



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

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

3

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18

19 **ABSTRACT**

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

43 **KEYWORDS**

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

45

46 **1. INTRODUCTION**

47 Microorganisms shape the environment they colonize through their physiology and metabolism,  
48 thus microbiome engineering has the potential to offer plenty of solutions for societal needs, as in  
49 the case of bioremediation strategies (Mapelli et al., 2017; Verstraete et al., 2007). However, the  
50 limited comprehension of bacterial communities' structure and dynamics in real context still  
51 hampers the development of novel microbiome-based solutions for environmental management  
52 (Bell et al., 2019). In particular, the response of bacterial communities to soil pollution and the  
53 identification of the environmental factors driving bacterial assembly can provide clues to steer the  
54 soil microbiome, enriching those populations endowed with the highest bioremediation potential.  
55 Bacterial communities are indeed primarily involved in the biodegradation processes and their  
56 composition, metabolic activity and degradation performances can be affected by several edaphic  
57 parameters, including soil texture, pH, organic matter content and contaminant fingerprint,  
58 concentration and bioavailability (Bell et al., 2013; Correa-García et al., 2021; Mukherjee et al.,  
59 2014). Many studies about microbiome selection factors in polluted soils refer to artificially  
60 contaminated and simplified systems (Chekol et al., 2004; Meggo and Schnoor, 2013; Qin et al.,  
61 2014, Halfadji et al., 2022), generating data (*e.g.*, degradation rates) useful as input parameters for  
62 fate modelling in the environment (Terzaghi et al., 2018). However, this approach does not  
63 contribute to fill in the gap of knowledge about the drivers of soil bacterial community structure  
64 and functionality in real contaminated sites. The National Priority Site for remediation (SIN) Brescia-  
65 Caffaro (Italy) is a vast historically contaminated site, characterized by high and uneven

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

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

87

## 88 **2. MATERIALS AND METHODS**

### 89 **2.1. Site description and soil sample collection**

90 The study sites are located inside the National Priority Site for remediation (SIN) Brescia-Caffaro  
91 (Italy). Soils were sampled in three former agricultural fields (*i.e.*, area A, area R and area T) where  
92 cultivation activities were banned in 2002 after the SIN establishment by the Italian Environment  
93 Ministry. Areas A, R and T are located south-west of the Caffaro plant, a former producer of PCB  
94 mixtures and other chlorinated chemicals. A detailed description of the site, including historical  
95 information on land use and cultivation was presented elsewhere (Di Guardo et al., 2020). The first  
96 sampling was conducted in the A, R and T areas along soil depth (October 2014). Nine stations were  
97 identified in the three areas (A1, A2, A3, R1, R2, R3, T1, T2, T3; Figure 1A). From each station, soil  
98 samples were collected by core drilling up to a depth of 100 cm, after removing the surface grass  
99 cover to limit the rhizosphere effect that can buffer the impact of edaphic factors on the soil  
100 microbiome structure (Mapelli et al., 2018). At each sampling station, three cores were collected  
101 within 1-meter distance and each core was then divided into fractions corresponding to seven  
102 intervals according to depth expressed in cm (0-10, 10-20, 20-30, 30-40, 40-60, 60-80, 80-100). For  
103 each sampling station (n=9), the replicated fractions of the same depth range of the three cores  
104 were merged, obtaining in total 63 samples, a procedure adopted to minimize the intrinsic soil  
105 heterogeneity (Di Guardo et al., 2020). A second independent sampling was performed (March  
106 2015) on a denser grid to cover the whole surface of the three sampling areas A (A1-A17), R (R1-  
107 R19) and T (T1-T28) (Figure 1B). Here, a total of 64 soil cores were collected to a depth of 40 cm,  
108 corresponding to the layer of soil mostly influenced by the former agricultural practices (*e.g.*, tillage)  
109 and characterized by higher contamination according to the chemical characterization of the soils  
110 collected during the first sampling (see the Results section). Topsoil samples (0-40 cm) were  
111 homogenized and, immediately after sampling, were stored at 4°C for hydrolytic activity analysis  
112 and at -20°C for chemical and molecular analyses.

113

## 114 **2.2. Soil physical and chemical analyses**

115 Soil chemical analyses were performed in outsource by a private company (Theolab S.p.A., now  
116 Mérieux NutriSciences, Volpiano TO, Italy). Soil pH, total nitrogen, total organic carbon, cation  
117 exchange capacity (CEC) and pollutant concentrations (*i.e.*, 79 PCB congeners and 11 metals and  
118 metalloids) were determined as respectively reported in Di Guardo et al. (2020) and Morosini et al.  
119 (2021). Polychlorinated biphenyl (PCB) concentration data were partially presented in a publication  
120 focused on the comparison of the different classes of PCB congeners, their distribution and  
121 movement across soil layers (Di Guardo et al., 2020). Samples were classified according to soil  
122 texture considering the silt, sand and clay contents and particle sizes, in agreement with the Soil  
123 Survey Manual (2017). Soils are defined as polluted according to the Italian legal threshold for  
124 agricultural areas (*i.e.*, PCBs: 0.02 mg/kg dry weight; As: 30 mg/Kg d.w.; Hg: 1 mg/Kg d.w.). Further  
125 information about the datasets generated through physical and chemical analyses and the statistical  
126 analyses applied for soil sample clustering are reported in the Supplementary Method 1.

127

## 128 **2.3. Evaluation of the soil hydrolytic activity**

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

133

## 134 **2.4. DNA extraction and quantification**

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



138

139 **2.5. Automated ribosomal intergenic spacer analysis (ARISA) and  $\beta$ -diversity of soil bacterial**  
140 **communities**

141 The automated ribosomal intergenic spacer analysis fingerprinting of 16S–23S ribosomal RNA  
142 (ARISA-PCR) was conducted on all soil samples using the primer set ITSF-FAM (5'-  
143 GTCGTAACAAGGTAGGCCGTA-3') and ITSReub (5'-GCCAAGGCATCCACC-3') in a PCR reaction mixture  
144 that contained 1X PCR buffer, 1.5 U of Taq DNA polymerase (Invitrogen), 0.2 mM (each) dNTPs, and  
145 0.25  $\mu$ M (each) primer in a final volume of 25  $\mu$ L (Cardinale et al., 2004). The mixture was held at  
146 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  
147 extension step at 72°C for 7 min (Mapelli et al., 2013). ARISA fragments were separated using the  
148 ABI3730XL genetic analyser applying the internal standard 1200-LIZ (Macrogen, Rep. of Korea) and  
149 the obtained fingerprints were analyzed using the Peak Scanner Software (Applied Biosystems).  
150 Information on raw data and statistical analyses is included in the Supplementary Methods 3.

151

152 **2.6. 16S rRNA gene sequencing and metataxonomic analysis of soil bacterial communities**

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

162

## 163 **2.7. Quantification of potential PCB-degrading bacteria by qPCR**

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

172

## 173 **3. RESULTS**

### 174 **3.1. Pollutant concentrations together with soil physical and chemical properties change with** 175 **depth and define soil bacterial community structure**

176 In this work, we initially characterized 63 soil samples collected down to 1 m depth from different  
177 sampling stations in area A (A1, A2, A3), area R (R1, R2, R3) and area T (T1, T2, T3), three former  
178 agricultural fields located inside the SIN Brescia-Caffaro (Figure 1A). According to the distribution of  
179 79 PCB congeners and 11 metal and metalloids detected in the samples (Supplementary Table 1)  
180 we identified the highest PCB and heavy metals (*e.g.*, Hg) concentrations in area A, particularly at  
181 the sampling station A1 (Supplementary Table 1). Physical and chemical parameters allowed the  
182 categorization of soils by CAP considering together the factors 'area' and 'depth' ( $\Delta_1^2$ :  
183 0.91894,  $p = 0.0001$ , Figure 2A) and 'site' and 'depth' ( $\Delta_1^2$ : 0.9992,  $p = 0.0004$ ) as the  
184 categorical explanatory variables of the analyses. Soil categories were further validated by  
185 PERMANOVA as indicated in Supplementary Table 5. According to SIMPER analysis, the soil diversity

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

192 Distinct clusters of bacterial communities could be identified in the analysed sampling sites at  
193 different depths, as shown by PCoA of the community profiles generated by ARISA fingerprinting  
194 (Figure 2B). PERMDISP analysis detected no statistical significance of the dispersion ( $p = 0.2826$ ),  
195 and the clustering of bacterial communities according to the interaction of the 'site' and 'depth'  
196 factors was confirmed by CAP ( $\Delta_1^2$ : 0.9971,  $p = 0.0013$ ) and PERMANOVA ( $F_{1,8} = 1.9615$ ;  $p =$   
197  $0.0001$ ) tests. A DistLM analysis was performed on the ARISA profiles using the physical and  
198 chemical data provided in Supplementary Table 2, where PCB congener concentrations were  
199 summed up according to the chlorination family (from mono- up to decachloro-biphenyls). DistLM  
200 sequential test recognised as significant driver of soil bacterial microbiota some of the considered  
201 physical and chemical parameters (DistLM, AICc = 405.22,  $R^2 = 0.30$ ; Supplementary Table 7),  
202 including those related to soil texture (*i.e.*, clay) and nutrient content (*i.e.*, total nitrogen) besides  
203 pollutant concentrations (*i.e.*, As, Hg, hexachloro-biphenyls). As shown by dbRDA, the pollutant  
204 concentrations played a key role in the separation of samples collected from sites A1, A2 and A3 at  
205 depth comprised between 0-40 cm (Figure 2C). Given the identification of As and Hg among the  
206 drivers of bacterial community composition, we performed a second DistLM analysis using all  
207 available metal and metalloid concentrations (Supplementary Table 1). The analysis confirmed the  
208 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

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

261

### 262 **3.4. Bacterial phylogenetic diversity and identification of possible marker taxa related to soil** 263 **contamination**

264 The bacterial community structure and taxonomy were further investigated by 16S rRNA gene  
265 sequencing to depict taxa distribution more in detail, focusing on topsoils sampled at 0-10 and 20-  
266 30 cm depth that correspond to the upper and most contaminated soil layers. 16S rRNA gene  
267 Illumina sequencing generated a number of non-chimeric denoised merged reads comprised  
268 between 67,318 and 115,766 (on average  $65,673 \pm 18,004$  reads per sample, Supplementary Table  
269 14), and a total of 934 ASVs were identified after classification at the 15<sup>th</sup> level of the SILVA  
270 taxonomic assignment (Supplementary Table 14). The rarefaction curves of the libraries were  
271 assessed, showing a coverage of more than 99% in all the samples (data not shown). Alpha-diversity  
272 indexes (*i.e.*, richness, Shannon diversity and dominance) were calculated and are indicated in  
273 Supplementary Table 15. Richness, expressed as the number of ASVs per sample, was not  
274 significantly different in soils collected from the areas A, R and T (ANOVA,  $F_{2,12} = 0.0975$ ;  $p = 0.9078$ )  
275 or at the 0-10 and 20-30 cm depth (ANOVA,  $F_{1,12} = 2.1816$ ;  $p = 0.1654$ ). On the opposite, bacterial  
276 community diversity, expressed as Shannon index, was significantly higher in soils collected at 0-10  
277 cm fraction compared to those at 20-30 cm (ANOVA,  $F_{1,12} = 7.0844$ ;  $p = 0.02073$ ) and coherently we  
278 detected a significantly lower dominance within the same bacterial communities (ANOVA,  $F_{1,12} =$

279 4.7928;  $p = 0.04907$ ). Following PERMDISP validation ( $p = 0.7806$ ), PCoA analysis applied to ASV  
280 distribution in the soil samples showed that bacterial communities were separated on the first axis  
281 according to depth of sampling (Figure 5A). CAP results demonstrated that bacterial communities  
282 could be significantly categorized according to depth ( $\Delta_1^2$ : 0.60738,  $p = 0.0338$ ).

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

301

#### 302 4. DISCUSSION

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

313 In addition, we outlined for the first time the bacterial community structure of one-hundred-twenty-  
314 seven soil samples collected on a broad x-y-z spatial grid to unravel the environmental drivers of soil  
315 microbiota diversity and function. The adopted molecular fingerprinting has been recognised as a  
316 valid and reliable method for the description of beta-diversity in complex microbial communities,  
317 including soil-dwelling ones, and remains a suitable tool for correlation analysis between biotic and  
318 abiotic datasets providing similar results to those obtained on high-throughput sequencing  
319 methods, though it has limitation in terms of richness detection (Gobet et al., 2014; van Dorst et al.,  
320 2014). Soil bacterial communities in this historically contaminated site were significantly different  
321 according to the collection area and depth. The role of depth on soil bacterial communities is known  
322 in natural ecosystems (Fierer et al., 2003) and depth can influence microbial community diversity  
323 also in agricultural soil, where crop roots play a selection effect on the bulk soil 'seed bank' far below  
324 the topsoil layer (Hao et al., 2021). The soils sampled in this study were located in former agricultural  
325 fields, where cultivation is forbidden since 2002 when the SIN Brescia-Caffaro perimeter was  
326 defined, and the plant cover is limited to meadow spontaneous species (Di Guardo et al., 2017).



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

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

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

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

377 In this study, we also quantified the putative aerobic PCB degraders (*i.e.*, soil bacterial populations  
378 harbouring the *bphA* gene), revealing an enrichment of putative PCB degraders over the total  
379 bacterial community in the more polluted topsoil layers. During a previous greenhouse experiment,  
380 higher hydrolytic activity and the selection of putative aerobic PCB degrading populations from soil  
381 microbiome were reported in response to biostimulation mediated by plant species (*i.e.*, *Medicago*  
382 *sativa*, *Festuca arundinacea*, *Cucurbita pepo* ssp. *pepo*) cultivated on the SIN Brescia-Caffaro  
383 polluted soil, which showed a decreased PCB concentration at the end of the trial (Terzaghi et al.,  
384 2019). In this framework, based on the current identification of autochthonous bacterial  
385 communities endowed with the highest bioremediation potential, it would be worthy to select  
386 specific soil plots in the SIN Brescia-Caffaro to realize a direct comparison of cultivated and control  
387 plots to measure the plant biostimulation effect in the topsoil under field condition. In fact,  
388 although contamination shifts are less sharp between different areas compared to those described  
389 according to depth, the data retrieved in this study from topsoils showed that hydrolytic activity and  
390 the abundance of putative aerobic PCB degraders changed according to the area of collection. In  
391 particular, the latter values were significantly lower in the topsoil of area T, possibly due to the lower  
392 average concentration of total PCBs occurring in this area. However, we did not detect a linear  
393 correlation between the relative abundance of putative aerobic PCB degraders and the  
394 concentration of PCBs (data not shown). This suggests that additional environmental factors play a  
395 pivotal role not only as driver of the overall bacterial community, but also in the selection of key  
396 populations for PCB degradation. For example, the higher abundance of plant secondary  
397 metabolites and other plant-derived compounds in the topsoil layers might explain the observed  
398 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

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

432

#### 433 **4. CONCLUSIONS**

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

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

449

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457

#### 458 **AUTHOR CONTRIBUTIONS**

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

468

469 **DECLARATION OF COMPETING INTERESTS.** The authors declare that they have no known  
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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

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

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

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

711 **Figure 5. Bacterial community structure and taxonomy in the topsoils of three areas located in the**  
712 **SIN Brescia-Caffaro according to 16S rRNA gene Illumina sequencing.** Principal Coordinate Analysis  
713 (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)**.