Journal of Hazardous Materials 511 (2026) 142224

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Region-specific patterns of soil bacterial communities' adaptation to hexachlorocyclohexane contamination

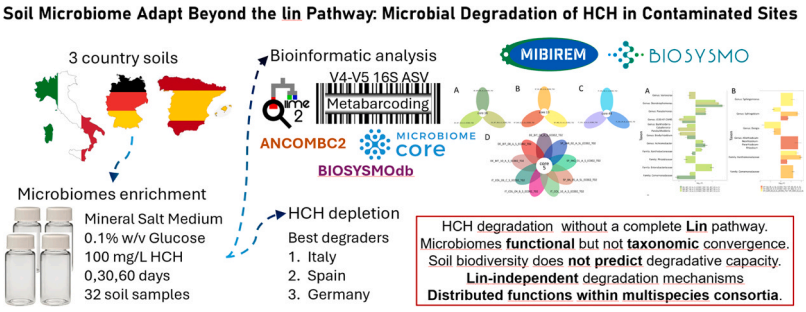
Giacomo Barnabei [a], Devaki Destri [a,b], Marta Franco-Benito [c], Miguel Cebrian-Aldana [c], Akanksha Mishra [c], Sara Gil-Guerrero [c], Jose Carlos Castilla-Alcantara [d], Blanca Velasco-Arroyo [d], Rocio Barros-Garcia [d], Riccardo Di Mambro [a], Nicola Bertelloni [a], Simona Di Gregorio [a,e,*]

[a] Department of Biology, University of Pisa, Via Luca Ghini 13, Pisa 56123, Italy
[b] BD Biodigressioni SRL, Lungarno Mediceo 40, Pisa 56127, Italy
[c] Biotechnology Division, IDENER.AI, Aeropolis Parque Tecnológico Aeroespacial de Andalucía, N24 Unit 8, C. Earle Ovington, Sevilla 41300, Spain
[d] International Research Centre in Critical Raw Materials for Advanced Industrial Technologies-ICCRAM, Universidad de Burgos, Plaza Misael Bañuelos s/n, Burgos 09001, Spain
[e] National Institute of Oceanography and Applied Geophysics, Via del Cedro, Livorno 57122, Italy

HIGHLIGHTS

- 64 bacterial consortia from DE, IT, ES were selected for HCH degradation capacity.
- All enrichments efficiently depleted all HCH isomers despite differing *lin* pathway.
- Suggested *lin*-independent pathway for non Sphingomonadacae taxa.
- Suggested HCH biodegradation by diverse taxa and *lin*-independent pathways.
- Bioremediation options expand via *lin* independent HCH degradation capacity.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Hexachlorocyclohexane (HCH)
Lin genes
Lin pathway
Stenotrophomonas
Pseudomonas
Achromobacter
Cupriavidus
Nocardioides
Pseudolabrys
Sphingobium

ABSTRACT

Hexachlorocyclohexane (HCH) is a persistent organochlorine pollutant whose attenuation at former production sites relies on microbial degradation. The canonical *lin* pathway, predominantly associated with Sphingomonadaceae, is considered the main aerobic route for HCH transformation, yet its environmental distribution remains limited. We investigated soil bacterial communities and enrichment-derived bacterial consortia from three historically contaminated sites in Germany, Italy, and Spain using HCH depletion assays, 16S rDNA metabarcoding, and functional inference, based on a curated BIOSYSMOdb dataset developed by the BIOSYSMO project. Based on the ASL-level functional inference, the Spanish samples uniquely encoded a complete *lin* pathway restricted to *Sphingobium* sp., whereas German and Italian communities harboured respectively partial (LinB–C) or single-step (LinB) modules. Despite these differences, efficient depletion of all HCH isomers occurred across all enrichment cultures. Core-microbiome and differential-abundance analyses identified several non-Sphingomonadaceae taxa, including *Stenotrophomonas*, *Pseudomonas*, *Achromobacter*, *Pseudolabrys*, and

* Corresponding author at: Department of Biology, University of Pisa, Via Luca Ghini 13, Pisa 56123, Italy.
E-mail address: simona.digregorio@unipi.it (S. Di Gregorio).

https://doi.org/10.1016/j.jhazmat.2026.142224
Received 6 December 2025; Received in revised form 24 April 2026; Accepted 26 April 2026
Available online 30 April 2026

Microbiome
lin-independent mechanisms

Cupriavidus, which consistently increased during selective enrichment and likely contribute to HCH depletion. Overall, our findings suggest that effective HCH degradation is not restricted to the canonical *lin* pathway nor to Sphingomonadaceae but it might be mediated also by diverse soil bacteria via alternative *lin*-independent mechanisms. These results broaden the known ecological and functional landscape of HCH biodegradation and support the exploration of non-Sphingomonadaceae taxa for bioremediation of legacy lindane-contaminated sites.

1. Introduction

Hexachlorocyclohexane (HCH) is a highly persistent and toxic organochlorine pesticide that was historically used on a large scale in agriculture and public health, primarily as technical-grade HCH (t-HCH) and its purified isomer, lindane (γ-HCH). Its extensive application has resulted in the accumulation of large quantities of residues in soils and groundwater worldwide, leading to severe and long-lasting contamination with significant ecological and human health risks [1,2]. HCH isomers are characterized by high hydrophobicity and chemical stability, properties that contribute to their environmental persistence and bioaccumulation in food webs [3,4].

Microbial communities inhabiting contaminated soils can play a crucial role in the natural attenuation of HCH through biodegradation. Aerobic lindane degradation is described as mediated by the *lin* catabolic pathway, a series of enzymatic reactions that progressively dechlorinate lindane, converting it into less toxic intermediates and ultimately mineralizing it to carbon dioxide and chloride ions [5–9]. The pathway comprises two major modules. The *upper pathway* module that includes LinA, a dehydrochlorinase initiating dechlorination; LinB, a haloalkane dehalogenase catalyzing hydrolytic dehalogenation of intermediates formed by LinA; and LinC, a dehydrogenase mediating subsequent oxidation steps [10,11]. The *lower pathway* module completes mineralization of the chlorinated intermediates generated upstream by LinA–LinC. Specifically, LinD (dehydrogenase) converts LinC products into chlorophenolic species, which are hydroxylated by LinE and LinF to yield chlorocatechols. These are subsequently cleaved and processed through LinG, LinH, LinI, and LinJ (chlorocatechol 1,2-dioxygenase–associated reactions), generating open-chain acids. Finally, LinK, LinL, LinM, and LinN catalyze reductive and rearrangement reactions that transform these intermediates into central metabolites (e.g., succinate and acetyl-CoA), achieving complete mineralization [7, 12–15] Evidence for the involvement of the *lin* pathway in the degradation of all HCH isomers, however, remains incomplete.

Several well-characterized HCH-degrading bacterial strains, such as *Sphingobium indicum* B90A, *Sphingobium japonicum* UT26, *Sphingobium francense* Sp+, and *Novosphingobium lindaniclasticum* LE124, carry *lin* genes and utilize this pathway [8,16,17]. These strains have been isolated from diverse HCH-contaminated sites worldwide, underscoring the ecological relevance of the *lin* pathway in HCH biodegradation. Even though the *lin* pathway has been reported within the family Sphingomonadaceae [18,19], several non-Sphingomonadaceae species have also been described as capable of degrading HCH [19–23], but the presence of the *lin* pathway in their genomes has not yet been confirmed. [19,24,25]

Given the central role of indigenous microbial communities in contaminant degradation and the potential for HCH attenuation through biodegradation, this study investigated the microbial ecology of three geographically distinct, historically contaminated t-HCH production sites located in Germany (Bitterfeld), Italy (Colleferro), and Spain (Sabiñánigo) respectively. All three sites hosted decommissioned production plants. The native bacterial populations potentially involved in HCH degradation were identified and characterized by 16S rDNA metabarcoding. Subsequently, a culturomics approach was adopted to recover and characterize bacterial consortia capable of HCH degradation. To maximize the likelihood of isolating effective degraders, up to ten soil samples from Germany, eleven from Spain and twelve from Italy were collected. An enrichment processes of HCH depleting bacterial consortia in presence of high concentration of HCH and an additional carbon source was adopted in parallel to the measurement of the kinetics of HCH depletion.

Initially, an investigation primarily aimed to identify bacterial taxa potentially harbouring the *lin* pathway was performed and a predictive profiling was adopted. The predictive profiling was based on the alignment of the bacterial consortia candidate proteomic sequences involved in HCH degradation to a curated protein dataset specifically developed by the BIOSYSMO project [26].

This dataset provides a reference framework for mapping candidate proteomes and identifying which functional steps of the *lin* pathway mediated HCH degradation are potentially encoded within each soil samples and derived bacterial consortia. By combining taxonomic identification with available genomic and proteomic information from the predicted candidate genomes, the approach allows confirmation of *lin* pathway associated protein sequences intrinsically linked to the detected taxa, contributing to a clearer taxonomic context for each soil area. Results obtained indicate that the Italian bacterial consortia putatively encoded only LinB, indicating a hydrolytic single-step configuration. German bacterial consortia putatively encoded LinB and LinC, supporting partial oxidative capability, while Spanish bacterial consortia putativelly retained the full LinA–F complement, representing the complete *lin* pathway, suggesting a discrete functional architecture among sites. The entire *lin* pathway was harboured only by the *Sphingobium* sp., whose presence was restricted to the Spanish soil samples and derived bacterial consortia. The HCH degradative capacity of the different bacterial consortia, even in the possible absence of the *lin* pathway, prompted a more detailed investigation of the most effective bacterial consortia in terms of their degradation performance. Three best performing bacterial consortia per site were selected for a comparative analysis of the core bacterial consortia, shared with their corresponding contaminated soils. This approach enabled the identification of taxa of potential relevance in HCH degradation, based on the assumption that taxa present in the original soils and persisting through the HCH degrading bacterial consortia enrichment process, particularly those increasing in abundance, represent strong candidates for HCH-degrading capability. Results demonstrated that at genus level *Stenotrophomonas*, *Pseudomonas*, and *Achromobacter*, increased significantly in abundance during the enrichment process of bacterial consortia deriving from the German site (Bitterfeld, DE); in the case of the Spanish site (Sabiñánigo, SP), *Sphingobium* and *Sphingomonas,* and of the Italian one (Colleferro, IT), *Pseudomonas*, *Pseudolabrys*, and *Cupriavidus* spp. increased in abundance during the enrichment process. Intermediates of degradation of HCH not referrable to the *lin* pathway have been identified. The involvement of an efficient, non *lin* pathway dependent HCH degradation capacity in non Sphingomonadaceae bacterial strains was proposed.

2. Materials and methods

2.1. Bacterial consortia enrichment and HCH depletion assays

Soil samples collected from the Germany, Bitterfeld (51°37'55.4"N 12°16'44.3"E), Italy, Colleferro (41°44'37.4"N 12°58'56.8"E), and Spain, Sabiñánigo (42°29'03.7"N 0°21'17.0"W) regions at dismissed industrial plants. The soil samples, deriving from historically contaminated sites,

were used as inocula in liquid culture, under selective medium conditions, to evaluate the degradation kinetics of HCH isomers, while concurrently enriching bacterial consortia able to grow under the imposed selective conditions. A total of 10 different soil samples were utilised from the German, 11 from the Spanish site, 12 from the Italian one. The HCH depletion kinetics of depletion were used as the criterion to select bacterial consortia with high degradation efficiency. A total of 576 sterile gas-tight 20-mL vials were prepared (6 vials per soil sample per time of analysis), each containing 5 mL of Minimum Salt Medium (MSM) (KH_2PO_4, 170 mg; Na_2HPO_4, 980 mg; $(NH4)_2SO_4$, 100 mg; $MgSO_4$, 4.87 mg; $FeSO_4$, 0.05 mg; $CaCO_3$, 0.2 mg; $ZnSO_4$, 0.08 mg; $CuSO4{\cdot}5\,H_2O$, 0.016 mg; $Na_2MoO_4{\cdot}2\,H_2O$, 0.05 mg; $MnSO_4$, 0.05 mg; H_3BO_3, 0.006 mg) supplemented with HCH at a final concentration of 100 $mg \bullet L^{-1}$ (α, β, γ, δ; 1:1:1:1) and glucose at a final concentration of 0.1% w/v, to facilitate potential co-metabolic processes during the degradation. In fact, these observations are validated by the ones seen in previous studies, that have shown that co-metabolism between glucose and HCH contributed to the capacities of soil microbial consortia to deplete all the HCH isomers [27]. Two grams of previously homogenised soil sample were sieved under 2 mm. The sieved soil was added to 30 mL of physiological solution (sterile deionized water with 0.9% w/v NaCl). The resulting suspension was sonicated in a water-bath sonicator (Elmasonic P70H, Elma Schmidbauer GmbH, Germany) at 37 kHz, 37 °C, and 100% power for 5 min 100 μL of the soils suspensions were added to the 567 vials containing 5 mL of MSM with HCH and glucose (0.1% w/v) to achieve an inoculum ratio of 2% v/v. As negative controls, three vials per time point were prepared containing MSM, 0.1% w/v glucose, and HCH at 100 $mg \bullet L^{-1}$, without soil inoculum, and analysed at the corresponding time points for residual HCH quantification. In addition, to confirm the absence of possible biotic removal in control vials, 100 μL from each vials were plated on LB agar plates at the time points of analysis to assess absence of microbial growth. LB is a complex (undefined) nutrient-rich medium that supports the growth of a broad range of culturable bacteria. Evan though not exhaustive supported the absence of not specific microbial growth in the control vials. All the vials (576 plus 9 control vials) were incubated at 25 °C ± 1 °C in the dark with orbital shaking at 130 rpm. Six biological replicates were prepared for each sample and for each time point. Every 15 days, the vials were opened and aerated under a sterile air flow (0.8 $NL \bullet min^{-1}$ for 10 s). Sampling times to evaluate HCH depletion kinetics were at time zero, and after 30 and 60 days of incubation.

2.2. Measurement of HCH depletion

All soil samples used in this study were analysed by AGROLAB Italia S.r.l. (Via Retrone 29/31, 36077 Altavilla Vicentina, VI, Italy) to determine the concentrations of all HCH isomers, the certified laboratory report result with a RSD% of 7–15%. Each liquid sample was analysed internally by GC-FID. The vials, inoculated or not inoculated with soil samples, were extracted with an equal volume of n-hexane containing 248 $\mu L \bullet L^{-1}$ of fluorene as an internal standard. The mixture was vortex-emulsified for 2 min, after which an aliquot of the non-aqueous phase was collected, treated by adding 0.1 g of anhydrous Na_2SO_4, allowed to settle for 2 min in the dark at room temperature to remove residual water, and analysed as follows: 1 μL of the extract was injected into an Agilent 7890B gas chromatograph (GC) equipped with a flame ionization detector (FID) and a 20 m column (0.18 mm i.d., 0.18 μm film) coated with a non-polar stationary phase containing 5% diphenyl-polysiloxane (HP-5MS, Agilent). Instrument settings were splitless injector mode, constant injector temperature 280 °C, septum purge flow 3 $mL \bullet min^{-1}$, inlet purge flow 40 $mL \bullet min^{-1}$ after 1 min; isocratic carrier gas (helium) flow 0.8 $mL \bullet min^{-1}$. The oven temperature program was: 80 °C for 2 min; ramp to 100 °C at 20℃ $\bullet$ min^{-1}, hold 1 min; ramp to 240 °C at 27.5℃ $\bullet$ min^{-1}, no hold; ramp to 250 °C at 14℃ $\bullet$ min^{-1}, hold 4 min. The total run time was 14 min. Detector settings were: filament voltage 1.8 V, delay 240 s, mass range 35–500 m/z, and acquisition rate 12 Hz. Transfer line temperature was 280 °C, source temperature 280 °C, and ionization potential −70 eV. Each extract was injected twice into the GC-FID, yielding three biological and three technical replicates per sample analyzed. For GC-FID calibration, dilutions were prepared from an HCH stock solution at 0.46 $g \bullet L^{-1}$ (α, β, γ, δ; 1:1:1:1; Sigma-Aldrich) dissolved in acetone. A calibration curve was generated by interpolating signals from five serial dilutions spanning 0–100 $mg \bullet L^{-1}$ per isomer. Calibration curves were fitted with a free intercept; an instrument blank containing hexane only was used to detect possible contamination. The R^2 values for the α-, β-, γ-, and δ-isomers were 0.999, 0.995, 0.986, and 0.999, respectively. From a fluorene stock solution at 1.02 $g \bullet L^{-1}$ in hexane, a spike was added to the extraction solvent to obtain a final concentration of 248 $\mu g \bullet L^{-1}$ fluorene in each sample, used as the internal standard. Chromatographic peak integration was performed by normalizing the baseline-integrated area of each analyte peak to the chromatographic peak area of the internal standard, thereby yielding four normalized areas, one for each isomer analysed. Post hoc comparisons use Dunn's test with Benjamini–Hochberg correction for multiple comparison (nparLD v2.2, rstatix v0.7.2) [28].

2.3. Identification of HCH metabolites of degradation

The identification of HCH metabolites of the degradation process by the different bacterial consortia was attempted by chromatographic analyses using an Agilent 7890B GC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a Bench-TOF time-of-flight mass analyzer (Markes International Ltd., Llantrisant, United Kingdom). Helium was used as the carrier gas. Injections were carried out in split mode: a split ratio of 1:20 was used for the quantification of analytes, while a split ratio of 1:2 was employed for the detection of metabolites. The installed capillary column was a DB-5MS (30 m × 0.25 mm i.d. × 0.25 μm film thickness) provided by Agilent Technologies. The temperature program was as follows: the initial temperature of 80 °C was held for 1 min, then increased to 280 °C at a rate of 10 °C/min. The Bench-TOF ionization source was set to −70 eV, and the transfer line temperature was maintained at 250 °C [29–31] Data acquisition was performed in Total Ion Current (TIC) mode to obtain a full scan of the ions generated during fragmentation. Metabolite identification was carried out using ChromSpace software dedicated to the integrated management of chromatographic and mass spectrometry data. Each significant signal detected in the chromatogram was analyzed by means of the mass spectrum associated with the corresponding peak and compared against the EPA-NIST-NIH spectral library installed in the system, based on the percentage of spectral overlap (match). Each spectrum was additionally subjected to manual verification, the expected fragmentation patterns were evaluated using, as the primary discriminating criterion, the presence of the characteristic isotopic pattern of chlorine: an element naturally occurring as ^{35}Cl (76%) and ^{37}Cl (24%), which generates characteristic signals in the mass spectrum (e.g., $[M]^+$, $[M+2]^+$, and $[M+4]^+$), thus representing a distinctive marker for the recognition of chlorinated compounds and their transformation products. In relation to the extraction procedure, each sample was taken from the freezer (−21 °C) and left to thaw at room temperature. A solution of fluorene in acetone was prepared at a concentration of 1000 ppm, and each sample was spiked with fluorene at 100 ppm; samples were then vortexed for 1 min to ensure homogeneity. A dispersive liquid-liquid extraction was adopted. 5 mL of n-hexane [31, 32] was added as extraction solvent and 1 g of NaCl was added to induce the "salting out" phenomenon of the analytes, to facilitate the process. After vortexing for 1 min, the sample was set aside to await proper phase separation. Once the correct separation between the aqueous and organic layers was achieved, 200 μL of the supernatant were collected and transferred into a 250 μL vial containing activated anhydrous

sodium sulfate, to completely dehydrate the sample.

2.4. Metagenomic DNA extraction

For each soil sample and each enrichment-derived bacterial consortia, three replicates were analyzed, for a total of 93 soil samples and 27 enrichment samples. Soil samples were manually homogenized, and 500 mg aliquots were collected. For bacterial consortia samples obtained from liquid cultures, 100 mg of cell pellet were collected. The cell pellet was obtained by serial centrifugation at 14,000 × g for 5 min until the entire 5 mL sample had been processed. Genomic DNA of soil and cell pellet samples was extracted using the FastDNA™ SPIN Kit for Soil (MP Biomedicals) and the FastPrep Instrument (MP Biomedicals), following the manufacturer's instructions. DNA integrity was evaluated by electrophoresis on a 1% agarose gel. DNA quantity was measured with a Qubit® 3.0 fluorometer (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's instructions. Sample purity was assessed by the 260/280 nm and 260/230 nm absorbance ratios.

2.5. 16S rDNA metabarcoding

Illumina sequencing libraries were prepared by Novogene (Novogene Company Limited, Rm. 19 C, Lockhart Ctr., 301–307 Lockhart Rd., Wan Chai, Hong Kong). Bacteria were identified by specific amplification of the V4–V5 hypervariable regions of the 16S rRNA gene using the forward primer 515 F (5′-GTGCCAGCMGCCGCGGTAA-3′) and the reverse primer 907 R (5′-CCGTCAATTCCTTTGAGTTT-3′). For both soil and cell-pellet samples, a total of 200 ng DNA was used to generate the metagenomic libraries. Samples were sequenced in triplicate on an Illumina NovaSeq 6000 platform with 250 bp paired end reads.

2.6. Bioinformatic analysis

Paired-end 250 bp reads from 16S libraries were demultiplexed by sample-specific barcodes and trimmed with Cutadapt v4.6 [33]. Forward and reverse reads were merged, filtered (minimum Phred $\geq$ 10), and screened for chimeras. Amplicon sequence variants (ASVs) were inferred using DADA2 v1.26 within QIIME2 v2023.2 [34–36]. Taxonomy was assigned with RESCRIPt v2023.2.0 in QIIME2 [37], using a classifier trained on the corresponding hypervariable region from the SILVA 138 99% 16S reference database. ASV tables were normalized by coverage-based standardization (0.9950 ± 0.0025) implemented with iNEXT v3.0.2. [38] α-Diversity was quantified using the Hill–Simpson and Hill–Shannon indices and the Chao1 richness estimator (metagMisc_R v0.5.0) [39–41]. Statistical significance was evaluated by Kruskal–Wallis test by ranks followed by Dunn's post-hoc tests (nparLD v2.2, rstatix v0.7.2) [28]. Rarefaction curves of observed species were generated at the specified coverage threshold.

The core microbiome for each microbial community was inferred from the complete ASV dataset through a parameterised filtering workflows with specific stringency, aligned to the specific analytical objective. Analyses were conceived as exploratory and hypothesis generating, relying on ASV based taxonomic assignments to inform downstream functional interpretation rather than direct functional inference from marker gene data. All statistical analyses were performed in R (v4.3.1) via RStudio and Pyhton (v3.12.3) via Pandas (v2.3.3) Bio.SeqIO (v1.85). For community composition analyses (diversity and abundance metrics), we applied a high-stringency criterion that retained only ASVs meeting both of the following requirements: (i) a prevalence $\geq$ 50% (detected in at least half of the biological triplicates) and (ii) a minimum detection threshold of 0.1% relative abundance. ASVs falling below this limit were classified as ultra-low-frequency taxa and excluded due to their limited representativeness of the core microbiome (microbiome v1.24.0). Shared taxa across samples were visualized with flower plots generated using the EVenn online tool, which summarizes set relationships [42]. Differential abundances between soil-derived bacterial communities and enriched bacterial consortia were assessed with ANCOM-BC2 (ANCOMBC v3.18). A less stringent filtering scheme was implemented to assess the functional potential of the microbial ecology of the soil samples and of the bacterial consortia. This approach employed a relative abundance threshold of $\geq$ 0.2% [43,44] and required ASVs to be present in at least 20% [45] of the samples, ensuring the retention of functionally relevant but potentially less dominant taxa. The filtered ASVs were compared against the NCBI nucleotide database [46] using the BLASTn algorithm [47] to infer potential genome-level affiliations. To explore potential functional traits related to pollutant degradation, exploration of functional traits related to pollutant degradation was therefore grounded on taxonomic affiliations inferred from 16S rDNA ASVs and their association with the proteomic sequences of the candidate genomes, instead of the detection of functional genes, providing putative, overall community-level assessment of the contribution of each consortium to HCH degradation at each site. The strength of this approach was that the retrieved protein datasets associated with each candidate genome were aligned against a curated subset of the BIOSYSMOdb v1.0 [26] specifically focused on lindane degradation pathways in combination with UniProt specific annotation entries (available in Supplementary Information) [26,48,49]. BLASTp searches were conducted using predefined similarity thresholds selected to balance sensitivity and specificity in a screening-oriented context (minimunm) identity of 60%, e-value $\leq$ 1e−5, and minimum query coverage of 80%) applied uniformly across all samples to ensure methodological consistency across the different sites. Although, the identified *lin* gene potential homologues were therefore considered putative, they are indicative of potential functional affiliation, which in future studies will be tested further for evidence of confirmed enzymatic activity or pathway completeness. The rationale and parameterisation of the filtering workflows is described in detail in the Supplementary Information (SF1 and SF2)

3. Results

3.1. Soil sample chemical characterization

The HCH contamination levels in the soil samples collected at the three sites are presented in Table 1. The data were the results of Agrolab lab's quantification (Agrolab group, Italia). Soils from Bitterfeld (Germany, DE) show uniformly quite elevated α-HCH.; notably, DE_BIT_03_A_S, DE_BIT_04_A_S, DE_BIT_08_A_S, and DE_BIT_10_A_S contain 2000, 7500, 204, and 310 $mg \bullet kg^{-1}$, respectively. β-HCH is likewise elevated in several DE samples—DE_BIT_04_A_S (135 $mg \bullet kg^{-1}$), DE_BIT_10_A_S (93 $mg \bullet kg^{-1}$), DE_BIT_03_A_S (77 $mg \bullet kg^{-1}$), and DE_BIT_08_A_S (74 $mg \bullet kg^{-1}$).

These four are the most contaminated German soils, also exhibiting the highest δ- and γ-HCH values (DE_BIT_04_A_S: 110 $mg \bullet kg^{-1}$ δ-HCH; 109 $mg \bullet kg^{-1}$ γ-HCH).

By contrast, soils from Colleferro (Italy, IT) display lower total HCH contamination. The α-HCH is comparatively high in IT_COL_02_B_S (58.7 $mg \bullet kg^{-1}$), IT_COL_04_B_S (45 $mg \bullet kg^{-1}$), and IT_COL_08_B_S (23.4 $mg \bullet kg^{-1}$). The β-HCH peaks at 305 $mg \bullet kg^{-1}$ in IT_COL_04_B_S, which is also among the highest total HCH concentrations (Σ-HCH = 355.3 $mg \bullet kg^{-1}$). The highest δ-HCH among Italian samples occurs in IT_COL_07_B_S (10 $mg \bullet kg^{-1}$), while γ-HCH reaches 8.5 $mg \bullet kg^{-1}$ in IT_COL_10_A_S. In Sabiñánigo (Spain, SP), contamination levels vary widely. The most contaminated sample, SP_SAR_02_A_SL, contains 1 750 $mg \bullet kg^{-1}$ α-HCH, 85 $mg \bullet kg^{-1}$ β-HCH, 3 700 $mg \bullet kg^{-1}$ γ-HCH, and 2 090 $mg \bullet kg^{-1}$ δ-HCH, yielding a total of 7 625 $mg \bullet kg^{-1}$ Σ-HCH. Other Spanish samples show much lower levels: for instance, SP_SAR_03_A_S has 4.5 $mg \bullet kg^{-1}$ α-HCH, 26 $mg \bullet kg^{-1}$ β-HCH, 0.48 $mg \bullet kg^{-1}$ γ-HCH, and 0.77 $mg \bullet kg^{-1}$ δ-HCH (Σ = 31.8 $mg \bullet kg^{-1}$); while SP_INQ_01_A_S records 0.2 $mg \bullet kg^{-1}$ α-HCH, 2.45 $mg \bullet kg^{-1}$ β-HCH, 0.027 $mg \bullet kg^{-1}$

Table 1
The soils concentration of each HCH-isomers is reported as $mg \bullet kg^{-1}$. RSD (7–15%).

Country	Samples	α-HCH	β-HCH	γ-HCH	δ-HCH	Σ-HCH
Spain	SP_BA_01_A_S	0,0029	0,00191	0,0027	0,01	0,01751
Spain	SP_BA_02_A_S	0,073	0,084	0,077	0,085	0,319
Spain	SP_BA_03_A_S	0,146	0,0077	0,161	0,3	0,6147
Spain	SP_BA_04_A_S	0,0199	0,021	0,023	0,165	0,2289
Spain	SP_BA_05_A_SL	0,31	0,92	0,0159	0,034	1,2799
Spain	SP_INQ_01_A_S	0,2	2,45	0,027	0,078	2755
Spain	SP_INQ_02_A_S	0,0098	0,3	0,0093	0,036	0,3551
Spain	SP_SAR_01_A_S	0,207	0,173	0,145	0,53	1055
Spain	SP_SAR_02_A_SL	1750	85	3700	2090	7625
Spain	SP_SAR_03_A_S	4,5	26	0,48	0,77	31,75
Germany	DE_BIT_01_A_S	0,62	0,95	0,0084	0,01	1,5884
Germany	DE_BIT_02_A_S	1,43	2,5	0,0097	0,0117	3,9514
Germany	DE_BIT_03_A_S	2000	77	24,7	9,5	2111,2
Germany	DE_BIT_04_A_S	7500	135	109	110	7854
Germany	DE_BIT_05_A_S	0,167	0,19	0,001	0,01	0,368
Germany	DE_BIT_06_A_S	3,5	1,59	0,025	0,01	5125
Germany	DE_BIT_07_A_S	0,92	1,08	0,0154	0,027	2,0424
Germany	DE_BIT_08_A_S	204	74	3,5	1,44	282,94
Germany	DE_BIT_09_A_S	13,9	41	0,144	0,239	55,283
Germany	DE_BIT_10_A_S	310	93	4	2,31	409,31
Italy	IT_COL_01_C_S	6,7	0,0617	0,452	0,01	7,2237
Italy	IT_COL_02_B_S	58,7	0,75	4,86	0,099	64,409
Italy	IT_COL_03_C_S	0,594	0,505	0,0612	0,83	1,9902
Italy	IT_COL_04_B_S	45	305	0,103	5,2	355,303
Italy	IT_COL_05_C_S	10,7	1,16	0,75	4,7	17,31
Italy	IT_COL_06_C_S	0,0014	0,0018	0,0019	0,01	0,0151
Italy	IT_COL_07_B_S	18,9	16,7	2,24	10	47,84
Italy	IT_COL_08_B_S	23,4	4,18	0,386	2,41	30,376
Italy	IT_COL_09_A_S	0,0259	0,0092	0,094	0,027	0,1561
Italy	IT_COL_10_A_S	0,067	0,132	8,5	1,25	9949
Italy	IT_COL_11_C_S	0,0023	0,003	0,0031	0,01	0,0184
Italy	IT_COL_12_C_S	0,79	0,14	0,087	0,04	1057

γ-HCH, and 0.078 $mg \bullet kg^{-1}$ δ-HCH (Σ = 2.76 $mg \bullet kg^{-1}$). Across all soils, maximum α-HCH occurs in DE_BIT_04_A_S (7 500 $mg \bullet kg^{-1}$), The β-HCH peaks in IT_COL_04_B_S (305 $mg \bullet kg^{-1}$), and δ-/γ-HCH attain their maxima in SP_SAR_02_A_SL (2 090 $mg \bullet kg^{-1}$ δ-HCH; 3 700 $mg \bullet kg^{-1}$ γ-HCH).

3.2. Enrichment of HCH degrading bacterial consortia from the three contaminated sites

The enriched bacterial consortia kinetics of HCH depletion were determined during their enrichment process to distinguish the bacterial consortia on the base of their efficiency in the depletion of all the different HCH isomers (Fig. 1). HCH isomer concentrations were quantified at the setup of the experimentation (day 0) and after 30 and 60 days of incubation. Fig. 1, panel A shows the HCH isomers concentrations over time of incubation for Bitterfeld bacterial consortia (Germany, DE). Any bacterial consortia reduced α-, δ-, or γ-HCH concentrations to below 10 $mg \bullet L^{-1}$by day 60, whereas several achieved levels as low as 5 $mg \bullet L^{-1}$for β-HCH. The most effective bacterial consortia were DE_BIT_03_A_S_EC002, DE_BIT_08_A_S_EC002, and DE_BIT_10_A_S_EC002, which also performed better than others across the other isomers. Notably, DE_BIT_08_A_S_EC002 showed the lowest residual α-HCH concentration (16.49 $mg \bullet L^{-1}$) by day 60, followed by DE_BIT_03_A_S_EC002 (18.83 $mg \bullet L^{-1}$) and DE_BIT_10_A_S_EC002 (17.98 $mg \bullet L^{-1}$). For β-HCH, the same consortia exhibited final concentrations of 11.99, 14.61, and 8.66 $mg \bullet L^{-1}$, respectively. Residual γ-HCH concentrations were 18.94 $mg \bullet L^{-1}$ for DE_BIT_08_A_S_EC002, 20.43 $mg \bullet L^{-1}$ for DE_BIT_03_A_S_EC002, and 19.45 $mg \bullet L^{-1}$ for DE_BIT_10_A_S_EC002, while δ-HCH remained at 18.25, 18.72, and 17.93 $mg \bullet L^{-1}$, respectively. Overall, the total HCH residuals at day 60 were 65.69, 72.58, and 63.90 $mg \bullet L^{-1}$.

Fig. 1, panel B summarizes HCH depletion by bacterial consortia enriched from Sabiñánigo soils (Spain, SP). Depletion patterns varied across consortia. The most efficient were SP_BA_01_A_S_EC002, SP_BA_05_A_SL_EC002, SP_INQ_01_A_S_EC002, SP_SAR_02_A_SL_EC002, and SP_SAR_03_A_SL_EC002. By day 60, these exhibited the lowest residual concentrations for α-HCH (ranging from 0.08 to 0.47 $mg \bullet L^{-1}$) and γ-HCH (0.32–0.37 $mg \bullet L^{-1}$). For δ-HCH, SP_BA_05_A_SL_EC002, SP_INQ_01_A_S_EC002, and SP_SAR_02_A_SL_EC002 reached values close to 0 $mg \bullet L^{-1}$ (complete depletion within measurement error), while SP_SAR_03_A_SL_EC002 remained at 0.72 $mg \bullet L^{-1}$ and SP_BA_01_A_S_EC002 at 4.84 $mg \bullet L^{-1}$. The β-HCH residual concentrations ranged between 4.27 and 13.66 $mg \bullet L^{-1}$, with the lowest levels again in SP_BA_05_A_SL_EC002 and SP_SAR_02_A_SL_EC002. Three consortia, the—SP_BA_05_A_SL_EC002, SP_SAR_02_A_SL_EC002, and SP_INQ_01_A_S_EC002—displayed mean total HCH residuals < 8 $mg \bullet L^{-1}$ after 60 days.

Fig. 1, panel C shows HCH isomer depletion by bacterial consortia isolated from Colleferro soils (Italy, IT). All Colleferro consortia displayed similar degradation patterns, with almost complete removal of α-, γ-, and δ-HCH. Residual α-HCH concentrations ranged from 0.26 to 0.69 $mg \bullet L^{-1}$ (average 0.41 $mg \bullet L^{-1}$), γ-HCH from 0.44 to 1.21 $mg \bullet L^{-1}$ (average 0.77 $mg \bullet L^{-1}$), and δ-HCH from 0.52 to 1.47 $mg \bullet L^{-1}$ (average 1.04 $mg \bullet L^{-1}$). β-HCH showed higher persistence, with residual values between 3.36 and 8.01 $mg \bullet L^{-1}$ (average 5.23 $mg \bullet L^{-1}$). The identified metabolites, in all the microbial consortia analyzed, were γ-pentachlorocyclohexene (Table S2, panel A), belonging the *lin* pathway. The intermediates of degradation δ-3,4,5,6-tetrachlorocyclohexene (Table S2, panel B) and 1,2,4-trichlorobenzene (Table S2, panel C), not belonging to the *lin* pathway, were also identified in all the microbial consortia analyzed. The reported intermediates were not detected in the control extracts.

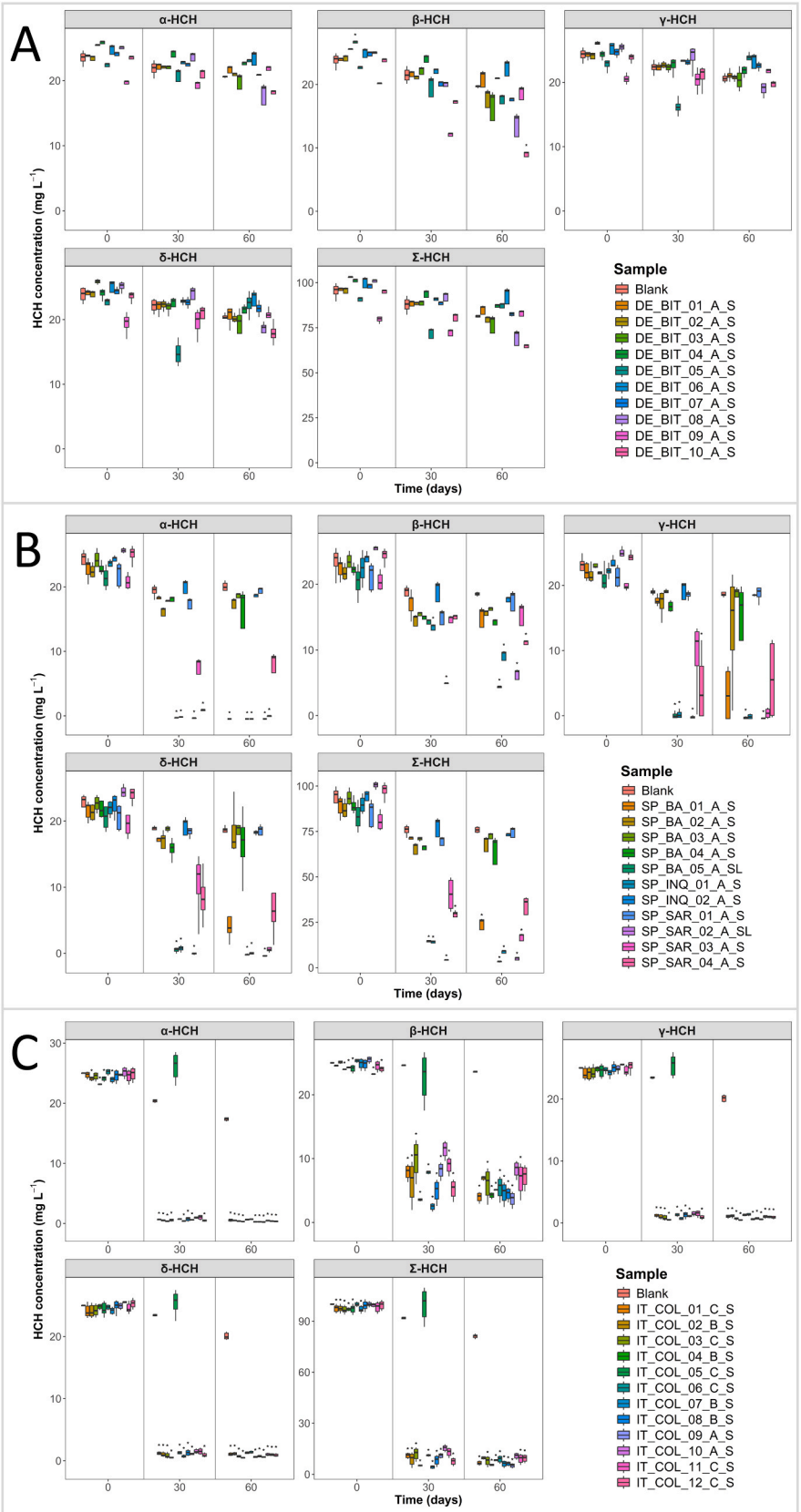


Fig. 1. Boxplots of α-, β-, γ-, and δ-HCH isomer concentrations, and their sum, measured at the enrichment protocol time points. The figure reports data for selected samples from Bitterfeld (Panel A), Sabiñánigo (Panel B), and Colleferro (Panel C) at setup (Day 0), Day 30, and Day 60. Post hoc comparisons use Dunn's test with Benjamini–Hochberg correction for multiple testing. Asterisks above the boxplots denote statistically significant differences ($p < 0.05$) between each sample and the control (Blank). All remaining pairwise comparisons are provided in the Supplementary Material for brevity and ease of interpretation (S3 for Germany, S4 for Italy and S5 for Spain).

3.3. Functional inference of the lin pathway of the three site soil samples and derived bacterial consortia

To assess a putative site-specific presence of the *lin* pathway as harboured by the bacterial communities of the soil samples and its stability during the enrichment process of the different bacterial consortia, metagenome-resolved inference was performed at the ASV level following the filtering criteria described in Supplementary materials (SF1) providing an indirect, community-level view of *lin*-associated functional potential. The inference aimed to minimize stochastic noise and retain ecologically stable variants for downstream functional profiling related to the identification of taxa potentially harbouring *lin* genes. The resulting datasets, as well as detailed counts of retained ASVs, candidate genomes, and protein sequences for each country, are reported in the Supplementary materials (SF2). From the German soil samples with no enrichment conditions, four ASVs were associated with ten candidates' genomes containing *lin* gene homologues, yielding eighteen protein hits assigned to LinB (haloalkane dehalogenase) and LinC (2,5-DDOL dehydrogenase). These ASVs were associated to *Mycobacterium*, *Bradyrhizobium*, *Kribella*, *Streptomyces* spp. The recovered enzymes correspond to the upper and intermediate modules of the *lin* pathway and collectively support hydrolytic and oxidative degradation potential. No hits were detected for the lower pathway enzymes (*LinD–LinF*), suggesting incomplete catabolic capacity by the *lin* pathway at the community level. Under enrichment conditions, only three bacterial consortia the DE_BIT_03_A_S_EC002_T02, DE_BIT_08_A_S_EC002_T02 and DE_BIT_10_A_S_EC002_T02 were associated with only one candidate genome, belonging to *Bradyrhizobium* sp., encoding only LinB hits, indicating a functional contraction during the enrichment process and the loss of oxidative capacity associated with LinC. In the Italian soils, three ASVs were linked to four candidate genomes. The taxonomical characterisation indicated *Variovorax*, *Bradyrhizobium*, *Mehtylorubrum*, *Geothrix* spp. encoding for *lin*B homologous genes, with no other *lin* genes detected. This configuration restricts the soil activity to the initial hydrolytic dehalogenation step of the pathway. All the enriched bacterial consortia showed a similar pattern, where four ASVs, belonging to *Variovorax*, *Methylorubrum, Geothrix and Bradyrhizobium* genera, produced six candidate genomes and six *lin*B hit, suggesting the conservation of the encoding sequence for *lin*B during the enrichment process. Spanish soils yielded five candidate genomes associated with three ASV taxonomically affiliated with *Sphingobium* sp., *Bradyrhizobium sp.* and *Kribbella sp*. These candidate genomes collectively encode twenty-five protein hits, spanning multiple gene families, including LinA, LinB, LinC, and LinF. In contrast to the German and Italian this configuration spans all major functional modules of the HCH degradation pathway from the initial dehydrochlorination (*linA*) through the hydrolytic and dehydrogenative steps (*linB–C*), to the final ring-cleavage (*linF*). Notably the same pattern was retained in the enriched bacterial consortia, suggesting potential conservation of the *lin* pathway during the enrichment process, rather than a contraction toward partial pathway components. Overall, these patterns point to distinct site specific configurations of *lin*-related functional potential across regions, which are interpreted as indicative architectures at the bacterial taxonomical distribution scale rather than strain-resolved complete pathways association.

3.4. Microbial ecology of soils and derived HCH depleting bacterial consortia

To tentatively depict the bacterial taxa responsible for the observed kinetics of HCH depletion, not necessarily restricted to the presence of the intact *lin* pathway, three better performing bacterial consortiafor site were selected for better characterisation by downstream analysis. For the Italian site, all consortia showed high degradation performance thus to ensure site representativeness, IT_COL_03_C_S_EC002_T02, IT_COL_04_B_S_EC002_T02, and IT_COL_10_A_S_EC002_T02 were selected, as they originated from distinct sampling areas. For the Spanish site, the chosen consortia were SP_BA_05_A_SL_EC002, SP_INQ_01_A_S_EC002, and SP_SAR_02_A_SL_EC002. For the German site the DE_BIT_09_A_S, DE_BIT_05_A_S and DE_BIT_05_A_S.

Fig. 2 shows the α-diversity profiles of the selected bacterial consortia. Based on Chao1 richness, the German (DE) and Spanish (SP) bacterial consortia exhibit higher richness than those from Colleferro (IT). An exception is SP_SAR_02_A_SL, which departs from the Spanish trend and resembles the Italian ones. Hill–Simpson evenness indicates that Italian bacterial consortia are less even, except IT_COL_10_A_S, whose evenness is comparable to SP_BA_05_A_SL. Within the Spanish bacterial consortia, SP_SAR_02_A_SL also shows low evenness, whereas the German bacterial consortia are more heterogeneous, with DE_BIT_05_A_S exhibiting the highest evenness. Consistent with these patterns, the Hill–Shannon index, which integrates richness and evenness, shows that the Italian bacterial consortia are, on average, less diverse and less evenly distributed than those from German and Spanish one.

Comparing the three soil α-diversity with that of the deriving bacterial consortia for each region it is evident that the selective growth conditions during enrichment markedly shaped the bacterial consortia ecology (Fig. 3). For the German site (Fig. 3, Panel A) the selected consortia, DE_BIT_03_A_S_EC002_T02, DE_BIT_04_A_S_EC002_T02, and DE_BIT_08_A_S_EC002_T02, display Chao1 values far below the soil averages one, indicating reduced richness. The strong selective pressure of the enrichment procedure narrowed the taxonomic breadth, lowering diversity. Evenness likewise dropped substantially, a trend corroborated by the Hill–Shannon index. Statistical analysis revealed significant differences in all indices between selected bacterial consortia and soils, whereas diversity among the soil themselves did not differ significantly. Notably, soil Chao1 values are up to three orders of magnitude higher than those of the bacterial consortia (near zero). In relation to evenness, the Hill–Simpson declines from values on the order of hundreds in soils to near zero in bacterial consortia. Overall, enrichment led to a pronounced decrease in both richness and evenness relative to the source soils.

For the Spanish site (Fig. 3, Panel B) the selected consortia exhibit marked reductions in both richness and evenness. Index values for the selected consortia approach zero, whereas the soils show Chao1 ~2000 and Hill–Simpson on the order of hundreds. The contrast is less pronounced for SP_SAR_02_A_S relative to its corresponding consortium. The strongest statistical differences concern evenness (Hill–Simpson), underscoring a clear separation between soils and selected consortia, particularly for evenness. These results indicate that selective growth conditions altered community ecology, reducing richness and especially evenness in the selected bacterial consortia compared to the originating soils.

For the Italian site (Fig. 3, Panel C) bacterial consortia show reduced richness, though Chao1 remains broadly comparable to the soils. Evenness is also similar between soils and bacterial consortia, with uniformly low Hill–Simpson values across samples. IT_COL_10_A_S showed the highest richness and evenness, diverging most from its corresponding bacterial consortia (IT_COL_10_A_S_EC002_T02). Significant differences were detected both between selected consortia and soils and among soils themselves. As highlighted in Fig. 3, the most relevant contrasts arise among soils, rather than between each soil and its matched consortium, with the exception of IT_COL_10_A_S for Hill–Simpson. Consistently across regions, the Hill–Shannon index corroborates these patterns, showing a clear separation between environmental soil bacterial communities and their post-enrichment counterparts at all three sites.

3.5. Core-microbiome characterization and composition analysis of the selected bacterial consortia

The core microbiomes of the selected HCH degrading bacterial consortia per site (site-specific core microbiomes) were determined and

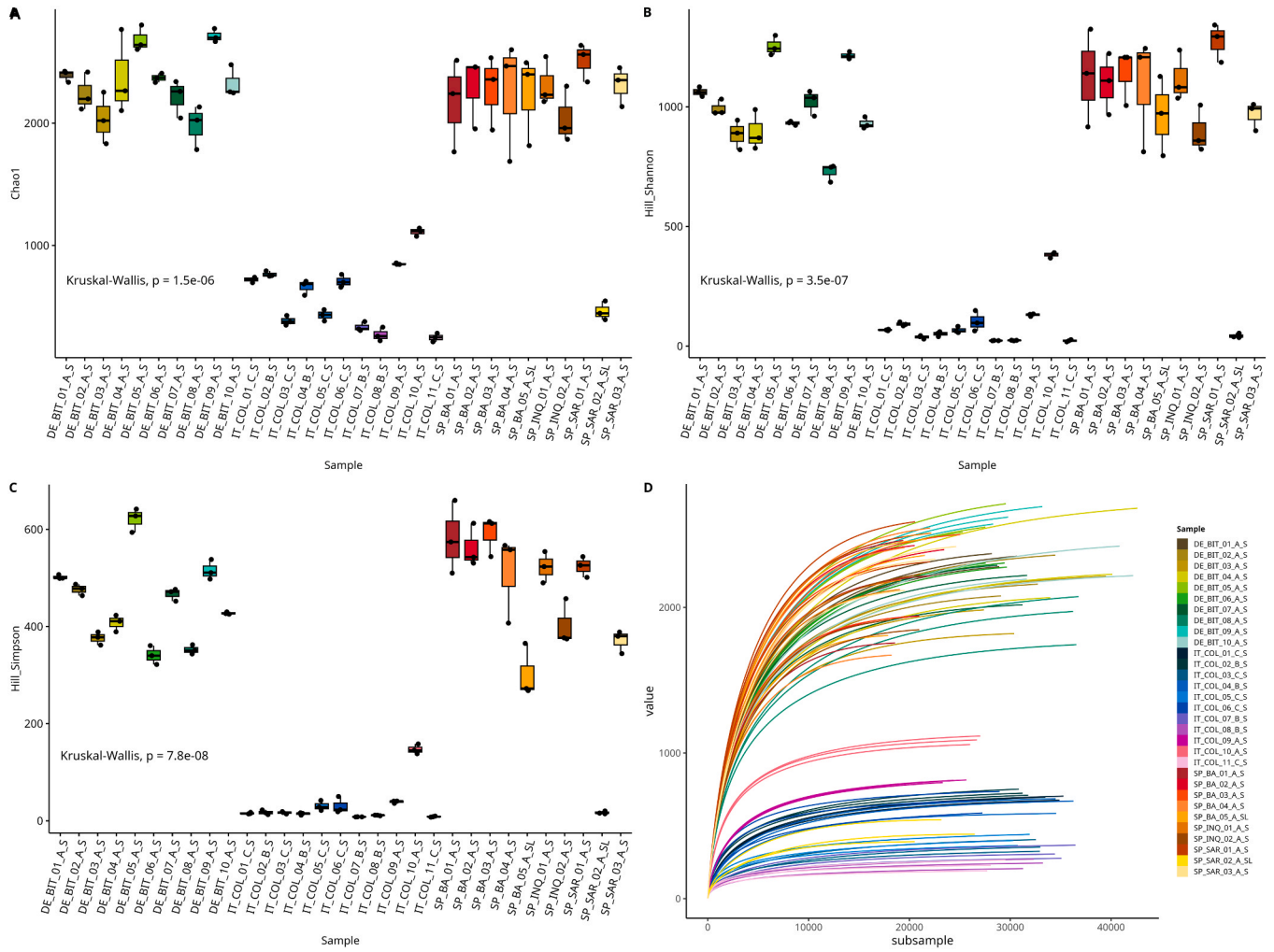


Fig. 2. α-diversity indexes of the soil samples of the tree country (Bitterfeld (DE), Colleferro (IT), Sabiñánigo (SP). Chao1 (A), Hill–Shannon (B), and Hill–Simpson (C) indices, each computed after coverage-based rarefaction at 98%, and rarefaction curves of observed species for the bacterial community (D). Boxplots show the minimum (Q0), first quartile (Q1), median (Q2), third quartile (Q3), and maximum (Q4) for each group. The reported p-value is from a Kruskal–Wallis test (α = 0.05). Post hoc comparisons use Dunn's test with Benjamini–Hochberg correction for multiple testing.

results are shown in Fig. 5, Panel A-C and associated Table S1. A flower plot was produced to visualize taxa shared by all bacterial consortia core microbiome, represented by the number at the centre of the flower plot. Table S1 reports the identification of the taxa constituting the core microbiome of each site-specific enriched microbial consortia core microbiome. The bacterial consortia selected from Colleferro are characterized by the highest number of core taxa, whereas those from Sabiñánigo contain the lowest. The Bitterfeld bacterial consortia show an intermediate number of core taxa but few unique taxa.

To determine whether the core taxa of the site-specific bacterial consortia could be involved in HCH degradation, an analytical approach was adopted to assess whether their abundance increased during the enrichment of the degrading bacterial consortia, assuming that such an increase is associated with a functional involvement of these taxa in the process of HCH depletion. The analytical approach was the Analysis of Composition of Microbiomes with Bias Correction (ANCOM-BC2) [42]. This group-wise comparison identifies taxa whose abundances differ significantly between two samples and quantifies the magnitude of those differences. We compared the soil bacterial comunities with bacterial consortia derived from the corresponding enrichment cultures to determine which soil taxa were retained during the enrichment phase and whether their relative abundances increased or decreased during the process. ANCOM-BC2 provides a statistically robust assessment of differential abundances for specific taxa. Taxa that increased significantly under selective growth conditions were considered candidate taxonomic markers of HCH-degrading bacterial consortia, as they are present in the soil and are subsequently favoured by the applied selective conditions. Pointing out that the composite genus names *Burkholderia–Caballeronia–Paraburkholderia* and *Allorhizobium–Neorhizobium–Pararhizobium–Rhizobium* follow the SILVA 138.2 classifier used in our analyses., results obtained from the analysis are shown in Fig. 6. In Fig. 6 panel A, the core taxa from Bitterfeld bacterial consortia (DE) *Stenotrophomonas*, *Pseudomonas, Variovorax,* JG30-KF-CM45, *Burkholderia–Caballeronia–Paraburkholderia*, *Bradyrhizobium* show statistical differences in abundance. Genera *Stenotrophomonas* and *Pseudomonas* are significantly more abundant in the three selected Bitterfeld bacterial consortia (DE), while the genus *Achromobacter* is more abundant in two out of three consortia (DE_BIT_03_A_S_EC002_T02 and DE_BIT_10_A_S_EC002_T02) than in the corresponding soils. The genera JG30-KF-CM45, Burkholderia–Caballeronia–Paraburkholderia, and Bradyrhizobium are less represented in the selected bacterial consortia than in their soils of origin. In Fig. 6, panel C, ANCOM-BC2 results for Colleferro are shown. *Pseudomonas*, *Cupriavidus* and *Pseudolabrys sp.* are all present in the bacterial consortia core microbiome of Colleffero. *Cupriavidus* increases significantly in all three selected bacterial consortia, while *Pseudomonas* and *Pseudolabrys* increases both in IT_COL_04_B_S_EC002_T02 and in IT _COL_10_A_S_EC002_T02 and IT_COL_03_C_S_EC002_T02 respectively. *Nocardioides* sp. is significantly

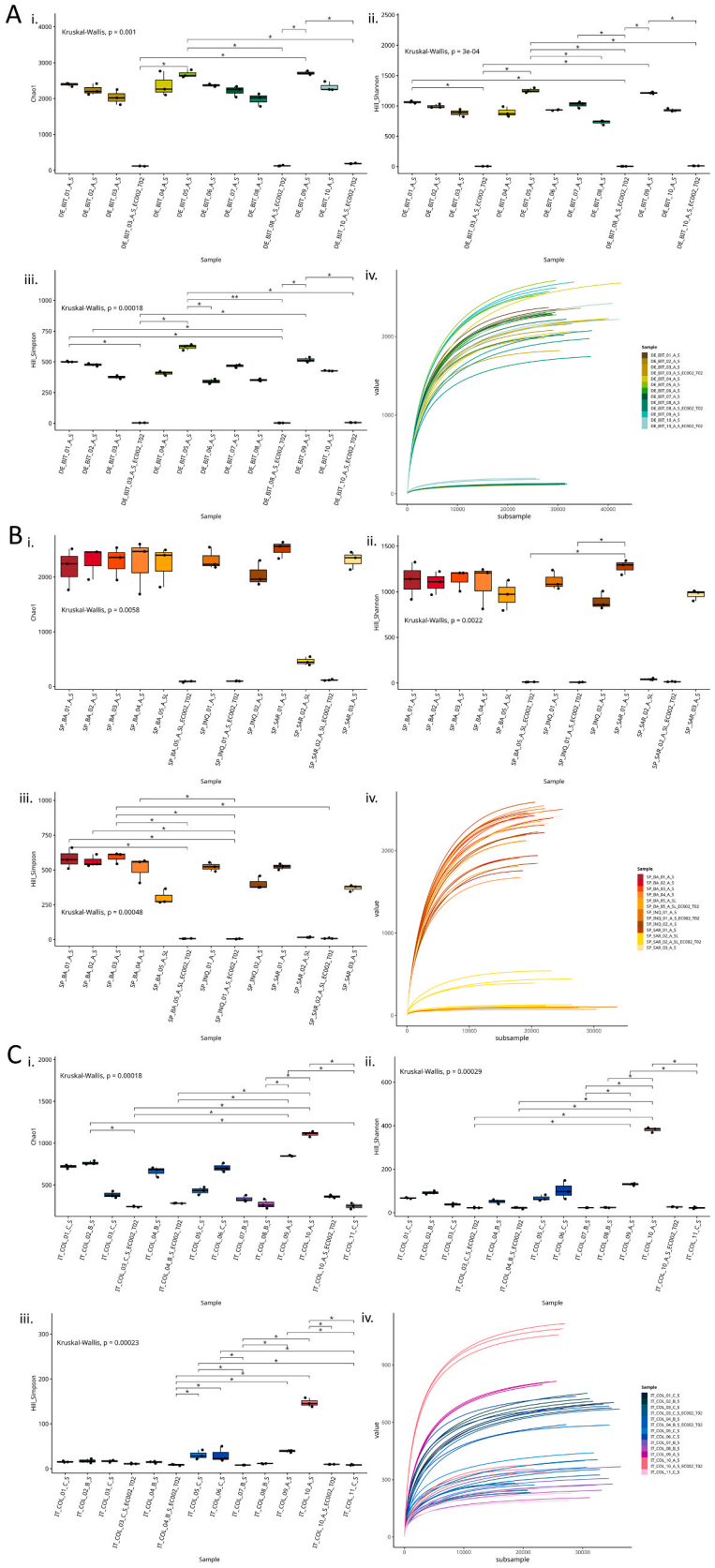


Fig. 3. α-diversity metrics of soils from the three contaminated sites and the deriving microbiomes. Panel A – Bitterfeld (DE), Panel B – Sabiñánigo (SP), Panel C – Colleferro (IT). Chao1 index (i), Hill–Shannon (ii), and Hill–Simpson (iii), each computed after 98% coverage-based rarefaction, and rarefaction curves of observed species in the bacterial community composition (iv). Boxplots show the minimum (Q0), first quartile (Q1), median (Q2), third quartile (Q3), and maximum (Q4) for each group. The reported p-value is from a Kruskal–Wallis test ($\alpha = 0.05$). Post hoc statistics are based on Dunn's test with Benjamini–Hochberg correction for multiple comparisons.

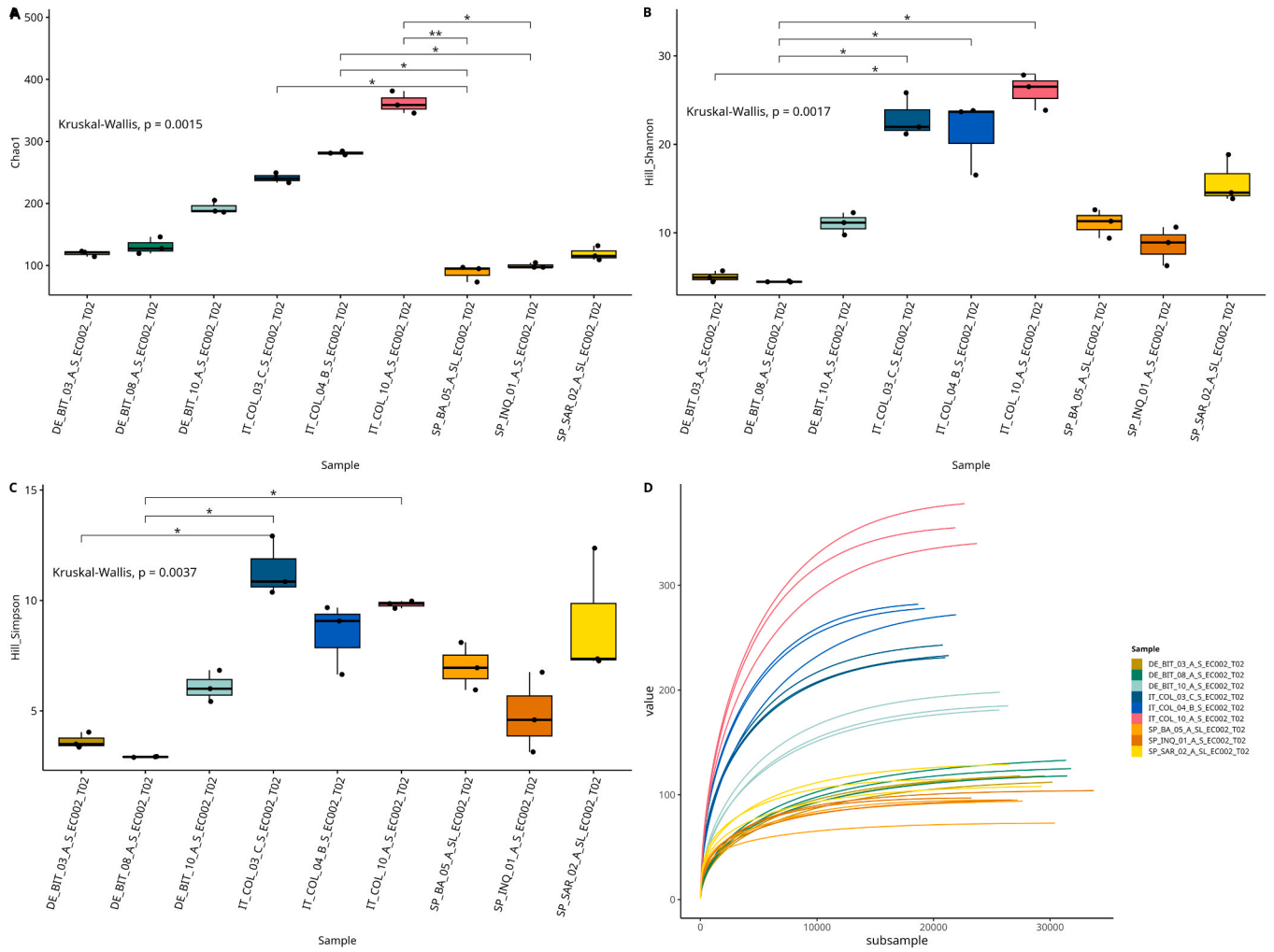


Fig. 4. α-diversity metrics of selected microbiomes from Bitterfeld, Colleferro, and Sabiñánigo. Chao1 index (A), Hill–Shannon (B), and Hill–Simpson (C), each computed after 98% coverage-based rarefaction, and rarefaction curves of observed species in the bacterial community composition (D). Boxplots show the minimum (Q0), first quartile (Q1), median (Q2), third quartile (Q3), and maximum (Q4) for each group. The reported p-value is from a Kruskal–Wallis test (α = 0.05). Post hoc statistics are based on Dunn's test with Benjamini–Hochberg correction for multiple comparisons.

more abundant in IT_COL_10_A_S_EC002_T02 and IT_COL_04_B_S_EC002_T02, whereas it shows the opposite behaviour, being significantly less abundant in IT_COL_03_C_S_EC002_T02. In Fig. 6 panel B, analysis of the Sabiñánigo-derived bacterial consortia highlights taxa well known for HCH degradation, namely *Sphingobium* and *Sphingomonas*, genera not found in soils from the other countries. *Sphingomonas* is significantly more abundant only in SP_SAR_02_A_SL_EC002_T02, accompanied by a decrease in *Sphingobium*, whereas *Sphingobium* increases in the selected bacterial consortia SP_INQ_01_A_S_EC002_T02 and SP_BA_05_A_SL_EC002_T02. The family Xanthomonadaceae is more abundant in enrichment-derived bacterial consortia than in source soils across all analyzed samples. In summary, any taxa significantly increasing in relative abundance during the bacterial consortia enrichment process are shared across all the HCH depleting bacterial consortia. However, site-specific candidates can be identified: in Bitterfeld (DE), *Stenotrophomonas*, *Pseudomonas*, and *Achromobacter* spp; in Colleferro (IT), *Pseudomonas*, *Pseudolabrys*, and *Cupriavidus* spp.; in Sabiñánigo (SP), *Sphingobium*, *Sphingomonas*, and the family Xanthomonadaceae.

4. Discussion

The decontamination of recalcitrant xenobiotics in environmental matrices poses several challenges. In matrices such as soils, it is desirable to adopt bio-based processes that exploit the catabolic capacities of indigenous microorganisms to degrade pollutants while preserving the biogeochemical cycles and the biodiversity of the treated matrix. Bacteria have demonstrated the ability to degrade recalcitrant organic compounds, enabling the development of detoxification strategies both in the laboratory and at field scale. In the case of HCH contamination, given the coexistence of four toxic isomers with different degrees of recalcitrance due to their structural features, it is reasonable to assume that the metabolic capacity of a bacterial consortia exceeds that of single strains. It is therefore crucial to isolate bacterial consortia with the metabolic traits of interest when designing bio-based approaches for the treatment of contaminated soils.

In this work, we developed a protocol to isolate bacterial consortia capable of degrading HCH, starting from historically contaminated soils collected from geographically distinct areas. The enrichment protocol here adopted enabled the isolation of bacterial consortia competent to degrade, even with different efficiency, all four HCH isomers from each region. This enrichment protocol overcame also the recalcitrance of the most recalcitrant isomers, δ and β [50]. Access to these bacterial consortia provided an opportunity to investigate the microbial ecology of both the enriched communities and their source soils, with the aim of identifying taxonomic and possibly functional markers associated with HCH degradation.

By analysing soils from distant geographical regions, we observed that differing physicochemical conditions significantly influence

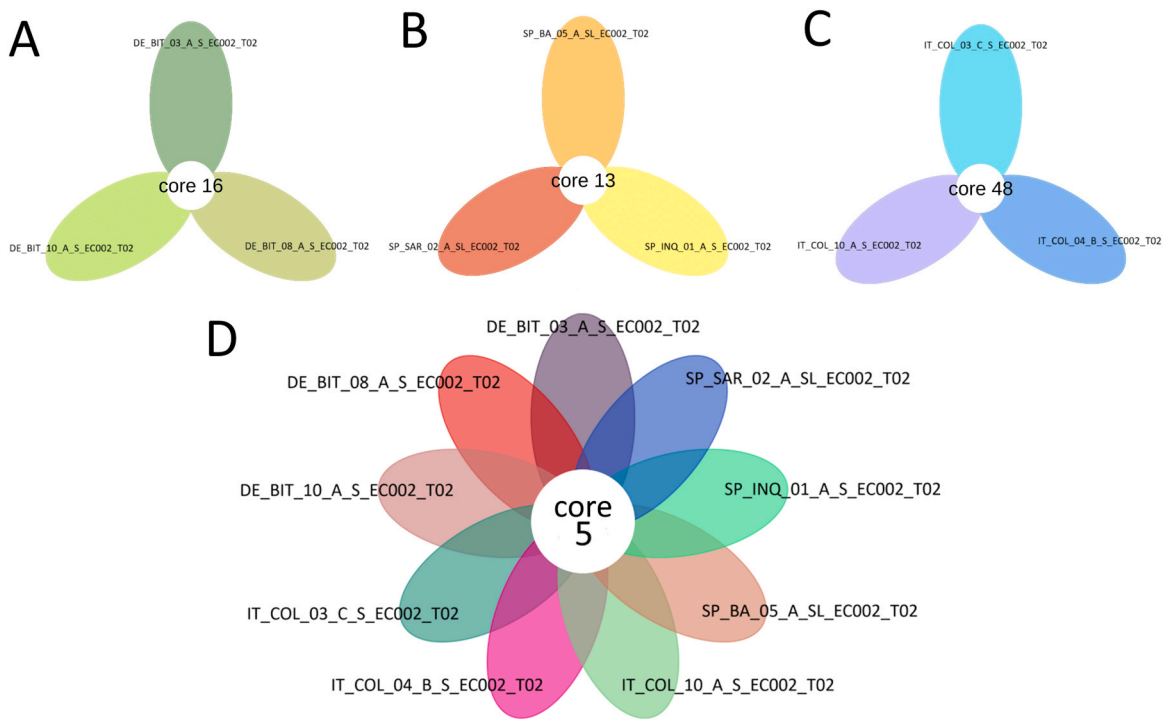


Fig. 5. Flower plot showing the intersection among the core microbiomes of microbiomes obtained by enrichment from Bitterfeld (A), Colleferro (B), and Sabiñánigo (C). The center indicates the number of taxa shared across all samples. The flower plot was generated with the EVenn software [1].

microbial community composition and structure, as shown by α-diversity analyses. Bacterial consortia from the Bitterfeld (DE) and Sabiñánigo (SP) regions displayed comparable values for the Chao1, Hill–Simpson, and Hill–Shannon indices, whereas Colleferro (IT) bacterial consortia were less diverse and less evenly distributed. Another key finding from comparing soil and isolated bacterial consortia is the marked reduction in biodiversity observed in the Bitterfeld (DE) and Sabiñánigo (SP) bacterial consortia relative to their source soils. This result, consistent with the strong selective stress applied during bacterial consortia enrichment process, is confirmed by the sharp decrease in the Hill–Shannon index, which drops from average values around 1000 in environmental bacterial consortia to values near zero in the selected consortia. In contrast, bacterial consortia isolated from Colleferro (IT) did not show a decrease in biodiversity as pronounced as that seen in their source soils, suggesting greater resilience of the isolated bacterial consortia to the selective pressure exerted during the enrichment process. This may reflect a better adaptation of the soil microbial ecology to environmental conditions dominated by HCH presence and other factors that might have altered the microbial community's response to HCH. Results obtained suggest that the observed biodiversity values may therefore reflect environmental conditions (e.g.: soil lithology, pH, water content) more than the selective pressure exerted by the contaminant. Moreover, when considering HCH isomer depletion kinetics, isolated bacterial consortia do not necessarily reflect the biodiversity of their source soils. Depletion kinetics were broadly comparable between Colleferro (IT) and Sabiñánigo (SP) bacterial consortias, while Bitterfeld (DE) exhibited lower levels of removal. Thus, biodiversity in historically contaminated environments might be a not a reliable indicator of greater metabolic potential toward the contaminant. Indeed, Bitterfeld (DE) yielded the least efficient degrading bacterial consortia; Sabiñánigo (SP) displayed uneven capacities, and Colleferro (IT) produced the most efficient bacterial consortia for oxidative HCH degradation. A high level of soil microbial biodiversity in the presence of such a toxic contaminant is not necessarily expected at historically contaminated sites. But, even when present, it cannot automatically be linked to enhanced degradative capacity, and results obtained more convincingly point to microbial communities that are tolerant to contamination rather than resistant because they can degrade the contaminant. Consequently, biodiversity descriptors for HCH-contaminated soils cannot be used as indices of the matrix's intrinsic degradation efficiency. More in details, Colleferro (IT) bacterial consortia achieved the highest overall removal of HCH isomers, consistent with good adaptation of local communities to the contaminant, possibly due to prolonged historical exposure that favoured the selection of degrading taxa. In contrast, Bitterfeld (DE) bacterial consortia, despite originating from highly contaminated soils, showed lower removal percentages, suggesting lower selective efficiency or a different ecological equilibrium. Sabiñánigo (SP) bacterial consortia behaved more heterogeneously, with SP_SAR_02_SL_EC002, SP_INQ_A_S_EC002, and SP_BA_05_A_S-L_EC002 particularly efficient and others less efficient in HCH depletion. A particularly noteworthy aspect concerns the recalcitrant β-HCH. In Bitterfeld (DE) bacterial consortias, β-HCH showed greater degradation relative to the other isomers, whereas in Colleferro (IT) and Sabiñánigo (SP), degradation was more efficient for the other isomers and β-HCH remained the most recalcitrant. At the same time, the comparison between the biodiversity indexes related to the source and the product of a microbial enrichment process, might be considered a proxy of the resilience and stability of the key players in the degradation of the contamination of interest during the enrichment process. Thus, the above mentioned key players might be good candidates for the bio-based approach of bioaugmentation. To identify specific microbial taxonomic markers that might potentially indicate the efficiency of HCH oxidation since associated to all the HCH degrading bacterial consortia, even though deriving from different sites, taxa shared across all selected enriched bacterial consortia have been identified. The identified one did not increase significantly in relative abundance during the bacterial consortia enrichment process, suggesting possible lack of direct competence for HCH depletion but possible involvement in co-metabolic processes. Possible candidate marker taxa for HCH depletion were identified since increasing during the enrichment process. The core microbiome analysis revealed a set of taxa shared across all enriched HCH-depleting consortia, indicating the presence of recurring

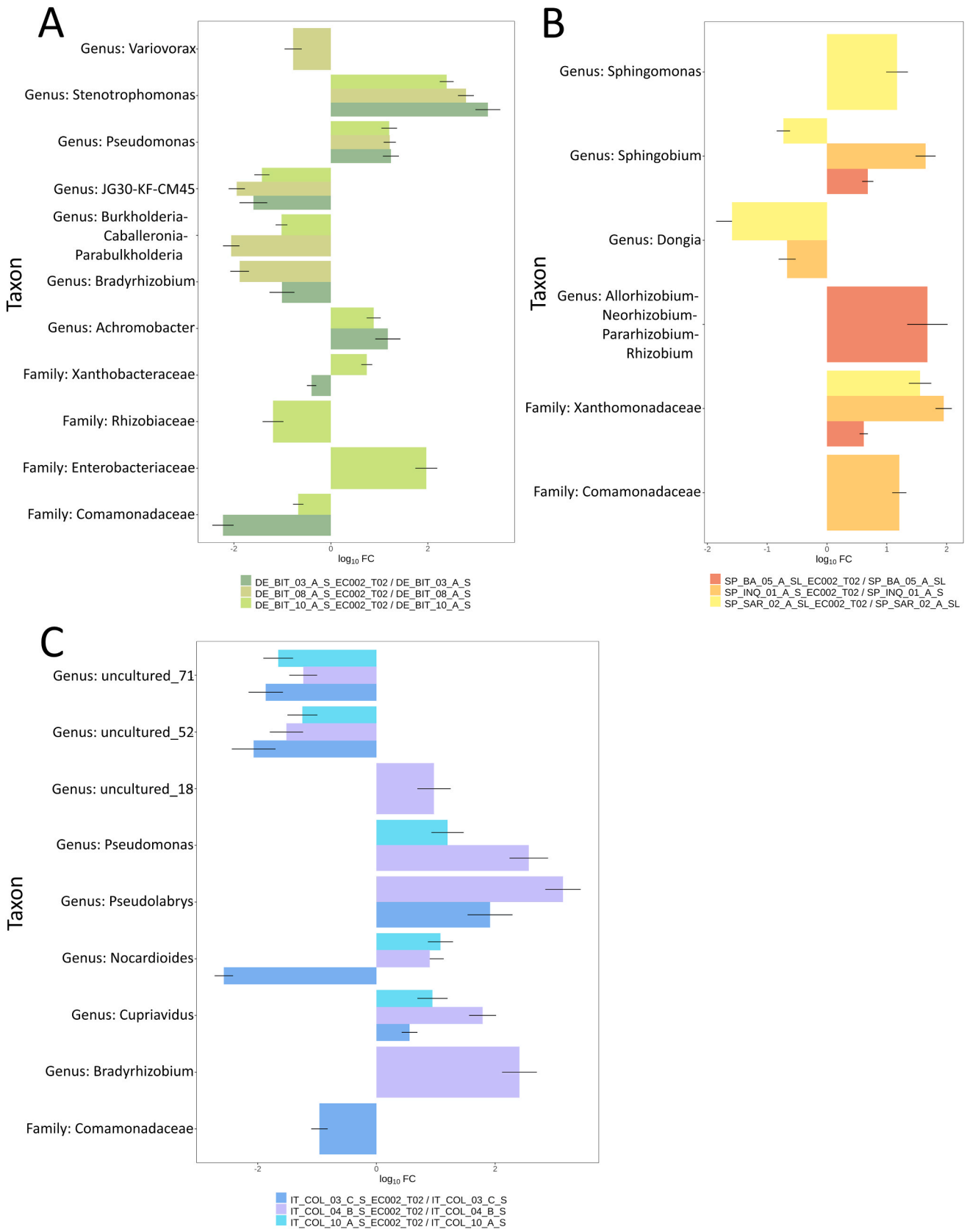


Fig. 6. Analysis of Composition of Microbiomes with Bias Correction (ANCOM-BC). On the horizontal axis are the log-transformed differential abundance values of each taxon between the two compared samples. Shown are comparisons between the environmental microbiomes and the corresponding selected microbiomes for Bitterfeld (Panel A), Colleferro (Panel B), and Sabiñánigo (Panel C).

community members despite their different site origins. However, core membership alone does not necessarily imply direct involvement in HCH depletion. For this reason, we interpreted core taxa considering their enrichment trajectories. Core taxa that were consistently detected across all bacterial consortia but did not significantly increase in relative abundance during enrichment, are less likely to represent primary HCH transforming bacteria under the applied selective conditions, i.e. high concentration of HCH in presence of less concentrated co-metabolic source of growth. Their persistence may instead reflect indirect or supportive roles within the community, such as co-metabolic interactions, cross-feeding on transformation by-products, provision of essential metabolites or cofactors, maintenance of redox balance and community stability, and/or detoxification of inhibitory intermediates. In this context, these taxa may contribute to consortium functioning without being directly competent for HCH depletion.

Conversely, taxa that significantly increased during enrichment are expected to be selectively favoured by the imposed conditions, i.e., the presence of HCH and the associated selective pressure. An enrichment-driven increase suggests a closer functional association with HCH depletion, either through direct participation in depletion pathways or through tightly coupled metabolic interactions that facilitate the process and benefit from it. Therefore, taxa showing consistent increases during enrichment were prioritized as candidate marker taxa associated with HCH depletion. Results obtained showed that for Bitterfeld (DE), the candidate markers are the genera *Pseudomonas*, *Stenotrophomonas*, and *Achromobacter* spp. *Stenotrophomonas* sp. has been extensively studied for its metabolic versatility and its ability to adapt to diverse environmental conditions; several species have been isolated from soils contaminated by various xenobiotics, including organophosphate pesticides, DDT, PAHs, and HCH. *Achromobacter* sp. increased significantly in two Bitterfeld bacterial consortia (DE_BIT_03_A_S_EC002 and DE_BIT_10_A_S_EC002), which did not display particularly high overall degradation but were able to degrade β-HCH, the most recalcitrant isomer. *Achromobacter* sp. has been associated with the degradation of haloaromatic compounds and PAHs, and genes involved in the metabolism of styrene, aminobenzoate, and benzoate have been described [51,52]. *Pseudomonas* sp. is well known for degrading HCH isomers, and *lin* genes have been sequenced in *Pseudomonas* spp., where they are associated with HCH isomer metabolism [53,54].

Pseudomonas was also present in the core bacterial consortia of Colleferro (IT) bacterial consortia, although it was significantly more abundant in only two consortia. Other genera that may be considered Colleferro (IT) markers include *Cupriavidus* sp., which was significantly more abundant in all selected bacterial consortia. *Cupriavidus* sp. has been isolated from microbial consortia capable of degrading all four HCH isomers, and members of the family Burkholderiaceae, to which it belongs, have been reported as degraders of a wide range of xenobiotics [55,56]. *Nocardioides* sp. was also observed as a Colleferro (IT) marker. *Nocardioides* spp are widespread in nature and have been isolated from sediments, soils, and aquatic environments and organisms; they can transform complex compounds and organic wastes, including crude oil, and many strains play important roles in bioremediation, degrading a variety of pollutants (alkanes, pyridine, phenols, phenanthrene, and others). Some *Nocardioides* strains degrade herbicides, including *N. jensenii*, which can degrade dinitro-o-cresol-based herbicides [57,58]. *Pseudolabrys* sp. has not been described for HCH isomer degradation nor characterized for *lin* genes, but it is involved in the degradation of chloroalkanes, chloroalkenes, and benzoate; dehalogenase functions have been inferred in both *Cupriavidus* and *Pseudolabrys*, suggesting potential activity on fluorotelomers (PFAS) [59].

Regarding possible taxonomic markers characteristic of Sabiñánigo (SP) bacterial consortia, *Sphingobium* sp. was significantly more abundant in SP_BA_05_A_S_EC002 and SP_INQ_01_A_S_EC002, while *Sphingomonas* sp. was significant only in SP_SAR_02_A_S_EC002. No taxa were simultaneously significantly more abundant across all three Sabiñánigo (SP) bacterial consortia and present in their shared core. Both *Sphingobium* and *Sphingomonas* spp. are well known for degrading HCH isomers. Strains such as *S. indicum* B90 and *S. japonicum* UT26 are archetypes for the *lin* gene pathway and the associated HCH degradation route. As previously assessed, the *lin* pathway is considered specific to HCH degradation [60,61]. To date, *lin* genes for lindane degradation have been identified mainly in Sphingomonadaceae (*Sphingobium*, *Sphingomonas*, *Sphingopyxis*, *Novosphingobium*). Some studies nonetheless suggest that these genes, or analogous enzymatic activities, may occur in other taxonomic groups (e.g., Pseudomonadales), although it is not always clear whether they use the same *lin* genes or alternative metabolic routes.

The observation that some consortia, e.g., Colleferro (IT) and a subset from Sabiñánigo (SP) reached similar levels of HCH depletion despite significantly different compositions suggests the action of degradation pathways arising from convergent adaptive mechanisms.

To our knowledge, degradative functions for HCH have not been framed in terms of the metabolic complexity of whole bacterial consortia; most pathways and enzymatic capacities implicated in HCH degradation have been characterized in single microorganisms, and little is known about the molecular mechanisms operating in competent consortia. Many taxa that constitute the core bacterial consortia of the degrading consortia are not strictly linked to HCH isomer degradation, even though they are reported to degrade other pollutants, including halogenated compounds such as chloroalkanes and chloroalkenes, often via haloacid dehalogenases. Because of their broad substrate recognition, these enzymes have been proposed as degraders of fluorinated compounds as well. Conversely, Sabiñánigo bacterial consortia show involvement of Sphingomonadaceae, which are absent in Colleferro (IT) and Bitterfeld (DE). The convergent adaptive mechanisms mentioned above, leading to similar degradation efficiencies in compositionally different bacterial consortia, may be explained by horizontal transfer of *lin* genes, as reported particularly within Sphingomonadaceae. This assumption, however, must be weighed against the observation that, in the bacterial consortia studied here, highly efficient HCH-degrading consortia (e.g., from Colleferro) lack Sphingomonadaceae, implying that additional, as yet undescribed, metabolic pathways may contribute to HCH degradation.

Consistent with this hypothesis, analyses guided by taxonomic assignments inferred from ASV data revealed distinct regional configurations of *lin*-associated functional potential. German bacterial consortia displayed an intermediate profile characterised by truncated *lin*-associated gene complements, suggestive of a putative partial oxidation process. Italian bacterial consortia exhibited a highly constrained *lin*-associated profile, restricted to LinB-related hydrolytic potential. Notably, taxa found in taxonomic assignments inferred from ASV data were not identified as site-specific candidates for HCH depletion in previous analyses, suggesting a decoupling between *lin* gene presence potential and observed degradation performance. On the other hand, the Spanish bacterial consortia potentially retained the complete *lin* pathway harboured by *Sphingobium* sp. Collectively, these patterns suggest that the metabolic potential for HCH transformation is geographically structured and reflects long-term adaptive responses to site-specific contamination histories but is not restricted to the presence of the *lin* pathway at least as a whole. The discrepancy between the high HCH degrading kinetics observed in the Italian and German consortia and the putative absence of complete *lin* gene set in these communities, in contrast to the Spanish consortia, suggests the presence of alternative or complementary catabolic routes. While the detection of the *lin* pathway remains the best characterised standard for HCH degradation, our taxonomically informed, ASV-level analyses, provides strong indirect evidence that these communities may rely on complex inter-species metabolic handovers to achieve HCH depletion, or employ different metabolic routes. The major adaptive strategy in microbes involves the activation of broad-specificity catabolic pathways that metabolize chemically diverse substrates, including xenobiotics, each of which can serve as a potential carbon source. The KEGG ko00361

("Chlorocyclohexane and chlorobenzene degradation pathway" https://www.kegg.jp/pathway/map00361), which also includes the *lin* genes, features dehalogenases not directly involved in HCH isomer transformation. By dehalogenating contaminants, the pathway channels metabolites into the tricarboxylic acid cycle or benzoate degradation. Thus, broad substrate recognition by such dehalogenases may be among the mechanisms contributing to HCH degradation, in addition to the *lin* pathway, which appears largely confined to Sphingomonadaceae. In this context, synergistic or competitive interactions among taxa in multi-species consortia can shape metabolic functions differently than in isolated microorganisms and may even enhance them via broadly distributed mechanisms across genera. In the present case, the synergy would relate to dehalogenation of chlorinated compounds.

Supporting this interpretation, candidate marker taxa for Bitterfeld (DE) include *Achromobacter* sp., associated with genes involved in the degradation of 2,4-dichlorophenoxyacetic acid (2,4-D) within ko00361, and *Stenotrophomonas* sp., reported to carry genes for the initial steps of the chlorocyclohexane and chlorobenzene pathway [62,63]. For Colleferro, *Cupriavidus* sp. harbors genes and enzyme sets associated with the degradation of mono- and dichlorinated compounds, including "tfd" genes also present in the cyclochlorohexane/chlorobenzene pathway, and *Pseudolabrys* sp. has been described as carrying genes for the early steps of cyclochlorohexane and chlorobenzene degradation [64,65]. Therefore, the bacterial genera that increase significantly during bacterial consortia isolation relative to the source soils are not necessarily carriers of the *lin* pathway but may possess enzyme repertoires capable of dehalogenating a broad range of chlorinated compounds, comprising HCH. To reinforce this assumption the metabolites of degradation of the HCH was attempted for all the microbial consortia analysed. The identification of the intermediates of degradation of HCH by the enriched microbial consortia was attempted assuming the involvement of the sole HCH degradation pathway described so far, the *lin* pathway, and the possible alternative routes described in the KEGG reference pathway map00361, the Chlorocyclohexane and chlorobenzene degradation, https://www.kegg.jp/pathway/map00361. The initial attack on HCH isomers pathway, by the *lin* pathway, includes for γ-HCH, the γ-pentachlorocyclohexene, successively to 1,3,4,6-tetrachloro-1,4-cyclohexadiene, followed by 1,2,4-trichlorobenzene and 1,4-dichlorobenzene, proceeding through chlorobenzene to 2,5-dichlorohydroquinone, then to chlorohydroquinone, hydroquinone, and finally to maleylacetate. In relation to β-HCH it is sequentially degraded to δ-3,4,5,6-tetrachlorocyclohexene, successively to 5,6-dichloro-1,3-cyclohexadiene and downstream intermediates of benzene and chlorobenzene. In the context of the present experimentation the search for metabolites belonging to the *lin* pathway focused on chlorinated metabolites, which necessarily derive from the degradation of hexachlorocyclohexane. The identification of γ-pentachlorocyclohexene within the microbiota confirmed the inferred presence of the *lin*B genes in all the microbial consortia. On the other hand, the detection of 1,2,4- trichlorobenzene and δ-3,4,5,6-tetrachlorocyclohexene, not described as intermediates of the *lin* pathway (KEGG pathway map00361, https://www.kegg.jp/pathway/map00361), further indicate that the consortia undertake alternative degradative pathways. In the near future, to deepen the analysis of the alternative routes of HCH degradation in the different consortia, labeled HCH will be adopted in experimentation tailored for each single candidate, since the corresponding pathways might be different in the different consortia.

5. Conclusions

This study demonstrates that indigenous soil bacterial consortia from three historically contaminated sites can be selectively enriched to achieve efficient degradation of all four HCH isomers, including the highly recalcitrant δ- and β-HCH. Despite substantial differences in soil properties and native microbial communities, the enrichment protocol consistently yielded competent degraders, confirming its robustness across heterogeneous matrices. Any taxa was identifies as common microbial marker for oxidative HCH degradation. Instead, site-specific taxa emerged: *Pseudomonas*, *Stenotrophomonas*, and *Achromobacter* in Bitterfeld; *Cupriavidus*, *Nocardioides*, and *Pseudolabrys* in Colleferro; and *Sphingobium* and *Sphingomonas* in Sabiñánigo. The absence of Sphingomonadaceae in the most efficient consortia (Colleferro), together with the predominance of truncated lin-associated gene complements in Bitterfeld and Colleferro, supports the interpretation that effective HCH degradation is not necessarily dependent on a complete lin pathway and may be facilitated by non-Sphingomonadaceae taxa. Functional inference further suggested geographically distinct configurations of the *lin* pathway and a long-term adaptive responses to site-specific contamination history. Taken together our data, captured as community-level signals inferred from exploratory taxonomic–functional analysis, indicate that HCH degradation arises from a combination of *lin*-dependent and broader *lin*-independent dehalogenation mechanisms. captured as community-level signals inferred from exploratory taxonomic–functional analysis. In this context, considering that functional inference and increases in relative abundance support selection and association with HCH depletion but not necessarily prove direct catabolic competence, additional functional evidence (e.g., detection/expression of degradation genes) would be collected in the near future, also to tentatively depict the extent to which each taxa contributes to HCH degradation within a bacterial community.

Environmental implications

Our findings show that hexachlorocyclohexane (HCH) degradation in historically contaminated soils extends beyond the canonical *lin* pathway. Robust HCH depletion in communities lacking a complete *lin* gene set reveals additional, still poorly resolved, but dominant microbial routes that can sustain natural attenuation at legacy lindane sites. The enrichment of multiple taxa in HCH-amended cultures points to several and new candidate degraders that could be tracked and selectively stimulated in bioremediation efforts. Together, these results underscore the importance of accounting for the broader functional potential of soil microbiomes when predicting HCH fate and designing management strategies for contaminated sites.

CRediT authorship contribution statement

Riccardo Di Mambro: Writing – review & editing, Visualization, Data curation. **Nicola Bertelloni:** Methodology, Investigation, Formal analysis, Data curation. **Rocio Barros-Garcia:** Writing – review & editing, Visualization, Validation, Formal analysis, Data curation. **Miguel Cebrian-Aldana:** Writing – review & editing, Visualization, Validation, Software, Methodology, Formal analysis, Data curation. **Marta Franco-Benito:** Writing – review & editing, Visualization, Validation, Software, Investigation, Formal analysis, Data curation. **Devaki Destri:** Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Simona Di Gregorio:** Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Bernabei Giacomo:** Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Blanca Velasco-Arroyo:** Writing – review & editing, Visualization, Validation, Formal analysis, Data curation. **Jose Carlos Castilla-Alcantara:** Writing – review & editing, Visualization, Validation, Formal analysis, Data curation. **Sara Gil-Guerrero:** Writing – review & editing, Visualization, Validation, Software, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Akanksha Mishra:** Writing – review & editing, Validation, Software, Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2026.142224.

Data availability

Data will be made available on request.

References

[1] Breivik, K., Pacyna, J.M., Münch, J., 1999. Use of α-, β- and γ-hexachlorocyclohexane in Europe, 1970–1996. Sci Total Environ 239 (1-3), 151–163. https://doi.org/10.1016/S0048-9697(99)00291-0.

[2] Vijgen, J., Abhilash, P.C., Li, Y.F., et al., 2011. Hexachlorocyclohexane (HCH) as new Stockholm Convention POPs-a global perspective on the management of Lindane and its waste isomers. Environ Sci Pollut Res 18 (2), 152–162. https://doi.org/10.1007/S11356-010-0417-9/TABLES/2.

[3] Nayyar, N., Sangwan, N., Kohli, P., et al., 2014. Hexachlorocyclohexane: Persistence, toxicity and decontamination. Rev Environ Health 29 (1-2), 49–52. https://doi.org/10.1515/REVEH-2014-0015/XML.

[4] Li, Y.F., Cai, D.J., Singh, A., 1998. Technical hexachlorocyclohexane use trends in China and their impact on the environment. Arch Environ Contam Toxicol 35 (4), 688–697. https://doi.org/10.1007/S002449900432/METRICS.

[5] Murthy, H.M.R., Manonmani, H.K., 2007. Aerobic degradation of technical hexachlorocyclohexane by a defined microbial consortium, 149 (1), 18–25. https://doi.org/10.1016/J.JHAZMAT.2007.03.053.

[6] Manonmani, H.K., Chandrashekaraiah, D.H., Sreedhar Reddy, N., Elcey, C.D., Kunhi, A.A.M., 2000. Isolation and acclimation of a microbial consortium for improved aerobic degradation of α-hexachlorocyclohexane. J Agric Food Chem 48 (9), 4341–4351. https://doi.org/10.1021/JF990712C/ASSET/IMAGES/LARGE/JF990712CF00008.JPEG.

[7] Nagata, Y., Endo, R., Ito, M., Ohtsubo, Y., Tsuda, M., 2007. Aerobic degradation of lindane (gamma-hexachlorocyclohexane) in bacteria and its biochemical and molecular basis. Appl Microbiol Biotechnol 76 (4), 741–752. https://doi.org/10.1007/S00253-007-1066-X.

[8] Nagasawa, S., Kikuchi, R., Nagata, Y., Takagi, M., Matsuo, M., 1993. Aerobic mineralization of γ-HCH by Pseudomonas paucimobilis UT26. Chemosphere 26 (9), 1719–1728. https://doi.org/10.1016/0045-6535(93)90115-L.

[9] Sahu, S.K., Patnaik, K.K., Sharmila, M., Sethunathan, N., 1990. Degradation of Alpha-, Beta-, and Gamma-Hexachlorocyclohexane by a Soil Bacterium under Aerobic Conditions. Appl Environ Microbiol 56 (11), 3620–3622. https://doi.org/10.1128/AEM.56.11.3620-3622.1990.

[10] Hynková, K., Nagata, Y., Takagi, M., Damborský, J., 1999. Identification of the catalytic triad in the haloalkane dehalogenase from Sphingomonas paucimobilis UT26. FEBS Lett 446 (1), 177–181. https://doi.org/10.1016/S0014-5793(99)00199-4.

[11] Nagata, Y., Miyauchi, K., Takagi, M., 1999. Complete analysis of genes and enzymes for γ-hexachlorocyclohexane degradation in Sphingomonas paucimobilis UT26. J Ind Microbiol Biotechnol 23 (4-5), 380–390. https://doi.org/10.1038/SJ.JIM.2900736.

[12] Nagata, Y., Ohtsubo, Y., Tsuda, M., 2015. Properties and biotechnological applications of natural and engineered haloalkane dehalogenases. Appl Microbiol Biotechnol 99 (23), 9865–9881. https://doi.org/10.1007/S00253-015-6954-X.

[13] Nagata, Y., Tabata, M., Ohtsubo, Y., Tsuda, M., 2015. Biodegradation of Organochlorine Pesticides. Published online September 1 Man Environ Microbiol 5.1.2-1–5.1.2-30. https://doi.org/10.1128/9781555818821.CH5.1.2.

[14] Miyauchi, K., Suh, S.K., Nagata, Y., Takagi, M., 1998. Cloning and sequencing of a 2,5-dichlorohydroquinone reductive dehalogenase gene whose product is involved in degradation of gamma-hexachlorocyclohexane by Sphingomonas paucimobilis. J Bacteriol 180 (6), 1354–1359. https://doi.org/10.1128/JB.180.6.1354-1359.1998.

[15] Endo, R., Ohtsubo, Y., Tsuda, M., Nagata, Y., 2006. Growth inhibition by metabolites of gamma-hexachlorocyclohexane in Sphingobium japonicum UT26. Biosci Biotechnol Biochem 70 (4), 1029–1032. https://doi.org/10.1271/BBB.70.1029.

[16] Verma, H., Kumar, R., Oldach, P., et al., 2014. Comparative genomic analysis of nine Sphingobium strains: Insights into their evolution and hexachlorocyclohexane (HCH) degradation pathways. BMC Genom 15 (1). https://doi.org/10.1186/1471-2164-15-1014/.

[17] Pal, R., Bala, S., Dadhwal, M., et al., 2005. Hexachlorocyclohexane-degrading bacterial strains Sphingomonas paucimobilis B90A. UT26 and Sp+, having similar lin genes, represent three distinct species, Sphingobium indicum sp. nov., Sphingobium japonicum sp. nov. and Sphingobium francense sp. nov., and reclassification of [Sphingomonas] chungbukensis as Sphingobium chungbukense comb. nov. Int J Syst Evol Microbiol 55 (5), 1965–1972. https://doi.org/10.1099/IJS.0.63201-0.

[18] Jariyal, M., Jindal, V., Mandal, K., Gupta, V.K., Singh, B., 2018. Bioremediation of organophosphorus pesticide phorate in soil by microbial consortia. Ecotoxicol Environ Saf 159, 310–316. https://doi.org/10.1016/J.ECOENV.2018.04.063.

[19] Lal, D., Jindal, S., Kumari, H., et al., 2015. Bacterial diversity and real-time PCR based assessment of linA and linB gene distribution at hexachlorocyclohexane contaminated sites. J Basic Microbiol 55 (3), 363–373. https://doi.org/10.1002/JOBM.201300211.

[20] Manickam, N., Mau, M., Schlömann, M., 2006. Characterization of the novel HCH-degrading strain, Microbacterium sp. ITRC1. Appl Microbiol Biotechnol 69 (5), 580–588. https://doi.org/10.1007/S00253-005-0162-Z.

[21] Manickam, N., Misra, R., Mayilraj, S., 2007. A novel pathway for the biodegradation of γ-hexachlorocyclohexane by a Xanthomonas sp. strain ICH12. J Appl Microbiol 102 (6), 1468–1478. https://doi.org/10.1111/J.1365-2672.2006.03209.X.

[22] Manickam, N., Reddy, M.K., Saini, H.S., Shanker, R., 2008. Isolation of hexachlorocyclohexane-degrading Sphingomonas sp. by dehalogenase assay and characterization of genes involved in γ-HCH degradation. J Appl Microbiol 104 (4), 952–960. https://doi.org/10.1111/J.1365-2672.2007.03610.X.

[23] Lal, R., Dogra, C., Malhotra, S., Sharma, P., Pal, R., 2006. Diversity, distribution and divergence of lin genes in hexachlorocyclohexane-degrading sphingomonads. Trends Biotechnol 24 (3), 121–130. https://doi.org/10.1016/J.TIBTECH.2006.01.005.

[24] Lal, R., Dadhwal, M., Kumari, K., et al., 2008. Pseudomonas sp. to Sphingobium indicum: A journey of microbial degradation and bioremediation of Hexachlorocyclohexane. Indian J Microbiol 48 (1), 3–18. https://doi.org/10.1007/S12088-008-0002-9.

[25] Sangwan, N., Lata, P., Dwivedi, V., et al., 2012. Comparative Metagenomic Analysis of Soil Microbial Communities across Three Hexachlorocyclohexane Contamination Levels. PLoS One 7 (9), e46219. https://doi.org/10.1371/JOURNAL.PONE.0046219.

[26] Gil-Guerrero, S., Division, I.D.E.N.E.R.A.B., Otero-Gonzalez, L., Franco de Benito, M., Hall, A., 2025. BIOSYSMOdb: Curated Database for Biodegradation and Bioremediation (Published online February). Zenodo. https://doi.org/10.5281/ZENODO.14795254.

[27] Bernabei, G., De Simone, G., Becarelli, S., Di Mambro, R., Gentini, A., Di Gregorio, S., 2024. Co-metabolic growth and microbial diversity: Keys for the depletion of the α, δ, β and γ-HCH isomers. Dec 5 J Hazard Mater 480, 135963. https://doi.org/10.1016/j.jhazmat.2024.135963.

[28] Noguchi, K., Gel, Y.R., Brunner, E., Konietschke, F., 2012. nparLD: An R Software Package for the Nonparametric Analysis of Longitudinal Data in Factorial Experiments. J Stat Softw 50 (12), 1–23. https://doi.org/10.18637/JSS.V050.I12.

[29] Portolés, T., Pitarch, E., López, F.J., Sancho, J.V., Hernández, F., 2007. Methodical approach for the use of GC-TOF MS for screening and confirmation of organic pollutants in environmental water. J Mass Spectrom 42 (9), 1175–1185. https://doi.org/10.1002/jms.1248.

[30] Gartner, Lesueur C., Mentler, M., Fuerhacker, A., 2008. M. Comparison of four extraction methods for the analysis of 24 pesticides in soil samples with gas chromatography–mass spectrometry and liquid chromatography–ion trap–mass spectrometry. Talanta 75 (1), 284–293. https://doi.org/10.1016/j.talanta.2007.11.031.

[31] U.S. Environmental Protection Agency, 2007. Method 8081B Organochlor Pestic Gas Chromatogr. ⟨https://www.epa.gov/sites/default/files/2015-12/documents/8081b.pdf⟩.

[32] Khan, M.I., Yoo, K., Schwab, L., Kümmel, S., Nijenhuis, I., 2024. Characterization of anaerobic biotransformation of hexachlorocyclohexanes by novel microbial consortia enriched from channel and river sediments. J Hazard Mater 476 (2), 135198. https://doi.org/10.1016/j.jhazmat.2024.135198.

[33] Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet J 17 (1), 10–12. https://doi.org/10.14806/EJ.17.1.200.

[34] Nearing, J.T., Douglas, G.M., Comeau, A.M., Langille, M.G.I., 2018. Denoising the Denoisers: an independent evaluation of microbiome sequence error-correction approaches. PeerJ 6 (8), e5364. https://doi.org/10.7717/peerj.5364.

[35] Prodan, A., Tremaroli, V., Brolin, H., Zwinderman, A.H., Nieuwdorp, M., Levin, E., 2020. Comparing bioinformatic pipelines for microbial 16S rRNA amplicon sequencing. PLoS One 15 (1), e0227434. https://doi.org/10.1371/JOURNAL.PONE.0227434.

[36] Bolyen, E., Rideout, J.R., Dillon, M.R., et al., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol 37 (8), 852–857. https://doi.org/10.1038/s41587-019-0209-9.

[37] Robeson, M.S., O'Rourke, D.R., Kaehler, B.D., et al., 2021. RESCRIPt: Reproducible sequence taxonomy reference database management. PLoS Comput Biol 17 (11), e1009581. https://doi.org/10.1371/JOURNAL.PCBI.1009581.

[38] Hsieh, T.C., Ma, K.H., Chao, A., 2016. iNEXT: an R package for rarefaction and extrapolation of species diversity (Hill numbers). Methods Ecol Evol 7 (12), 1451–1456. https://doi.org/10.1111/2041-210X.12613.

[39] Chao, A., Gotelli, N.J., Hsieh, T.C., et al., 2014. Rarefaction and extrapolation with Hill numbers: A framework for sampling and estimation in species diversity studies. Ecol Monogr 84 (1), 45–67. https://doi.org/10.1890/13-0133.1.

[40] Alberdi, A., Gilbert, M.T.P., 2019. A guide to the application of Hill numbers to DNA-based diversity analyses. Mol Ecol Resour 19 (4), 804–817. https://doi.org/10.1111/1755-0998.13014.

[41] Gotelli, N.J., Chao, A., 2013. Measuring and Estimating Species Richness, Species Diversity, and Biotic Similarity from Sampling Data. Encycl Biodivers Second Ed. https://doi.org/10.1016/B978-0-12-384719-5.00424-X.

[42] Lin, H., Peddada, S.Das, 2023. Multigroup analysis of compositions of microbiomes with covariate adjustments and repeated measures. *2023 21:1* Nat Methods 21 (1), 83–91. https://doi.org/10.1038/s41592-023-02092-7.
[43] Edwin, N.R., Fitzpatrick, A.H., Brennan, F., Abram, F., O'Sullivan, O., 2024. An in-depth evaluation of metagenomic classifiers for soil microbiomes. Environ Micro 19 (1), 19. https://doi.org/10.1186/S40793-024-00561-W.
[44] Reitmeier, S., Hitch, T.C.A., Treichel, N., et al., 2021. Handling of spurious sequences affects the outcome of high-throughput 16S rRNA gene amplicon profiling. ISME Commun 1 (1), 31. https://doi.org/10.1038/S43705-021-00033-Z.
[45] Chen, H., Ma, K., Lu, C., et al., 2022. Functional Redundancy in Soil Microbial Community Based on Metagenomics Across the Globe. Front Microbiol 13, 878978. https://doi.org/10.3389/FMICB.2022.878978/BIBTEX.
[46] Sayers, E.W., Bolton, E.E., Brister, J.R., et al., 2022. Database resources of the national center for biotechnology information. Nucleic Acids Res 50 (D1), D20–D26. https://doi.org/10.1093/NAR/GKAB1112.
[47] Camacho, C., Coulouris, G., Avagyan, V., et al., 2009. BLAST+: architecture and applications. *2009 10:1* BMC Bioinforma 10 (1), 421. https://doi.org/10.1186/1471-2105-10-421.
[48] Bateman, A., Martin, M.J., Orchard, S., et al., 2023. UniProt: the Universal Protein Knowledgebase in 2023. Nucleic Acids Res 51 (D1), D523–D531. https://doi.org/10.1093/NAR/GKAC1052.
[49] Cock, P.J.A., Antao, T., Chang, J.T., et al., 2009. Biopython: freely available Python tools for computational molecular biology and bioinformatics. Bioinformatics 25 (11), 1422–1423. https://doi.org/10.1093/BIOINFORMATICS/BTP163.
[50] Willett, K.L., Ulrich, E.M., Hites, R.A., Willett, K., 1998. EURH. Differential toxicity and environmental fates of hexachlorocyclohexane isomers, 32 (15), 2197–2207. https://doi.org/10.1021/ES9708530.
[51] Marzec-Grządziel, A., Gałązka, A., 2023. Sequencing of the Whole Genome of a Bacterium of the Genus Achromobacter Reveals Its Potential for Xenobiotics Biodegradation. *2023, Vol 13, Page 1519* Agriculture 13 (8), 1519. https://doi.org/10.3390/AGRICULTURE13081519.
[52] Gao, S., Seo, J.S., Wang, J., Keum, Y.S., Li, J., Li, Q.X., 2013. Multiple degradation pathways of phenanthrene by Stenotrophomonas maltophilia C6. Int Biodeterior Biodegrad 79, 98–104. https://doi.org/10.1016/J.IBIOD.2013.01.012.
[53] Lodha, B., Bhat, P., Kumar, M.S., et al., 2007. Bioisomerization kinetics of γ-HCH and biokinetics of Pseudomonas aeruginosa degrading technical HCH. Biochem Eng J 35 (1), 12–19. https://doi.org/10.1016/J.BEJ.2006.12.015.
[54] Singh, A.K., Chaudhary, P., Macwan, A.S., Diwedi, U.N., Kumar, A., 2007. Selective loss of lin genes from hexachlorocyclohexane-degrading Pseudomonas aeruginosa ITRC-5 under different growth conditions. Appl Microbiol Biotechnol 76 (4), 895–901. https://doi.org/10.1007/s00253-007-1056-z.
[55] Singh, A.K., Chaudhary, P., Macwan, A.S., Diwedi, U.N., Kumar, A., 2007. Selective loss of lin genes from hexachlorocyclohexane-degrading Pseudomonas aeruginosa ITRC-5 under different growth conditions. *2007 76:4* Appl Microbiol Biotechnol 76 (4), 895–901. https://doi.org/10.1007/S00253-007-1056-Z.
[56] Srivastava, V., Dhuliya, S., Kumar, M.S., 2022. Biodegradation of technical hexachlorocyclohexane by Cupriavidus malaysiensis. *2022 38:6* World J Microbiol Biotechnol 38 (6), 108. https://doi.org/10.1007/S11274-022-03284-7.
[57] Zhu, L., Yang, B., Guo, W., et al., 2024. Nocardioides limicola sp. nov., an alkaliphilic alkane degrading bacterium isolated from oilfield alkali-saline soil. Antonie Van Leeuwenhoek 117 (1), 14. https://doi.org/10.1007/S10482-023-01907-Z.
[58] Kumar, D., 2018. Biodegradation of γ-Hexachlorocyclohexane by Burkholderia sp. IPL04. Biocatal Agric Biotechnol 16, 331–339. https://doi.org/10.1016/J.BCAB.2018.09.001.
[59] Manucharova, N.A., Bolshakova, M.A., Babich, T.L., et al., 2021. Microbial Degraders of Petroleum and Polycyclic Aromatic Hydrocarbons from Sod-Podzolic Soil. *2021 90:6* Microbiology 90 (6), 743–753. https://doi.org/10.1134/S0026261721060096.
[60] Zheng, G., Selvam, A., Wong, J.W.C., 2011. Rapid degradation of lindane (γ-hexachlorocyclohexane) at low temperature by Sphingobium strains. Int Biodeterior Biodegrad 65 (4), 612–618. https://doi.org/10.1016/J.IBIOD.2011.03.005.
[61] Dong, S., Yan, P.F., Mezzari, M.P., Abriola, L.M., Pennell, K.D., Cápiro, N.L., 2024. Using Network Analysis and Predictive Functional Analysis to Explore the Fluorotelomer Biotransformation Potential of Soil Microbial Communities. Environ Sci Technol 58 (17), 7480–7492. https://doi.org/10.1021/ACS.EST.4C00942.
[62] Raina, V., Mrutyunjay, A.E., Ae, S., et al., 2007. Enhanced biodegradation of hexachlorocyclohexane (HCH) in contaminated soils via inoculation with Sphingobium indicum B90A. *2007 19:1* Biodegradation 19 (1), 27–40. https://doi.org/10.1007/S10532-007-9112-Z.
[63] Vedler, E., Vahter, M., Heinaru, A., 2004. The completely sequenced plasmid pEST4011 contains a novel IncP1 backbone and a catabolic transposon harboring tfd genes for 2,4-dichlorophenoxyacetic acid degradation. J Bacteriol 186 (21), 7161–7174. https://doi.org/10.1128/JB.186.21.7161-7174.2004;JOURNAL:JOURNAL:JB;WGROUP:STRING:PUBLICATION.
[64] Pérez-Pantoja, D., De La Iglesia, R., Pieper, D.H., González, B., 2008. Metabolic reconstruction of aromatic compounds degradation from the genome of the amazing pollutant-degrading bacterium Cupriavidus necator JMP134. FEMS Microbiol Rev 32 (5), 736–794. https://doi.org/10.1111/J.1574-6976.2008.00122.X.
[65] Vera, A., Wilson, F.P., Cupples, A.M., 2022. Predicted functional genes for the biodegradation of xenobiotics in groundwater and sediment at two contaminated naval sites. *2022 106:2* Appl Microbiol Biotechnol 106 (2), 835–853. https://doi.org/10.1007/S00253-021-11756-3.