Core ideas

- The number of active phages decreases upon exposition to microplastics.
- The "disappearance" of phages is based on the adsorption of virions to plastic.
- Polymer leachable additives have a negative effect on the activity of bacteriophages.
- Adsorbed phages can be recovered by incubating with TWEEN-20.
- The heteroaggregation of virions with microplastic is governed by DLVO theory.

Heteroaggregation of Virions and Microplastics Reduces the Number of Active Bacteriophages in Aqueous Environments

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ABSTRACT

The objective of this study is to explore the effects of microplastics on the viability of the bacteriophages in an aqueous environment. Bacteriophages (phages), i.e., viruses of bacteria, are essential in homeostasis. It is estimated that phages cause up to 40% of the death of all bacteria daily. Any factor affecting phage activity is vital for the whole food chain and the ecology of numerous niches. We hypothesize that the number of active phages decreases due to the virions' adsorption on microplastic particles or by the released leachables from additives used in the production of plastic, e.g., stabilizers, plasticizers, colorants, and reinforcements. We exposed three diverse phages, namely T4 (tailed), MS2 (icosahedral), and M13 (filamentous), to 1 mg/mL suspension of twelve industrial grade plastics [acrylonitrile butadiene styrene (ABS), high-impact polystyrene (HIPS), poly-ε-caproamide (PA6), polycarbonate (PC), polyethylene (PE), polyethylene terephthalate (PET), poly(methyl methacrylate) (PMMA), polypropylene (PP), polystyrene (PS), polytetrafluoroethylene (PTFE), polyurethane (PUR), polyvinyl chloride (PVC)] shredded to obtain microparticles of radius ranging from 2 to 50 μm. The effect of leachables was measured upon exposure of phages not to particles themselves but to the buffer pre-incubated with microplastics. A double-overlay plaque counting method was used to assess phage titers. We employed a classical linear regression model to verify which physicochemical parameters (65 variables were tested) govern the decrease of phage titers. The key finding is that adsorption mechanisms result in up to complete scavenging of virions, whereas leachables deactivate up to 50% of phages. This study reveals microplastic pollution's plausible and unforeseen ecotoxicological effect causing phage deactivation. Also, phage transmission through adsorption

can alter the balance of the food chain in the new environment. The effect depends mainly on the zeta potentials of the polymers and the phage type.

Abbreviations

ABS, acrylonitrile butadiene styrene; CLRM, Classical Linear Regression Model; HIPS, high-impact polystyrene; PA6, poly-ε-caproamide; PC, polycarbonate; PE, polyethylene; PET, polyethylene terephthalate; PMMA, poly(methyl methacrylate); PP, polypropylene; PS, polystyrene; PTFE, polytetrafluoroethylene; PUR, polyurethane; PVC, polyvinyl chloride; TOC, total organic carbon.

1. INTRODUCTION

It is estimated that approximately 10^{31} bacteriophage virions, i.e., individual viral particles, are present in the world at any given time (Keen, 2015). They exist in virtually all environments, from ocean waters to highly urbanized zones (Mc Grath and von Sinderen, 2007). The number of virions and their distribution is correlated to the presence of host bacteria. Bacteriophages are an essential contributing factor in the maintenance of homeostasis in the bacterial community. Phages terminate about 40% of bacterial biomass daily (Czajkowski et al., 2019). Herein, we demonstrate the influence of microplastics on the viability of the bacteriophages in aqueous environments. This might be yet another unforeseen mechanism explaining the impact of microplastic on the environment.

Plastics, which are synthetic organic polymers, are produced at a rate of 380 million tons annually (Zhao et al., 2022). Mittal *et al.* predicted world plastic waste would be doubled by 2030 (Mittal et al., 2022). A study conducted in 2015 found 5 billion tons of plastic wastes are accumulated in the environment. (Geyer et al., 2017). Due to minimal biological degradation, they remain in the environment for centuries, eventually ending in water (Cole et al., 2011). Plastics

are broken down into fragments in aquatic systems over time due to wave action, oxidation, and ultraviolet radiation (Ekvall et al., 2019). All this contributes to the overall concentration of microplastic reaching from 10^{-3} to 10 particles per liter in aqueous environments (Kataoka et al., 2019).

Microplastics are defined as a plastic particle with a diameter less than 5 mm divided into primary and secondary categories. Primary microplastics are directly released into the environment from daily-use consumer plastic products. Further fragmentation creates secondary plastic due to exposure to unfavorable physical, chemical, and biological conditions (Julien and Friot, 2017). Microplastic particles do not remain inactive in an aqueous environment. Often, their surface adsorbs nutrients and organic materials, thus providing the necessary ingredients for the formation of microbial biofilms (Shen et al., 2019). The biofilm layer formed on the surface of the microplastics can create an environment where other organisms can colonize. The formed biofilm can significantly affect the substrate's physicochemical properties, biodegradability rate, and, most importantly, its destination and path in the aquatic environment (Tu et al., 2020). Together, these elements change the degree of microplastic immersion and access to air and weather factors and increase the risk of its trophic transport (Feng et al., 2022). Research shows that heterotrophic bacteria colonizing the surfaces of polymers such as polyethylene (PE) or poly(ethylene terephthalate) (PET) in seawater can survive in a submerged microplastic much longer than free-living bacteria in the surrounding waters (Lobelle and Cunliffe, 2011). Evidence shows that microplastics significantly influence the natural evolution of microorganisms by creating an imbalance between the groups of microorganisms that form biofilms. Moreover, microplastics might cause evolutionary changes in microorganisms (Yang et al., 2020).

Microplastics can enter the gastrointestinal tract of aquatic organisms from various trophic levels. Contamination can be passed along the food chain, causing adverse effects and damage to

the health of many marine organisms. It is estimated that, by consuming seafood, humans ingest around 7.7 ± 20 microplastic particles per kilogram of food, which translates into an average of 13 ± 58 microplastic particles per year for a person (Daniel et al., 2021).

Bacteriophages are a major balancing factor in the microbial food web. However, phages are very susceptible to external factors, and their infectivity rates can vary dramatically with environmental changes (Jończyk et al., 2011). Despite the fact that microplastics are known to affect diverse niches and organisms varying from bacteria to humans, there was no link between microplastics and bacteriophages. In this study, we explored the effects of various microplastics upon different types of phages in liquid samples and provided possible explanations regarding the observed decrease in phage titers. We estimated that the accumulated plastic debris, upon degradation to particles of a diameter of 1 μm could scavenge between 10^{30} to 10^{31} virions (i.e., virtually all) *via* adsorption.

2. MATERIALS AND METHODS

2.1 Microplastic preparation

Twelve types of plastic were used for the preparation of microplastic samples: acrylonitrile butadiene styrene (ABS), high-impact polystyrene (HIPS), poly-ε-caproamide (PA6), polycarbonate (PC), polyethylene (PE), polyethylene terephthalate (PET), poly(methyl methacrylate) (PMMA), polypropylene (PP), polystyrene (PS), polytetrafluoroethylene (PTFE), polyurethane (PUR), polyvinyl chloride (PVC). Eleven polymer pieces were purchased from a local commercial service that offers polymer products. PTFE was purchased from a local store selling building materials. All of the used materials are genuine potential sources of microplastics in the environment.

The polymer pieces of a few cubic centimeters were cleaned with paper towels soaked in ethanol and rinsed with ultrapure water. Next, a sharp scalpel was used to remove the surface layer of the polymer. Such exposed materials were next mechanically scraped using a scalpel or rotary tool (Dremel). The fraction of smallest particles generated in this process were collected in glass containers, rinsed with ultrapure water, and dried.

2.2 Incubation of bacteriophages with plastic samples

To assess the impact of microplastic on bacteriophages, we judiciously choose to use MS2, M13, and T4 phages suspended in TM buffer. The bacteriophage preparation method is described in the Supporting Information. The concentration of phage titers was around 5×10^5 PFU/mL. The number of marine viruses in the oceans varies from about 10^6 viruses per mL in the deep sea to around 10^8 viruses per mL in productive coast waters (Suttle, 2005). The number of phages in aquatic environments differs depending on the place and the season (Bergh et al., 1989). In the same spot (Raunefjorden), February's total number of virus particles was below the detection limit (i.e., around 10^4 viruses per mL), but it was about 10^7 viruses per mL in August. We chose 5×10^5 PFU/mL to balance two factors: 1) too high number of virions could "saturate" studied microplastic particles obscuring the titer decrease, and 2) too low number of phages could result in inefficient scavenging, as the adsorption rate could be low because of kinetic (low number of collisions) and thermodynamic (equilibrium shifted towards free virions) reasons.

The experiment examining the heteroaggregation of phages and microplastics was prepared in the following order: 1 mg of the given polymer sample was weighed into 1.5 mL Eppendorf tubes. Then, 1 mL of phage suspension was added. Phage suspensions without microplastic were tested as controls. Samples were first briefly vortexed and then shaken for 1 h, 24 h, or seven days using an orbital shaker (115 rpm) at room temperature. The double-overlay

method was used to evaluate the number of remaining phages in the suspension. The methodology is described in the Supporting Information.

We aimed to verify whether phages were adsorbed onto the polymer particles. First, phages were incubated with selected microplastic samples (1 mg/mL) for 24 h at room temperature with mixing (orbital shaker, 115 rpm). Next, the microplastic particles were separated from the liquid using centrifugation and rinsed twice with TM buffer. Two experiments were conducted on such samples. 1) Pellet was placed onto the surface of the double overlay method agar plate prepared for phage titration. The appearance of inhibition zones indicated the presence of active virions at the surface of the microplastic (**Figure 1B**). 2) TM buffer containing 0.002% v/v TWEEN-20 (termed TM_T) was added to the microplastics, incubated with phages, centrifuged, and rinsed. Such samples were shaken for 24 h using an orbital shaker (115 rpm) at room temperature. The number of phages recovered from microplastic was evaluated using the double overlay method (**Figure 1A**).

For evaluating the effect of leachables, 10 mg of each polymer microplastic was placed in a 15 mL Falcon tube, to which 10 mL of TM buffer was added. Such samples were briefly vortexed and then shaken for 24 h using an orbital shaker (115 rpm) at room temperature. Afterward, microplastic pellets were removed, and the TM buffer samples exposed to polymers (marked TM_P) were used to prepare dilutions of each bacteriophage. Phages in TM_P were briefly vortexed and then shaken for 24 h using an orbital shaker (115 rpm) at room temperature (**Figure 1C**). Phage solution prepared with a buffer that was not exposed to microplastics was used as controls. Double overlay method was used to evaluate the number of remaining phages in the suspension.

2.3 Statistical analysis

Statistical analysis was performed with STATA/MP 17.0. Supporting information contains a database (Database.xlsx) providing the description of the used variables and collected data used for the Classical Linear Regression Models. We presented in detail description of the process of modification of variables, calculation of radii of particles, coarse estimation, and introducing functions derived from the DLVO theory, and regarding wetting angles. The final models are presented with corresponding diagnostics of them (Supporting Information).

More detailed information (chemicals used in the experiments, microplastic BET characterization, preparation of the bacteriophages, evaluation of number of active phages in the suspensions – double-overlay method, total organic carbon (TOC) measurement, and SEM pictures) is described in the supplemental materials section.

3. RESULTS AND DISCUSSION

We judiciously choose T4, MS2, and M13, as examples of very distinct bacteriophages. T4 is a representative of tailed phages. In the study by Ackermann, around 96% among around 5500 inspected phages are tailed (Ackermann, 2007). T4 has three main structural elements: head, tail, and long tail fibers. The genome (dsDNA) is stored in the icosahedral capsid. The tail is composed of a contractile sheath surrounding the tail tube ending in a hexagonal base plate (Maghsoodi et al., 2019). M13 is ssDNA filamentous phage. This phage is vital in biotechnology as it is often used in the *phage display* method (Harada et al., 2018). MS2 bacteriophage has an icosahedral structure, and its genetic material is ssRNA (Farafonov and Nerukh, 2019). MS2 serves as a surrogate for eukaryotic viruses (Turgeon et al., 2014). Despite differences (**Table S1**), all three studied phages share a common host – *Escherichia coli*.

We obtained twelve polymeric materials from commercial sellers. We judiciously chose industrial-grade polymers to reflect the real sources of microplastic in the environment. We prepared polymer samples by mechanically crumbling larger pieces of commercial-grade plastics. This process simulates how plastic fragments are created in the environment. We performed BET analysis to find the surface area per unit mass and the porosity of the studied microplastics. All samples were non-porous with a rather low surface area. Nine samples had a surface area below 0.1 m^2/mg. PE and PUR surface areas were around 0.15 m^2/mg. Significantly larger surface areas were found for PC (0.32 m^2/mg) and PMMA (0.93 m^2/mg). Knowing the density of polymers, we calculated the average radius of the microplastic particles (Supporting Information, equation 10). The radius ranged from 20-50 μm, except for PC (7 μm) and PMMA (2 μm). We also evaluated the size of particles directly by analyzing optical microscopy pictures. Both sets of data were in good agreement (Figure S1, **Table S2,** Supporting Information). Full data, characterization, and exact values of BET analysis are provided in the Database file, Supporting information.

Our recent publication found that virions "disappear" from the suspension by adsorbing onto the surface of plastic labware, resulting in a phage titer decrease of up to 5 logs(Richter et al., 2021). The effect is governed by the hydrophobic/hydrophilic interactions between the surface, water molecules, and virions. For more hydrophobic materials, it is more favorable to "cover" such surfaces with virions than to allow direct contact with water molecules. As a result, water is in contact with more hydrophilic parts of virions, reducing the system's overall energy. This was in line with findings on the sorption of viruses onto mineral particles (Chattopadhyay and Puls, 1999). Reports concerning other biomolecules assume the conformation in which biomolecular hydrophobic parts are in contact with the hydrophobic surface while the more hydrophilic regions of the molecule are exposed to bulk (and water)(Rabe et al., 2011). O'Connell *et al*. showed the

effect of containers on phage titer, but they suggested that the topology of the surface governed the adsorption of phage virions on plastic surfaces (O'Connell et al., 2022). Based on these reports, we hypothesized that virions can be scavenged also by microplastic particles. We ensured that the labware used for the experiments did not influence the phage titers. Phage concentration remained the same after 7 days of incubation in the room temperature. Hence, if there is a titer loss in the results, it is due to phage interaction with microplastic particles.

We found statistically significant differences in T4, MS2, and M13 phages titers upon incubation with microplastic samples. In a few cases, the effect was visible even after 1 h of incubation. After 24 h, we observed up to around 97% decrease in titer (MS2 and PET), with exceptional complete scavenging i.e., by around 5 logs, when M13 was incubated with PET or PVC. The results are summarized in **Table 1**. There were at least three possible mechanisms explaining titer loss: i) virions were scavenged due to the adsorption at the microplastic particles, ii) virions were inactivated due to the contact with the microplastic particles, and iii) virions were deactivated due to leachables released from the microplastic particles.

3.1 The effects of leachables on bacteriophages

Additives are common in the polymer industry. They are used to stabilize and modify end-product properties. For this reason, various kinds of leachables are used depending on plastic producers. Often additives are not chemically bound to the polymer. Instead, they form a solid mixture (Hahladakis et al., 2018), from which a variety of potentially toxic compounds (e.g., plasticizers, slip agents) (Grzeskowiak et al., 2015) washed out into the liquid. Even medical-grade syringes and syringe filters released leachables (Lee et al., 2015).

We evaluated the effect of possible leachables from microplastics on MS2, M13, and T4 bacteriophages. Firstly, TM_P, i.e., TM buffers incubated with 1 mg/mL of microplastics for 24 h,

was prepared (**Figure 1C**). Next, TM_P samples were used to prepare proper dilutions of phage suspensions. After consecutive 24 h incubation, the number of active virions was evaluated. No statistically significant effect was found in the case of MS2. M13 was affected by leachables from five (PC, PS, PTFE, PUR, PVC), whereas T4 from eight (ABS, PA6, PC, PE, PET, PMMA, PP, PTFE) samples. Mostly, the effect was not very pronounced but reached around 50% for M13 (PUR, PS, and PTFE). There was no correlation between the color of the microplastic (ABS, HIPS, PUR were colored) and the adverse effect of leachables. Leachables from HIPS were not affecting any of the studied phages, ABS affected only T4, and PUR only M13. Such "selectivity" of leachables against specific bacteriophages is intriguing and needs further investigation to assess the mechanisms of action. The SEM pictures (Fig. S2, Supporting Information) showed that, at least in some cases, microplastic fragmented further during the experiment. We did not find a correlation between the phage titer decreases and the presence of such nano- or sub-microparticles. We demonstrated that even though leachables affected phage, this was not a primary mechanism of action of microplastic.

We aim to correlate TOC measurements with the decrease in the phage titers due to the presence of leachables. Only in two samples, namely PA6 (4.5 ± 0.7 ppm) and PUR (3.5 ± 0.5 ppm) the content of total organic carbon was above the detection limit (2 ppm). The highest number of leachables resulted in the highest deactivation of phages. Namely, the highest deactivation of T4 phages was caused by leachables from PA6 polymer, whereas PUR caused the highest deactivation of M13. Therefore, the results on phages were in line with TOC measurements. The specific leachables showed varying potency against different phages. Leachables from PA6 did not affect M13 nor MS2, whereas leachables from PUR did not affect T4 nor MS2. Leachables from other polymers might be potent against phages at concentrations

below the detection limit, i.e., 2 ppm. We concluded that leachables have a different impact depending on the phage structure and polymer type.

3.2 The effects of microplastics on bacteriophages

We verified the mechanism beyond the disappearance of phages when leachables alone did not have any statistically significant effect on the phage titer, but microplastic particles did. After incubation with phages, particles were separated from the liquid and washed carefully with fresh TM buffer. Afterward, they were placed onto a double overlay agar plate with bacteria. Clear inhibition zones were visible in samples exposed to phages, whereas pristine microplastic was not causing such an effect (**Figure 1B**). The same microplastic particles with adsorbed virions were resuspended in a fresh TM buffer containing TWEEN-20 (TM_T) (**Figure 1A**). TWEEN-20 compound was used as a detergent to separate phages from the surface of a microplastic. Active virions were detected after 24 h incubation of microplastic particles in TM_T, but not in just TM buffer. The only source of virions was microplastic, i.e., the reappearance of phages was caused by the desorption of virions from microplastic particles.

We assumed that for cases where leachables did not have a significant effect, phages that "disappeared" were bound to microplastic. We consistently observed active phages for MS2 (adsorbed on PUR), M13 (adsorbed on PS), and T4 (ABS, HIPS, PVC, PUR, PS), but the experimental recovery rate ranged from a few percent to below 90% of the predicted values. Not all phages scavenged from the suspension were active at the surface of microplastics. It was shown before that heteroaggregation of phages with colloidal sediments affects the viability of enveloped phages (Katz et al., 2018). We studied non-enveloped phages, and it was unclear if the contact with microplastic caused the deactivation of virions. The high local concentration of leachables (higher than in bulk) could also lower apparent recovery rates.

In **Figure 2**, we plotted data from **Table 1**, but only where Student's t-test suggested a statistically significant difference from the control samples at $p < 0.05$. The t-Student test was performed (using Origin software) to get the p-values. The statistical significance of the drop of the phage titer is tested against the control, which was set as 100%. Next, we marked the cases (hashed columns) where leachables (TM_P) caused a similar decrease as microplastic after 24 h incubation. Formally, we checked if the percentage of survivors in TM_P minus the standard deviation of TM_P was smaller than the percentage of survivors upon exposition to microplastic.

The collected data suggested that the efficacy of both mechanisms (leachables and scavenging) varies depending on bacteriophage type. After 24 h of incubation with microplastic particles, the average drops in phage titer (calculated for all 12 studied polymers) were around 70% for T4, 60% for MS2, and 50% for M13. Leachables also appeared selective against phages, with MS2 showing no significant titer decrease in TM_P, as opposed to T4 and M13.

We aimed to draw more general conclusions on the relations between the physicochemical parameters of microplastics and their impact on phages. However, simple characteristics, such as the wetting angle or the size of particles, were not sufficient to describe the process. For instance, HIPS and PS microplastic samples had similar particle sizes (around 30 μm and 39 μm, respectively) and similar wetting angles (approximately 82°). HIPS usually comprises some additives, e.g., 5 to 10% rubber or butadiene copolymer (Wang et al., 2019). Hence, we expected that HIPS might have a more significant effect on phage titers due to additives compared to PS. Surprisingly, leachables from HIPS did not show any adverse effect, whereas TM_P (PS) resulted in around 45% decrease in M13 titer. The effect of adsorption was similar in the case of T4 (approximately 40% and 30% decrease upon 24 h incubation with HIPS and PS particles, respectively). Still, PS had more impact on M13 (around 75% *versus* about 40% decrease) and

MS2 (around 70% decrease *versus* almost no decrease). Based on this comparison, it is clear that the reduction of phage titer upon exposition to microplastic is a multivariable phenomenon.

3.3 Classical Linear Regression Model

To analyze the importance of various parameters on phage scavenging by microplastics, we utilized models often applied for the quantitative measurement of economic phenomena (Mycielski, 2010). The Classical Linear Regression Model (CLRM) is widely used to estimate the relations between a dependent variable and explanatory variables. It is especially useful in big data analysis. The model in the form:

$$y = X\beta + \varepsilon \tag{1}$$

$$y = \begin{bmatrix} y_1 \\ \vdots \\ y_\mathrm{i} \end{bmatrix}, X = \begin{bmatrix} x_{1,1} & \cdots & x_{1\mathrm{j}} \\ \vdots & \ddots & \vdots \\ x_{\mathrm{i}1} & \cdots & x_{\mathrm{ij}} \end{bmatrix}, \beta = \begin{bmatrix} \beta_1 \\ \vdots \\ \beta_\mathrm{j} \end{bmatrix}, \varepsilon = \begin{bmatrix} \varepsilon_1 \\ \vdots \\ \varepsilon_\mathrm{i} \end{bmatrix} \tag{2}$$

explains the value of a dependent variable y in terms of a set of explanatory variables x_{ij}.and a random variable ε. The vector β contains the parameters of a linear combination of variables x_{ij}. The value of β was found according to the principle of ordinary least squares regression. The dependent variable y_i was the percentage of surviving phages after 24 h incubation with microplastic and taken as the mean value of three biological repetitions (**Table 1**). By changing the variables x_{ij} we search for the set of parameters that allowed for the best fit giving the highest R^2 value.

We built a database of the physicochemical variables based on the literature (i.e., density, zeta potential, contact angle) and our experimental data (i.e., BET measurements, wetting angle). We also modified them by considering their functions (i.e., tanh x, x^2), binary representations (i.e., hydrophobic/hydrophilic), or synergy effects (interactions between the variables). For all the details, see the Database file. Supporting information contains detailed descriptions of the variable

selection, construction of the model, and diagnoses of the selected models (**Figure S3 – S14, Table S3 – S4**).

3.3.1 Coarse analysis and the impacts of the polymers

As an initial benchmark, we used a model in which categorical binary variables describing the bacteriophages and the polymers were used. This was not meaningful from the physicochemical point of view but allowed us to estimate the R^2 of the scenario, with multiple variables corresponding to the combination of virtually all properties of phages and polymers. In such a case R^2 value was 0.80. Therefore, we expected the linear model to explain the variance of the observed phenomenon (y_i) in around 80%. The rest might come from data scattering due to experimental inaccuracy as estimated parameters had a relatively high standard error (Supporting Information).

To calculate the polymer's average influence on the phages' activity, we performed the regression with a categorical variable describing the type of the polymer. We compared the β coefficients for all of the polymers (**Figure 3A**, Supporting Information). Subsequently, we categorized them depending on very high ($\beta_i < -60$), high ($-60 < \beta_i < -45$), medium ($-40 < \beta_i < -25$) or low-impact ($\beta_i < -25$) on phages. PET and PVC were classified into the "very high impact" category, ABS, PS, and PUR into the "high impact", PP and HIPS into the "medium impact" and PA6, PC, PE, PMMA, and PTFE into the "low impact". Such categorization was surprisingly in line with recent data providing a risk ranking of the 36 microplastics(Yuan et al., 2022). In this report, the baseline model ranked the polymers according to the calculated risk factor. The positions of the polymers studied by us were as follows: (1) PUR, (2) PVC, (4) ABS, (5) PMMA, (9) PET, (10) PS, (12) HIPS, (13) PP, (15) PC, (16) LDPE, (19) PTFE, (23) PA6. We underline that the authors also considered the amount of production of the given polymer as one of the parameters influencing risk factors. The correlation (but not causation) between the most "risky"

polymers and the magnitude of impact on phages underlines the need to investigate phage scavenging by microplastic.

3.3.2 Introducing physicochemical factors into the model

In a recent paper, Hicks and Wiesner studied bacteriophage and kaolinite heteroaggregation. Regardless of the ionic strength or the tested phage (T4) to kaolinite ratios, the phenomenon occurred rapidly and was likely driven by Derjaguin—Landau—Verwey—Overbeek (DLVO) forces (Hicks and Wiesner, 2022). This was in agreement with a study from 2012 by Chrysikopoulos and Syngouna. They used extended-DLVO interaction energy calculations and showed that the attachment of viruses (MS2 and PhiX174) onto model clay colloids (including kaolinite) was primarily caused by hydrophobic interactions(Chrysikopoulos and Syngouna, 2012). Later works showed that hydrophobic and electrostatic interactions governed the deposition process (Armanious et al., 2016; Dang and Tarabara, 2019).

DLVO theory describes the stability of identical spheres (colloid), interaction energy as a sum of the van der Waals attraction energy, and double-layer repulsion energy (Derjaguin et al., 1987; Ohshima, 2012). Repulsion free energy of two spheres can be described as:

$$E = \frac{64\pi k_B T r \rho_\infty \gamma^2}{\kappa^2}\, e^{-\kappa d} \tag{3}$$

$$\gamma = \tanh\left(\frac{z e_0 \psi_0}{4 k_B T}\right) \tag{4}$$

where, r – radius of the sphere, ρ_∞- the number density of ions in the bulk solution, γ – reduced surface potential, d – the distance between spheres, z – valency of the ion.

We searched for variables that might have physicochemical meaning for the adsorption process. We tested almost two hundred variables and their interactions (realized as a function of two variables, e.g., $x_i = x_a x_b$). Throughout the selection process of the variables, we came to the conclusion that the model based on the interaction between type of the phage and squared zeta

potential of polymer, average radius of the particle and cosine of the contact angle of the polymer may describe the phenomenon to the certain level ($R^2 = 0.655$).

We found a strong dependence between the number of survivors (in percent) and the zeta potential of polymers. We assumed that ψ_0 (Equation 4) is related to zeta potential ζ, and therefore $\gamma = \tanh(a\zeta)$. For small values of parameter a and in the range of ζ from our data, the distribution of values of $\tanh(a\zeta)$ is proportional to the distribution of values of $a\zeta$. In other words, for small a and within the certain range of ζ, $\tanh(a\zeta) = Ca\zeta$. This assumption also gave a better correlation in the linear regression. That confirmed our conviction to simplify γ to ζ. The constant Ca will be included in β (Equation 1). Because E depends on γ^2, therefore we tested ζ^2 as a variable. To consider the interaction between non-identical objects (microplastic particles and phages of different morphologies), we introduced interactions between ζ^2 of the polymers and a categorical variable describing the phage type.

To include characteristics related to hydrophobicity, we introduced the cosine of water wetting angle as a parameter in the model. This parameter is essential in the Young equation to characterize all interfacial energies (γ_{SG} solid-vapor; γ_{SL} solid-liquid; γ_{LG} liquid-vapor interfacial energy):

$$\cos\theta = \frac{\gamma_{SG} - \gamma_{SL}}{\gamma_{LG}} \quad (5)$$

We found that the size of the particles contributed to the overall quality of the fit when it was analyzed as a combination of r (radius of particles) and r^2. For the analysis, we used values of r based on BET surface area per unit mass and macroscopic density of polymers ρ assuming the spherical shape of the particles:

$$r = \frac{3}{BET \cdot \rho} \quad (6)$$

BET and density themselves were not significant parameters. According to the model, the most pronounced decrease in phage titers was suggested for particles from around 20 to 40 μm. Both small and large particles did not cause a significant titer drop. Small particles have a limited number of active sites where virions could adsorb, whereas large particles have a small overall surface area.

Using only variables related to ζ^2, $cos\theta$, r, and r^2 resulted in the model that explained the analyzed phenomenon in around 65% (R^2 = 0.655). However, this model did not pass some diagnostic tests usually performed in such analysis (Supporting Information). This was most likely due to a limited number of data points, or the functional character of the model was not sufficient to fully explain the phenomenon. The model was based only on 36 experiments (each experiment consisted of 3 biological replicates) and an additional value representing only buffer (base 100% activity of the given bacteriophage after 24 h without polymer). Therefore, we treat these results as suggestions and not proofs.

3.3.3 Combined approach toward physicochemical and other factors

The best model that we found (and a model which passed all the diagnostic tests, Supporting information) showed the importance of ζ^2 and a categorical parameter related to ABS, PET, PS, and PUR (**Figure 3B**). Such model gave R^2 = 0.813 and adjusted R^2 = 0.767, which is remarkably high. In other words, those results suggest that DLVO theory expressed by the dependence on the interaction between ζ^2 and the type of the phage is sufficient to describe the deactivation of phages with most of the polymers. In the case of ABS, PET, PS, and PUR there is an additional factor decreasing the activity of the phages, which cannot be neglected. We searched for the feature that differentiated these four polymers. We hypothesized that it could be related to the aromatic character of these polymers. HIPS, which also possesses aromatic domains, contains substantial

amounts of additives, which might modify interactions with virions and thus did not appear significant in the analysis.

4. Summary

We found two mechanisms causing the reduction of phage titers: 1) action *via* leachables or generated nano- and sub-microparticles, and 2) adsorption of virions at the surface of microplastic particles. Virions scavenging *via* adsorption has a more pronounced effect than leachables and secondary, small particles.

Data were fitted using the CLRM to verify which parameters are significant to describe the phenomenon. We were able to find a set of parameters giving $R^2 = 0.813$. We revealed that the primary parameter is the zeta potential of polymers. This was in line with previous studies describing the heteroaggregation of phages (predominantly icosahedral, e.g., MS2) and microparticles (usually mineral or clay) using DLVO or extended DLVO theory (Chrysikopoulos and Syngouna, 2012; Armanious et al., 2016; Dang and Tarabara, 2019; Hicks and Wiesner, 2022).

There are, however, limitations of the presented results, which need to be addressed in future works:

- We did not aim at identifying the leachables. Others make considerable efforts in this respect (Cooper and Tice, 1995; Vandenberg et al., 2007; McDonald et al., 2008; Grzeskowiak et al., 2015; Lee et al., 2015; Zhang et al., 2016; Hahladakis et al., 2018), but the formulation used by local producers might vary, influencing the release rate and types of leachables. Also, the post-processing of plastics might have an impact, e.g., by altering roughness or brittleness.

- The concentration of microplastic used in the study was relatively high. Such conditions are possible upon debris accumulation as in the case of the "Great Pacific garbage patch" (Lebreton et al., 2018), or upon further careless disposal of plastic wastes.
- The studied concentration of phages was fixed and was lower than average but still higher than previously reported in specific regions or seasons (Bergh et al., 1989; Suttle, 2005).
- Ionic strength is crucial for electrostatic interactions in the case of virions(Schaldach et al., 2006). We used a relatively low ionic strength buffer (ionic strength equal to around 50 mM). The interaction energy between virions and the charged surface is more extensive for lower ionic strengths (Schaldach et al., 2006). However, the ionic strength of seawater is around 0.7 M. It was found that high ionic strength facilitates the deposition of nanoparticles at the surface (Winkler et al., 2011). In the experiments on nanoparticles (NPs) by Winkler *et al*., ionic strength similar to that of seawater corresponded to the regime in which "NPs adsorb and form dense layers". Therefore, it is likely that higher ionic strengths could facilitate the scavenging of virions (especially small ones, e.g., MS2 (Farafonov and Nerukh, 2019)). Moreover, Schaldach et al. showed significant differences in the interactions due to changes in pH (Schaldach et al., 2006).

To conclude, microplastic has become a significant concern. It was found to affect numerous environmental niches and organisms. In this study, we showed the link between microplastic and bacteriophages. The presence of microplastic results in a decrease in the number of active bacteriophages in aquatic environments. Bacteriophages cause the death of around 20% to 40% of all bacteria every day ("Microbiology by numbers," 2011), participating in the homeostasis of numerous niches (Czajkowski et al., 2019). The effect of microplastic on such a

basic level might propagate to more complex environmental systems where bacteria are of importance.

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SUPPLEMENTAL MATERIAL

Supplementary material includes:

- Materials and methods section: chemicals used in the experiments; microplastic BET characterization; preparation of the bacteriophages; evaluation of number of active phages in the suspensions – double-overlay method; total organic carbon (TOC) measurement; SEM pictures.

- Analysis of the results using Classical Linear Regression Model: coarse estimation; building the model; Model 1A; Model 1B; Model 2 (XLSX).

Author Contributions

‡ These authors contributed equally. The manuscript was written through the contributions of all authors. All authors have given approval for the final version of the manuscript.

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Data availability

The data described in this article are available in Dryad at https://doi.org/10.5061/dryad.63xsj3v6k.

Conflict of interest

The authors declare no competing financial interest.

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Figure 1. A) The scheme shows the experimental design that indicated the adsorption of virions at the surface of microplastic particles. Phages were incubated with microplastic particles.

Afterward, the liquid suspension was discarded, and fresh TM or TM_T buffer (TM supplemented with TWEEN-20) buffer was added to microplastic samples. The addition of TWEEN-20 allowed for the reappearance of phages in the liquid suspension. B) Pictures of double overlay agar plates onto which microplastic and microplastic incubated with T4 phages, separated from the liquid and rinsed, were placed. Bacteria-free zones are visible in samples incubated with phages, proving that active virions adsorbed at the surface of microplastic. C) The experimental design aims to evaluate the influence of leachables. First, microplastics were incubated in TM buffer, allowing leachable to be washed out. The liquid was separated from the solid particles. Such buffer enriched in leachables was termed TM_P and was used to prepare final dilutions of phages. Titer drop was measured after incubation with TM_P.

Figure 2. Graphs showing the influence of twelve microplastic samples and leachables (TM_P) on T4, MS2, and M13 bacteriophages. Schemes on the left show structures of studied phages. The percentage of active phages is relative to the control experiment (without plastics; 100%). Results where $p < 0.05$ (estimated with Student t-test) were plotted. PUR and PVC caused the decrease of M13 titer below the detection limit (below 25 PFU/mL, which corresponds to around 0.01%). Hashed bars represent the situations in which leachables (TM_P) caused a similar or more significant titer decrease compared to microplastic after 24 h incubation.

Figure 3. A) The average decrease of activity (expressed in percentage) of the phage caused by the certain polymer after 24 h. Linear regression with the categorical variable describing the type of the polymer. B) Fitted activity decrease according to our model. The model was based on the influence of the squared zeta potential for every phage and the categorical variable describing the type of the polymer.

644 **Table 1.** Changes in phage titer upon incubation with leachables and exposition to microplastic samples for 1 h and 24 h are shown (the
645 number of active phages is expressed in percentages). Samples showing statistically significant differences upon exposition to
646 microplastic particles are in italics. The concentrations (expressed in PFU/mL) in control samples (averages from three biological
647 replicates) were as follows: T4 1 h $4.12\times10^5\pm2.56\times10^4$; T4 24 h $4.03\times10^5\pm1.57\times10^4$; T4 leachables $3.42\times10^4\pm2.91\times10^3$; M13 1 h
648 $4.48\times10^5\pm2.96\times10^4$; 24 h $2.30\times10^5\pm1.01\times10^4$; M13 leachables $1.64\times10^5\pm1.39\times10^4$; MS2 1 h $3.29\times10^5 \pm 1.82\times10^4$; 24 h $1.92\times10^5 \pm$
649 1.22×10^4; MS2 leachables $4.63\times10^4 \pm 6.76\times10^3$.

	T4			**M13**			**MS2**		
	1 h	**24 h**	**leachables**	**1 h**	**24 h**	**leachables**	**1 h**	**24 h**	**leachables**
ABS	89.68±6.74%	*61.49±3.22%* ***	*80.24±9.29%* **	*63.75±5.51%* ***	*38.04±4.59%* ***	92.59±10.81%	*84.30±6.59%* *	*44.35±5.56%* ***	81.76±23.77%
HIPS	100.00 ± 7.61%	*60.59±3.45%* ***	91.46±9.29%	*68.03±5.77%* ***	*57.97±5.86%* ***	84.70±8.90%	88.61±6.81%	99.13±8.48%	111.49±31.37%
PA6	90.08±7.05%	*70.60±4.39%* ***	*61.71±6.54%* ***	*71.76±5.79%* ***	*80.98±4.03%* **	115.45±13.11%	85.32±7.17%	*80.87±7.93%* *	69.37±20.55%
PC	96.15±7.39%	*80.54±5.2%* **	*81.46±1.59%* **	*71.38±5.87%* ***	*77.17±5.44%* ***	*73.10±8.36%* *	85.57±6.03%	*78.70±6.73%* *	106.53±31.22%
PE	89.88±7.07%	*71.43±4.51%* ***	*72.20±8.91%* **	*65.99±5.62%* ***	*74.46±5.11%* **	106.88±11.78%	115.19±9.92%	91.74±7.96%	81.76±26.05%
PET	102.23±7.68%	*42.65±3.38%* ***	*78.54±7.45%* **	*81.60±7.13%* *	*0.00%* ***	112.35±14.22%	93.67±7.49%	*2.93±0.33%* ***	106.53±30.48%
PMMA	*84.82±6.01%* *	*77.43±4.39%* ***	*79.51±11.27%* *	*65.24±5.21%* ***	*76.09±6.72%* **	82.71± 9.47%	90.38±6.52%	*81.30±8.54%* *	104.05±29.04%
PP	88.26±7.38%	*80.75±4.34%* ***	*78.29±7.85%* ***	*70.26±5.96%* ***	*43.48±6.89%* ***	106.32±11.08%	108.61±7.69%	*72.61±7.50%* **	84.23±25.71%
PS	85.22±6.96%	*67.08±4.15%* ***	89.27±9.53%	*80.11±6.66%* **	*34.78±4.54%* ***	*55.84±8.73%* ***	*51.9±4.23%* ***	*32.61±3.11%* **	123.87±33.34%
PTFE	91.09±7.01%	*85.09±5.2%* *	*79.02±8.83%* **	*64.87±5.08%* ***	*56.52±4.20%* ***	*52.28±7.48%* ***	89.87±7.64%	100.00±8.54%	109.01±31.70%
PUR	*80.97±6.12%* *	*66.67±3.88%* ***	95.85±9.84%	*68.40±5.57%* ***	*46.74±5.39%* ***	*51.78±6.3%* ***	91.14±7.61%	*52.61±6.65%* ***	85.47±26.31%
PVC	90.89±7.04%	*60.87±4.23%* ***	90.00±10.01%	*74.72±5.69%* **	*0.00%* ***	*72.54±8.58%* *	*80.51±5.53%* **	*10.83±1.95%* ***	89.19±25.52%

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