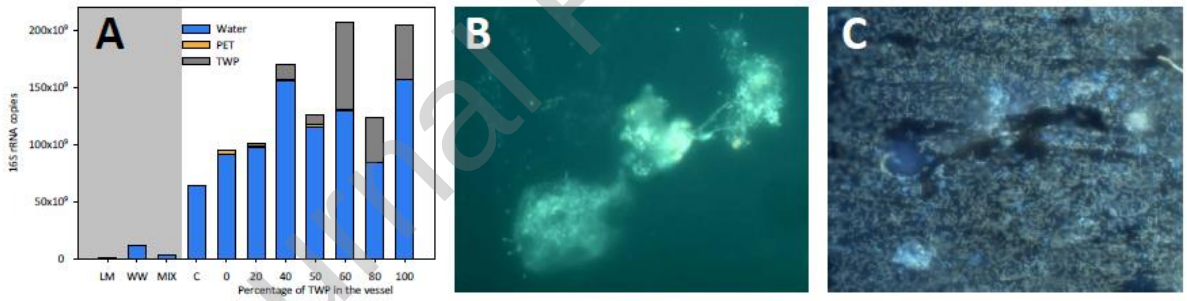


Figure 2. (A) Number of bacteria in each vessel at Day 28, expressed as number of 16SrRNA gene copies per substrate (water, PET, TWP). (B, C) Microphotographs of the biofilm on MPs. MPs were collected at Day 28 and observed by epifluorescence microscopy (DAPI stained, each image mean of 4 photos, 1250x). (B): bacterial colonies on PET (V5) characterized by limited colonization of the surface and high morphological plasticity of the community. (C): bacterial colonies on TWP (V9) characterized by high colonization of the surface and low morphological plasticity of the community.

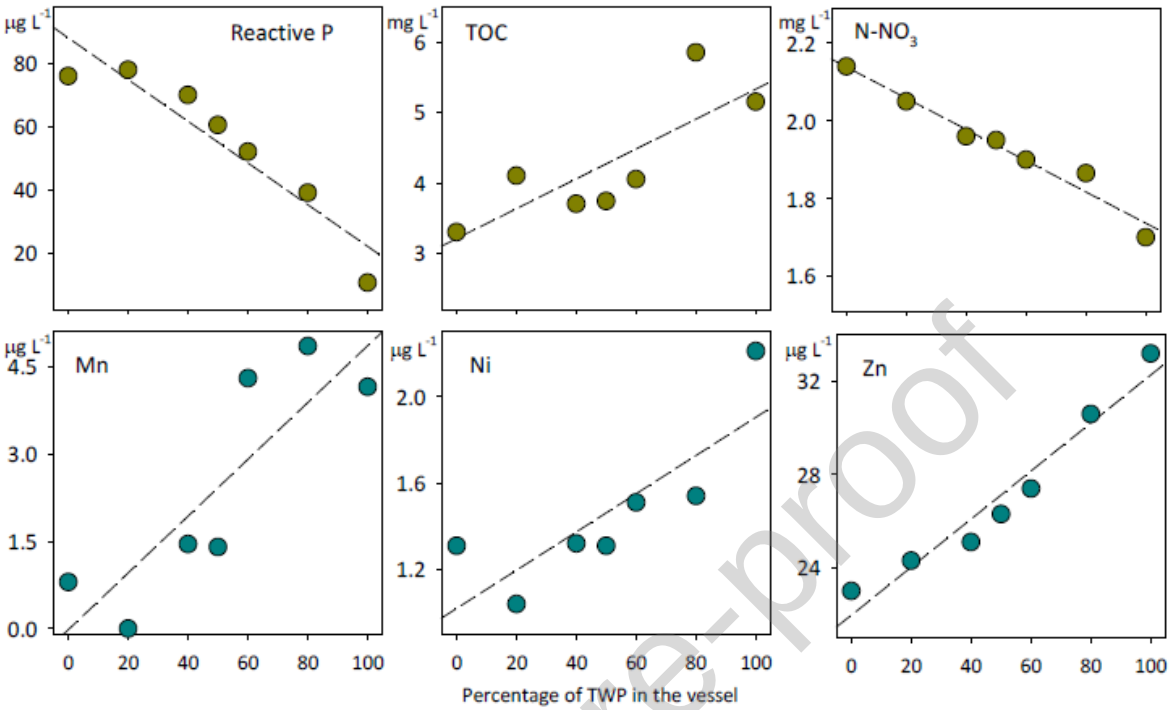


Figure 3. Nutrients and trace metals showing a significant correlation with TWP proportions at the end of the experiment. Specifically, TOC and some metals (Mn, Ni, and Zn) increased in the water with higher TWP quantities; N-NO_3 and RP decreased with higher TWP proportions.

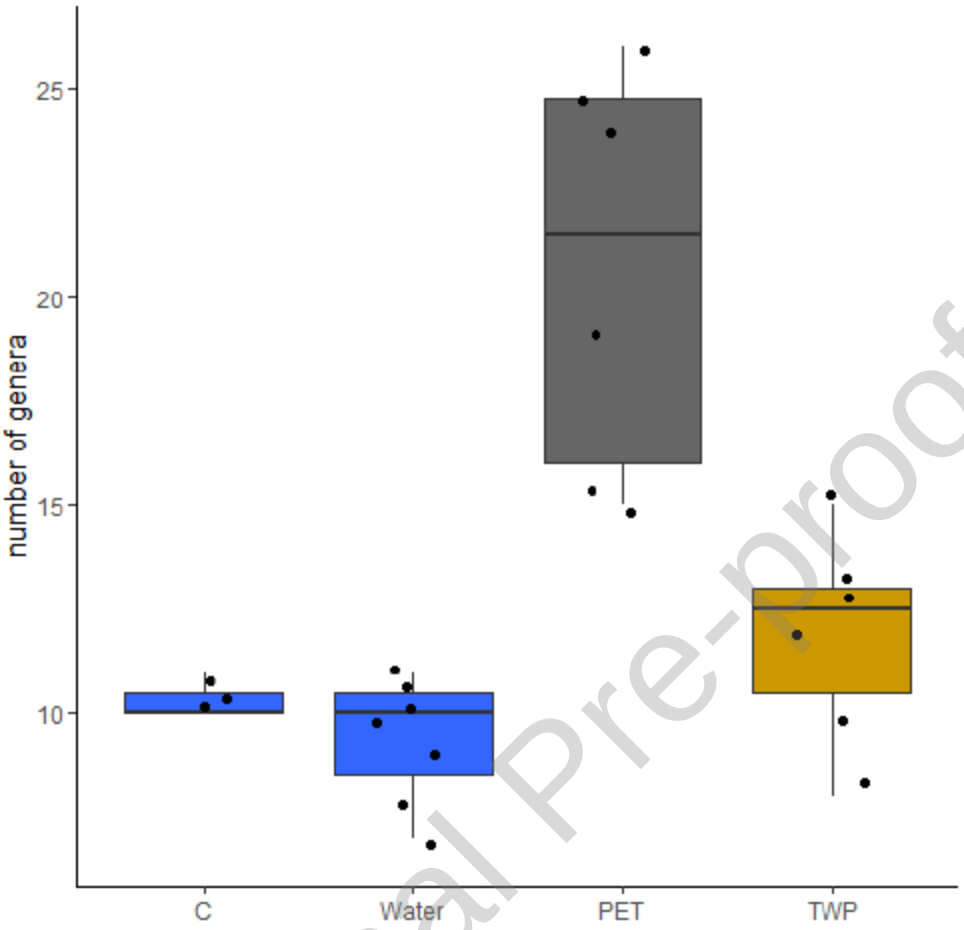


Figure 4. Boxplots of overall genera richness of potential pathogenic bacteria in the different experimental groups.

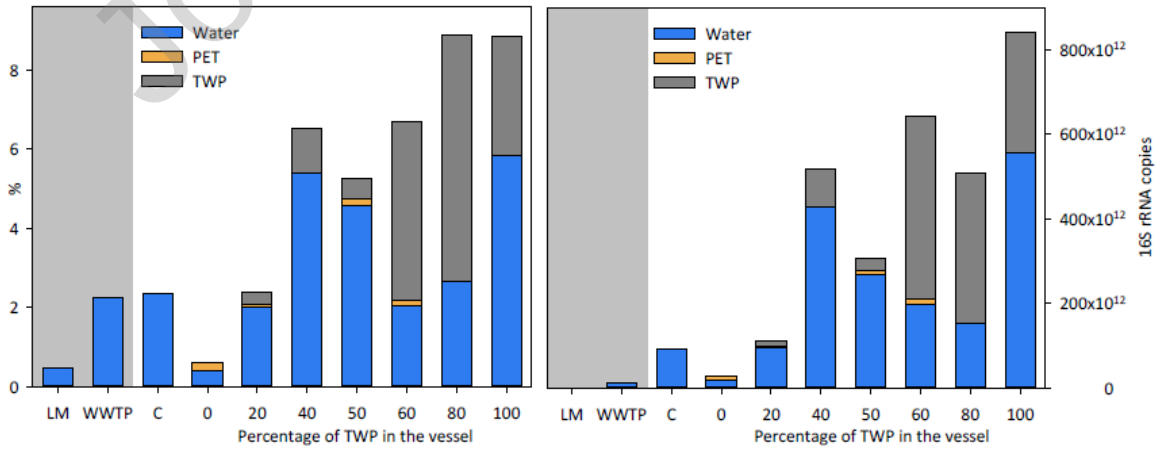


Figure 5. Pathobiome composition. Percentage of sequences on the total reads number belonging to the pathobiome (left panel) and overall reads number of the pathobiome(right panel) in the lake community (LM), in the WWTP community (WWTP), in the controls (C, average of 3 vessels), and in the different experimental treatments (V4-V10) measured on Day 28. N-NO_3

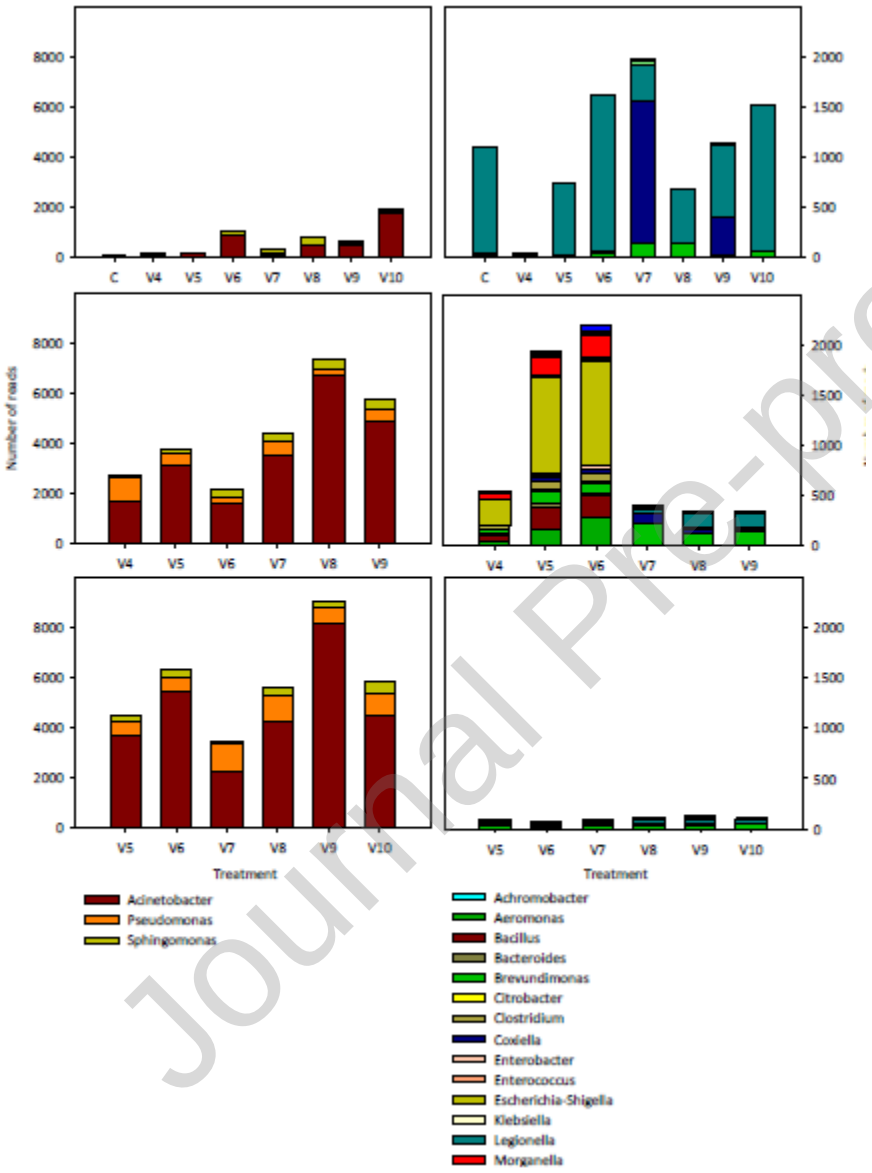


Figure 6: Abundances of the different genera of potential pathogens (overall number of 16S rRNA genes per extraction) at the end of the experiment (Day 28) in water (upper row), on PET (middle row) and on TWP (lower row). Potential pathogenic genera are depicted separately: Acinetobacter, Pseudomonas and Sphingomonas (natural r-strategists) on the left, the other potential pathogens on the right.

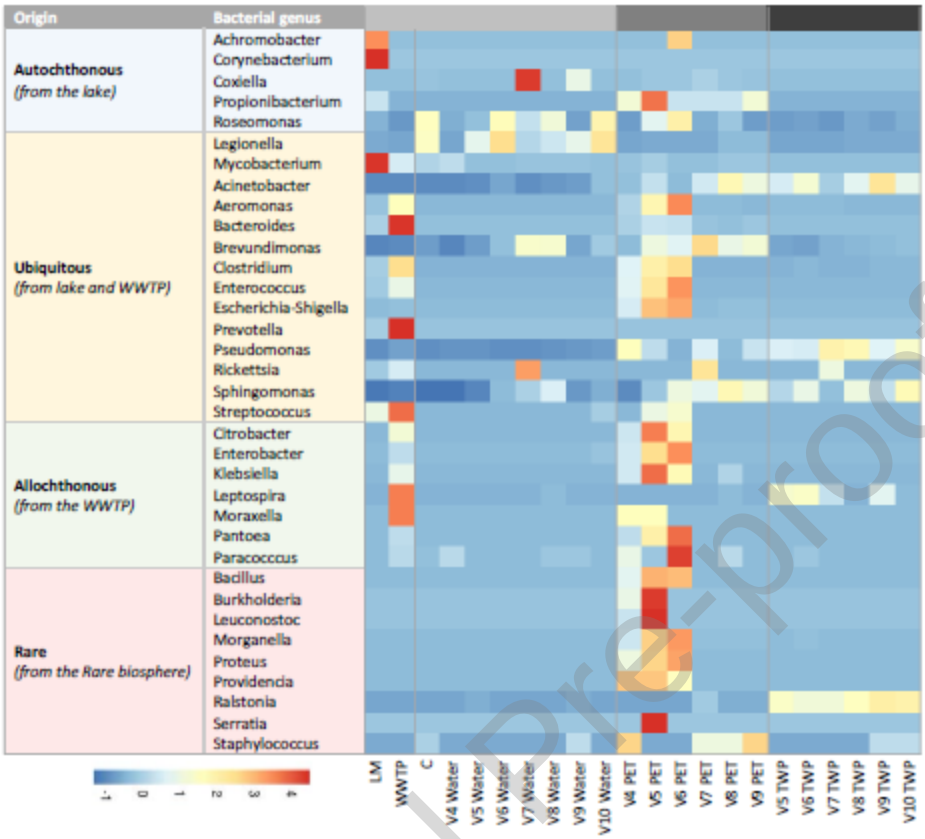


Figure 7. List of the potential pathogenic genera according to their origin and heatmap of their proportion in the different treatments/substrates. Colour scheme red/yellow/blue in order of relative abundance (high to low).

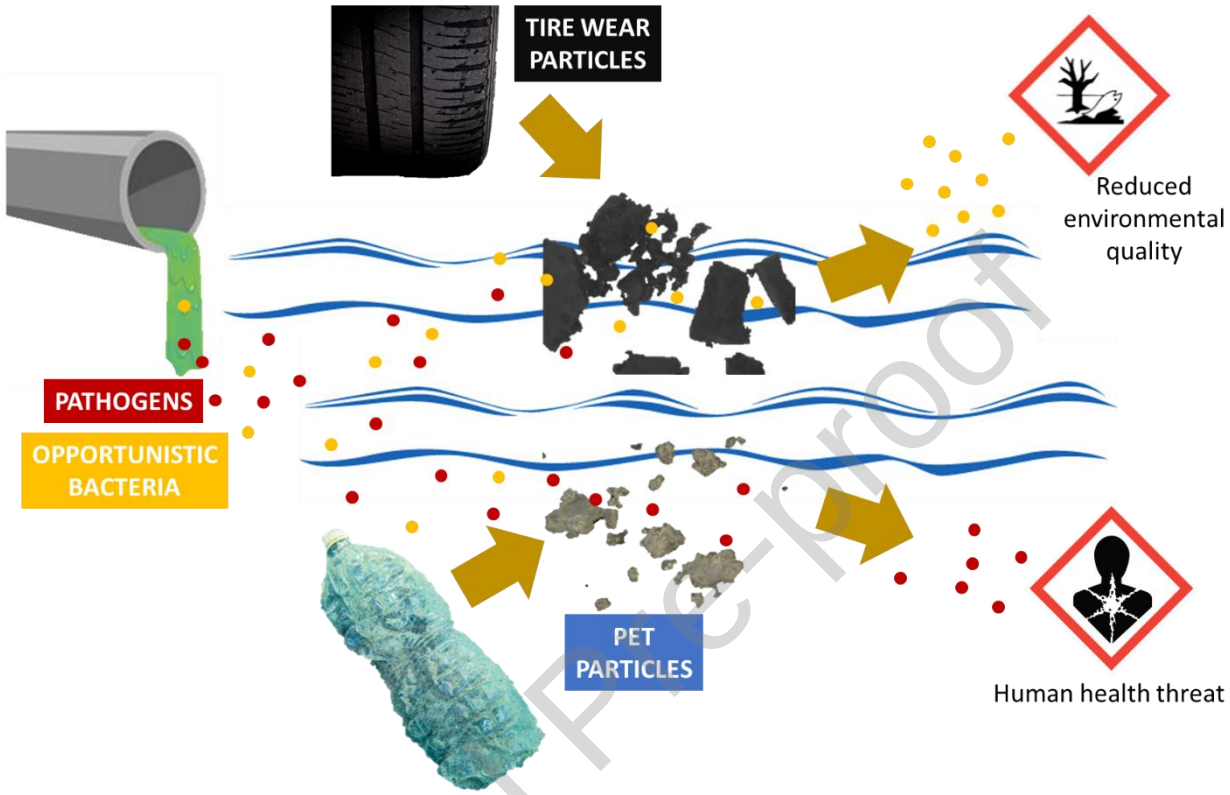
CRediT authorship contribution statement

MB Sathicq: Methodology, Validation, Formal analysis, Investigation, Writing - review & editing, Funding acquisition. R Sabatino: Methodology, Validation, Formal analysis, Data curation, Writing - review & editing. A Di Cesare: Conceptualization, Methodology, Writing - review & editing. EM Eckert: Methodology, Validation, Writing - review & editing. D Fontaneto: Formal analysis, Writing - review & editing. M Rogora: Formal analysis, Writing - review & editing. G Corno: Conceptualization, Formal analysis, Investigation, Data curation, Writing - original draft, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Graphical abstract



HIGHLIGHTS:

4. Microplastic particles of different origin select for a specific pathobiome
5. PET particles offer a refuge for allochthonous/rare potential pathogens in water
6. Tyre wear particles are an additional source of nutrients for fast growing bacteria