

Supporting Information for

Tire wear particle microbiome-derived extracellular vesicles

function as potent vectors amplifying antimicrobial

resistance risk in soil ecosystems

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Text S1 Co-incubation of EVs and recipient bacteria

E. coli DH5 α and *P. aeruginosa* PAO1 were used as recipient strains. Bacterial suspensions ($\sim 10^5$ CFU in 100 μ L) were mixed with an equal volume of DNase I (1 U) and proteinase K-treated EVs ($\sim 10^8$ particles). Specifically, Proteinase K treatment was applied to remove surface-associated proteins and phages, while DNase I was used to degrade free DNA, helping to ensure that any horizontal gene transfer was mediated by the protected internal cargo of EVs. The mixtures were incubated at 37°C for 4 h, followed by dilution to 1 mL with fresh medium and continued shaking incubation (220 rpm, 37°C) for another 4 h. The cultures were subsequently expanded to 10 mL with LB broth and incubated under the same conditions for 24 h. The harvested cells were then collected for antibiotic resistance assessment.

Text S2 DNA isolation

DNA from environmental samples and EVs was extracted separately using the FastDNA Spin Kit for Soil (Cat no 6560200, MP Biomedicals, France) and the QIAamp® DNA Mini Kit (Cat no 51304, QIAGEN, Germany), respectively. To ensure that the DNA was originated from EVs, samples were pretreated with Turbo DNase (2 U; Invitrogen, USA) in 1 $\times$ DNase buffer for 30 min. DNA concentration was measured using a Qubit 3.0 fluorometer (Thermo Fisher Scientific, USA), and integrity was verified by agarose gel electrophoresis. Purified DNA was stored at -20°C until future analysis.

Text S3 Details of metagenomic sequencing

Each sample produced approximately 8 Gb of clean 150 bp paired-end reads after quality control, corresponding to a sequencing depth of ~ 15 Gb. We used the

MEGAHIT assembler (v1.2.9) with default parameters to assemble the cleaned reads into contigs¹. To construct a non-redundant gene catalog, ORFs were first identified from contigs longer than 800 bp using Prodigal (v2.6.3)². Following this, the predicted gene sequences were clustered with CD-HIT (v4.8.1) at 95% identity and 90% coverage³. The quality-controlled reads were aligned to the non-redundant gene catalog using Bowtie 2 (v2.4.2)⁴. Subsequently, read counts for each gene per sample were generated with SAMtools⁵ to determine abundance.

1) Taxonomy annotation

Representative sequences from the nonredundant gene catalog were taxonomically annotated against the NCBI NR database using DIAMOND BLASTp (e-value $< 1 \times 10^{-10}$)⁶. Taxonomic information was classified from domain to species level. Potential pathogens were identified by cross-referencing the species-level assignments with a previously curated pathogen database⁷.

2) Gene annotation

ARGs were identified by DIAMOND BLASTX searches against the Structured Antibiotic Resistance Gene (SARG) database⁸, and virulence factor genes (VFGs) were annotated using the VFDB core dataset⁹. Only alignments > 75 bp with identity $> 80\%$ and e-value $< 1 \times 10^{-7}$ were retained¹⁰. ARG abundance was normalized using the ARGs-OAP 3.0 pipeline and expressed as reads per kilobase per million mapped reads (RPKM).

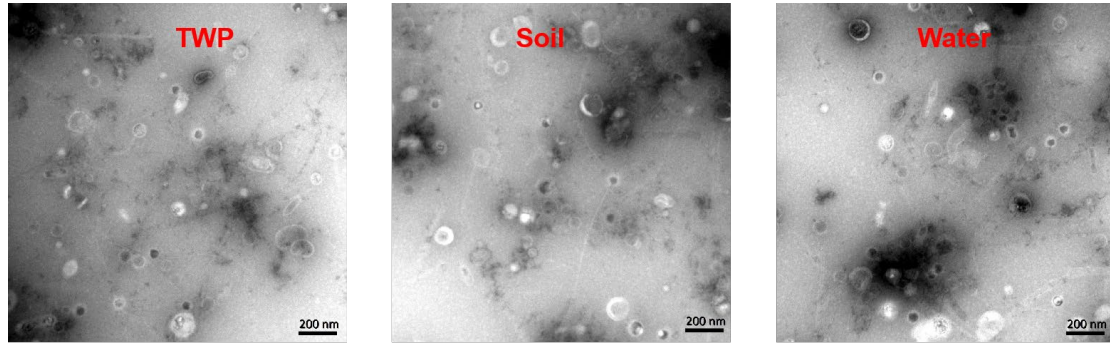


Fig. S1. Characterization of EVs released from TWPs and their surrounding environments. Transmission electron microscopy (TEM) images of EVs isolated from soil (a), water (b), and TWP surfaces (c). Scale bar = 200 nm.

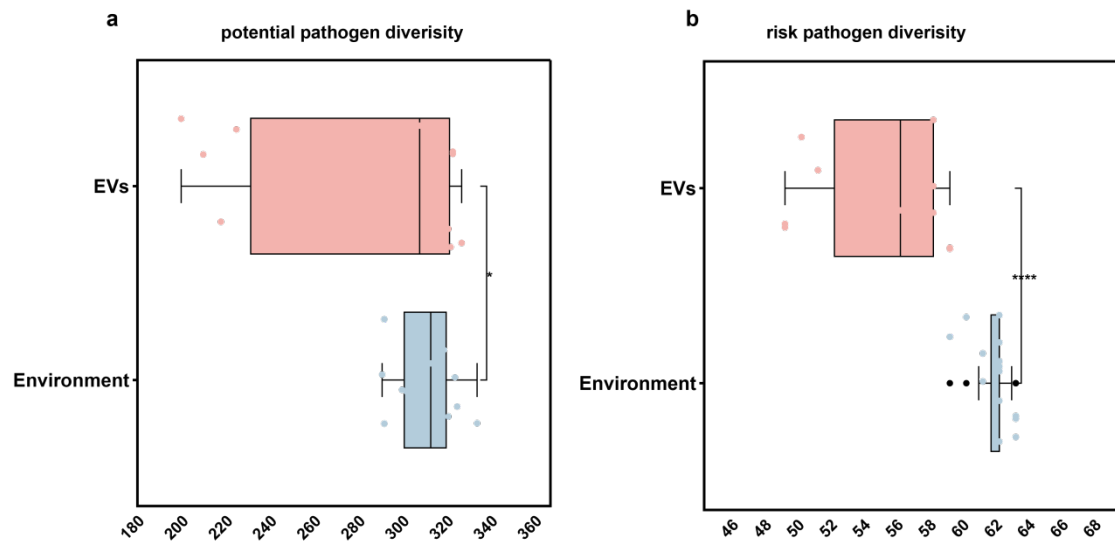


Fig. S2. Comparison of pathogen community diversity between TWP-derived EVs and the environment. Box plots comparing the (a) potential pathogen diversity and (b) risk pathogen diversity in TWP-derived extracellular vesicles (EVs) and the surrounding environment (Environment). Statistical significance was assessed using t test. Asterisks denote the level of statistical significance: * $p < 0.05$; **** $p < 0.0001$.

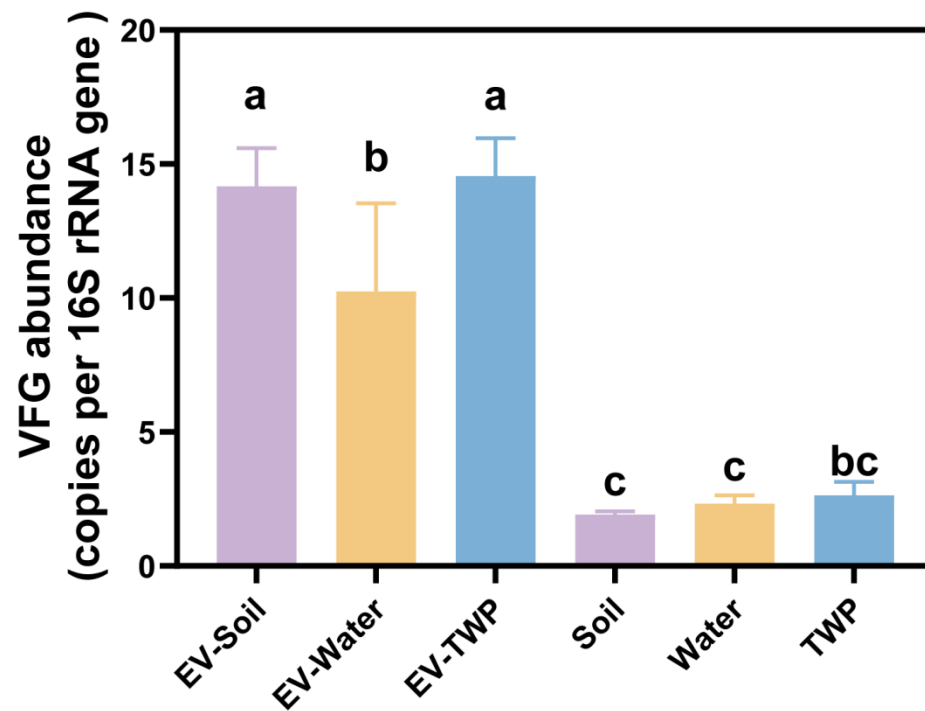


Fig. S3. Total relative abundance of VFGs in EVs and microbial communities from TWPs and other habitats. Different letters above the bars indicate significant differences among groups ($p < 0.05$), as determined by one-way ANOVA followed by Tukey's post hoc test.

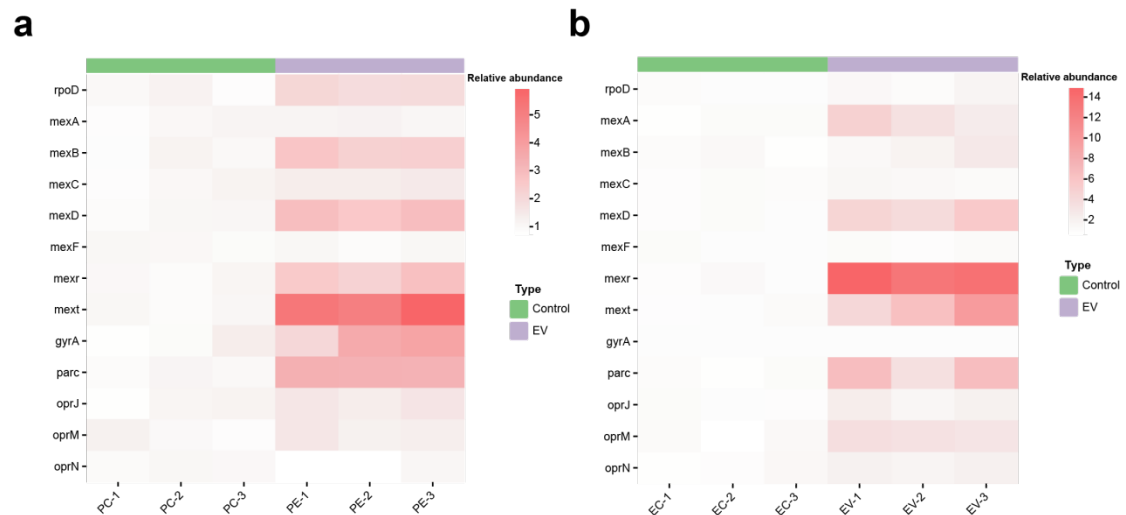


Figure S4. Transcriptional response of resistance-related genes in recipient bacteria. Heatmap shows relative expression ($2^{-\Delta\Delta C_t}$) of genes involved in multi-drug resistance mechanisms, specifically efflux pumps and membrane permeability. Control: control groups; EV: TWP-EV treated groups. For *P. aeruginosa* (a), groups are labeled as PC (control) and PE (TWP-EV treated). For *E. coli* (b), groups are labeled as EC (control) and EV (TWP-EV treated).

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2026-05-12

American Chemical Society (ACS)

<https://doi.org/10.1021/acs.estlett.6c00328>

Qin Y-F, Pang J-Y, Li Y, et al., (2026) Tire wear particle microbiome-derived extracellular vesicles function as potent vectors amplifying antimicrobial resistance risk in soil ecosystems. *Environmental Science & Technology Letters*, Volume 13, Issue 5, May 2026, pp. 684-690