

Image Cytometry and Kinetic Modelling Reveal How Aged Leaf Biomass Dose Regulates Microbial Physiology and PAH Degradation

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Abstract

Purpose:

Organic amendments are widely applied to enhance bioremediation of PAH-contaminated soils; however, remediation performance varies with amendment dosage, and the mechanisms linking dose, microbial physiological state, and degradation kinetics remain unclear. This study integrated kinetic modelling with imaging-enabled cytometry and machine learning to determine how aged leaf-biomass dose regulates microbial physiology and PAH degradation efficiency.

Methods:

PAH-contaminated soil containing phenanthrene and pyrene (249.15 mg kg⁻¹ combined) was amended with pine–poplar leaf litter at 10%, 30%, and 70% (w/w), alongside unamended (SS) and leaf-only (AL) treatments, followed by 30-day aerobic aging. Enriched consortia and tolerant isolates were evaluated in liquid minimal salt medium (40 mg L⁻¹ PAH mixture). Degradation kinetics were modelled using first-order decay and validated by GC–FID. Imaging flow cytometry coupled with supervised machine learning classified viable and debris-associated cells using morphology- and fluorescence-derived features. Multivariate analyses quantified associations between cellular phenotypes and degradation rate constants.

Results:

Amended systems achieved 60–80% PAH removal within 10 days, whereas unamended soil retained approximately 70% residual PAHs. The 10% amendment exhibited the highest degradation rate ($k \approx 0.153 \text{ day}^{-1}$; $t_{1/2} \approx 4.5 \text{ days}$), compared with slower kinetics in unamended soil ($k \approx 0.052 \text{ day}^{-1}$). Imaging cytometry revealed sustained viable-cell fractions in amended treatments and elevated mortality under PAH-only conditions. Machine learning classifiers achieved near-perfect discrimination (ROC–AUC ≈ 1.00). Morphology-derived cellular metrics showed the strongest correlation with degradation rates ($\rho \approx 0.89$), exceeding viability-based associations ($\rho \approx 0.83$).

Conclusion:

Moderate aged-leaf biomass doses optimized PAH degradation by preserving microbial physiological integrity rather than merely increasing biomass. Imaging-derived cellular phenotypes quantitatively predicted degradation kinetics, demonstrating that amendment dose regulates remediation efficiency through microbial physiological control. Integrating amendment optimization with cytometry-based physiological monitoring provides a scalable framework for predictive and cost-efficient PAH bioremediation.

Introduction

Soils impacted by industrial activity, petroleum handling, and urban emissions frequently accumulate persistent organic contaminants, among which polycyclic aromatic hydrocarbons (PAHs) represent a major concern due to their environmental persistence, ecological risks, and toxicity to soil ecosystems and human health (Sumathi and Manian 2023; Wang et al. 2024). PAHs are semi-volatile hydrophobic organic compounds that tend to adsorb strongly to soil organic matter and mineral surfaces, leading to long-term persistence in contaminated soils (Teixeira et al. 2024). Although microbial degradation constitutes a principal natural attenuation pathway, degradation efficiency varies widely across sites and treatment strategies, often limiting remediation success (Zhou et al. 2023). Microbial bioremediation is considered an effective and sustainable strategy for PAH-removal due to low energy requirements and reduced secondary pollution, but site-specific factors such as bioavailability and microbial community composition heavily influence outcomes (Sumathi and Manian 2023).

Organic amendments are widely applied to stimulate microbial activity and enhance contaminant removal; however, remediation performance often varies with amendment dosage, and degradation efficiency does not consistently increase with amendment input, leaving the biological mechanisms underlying dose-dependent responses insufficiently understood. Organic wastes supply nutrients and soluble carbon that promote microbial biomass and diversity, but excessive inputs may reduce degradation efficiency through contaminant sequestration, microbial competition, oxygen limitation, or unfavourable redox conditions (Omoni et al. 2025; Larney and Angers 2012). Among these materials, leaf litter represents an abundant renewable carbon source capable of stimulating microbial metabolism and modifying contaminant bioavailability, often promoting rhizosphere-like microbial dynamics, although responses vary with litter composition and soil conditions (Akinseye and Knapp 2026; Zhang et al. 2021).

A major limitation in existing remediation studies is the reliance on bulk microbial or chemical measurements, which obscure physiological heterogeneity within microbial communities. Microbial abundance alone does not necessarily translate into degradation performance, since contaminant stress can impair metabolic pathways, enzyme activity, and overall cellular function even when cells are detectable in bulk assays (Wang et al. 2024). Advanced single-cell technologies like imaging flow cytometry and associated computational approaches now permit high-throughput characterization of microbial phenotypes, linking viability, stress responses, and morphological traits to functional potential, though applications in soil remediation studies remain limited (Shan et al. 2023). Integrating these tools with degradation kinetics offers a promising path to mechanistically explain how treatment-dependent changes in microbial physiology drive remediation outcomes.

In this study, PAH-contaminated soil containing phenanthrene and pyrene was amended with graded levels of pine–poplar leaf litter (10%, 30%, and 70% w/w) and incubated to promote microbial acclimation. Subsequent analyses combined microbial enumeration, enrichment and isolation of PAH degraders, tolerance assays, liquid-phase biodegradation experiments, first-order kinetic modelling, and GC–FID validation. Imaging flow cytometry coupled with automated image segmentation and supervised machine learning was used to quantify viable, damaged, and debris-associated populations and to

extract morphology-derived cellular features. Multivariate analyses were then applied to link imaging-derived microbial phenotypes with degradation rate constants across treatments. This integrated framework provides a systems-level understanding linking amendment dosing, microbial physiology, and PAH degradation performance.

We hypothesized that amendment dosage regulates microbial physiological stability, and that this regulation quantitatively determines PAH degradation kinetics. Specifically, moderate amendment levels were expected to maintain structurally intact and metabolically competent populations capable of sustaining contaminant turnover, whereas excessive amendment levels would reduce degradation performance through physiological stress or competitive inhibition. By integrating degradation kinetics with single-cell phenotyping and automated classification, this work establishes a mechanistic framework linking amendment dosing, microbial cellular state, and treatment-dependent biodegradation efficiency, providing new insight for physiology-guided remediation design.

Materials and Methods

Experimental design and setup

Deciduous leaf litter derived from pine and poplar trees was collected, air-dried, and milled to obtain a homogeneous organic amendment. PAH-contaminated soil containing phenanthrene and pyrene at a combined concentration of $249.15 \pm 15.6 \text{ mg kg}^{-1}$ was used as the experimental substrate. For each treatment, 200 g of contaminated soil was amended with a pine–poplar leaf mixture (1:1, w/w) at defined concentrations to generate amendment levels of 10% (10AL), 30% (30AL), and 70% (70AL) w/w. Additional treatments consisted of contaminated soil without amendment (SS), and leaf litter only (AL). All treatments were prepared in triplicate and incubated aerobically at ambient temperature for 30 days to allow microbial acclimation, organic matter decomposition, and adaptation to PAH contaminants. After aging, samples were subjected to microbial enumeration, enrichment and isolation of PAH degraders, tolerance testing, liquid-phase biodegradation assays, imaging flow cytometry, and automated image-based analyses.

Enumeration of microbial communities

Culturable microbial populations were quantified using standard plate count techniques. Soil suspensions were prepared using sixfold serial dilutions in sterile PBS, and aliquots were plated in triplicate onto selective and non-selective media. Nutrient Agar was used for enumeration of aerobic heterotrophic bacteria, Rose Bengal Agar for fungi, Kenknight and Munaier's medium for actinomycetes, and Jensen's medium for asymbiotic nitrogen-fixing bacteria. PAH-degrading bacteria were enumerated on minimal salt agar plates supplemented with phenanthrene and pyrene as sole carbon sources. Plates were incubated following established protocols (Khatoon and Malik 2019): heterotrophic bacteria at 37°C for 24 h, fungi at 28°C for 3–5 days, and actinomycetes and nitrogen fixers at 30°C for 2–4 days. Colonies were counted on plates containing 30–300 colonies, and results were expressed as colony-

forming units per gram of dry soil (CFU g⁻¹). Colony morphotypes were recorded qualitatively to guide isolate selection.

Enrichment and isolation of PAH-degrading microorganisms

Enrichment cultures targeting PAH degraders were prepared following Darmawan et al. (2015) with modification, by inoculating 10 g of aged soil into 190 mL MSM containing phenanthrene and pyrene (1:1, w/w) at a final concentration of 40 mg L⁻¹ as sole carbon sources. Cultures were incubated at 37°C with shaking at 200 rpm for 7 days. Three sequential enrichment transfers were performed at 7-day intervals using 1% (v/v) inoculum to increase selective pressure. After enrichment, aliquots were plated onto MSM agar supplemented with PAHs and incubated at 30°C until colony formation. Morphologically distinct colonies were purified through successive streaking steps, and four dominant isolates were retained and designated PC1–PC4.

PAH tolerance assay

Tolerance of purified isolates to PAHs was evaluated using surface-loaded Minimal Salt Agar plates, a modified surface plate diffusion assay adapted from Zafra et al. (2015). A phenanthrene–pyrene mixture dissolved in hexane–acetone (1:1) was evenly distributed across plate surfaces to achieve loadings ranging from 10 to 70 mg per dish. Plates were dried to remove solvent prior to inoculation. Isolates grown to mid-log phase were standardized to OD₆₀₀ = 0.14, and 100 µL aliquots were spread across agar surfaces supplemented with 1% glucose. Plates were incubated at 30°C for 24–158 h depending on growth rates. Tolerance was quantified by measuring radial colony expansion relative to PAH deposition zones. The isolate exhibiting highest tolerance (PC1) was selected for downstream experiments.

Liquid-phase PAH degradation assay

Biodegradation capacity of enriched microbial consortia and isolate PC1 was evaluated in liquid MSM containing phenanthrene and pyrene at a combined concentration of 40 mg L⁻¹. Cultures were incubated at 37°C with shaking at 150 rpm. Abiotic controls lacking microbial inoculum were included. Samples were collected at 0, 3, 5, and 10 days, centrifuged to remove biomass, and extracted with n-hexane. Residual PAH concentrations were quantified by UV–visible spectrophotometry at 251 nm and 334 nm for phenanthrene and pyrene, respectively. Degradation percentages were calculated relative to initial concentrations.

Selected samples were additionally analysed using Gas Chromatography–Flame Ionization Detection (GC-FID) to validate spectrophotometric measurements.

GC-FID analysis

The extracted samples were dried over anhydrous sodium sulphate to remove residual moisture prior to analysis. Quantitative analysis was performed using GC-FID operating at a carrier gas pressure of 20 psi and fitted with a non-polar capillary column appropriate for the separation of polycyclic aromatic

hydrocarbons (PAHs). The injector and detector temperatures were maintained in accordance with standard operating conditions. The oven temperature program was optimized to ensure efficient compound resolution, beginning at 50°C (initial temperature), ramped to 250°C at a rate of 10°C min⁻¹, and held at 250°C for 10 minutes. Compounds were identified using retention times of analytical standards, and quantification was based on peak area integration. GC-FID data were used to confirm PAH removal trends observed spectrophotometrically.

Imaging flow cytometry acquisition

Samples from degradation experiments were collected at Days 1, 3, 5, and 7. Cells were centrifuged, washed twice with PBS, and resuspended to concentrations of approximately 10⁵–10⁶ cells mL⁻¹. Viability staining employed SYBR Green I and propidium iodide to distinguish viable and membrane-compromised cells, followed by incubation for 15 min in darkness prior to acquisition.

Data were acquired using a CytoPpix imaging flow cytometer equipped with lasers for fluorescence excitation and bright-field imaging. Approximately 1,000–1,500 imaging events were acquired per sample. Following image segmentation, individual cellular objects were extracted from each event, resulting in a total dataset of approximately 4.5×10^5 segmented cellular objects across all treatments and timepoints. Image data and event statistics were exported for downstream analysis. Instrument calibration was verified daily using fluorescent bead standards.

Image analysis and feature extraction

Image analysis was conducted using a Python-based pipeline. Images underwent preprocessing including background correction and noise reduction before segmentation. Adaptive thresholding and watershed algorithms were applied to separate individual cellular objects. Morphological, fluorescence, texture, and focus-related features were extracted for each object, generating multidimensional per-cell descriptors.

Quality-control filtering using interquartile range thresholds removed segmentation artifacts and optical outliers, resulting in retention of approximately 4.3×10^5 high-quality cellular measurements used for all downstream analyses (Supplementary Table S3).

Machine learning and statistical analysis

A manually annotated subset of approximately 5,000 representative events was used to train supervised classifiers for discrimination of viable and debris-associated cells and fluorescence-defined states. Random Forest, Gradient Boosting, Support Vector Machine, and Logistic Regression models were trained using standardized features and evaluated using stratified training–test splits and cross-validation. Performance metrics included accuracy, precision, recall, F1-score, ROC–AUC, and PR–AUC (Supplementary Tables S4 & S5). Reliability diagrams further evaluated probability calibration performance across morphological classification tasks, confirming well-calibrated probability outputs following Platt and isotonic calibration procedures (Supplementary Fig. 2).

Statistical analyses were performed using Python libraries including SciPy, statsmodels, and scikit-learn. Non-parametric tests were used where assumptions of normality were violated. Treatment and temporal effects were assessed using Kruskal–Wallis tests and mixed-effects models, and correlation analyses evaluated relationships among morphological, fluorescence, and degradation variables. Statistical significance was accepted at $p < 0.05$.

Results

Dose-Dependent PAH Degradation Kinetics Across Treatments

Residual phenanthrene–pyrene concentrations decreased over time in a treatment-dependent manner (Fig. 2A). Leaf-litter–amended soils (10AL, 30AL, and 70AL) and the pure culture isolate (PC) exhibited rapid PAH removal, achieving approximately 60–80% reduction by Day 10. In contrast, the spiked soil treatment (SS) showed slower degradation, retaining approximately 70% residual PAHs, while the aged-leaf–only treatment (AL) displayed intermediate removal with approximately 50% PAHs remaining. No significant PAH loss was observed in the abiotic control (CTRL (MSM + PAH)). Among amended treatments, 10AL exhibited the highest degradation rate, while 30AL showed slightly lower but comparable kinetics, indicating that moderate amendment levels were sufficient to achieve near-maximal degradation efficiency without requiring higher organic inputs. The pure culture exhibited degradation rates comparable to amended soil communities, whereas unamended soil showed reduced removal efficiency, confirming the stimulatory effect of organic amendment on microbial PAH degradation.

GC-FID chromatographic analysis confirmed treatment-dependent degradation of phenanthrene and pyrene (Fig. 2B). Characteristic peaks corresponding to both compounds were detected across samples, confirming compound identity. The pure culture retained detectable PAH peaks throughout incubation, whereas the 10AL treatment showed progressive peak reduction with near-complete disappearance by Day 10, indicating enhanced degradation under leaf-litter amendment. These chromatographic trends corroborate kinetic analyses demonstrating accelerated PAH removal in amended systems relative to unamended and control treatments.

B. GC-FID confirmation of PAH degradation in PC and 10AL treatments. *Representative GC-FID chromatograms of phenanthrene and pyrene from (A) pure culture (PC) and (B) 10% aged-leaf–amended soil (10AL) at Days 1, 3, 5, and 10. Peaks at approximately 21.2 min and 24.5 min correspond to phenanthrene and pyrene, respectively. The 10AL treatment shows pronounced and progressive peak diminution with near-complete disappearance by Day 7, whereas PC retains detectable peaks, confirming enhanced PAH biodegradation in the leaf-litter–amended system.*

First-order kinetic modelling confirmed treatment-dependent differences in degradation rates (Table X). The 10AL treatment exhibited the highest degradation rate constant ($k \approx 0.153 \text{ day}^{-1}$) and shortest half-life (~ 4.5 days), whereas the abiotic control showed negligible degradation. Leaf-litter–amended treatments and the pure culture displayed substantially higher degradation rates than unamended soil,

while spiked soil exhibited slower kinetics ($k \approx 0.052 \text{ day}^{-1}$). Statistical analyses indicated significant treatment differences in residual PAH concentrations from Day 3 onward, with amended treatments and the pure culture sustaining faster degradation than unamended systems.

Table 1
First-order kinetic parameters describing phenanthrene–pyrene degradation across treatments.

Treatment	$k \text{ (day}^{-1}) \pm \text{SE}$	Half-life $t_{1/2} \text{ (days)} \pm \text{SE}$	Mean lifetime $\tau \text{ (days)} \pm \text{SE}$	Model fits (R^2)
PC	0.1368 ± 0.00915	5.06 ± 0.34	7.31 ± 0.49	0.9579
10AL	0.1533 ± 0.02499	4.52 ± 0.74	6.52 ± 1.06	0.9706
30AL	0.1309 ± 0.01025	5.29 ± 0.41	7.64 ± 0.60	0.9143
70AL	0.1195 ± 0.00583	5.80 ± 0.28	8.37 ± 0.41	0.9316
SS	0.0520 ± 0.00365	13.33 ± 0.94	19.23 ± 1.35	0.9566
AL	0.0884 ± 0.00045	7.84 ± 0.04	11.31 ± 0.06	0.9108
CTRL (MSM + PAH)	0.00063 ± 0.00021	-	-	-

Values expressed as mean $\pm$ standard error (SE), $n = 3$.

Degradation rate constants (k) and corresponding half-lives ($t_{1/2}$) were estimated using first-order decay modelling of residual PAH concentrations over time. Values are reported as mean $\pm$ standard error (SE) from triplicate experiments. Leaf-litter–amended treatments and the pure culture isolate exhibited higher degradation rates and shorter half-lives compared with unamended soil and abiotic control treatments, indicating enhanced microbial PAH biodegradation under amendment conditions.

Microbial Community Structure Following Leaf-Litter Amendment

Quantitative plate counts demonstrated treatment-dependent shifts in microbial community composition following leaf-litter amendment of PAH-contaminated soils (Fig. 3). Leaf-amended treatments exhibited higher abundances of PAH-degrading bacteria and associated functional groups, including heterotrophs, fungi, actinomycetes, and nitrogen-fixing bacteria, compared with unamended controls. The 10AL treatment showed the strongest microbial enrichment, while 30AL and 70AL also supported elevated microbial populations. In contrast, spiked soil exhibited reduced community-wide proliferation, and the aged-leaf treatment maintained intermediate microbial abundances. Greater colony morphotype diversity was observed in amended treatments relative to controls (Supplementary Table S6).

Dose-Dependent PAH Tolerance of Purified Isolates

Dose–response assays revealed isolate-specific tolerance to increasing PAH surface loadings (Fig. 4). Colony expansion increased with PAH dose for all isolates; however, PC1 showed substantially greater tolerance, reaching colony radii of approximately 17 mm at 70 mg PAH per dish, whereas PC2–PC4 plateaued at approximately 6–7 mm at the highest loadings. Divergence among isolates became pronounced at PAH concentrations ≥ 30 mg dish⁻¹, indicating differential tolerance capacities. Two-way ANOVA confirmed significant effects of PAH dose, isolate identity, and their interaction on colony expansion ($p < 0.0001$). Based on its consistently superior growth across concentrations, PC1 was selected as the reference isolate for subsequent analyses.

Cytometric Distribution of Microbial Cell States Across Treatments

Imaging flow cytometry revealed treatment-dependent differences in microbial cell-state distributions based on GFP/PI staining (Fig. 5A&B). Leaf-litter–amended treatments maintained high fractions of viable cells, whereas PAH-only and aged-leaf treatments showed increased proportions of dead and debris-associated populations. Heatmap summaries confirmed reduced viability and elevated mortality under PAH stress, while amended systems sustained higher viable-cell fractions. These patterns demonstrate treatment-specific shifts in microbial physiological states consistent with observed biodegradation performance.

Dose–Response Effects of Aged-Leaf Concentration on Microbial Viability. Flow cytometry analyses demonstrated concentration-dependent effects of aged-leaf amendments on viable-cell abundance (Fig. 5C&D). Viable-cell counts declined with increasing amendment concentration, with the strongest reductions observed at higher concentrations during later exposure periods, whereas lower amendment levels maintained higher viable-cell populations and partial recovery at later time points. Estimated EC_{50} values decreased during early exposure and stabilized thereafter, indicating progressive physiological adaptation. Correlation analysis showed a strong inverse relationship between dead-cell accumulation and viability ($r = -0.9988$, $p < 0.001$), while total cell abundance positively tracked viable-cell dynamics ($r = 0.87$, $p < 0.001$), linking cellular responses to treatment-dependent functional performance.

(D) *Table summarizing estimated EC_{50} concentrations and corresponding half-maximal viable-cell counts (with standard deviations) derived from dose–response analyses across exposure days, illustrating temporal variation in concentration-dependent viability responses.*

Image-Enabled Cytometry and Machine Learning–Based Classification of Microbial Cell States

Image-enabled flow cytometry combined with supervised machine learning enabled accurate discrimination of viable, dead, and debris-associated populations across treatments using integrated morphological and fluorescence features (Fig. 6A&B). Ensemble classifiers demonstrated near-perfect performance, with Random Forest and Gradient Boosting achieving ROC–AUC values of 1.00 and classification accuracies exceeding 0.999, while Support Vector Machine and Logistic Regression

models also showed strong discrimination performance ($\text{ROC-AUC} \geq 0.995$), confirming robust separability of microbial cell states within the multidimensional feature space.

Correlation analysis of extracted features indicated strong redundancy among size-related descriptors, including area, perimeter, diameter, and axis lengths, while shape-related metrics showed moderate interrelationships. In contrast, fluorescence intensity and focus parameters exhibited weak associations with morphology metrics, demonstrating that optical and geometric descriptors capture largely independent aspects of cellular state. Representative non-redundant metrics were therefore retained for downstream modelling to reduce dimensional redundancy while preserving biologically relevant variation (Supplementary Table S1).

Feature importance analysis indicated that classification performance was governed primarily by morphological descriptors rather than fluorescence metrics. Compactness, shape index, and solidity were the strongest contributors to model decisions, followed by circularity and perimeter, whereas fluorescence intensity and focus-related parameters contributed minimally. These results indicate that cell-state discrimination is predominantly driven by geometric integrity and boundary regularity rather than intensity-based features.

Application of the trained models to treatment-level datasets revealed pronounced shifts in microbial physiological states across treatments (Fig. 5A&B). Leaf-litter-amended systems maintained higher proportions of viable cells, whereas PAH-stressed and aged-leaf treatments exhibited increased dead and debris-associated populations. These treatment-dependent cytometric patterns closely paralleled observed degradation performance, linking microbial physiological status to functional biodegradation outcomes.

Morphological Differentiation Between Viable Cells and Debris

Principal Component Analysis (PCA) of image-derived morphological features showed clear separation between viable cells and debris populations (Fig. 7A, Supplementary Fig. 1), with PC1 and PC2 explaining 47.3% and 20.5% of total variance, respectively (67.8% cumulative). Separation occurred primarily along PC1, and consistent overlap across sampling days indicated stable morphological discrimination over time. PCA of viable cells across treatments showed overlapping 95% confidence ellipses and closely clustered centroids, demonstrating conserved viable-cell morphology with only minor treatment-related variation. Hence, morphology robustly distinguished viable cells from debris while viable-cell structure remained stable across amendment conditions.

Quantitative comparisons confirmed significant morphological differences between viable cells and debris across evaluated parameters ($p < 0.001$) (Fig. 7B and Supplementary Table S2). Debris particles exhibited substantially larger size metrics, with mean area values approximately 40-fold greater than viable cells, together with markedly lower circularity and solidity. Effect size analysis indicated that circularity and compactness showed the strongest discrimination (Cohen's $d \approx 2.39$), followed by solidity

($d \approx 2.22$), confirming that shape integrity and boundary regularity are primary determinants of cell-type separation, whereas intensity-based features showed minimal differences between populations.

Linking Cellular Phenotypes to PAH Degradation Rates

Comparison of degradation rate constants with imaging-derived cellular metrics revealed treatment-dependent associations between microbial physiological state and biodegradation performance (Fig. 8). Degradation rates increased with viable-cell fractions ($\rho = 0.83$, $p < 0.05$), indicating faster PAH removal in treatments sustaining higher viability. Fluorescence-derived activity metrics showed a similar but weaker positive association with degradation rates. Morphological principal component summaries of viable cells exhibited the strongest relationship with degradation kinetics ($\rho = 0.89$, $p < 0.05$), with treatments characterized by compact, regular cell morphologies corresponding to higher degradation rates. Collectively, these results demonstrate that microbial physiological and morphological states quantitatively predict PAH degradation efficiency across treatments.

Relationships between treatment-level microbial cellular characteristics derived from imaging flow cytometry and first-order degradation rate constants (k) for phenanthrene–pyrene biodegradation. (A) Degradation rate constants increase with higher mean viable-cell fractions, indicating that treatments maintaining physiologically stable populations support faster PAH removal. (B) Mean fluorescence intensity, used as a proxy for cellular metabolic activity, shows a positive association with degradation rates, although with weaker separation among treatments compared with viability metrics. (C) Median PC1 scores derived from morphology-based principal component analysis of viable cells demonstrate the strongest relationship with degradation performance, with treatments exhibiting compact, regular cell morphologies corresponding to higher degradation rates. Each point represents a treatment-level summary across sampling days, and lines indicate linear regression fits provided as visual guides. Collectively, these results demonstrate that microbial physiological state and morphology quantitatively predict PAH degradation efficiency under different amendment conditions.

Representative Morphotypes and Division Dynamics

Representative bright-field micrographs show progressive cell division patterns in the pure-culture isolate and morphological heterogeneity in treatment-derived communities (Fig. 9). In the pure culture, Day 1 fields were dominated by single cells and doublets, while Days 3–5 showed increasing formation of small clusters indicative of active replication. By Day 7, multicellular aggregates predominated, demonstrating sustained proliferation and progressive microcolony development. Division patterns included paired and clustered cells consistent with sequential cell division, while overall cell morphology remained compact and regular throughout incubation. Quantitative image counts further indicated a temporal shift from predominantly single-cell states at early time points to cluster- and aggregate-dominated populations by Day 7, consistent with sustained proliferation.

In contrast, treatment-derived samples exhibited diverse morphotypes, including irregular single cells, elongated dividing forms, budding or clustered microcolonies, dense aggregates, filamentous and

branched cells, filament-associated clusters, paired dividing cells, coccoid forms, and non-cellular debris. This morphological heterogeneity reflects coexistence of multiple physiological states and microbial populations within amended and stressed systems. These observations align with cytometric and image-derived analyses showing treatment-dependent variation in cellular integrity and population structure across samples.

Representative bright-field micrographs illustrating temporal division patterns in the pure-culture isolate (A) and morphological diversity in environmental and treatment-derived samples (B). Pure-culture images show a progressive transition from single cells and doublets (Day 1) to small clusters (Day 3) and larger multicellular aggregates (Days 5 and 7), reflecting active proliferation and sustained division cycles. Representative morphotypes from mixed samples include irregular single cells, elongated dividing cells, budding or clustered microcolonies, dense cell aggregates, filamentous and branched forms, filament-associated aggregates, paired dividing cells, small coccoid cells, and debris or non-cellular particles. Scale bars = 5 μm . Images highlight both controlled division dynamics in the pure culture and pronounced morphological heterogeneity in treatment-derived microbial communities.

Discussion

Despite extensive application of organic amendments in PAH-contaminated soils, uncertainty remains regarding why amendment performance varies with dosage and how microbial physiological responses translate into differences in degradation efficiency (Ejileugha and Semple 2025; Omoni et al. 2020). Many remediation studies demonstrate amendment-driven improvements in contaminant removal but often treat microbial communities as bulk populations, without resolving how cellular health, structural integrity, and physiological state determine functional outcomes (Zhang et al. 2023; Chen et al. 2024). As a result, the mechanisms linking amendment dosing, microbial population dynamics, and degradation kinetics remain insufficiently understood (Rusănescu et al. 2024; Sayara and Sánchez 2020). The present study addresses this gap by integrating degradation kinetics, chromatographic validation, microbial community analyses, and imaging-enabled cytometry to elucidate how amendment-driven physiological stabilization of microbial populations governs treatment-dependent PAH degradation performance (Chen et al. 2024; Kieps et al. 2023).

Intermediate Amendment Levels Drive Optimal Degradation – Leaf-litter amendment consistently accelerated phenanthrene and pyrene degradation relative to unamended soil, thereby confirming that organic inputs stimulate biologically mediated PAH removal through the provision of bioavailable carbon and nutrients that support hydrocarbon-degrading microorganisms, as similarly reported by Omoni et al. (2020) and Sayara and Sánchez (2020). Comparable stimulation effects have been widely reported, with organic amendments enhancing microbial biomass, enzymatic activity, and contaminant bioavailability in PAH-contaminated soils (Rusănescu et al. 2024; Zhang et al. 2023). However, the present findings demonstrate that degradation efficiency does not increase proportionally with amendment dosage; rather, intermediate amendment levels achieved degradation kinetics comparable to or exceeding those observed at higher inputs.

This pattern indicates that microbial metabolic demands may be satisfied at moderate amendment levels, beyond which additional organic inputs do not yield proportional gains in degradation performance (Wang et al. 2021). Elevated amendment loading has been associated with inhibitory effects, including competition from rapidly proliferating non-degrading heterotrophs, accumulation of metabolic byproducts, and oxygen limitation that can suppress aerobic degradation pathways (Ejileugha and Semple 2025; Zhang et al. 2023). Conversely, unamended soils typically remain nutrient-limited, restricting microbial proliferation and enzymatic activity despite contaminant availability (Rusănescu et al. 2024).

Notably, degradation trajectories in amended soils closely approached those of the purified isolate, indicating that amendment-driven community restructuring effectively enriches functional degraders capable of performance comparable to laboratory-selected strains, as similarly observed by Chen et al. (2024) and Miralles et al. (2021). Organic amendments have likewise been shown to promote hydrocarbonoclastic populations that dominate active degradation phases under favorable environmental conditions (Assil et al. 2021). These observations support the conclusion that remediation enhancement arises not solely from increased microbial abundance but from selective stimulation of metabolically competent populations (Mayans et al. 2025; Bala et al. 2022). Collectively, these findings reinforce that optimized amendment dosing, rather than maximal input, is critical for efficient and economically viable remediation strategies (Jia et al. 2025; Hao et al. 2025; Akinseye et al. 2026).

Importantly, this study extends conventional demonstrations of amendment-enhanced biodegradation by mechanistically linking amendment dosage to microbial physiological stability and degradation outcomes. Through integration of kinetic modelling, imaging-based cytometry, and automated classification, the results demonstrate that treatment-dependent degradation performance is governed not merely by microbial abundance but by the maintenance of structurally intact and metabolically competent populations. This quantitative linkage between morphology-derived cellular metrics and kinetic variability across amendment treatments provides a mechanistic bridge between microbial physiology and ecosystem-scale remediation performance, consistent with emerging systems-level approaches in environmental biotechnology (Kieps et al. 2023).

Microbial Survival Alone Does Not Guarantee Degradation Performance – Flow cytometric analyses provided further insight into the influence of amendment treatments on microbial populations under PAH stress. In PAH-only systems, increased proportions of dead and debris-associated cells were observed, consistent with membrane damage and metabolic inhibition associated with hydrocarbon toxicity, where oxidative stress and toxic intermediates impair microbial survival (Idowu et al., 2019; Ghosal et al., 2016). In contrast, amendment treatments mitigated these stress responses, maintaining higher viable-cell fractions and supporting populations capable of sustained substrate turnover (Zhang et al., 2024).

Despite this, viability measurements alone did not fully account for treatment-dependent differences in degradation kinetics. Certain treatments displayed comparable viable-cell proportions yet differed in

degradation rates, indicating that microbial survival does not necessarily equate to functional degradation capacity (Jiang et al., 2019). This observation underscores a limitation of viability-based assessments, which quantify membrane integrity but do not capture the structural and physiological attributes required for sustained metabolic performance, as similarly noted by Claveau et al. (2025).

Imaging-based analyses demonstrated that treatments characterized by compact, regular cellular morphologies corresponded to higher degradation rates, whereas systems dominated by irregular or fragmented morphotypes exhibited slower kinetic progression. Morphological descriptors effectively captured stress-associated structural alterations—including swelling, aggregation, and fragmentation—that are not fully reflected in fluorescence-based viability metrics. Comparable morphological changes under hydrocarbon stress have been linked to compromised membrane functionality and reduced metabolic efficiency (Zhou et al., 2024; Vuorio et al., 2021; Zdarta et al., 2020).

Collectively, these findings indicate that biodegradation efficiency depends not only on maintaining viable populations but also on preserving structurally intact cells capable of sustained enzymatic activity (Zhang et al., 2024). Morphological integrity therefore provides explanatory value beyond conventional viability metrics, supporting the integration of structural phenotyping into comprehensive assessments of remediation performance (Claveau et al., 2025; Hernández-López et al., 2024).

Imaging Cytometry Explains Treatment-Dependent Kinetic Variability – Machine learning analysis of imaging flow cytometry data provided further mechanistic insight into how microbial physiological states govern degradation outcomes. Ensemble classifiers achieved near-perfect performance in distinguishing cell states, demonstrating that microbial populations subjected to PAH stress occupy well-separated morphological and fluorescence feature spaces. Comparable studies have shown that machine learning approaches substantially improve classification robustness when analysing heterogeneous microbial communities exposed to environmental stressors (Blasi et al., 2022; Jeong et al., 2025; Eulenberg et al., 2023). Importantly, morphological features dominated model decision-making, indicating that structural integrity is a stronger indicator of physiological condition than fluorescence intensity alone. Fluorescence-based stains capture membrane permeability or metabolic activity but may overlap among transitional physiological states, whereas morphological changes more directly reflect physical cellular damage or adaptation (Power et al., 2021; Kieps et al., 2023). Treatment-dependent shifts detected by automated classification closely paralleled degradation kinetics, demonstrating that imaging-derived physiological metrics provide predictive insight into functional degradation outcomes. Similar relationships between microbial phenotyping and contaminant degradation have been reported in systems combining high-content imaging and metabolic profiling (Koblitz et al., 2025; Nerini et al., 2024).

Direct microscopic observation further supported these interpretations. Pure isolate cultures exhibited orderly transitions from single cells to aggregates, reflecting active proliferation under favourable conditions. In contrast, treatment-derived communities displayed diverse morphotypes, including elongated, filamentous, and aggregated forms, reflecting adaptive responses and coexistence of multiple physiological states within mixed consortia (Zhang and Zhang, 2022; Paniagua-Michel and

Fathepure, 2018). While such diversity may confer ecological resilience, excessive morphological disruption signals physiological stress that constrains degradation performance (Jiang et al., 2026; Hong et al., 2021).

Together, these findings demonstrate that imaging-enabled cytometry explains why degradation kinetics differ across treatments: amendment dosing shapes microbial physiological states, which in turn determine the capacity for sustained PAH turnover (Eulenberg et al., 2017; Kieps et al., 2023).

Implications for Engineered Bioremediation – These findings carry important implications for engineered remediation systems. First, amendment strategies should aim for optimal nutrient stimulation rather than maximal organic input, as excessive amendment may inadvertently suppress degradation efficiency while increasing operational costs (Singha et al., 2023; Guo et al., 2024). Field-scale remediation programs therefore benefit from dosing strategies guided by microbial physiological responses rather than solely by contaminant concentration. Second, monitoring strategies must move beyond total microbial counts or viability estimates (Priyadarsini et al., 2023). Imaging-enabled cytometry combined with automated classification offers scalable tools for tracking microbial physiological states in real time, enabling early detection of population stress before degradation rates decline (Blasi et al., 2022; Eulenberg et al., 2023). Such approaches can support adaptive management strategies in large-scale remediation projects (Price et al., 2017). Third, isolate tolerance analyses highlight the importance of resilient keystone degraders capable of sustaining metabolism under fluctuating contaminant loads. Effective remediation systems depend on cooperative microbial consortia where tolerant degraders operate within broader metabolic networks supporting nutrient cycling and stress mitigation (Ruan et al., 2024; Banerjee et al., 2018). Hence, integrating degradation kinetics with cytometric and imaging-derived physiological metrics establishes a framework connecting cellular-scale processes with ecosystem-level contaminant removal (Yang et al., 2021). Such multiscale understanding advances the development of predictive remediation systems capable of sustaining efficient contaminant removal under variable environmental conditions (Zhou et al., 2024).

While the controlled laboratory framework employed here enables mechanistic resolution of treatment effects, caution is required when extrapolating findings to field-scale remediation, where soils exhibit spatial heterogeneity, fluctuating moisture and temperature regimes, and complex contaminant mixtures that may alter amendment responses and microbial community dynamics (Heintz-Buschart et al., 2020; Jing et al., 2024). In addition, imaging cytometry, although highly informative, remains more resource-intensive than routine field monitoring approaches, potentially limiting immediate large-scale deployment (Power et al., 2021). Future studies should therefore validate amendment optimization and physiology–kinetic relationships under field conditions and develop simplified or automated imaging workflows suitable for operational remediation monitoring across diverse soil types and contaminant settings (Claveau et al., 2025).

Taken together, the results demonstrate that intermediate amendment levels maximize degradation efficiency and that microbial survival must be interpreted alongside morphological integrity. These

findings consistently indicate that physiological integrity, rather than microbial abundance alone, governs degradation performance (Banerjee et al., 2023). By directly linking cellular state to degradation kinetics, this study advances understanding of how amendment strategies shape remediation outcomes and provides a foundation for designing more effective and controllable bioremediation systems.

Conclusion

This study demonstrates that leaf-litter amendment regulates PAH degradation primarily through its effects on microbial physiological stability rather than through simple increases in microbial abundance. Moderate amendment levels preserved viable and structurally intact degraders capable of sustaining contaminant turnover, confirming that balancing nutrient stimulation with cellular stress determines remediation efficiency.

By integrating degradation kinetics with imaging-enabled cytometry, this work shows that morphology-derived single-cell metrics provide mechanistic insight into treatment-dependent degradation performance, revealing how amendment dosing shapes microbial functionality under contaminant pressure. These findings establish microbial physiological state as a central determinant of biodegradation outcomes and highlight the value of imaging-based phenotyping for resolving functional dynamics within complex remediation systems. Importantly, this work demonstrates that imaging-derived cellular phenotypes can quantitatively predict degradation kinetics, providing a scalable framework for physiology-guided remediation design.

More broadly, the results emphasize that effective bioremediation requires amendment strategies that maintain functional microbial communities rather than simply maximizing organic input. Integrating amendment optimization with microbial physiological monitoring offers a practical path toward predictable, cost-efficient, and resilient PAH bioremediation strategies.

Declarations

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Availability of Data and Materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request. Machine learning models, scripts, and processed imaging datasets are archived in Zenodo at <https://doi.org/10.5281/zenodo.18802989>.

Code Availability

Custom code used for image preprocessing, feature extraction, and supervised classification is available at <https://doi.org/10.5281/zenodo.18802989> (or upon request from the corresponding author).

Competing Interests

The authors declare that they have no competing interests.

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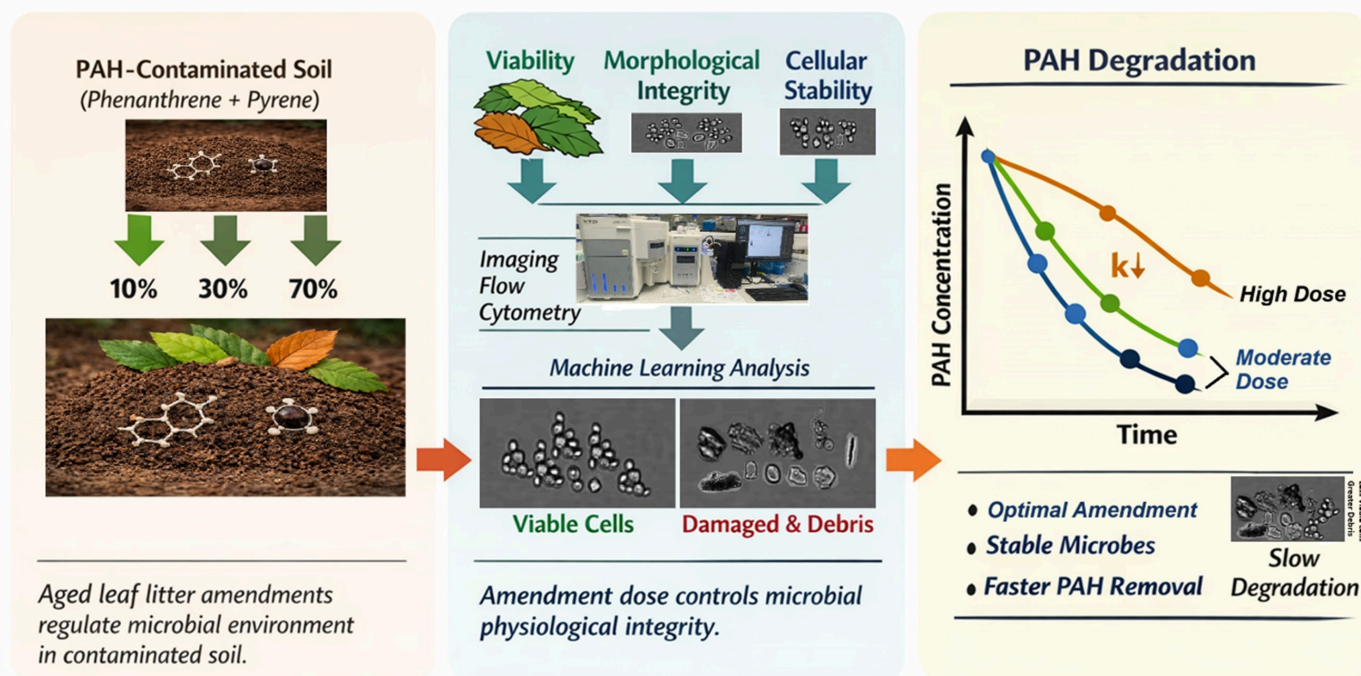
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Figures

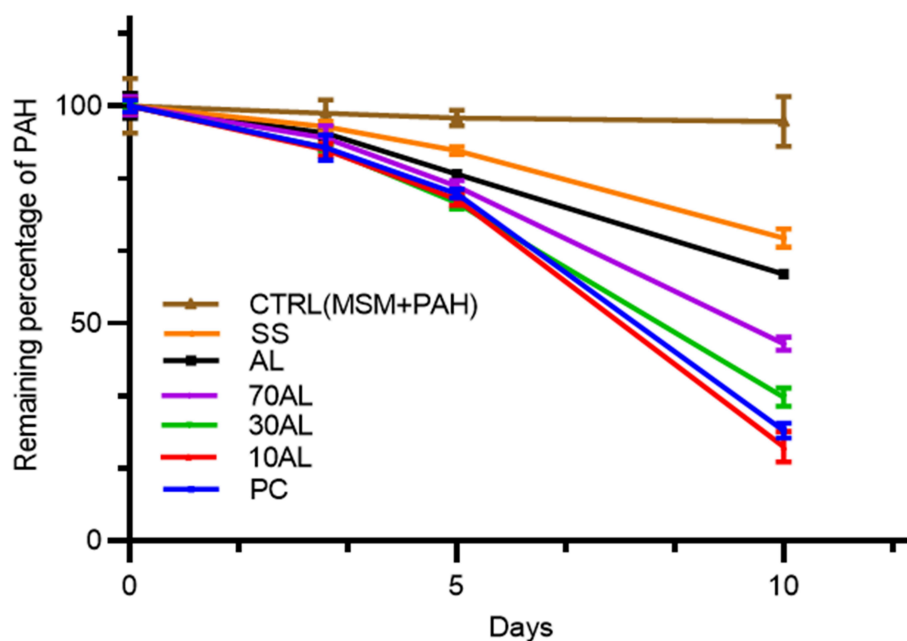
Dose-Dependent Regulation of PAH Degradation via Microbial Physiological Stability



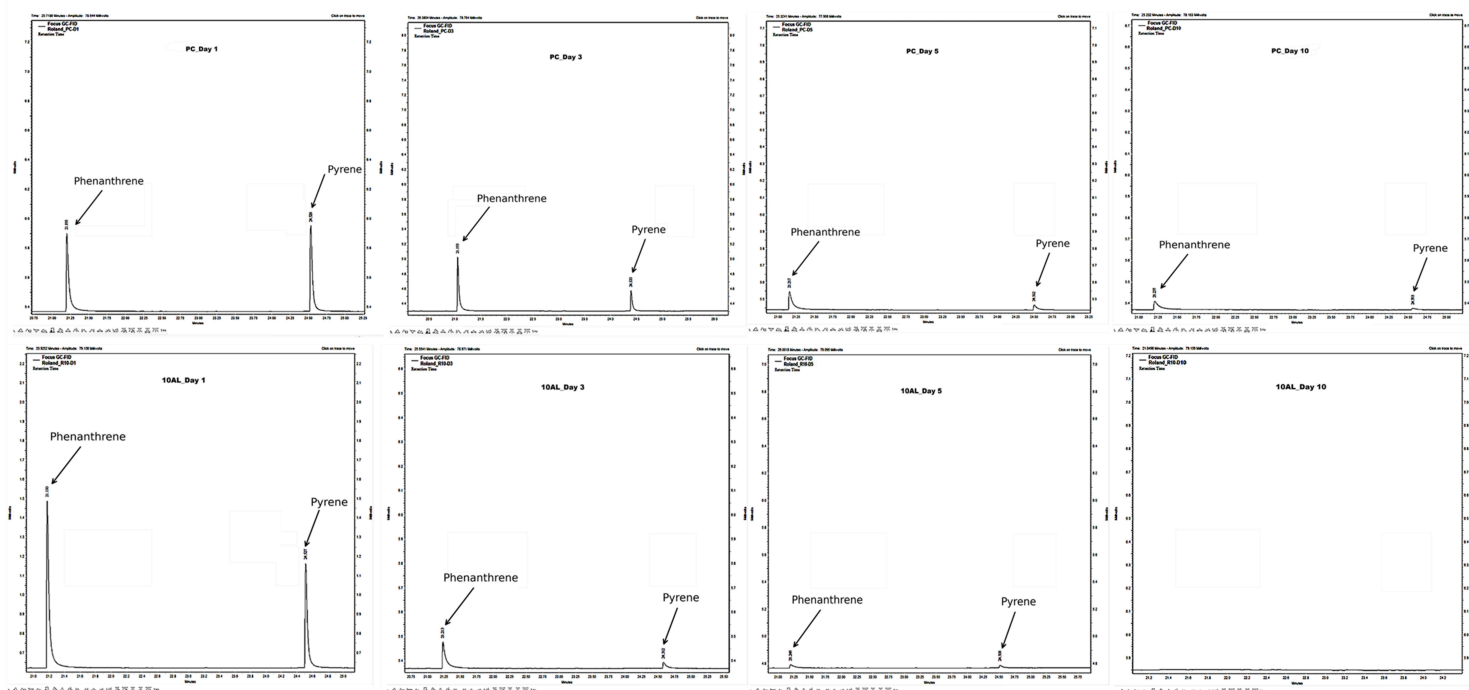
Physiology-guided amendment dosing enables efficient PAH bioremediation.

Figure 1

Conceptual overview of dose-dependent regulation of PAH biodegradation in contaminated soil following aged leaf-litter amendment. Pine–poplar leaf additions at graded levels modify microbial physiological stability, influencing cell viability and morphological integrity as quantified by imaging flow cytometry and machine learning–based classification. Moderate amendment levels sustain structurally intact microbial populations and result in faster phenanthrene–pyrene degradation, whereas excessive amendment leads to reduced microbial stability and slower contaminant removal. The framework demonstrates how physiology-guided amendment optimization enhances PAH bioremediation efficiency.



A



B

Figure 2

A Time-resolved biodegradation of phenanthrene–pyrene across treatments. *Residual phenanthrene–pyrene (%) as a function of incubation time for pure culture (PC), leaf-litter–amended treatments (10AL, 30AL, 70AL), aged leaf (AL), spiked soil (SS), and abiotic control (CTRL (MSM+PAH)). Data points represent mean $\pm$ standard deviation ($n = 3$). Leaf-litter–amended treatments and the pure culture exhibited rapid and*

sustained PAH removal, whereas the abiotic control showed no measurable degradation. Lines indicate first-order kinetic fits.

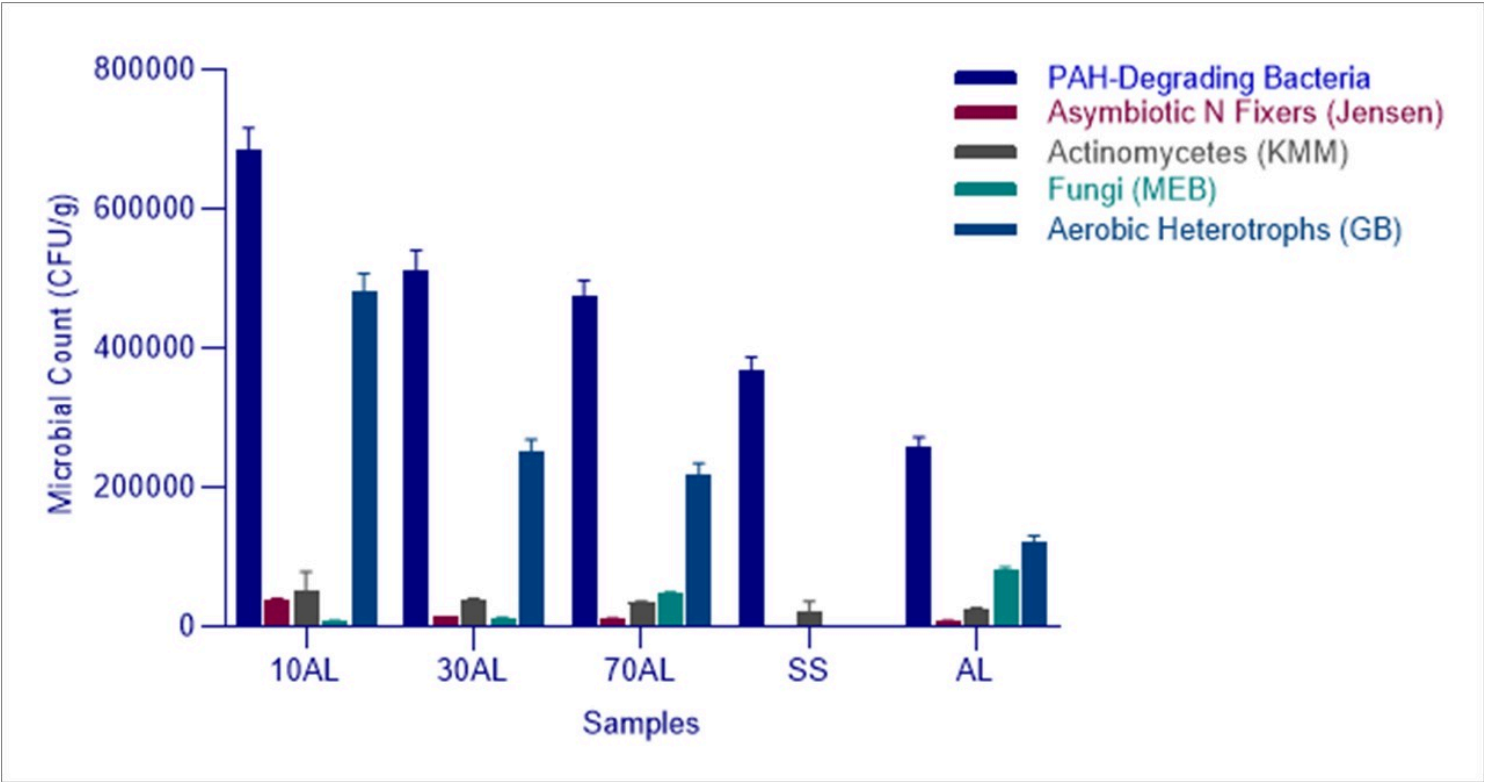


Figure 3

Quantitative microbial community structure in leaf-litter–amended PAH-contaminated soils. Mean colony-forming units (CFU g⁻¹ dry soil) ± SE (n = 3) is shown for PAH-degrading bacteria, asymbiotic nitrogen fixers, actinomycetes, fungi, and aerobic heterotrophs across treatments (10AL, 30AL, 70AL, SS, AL). Leaf-litter amendments significantly increased microbial abundance, with 10AL showing the strongest enrichment of PAH degraders and supporting microbial guilds.

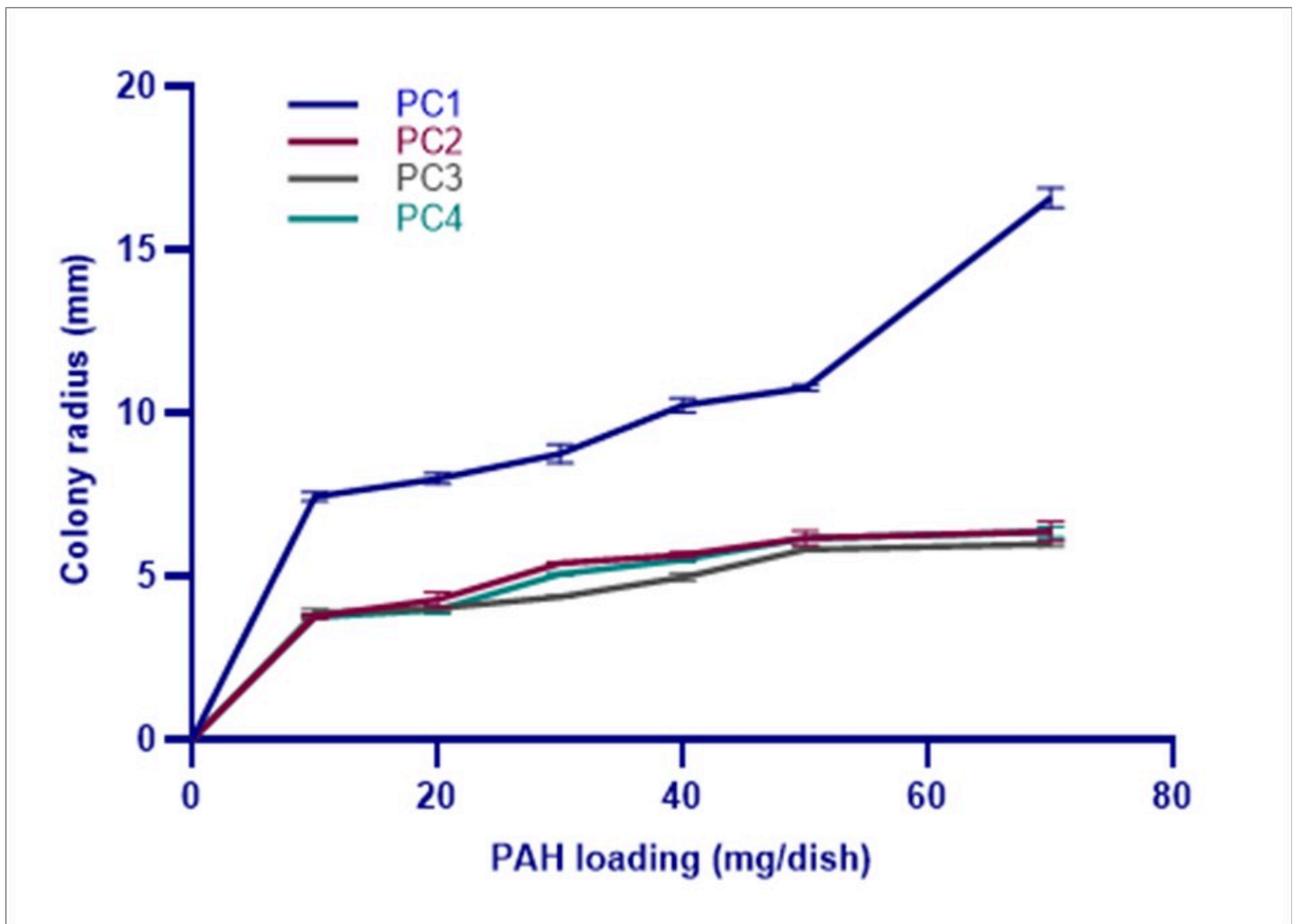


Figure 4

Dose–response tolerance of purified microbial isolates (PC1–PC4) to increasing PAH surface loadings. Colony expansion radius (mm; mean $\pm$ SE) is shown as a function of PAH dose (mg dish⁻¹). PC1 exhibits significantly higher tolerance across all concentrations, whereas PC2–PC4 show moderate, plateauing responses at high PAH loadings.

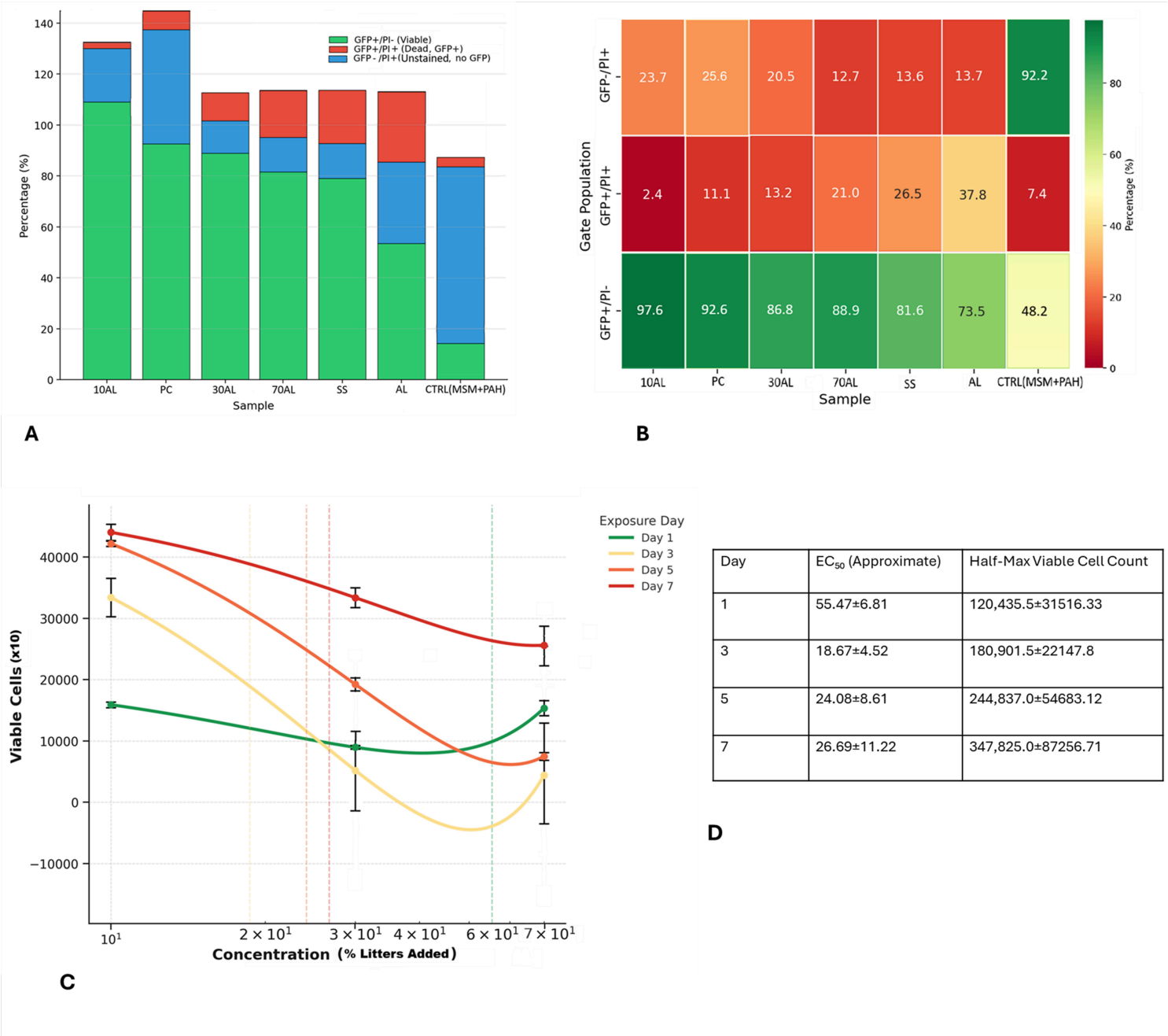


Figure 5

Cytometric distribution of microbial cell states across treatments and dose–response–correlation analysis of aged-leaf amendment effects on microbial viability. **(A)**Stacked bar plots showing proportions of viable (GFP^+/PI^-) and debris (dead or membrane-compromised (GFP^+/PI^+), and unstained-associated (GFP^-/PI^-)) populations across treatments derived from imaging flow cytometry classification. Leaf-litter-amended treatments maintained higher viable-cell fractions, whereas PAH-only and aged-leaf treatments exhibited increased non-viable and debris-associated populations. **(B)** Heatmap representation summarizing treatment-level distributions of viable and debris-associated (dead and unstained) populations, with colour intensity indicating population percentages. The heatmap highlights reduced viability and increased mortality under PAH stress compared with sustained survival in amended systems, consistent with observed biodegradation trends. **(C)** Dose–response curves showing viable-cell abundance

as a function of aged-leaf amendment concentration across exposure days, with error bars representing standard deviations. Curves illustrate concentration-dependent reductions in viable-cell populations, and dashed vertical lines indicate approximate EC_{50} values estimated for each sampling day.

(D) Table summarizing estimated EC_{50} concentrations and corresponding half-maximal viable-cell counts (with standard deviations) derived from dose–response analyses across exposure days, illustrating temporal variation in concentration-dependent viability responses.

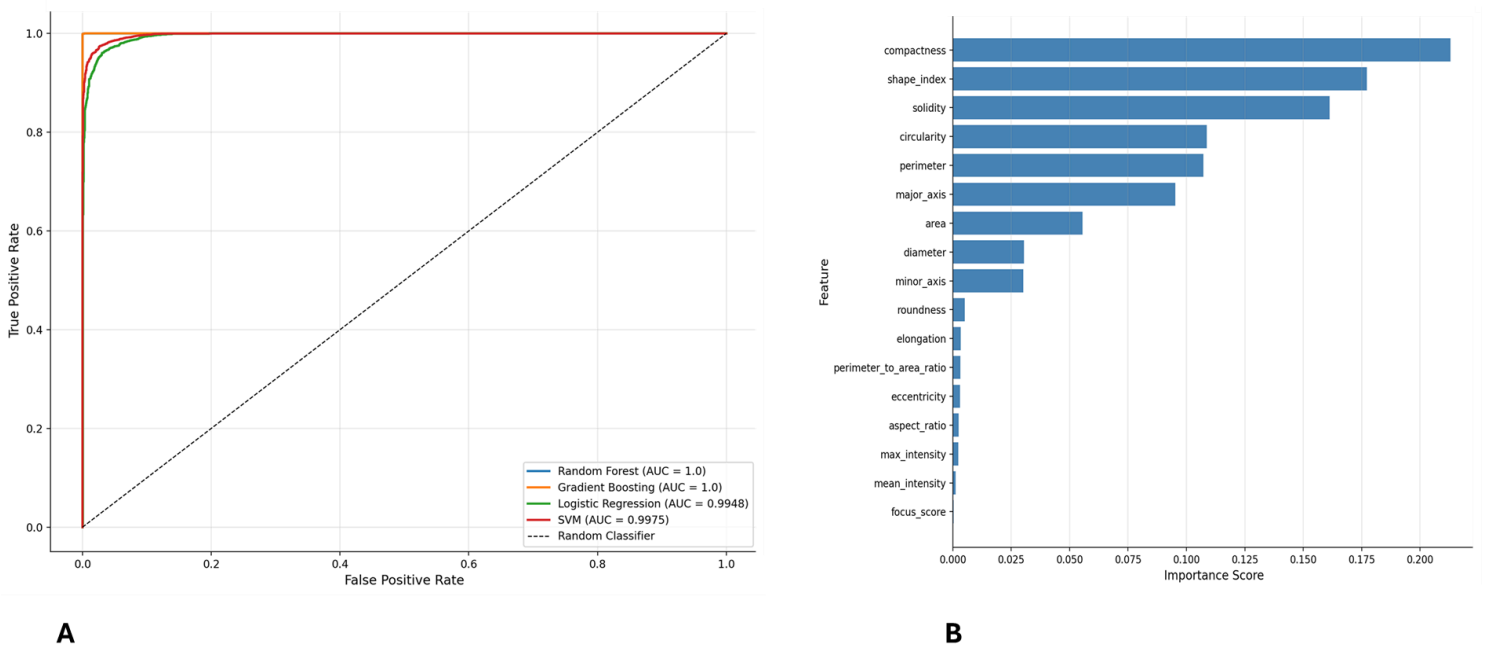


Figure 6

Machine learning–based classification and feature importance for microbial cell-state discrimination. **(A)** Receiver operating characteristic (ROC) curves showing classification performance of Random Forest, Gradient Boosting, Support Vector Machine, and Logistic Regression models for discrimination of viable and debris-associated cells using image-derived morphological and fluorescence features. Ensemble models achieved near-perfect discrimination (ROC–AUC = 1.000), with alternative classifiers also demonstrating strong predictive performance. **(B)** Feature importance ranking derived from the Random Forest model, indicating that morphological descriptors, particularly shape-related features such as compactness, solidity, and circularity, contributed most strongly to classification, whereas fluorescence intensity features showed comparatively minor contributions, highlighting the dominant role of morphological integrity in cell-state discrimination.

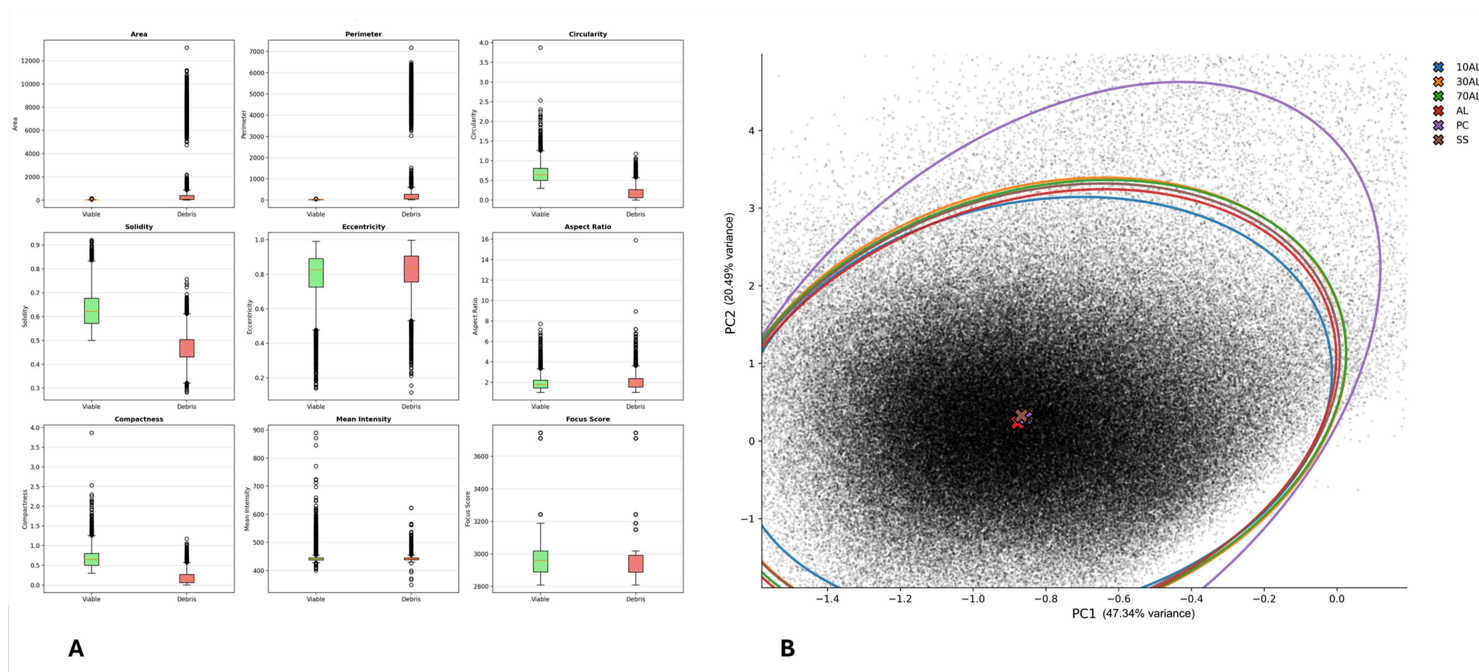


Figure 7

Principal Component Analysis (PCA) of image-derived morphological features separating viable cells from debris populations, alongside distributions of morphological and intensity features across both groups. **(A)** PCA of viable-cell morphology across treatments showing treatment centroids with 95% confidence ellipses, indicating largely conserved viable-cell morphology across treatments with only minor treatment-dependent variation. **(B)** Boxplots compare distributions of key morphological and intensity parameters between viable cells and debris across all samples. Size-related metrics (area and perimeter) show substantially larger values for debris, while shape descriptors including circularity, solidity, and compactness indicate greater structural regularity in viable cells. Eccentricity and aspect ratio reveal increased elongation and irregularity in debris populations. In contrast, intensity-based and focus-related metrics show comparatively smaller differences between groups. Collectively, these distributions demonstrate that geometric and shape-related characteristics primarily distinguish viable cells from debris populations.

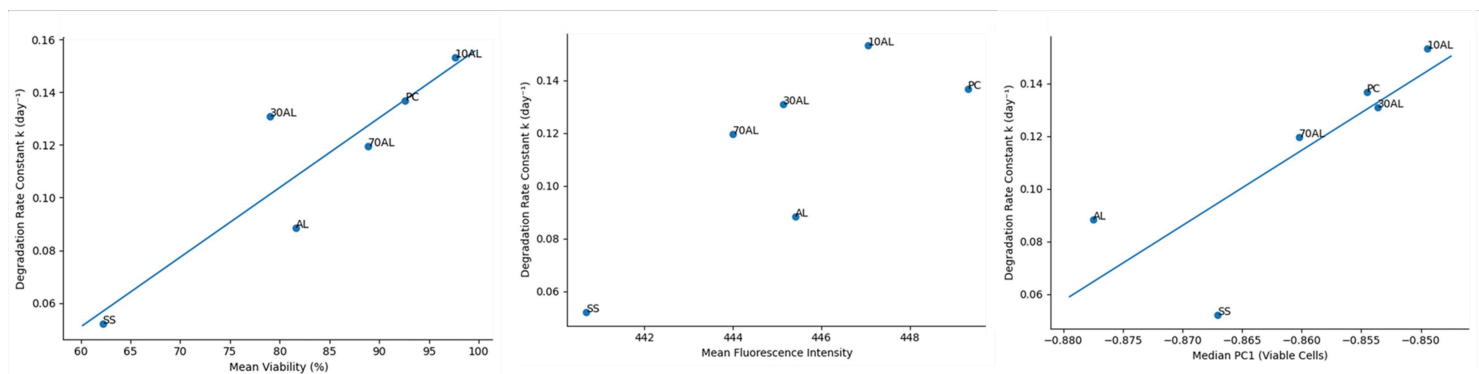


Figure 8

Linking cellular phenotypes to PAH degradation kinetics across treatments.

Relationships between treatment-level microbial cellular characteristics derived from imaging flow cytometry and first-order degradation rate constants (k) for phenanthrene–pyrene biodegradation. (A) Degradation rate constants increase with higher mean viable-cell fractions, indicating that treatments maintaining physiologically stable populations support faster PAH removal. (B) Mean fluorescence intensity, used as a proxy for cellular metabolic activity, shows a positive association with degradation rates, although with weaker separation among treatments compared with viability metrics. (C) Median PC1 scores derived from morphology-based principal component analysis of viable cells demonstrate the strongest relationship with degradation performance, with treatments exhibiting compact, regular cell morphologies corresponding to higher degradation rates. Each point represents a treatment-level summary across sampling days, and lines indicate linear regression fits provided as visual guides. Collectively, these results demonstrate that microbial physiological state and morphology quantitatively predict PAH degradation efficiency under different amendment conditions.

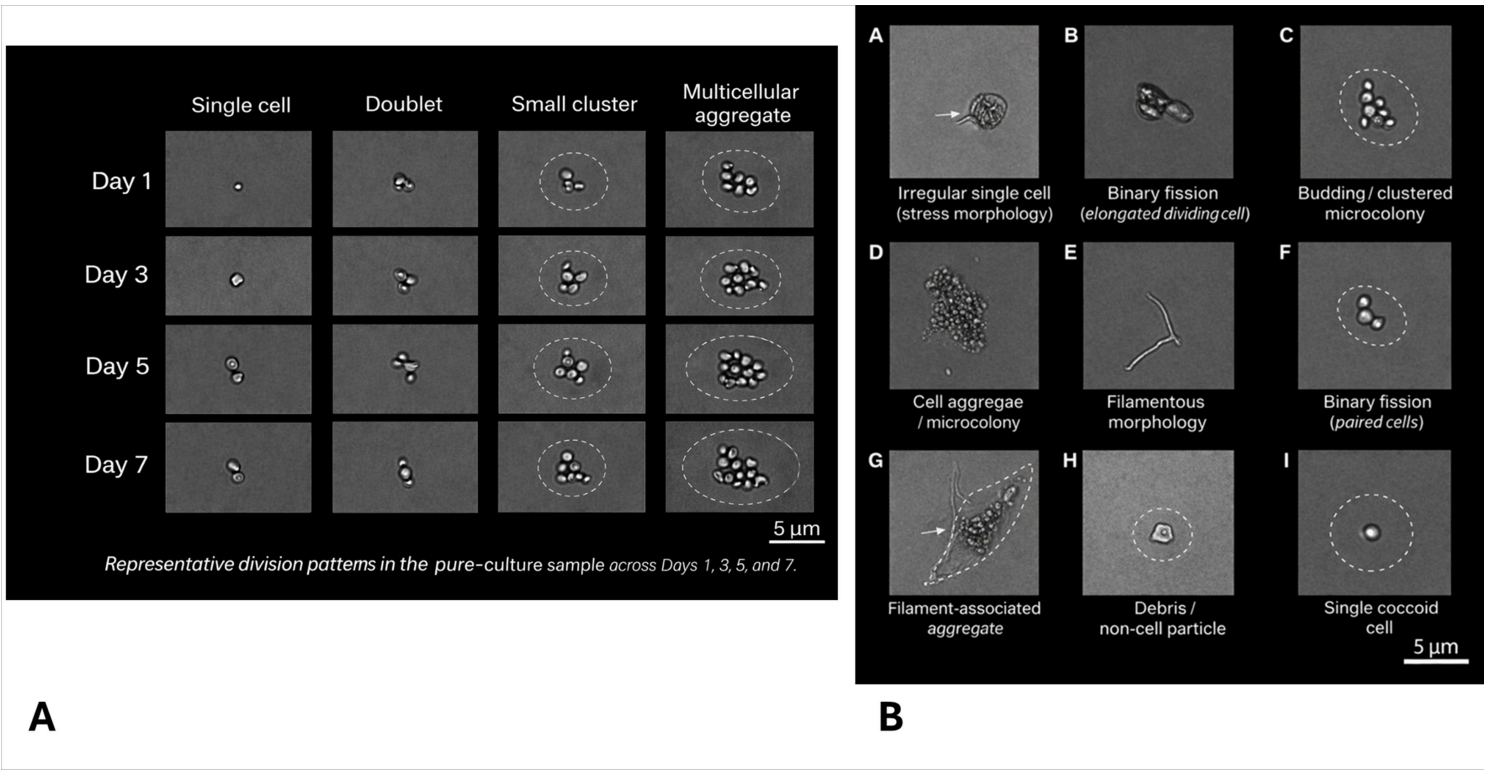


Figure 9

Division dynamics and representative microbial morphotype across pure culture and treatment-derived samples.

Representative bright-field micrographs illustrating temporal division patterns in the pure-culture isolate (A) and morphological diversity in environmental and treatment-derived samples (B). Pure-culture images show a progressive transition from single cells and doublets (Day 1) to small clusters (Day 3) and larger multicellular aggregates (Days 5 and 7), reflecting active proliferation and sustained division cycles.

Representative morphotypes from mixed samples include irregular single cells, elongated dividing cells, budding or clustered microcolonies, dense cell aggregates, filamentous and branched forms, filament-associated aggregates, paired dividing cells, small coccoid cells, and debris or non-cellular particles. Scale bars = 5 μ m. Images highlight both controlled division dynamics in the pure culture and pronounced morphological heterogeneity in treatment-derived microbial communities.

Supplementary Files

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