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Engineering a microbial 'trap and release' mechanism for microplastics removal

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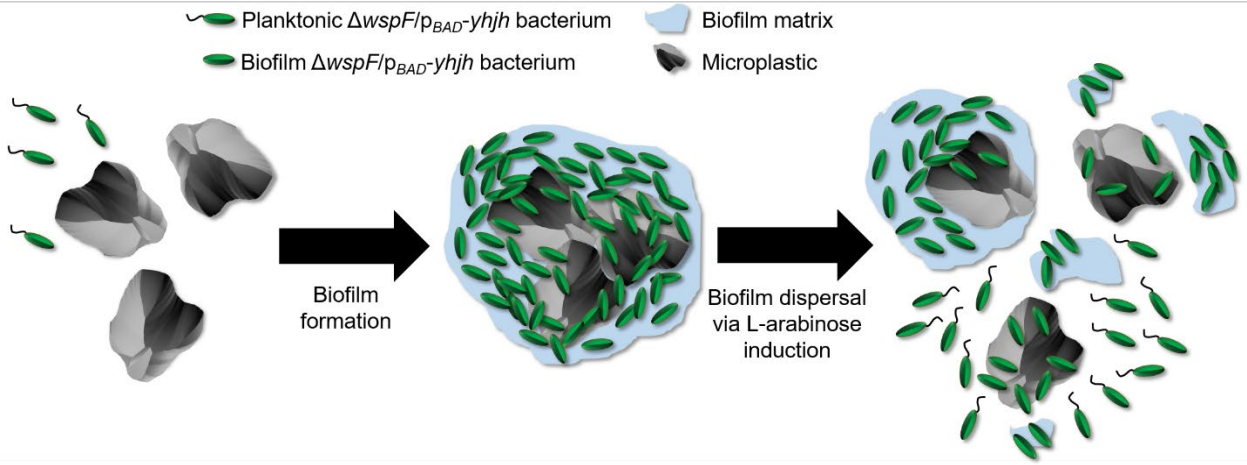
Abstract:

Plastics are discarded and accumulated in the environment at an alarming rate. However, their resistance to biodegradation allows them to persist in the environment for prolonged durations. While large plastics are easier to remove, microplastic particles from cosmetics or fragments from larger pieces are extremely difficult to remove from the environment. Furthermore, current techniques such as filters poorly retain microplastics or require harsh chemical treatments in wastewater treatment plants. Hence, microplastics enter the natural environment easily even after effluent treatments, thereby endangering aquatic life and human who consumed seafood. It is imperative to develop novel cheap and sustainable bioaggregation processes to trap microplastics quickly for easier removal from the environment. Here, we showed that microplastics can be trapped and aggregated in the sticky exopolymeric substances produced by biofilms. As proof-of-concept, we engineered a bacterial biofilm with a 'capture-release mechanism', whose EPS can first cause bioaggregation of microplastics for easier isolation, followed by an inducible biofilm dispersal mechanism that releases trapped microplastics for downstream resource recovery. We also demonstrated the potential application of the engineered biofilm in mitigating microplastics pollution in seawater samples collected in the vicinity of a sewage outfall. We have demonstrated that this capture-and-release approach should prove widely applicable to remove other micropollutants or used in other biofilm-enabled catalysis.

Keywords: Microplastics; *Pseudomonas aeruginosa*; biofilms; bioaccumulation; exopolymeric substances



Graphical Abstract. Schematic illustration of 'capture-and-release' mechanism of engineered *P. aeruginosa*.

1. Introduction:

Pollution caused by plastics, especially microplastics, is a major environmental concern as the world becomes increasingly industrialized. Microplastics are synthetic hydrocarbon-based particles with a size range between 1 μm to 5 mm, with diverse sources from cosmetics, synthetic textile, packaging and broken pieces of larger plastic items. Their recalcitrance to biodegradation allows microplastics to persist in the natural environment, especially in water bodies. These pollutants can pass through even the most efficient water filtration systems and end up being released into water bodies. Furthermore, the highly varied composition, size and pollutants attached to the surface of microplastics pose a multitude of problems to the biota. Hence, the effects of microplastics on organisms could be dire, where humans or animals down the food chain could ingest them or suffer from toxic pollutants attached to the microplastics [1-3].

There are multiple challenges to be addressed in the removal of microplastics from waste and the polluted environment. Firstly, common microplastics such as polyethylene (PE), polypropylene (PP) and polystyrene (PS) are positively or neutrally buoyant that exist as dispersed or suspended solid particles, making meaningful isolation for separate disposal difficult. Even with the use of harsh chemical treatments or expensive filters [4] in wastewater treatment processes, a significant amount of microplastics remains in the effluent, rendering wastewater treatment plants as a main source of microplastics pollution [5-7]. Next, bioremediation of plastics is currently limited to specific enzymes such as the PETase which could degrade polyethylene terephthalate (PET) [8], but is inefficient in removing a mixture of microplastics which comprise various polymer types and sizes. Lastly, incineration of plastic debris such as polyvinyl chloride (PVC) together with other waste can emit toxic dioxins [9]. Hence, PVC recovery and recycling processes are assessed to be environmentally friendlier

than primary PVC production [10]. The European Commission had set new rules on PVC waste recovery and reuse in construction projects in its Waste Framework Directive [11].

Bioremediation strategies have the potential to address the challenge of microplastics contamination, but the biological processes to degrade microplastics may take a long time. There is a paucity of studies on the interactions of biological materials on microplastics. Environmental microbes could form multicellular biofilms with their self-produced exopolymeric matrix on microplastics, often with altered diversity, metabolism and function [12, 13], allowing microbes to colonize on the microplastic surfaces. Metagenomic studies had been conducted to identify microbes that colonize and grow biofilms on microplastics, with the *Pseudomonad* genus prevalently isolated [13-16]. Furthermore, microplastics could aggregate with marine biogenic particles comprising live or dead biomatter and sink into the deep-sea sediment [17].

Biofilm formation and dispersal in many bacterial species are controlled by the intracellular c-di-GMP secondary messenger signaling. Typically, synthesis of c-di-GMP by GGDEF-containing diguanylate cyclases (DGCs) leading to high c-di-GMP levels will promote biofilm formation, while degradation of c-di-GMP by EAL/HYP-containing phosphodiesterases (PDEs) lead to biofilm dispersal [18]. Many bacterial species contain multiple DGCs and PDEs, reflecting their redundancy is key to fine tune metabolism and biofilm formation for survival [19]. One example of c-di-GMP signaling is the *wsp* chemosensory pathway in *P. aeruginosa*, where WspR is a DGC involved in c-di-GMP synthesis and production of exopolysaccharides for biofilm formation [20]. The WspA is involved in sensing surfaces, leading to autophosphorylation of WspE, which in turn phosphorylates and activates downstream WspR. Deletion of the *wspF* methylesterase gene can cause overmethylation of WspA and results in constitutive activation of WspR, leading to enhanced biofilm formation [20].

One potential way to remove microplastics is bioaggregation, which has been utilized in the gradual accumulation of toxic substances such as pesticides or metalloids by an organism from the polluted environment [21, 22]. Furthermore, convenient recovery of plastic from bioaggregation may promote the recycling of retrieved plastics as end-of-life alternatives, instead of choosing landfills or incineration which are highly polluting to the environment. It is estimated that nearly 80% of plastics ended up in landfills and 12% was incinerated, but a meagre 9% was recycled [23]. Hence, recycling was ranked as the preferred choice over incineration and landfills [24], warranting the need for improved recycling efforts.

As a proof-of-concept, we engineered a ubiquitous environmental bacterium, *P. aeruginosa* biofilm which can efficiently aggregate microplastics within its sticky matrix and be later induced to release trapped microplastics for convenient downstream retrieval and recycling. This 'trap-and-release' bioaggregation strategy has several advantages: firstly, microplastics can be aggregated irrespective of their material, size or composition and thereby circumvent the specificity issue. Next, microplastics are localized at high concentrations and can be cumulatively removed from the environment at ease. The increased total mass will promote easier removal by filtering or sedimentation in tanks. Finally, to release microplastics from the sticky exopolymeric matrix of biofilms, treatment with an inducible stimulus could disperse biofilms and release the microplastics for convenient retrieval.

2. Materials and Methods:

2.1 Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* DH5a strain was used for standard DNA manipulations. For bacterial growth, LB medium was used to cultivate *E. coli* and *P. aeruginosa* strains. For experiment, *P. aeruginosa* strains were grown in ABTGC (ABT minimal medium supplemented with 2 g L^{-1} glucose and 2 g L^{-1}casamino acids) [18]; artificial seawater (Instant Ocean Reef Crystals, USA) supplemented with 1 μM $FeCl_3$, 2 g L^{-1} glucose and 2 g L^{-1}casamino acids; or freshwater supplemented with 1 μM $FeCl_3$, 2 g L^{-1} glucose and 2 g L^{-1}casamino acids. Both glucose and amino acids were added at environmentally-relevant levels as carbon and nitrogen sources respectively to simulate the presence of organic matter in seawater and freshwater, thus such media were routinely used in experimental studies of environmental microbes and bioremediation [25-29]. For plasmid maintenance in *E. coli*, the medium was supplemented with 100 μg ml^{-1} ampicillin and 15 μg ml^{-1} gentamicin. For marker selection in *P. aeruginosa*, 30 μg ml^{-1} gentamicin, were used, as appropriate.

Table 1 Bacterial strains used in this study.

Strain/ plasmid	Description	Source/ Reference
P. aeruginosa		
PAO1	Prototypic nonmucoid wild-type strain	[30]
PAO1/p_{lac}-*gfp*	Gm^r; PAO1 containing the Tn7-p_{lac}-*gfp* vector	[31]
PAO1/p_{cdrA}-*gfp*	Gm^r; PAO1 containing the p_{cdrA}-*gfp* vector	[32]
Δ*wspR*	*wspR* knockout of PAO1 constructed by allelic exchange	This study

Δ*wspF*	*wspF* knockout of PAO1 constructed by allelic exchange	[33]
Δ*wspF*/p_{BAD}-*yhjH*	Gm^r; Δ*wspF* containing the p_{BAD}-*yhjH* plasmid	This study
PAO1/p_{lac}-*yedQ*/p_{BAD}-*yhjH*	Gm^r and $Carb^r$; OUS82 containing the p_{lac}-*yedQ* and p_{BAD}-*yhjH* insertion vector	This study
P. putida		
OUS82	Prototypic nonmucoid wild-type strain	[34]
OUS82/p_{lac}-*yedQ*/p_{BAD}-*yhjH*	Gm^r and $Carb^r$; OUS82 containing the p_{lac}-*yedQ* and p_{BAD}-*yhjH* insertion vector	This study
E. coli		
DH5α	F^-, ø80d*lacZ*ΔM15, Δ(*lacZYA*-*argF*)U169, *deo*R, *recA*1, *endA*1, *hsdR*17(rK^-, mK^+), *phoA*, *supE*44, λ–, *thi*-1, *gyrA*96, *relA*1	Laboratory collection
Plasmid		
pJN105	Gm^r; broad-host-range vector carrying the *araBAD* promoter	[35]
p_{BAD}-*yhjH*	Gm^r; pUCP22 carrying the *yhjH* gene	[36]
p_{lac}-*yedQ*	$Carb^r$; pUCP18 carrying the *yhjH* gene	This study
Tn7-p_{lac}-*gfp*	Apr Gm^r; tn7 transposon vector carrying the p_{lac}-*gfp* fusion	[31]
p_{cdrA}-*gfp*	Apr Gm^r; pUCP22 carrying the p_{cdrA}-*gfp* fusion	[32]

2.2 Preparation of microplastics

Microplastics of poly(ethylene terephthalate) (PET), poly(methyl methacrylate) (PMMA), nylon 6/6 and polyvinyl chloride (PVC) were made using a Retsch CryoMill cryogenic grinder (Haan, Germany). The precooling stage lasted 7 min at 5 shakes s^{-1}, followed by the grinding stage for

1.5 min at 25 shakes s^{-1} in the presence of liquid nitrogen. To collect microplastics (<100 μm), the grinded plastics were sieved through a filter (pore size = 100 μm) before use.

2.3 Growth of biofilms on microplastics

Experimental cultivation of *P. aeruginosa* strains was carried out in ABTGC (ABT minimal medium supplemented with 2 g L^{-1} glucose and 2 g L^{-1} casamino acids) [37], artificial seawater (Instant Ocean Reef Crystals) supplemented with 2 g L^{-1} glucose and 2 g L^{-1} casamino acids) or freshwater supplemented with 2 g L^{-1} glucose and 2 g L^{-1} casamino acids).

Microplastics of varying materials at sizes were tested: polyvinyl chloride (PVC), nylon, polystyrene, poly(methyl methacrylate) (PMMA) and Polyethylene terephthalate (PET) at 100<particle size<300 μm or <100 μm. Bacterial cultures were grown to exponential phase in LB for 4 hrs and then diluted 1000-fold for experimental growth in 10 ml medium containing 1, 3 or 5 μg ml^{-1} of microplastics in 50-ml tubes at 25 °C or 30 °C, shaken at 200 rpm, for 24 hrs until sample processing.

2.4 Quantification of dry weight mass of microplastics

Biofilms which accumulated microplastics were disrupted by vortexing and bacterial cells were lysed by sterile ddH_2O + 1% Triton-X (v/v). The microplastics were pelleted by brief centrifugation and the supernatant with bacterial lysate were discarded. The microplastics were washed twice with ddH_2O to remove any bacterial remnants and dried at 40 °C for 8 hrs. The microplastics were weighed on the analytical balance with accuracy of ±0.0001g. Experiments were performed in triplicate, and the results are shown as the mean±s.d.

2.5 Quantification of bacterial numbers by colony-forming units (CFU)

Biofilm cells were homogenized by vigorous vortexing in 0.9% (w/v) NaCl saline solution and the cell suspension was diluted serially in 0.9% (w/v) NaCl solution. The diluted samples were then

transferred to LB agar plates in 5 replicates and incubated for 16 hrs at 37 °C. The colonies grown on the agar plate were then enumerated, with the CFU ml^{-1} tabulated by colony number X dilution factor X volume. Experiments were performed in triplicate, and the results are shown as the mean±s.d.

2.6 Scanning electron microscopy (SEM) sample preparation and image acquisition

As previously described [38], the PAO1 and Δ*wspF* biofilms which contained PVC microplastics were fixed on holders by 0.1M PBS buffer + 5% glutaraldehyde for 4 hrs. Next, the samples were dehydrated through ethanol series, 35% (v/v), 50% (v/v), 75% (v/v), 95% (v/v) once for 10 mins and absolute ethanol (>99% v/v) for 10 mins twice, followed by drying at room temperature for 16 hrs. Before the scanning, the samples were sputtered by gold particles (Nanoimages, MCM-200) and wiped with the conductive glue on the holders' corners. Microscopy images were captured and acquired by using Tescan VEGA3 Scanning electron microscopy (voltage= 20 kv and the magnification= 500× to 15000×). Experiments were performed in triplicate, and representative image was presented.

2.7 Epifluorescence imaging of biofilms and microplastics

As described in the previous section, PAO1/p_{lac}-*gfp* or PAO1/p_{cdrA}-*gfp* strains were grown in 1 mg ml^{-1} PVC (size <106 μm, gray for easier observation). The biofilms which contained the microplastics were transferred carefully to an 8-well chamber (μSlide, ibiTreat, Ibidi, Germany). As control, planktonic PAO1/p_{cdrA}-*gfp* which did not trap any PVCs were directly placed into the 8-well chamber. All microscopy images were captured and acquired by using Nikon Eclipse Ti2-E Live-cell Fluorescence Imaging System with 40× objectives through two channels and Z-stack project at bright field and GFP fluorescence field. At least 5 images were captured for every replicate well. All the images were exported by the NIS (Nikon) program. Experiments were performed in triplicate, and representative image was presented.

2.8 Quantification of c-di-GMP levels in bacterial biofilms

As previously described [39], c-di-GMP were extracted from PAO1 biofilms containing trapped PVC (size <106 μm). Briefly, samples were centrifuged at 16000 g for 2min at 4 ℃, and washed three times by ice-cold PBS buffer. The cell pellet was resuspended in ice-cold PBS buffer and were immediately transferred to incubator at 100℃ for 3 minutes. Ice-cold ethanol was then added into the sample (final concentration = 65% (v/v)) and the sample was vortexed for 15 secs. The treated samples were centrifuged at 16000 g for 2 min, 4 ℃), and the supernatant containing extracted c-di-GMP was collected. The samples were then lyophilized and resuspended in 50 μl ddH_2O. The c-di-GMP in each sample was quantified by using the c-di-GMP ELISA kit (LMAI, Shanghai) per manufacturer's instructions and measuring OD_{450nm} with the microplate reader (Tecan, Infinite M1000 Pro). The c-di-GMP concentration was normalized by protein concentration, where protein concentration (OD_{280nm}) was measured by Nanodrop (Thermofisher, NanoDrop One, ND-One-W).

2.9 Extraction and Quantification of exopolysaccharides

As previously described [40], PAO1 planktonic cells and biofilms were collected from bacterial cultures grown in ABTGC + 1 μg ml^{-1} grey PVC at 30 °C for 24 hrs. The PVC-containing biofilms were decanted and separated from planktonic cells in supernatant by sedimentation for 10 mins. The PVC-containing biofilms were re-suspended in 0.9% (w/v) NaCl and treated with mild water-bath sonication (Elmasonic P120H, Power=50%, Frequency=37 KHz, 5 mins) to separate the cells and PVC from the surface-associated matrix. The cells were then separated from the matrix by centrifugation, leaving behind the crude matrix extract.

The crude extract was then further treated by removal of eDNA by precipitation with 25% (v/v) ethanol and 0.1 M $CaCl_2$. Extracellular proteins were then removed from the extract with 0.5 mg

ml^{-1} proteinase K at 60 °C for 1 hr and inactivation at 80 °C for 30 mins. The extract was then filtered with centrifugal filter (<3 kDa) to remove the metabolites. The extract was then lyophilized and re-suspended in sterile ddH_2O. To quantify exopolysaccharide concentration, the phenol-chloroform assay was used and OD420nm was measured by microplate reader Infinite Pro (Tecan, Denmark). . Experiments were performed in triplicate, and the results are shown as the mean±s.d.

2.10 Screening of DGC/PDE mutant library

As described in the previous section, DGC/PDE mutants from the Seattle Transposon Library [39] were grown in 10 ml ABTGC + 1 mg ml^{-1} PVC (size <106 μm, gray for easier observation) at 37 ℃, shaking at 200 rpm for 24 hrs. The biofilms and microplastics were transferred carefully into microscopic dishes (35 mm diameter). At least 5 microscopy images of each mutant were captured and acquired by stereomicroscope (Nikon SMZ1270i, Japan) at 15× magnification. Experiments were performed in duplicate, and representative image was presented.

2.11 Preparation of seawater samples

Surface seawater was collected from Kwun Tong, Hong Kong (22°18’30”N; 114°13’11”E) close to a sewage outfall, a sampling site which had been identified as a hotspot of microplastics [7]. Collected seawater (150 L) was sieved through 300 μm and 106 μm. Retained materials including microplastics (106–300 μm) were resuspended and concentrated in 1 L of seawater, which remained undisturbed for 3 h to precipitate and discard sand particles. The water column containing microplastics was used here and was well mixed and divided into three seawater samples as replicates to evaluate our bioaggregation approach. The amounts of microplastics in these samples, before and after the bacterial treatments described above, were determined using Raman microspectrometry.

2.12 Raman microspectroscopy of microplastics

Microplastics in each seawater sample were retrieved on a stainless-steel filter paper (31 μm pore size) and assessed by a Renishaw inVia confocal Raman microspectrometer (Wotton-under Edge, England) using 785 nm excitation at 10% laser power for 5 s to acquire Raman spectra (675–1767 cm^{-1}). The whole area coated with microplastics (8 mm in diameter) was scanned at a spatial resolution of 28.4 μm, which is a mapping technique to yield two-dimensional and colour-coded illustration of Raman spectra, to facilitate identification of polymer types as well as sizes and shapes of the microplastics. Baseline correction and smoothing of the acquired spectra were performed with the Renishaw WiRE software. The polymer types of microplastics were identified by matching their Raman spectra with those in the Renishaw Polymeric Materials Database.

2.14 Statistical analysis

All experiments were performed in triplicates. Averages, standard deviations, and independent-group t-tests were carried out in Microsoft Excel. One-way ANOVA, followed by Tukey's multiple comparison tests if required, were carried out in Graphpad Prism.

3. Results:

3.1 P. aeruginosa can form biofilms to bioaggregate microplastics

We first asked if microbes can perform the function of accumulating microplastics by testing the ability of *P. aeruginosa* to accumulate microplastics. The planktonic cells were grown with microplastics in suspension, where biofilms could start colonizing on microplastics and produce exopolymeric matrix that embed microplastic particles. We first tested a variety of microplastics of various sizes and materials. By quantifying the microplastic mass trapped in biofilms and measuring the biofilm cell number via CFU, we showed that *P. aeruginosa* could accumulate large microplastics (100<particle size<300 μm) and small microplastics (<100 μm) of varying materials at 24 hrs (Supplementary Figure 1a-c).

Since bioaccumulation of microplastics by *P. aeruginosa* can be applied to all tested plastic types, we focused on using PVC for our downstream applications. *P. aeruginosa* could trap PVCs of <106 μm diameter (grey color for easier observation) into a bolus-like aggregate (Figure 1a). Its efficiency to trap PVCs improved over time (0, 3, 6, 12 and 24 hrs) where nearly all microplastics were accumulated at 24 hrs, so that dispersed microplastics remaining in free suspension would be reduced (Figure 1b). The mass of trapped microplastics was also correlated to bacterial numbers (Figure 1c), implying that *P. aeruginosa* biofilm was growing and entrapping microplastics. Further examination of the aggregates using CLSM and SEM revealed that bacterial populations localized directly on microplastics (Figure 1d and 1e).

3.2 C-di-GMP signaling is key to biofilm accumulation of microplastics

We next showed that *P. aeruginosa* employed c-di-GMP-mediated biofilm formation to bioaccumulate PVCs. This was reflected by direct quantification of c-di-GMP by ELISA (Figure 2a) and observation of biofilms containing a GFP-based biosensor (p_{cdrA}-*gfp* reporter) whose

GFP expression correlated to c-di-GMP expression [32] (Figure 2b). Accumulation of microplastics was attributed to the production of biofilm-associated exopolysaccharides, where we observed higher polysaccharide levels in microplastic-containing biofilms than in planktonic cells from the remaining media (Figure 2c). Mutations of exopolysaccharides production in the Δ*pelA*Δ*pslBCD* mutant [41] could lead to near-complete loss of microplastics-accumulating biofilms, confirming that exopolysaccharides are key to accumulating microplastics (Supplementary Figure 2).

With the aim of enhancing the microplastics bioaccumulation process, we aimed to develop a *P. aeruginosa* strain with a propensity to form biofilms and accumulate microplastics. We first screened an in-house mutant library of DGCs to identify which gene is important in PVC-mediated biofilm formation (Supplementary Figure 3) and identified that a few mutants, especially Δ*wspR* (PA3702) mutant which could not accumulate microplastics. The Δ*wspR* mutant formed significant lesser biofilms (Figure 2d) and accumulated lesser microplastics (Figure 2e) as compared to the wild-type PAO1.

3.3 Overexpression of wsp operon can boost accumulation of microplastics by biofilms

To improve the capture efficiency of *P. aeruginosa* biofilms, we engineered the expression of *wsp* operon in *P. aeruginosa* by using the Δ*wspF* mutant. We observed that the Δ*wspF* mutant could grow more biofilms (Figure 3a-b) incorporate higher concentrations of PVC microplastics faster than wild-type PAO1 (Figure 3c). By 24 hrs, the Δ*wspF* mutant could accumulate higher mass of PVCs than PAO1. We also tested the ability of the engineered strain to accumulate high levels of PVCs (3 and 5 mg ml^{-1}) and found that it could form biofilm of sufficient size to accumulate more than 90% of PVCs (Figure 3d-e). Furthermore, the Δ*wspF* mutant could accumulate low-density floating microplastics (polystyrene), resulting in the sinking of polystyrene to the bottom of the container due to increased bulk (Supplementary Figure 4). The

3.4 Engineering P. aeruginosa biofilms for 'capture and release' of microparticles

To incorporate the 'release' component in our strain, we inserted a PDE gene with an L-arabinose-inducible promoter (p_{BAD}-*yhjh* plasmid) in the Δ*wspF* mutant. We had previously shown that the YhjH PDE could lower c-di-GMP levels and cause biofilm dispersal [42], thus addition of arabinose to the engineered Δ*wspF*/p_{BAD}-*yhjh* strain could negate the pro-biofilm effects of *wsp* operon and cause biofilm dispersal. This was mediated by the production of glycosidases and proteases which can degrade the biofilm matrix [43].

To test if the engineered strain could trap and release microplastics, we first grew Δ*wspF*/p_{BAD}-*yhjh* biofilms to accumulate microplastics in the absence of arabinose, followed by retrieval and arabinose treatment of biofilm-microplastics aggregates. In the initial growth of biofilms without arabinose, Δ*wspF*/p_{BAD}-*yhjh* could accumulate microplastics similarly to Δ*wspF*, implying that the biofilm dispersal was not induced in the absence of arabinose (Figure 4a-b). After the microplastics-containing biofilms were treated with varying concentrations of arabinose for 7 hrs, we quantified the recovery rate of freed microplastics from the biofilms. We found that increasing arabinose concentrations could effectively induce biofilm dispersal (Figure 4c) and release of microplastics for recovery (Figure 4d). To show that shear stress from shaking incubation of biofilms was not involved in biofilm dispersal, we performed a negative control where no arabinose was added to the biofilms and found no significant changes to biofilm mass and loss of trapped microplastics (Figure 4c-d). Furthermore, to show that arabinose was not a chemical stimulus for biofilm dispersal per se, but an inducible agent for p_{BAD}-*yhjh* expression, we treated Δ*wspF* strain with 1% arabinose and showed no significant changes to biofilm mass and loss of trapped microplastics (Figure 4c-d).

3.5 Bioaggregation of microplastics from environmental samples

To show the Δ*wspF*/p_{BAD}-*yhjh* strain could be used to aggregate microplastics from environmental samples, we collected seawater samples in the vicinity of a sewage outfall where microplastics pollution was rampant and conducted our studies in bioreactors. We first tested the ability of our engineered strain to grow in freshwater and seawater supplemented with glucose and amino acids at various temperatures, and found little differences in the microplastics accumulated in the biofilms (Supplementary Figure 5), implying that our proof-of-concept could be applied to different water sources.

Our engineered strain could form biofilms and accumulate most microplastics from seawater samples over time, with a few loose microplastics which were not trapped by biofilms (Figure 5a). Addition of arabinose which activated the release mechanism caused dispersal of biofilms (Figure 5b) and release of microplastics (Figure 5c). The bioaggregation of microplastics was not discriminatory, where the microplastics of different materials were recovered from the biofilms (Figure 5d).

3.6 Bioaggregation of microplastics by P. putida

Taking a step further for our proof-of-concept, we test its applicability to other bacterial species by employing the *P. putida* species known to colonise and form biofilms on microplastic surfaces *in situ* [13-16]. *P. putida* is an environmental bacterium which can degrade different types of pollutants and plastics [44], rendering it as another choice organism for bioremediation and bioaccumulation [45, 46]. Since many bacterial species do not contain the *wspF* mutation, we were also interested in employing other biofilm-inducing mechanisms which can be conveniently applied to different microbes for trapping microplastics. One example is the use of an exogenous plasmid p_{lac}-*yedQ*, which encoded the YedQ DGC involved in c-di-GMP signaling [47]. We had previously employed p_{lac}-*yedQ* to study the induction of exopolysaccharide production and biofilm formation by YedQ DGC in different Gram-negative bacterial species [48-

50], which provides us the rationale that it could be applied to accumulate microplastics in a similar fashion.

In this case, we engineered the trap mechanism in *P. putida* OUS82 and *P. aeruginosa* PAO1 by inserting p_{lac}-*yedQ*, and incorporated the p_{BAD}-*yhjh* plasmid for release mechanism. Similar to Δ*wspF*/p_{BAD}-*yhjh*, we first grew PAO1/p_{lac}-*yedQ*/p_{BAD}-*yhjh* and OUS82/p_{lac}-*yedQ*/p_{BAD}-*yhjh* biofilms to accumulate microplastics in the absence of arabinose, followed by retrieval and arabinose treatment of biofilm-microplastics aggregates. In the initial growth of biofilms without arabinose, PAO1/p_{lac}-*yedQ*/p_{BAD}-*yhjh* and OUS82/p_{lac}-*yedQ*/p_{BAD}-*yhjh* could accumulate microplastics, leaving low levels of free microplastics in the media (Figure 6a). After the microplastics-containing biofilms were treated with varying concentrations of arabinose for 7 hrs, we quantified the recovery rate of freed microplastics from the biofilms. We found that increasing arabinose concentrations could effectively induce biofilm dispersal (Figure 6b) and release of microplastics for recovery (Figure 6c). This indicated that the trap and release mechanism could be applied flexibly to another microbial species.

4. Discussion:

The United Nations' Sustainable Development Goals (SDGs) have recently set the challenge of employing biotechnology in remediation and pollution control, so we should exploit the potential of microbial biotechnology in the removal of pollutants especially microplastics. While bacterial species with the ability to degrade microplastics have been identified, such as *P. aeruginosa* can degrade polystyrene and polythene [51, 52], their varying plastic-degrading efficiency may pose a problem in the effective removal of microplastics from the sewage or water bodies. Furthermore, biodegradation of plastic may release toxic wastes, warranting the need to first capture microplastics efficiently, remove from the system (wastewater treatment plants or environment) for separate processing in isolated chambers. Hence, accumulation of microplastics is a viable way to remove microplastics safely. However, biofilms in nature also lack the bulk to cause flocculation or coagulation of microplastics from liquid suspension [53], thereby preventing the formation of larger aggregates which can sink to the bottom for convenient removal. While plastics such as PVCs and nylon have higher densities which allow them to sink to the bottom, low-density plastics such as polystyrene remain buoyant in liquids, which render their physical removal difficult [54].

We report the engineering of a *P. aeruginosa* strain with the 'capture-and-release' mechanism by harnessing the power of c-di-GMP signaling for biofilm and demonstrate its potential application in accumulating microplastic pollutants from polluted environmental samples. The engineered strain could accumulate microplastics at higher concentrations and of varying densities. The engineered strain could aggregate low-density microplastics which would lead to sinking and easier removal of microplastics in bioreactor tanks.

While biofilms are good at accumulating microplastics, their sticky exopolymeric matrix rendered separation of microplastics from biofilms and its recovery difficult. While it is currently not commercially feasible to recycle microplastics from the environment, improving its recovery can drive recycling efforts of plastics. Hence, we propose and incorporate the release mechanism where the Δ*wspF*/p_{BAD}-*yhjh* strain could activate its PDE activity via the addition of arabinose. By reducing the c-di-GMP levels in biofilms, biofilms are driven towards dispersal, where glycosidases and proteases are produced to degrade the biofilm matrix [55] and release microplastics for convenient recovery of particulate plastic wastes.

This controllable biofilm development approach we have demonstrated here should prove widely applicable for other biofilm-enabled applications, such as bioremediation or biofilm-mediated biocatalysis of chemicals. Our proof-of-concept study exemplifies the potential for translation from biofilm biology to biofilm engineering for environmental applications. In the context of application in wastewater treatment plants, microplastics are difficult to remove conventionally by filters which poorly retain microplastics or harsh chemical treatment (alum salts) [4], resulting in the release of microplastics into the environment. Our work serves as proof-of-concept where microplastics are accumulated by biofilms, where they will sink to the bottom of the bioreactors for convenient removal. Upon transfer to new tanks, the release mechanism could be activated to break down biofilms into suspensions of planktonic bacteria and release microplastics for easier recovery. While our work may not be directly used for industrial applications due to safety concerns attributing to genetically-modified bacteria, this provides the basis for future work in identifying pro-biofilm-forming isolates from sewage which can trap microplastics efficiently. The microplastic-laden biofilms could then be separated and treated with safe anti-biofilm agents, such as nitric oxide or glycosidases [43, 56, 57], for biofilm disruption and microplastics release.

5. Conclusions:

P. aeruginosa formed biofilms which could accumulate microplastics of varying sizes and materials within the exopolymeric matrix. Screening of biofilm mutants revealed the role of *wsp* operon in accumulating microplastics within the biofilm. Engineering a pro-biofilm strain based on *wsp* operon enhanced bioaccumulation of microplastics, while incorporating a release mechanism via dispersal promoted efficient release and recovery of microplastics.

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Author Contributions:

S.L.C and J.K.H.F designed methods and experiments. Y.S.L and M.M.L.L carried out laboratory experiments, analysed the data and interpreted the results. All authors wrote the paper. All authors have contributed to, seen and approved the manuscript.

Competing interests:

The authors declare no competing financial interests.

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Figures:

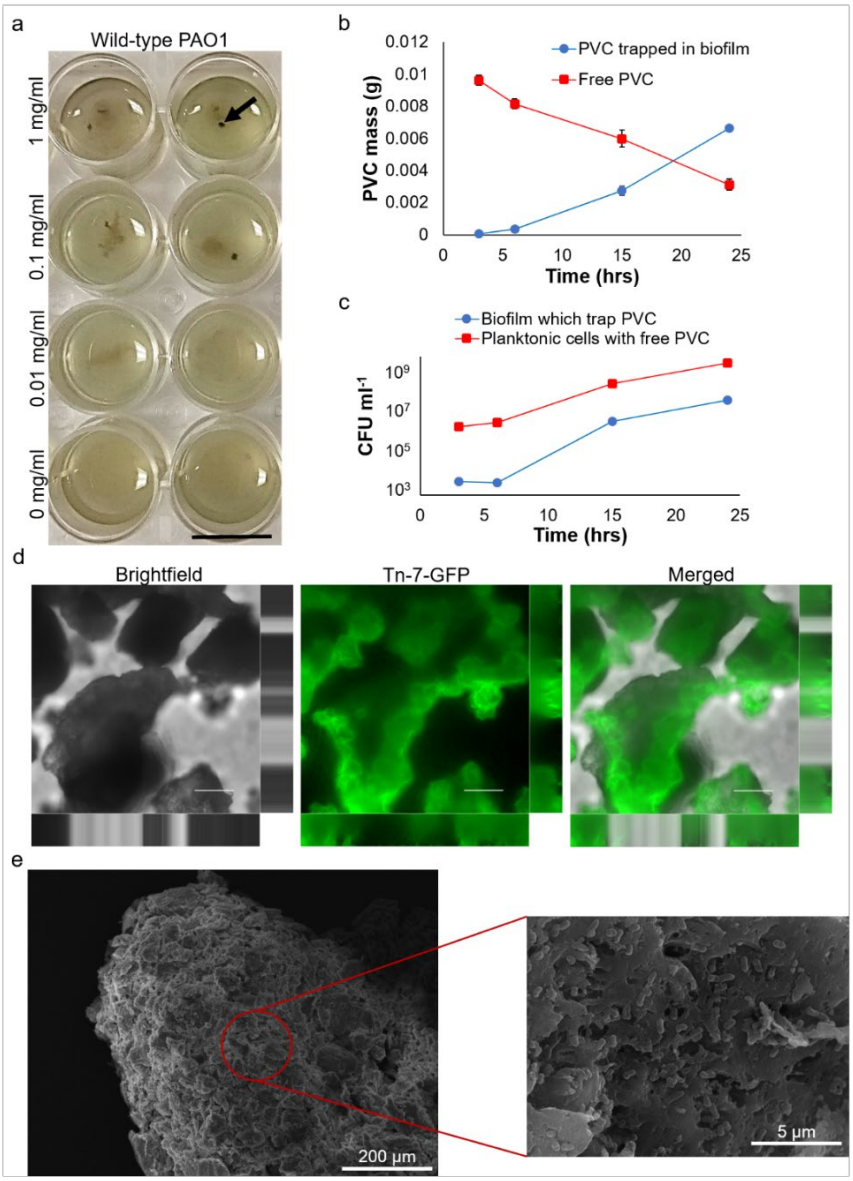

Figure 1. *P. aeruginosa* can form biofilms on microplastics. (a) Image of *P. aeruginosa* biofilm accumulating PVC (<106 µm) into a bolus (shown with black bold arrow). Scale bar (bottom right): 1 cm. (b) Mass of microplastics accumulated and lost by biofilm. (c) CFU of bacteria within and outside of biofilm. (d) Representative image of *P. aeruginosa* PAO1 biofilm cells on PVC particles (Scale bar: 50 µm). (e) Representative electron microscopy image of *P. aeruginosa* biofilm cells on PVC particles (Scale bar: 1 nm). Means and s.d. from triplicate experiments are shown. **P < 0.01, ***P < 0.001, n.s (not significant), One-Way ANOVA.

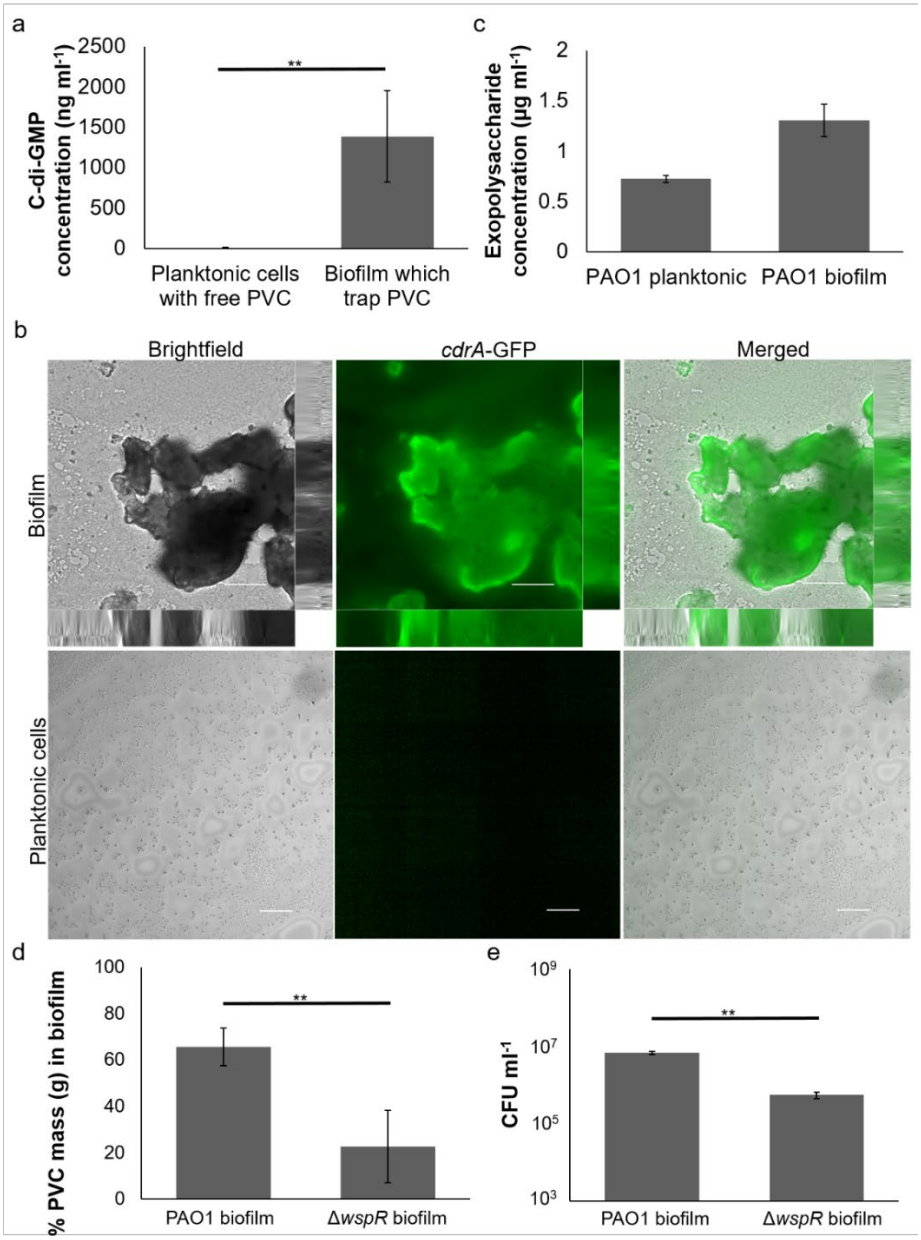

Figure 2. C-di-GMP signaling is key to biofilm accumulation of microplastics. (a) Quantification of c-di-GMP levels in microplastics-accumulated biofilms by ELISA. (b) Representative image of PAO1/p_{cdrA}-*gfp* biofilms and planktonic bacteria on microplastics. (Scale bar: 50 µm). (c) Quantification of exopolysaccharides in microplastics-accumulated biofilms. (d) Mass of microplastics accumulated and lost by PAO1 and Δ*wspR* biofilm. (e) CFU of PAO1 and Δ*wspR* within and outside of biofilm. Means and s.d. from triplicate experiments are shown. **P < 0.01, ***P < 0.001, n.s (not significant), One-Way ANOVA.

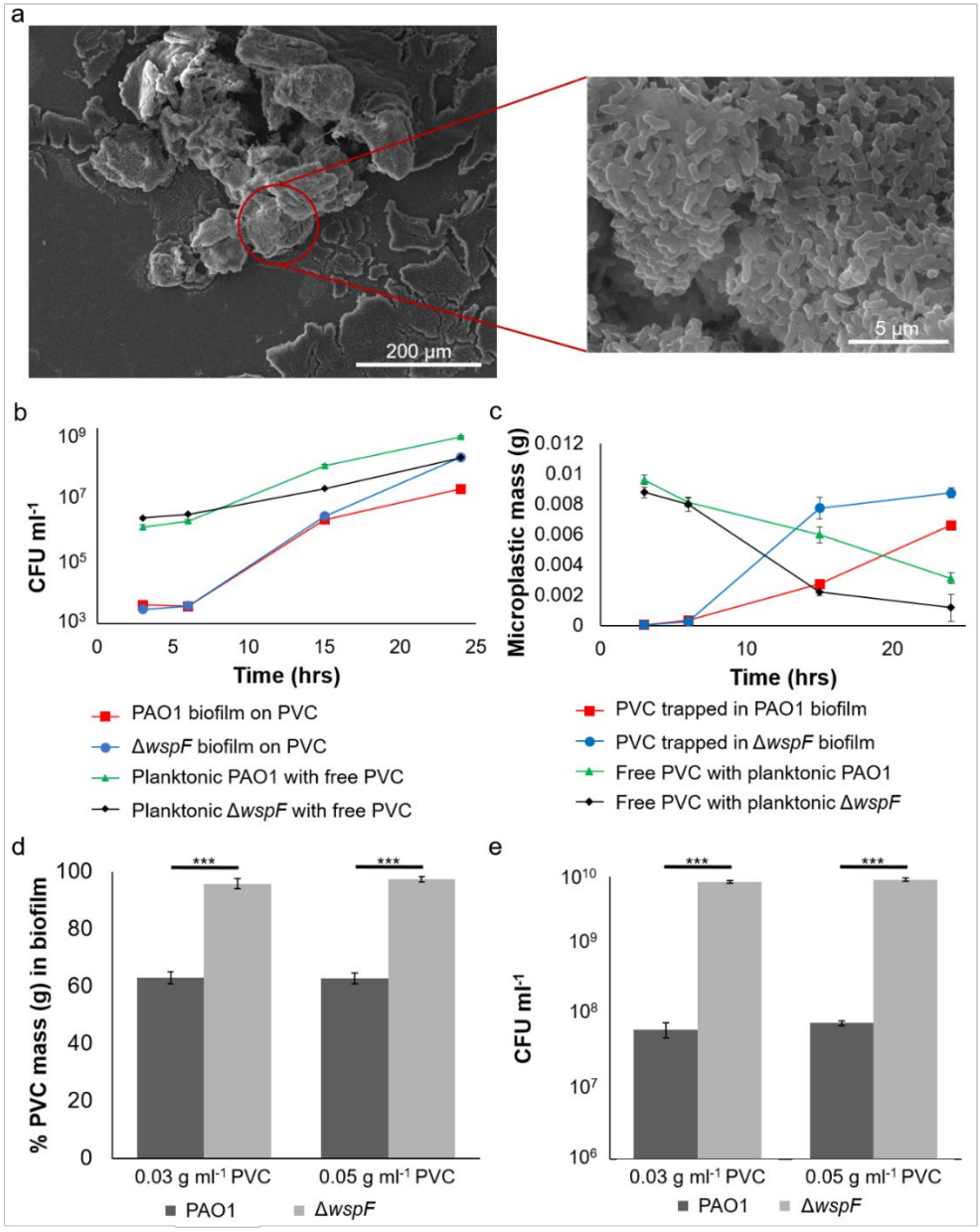

Figure 3. Overexpression of wsp operon can boost accumulation of microplastics by biofilms. (a) Representative electron microscopy image of Δ*wspF* biofilm cells on PVC particles. (b) Bacterial CFU over time. (c) Mass of microplastics accumulated by biofilms over time. (d) Δ*wspF* can accumulate microplastics (3 and 5 mg ml^{-1}) at higher efficiency than PAO1. (e) CFU of Δ*wspF* biofilms on microplastics (3 and 5 mg ml^{-1}) is higher than PAO1. Means and s.d. from triplicate experiments are shown. **P < 0.01, ***P < 0.001, n.s (not significant), One-Way ANOVA.

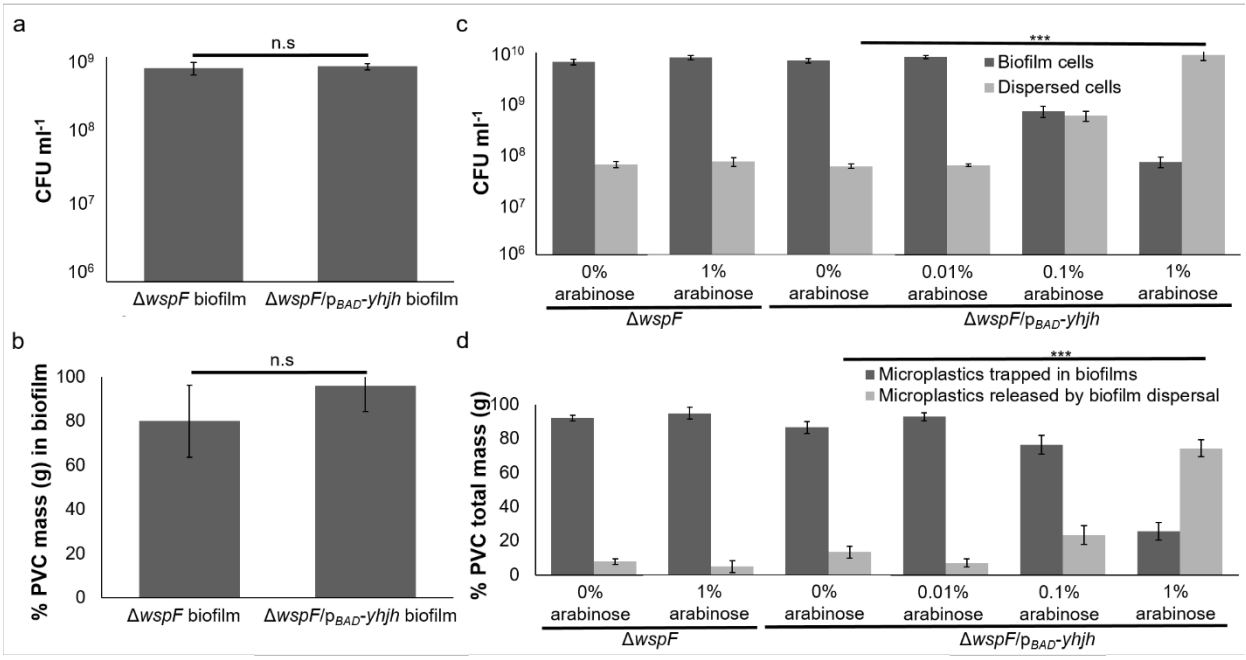

Figure 4. Trap and release mechanism of engineered Δ*wspF*/p$_{BAD}$-*yhjh* strain. (a) No difference in biofilm CFU of Δ*wspF*/p$_{BAD}$-*yhjh* and Δ*wspF* which trap microplastics. (b) No difference in mass of microplastics accumulated by Δ*wspF*/p$_{BAD}$-*yhjh* and Δ*wspF* biofilms over time. (c) Bacterial CFU of biofilm released by arabinose induction of engineered strain. (d) Mass of microplastics released by arabinose induction of engineered strain. ***P < 0.001, n.s (not significant), One-Way ANOVA.

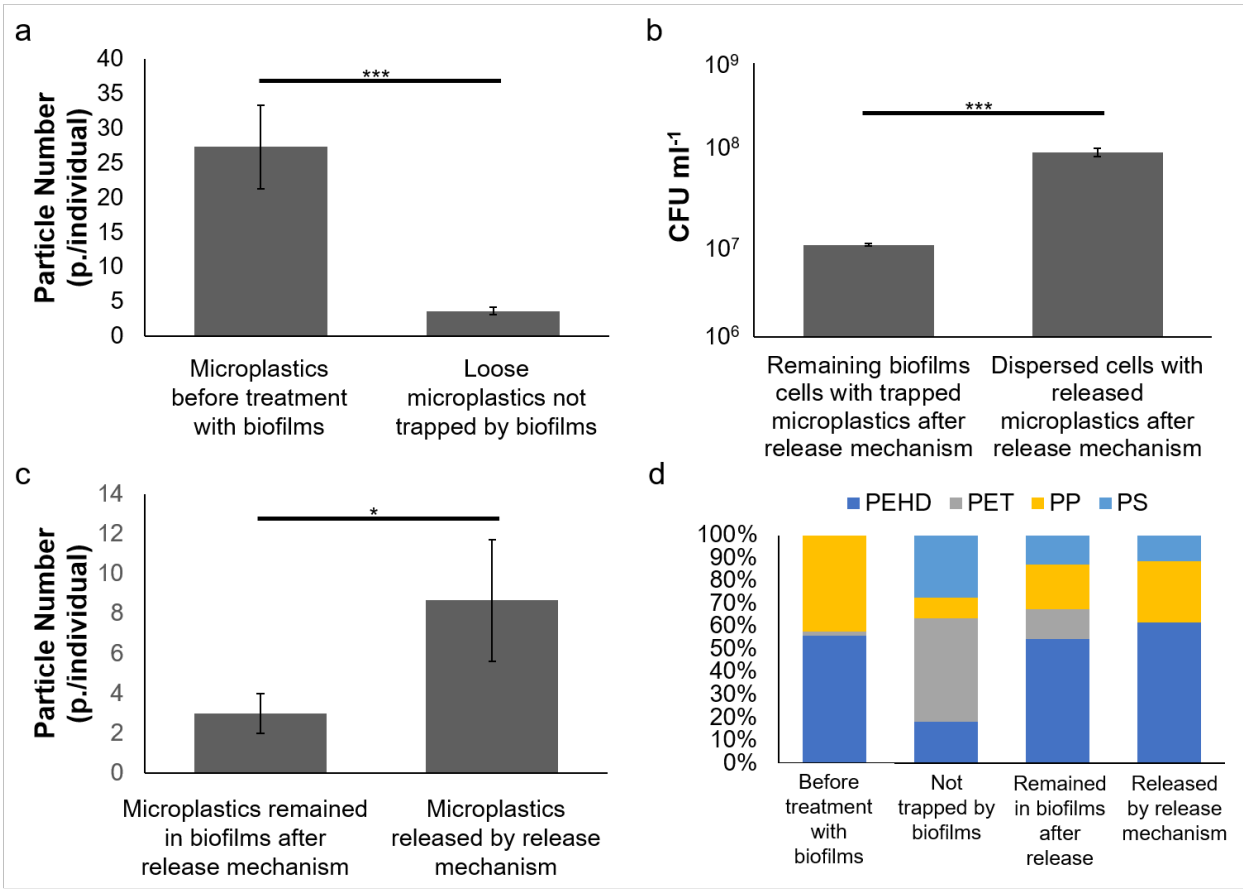

Figure 5. Biofilms can bioaccumulate microplastics from freshwater and seawater samples. (a) Number of microplastic particles before and after microbial treatment. (b) Bacterial CFU of biofilms and dispersed cells after 8 hrs of activated release mechanism. (c) Number of microplastic particles before and after 8 hrs of activated release mechanism. *P < 0.05, ***P < 0.001, One-Way ANOVA. (d) material type collected and released by biofilms from environmental samples. PEHD: high-density polyethylene; PET: polyethylene terephthalate; PP: polypropylene; PS: polystyrene.

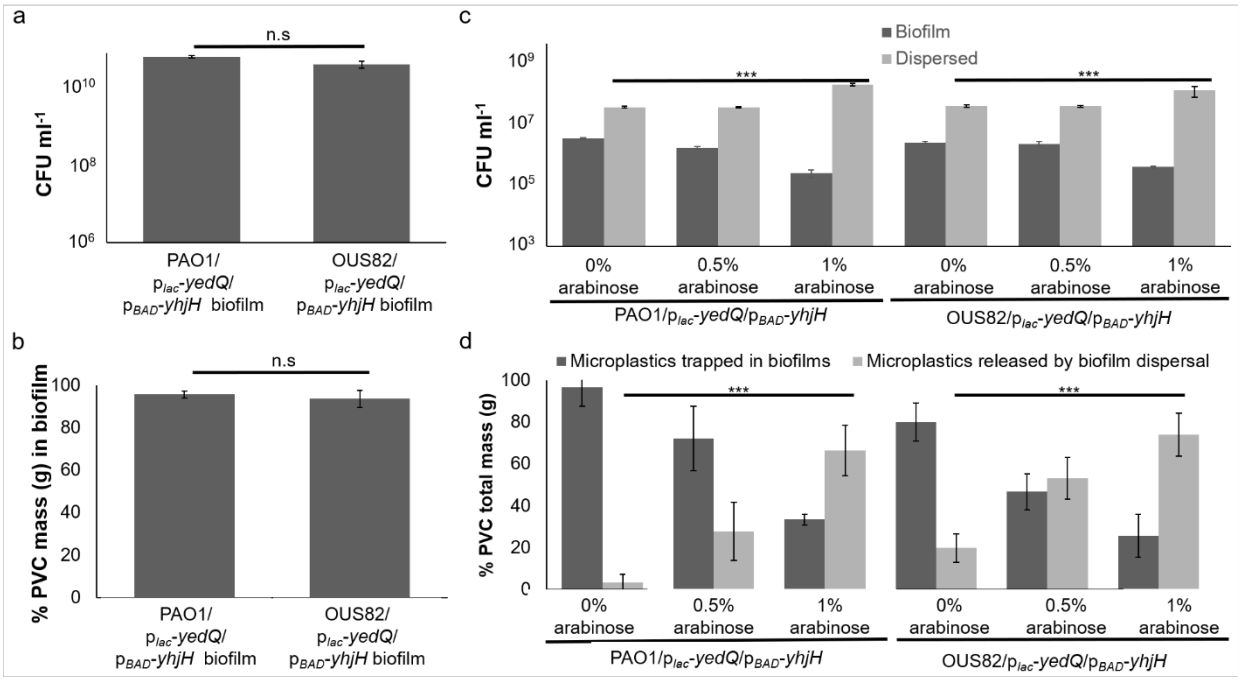

Figure 6. Trap and release mechanism of engineered *P. putida* OUS82/p*lac*-*yedQ*/p*BAD*-*yhjh* and PAO1/p*lac*-*yedQ*/p*BAD*-*yhjh* strain. (a) Both strains could trap microplastics in their biofilms. (b) Bacterial CFU of biofilm released by arabinose induction of engineered strain. (c) Mass of microplastics released by arabinose induction of engineered strains.