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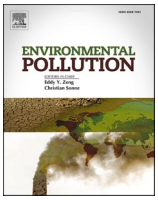

Impact of ionizing radiation on the environmental microbiomes of Chornobyl wetlands☆

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ABSTRACT

Radioactive contamination has the potential to cause damage to DNA and other biomolecules. Anthropogenic sources of radioactive contamination include accidents in nuclear power plants, such as the one in Chornobyl in 1986 which caused long-term radioactive pollution. Studies on animals within radioactive zones have provided us with a greater understanding of how wildlife can persevere despite chronic radiation exposure. However, we still know very little about the effects of radiation on the microbial communities in the environment. We examined the impact of ionizing radiation and other environmental factors on the diversity and composition of environmental microbiomes in the wetlands of Chornobyl. We combined detailed field sampling along a gradient of radiation together with 16S rRNA high-throughput metabarcoding. While radiation did not affect the alpha diversity of the microbiomes in sediment, soil, or water, it had a significant effect on the beta diversity in all environment types, indicating that the microbial composition was affected by ionizing radiation. Specifically, we detected several microbial taxa that were more abundant in areas with high radiation levels within the Chornobyl Exclusion Zone, including bacteria and archaea known to be radioresistant. Our results reveal the existence of rich and diverse microbiomes in Chornobyl wetlands, with multiple taxonomic groups that are able to thrive despite the radioactive contamination. These results, together with additional field and laboratory-based approaches examining how microbes cope with ionizing radiation will help to forecast the functionality and re-naturalization dynamics of radiocontaminated environments.

1. Introduction

Human activities are transforming natural ecosystems at an unprecedented rate (Steffen et al., 2007). Intense use and transformation of natural habitats over the last decades have had a severe impact on biodiversity (Palumbi, 2001, IPBES et al., 2019). Habitat destruction and fragmentation, climate alteration, invasive species, and the release of numerous pollutants into the environment are the main factors behind biodiversity decline (Rands et al., 2010). Pollutants, in particular, can affect species distribution and abundance, lead to the extirpation of the most susceptible ones, and alter biological functions, ecological networks and ecosystem services (Edwards, 2002).

Ionizing radiation can act as a rare but potentially devastating pollutant. This type of radiation is present in the environment at low levels as a natural phenomenon (e.g. cosmic and terrestrial radiation), and generally does not cause damage to living organisms. However, certain human activities, such as weapons testing and accidents at nuclear power plants, can involve releases of ionizing radiation above safety levels. Ionizing radiation may damage organic molecules, including DNA, and cause malfunctions in cell processes that lead to

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cellular and organismal death (Han & Yu, 2010; Reisz et al., 2014). Indeed, the effects of acute exposure to ionizing radiation are acknowledged to negatively impact organisms and ecosystems (Møller & Mousseau, 2006, 2015).

The accident at the Chornobyl nuclear power plant, on the April 26, 1986, led to the largest release of radioactive material in human history (UNSCEAR, 1988). An exclusion zone of ca. 4,700 km^2 was created around the power plant (Chornobyl Exclusion Zone, CEZ), to prevent human settlement in the area; these restrictions remain in effect. Exposure to the acute radiation levels generated by the Chornobyl accident caused a severe impact on the organisms in the area, including humans (Smith & Beresford, 2005; Møller & Mousseau, 2015). Studies on wildlife conducted after the accident reported that the radioactive contamination led to reductions in species diversity, multiple physiological costs, and increased DNA damage (Møller & Mousseau, 2006, 2015). However, the effects of ionizing radiation are far from generalized; while some studies reported negative consequences on wildlife populations currently living in the area (e.g. Beaugelin-Seiller et al., 2020), others have reported population recoveries (e.g. Deryabina et al., 2015; Schlichting et al., 2019), and signs of adaptation to the chronic exposure (e.g. Galván et al., 2014; Møller & Mousseau, 2016; Burraco & Orizaola, 2022). There is still an intense scientific debate about the long-lasting effects of chronic exposure to moderate levels of ionizing radiation on biodiversity (e.g. Møller & Mousseau, 2006; Beresford et al., 2016, 2020).

Microbial communities are crucial for maintaining ecosystem functions due to their role in the cycling, retention, and release of major nutrients and soil carbon (Gucht et al., 2007; Newton et al., 2011; McKenney et al., 2018). Chronic exposure to pollutants, including ionizing radiation, can compromise the diversity and composition of microbial communities (Chapin et al., 2000; Ager et al., 2010; Zhu & Penuelas, 2020). Furthermore, a host-associated microbiome is predominantly constrained by the microbes they can to recruit from their environment, and the composition and diversity of the resulting microbial community in the host can have important effects on their health (Liu et al., 2019). Changes in the composition of environmental microbiomes as a consequence of chronic exposure to radiation can therefore have indirect effects on local wildlife by changing the available symbionts present in the environment.

Despite the crucial ecological role of microbes in the environment, the impact of ionizing radiation on environmental microbiomes has not been comprehensively explored, and the Chornobyl accident represents an ideal opportunity in this regard (IAEA, 2006). Microbes are often considered to have a greater resistance to ionizing radiation than other organisms (ICRP, 2014). Some microbial taxa have been recovered from highly radio-contaminated environments, and, in some cases, their radioresistance capacity has been demonstrated under laboratory conditions (Ryabova et al., 2020). However, these studies have been restricted to a handful of taxa while the majority of environmental microbes have never been studied in relation to radiation, neither in the laboratory nor in their natural environment. Shortly after the Chornobyl accident, studies on soil samples from the central area of the Chornobyl Exclusion Zone reported a two-fold lower abundance in bacteria, compared to control non-contaminated areas outside the Zone (Romanovskaya et al., 1998; Yablokov, 2009). Some soil bacteria from the Chornobyl area were able to accumulate high concentrations of radioactive substances (e.g. ^{137}Cs), as in the case of *Agrobacterium* sp., *Enterobacter* sp., and *Klebsiella* sp. (Yablokov, 2009). Chapon et al. analyzed highly contaminated areas in the CEZ and identified a high diversity of soil bacteria using a mix of culturing techniques and sequencing tools (Chapon et al., 2012), whereas Hoyos-Hernandez et al. identified genes potentially associated with radiation resistance in prokaryotes (Hoyos-Hernandez et al., 2019). Recent studies have also examined the effects of radiation on the gut microbiome of wild vertebrates (e.g. Lavrinienko et al., 2018a,b; Antwis et al., 2021) and earthworms (Newbold et al., 2019). Therefore, despite some progress, information on the microbial communities present along the gradient of radioactive contamination in Chornobyl is still scarce. Acquiring this knowledge is not only relevant for evaluating the impact of radiation on microbes themselves, but also for further comprehensive assessments of the impact of radiation on multicellular organisms' host-associated microbiota.

In this study, we examined the role of radiation on the microbial communities in Chornobyl wetlands. Wetlands are essential for nutrient and water cycles, for climate regulation, as well as for a large number of aquatic and terrestrial plants and animals. We sampled pond water, sediment, and soil at multiple locations inside and outside the Chornobyl Exclusion Zone, and evaluated environmental variables that might have effect on the microbial composition. Specifically, our hypothesis were: a) long-term exposure to radioactive pollution may have affected the microbial diversity of Chornobyl wetlands, although it may have also driven the proliferation of bacteria resistant to radiation; b) the chronic exposure to radiation will have altered the structural and phylogenetic composition of the environmental microbiomes, and c) radioactive pollution may have affected some taxa more than others, and some radio-resistant taxa may be actually overrepresented in sites with high radiation. Understanding how ionizing radiation alters the microbial communities associated with these environments is crucial for a comprehensive evaluation of radio-contaminated ecosystems.

2. Material and methods

2.1. Field work

Sampling was conducted in Northern Ukraine, inside and outside the Chornobyl Exclusion Zone between the 29th of May and the June 3, 2019 (Fig. 1, Table S1). In total, we selected 21 permanent wetlands: 16 within the Chornobyl Exclusion Zone and 5 in a nearby control area with background radiation levels typical of the region (Fig. 1, Table S1). All the sampled wetlands shared similar characteristics: small to medium size wetlands with reed beds, situated within a matrix of forest and meadows on sandy soils (soddy-podzolized sandy and clay-sandy soils; Soil Map of Ukraine accessed from https://esdac.jrc.ec.europa.eu/content/title-russia-soil-map-ukraine). To examine the diversity and composition of microbial communities, at each site we collected samples from three *environment types*: water, pond sediment, and soil near the banks of the wetland. Each environment type was sampled at three randomly selected points within each site (distance between sampling points ranging 5–10 m) in order to maximize the chances of sampling scarce, localized, microbes at each site. In total, we collected 189 samples using tubed sterile Dryswab MW100 swabs with rayon tip. We collected water samples by swirling a swab on the water surface for 25 s in areas about 1-m depth, and about 2 m from the shore (similar to e.g. Walke et al., 2014). Sediment was collected at ca. 0.5 m depth with a sampler that removes the top 10 cm of sediment, and swabs were inserted into the sediment five times for 5 s each time. Soil samples were collected on land, 5–10 m from the water edge, by first removing the top 5 cm of soil and then twirling a swab inside the exposed soil for 15 s. The use of swabs allowed us to sample a standardized amount from different environments without the need to collect and transport large amounts of material, which is not advisable in radio-contaminated areas. The swabs were placed in individual plastic vials on-site and stored in a portable cooler until arrival at our laboratory in Chornobyl, where samples were stored in a fridge at 4 °C for three days. Finally, we transported the samples under low temperature to our laboratory at the University of Oviedo (Spain), where they were stored at −20 °C until further processing.

At each site, we measured common water chemical characteristics with a Hanna multiparametric portable meter HI9811-5: temperature, pH, total dissolved solids (TDS; ppm/mg/L), and electrical conductivity (EC; μS/cm), at the same three sampling points where we collected water and sediment samples. We measured radiation levels (in μSv/h)

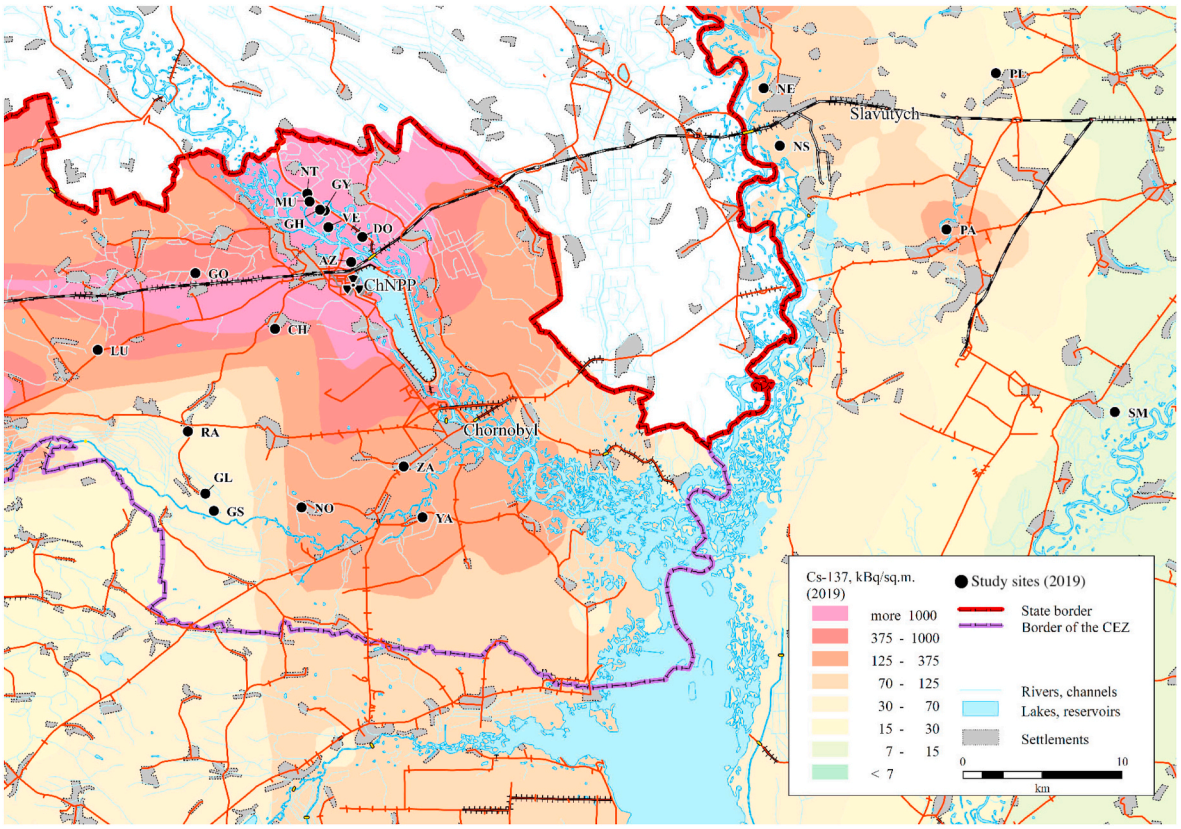

Fig. 1. Map showing the sampling sites in Northern Ukraine. Abbreviations refer to the location name (see Table S1 for details). The underlying ^{137}Cs soil data (decay corrected to spring 2019) are derived from the Atlas of Radioactive Contamination of Ukraine (Intelligence Systems GEO, 2011).

with an MKS-AT6130 handheld radiometer by placing the radiometer 5 cm above the sampled area at five points in each environment type and site: in water, measured at ca. 5 cm above the water surface in pond areas with ca. 0.5 m depth; in sediment, measured along the shoreline (defined as the water-land interface); and in soil, measured in the terrestrial environment, covering the pond surroundings and not further than 10 m from the shoreline (see Burraco et al., 2021 for a similar approach, Table S3). Ambient radiation levels in a given locality are repeatable among days and even years, once corrected by radioactive decay, and are an accurate proxy for assessing the real exposure to biota (Lavrinienko et al., 2018a; Antwis et al., 2021).

2.2. DNA isolation, library preparation, and sequencing

We used the DNeasy PowerSoil DNA isolation kit (Qiagen) to isolate DNA from soil and sediment samples, and the NZY Tissue gDNA isolation kit (NZYTech) to isolate DNA from water samples. These kits were recommended by the sequencing company to match the different substrates. We resuspended DNA in a final volume of 100 or 50 μL when Qiagen or NZY kit were used, respectively. We included an extraction blank in every DNA extraction round and treated it as a regular sample to check for cross-contamination.

For library preparation, we amplified a fragment of the bacterial 16S rRNA region (V4) of around 300 bp using the standard primers 515 F (5′ GTG YCA GCM GCC GCG GTA A 3′) (Parada et al., 2016) and 806 R (5′ GGA CTA CNV GGG TWT CTA AT 3′) (Apprill et al., 2015). We ran PCRs using a final volume of 25 μL, containing 2.5 μL of template DNA (except for 11 samples with low concentration for which we used 5 μL), 0.5 μM of the primers, 12.5 μL of Supreme NZYTaq 2x Green Master Mix (NZYTech), and ultrapure water up to 25 μL. The reaction mixture was incubated as follows: an initial denaturation at 95 °C for 5 min, followed by 25 cycles of 95 °C for 30 s, 46 °C for 45 s, 72 °C for 45 s, and a final extension step at 72 °C for 7 min.

For each sampled site, we pooled the three PCR replicates from each environment type (e.g. 3 × soil samples at each location). Soil sampling strategies typically combine multiple small samples from different locations or depths within the site of interest into a single homogenized sample that is then used for analysis (Ellingsøe & Johnsen, 2002; Ranjard et al., 2003). This procedure is done to minimize spatial variation among samples and incorporate the vast majority of the microbial diversity present in the target sample while maximizing the value of research funds. Meta-analyses have found no biases using this approach (Allen et al., 2021). Once pooled, we attached the oligonucleotide indices required for multiplexing in a second PCR with identical conditions as previously but during 5 cycles and using 60 °C as the annealing temperature (see Vierna et al., 2017). We included a negative control in every PCR run to check for contamination during library preparation. The libraries were run on 2% agarose gels stained with GreenSafe (NZYTech), and imaged under UV light to verify the library size. Libraries were purified using the Mag-Bind RXNPure Plus magnetic beads (Omega Biotek), and then pooled in equal concentrations according to the quantification data provided by a Qubit dsDNA HS Assay (Thermo Fisher Scientific). The pool was sequenced in a fraction of an Illumina NovaSeq paired end 250bp run by AllGenetics & Biology SL (A Coruña, Spain).

2.3. Data processing

We obtained a total of 3.7 M reads and evaluated the read quality using FastQC (Andrews, 2010) in combination with MultiQC (Ewels et al., 2016). We next used DADA2 (Callahan et al., 2016) implemented in QIIME2 (v. 2020.2; Bolyen et al., 2019) to remove PCR primers,

quality-filter reads, denoise, merge the pairs, remove chimaeric reads, and construct amplicon sequence variants (ASVs). Forward and reverse reads were truncated at position 249 before merging with a default minimum overlapping region of 12 identical base pairs. After these filtering steps there were 2.7 M reads in total. Taxonomy was assigned using a classifier trained on the SILVA reference database (Quast et al., 2013, release 138 December 2019), with the feature-classifier classify-sklearn approach, implemented in QIIME2 (Bokulich et al., 2018). A phylogenetic tree was constructed in QIIME2 (v. 2020.2), using MAFFT (Katoh & Standley, 2013) and FastTree2 (Price et al., 2010) for phylogenetic analyses.

The ASV table was imported into R (v. 4.0.2; R-Team-Core, 2020), and the packages *phyloseq* (v. 1.32.0; McMurdie & Holmes, 2013) and *vegan* (v. 2.5–6; Oksanen et al., 2019) were used for statistical analysis. From downstream analyses, we excluded the ASVs with a single read in the whole dataset (singletons), the completely unassigned sequences (no bacterial classification), and those from chloroplast and mitochondrial origin. We also removed ASVs occurring at a frequency below 0.01% in each sample to account for potential misassignments during library preparation and low-frequency contaminants. Furthermore, we used decontam (v. 1.6.0; Davis et al., 2018) to identify and eliminate 9 potential contaminating ASVs in the reagents by analyzing the blank samples that were sequenced simultaneously as negative controls. A high read depth of high-quality sequences (average number of reads per sample = 41,210) allowed us to safely rarefy the data to 30,000 reads without losing any samples or reducing statistical power.

2.4. Statistical analysis

We calculated Alpha diversity for the microbial communities in water, pond sediment, and soil using three different metrics: Richness (i.e. total number of unique ASVs), Shannon index (which takes into account both richness and evenness), and Faith's index (i.e. phylogenetic diversity). Beta diversity was measured with the Bray-Curtis distance metric, which accounts for both the presence/absence and abundance of microbes, and with unweighted UniFrac, which measures phylogenetic distances between microbes. We used *betadisper* and *adonis* in the R package *vegan* to calculate homogeneity of group dispersion and to perform PERMANOVAs to assess variation. Radiation levels (μSv/h), Total Dissolved Solids (TDS; ppm/mg/L), and Electrical Conductivity (EC; μS/cm) were log-transformed prior to analysis. Longitude and latitude values were included in the PERMANOVA to account for variation due to geographical distance. Finally, we used ANCOM with bias correction (ANCOM-BC, v. 0.99.1; Lin & Peddada, 2020) on each environment type (water, sediment, soil), to identify specific taxa that were associated with higher or lower radiation levels (continuous log-transformed radiation levels). In ANCOM-BC, we used a conservative variance estimate and accounted for site location within or outside

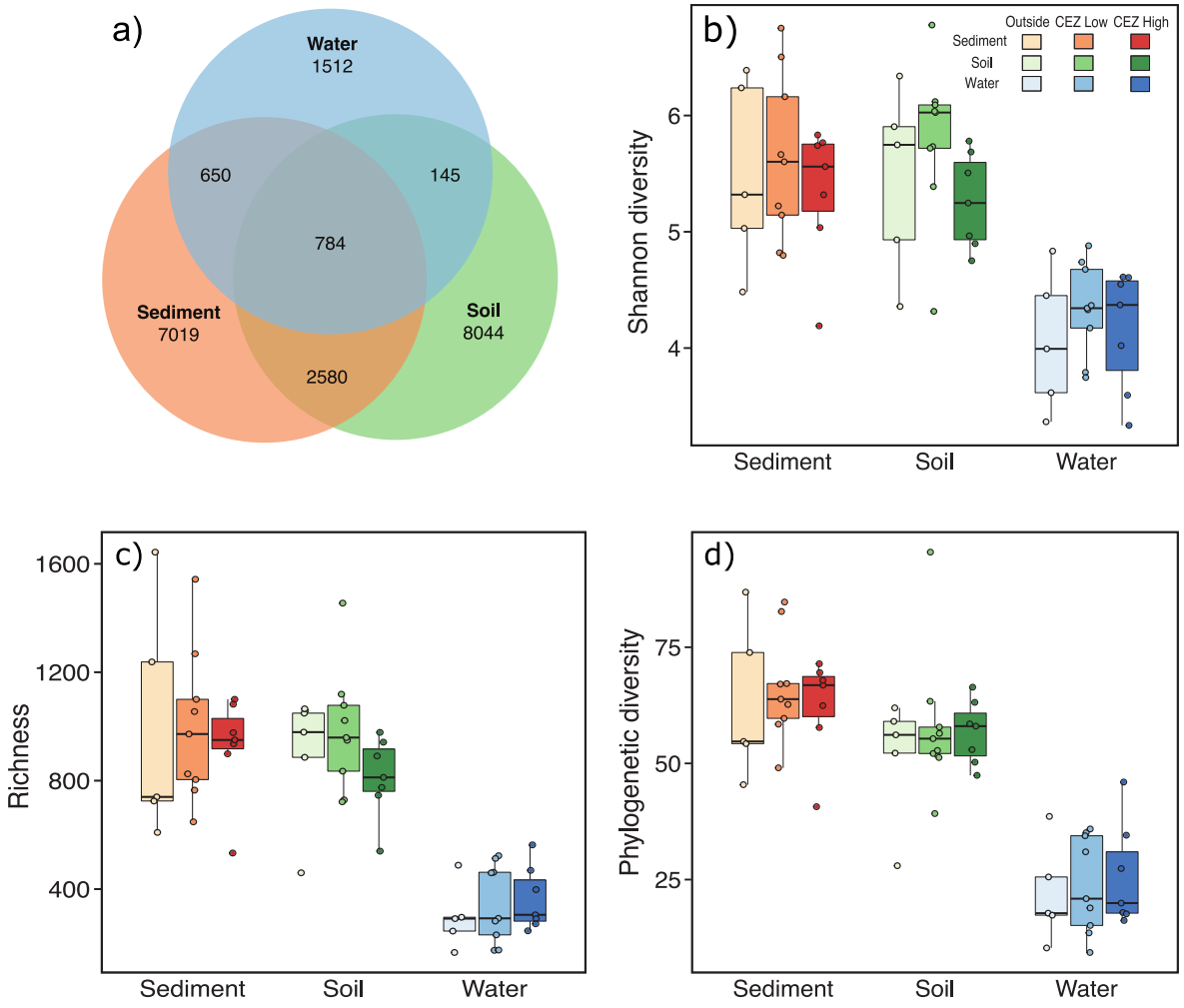

Fig. 2. Diversity of microbial communities in wetlands within and outside Chornobyl Exclusion Zone. (a) Number of unique and shared amplicon sequence variants (ASVs) in each environment type (all localities, inside and outside Chornobyl Exclusion Zone, combined), (b) Shannon diversity, (c) ASV richness, and (d) phylogenetic diversity.

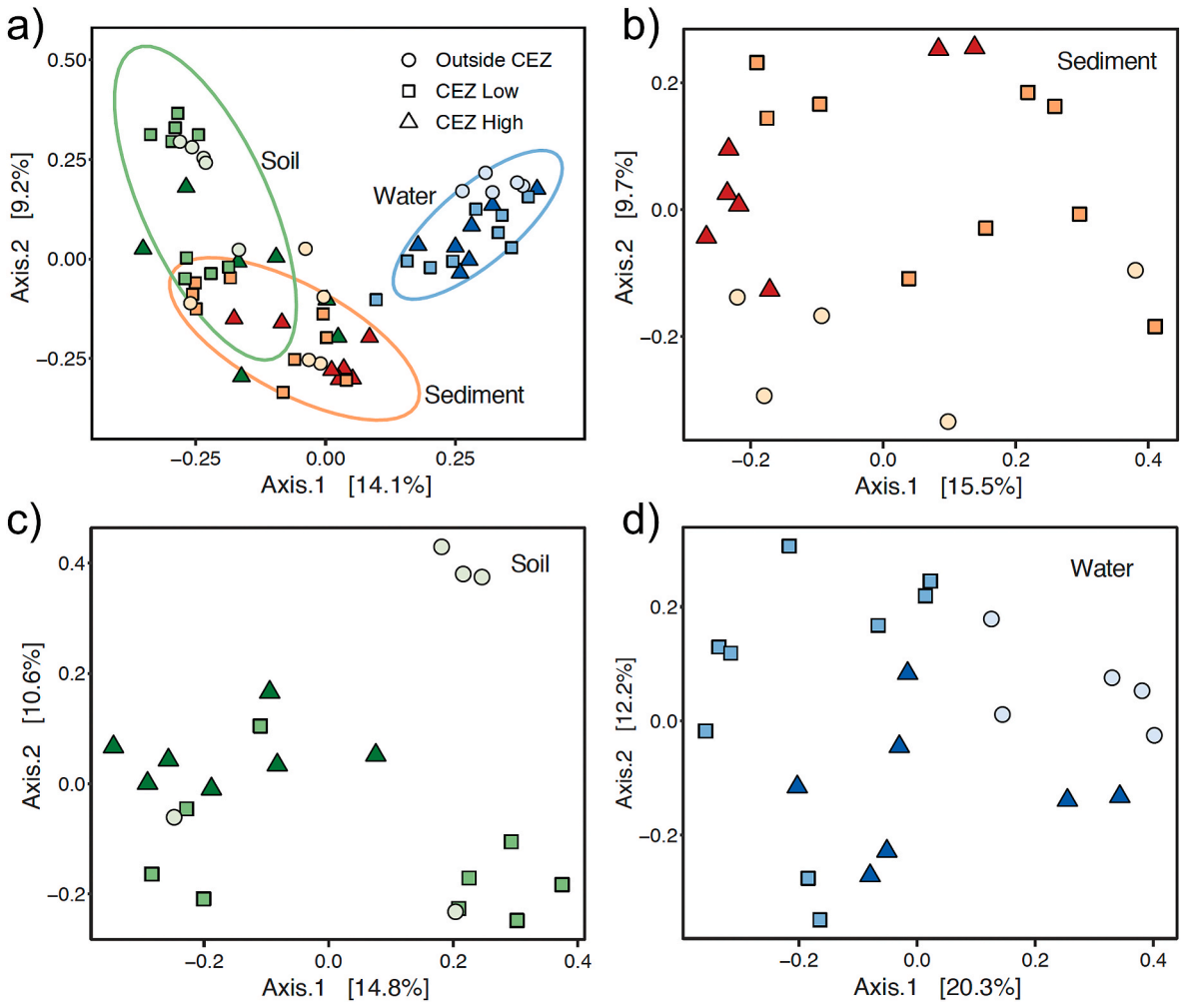

Fig. 3. Principal Coordinate Analysis (PCoA) on Bray–Curtis dissimilarity distances of the Chornobyl wetland microbiomes in (a) all samples colored by environment type; and each environment type: (b) sediment, (c) soil, and (d) water. Each point represents a locality and symbols show radiation category. Ellipses denote the 90% confidence intervals. Related plots of UniFrac distances can be found in Supplementary Figs. S2–S4.

the CEZ (group parameter). P-values in ANCOM-BC were corrected for multiple testing according to the default Holm method (Lin & Peddada, 2020).

For visualization purposes, sampling sites were assigned to three different areas regarding their location and radiation levels: *CEZ-high* for sites located inside the Chornobyl Exclusion Zone in environments with soil and sediment radiation levels >2.0 μSv/h; *CEZ-low* for sites located inside Chornobyl with radiation levels <0.5 μSv/h; and *Outside-CEZ* for sites outside Chornobyl, where radiation levels were <0.2 μSv/h (Table S1).

3. Results

We identified a total of 20,816 unique ASVs across the three environment types (surface water, pond sediment, and soil) from the 21 sampled sites. Soil and pond sediment had the highest numbers of ASVs (soil = 11,553, sediment = 11,033), whereas the water samples had much fewer ASVs (water = 3091; Fig. 2a). Despite the large diversity of microbes in both soil and pond sediment, the majority of ASVs was not shared across the sample types (Fig. 2a). Water had substantially lower Shannon diversity (25% lower) and richness (70% lower) than sediment and soil. There were no differences in alpha diversity among the sites located within the CEZ (high and low radioactivity) and outside the CEZ, for any environment type (sediment, soil, water) (ANOVA of Shannon index: sediment, $F_{2,16} = 0.29$, $P = 0.755$; soil, $F_{2,16} = 1.51$, $P = 0.252$; water, $F_{2,16} = 0.55$, $P = 0.588$; Fig. 2b). Similar results were obtained for ASV richness (sediment, $F_{2,16} = 0.12$, $P = 0.891$; soil, $F_{2,16} = 1.24$, $P = 0.315$; water, $F_{2,16} = 0.37$, $P = 0.698$; Fig. 2c) and Faith's phylogenetic diversity (sediment, $F_{2,16} = 0.18$, $P = 0.834$; soil, $F_{2,16} = 0.41$, $P = 0.668$; water, $F_{2,16} = 0.17$, $P = 0.847$; Fig. 2d). The effects of temperature and pH were not significant for any of these analyses ($P > 0.159$, in all cases). We also found no correlation between radiation levels and alpha diversity in either of the three sample types (Spearman's correlation test: $P > 0.32$, in all cases; Fig. S1).

The composition of environmental microbiomes differed substantially between the three environment types ($R^2 = 17.5\%$, $P < 0.001$) with the water microbiome being the most different (Fig. 3a). Analyses of sources of variance for each of the sample types using Bray-Curtis and UniFrac distances showed that radiation had the largest effect of all variables measured on the composition of all three environmental microbiomes (Table 1, Fig. 3b–d). However, the explained variance in the first two PCoA axes was relatively low, suggesting that other environmental variables, not measured here, may also explain part of the observed variance. Radiation significantly influenced the microbial membership and relative abundance in sediment (Bray-Curtis: $R^2 = 6.8\%$; Fig. 3b) and soil (Bray-Curtis: $R^2 = 7.5\%$; Fig. 3c). However, in water, radiation primarily affected the phylogenetic composition of the microbiome (UniFrac: $R^2 = 7.8\%$; Fig. 3d). Radiation also showed strong significant effects on the phylogenetic composition of soil ($R^2 = 7.2\%$, $P = 0.004$), but not sediment ($R^2 = 6.3\%$, $P = 0.09$; Table 1). Longitude

Table 1
Permanova of the effects of radiation and environmental factors on the three types of environment microbiomes. Bray-Curtis dissimilarities (top) and UniFrac distances (bottom) are reported. TDS = Total Dissolved Solids, EC = Electrical Conductivity.

	Sediment			Soil			Water		
	F	R^2	*P*	*F*	R^2	*P*	*F*	R^2	*P*
Bray-Curtis									
Radiation	1.46	**0.068**	**0.045***	1.58	**0.075**	**0.018***	1.50	0.063	0.079
Longitude	1.72	**0.080**	**0.010****	1.05	0.050	0.359	2.01	**0.084**	**0.007****
Latitude	1.08	0.050	0.319	1.29	0.062	0.072	2.13	**0.089**	**0.003****
Temperature	0.92	0.043	0.607	1.32	0.063	0.063	1.17	0.049	0.225
pH	1.14	0.053	0.211	1.08	0.051	0.302	1.21	0.051	0.187
TDS	1.29	0.060	0.097	0.80	0.038	0.881	1.87	**0.078**	**0.011***
EC	0.89	0.041	0.671	0.81	0.039	0.836	0.98	0.041	0.450
UniFrac									
Radiation	1.29	0.063	0.087	1.48	**0.072**	**0.004****	1.68	**0.078**	**0.011***
Longitude	1.19	0.058	0.132	1.12	0.055	0.136	1.12	0.052	0.221
Latitude	0.82	0.040	0.860	1.09	0.053	0.182	1.06	0.050	0.319
Temperature	0.90	0.044	0.638	1.05	0.051	0.256	1.12	0.053	0.240
pH	0.95	0.047	0.544	0.95	0.046	0.633	1.35	0.063	0.076
TDS	1.14	0.056	0.186	0.88	0.043	0.874	1.01	0.047	0.419
EC	1.07	0.053	0.276	1.00	0.049	0.449	1.07	0.050	0.297

and latitude had a significant effect on the water microbiome in particular, but only with Bray-Curtis dissimilarities (R^2 = 8.4% and 8.9%; Table 1), and this was true for dissolved solids as well (R^2 = 7.8%; Table 1). All group dispersion tests (function *betadisper* within vegan package) discarded any significant heterogeneity of environment microbiomes ($P > 0.1$), indicating that the beta diversity differences in Table 1 and Fig. 3 could not be explained simply by sample dispersion.

The taxonomic analysis of environmental microbiomes showed that sediment and soil communities had a diverse composition consisting of multiple abundant phyla, with Proteobacteria as the most abundant group, overall. Actinobacteriota were more abundant in soil and Desulfobacterota more common in sediment (Fig. 4). Water communities consisted almost exclusively of Proteobacteria and Bacteroidota (Fig. 4). The effects of radiation on specific members of the microbiomes evaluated using ANCOM-BC revealed several microbes with higher abundances in the localities with higher radioactivity (Fig. 5). The majority of the taxa that were positively associated with radiation levels were unique to their respective environment microbiome; for example,

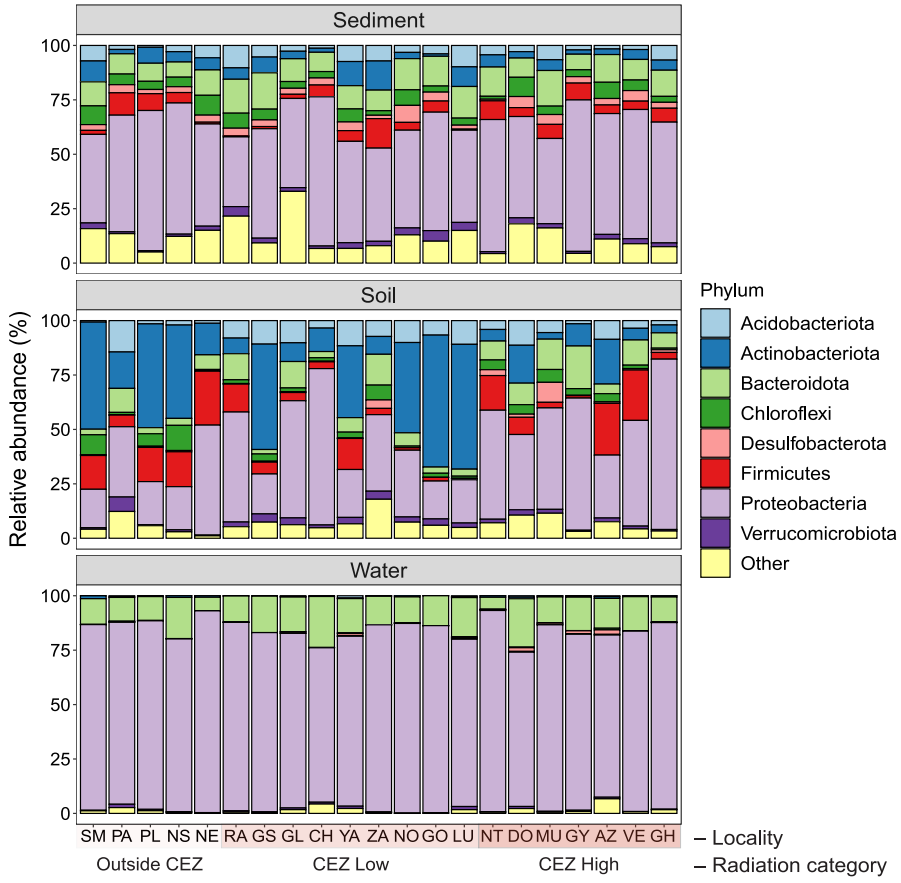

Fig. 4. Taxonomic composition of Chornobyl wetland microbiomes in sediment, soil, and water. Localities (x-axis) are arranged according to radiation category with the right-most localities (CEZ High) having the highest radioactivity.

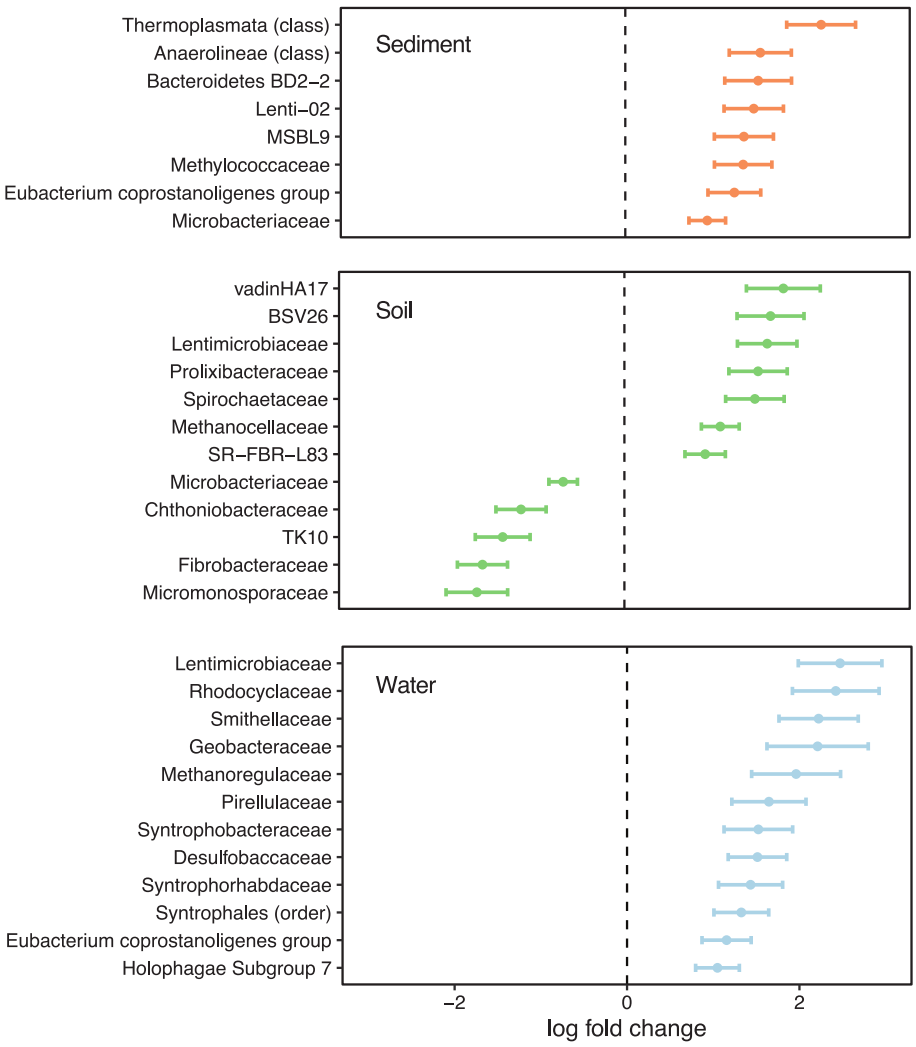

Fig. 5. Differentially abundant taxa in Chornobyl wetland microbiomes in response to radiation levels in sediment, soil, and water. The error bars show the unstandardized effect size (beta) ± standard error (SE). The Y-axis lists significantly differentially abundant microbial families (or the closest taxonomic order assigned). Positive log fold change indicates higher abundance in localities with higher radioactivity. For further details see Supplementary Table S2.

Prolixibacteraceae in soil, *Methylococcaceae* in sediment, and *Rhodocyclaceae* in water. However, some taxa were differentially abundant across sample types, e.g. *Lentimicrobiaceae* was more abundant at the high-radiation localities in both soil and water microbiomes, and *Eubacterium coprostanoligenes* group was more abundant at the high-radiation localities in both sediment and water. Families characterized by higher abundance in high-radiation areas also included *Anaerolineae* and Thermoplasmata in sediment or *Smithellaceae* and *Geobacteraceae* in water; whereas families with lower abundances in high-radiation soil microbiomes included *Micromonosporaceae*, *Microbacteriaceae*, TK10, *Fibrobacteraceae*, and *Chthoniobacteraceae* (Fig. 5).

4. Discussion

Chornobyl wetlands maintain rich and diverse microbial communities three decades after the accident in the nuclear power plant. Our study reveals that, overall, the diversity of microbial communities in pond sediment, soil, and water were similar between wetlands sampled inside and outside the Chornobyl Exclusion Zone, and that the alpha diversity parameters were not influenced by radiation levels. However, the composition and phylogeny of the microbial communities in Chornobyl wetlands were affected primarily by radioactivity, among the examined factors. In addition, as predicted, we discovered several microbes with higher abundance in sites with high radiation levels. Understanding how microbial communities are influenced by radioactive contamination is crucial to forecast the future development of the ecosystems in the Chornobyl area, as well as for evaluating the impact of radioactive substances in the environment.

Our study presents the most comprehensive assessment of environmental microbial communities in areas affected by the Chornobyl accident to date, reporting more than 20,000 unique ASVs. It effectively builds upon previous studies of the area, many of which used other techniques to identify microbes or were restricted to small areas within Chornobyl (Chapon et al., 2012; Theodorakopoulos et al., 2017; Lavrinienko et al., 2018a, b; Hoyos-Hernandez et al., 2019). By using metabarcoding techniques and extensive field sampling, we found substantial differences between environments, with very high microbial richness and diversity across all the examined soil and sediment microbiomes. These results corroborate the findings of the Earth Microbiome Project (EMP), where sediment and soil microbiomes were shown to vastly outnumber all other free-living microbial communities in terms of bacterial richness (Thompson et al., 2017). Similar to the EMP, our water microbiomes had much lower richness and differed in community composition, although this could potentially be influenced by the use of a different extraction protocol for water. Stark microbial differences across environment types are not surprising given their highly differentiated characteristics (e.g. microclimate and environmental components).

Microbial diversity of the studied sites did not change across the gradient of radioactive contamination, or between samples collected inside Chornobyl and in areas with background radiation levels outside the Exclusion Zone. This finding agrees with two previous studies that sampled biofilm communities and bacteria in trenches for radioactive waste disposal in Chornobyl, both reporting that the diversity of bacteria did not change between sites with high and low levels of radiation, or even when compared with remote non-contaminated areas, although these studies were only able to examine a low number of OTUs (Ragon et al., 2011; Chapon et al., 2012). A small localized study found more bacterial OTUs in the high-radiation samples than in the low-radiation samples (Theodorakopoulos et al., 2017). Other studies, however, have reported a lower diversity of bacteria from the most highly radio-contaminated sites. For example, the diversity of some cultured bacteria sampled in the 10-km zone around the Chornobyl Nuclear Power Plant was two orders of magnitude lower than in control non-contaminated areas (Romanovskaya et al., 1998). Similarly, the diversity of soil bacterial communities was lower in samples from the most highly radio-contaminated areas within Fukushima (Ihara et al., 2021). There are several possible factors behind this large variation in microbial diversity results across studies. The combination of a metabarcoding technique including high-throughput sequence data (Illumina NovaSeq) with a large environmental sampling within the Chornobyl Exclusion Zone allowed us to conduct a comprehensive evaluation of the microbial communities in the area. This methodological implementation led to a massive improvement in the detection of microbes compared to previous studies, including lineages that are rare or hard to culture (Woo et al., 2008; Joos et al., 2020). In addition, the microbial communities in the area are likely to differ between wetlands and terrestrial environments, which have been targeted in previous studies (e.g. Romanovskaya et al., 1998; Chapon et al., 2012; Theodorakopoulos et al., 2017). More remarkably, over thirty years have passed since the accident in Chornobyl, and thus chronic exposure to low-dose radiation may have facilitated the proliferation of bacteria resistant to radiation (already suggested by Zavilgelsky et al., 1998). This would explain why some of the studies conducted closer in time to the nuclear accidents in Chornobyl (Romanovskaya et al., 1998) or Fukushima (Ihara et al., 2021) found lower bacterial diversity measures. Bacterial adaptation to radioresistance can result from relatively small genetic changes affecting DNA repair and metabolic functions (DeVeaux et al., 2007; Harris et al., 2009; Byrne et al., 2014), and these evolutionary responses are much more likely to appear with increasing time after radiation exposure.

The structure and phylogenetic composition of the microbial communities in Chornobyl wetlands were mainly affected by radiation, among the studied factors. Overall, soil communities were characterized by a higher relative abundance of Actinobacteriota, a phylum typically dominant in this environment (Hill et al., 2011), abundant also in previous studies in Chornobyl (Theodorakopoulos et al., 2017). Water communities were dominated by Proteobacteria and Bacteroidota, two of the most common phyla of bacteria in freshwater environments (Newton et al., 2011). When examined using Bray-Curtis dissimilarity, the composition of microbial communities in water was affected by latitude, longitude, and also by the amount of dissolved organic matter, which has been previously recognized as potential driver of bacterial community structure (e.g. Judd et al., 2006). Although pH has been shown to be a key determinant of the composition of bacteria communities in Chornobyl (Newbold et al., 2019) and elsewhere (e.g. Griffiths et al., 2011; Zhalnina et al., 2015), we did not detect any significant effect of pH. When evaluated using UniFrac phylogenetic distances, the effect of radiation was significant in soil and water, indicating that radiation has a notable effect on the phylogenetic composition of the microbiome in these environments.

The main influence of radiation on the microbial communities of Chornobyl was detected when examining the taxonomic composition of the different microbiomes. In particular, we identified several microbes with higher abundances in sites with higher radioactivity. Among them, many taxa are reported as common in radioactive environments, with some being able to reduce uranium and other radioactive metals. For example, several of the taxa that were more abundant in water with high radiation levels included *Rhodocyclaceae*, *Smithellaceae*, *Geobacteraceae*, Synthrophales and *Desulfobaccaceae*. *Rhodocyclaceae* is a family that includes UV-radiation resistant members (Han et al., 2020) and has been detected also in the Handford 300 area, a former complex for radioactive fuel manufacture (Converse et al., 2015). *Geobacteraceae* are metal-reducers that have been frequently found in areas rich in uranium (Suzuki et al., 2005; Simonoff et al., 2007; N'Guessan et al., 2010; Zachara et al., 2013; Sutcliffe et al., 2018). *Smithellaceae*, *Desulfobaccaceae* and Synthrophales have also been detected in disposal sites for liquid radioactive waste and experimental areas with radionuclide contamination (Nazina et al., 2010, Vikman et al., 2019; Gihring et al., 2011). In our study, among the microbes that were more abundant in high radiation sediment samples were *Anaerolineae*, *Microbacterium* and the archaea Thermoplasmata. These three taxa have also been previously associated with uranium-rich soils (Mondani et al., 2011), groundwater from nuclear waste depositories (Nedelkova et al., 2007), and radioactive legacy sites (Vazquez-Campos et al., 2021), respectively. A uranium-tolerant strain of Microbacterium has been also isolated from Chornobyl soil (Gallois et al., 2018). *Prolixibacteraceae,* which were associated with high soil radiation levels in our study, have also been detected in bogs with high levels of radioactive selenium, cesium, thorium and uranium (Lusa & Bomberg, 2021). Several of the groups that were abundant in high radiation soils, e.g. *Lentimicrobiaceae*, are slow-growing bacteria associated with polluted environments. Radioresistant bacteria often have slow growth cycles, diverting their resources from growth to DNA repair and stress resistance mechanisms (Zakrzewska et al., 2011). Different studies have revealed that the activation of genes linked to DNA repair or the cellular response to oxidative damage is often crucial for the survival of microbes exposed to ionizing radiation (e.g. Byrne et al., 2014. Jung et al., 2017). For many of the other taxa identified in the study, however, we still have little information about their ecological requirements, in particular any potential resistance to radiation. Further field and empirical studies on the effects of radioactivity on specific microbial taxa are therefore necessary.

5. Conclusions

We detected rich and diverse microbial communities in the wetlands of the Chornobyl Exclusion Zone, and the structure of these communities was mainly shaped by radiation levels, among the studied factors. Multiple bacterial and archaeal taxa had higher abundance in the high-radiation sites, many of which were previously reported to thrive in other areas with high radioactivity. By using detailed field sampling together with metabarcoding and high-throughput sequencing, our study significantly contributes to our understanding of the drivers that affect the diversity and composition of microbial communities in radio-contaminated environments. The host-associated microbiomes of multicellular organisms (plants and animals) that live in these highly radioactive areas may be directly sourced from the microbial communities present in the local environment (as suggested by Jones et al., 2004). Thus, an improved knowledge of how environmental microbiomes respond to a gradient of radioactivity is therefore needed to estimate the future functionality and re-naturalization potential of radio-contaminated environments. On a more broader perspective, understanding the long-term effects of radioactive pollution in natural systems is crucial to accurately forecast the putative environmental impact of the accidental or intentional release of radioactive substances to the environment.

Author contributions

G.O. conceived the study. P.B. and G.O. conducted field surveys and sample collection. E.V. analyzed the data. E.V. and G.O. wrote the manuscript with significant contributions from P.B.

Declaration of competing interest

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

Data availability

Detailed information on all sampling sites is available in the supplementary information. The sequence reads have been uploaded to EMBL-EBI ENA under the accession number PRJEB50310.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.121774.

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