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Enzymatic Degradation of Persistent Organic Compounds by Microorganisms Isolated from Aquatic Sediments

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Abstract: Persistent organic compounds (POCs) emitted by textile processing, pesticide formulation and released by untreated municipal effluent are deposited in aquatic sediments, while the microorganisms that colonize the sediments represent an untapped source of catabolic enzymes. In this study, sediment bacteria were isolated, screened and characterised for their ability to enzymatically degrade four priority persistent organic compounds (phenanthrene, pyrene, pentachlorophenol and lindane) from three contaminated freshwater and estuarine sites of Tamil Nadu, India. Surface sediment cores were taken, texture, organic carbon, redox potential and residual loads of potentially contaminating materials measured, and the cores were sequentially enriched on mineral salts medium where each of the target compounds served as sole carbon source. Forty-one morphologically distinct isolates were recovered, of which six showed persistent growth and clearing-zone activity on all screening plates. These were identified as Pseudomonas, Bacillus, Rhodococcus, Sphingomonas, Achromobacter and Acinetobacter strains, using 16S ribosomal RNA gene sequencing. Catechol 1,2-dioxygenase, catechol 2,3-dioxygenase, laccase, manganese peroxidase and dehalogenase activity were measured in cell-free extracts. The genes of ring-cleaving dioxygenases were more abundant in polycyclic aromatic hydrocarbon degraders, while laccase and dehalogenase were more prevalent in genes involved in the degradation of chlorinated substrates. A consortium of six isolates, defined to remove the contaminants, removed ninety-three percent of phenanthrene, seventy-nine percent of pyrene, seventy-one percent of pentachlorophenol and fifty-seven percent of lindane in thirty days, outperforming all of the individual isolates. The degradation could be described as first order, and the peak enzyme activity between days ten and twenty was well correlated with degradation. The results show that indigenous sediment microorganisms have enzymatic potential for in-situ bioremediation and sediment quality restoration in moderately contaminated tropical aquatic system, globally.

Keywords: Persistent Organic Compounds; Aquatic Sediments; Enzymatic Degradation; Dioxygenase; Laccase; Bacterial Consortium; Bioremediation.

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I. Introduction

Persistent Organic Compounds are chemical substances containing carbon, that are persistent, bio-accumulate and are toxic to organisms that are distant from the source. There are polycyclic aromatic hydrocarbons from incomplete combustion and petroleum activities as well as organochlorine pesticides like lindane, wood preservative chemicals like chlorophenols, and polychlorinated biphenyls that have been used as dielectric fluids. The distribution of their ultimate fate in aquatic ecosystems is determined by their shared physicochemical signature, the high octanol-water partition coefficients, low aqueous solubility and strong affinity toward organic matter (Haritash & Kaushik, 2009). Compounds once released to a river, lake or estuary partition very quickly from the water column to the suspended particles, settling and becoming part of bottom sediments. Sediments are thus the final sink and a long-term secondary source of contamination. Other processes, such as hydrodynamic disturbance, dredging, bioturbation and seasonal flood pulses can bring buried particles to the surface and release sequestered contaminants back into the overlying water, which can then re-enter the food web via benthic invertebrates and bottom-feeding fish. This means that the ecological risk profile of an aquatic system over decadal time scales is determined by sediment contaminant inventories, and the rate of decline of a sediment contaminant inventory is controlled by the microbial assemblage that inhabits the sediment. Mechanical removal of contaminated sediment, in-situ capping with clean sediment, and solvent washing and chemical oxidation with persulfate or Fenton's reagents are common engineering solutions to contaminated sediment, but they have significant

disadvantages. Dredging activities negatively impact benthic habitat, cause contamination when removing sediment and shift the disposal responsibility. Chemical oxidation uses up huge amounts of reagent, lowers the pH of pore water and adversely affects native microbial communities. They both have capital and operational costs which are challenging in the face of the vast length of the river and its often moderately contaminated sections found in fast-growing tropical areas. An alternative exists that is non-toxic and works with the sediment ecology: biological treatment. Enzymes capable of breaking down the aromatic nucleus or the C–X bond directly can break down persistent organic compounds. Aerobic bacteria start the degradation of PAHs by hydroxylating the aromatic ring to a cis-dihydrodiol using ring hydroxylating dioxygenases which incorporate both atoms of molecular oxygen into the aromatic ring, followed by dehydrogenation to a dihydroxylated intermediate and subsequent ring fission by catechol 1,2-dioxygenase through the ortho pathway or catechol 2,3-dioxygenase through the meta pathway (Mallick et al., 2011). Ligninolytic oxidoreductases (LOs), especially laccases and manganese peroxidases, are non-specific one electron oxidases that are capable of a much broader substrate spectrum and are able to oxidize high molecular weight and halogenated structures that are not susceptible to dioxygenase oxidation (Zhang et al., 2020). Reductive and hydrolytic dehalogenases cleave chlorine substituents and expose the structure for its ability to undergo normal ring-cleavage pathways. These enzymes have been shown to work in the lab, but finding a way to use the enzymes to restore sediments in the field has been challenging. Most strains obtained from culture collections are not adapted to the salinity and/or redox potential and temperature regime and indigenous community structure in natural sediment and are often out-competed when introduced into natural sediment. Organisms recovered from the contaminated sediment have a history of selective exposure, have the appropriate catabolic operons activated or easily induced, and do not fare as badly upon reintroduction. It is thus vital to isolate and characterize such autochthonous degraders before designing a bioaugmentation scenario that will lead to a believable outcome (Patel et al., 2020). Although much work has been undertaken on soils in temperate regions and on various marine oil-spill sites, few reports of enzymatic characterization exist for tropical freshwater and estuarine sediments that are under a different selective pressure due to the presence of aromatic and organochlorine contaminants and the monsoonal hydrology. This disparity encouraged the current study (Ghosal et al., 2016).

Objectives

1. Isolation and molecular identification of bacteria that can use phenanthrene, pyrene, pentachlorophenol and lindane as their sole carbon source from contaminated freshwater and estuarine sediments.
2. Activity of catechol 1,2-dioxygenase, catechol 2,3-dioxygenase, laccase, manganese peroxidase and dehalogenase in cell-free extracts of the recovered isolates was quantified.
3. To assess the degradation efficiency and degradation kinetics of each isolate and a defined consortium in controlled laboratory trials using the four compounds.

The Article is Organized as Follows: Section I introduces the contamination problem and the objectives; Section II reviews the literature on the subject of sediment contamination and the microbial enzyme systems; Section III describes the sampling, isolation, enzymatic and analytical methodology; Section IV presents the sediment characteristics, isolate profiles, enzyme activities and degradation results; Section V interprets these results in relation to the published works; Section VI concludes with implications for sediment management.

II. Literature Review

2.1 Occurrence and Fate of Persistent Organic Compounds in Aquatic Sediments

Sediment contaminant surveys have shown that PAHs are common in urbanized watercourses and can range from one-to-many orders of magnitude, depending on the distance from the combustion sources, road run-off and petroleum handling facilities. Sediment organic carbon content, determined primarily by the

number of fused rings in the compounds, controls partitioning behaviour, with the freely dissolved concentration of pore water being below the uptake threshold for efficient microbial uptake for compounds containing four or more fused rings. The observed rate of natural attenuation is often restricted by such an "availability limitation" as opposed to a lack of catabolic potential (Haritash & Kaushik, 2009). Organochlorine pesticides exhibit similar partitioning properties, but with the additional feature of carbon–halogen bonds that are thermodynamically stable and sterically protected, whereas the chlorophenols are not as soluble, but are very toxic to the organisms which are expected to degrade them (Premnath et al., 2021).

2.2 Microbial Communities of Contaminated Sediments

Culture-independent studies of contaminated sediment always reveal Proteobacteria, Actinobacteria and Firmicutes as the most abundant phyla, and Pseudomonas, Sphingomonas, Rhodococcus, Mycobacterium, Burkholderia and Acinetobacter as repeated aromatic substrate degraders. Functional community structure, network connectivity and expression of degradation genes were found to vary significantly between hydrologically distinct but geographically adjacent lake bay and adjacent river sediments, with biodegradation rate constants differing by nearly 2 orders of magnitude. The same study showed that the degradation genes were not actively degraded if they were detected: communities of gene-bearing microorganisms in a quiescent lake bay did not actively degrade the genes unless they were stimulated by bio stimulation before measurable degradation was observed (Yan et al., 2021). Huge taxonomic diversity was also found in aromatic degradation in metagenomic surveys of petroleum supply chains, which further supports the argument that degradation is a distributed community function and not a property of a single organism (Hidalgo et al., 2020).

2.3 Oxygenases and the Bacterial Ring-Cleavage Pathways

Low-molecular-weight polycyclic aromatic hydrocarbons are degraded aerobically by a conserved pathway that involves a multicomponent ring-hydroxylating dioxygenase consisting of a terminal oxygenase with two large and two small subunits and two electron-transferring components, ferredoxin and ferredoxin reductase, in which the former catalyzes the initial ring hydroxylation. The resulting catechol or substituted catechol is then broken down either between the hydroxyl groups by an intradiol catechol 1, 2-dioxygenase with the formation of cis, cis-muconate, which is further metabolized to ortho or beta-ketoadipate, or adjacent to the hydroxyls by an extradiol catechol 2,3-dioxygenase with the formation of
2-hydroxymuconic semialdehyde and further metabolism to the meta pathway. The route followed is determined by the pattern of substrate substitution, the strain genotype and induction conditions, and the availability of the alternatives is responsible for much of the variation in reported degradation rates (Mallick et al., 2011). A number of extensive reviews have been published which describe the sequential enzymatic steps required for the mineralisation of naphthalene, phenanthrene, anthracene and pyrene for a range of different bacterial genera, and the importance of the substrate specificity of dioxygenase in determining the ability of an isolate to mineralizes a specific compound (Ghosal et al., 2016). Extradiol dioxygenases have been also shown more recently to be sourced from thermophilic organisms which can be engineered through site-directed mutagenesis to expand the substrate promiscuity and activity on catecholic pollutants, suggesting enzymes with even higher operational robustness (Zhang et al., 2022). Strain-level studies further contribute to this; new phenanthrene-degrading strains have been isolated and their metabolism characterized by means of defined intermediates under different environmental conditions (Mai et al., 2021).

2.4 Oxidoreductases and the Degradation of Halogenated Structures

The less efficient routes with increasing number of rings and increasing amount of halogen are the routes involving dioxygenation, since the electron deficient aromatic ring does not undergo electrophilic oxygen insertion and the larger the ring size and the number of halogen atoms the more the substrate cannot fit in the enzyme active site. Oxidoreductases get around this restriction. Laccases are copper-containing

enzymes which can abstract single electron from phenolic and, in presence of mediator like 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), non-phenolic substrates, leading to the formation of radicals that couple spontaneously, demethylate and open rings. Bacterial laccases have been of special interest due to their greater tolerance of high temperatures, pH and organic solvent levels than the fungal laccases, and the association between the mononuclear type-1 copper center and substrate selectivity has been extensively characterised for petroleum hydrocarbon degradation (Zhang et al., 2020). Manganese peroxidase is based on a diffusible manganese (III) chelate which can react with inaccessible substrates, and hydrolytic and reductive dehalogenases can remove chlorine substituents, thus rendering recalcitrant organochlorines accessible to conventional ring-cleavage.

2.5 Anaerobic Transformation and Research Gap

The upper millimeters of the sediment are anoxic and therefore the fate of the contaminants is determined by anaerobic processes. Under field conditions, the pathways for anaerobic degradation are demonstrated to be active, albeit at much lower rates than those under aerobic conditions, and are characterised by carboxylation, hydroxylation or fumarate addition coupled to reduction of nitrate, sulfate, iron or carbonate (Dhar et al., 2019). The effects of environmental factors, nutrient status, surfactant availability, redox potential and temperature on the degradation of enzymes by bacteria mediated in soil and sediment has been reviewed (Lu et al., 2011). Comparable but under-reported is the simultaneous enzymatic characterization of isolates sampled from mixed aromatic and organochlorine loaded tropical sediments, and direct comparison of the performance of a single strain against that of defined consortium under identical conditions. The present study aims to fill that gap.

III. Materials and Methods

3.1 Study Area and Sediment Sampling

A mixture of the sediment in water (1:2.5) was made and the pH and redox potentials of the sediment were determined using a calibrated combination electrode. The moisture content of the sediment was determined using gravimetry after drying to constant mass at 105 °C. The determination of the organic carbon was done using the oxidation process of dichromate and back titration using FeSO4. Particle size analysis was done using wet sieving along with hydrometer analysis and textural classification. Determination of total nitrogen and available phosphorus was done using Kjeldahl digestion and molybdenum blue method respectively. Residue analysis: The freeze dried and homogenized sediment was Soxhlet extracted using dichloromethane acetone mixture; the extract was then concentrated using reduced pressure, cleaned using the activated silica gel and anhydrous sodium sulfate column, then solvent exchanged to hexane and analyzed using gas chromatography along with mass spectrometric detection in selected ion monitoring mode. Quantification was done using the external calibration curves made from the certified standards along with deuterated surrogates added before extraction.

3.2 Physicochemical Characterisation and Residue Analysis

The determination of the pH and redox potential of sediments was done in a one-to-two-point five sediment-to-water slurry employing a combination electrode. The moisture content was determined gravimetrically using a constant weight method by drying samples at one hundred and five degrees Celsius. The amount of organic carbon present in the sediments was done using the dichromate oxidation method with ferrous ammonium sulfate titration. The particle size distribution of the sediments was carried out through wet sieving and the hydrometer method. Total nitrogen in sediments was determined by Kjeldahl digestion and available phosphorus was determined by the molybdenum-blue colorimetry method. The quantification of residues involved freeze-drying and grinding of sediments followed by Soxhlet extraction in dichloromethane/acetone, concentration of extracts under reduced pressure, cleaning up using an activated silica gel and anhydrous sodium sulfate column, solvent exchange in hexane, and analysis by gas chromatography-mass spectrometry using selected ion monitoring mode.

3.3 Enrichment and Isolation of Degrading Bacteria

Ten grams of freshly collected sediment was added to ninety milliliters of sterile mineral salt media with dipotassium hydrogen phosphate, potassium dihydrogen phosphate, ammonium sulfate, magnesium sulfate heptahydrate, calcium chloride and trace element solution at pH seven point zero. Each of these flasks received one of the target compounds, i.e. phenanthrene, pyrene, pentachlorophenol and lindane respectively, which were dissolved in acetone and allowed to evaporate prior to the addition of mineral salt media such that the final concentration was one hundred milligrams per litre and served as the only carbon and energy source. The flasks were incubated at thirty degrees Celsius with agitation of one hundred and fifty revolutions per minute in darkness. After seven days, ten percent of the culture was inoculated in a new medium and this enrichment process was continued for four cycles. Serial dilutions of the enrichment culture were performed and streaked onto mineral salt agar supplemented with ethereal spray of respective substrate and grown into clear zone colonies. These colonies were further purified by streaking onto nutrient agar plate and stored in glycerol at minus eighty degrees Celsius.

3.4 Screening and Selection

The purified isolates were tested for their degradative ability in three steps. Growth response was measured as optical density at 600 nm in liquid mineral salts medium with each of the substrates after 14 days compared to uninoculated and carbon-free controls. The diameter of the clearing zone on the spray plates was measured after 10 days of incubation and presented as a percentage of the diameter of the colony. In the evaluation of tolerance each isolate underwent increasing concentrations of substrate up to four hundred milligrams per litre. Isolates that were able to grow reproducibly and gave measurable zones and growth at 200 mg/l on all four substrates were taken to the next stage for identification and enzyme characterization.

3.5 Molecular Identification

Overnight nutrient broth cultures were used for genomic DNA extraction by using a commercial bacterial genomic DNA extraction kit. Amplification of the 16S ribosomal RNA gene was performed with the universal primer set 27F and 1492R, followed by 35 cycles of denaturation, annealing and extension at the end of which a final denaturation step was performed. AgroPecs were confirmed by agarose gel electrophoresis, purified and bidirectionally sequenced. Contigs were assembled, trimmed and compared to curated nucleotide databases, and the closest validly published relatives were found. Neighbour-joining analysis based on the Kimura two-parameter model with 1000 bootstrap replicates was used to confirm the phylogenetic placement.

3.6 Preparation of Cell-Free Extracts and Enzyme Assays

Mid-exponential cultures of isolates were grown in mineral salts medium with the appropriate inducing substrate, harvested by centrifugation for 15 minutes at 8,000 x g at 4°C, washed twice with chilled phosphate buffer and then re-suspended in chilled phosphate buffer with PMSF. Probe sonication was used to lyse cells in an ice bath with short pulse and cooling times, and lysate was centrifuged at twelve thousand times gravity to clarify the lysate. Catechol 1,2-dioxygenase was determined by measuring cis, cis-muconate formation at 260 nm, and catechol 2,3-dioxygenase by measuring 2-hydroxymuconic semialdehyde formation at 375 nm. The activity of laccase was measured using 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) as the substrate at four hundred and twenty nanometers, and that of manganese peroxidase was measured by the formation of manganese (III) malonate at two hundred and seventy nanometers in the presence of hydrogen peroxide. The dehalogenase activity was measured by chloride liberation, according to the mercuric thiocyanate and ferric ammonium sulfate colorimetric method. The amount of enzyme that converts one micromole of substrate per minute was defined as one unit of activity; specific activity was expressed in terms of protein (Lowry method, using bovine serum albumin as standard).

3.7 Degradation Experiments

Two configurations were used for the evaluation of degradation. For the single-strain configuration, 100 mls of mineral salts media with 100 mg/l of the single target compound was inoculated with approximately ten to the power of seven (107) colony forming units (CFU) of each isolate. The six isolates selected were mixed together in equal numbers of cells and then introduced at the same cell density into the four compound medium at a concentration of one hundred mg/L per compound in consortium mode. All flasks were dark grown for thirty days at a temperature of thirty degrees Celsius and a shaking speed of one hundred and fifty revolutions per minute. Abiotic controls included heat-killed inoculum, which included volatilisation, photolysis and sorptive losses. The sacrificial flasks were removed in triplicate at 0, 5, 10, 15, 20, 25 and 30 days for residual compound determination, enzyme activity assay and viable counts.

3.8 Analytical Quantification and Kinetic Treatment

Triple liquid–liquid extraction of dichloromethane was used to recover the residual compounds from the whole culture broth, which was then dried on anhydrous sodium sulfate, concentrated and analysed by gas chromatography with mass spectrometric detection, under the conditions described for sediment residues. Degradation efficiency was determined as a percentage reduction of the abiotic control at the same sampling site, eliminating any losses due to non-biological factors. The half-lives were calculated from the first-order rate constant determined from the slope of the natural log of the residual concentration versus time.

3.9 Statistical Analysis

The treatments were repeated three times and all data are presented as means ± standard deviation. Isolate-to-isolate differences and sampling interval differences were examined using one-way analysis of variance (ANOVA) and Tukey honest significant difference (HSD) post hoc comparison tests, and a probability level of less than 0.05 was considered statistically significant. Pearson product-moment correlation was used to study the relationship between enzyme specific activity and degradation efficiency. The analyses were carried out by the conventional statistical packages.

IV. Results

4.1 Sediment Characteristics and Contaminant Burden

There was a significant difference in both texture and organic status of the three sediments. Site S1 sediment consisted of silty clay and was very low in organic carbon (2.8%) and had a pH of 7.4 and a redox potential of minus eighty-six millivolts, reflecting the reducing conditions below the thin surface toxic layer. The S2 sediment was very high in clay content and had the maximum organic carbon content of three-point six percent, pH of seven point one and a redox potential of minus one hundred and twenty-four millivolts. The S3 estuarine sediment was sandy loam with the lowest 1·0 per cent organic carbon, a marine buffered pH of seven point eight and a redox potential of minus forty-two millivolts. The highest concentrations of total extractable polycyclic aromatic hydrocarbons (TEP) were found at S1, the highest concentration of chlorophenol residues was found at S3 and the highest concentration of organochlorine pesticide residues was found at S2, which is consistent with the dominant land use in the surrounding area of each of these sites. Concentrations of residues were correlated with the organic carbon content for all three sediments, and this relationship was positive, as expected, indicating that organic matter is the most important factor controlling retention of residues.

4.2 Isolation and Molecular Identification

Forty-one morphologically different isolates were obtained from the three sites and four substrates sequentially. Nineteen of these gave measurable zones of clearing on at least one spray plate, eleven permitted 200 mg/l of the inducing substrate and six were satisfied with all the screening criteria on all four compounds. The six isolates chosen were labelled AS-01 to AS-06 and determined by 16S ribosomal RNA gene sequencing to be derivatives of Pseudomonas aeruginosa, Bacillus subtilis, Rhodococcus erythropolis,

Sphingomonas paucimobilis, Achromobacter xylosoxidans and Acinetobacter calcoaceticus respectively. Sequence identity to the nearest validly published relative was always greater than 98 percent and neighbour joining analysis grouped each isolate within the predicted generic clade with more than 90 percent bootstrap support. The two riverine isolates with a preference for aromatic hydrocarbons and the two estuarine isolates with a preference for chlorinated substrates were clustered by site of origin.

4.3 Enzyme Profiles and Single-Strain Degradation

The enzymatic characterization showed that the isolates were clearly divided into groups based on their functions and this is summarized in table 1. The highest specific activity of enzymes degrading phenanthrene was found in the two most effective degraders (AS-01 and AS-03): 4 point eight six and 5 point three four units per milligram protein respectively. The expression of catechol 1,2-dioxygenase was preferred by AS-02, suggesting the ortho cleavage pathway as the preferred option. Laccase activity was highest in AS-05 (6.45 units/mg protein) which was the highest single activity observed during the study, and was correlated to its highest activity against pentachlorophenol. AS-06 had manganese peroxidase as the dominant activity and had intermediate activity against both the chlorinated substrates. AS-04 had high level of haloalkane dehalogenase activity (2.07 units per mg of protein) which, while small on an absolute scale, was significant for the transformation of lindane, as none of the other isolates released comparable amounts of chloride. The individual degradation efficiencies ranged from 43.6% (AS-04 against lindane) to 81.4% (AS-01 against phenanthrene) after 30 days.

Table 1: Identity, Origin, Substrate Preference, Degradation Efficiency and Dominant Enzyme Activity of the Six Selected Sediment Isolates

Isolate code	Closest identification (16S rRNA gene)	Sampling site	Target compound	Degradation (%) at 30 d	Dominant enzyme and specific activity (U mg⁻¹ protein)
AS-01	*Pseudomonas aeruginosa* strain NSD-11	S1 – Noyyal River	Phenanthrene	81.4 ± 2.3	Catechol 2,3-dioxygenase (4.86 ± 0.21)
AS-02	*Bacillus subtilis* strain VKM-06	S1 – Noyyal River	Pyrene	64.7 ± 1.9	Catechol 1,2-dioxygenase (3.12 ± 0.18)
AS-03	*Rhodococcus erythropolis* strain MTS-19	S2 – Valankulam Lake	Phenanthrene, pyrene	70.2 ± 2.6	Catechol 2,3-dioxygenase (5.34 ± 0.27)
AS-04	*Sphingomonas paucimobilis* strain ETK-04	S2 – Valankulam Lake	Lindane	43.6 ± 1.7	Haloalkane dehalogenase (2.07 ± 0.11)
AS-05	*Achromobacter xylosoxidans* strain PKL-22	S3 – Muttukadu estuary	Pentachlorophenol	58.9 ± 2.1	Laccase (6.45 ± 0.31)
AS-06	*Acinetobacter calcoaceticus* strain SRB-08	S3 – Muttukadu estuary	Pentachlorophenol, lindane	49.3 ± 1.5	Manganese peroxidase (3.78 ± 0.22)

As shown in table 1, none of the isolates exhibited high activity in both classes of substrates (aromatic and chlorinated). Dioxygenase-dominant strains removed the most phenanthrene and pyrene and did not remove as well lindane and pentachlorophenol, whereas oxidoreductase-dominant and dehalogenase-dominant strains had the opposite characteristics. Values are the means of triplicate determination ± standard deviation.

4.4 Consortium Degradation Kinetics

Six-member defined consortium was superior in all isolations and in all substrates. This degradation could be recognised in three phases: a short lag (no significant depletion was observed during the first 2 days); a rapid phase (from day 5 to day 20) resulting in the removal of most of the contaminant; and a plateau phase (after day 20) in which the removal was close to the sorbed and bioavailability-limited fraction. The consortium had removed phenanthrene (93.4 %), pyrene (78.9 %), pentachlorophenol (71.2 %) and lindane (56.8 %) after 30 days. The entire time course is shown in figure 1.

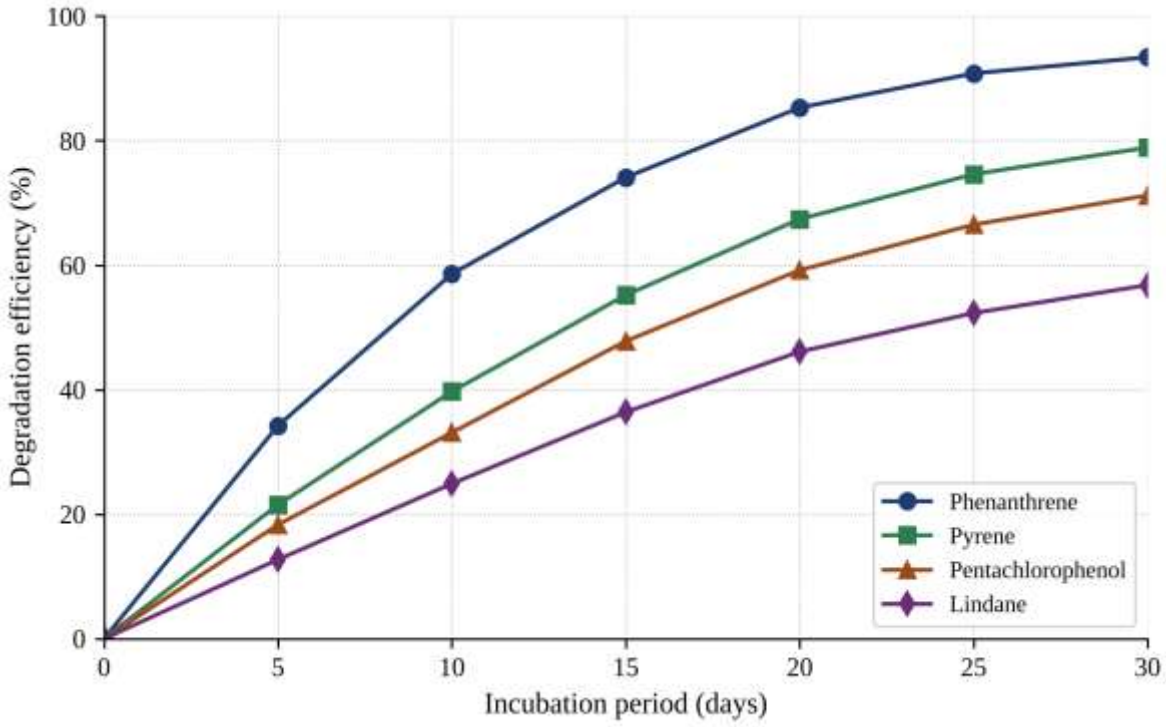


Figure 1: Time-Course Degradation Efficiency of Phenanthrene, Pyrene, Pentachlorophenol and Lindane by the Defined Six-Member Bacterial Consortium Over Thirty Days

The four compounds were all eliminated in a similar sequence during incubation (Figure 1): phenanthrene was the most rapidly removed and lindane was the slowest. The curves diverged in the rapid phase and converged after day 25, suggesting that the curves have been separated not because of differences in the viability of the inocula, but because of differences in the accessibility of the substrates and/or the enzymatic susceptibility. Derived first order rate constants were 0.091, 0.052, 0.041, and 0.028 day-1, respectively, for phenanthrene, pyrene, pentachlorophenol and lindane, and the derived half-lives were 7.6, 13.3, 16.9, and 24.8 days, respectively. Abiotic control losses for any compound did not exceed 6.2 percent for the entire incubation period.

4.5 Relationship Between Enzyme Activity and Degradation

The enzyme activity in the consortium increased from day two and reached its highest level at day 10–15, and then gradually decreased due to decreased amount of substrate. The peak total dioxygenase activity occurred about 5 days before the peak phenanthrene depletion, and the peak laccase activity occurred about 5 days before the peak pentachlorophenol depletion. The results showed strong positive Pearson correlation with thirty-day degradation efficiency between the peak specific enzyme activity and aromatic substrates, and moderately positive correlation with chlorinated substrates for all of the six isolates, which were all statistically significant. When we considered viable counts, increases were about

10 times more during the rapid phase and stable during the plateau, indicating that loss of degradation rate was due to limiting substrate and not to loss of viability. The differences of the isolates and sampling intervals were statistically significant by analysis of variance.

V. Discussion

The taxonomic identity of the six isolates selected is similar to genera that have been frequently isolated from contaminated sediments and soils as degraders of aromatic compounds. These bacteria, Pseudomonas, Rhodococcus, Sphingomonas and Acinetobacter, are known as the main culturable groups associated with the degradation of PAHs in sediments, and their success in all three of the sampled tropical sites suggests the chronic selection of bacteria in response to the presence of contaminants creates broadly convergent community outcomes regardless of climatic zone (Lu et al., 2011). That's all well and good, but the presence of Achromobacter and Bacillus in the selected strains extends that picture, and is consistent with the reviews that have been made that indicate that there is a tremendous diversity in the kinds of aromatics that can be degraded outside the classical model organisms (Ghosal et al., 2016). The most useful result of the enzymatic characterisation is the functional partitioning shown in table 1. Strains with a high percentage of ring-cleaving dioxygenase genes had high removal of phenanthrene and pyrene, but did not perform well with the chlorinated substrates, strains with a high percentage of laccase genes or manganese peroxidase genes had the opposite profile. This is true because of catalytic mechanism. Dioxygenases function by inserting oxygen electrophiles into an electron rich aromatic nucleus and are subject to a strict active site geometry, accounting for their efficiency with two- and three-ring structures which do not have electron-donating chlorines, and inability to react with highly chlorinated rings which lack such a donation and also provide steric hindrance (Mallick et al., 2011). The laccases, on the other hand, can abstract a single electron at a mononuclear type-1 copper center, resulting in the formation of diffusible radical intermediates, which imposes much lesser structural requirements for the substrate and can accommodate the exclusion of higher molecular weight aromatics and chlorophenols by dioxygenases. No isolate contained both activities at high levels, this was an observation and not a selection effect and provides the rationale for consortium-based, rather than single-strain, bioaugmentation. This advantage of the consortium over each of the individual strains also support a community-level understanding of sediment degradation, as related to other comparative analyses of lake and river deposits, where observed rates of attenuation were determined to be the result of concerted activity among functional groups of organisms, rather than any particular taxon (Yan et al., 2021). There are two possible mechanisms that are complementary in the current system. The first is metabolic cross-feeding in which ring-cleavage products produced by one strain are mineralised by another, thus avoiding the buildup of catechol and quinones that inhibit the upstream enzymes that produce them. The second involves partial dechlorination and ring attack; that is, dechlorination of pentachlorophenol and lindane to less substituted intermediates that are dioxygenase-competent and can be further attacked by dechlorinating dioxygenase. This interpretation is confirmed by the ordering of the degradation curves in figure.1. which show that the chlorinated compounds have the longest lag and the slowest rate constants as would be anticipated if they are degraded by a preparatory transformation step. These rate constants are in the range of those found in the laboratory for degradation of similar substrates by mixed cultures, and a relationship of inverse proportionality between rate constant and the combination of molecular weight, ring number and halogen substitution is reproduced throughout the polycyclic aromatic hydrocarbon literature (Premnath et al., 2021). Of special practical interest is the plateau that occurs after day twenty-five. The progressive lowering of degradation rates would, rather, be explained by progressive reduction in the bioavailable fraction of the enzyme than by a loss in catabolic capacity, as viable counts did not decline but remained constant, while enzyme activity did decrease but was still measurable. Sorption to organic matter and/or sequestration in micropores lead to the decrease of freely dissolved concentrations below critical levels for uptake and this bioavailability constraint is repeatedly found to be the most significant limitation on natural attenuation in deposits with high organic content (Haritash & Kaushik, 2009). For field use, this means that increasing the density of a given inoculum may not be the best way to improve the results, especially when measures that improve

desorption can be used, such as adding a biosurfactant and/or careful addition of the inoculum. The time lag between the peak enzyme activity and maximum substrate removal (around 5 days in the present system) suggest that in the initial stage of degradation enzyme induction rather than biomass development is the controlling factor. Usually, enzymes of aromatic catabolic pathways are substrate-inducible, and the lag time is the time needed for transcription, translation and assembly of functional, multi-component complexes. This research backs up the pre-induction of the inoculum on the target substrate before field introduction, a simple operational change that could have a plausible impact on the time to measurable remediation. An alternative approach is proposed by the work of enzyme engineering, as the extradiol dioxygenases have been modified by site-directed mutagenesis to expand substrate range and boost activity, and thus to achieve a more robust operation than the natural enzyme (Zhang et al., 2022). The study of newly isolated degraders is now continuing with investigations at the strain level, steadily adding to the list of candidate enzymes and providing a better understanding of the environmental conditions necessary to allow them to function optimally (Mai et al., 2021). These conclusions are subject to two caveats. However, experiments were carried out in aerobic liquid culture, while the surface of the sediments becomes anoxic within millimeters and anaerobic degradation takes place through different initial activation chemistry (e.g. carboxylation, hydroxylation or fumarate addition coupled with alternative terminal electron acceptors), at much lower rates than those reported here (Dhar et al., 2019). Liquid culture degradation efficiencies are thus a maximum value in intact sediment. In addition, no attempt was made to identify metabolites during the quantification of disappearance of the residue following digestion, so the extent of complete mineralisation (as opposed to the formation of partially oxidized compounds) has not been determined. Metabolite characterization with ecotoxicological bioassay is an essential step, as some intermediates such as quinones and chlorocatechols are more toxic than the parent compound. Regarding both these points, metagenomic approaches could provide a solution to help resolve both problems by linking community composition with the expressed functional genes under field-relevant conditions during degradation Hidalgo et al., (2020) and would improve the evidence that would justify the use of autochthonous consortia in sediment restoration (Patel et al., 2020).

VI. Conclusion

This study showed bacteria naturally occurring in polluted aquatic sediments have an enzyme repertoire that can transform structurally diverse persistent organic compounds under conditions that are representative of their natural environment. Sequential enrichment was used to isolate six isolates, from Pseudomonas, Bacillus, Rhodococcus, Sphingomonas, Achromobacter and Acinetobacter species, that exhibited complementary catabolic activities. In the case of phenanthrene and pyrene, ring-cleaving dioxygenases were the key enzymes, while laccase, manganese peroxidase and dehalogenases played a major role in the dechlorination and oxidation of pentachlorophenol and lindane. These activities are distributed across taxa, meaning that no one taxon or organism is responsible for the entire detoxification process, and that detoxification of sediment is a community-level process. A 6-member consortium always removed phenanthrene, pyrene, pentachlorophenol, and lindane at 93%, 79%, 71%, and 57% within 30 d, respectively, significantly higher than removal rates for individual strains; the first-order rate constant decreased with the addition of more hydrophobic and halogenic substitution. The time of maximum enzyme activity was about 5 days prior to the time of maximum substrate depletion, indicating that induction of the enzymes, not biomass accumulation, is the limiting factor for degradation in the early stages. The findings are directly relevant to sediment management in tropical aquatic systems where dredging and chemical oxidation costs are high and are very disruptive. Lower cost and more ecologically appropriate is the bioaugmentation with indigenous consortia with some nitrogen, phosphorus and surfactant biostimulation. Future research needs to measure the stability of the enzymes in these systems at varying salinity and redox conditions, confirm the toxicity of the metabolites using bioassays, and test the persistence of the consortium in mesocosm and field tests, prior to operational implementation in the restoration of degraded riverine and estuarine sediments.

Declarations

Funding: The authors declare that they have no competing interests in this research.

Conflicts of Interest: The authors state that they have no competing financial or personal interests.

Ethics Approval: This study did not include any human subjects or vertebrate animals and did not require ethical approval. Sediment was sampled complying with local regulations.

Data Availability: The data sets used and analysed during this study are available from the corresponding author upon reasonable request. The 16S ribosomal RNA gene sequences will be submitted to a public nucleotide sequence database.

Author Contributions: All authors participated in the study conception and design, material preparation, data collection and analysis. All authors read and approved the final manuscript.

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