Pollution and edaphic factors shape bacterial community structure and functionality in historically contaminated soils

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ABSTRACT

Studies about biodegradation potential in soils often refer to artificially contaminated and simplified systems, overlooking the complexity associated with contaminated sites in a real context. This work aims to provide a holistic view on microbiome assembly and functional diversity in the model site SIN Brescia-Caffaro (Italy), characterized by historical and uneven contamination by organic and inorganic compounds. Here, physical and chemical analyses and microbiota characterization were applied on one-hundred-twenty-seven soil samples to unravel the environmental factors driving bacterial community assembly and biodegradation potential in three former agricultural fields. Chemical analyses showed a patchy distribution of metals, metalloids and polychlorinated biphenyls (PCB) and allowed soil categorization according to depth and area of collections. Likewise, the bacterial community structure, described by molecular fingerprinting and 16S rRNA gene analyses, was significantly different according to collection site and depth. Pollutant concentrations (*i.e.*, hexachloro-biphenyls, arsenic and mercury), nitrogen content and parameters related to soil texture were identified as main drivers of microbiota assembly, being significantly correlated to bacterial community composition. Moreover, bacteria putatively involved in the aerobic degradation of PCBs were enriched over the total bacterial community in topsoils, where the highest activity was recorded using fluorescein hydrolysis as proxy. Metataxonomic analyses revealed the presence of bacteria having metabolic pathways related to PCB degradation and tolerance to heavy metals and metalloids in the topsoil samples collected in all areas. Overall, the provided dissection of soil microbiota structure and its degradation potential in the SIN Brescia-Caffaro can contribute to target specific areas for rhizoremediation implementation. Metagenomics studies could be implemented in the future to understand if specific degradative pathways are present in historically polluted sites characterized by the co-occurrence of multiple classes of contaminants.

KEYWORDS

Soil microbiota, Environmental selection, PCB, Heavy metals, *bphA*

1. INTRODUCTION

Microorganisms shape the environment they colonize through their physiology and metabolism, thus microbiome engineering has the potential to offer plenty of solutions for societal needs, as in the case of bioremediation strategies (Mapelli et al., 2017; Verstraete et al., 2007). However, the limited comprehension of bacterial communities' structure and dynamics in real context still hampers the development of novel microbiome-based solutions for environmental management (Bell et al., 2019). In particular, the response of bacterial communities to soil pollution and the identification of the environmental factors driving bacterial assembly can provide clues to steer the soil microbiome, enriching those populations endowed with the highest bioremediation potential. Bacterial communities are indeed primarily involved in the biodegradation processes and their composition, metabolic activity and degradation performances can be affected by several edaphic parameters, including soil texture, pH, organic matter content and contaminant fingerprint, concentration and bioavailability (Bell et al., 2013; Correa-García et al., 2021; Mukherjee et al., 2014). Many studies about microbiome selection factors in polluted soils refer to artificially contaminated and simplified systems (Chekol et al., 2004; Meggo and Schnoor, 2013; Qin et al., 2014, Halfadji et al., 2022), generating data (*e.g.*, degradation rates) useful as input parameters for fate modelling in the environment (Terzaghi et al., 2018). However, this approach does not contribute to fill in the gap of knowledge about the drivers of soil bacterial community structure and functionality in real contaminated sites. The National Priority Site for remediation (SIN) Brescia-Caffaro (Italy) is a vast historically contaminated site, characterized by high and uneven

concentration of organic and inorganic pollutants derived from the activity of the former Caffaro chemical factory, and represents a model to study soil microbial ecosystems and design eco-friendly solutions aimed at the remediation of aged and heterogeneously contaminated areas (Di Guardo et al., 2017). The SIN Brescia-Caffaro includes more than 100 ha of soil previously used for agriculture where PCBs, heavy metals and metalloids occur in concentration often higher than the legal threshold (Di Guardo et al., 2017). PCBs are highly toxic synthetic compounds recalcitrant to degradation and threatening environmental safety, animal and human health at global scale. Their production is now banned but they are found as main contaminants in many sites worldwide (Desforges et al., 2018; Quinete et al., 2014; Undeman et al., 2018). In the SIN Caffaro-Brescia, the degree of mobilization of PCBs from soil resulted by the specific land use history of each area (Di Guardo et al., 2020), thus a snapshot of the current pollutant and microbiota distribution is a priority to develop *in situ* interventions based on the stimulation of the biodegradation potential of the soil (*i.e.*, natural attenuation (Terzaghi et al., 2019; Vergani et al., 2017).

Our hypothesis was that the complex contamination pattern in this historically polluted site, besides physical and chemical parameters, has a major role in driving the soil bacterial community structure and the enrichment of populations involved in aerobic PCB degradation, biosignatures of soil natural attenuation potential. In this work, we characterized one-hundred-twenty-seven soil samples for metal, metalloid and PCB concentrations and we finely dissected the distribution of the resident bacterial populations. Soil physical and chemical analyses were statistically correlated with the bacterial community profiling, the assessment of soil hydrolytic activity and the quantification of putative aerobic PCB degraders over the total bacterial community.

2. MATERIALS AND METHODS

2.1. Site description and soil sample collection

The study sites are located inside the National Priority Site for remediation (SIN) Brescia-Caffaro (Italy). Soils were sampled in three former agricultural fields (*i.e.*, area A, area R and area T) where cultivation activities were banned in 2002 after the SIN establishment by the Italian Environment Ministry. Areas A, R and T are located south-west of the Caffaro plant, a former producer of PCB mixtures and other chlorinated chemicals. A detailed description of the site, including historical information on land use and cultivation was presented elsewhere (Di Guardo et al., 2020). The first sampling was conducted in the A, R and T areas along soil depth (October 2014). Nine stations were identified in the three areas (A1, A2, A3, R1, R2, R3, T1, T2, T3; Figure 1A). From each station, soil samples were collected by core drilling up to a depth of 100 cm, after removing the surface grass cover to limit the rhizosphere effect that can buffer the impact of edaphic factors on the soil microbiome structure (Mapelli et al., 2018). At each sampling station, three cores were collected within 1-meter distance and each core was then divided into fractions corresponding to seven intervals according to depth expressed in cm (0-10, 10-20, 20-30, 30-40, 40-60, 60-80, 80-100). For each sampling station (n=9), the replicated fractions of the same depth range of the three cores were merged, obtaining in total 63 samples, a procedure adopted to minimize the intrinsic soil heterogeneity (Di Guardo et al., 2020). A second independent sampling was performed (March 2015) on a denser grid to cover the whole surface of the three sampling areas A (A1-A17), R (R1-R19) and T (T1-T28) (Figure 1B). Here, a total of 64 soil cores were collected to a depth of 40 cm, corresponding to the layer of soil mostly influenced by the former agricultural practices (*e.g.*, tillage) and characterized by higher contamination according to the chemical characterization of the soils collected during the first sampling (see the Results section). Topsoil samples (0-40 cm) were homogenized and, immediately after sampling, were stored at 4°C for hydrolytic activity analysis and at -20°C for chemical and molecular analyses.

2.2. Soil physical and chemical analyses

Soil chemical analyses were performed in outsource by a private company (Theolab S.p.A., now Mérieux NutriSciences, Volpiano TO, Italy). Soil pH, total nitrogen, total organic carbon, cation exchange capacity (CEC) and pollutant concentrations (*i.e.*, 79 PCB congeners and 11 metals and metalloids) were determined as respectively reported in Di Guardo et al. (2020) and Morosini et al. (2021). Polychlorinated biphenyl (PCB) concentration data were partially presented in a publication focused on the comparison of the different classes of PCB congeners, their distribution and movement across soil layers (Di Guardo et al., 2020). Samples were classified according to soil texture considering the silt, sand and clay contents and particle sizes, in agreement with the Soil Survey Manual (2017). Soils are defined as polluted according to the Italian legal threshold for agricultural areas (*i.e.*, PCBs: 0.02 mg/kg dry weight; As: 30 mg/Kg d.w.; Hg: 1 mg/Kg d.w.). Further information about the datasets generated through physical and chemical analyses and the statistical analyses applied for soil sample clustering are reported in the Supplementary Method 1.

2.3. Evaluation of the soil hydrolytic activity

Hydrolytic activity on fluorescein diacetate (FDA) was quantified in the soil samples as a proxy for the soil microbiota activity. FDA hydrolysis was measured following a protocol optimized for soil (Green et al., 2006) and reported in detail in the Supplementary Method 2 together with the information about FDA results' analysis.

2.4. DNA extraction and quantification

Total DNA extraction was performed from 0.5 grams of each soil sample using the PowerSoil DNA kit (Qiagen) according to the manufacturer's protocol. DNA concentration was assessed fluorometrically using the Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific).

2.5. Automated ribosomal intergenic spacer analysis (ARISA) and β-diversity of soil bacterial communities

The automated ribosomal intergenic spacer analysis fingerprinting of 16S–23S ribosomal RNA (ARISA-PCR) was conducted on all soil samples using the primer set ITSF-FAM (5'-GTCGTAACAAGGTAGGCCGTA-3') and ITSReub (5'-GCCAAGGCATCCACC-3') in a PCR reaction mixture that contained 1X PCR buffer, 1.5 U of Taq DNA polymerase (Invitrogen), 0.2 mM (each) dNTPs, and 0.25 µM (each) primer in a final volume of 25 µL (Cardinale et al., 2004). The mixture was held at 94°C for 3 min, followed by 30 cycles of 94°C for 45 s, 55°C for 1 min, 72°C for 2 min, and a final extension step at 72°C for 7 min (Mapelli et al., 2013). ARISA fragments were separated using the ABI3730XL genetic analyser applying the internal standard 1200-LIZ (Macrogen, Rep. of Korea) and the obtained fingerprints were analyzed using the Peak Scanner Software (Applied Biosystems). Information on raw data and statistical analyses is included in the Supplementary Methods 3.

2.6. 16S rRNA gene sequencing and metataxonomic analysis of soil bacterial communities

Based on the results of pollutant concentration profiling along soil depth, we analyzed the soil bacterial assemblages in 18 selected topsoil samples, collected during the first sampling campaign at 0-10 and 20-30 cm depth, by applying Illumina MiSeq sequencing of the V3-V4 hypervariable regions of the bacterial 16S rRNA gene using the primers 341F and 785R (Klindworth et al., 2013) at Macrogen Korea. The obtained sequences were processed and analyzed using QIIME2 version 20.8 (Bolyen et al., 2019; https://qiime2.org/) software. The DADA2 workflow was followed to assemble the reads. The sequence reads were deposited in the NCBI SRA database under the BioProject ID: PRJNA809934. All details about raw data and statistical analyses are reported in the Supplementary Methods 4.

2.7. Quantification of potential PCB-degrading bacteria by qPCR

Quantitative PCR (qPCR) reactions were conducted on all the soil samples (n=127), targeting 16S rRNA and biphenyl dioxygenase (*bphA*) genes to assess the relative abundance of potential PCB degraders in the overall bacteria population. Biphenyl dioxygenase is a key enzyme involved in the upper pathway of PCBs aerobic biodegradation, taking part to their transformation in chlorobenzoates and chlorinated aliphatic acids. All the reactions were set up in polypropylene 96-well plates using an epMotion® 5070 (Eppendorf) and run in a BIORAD CFX Connect™ Real-Time PCR Detection System. Detailed information on qPCR reagents, protocols and data analysis is reported in the Supplementary Method 5.

3. RESULTS

3.1. Pollutant concentrations together with soil physical and chemical properties change with depth and define soil bacterial community structure

In this work, we initially characterized 63 soil samples collected down to 1 m depth from different sampling stations in area A (A1, A2, A3), area R (R1, R2, R3) and area T (T1, T2, T3), three former agricultural fields located inside the SIN Brescia-Caffaro (Figure 1A). According to the distribution of 79 PCB congeners and 11 metal and metalloids detected in the samples (Supplementary Table 1) we identified the highest PCB and heavy metals (*e.g.*, Hg) concentrations in area A, particularly at the sampling station A1 (Supplementary Table 1). Physical and chemical parameters allowed the categorization of soils by CAP considering together the factors ‘area’ and ‘depth’ (delta_1^2: 0.91894, p = 0.0001, Figure 2A) and ‘site’ and ‘depth’ (delta_1^2: 0.9992, p = 0.0004) as the categorical explanatory variables of the analyses. Soil categories were further validated by PERMANOVA as indicated in Supplementary Table 5. According to SIMPER analysis, the soil diversity

in each ‘area’ and ‘depth’ was explained by different physical and chemical parameters (Supplementary Table 6). All different PCB chlorination families, As and Hg concentrations, nutrient concentrations (N, C), CEC and clay, silt and sand contents contributed to a different extent to discriminate the 63 soil samples collected at increasing depths, defining two separate layers (0-40 and 40-100 cm) in each area, and permitting to identify the topsoil layers (0-40 cm) as the most polluted.

Distinct clusters of bacterial communities could be identified in the analysed sampling sites at different depths, as shown by PCoA of the community profiles generated by ARISA fingerprinting (Figure 2B). PERMDISP analysis detected no statistical significance of the dispersion (p = 0.2826), and the clustering of bacterial communities according to the interaction of the ‘site’ and ‘depth’ factors was confirmed by CAP (delta_1^2: 0.9971, p = 0.0013) and PERMANOVA ($F_{1,8}$ = 1.9615; p = 0.0001) tests. A DistLM analysis was performed on the ARISA profiles using the physical and chemical data provided in Supplementary Table 2, where PCB congener concentrations were summed up according to the chlorination family (from mono- up to decachloro-biphenyls). DistLM sequential test recognised as significant driver of soil bacterial microbiota some of the considered physical and chemical parameters (DistLM, AICc = 405.22, R^2 = 0.30; Supplementary Table 7), including those related to soil texture (*i.e.*, clay) and nutrient content (*i.e.*, total nitrogen) besides pollutant concentrations (*i.e.*, As, Hg, hexachloro-biphenyls). As shown by dbRDA, the pollutant concentrations played a key role in the separation of samples collected from sites A1, A2 and A3 at depth comprised between 0-40 cm (Figure 2C). Given the identification of As and Hg among the drivers of bacterial community composition, we performed a second DistLM analysis using all available metal and metalloid concentrations (Supplementary Table 1). The analysis confirmed the significance of As and Hg and revealed that other metals/metalloids (*i.e.*, Mn, Cr(VI), Pb, Cr tot)

209 included as predicting factors were significantly related to bacterial population distribution in the
210 samples (Figure 2D; Supplementary Table 8).
211
212 **3.2 Microbiota activity and PCB degradation potential increase in the topsoils where the pollution**
213 **levels are higher**
214 We evaluated in the soil samples the fluorescein diacetate (FDA) hydrolysis activity, as a proxy for
215 the overall microbiota activity on soil organic matter. The data presented in Figure 3A indicated a
216 consistent trend of higher hydrolytic activity values in the topsoils, collected at depth comprised
217 between 0-40 cm, while subsoil samples (40-100 cm) showed lower values in all the sampling sites.
218 Statistical analysis confirmed that the 'depth' factor is significantly related to the soil hydrolytic
219 activity, differentiating soil samples characterized by higher pollution level (ANOVA, $F_{1,8} = 86.9251$;
220 $p < 0.0001$). A similar trend was observed at each sampling site comparing the ratio between the
221 copy number of *bphA* gene, encoding for the key enzyme of the upper pathway of aerobic PCB
222 degradation, and 16S rRNA gene, encoding for the universal prokaryote small subunit ribosome
223 (Figure 3B). This ratio is an indication of the abundance of aerobic PCB degraders over the total
224 bacterial community. The *bphA*/16S rRNA gene copy ratio values were higher in the topsoil samples
225 compared to the subsoil ones collected between 40 and 100 cm (ANOVA, $F_{1,8} = 28.5686$; $p < 0.0001$)
226 and were significantly different according to the site of sampling (ANOVA, $F_{1,8} = 3.9384$; $p = 0.0016$)
227 with the overall highest values occurring in the samples collected from the most polluted area A.
228
229 **3.3. Pollutant distribution is scattered in the topsoil of the SIN Brescia-Caffaro, which host distinct**
230 **bacterial assemblages according to the sampling areas**

231 Given the highly heterogeneous nature of the soil matrices and the patchy distribution of
232 contamination, a second sampling was realized to collect, in each field (A: n=17; R: n=19; T: n=28),
233 a higher number of topsoil samples in a finer x-y grid (Figure 1B, Table 1).
234 Basing on physical and chemical data, CAP analysis allowed the separation of topsoil samples
235 according to the area of collection (delta_1^2 = 0.94612, *p* = 0.0001; Figure 4A). SIMPER analysis
236 identified different physical and chemical parameters (*i.e.*, all PCB chlorination family, As and Hg
237 concentrations, N and C concentrations, pH, CEC and clay, silt and sand contents) as explanatory
238 variable of soil diversity, showing that PCB concentration was particularly important in
239 differentiating samples of area A from those collected in areas R and T (Supplementary Table 9).
240 Conversely, PCB chlorination families contribute to a less extent to explain the differences between
241 samples of areas R and T (Supplementary Table 9). Following PERMDISP validation (*p* = 0.0807), CAP
242 analysis was applied on the bacterial community profiles generated by ARISA fingerprinting
243 demonstrating their significant clustering according to the 'area' factor (delta_1^2 = 0.99281, *p* =
244 0.0001; Figure 4B), as also confirmed by PERMANOVA test ($F_{2,58}$ = 6.4466; *p* = 0.0001). DistLM
245 analysis was performed using the physical and chemical data reported in Supplementary Table 4
246 and the test indicated soil texture-related parameters (*i.e.*, clay and coarse sand content), nutrient
247 content (*i.e.*, total nitrogen) and As concentration as the selection factors significantly correlated to
248 bacterial assemblages in the topsoils of the three analysed areas (DistLM, AICc = 405.16, R^2 = 0.20;
249 Figure 4C; Supplementary Table 10). A second DistLM analysis was performed using all available
250 metal and metalloid concentrations as predictive factors and pointed out Mn, As and Zn
251 concentrations among the drivers of soil bacterial community structure (Figure 4D; Supplementary
252 Table 11).
253 FDA test evidenced greater soil hydrolytic activity values in area A than in the other two areas (Figure
254 3C). Statistical analysis indicated that the 'area' factor significantly influenced soil hydrolytic activity

(ANOVA, $F_{2,61}$ = 17.219; p < 0.0001), though the pairwise test revealed that only area R values were significantly lower than those measured in areas A and T (Supplementary Table 12). Likewise, ANOVA test recognised the values of *bphA*/16S rRNA gene copy ratio as significantly diverse according to the factor 'area' (ANOVA, $F_{2,59}$ = 30; p < 0.0001), but such differences were recognizable only for the soils collected from area T (Figure 3D) that displayed lower values compared to the other two areas (Supplementary Table 13).

3.4. Bacterial phylogenetic diversity and identification of possible marker taxa related to soil contamination

The bacterial community structure and taxonomy were further investigated by 16S rRNA gene sequencing to depict taxa distribution more in detail, focusing on topsoils sampled at 0-10 and 20-30 cm depth that correspond to the upper and most contaminated soil layers. 16S rRNA gene Illumina sequencing generated a number of non-chimeric denoised merged reads comprised between 67,318 and 115,766 (on average 65,673±18,004 reads per sample, Supplementary Table 14), and a total of 934 ASVs were identified after classification at the 15th level of the SILVA taxonomic assignment (Supplementary Table 14). The rarefaction curves of the libraries were assessed, showing a coverage of more than 99% in all the samples (data not shown). Alpha-diversity indexes (*i.e.*, richness, Shannon diversity and dominance) were calculated and are indicated in Supplementary Table 15. Richness, expressed as the number of ASVs per sample, was not significantly different in soils collected from the areas A, R and T (ANOVA, $F_{2,12}$ = 0.0975; p = 0.9078) or at the 0-10 and 20-30 cm depth (ANOVA, $F_{1,12}$ = 2.1816; p = 0.1654). On the opposite, bacterial community diversity, expressed as Shannon index, was significantly higher in soils collected at 0-10 cm fraction compared to those at 20-30 cm (ANOVA, $F_{1,12}$ = 7.0844; p = 0,02073) and coherently we detected a significantly lower dominance within the same bacterial communities (ANOVA, $F_{1,12}$ =

4.7928; p = 0.04907). Following PERMDISP validation (p = 0.7806), PCoA analysis applied to ASV distribution in the soil samples showed that bacterial communities were separated on the first axis according to depth of sampling (Figure 5A). CAP results demonstrated that bacterial communities could be significantly categorized according to depth (delta_1^2: 0.60738, p = 0.0338).

The results of 16S rRNA gene sequencing and ASVs phylogenetic classification (Supplementary Table 16) indicated Proteobacteria, Acidobacteria, Bacteroidetes and Actinobacteria as dominant phyla in all the soil bacterial communities (Figure 5B). Moreover, Planctomycetes, Verrucomicrobia and Chloroflexi were abundantly present in the analysed soil samples while other 21 phyla contributed, on average, less than 2.5 % over total bacterial community. The differential abundance analysis identified six genera significantly responding to sampling depth (p < 0.05) in terms of relative abundance (Supplementary Figure 1). *Stenotrophomonas*, *Flavobacterium* and an uncultured *Saprospiraceaea* genus were more abundant in the surface (0-10 cm soil depth) while *Bacillus*, the *Nitrosomonadaceae* genus MND1 and the uncultured genus TRA3-20 were significantly more represented in soils collected at higher depth (20-30 cm below soil surface). In the attempt to gain information on the natural attenuation potential of the soil bacterial community, we investigated its functional diversity inferring metabolic properties related to hydrocarbon degradation, arsenic and heavy metal resistance based on the data generated by 16S rRNA Illumina sequencing. Several KEGG (Kyoto Encyclopedia of Genes and Genomes) orthologues related to different degradation/resistance pathways (*e.g.*, chloroalkane and chloroalkene degradation, benzoate degradation, arsenate reduction, arsenite oxidation and methylation) were present in the samples, but no statistically significant shifts could be observed when comparing their distribution in the soil samples collected at different depth (0-10 cm and 20-30 cm) or area (Supplementary Table 17).

4. DISCUSSION

In this study, we found that, based on physical and chemical features, soil samples of the SIN Brescia-Caffaro cluster according to the area and depth of collection and we demonstrated that contaminants played a primary role in the separation of soil samples collected at different depths in all areas, further discriminating those sampled from the most polluted area A. Integrating chemical and geospatial analyses, we recently reported in the same former agricultural fields that PCB concentration shifts were related to land use, field cultivation history and soil properties (Di Guardo et al., 2020). This study, including a higher number of soil samples clustered based on a wider physical and chemical characterization encompassing both organic and inorganic pollutants, confirmed the indications obtained from previous studies looking individually at PCB concentration gradients (Di Guardo et al., 2020) or Hg concentration (Morosini et al., 2021).

In addition, we outlined for the first time the bacterial community structure of one-hundred-twenty-seven soil samples collected on a broad x-y-z spatial grid to unravel the environmental drivers of soil microbiota diversity and function. The adopted molecular fingerprinting has been recognised as a valid and reliable method for the description of beta-diversity in complex microbial communities, including soil-dwelling ones, and remains a suitable tool for correlation analysis between biotic and abiotic datasets providing similar results to those obtained on high-throughput sequencing methods, though it has limitation in terms of richness detection (Gobet et al., 2014; van Dorst et al., 2014). Soil bacterial communities in this historically contaminated site were significantly different according to the collection area and depth. The role of depth on soil bacterial communities is known in natural ecosystems (Fierer et al., 2003) and depth can influence microbial community diversity also in agricultural soil, where crop roots play a selection effect on the bulk soil 'seed bank' far below the topsoil layer (Hao et al., 2021). The soils sampled in this study were located in former agricultural fields, where cultivation is forbidden since 2002 when the SIN Brescia-Caffaro perimeter was defined, and the plant cover is limited to meadow spontaneous species (Di Guardo et al., 2017).

Here, previously performed agricultural practices (*e.g.*, tillage) acting over time on soil structure and features (*e.g.*, organic matter content) differentiated topsoil from subsoil layers and as a consequence likely played a selection effect on the current soil bacterial community composition. Besides microbiota structure, agricultural practices like tillage can influence the hydrolytic activity of the soil microbial community (Vazquez et al., 2017). Soil hydrolytic activity has been used as a proxy to estimate the biological reactions potentially occurring in polluted soil (Doni et al., 2012; Macci et al., 2013). Boosting the organic matter turnover, hydrolytic activity impacts the bioavailability of hydrophobic molecules like PCBs, which movement in soils is influenced by dissolved organic matter and particle/colloid transport (Vitale et al., 2018). In the SIN Brescia-Caffaro, topsoil samples showed higher hydrolytic activity values, suggesting that in this layer the bioavailability of hydrophobic PCBs likely embedded in the recalcitrant soil organic matter (Terzaghi et al., 2018) is potentially increased. This result could partly be ascribed to the lower microbial biomass in the subsoil (Blume et al., 2002), nonetheless it agrees with previous studies reporting higher values of FDA hydrolysis in soils collected from areas deeply contaminated by different hydrocarbons in comparison to those characterized by lower contamination (Margesin et al., 2003; Mukherjee et al., 2014).

The primary role of the changing edaphic factors in structuring the microbial community was previously demonstrated on soils differentiated by land use (Kuramae et al., 2012) and should not be neglected in historically polluted sites. Indeed, given their influence on pollutant mobility and bioavailability, edaphic factors and contaminant distribution are interconnected parameters acting together in the selection of the soil inhabiting bacterial communities (Rogiers et al., 2021). Accordingly, the results of this study showed that both edaphic properties and the uneven mixed contamination shape soil bacterial communities along depth and area in the SIN Brescia-Caffaro. Among environmental factors, nutrients' content and parameters related to soil texture are

recognised among those most remarkably influencing the soil microbiome structure (Fierer, 2017), as we observed here for the soil nitrogen content and for clay and sand content. Noteworthy, clays are soil colloids that can influence the availability and the biodegradation of hydrophobic PCB congeners in soil (Li et al., 2021), eventually influencing the beta-diversity detected in the polluted soils object of this study. Among the different PCB congener families present in the analysed soils, only hexachloro-biphenyls resulted significantly related to the bacterial community composition. The selective pressure given by PCBs on the microbiota diversity could be ascribed to the enrichment of PCB degrading population and, on the other hand, to the inhibition of a large fraction of the bacterial communities mediated by the accumulation of metabolites generated by the biphenyl pathway and potentially even more toxic than parental molecules (Cámara et al., 2004). Apart the decachloro-biphenyls, characterized by the lowest bioavailability and biodegradability (Borja et al., 2005), hexacloro-biphenyls were the most abundant PCB chlorination family in the soils collected at SIN Brescia-Caffaro and this could explain their significant impact on the bacterial community structure. PCBs up to five atoms of chlorine are those less recalcitrant to biodegradation (Borja et al., 2005) and they could be more rapidly consumed by the autochthonous PCB degrading microbiome, selected over time in highly and historically contaminated sites. In addition to organic pollutants, the correlation analysis identified different heavy metals and metalloids (*i.e.*, zinc, lead, arsenic and chromium) among the factors steering bacterial communities structure in the SIN Brescia-Caffaro soils, in agreement with previous results (Abdu et al., 2017; Ma et al., 2020). The toxicity of heavy metals and metalloids to microbes is due to protein denaturation and structural disturbance of membranes (Li et al., 2021) and depends on their speciation, which influences metal bioavailability in soil (Macci et al., 2013). Among soil characteristics, pH values influence heavy metal solubilization, being inversely correlated to the soil available concentrations of copper, zinc and lead (Ma et al., 2020). However, the SIN Brescia-Caffaro soils analyzed in this study were mostly

categorized as alkaline with few sub-alkaline ones and, accordingly, correlation analyses confirmed pH influence on bacterial community structure as neglectable.

In this study, we also quantified the putative aerobic PCB degraders (*i.e.*, soil bacterial populations harbouring the *bphA* gene), revealing an enrichment of putative PCB degraders over the total bacterial community in the more polluted topsoil layers. During a previous greenhouse experiment, higher hydrolytic activity and the selection of putative aerobic PCB degrading populations from soil microbiome were reported in response to biostimulation mediated by plant species (*i.e.*, *Medicago sativa*, *Festuca arundinacea, Cucurbita pepo* ssp. *pepo*) cultivated on the SIN Brescia-Caffaro polluted soil, which showed a decreased PCB concentration at the end of the trial (Terzaghi et al., 2019). In this framework, based on the current identification of autochthonous bacterial communities endowed with the highest bioremediation potential, it would be worthy to select specific soil plots in the SIN Brescia-Caffaro to realize a direct comparison of cultivated and control plots to measure the plant biostimulation effect in the topsoil under field condition. In fact, although contamination shifts are less sharp between different areas compared to those described according to depth, the data retrieved in this study from topsoils showed that hydrolytic activity and the abundance of putative aerobic PCB degraders changed according to the area of collection. In particular, the latter values were significantly lower in the topsoil of area T, possibly due to the lower average concentration of total PCBs occurring in this area. However, we did not detect a linear correlation between the relative abundance of putative aerobic PCB degraders and the concentration of PCBs (data not shown). This suggests that additional environmental factors play a pivotal role not only as driver of the overall bacterial community, but also in the selection of key populations for PCB degradation. For example, the higher abundance of plant secondary metabolites and other plant-derived compounds in the topsoil layers might explain the observed enrichment of *bphA* gene, which is involved in their degradation (Rolli et al., 2021). On the other

399 hand, the possible enrichment of microorganisms having degrading pathways different from the
400 biphenyl operon could be further elucidated by metagenomics sequencing analyses.

401 By means of 16S rRNA gene sequencing we showed at a fine resolution scale the influence of soil
402 depth both on the structure and the diversity of bacterial communities. ASV number retrieved in
403 this study are in line with the results of previous metataxonomic investigations on highly
404 contaminated soils (Guo et al., 2017; Hong et al., 2015). Bacterial diversity was lower in the most
405 polluted soils, in agreement with studies correlating Shannon and Chao1 diversity indexes with
406 metal concentrations (Ma et al., 2020). Pollutants and environmental factors influence the
407 community structure at taxonomic level, opening the possibility to identify specific taxa as microbial
408 indicators of contamination in a given context (Simonin et al., 2019). The main components of SIN
409 Brescia-Caffaro soils were bacterial phyla (*i.e.,* Proteobacteria, Acidobacteria, Bacteroidetes and
410 Actinobacteria) largely present in soil at global scale (Delgado-Baquerizo et al., 2018) and,
411 coherently with our results, were also highly abundant in soils characterized by a similar pollution
412 in a French abandoned wasteland (Girardot et al., 2020). Metatranscriptome analyses recently
413 indicated Actinobacteria among the putative key players in a phytoremediation trials for the
414 removal of organic pollutant and metals (Tartaglia et al. 2022). The phylum Chloroflexi was among
415 a second group of dominant soil phyla in the analysed soils and it includes key players in anaerobic
416 dehalogenation of PCBs (Wang et al., 2014). Given the presence of a high number of diverse
417 microhabitats in soil (Fierer, 2017), the presence of anaerobic micro-niches where dechlorination
418 could take place likely occur in the SIN Brescia-Caffaro soils and can notably contribute to the natural
419 attenuation processes. 16S rRNA gene analysis allowed the identification of six sequences
420 differentially distributed in soils according to depth and belonging to taxa previously indicated as
421 positively correlated to heavy metal concentration in soil, *i.e.*, *Stenotrophomonas* (Ma et al., 2020),
422 or including members described for soil remediation potential toward metals and organic

contaminants as in the case of *Bacillus* genus (Leigh et al., 2007; Ma et al., 2020). Limitations exist when functional diversity is inferred from 16S rRNA gene sequences datasets, and horizontal gene transfer mechanisms can particularly hamper drawing conclusions on degradation potential since genes involved in metal detoxification or organic pollutant degradation are often present on plasmids and other mobile genetic elements (Fierer, 2017), nonetheless the approach is validated to ascertain metabolic pathways at the community scale (Raes et al., 2021). In this work, we could not determine the correspondence with specific phylogenetic lineages, however we report that bacterial communities endowed with PCB degradation potential and resistance to different heavy metals stably occurred in the SIN Brescia-Caffaro soils.

4. CONCLUSIONS

In former agricultural fields of the highly and historically polluted SIN Brescia-Caffaro, topsoils showed the highest pollutant concentrations and were characterized by higher organic matter hydrolysis activity and enrichment of putative aerobic PCB-degrading bacteria populations. Heavy metal, metalloid and PCB concentrations, together with edaphic properties were identified as drivers of the bacterial community structure both along depth and in topsoils collected from different sites having a different contamination fingerprint. Our data suggest that the soil natural attenuation potential results, besides pollutant concentration, from the complex interaction of different environmental factors. The widespread detection of degradation potential in topsoil layers, where we retrieved the higher pollution level, indicates that the autochthonous bacterial communities of large and historically polluted sites like the SIN Brescia-Caffaro could be exploited within the rational design of soil reclamation strategies based on plant biostimulation. In this framework, future perspectives include metagenomics and metatrascriptomic studies to unravel the possible selection of specific degradation pathways in historically polluted sites characterized

by the co-occurrence of multiple classes of contaminants which were demonstrated to be among the drivers for soil bacterial succession.

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AUTHOR CONTRIBUTIONS

Francesca Mapelli: Methodology, Formal analysis, Investigation, Data Curation, Writing-Original Draft, Visualization, Supervision. **Lorenzo Vergani**: Formal analysis, Investigation, Data Curation, Writing- Reviewing and Editing, Visualization. **Sarah Zecchin**: Formal analysis, Resources, Writing-Reviewing and Editing. **Ramona Marasco, Eleonora Rolli, Elisabetta Zanardini, Cristiana Morosini**: Writing- Reviewing and Editing. **Simone Anelli, Paolo Nastasio, Stefano Armiraglio**: Resources. **Vanna Maria Sale**: Resources, Writing- Reviewing and Editing. **Giuseppe Raspa, Elisa Terzaghi, Antonio Di Guardo**: Formal analysis, Writing- Reviewing and Editing. **Sara Borin**: Conceptualization, Methodology, Writing- Reviewing and Editing, Supervision, Project administration, Funding acquisition.

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668

669 **TABLE**

670 **Table 1. Overview of pollutant presence in the SIN Brescia-Caffaro topsoils.** Average value of
671 pollutant concentrations is reported for A, R and T areas. As and Hg concentrations are indicated as
672 mg/kg. PCB chlorination family concentrations are indicated as µg/kg. MoCB: monochlorinated
673 biphenyls; DiCB: dichlorinated biphenyls; TriCB: trichlorinated biphenyls; TCB: tetrachlorinated
674 biphenyls; PeCB: pentachlorinated biphenyls; HxCB: hexachlorinated biphenyls; HpCB:
675 heptachlorinated biphenyls; OCB: octachlorinated biphenyls; NoCB: nonachlorinated biphenyls;
676 DCB: decachlorinated biphenyls.

	As	Hg	MoCB	DiCB	TCB	TrCB	PeCB	HxCB	HpCB	OCB	NoCB	DCB
A	89,26	3,54	0,51	8,98	68,36	27,69	198,65	291,94	295,32	142,28	26,16	920,27
R	43,33	2,52	0,68	7,57	32,28	14,52	106,72	219,91	151,51	67,69	20,05	764,72
T	53,75	3,33	0,47	5,59	35,64	11,14	122,04	171,23	132,87	70,34	14,15	217,22

677

678 **FIGURE LEGENDS**

679 **Figure 1. Map of soil sampling in the in areas A, R and T of SIN Brescia-Caffaro.** Location of the 9
680 sampling stations of the first sampling campaign (October 2014). Sampling was conducted down to
681 1-meter depth dividing soils in fractions corresponding to seven depth intervals (0-10, 10-20, 20-30,
682 30-40, 40-60, 60-80, 80-100 cm) (**a**). Location of the sixty-four sampling stations of the second
683 sampling campaign (March 2015). From each station, one soil sample was collected and
684 homogenized covering the 0-40 cm range of depth (**b**).

685 **Figure 2. Distribution of SIN Brescia-Caffaro soils collected down to 1-meter depth (first sampling**
686 **campaign) according to physical and chemical characterization, bacterial community profiling and**
687 **their correlation.** Canonical analysis of principal coordinates (CAP) of the soil physical and chemical
688 data according to the interaction of 'area' and 'depth' factors (**a**). Principal Coordinate Analysis
689 (PCoA) of soil bacterial communities (ARISA fingerprinting) illustrating the clustering of samples

according to site and depth of collection (**b**). Distance-Base Redundancy Analysis (dbRDA) visualizing the significant correlation ($p < 0.005$) between bacterial communities in soil samples collected at increasing depth down to 1 meter from soil surface and physical and chemical properties (**c**)and heavy metals and metalloid concentrations (**d**) (data sets are included in Supplementary Table 3). In all panels, filled circles indicate soil samples collected at 0-40 cm and empty circles indicate those collected at 40-100 cm. Pink, black, and blue colors indicate A, R and T areas, respectively.

Figure 3. Soil microbial activity and abundance of aerobic PCB degraders over total bacterial community. Results of the fluorescein diacetate (FDA) hydrolytic activity test (**a**-**c**) and evaluation of the relative abundance of PCB-degradative bacteria with respect to the total bacterial population, quantified through amplification of the *bphA* and 16S rRNA genes respectively (**b**-**d**). Data in the upper and lower panels refer to the first and second sampling campaigns, respectively. Asterisks in the lower panels indicate which area was significantly different compared to the other areas.

Figure 4. Distribution of SIN Brescia-Caffaro soils collected in the topsoil layers (second sampling campaign) according to physical and chemical characterization, bacterial community profiling and their correlation. Canonical analysis of principal coordinates (CAP) of the soil physical and chemical data (**a**). Principal Coordinate Analysis (PCoA) of soil bacterial communities (ARISA fingerprinting) illustrating the clustering of samples according to the area of collection (**b**). Distance-Base Redundancy Analysis (dbRDA) visualizing the significant correlation ($p < 0.005$) between bacterial communities in soil samples collected from different areas and physical and chemical properties(**c**) and heavy metals and metalloid concentrations (**d**) (data sets are included in Supplementary Table 4). In all panels: pink, black, and blue colors indicated areas A, R and T, respectively.

Figure 5. Bacterial community structure and taxonomy in the topsoils of three areas located in the SIN Brescia-Caffaro according to 16S rRNA gene Illumina sequencing. Principal Coordinate Analysis (PCoA) on the ASV distribution showing the soil clustering according to depth. Soil samples collected

714 at 0-10 cm are indicated by filled circles and those collected at 20-30 cm by empty circles. Pink,
715 black, and blue colors indicate A, R and T area, respectively (**a**). Bar charts analysis showing the
716 relative abundance of the main phyla associated with contaminated soils. 'Other' represent the 21
717 phyla that contributed on average less than 0.5% of the total bacterial communities (**b**).