Modified clays alter diversity and respiration profile of microorganisms in long-term hydrocarbon and metal co-contaminated soil

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Summary
Clays and surfactant-modified clays (organoclays) are becoming popular as pollutant sorbents due to their high reactivity and low-cost availability. However, the lack of field testing and data on ecotoxicity limits their application. Considering such aspects, this study assessed the impact of clay amendments to polycyclic aromatic hydrocarbons (PAHs)/cadmium (Cd)-contaminated soil on microbial respiration profiles (active vs. inactive cells) using redox staining and the relative abundance and diversity of bacteria and archaea. These clay products are bentonite, cationic surfactant-modified bentonite and palmitic acid-grafted surfactant-modified bentonite. After 70 days, the addition of bentonite and its modified forms altered microbial community structure mainly among dominant groups (Actinobacteria, Proteobacteria, Firmicutes and Chloroflexi) with effects varying depending on material loading to soil. Among amendments, fatty acid (palmitic acid) tailored cationic surfactant-modified bentonite proved to be microbial growth supportive and significantly increased the number of respiration-active microbial cells by 5% at a low dose of material (e.g. 1%). Even at high dose (5%), the similarity index using operational taxonomic units (OTUs) also indicates that this modified organoclay-mixed soil provided only slightly different environment than control soil, and therefore, it could offer more biocompatibility than its counterpart organoclay at similar dose (e.g. cationic surfactant-modified bentonite). This study promotes designing ‘eco-safe’ clay-based sorbents for environmental remediation.

Introduction
Material-based remediation of soil contaminated with PAHs and metals (e.g. Cd) is well documented at laboratory and field scale (Lohmann et al., 2005; Qu et al., 2008; Chen and Yuan, 2011; Zhang et al., 2013). In most cases, sorption of contaminants is the focus, but the impact of these sorbents on native soil microbial communities remains unclear. Research has shown that the direct application of these materials (e.g. biochar, zeolite, red mud) may alter microbial community structure (Pietikäinen et al., 2000; Garau et al., 2007; Ahmad et al., 2016), and thus, the use of soil amendments may impact microbial community function, biodegradation potential and ecosystem health.

Clay and modified clays are potential sorbents for remediating both metal and organic contaminants from soil and sediment (Chaerun Siti et al., 2005; Yuan et al., 2013). In particular, bentonite and its organically modified products (i.e. organoclays) are useful materials for environmental remediation (de Paiva et al., 2008; He et al., 2014). However, different cationic surfactants often used for preparing organoclays have the potential to exert toxicological effects on microorganisms (Ying, 2006). This could limit the application of these organoclays because microbial degradation is an environmentally friendly, efficient and cost-effective strategy for remediating contaminants, especially PAHs (Xiong et al., 2008; Megharaj and Naidu, 2017). However, toxicity may vary depending on the type of surfactant utilized. For example, Arquad® 2HT-75-modified bentonite (AB) was less toxic to soil (micro)organisms than hexade- cytrimethylammonium and octadecytrimethylammonium-modified counterparts (Sarkar et al., 2013), but more
and redox-based respiration (active is necessary. Coupling 16S rRNA sequence analysis and redox indicator, which did not provide a comprehensive assessment of microbial health and vitality (Abbate et al., 2013). The presence of organoclays may reduce the metabolic activity of particular microbial groups, whilst stimulating the growth of others that are more adaptable to material supplements (Abbate et al., 2013). In the aforementioned, microbial metabolic profiles in organoclay-amended compost were investigated using single redox indicator, which did not provide a comprehensive assessment of microbial health and vitality (Abbate et al., 2013).

To understand the fate and function of microbial communities associated with PAH biodegradation and Cd resistance, a comprehensive assessment of soil microbial DNA, diversity and active respiration profiling is necessary. Coupling 16S rRNA sequence analysis and redox-based respiration (active vs. inactive cells) of microbial populations in clay-amended field-contaminated soil would provide an approach for studying the effect of (un)modified clays on PAH-degrading microbial communities (Bowsher et al., 2019). As a consequence, the aim of this study was to evaluate the impact of a surface-tailored organoclay and its parent clays on native microbial activity in a long-term PAH/Cd-contaminated soil. This was achieved using soil microcosms incubated over a 70 days time course period and the assessment of (i) microbial relative abundance and diversity using 16S rRNA and (ii) active respiration i.e., metabolically active microbial cells using fluorescence technology.

Results

Microbial DNA mass in soil

At the end of the 70 days incubation period, microbial DNA concentrations were significantly higher ($P < 0.05$) in soil supplemented with ABP compared with soil without clay amendments (NC hereafter), with unmodified clay (B) or organoclay (AB) (Fig. 1).

In the control (PAH/Cd-contaminated soil without clay amendments), the concentration of soil DNA was 22.43 ± 1.04 µg g$^{-1}$ soil. Similarly, the addition of B or AB to soil (clay loading 1%) resulted in significantly higher DNA concentrations compared with unamended soil ($P < 0.05$) (B = 30.82 ± 0.77 µg DNA g$^{-1}$ soil; AB = 41.41 ± 2.01 µg DNA g$^{-1}$ soil). However, in the presence of ABP, DNA of soil native microorganisms was three- to fourfold higher compared with the control and other clay-treated soils (ABP = 95.73 ± 4.0 µg DNA g$^{-1}$ soil) ($P < 0.05$). At a higher ABP loading (5%), DNA concentrations increased to 134.58 ± 3.90 µg DNA g$^{-1}$ soil ($P < 0.05$), whereas when the parent product (AB, 5%) was assessed, there was no significant increase in DNA concentration ($P > 0.05$) (Fig. 1).

Metabolically active microorganisms in clay-amended soil

Metabolic profiles indicate that the addition of clay and clay products at the lower loading (1%) increased metabolically active microbial cells (35.41 ± 3.87, 31.42 ± 1.89 and 28.54 ± 5.0% in B, AB and ABP-amended soil, respectively, vs. 23.56 ± 2.28% in the control soil) (Fig. 2). However, when clay loading was
increased to 5%, the percentage of active cells decreased irrespective of types of clay amendment (17.14–24.07% active cells). The addition of AB at 5% had the greatest impact on microbial metabolic activity (17.14 ± 3.54%), which was significantly lower than its counterparts (B and ABP) at the corresponding application rate \((P < 0.05)\). In contrast, although lower, the metabolic impact of ABP at 5% was not significantly
different (23.81% in ABP treatment) to the unamended control soil (Fig. 2).

The relative abundance of soil microorganisms

Phylum with the relative abundance of > 2% will be reported in this section, whilst those less than that are listed in the Supporting Information (Fig. S1). The major bacterial taxa in the PAH/Cd-contaminated field soil were Actinobacteria, Proteobacteria, Firmicutes and Chloroflexi, accounting for 94.84% of the total microbial population (archaea and unassigned bacteria accounted for 0.04%). Among these taxa, Proteobacteria was the dominant group (39.85 ± 0.43%), followed by Firmicutes (28.85 ± 0.43%), Actinobacteria (20.11 ± 1.24%) and Chloroflexi (6.04 ± 0.37%). Whilst these groups contributed relative abundance of 94.84 ± 0.76%, the addition of B at the low dose (1%) increased the relative abundance to 96.60 ± 0.40% mainly by the addition of Gemmatimonadetes, which further increased (99.00 ± 0.70) at its higher loading (5%) by the contribution of Bacteroidetes. Although the application of AB and ABP did not increase the relative abundance of the total microbial population (i.e. values ranged between 95 and 97%), specific bacterial groups shifted significantly as a consequence of these soil amendments ($P < 0.05$). For example, Actinobacteria (33.99 ± 0.13%) was the dominant microbial group followed by Proteobacteria (27.47 ± 0.82%) and Firmicutes (26.27 ± 0.61%) when the raw clay (B, 1%) was used as the soil amendment ($P < 0.05$). The relative abundance of these groups changed (Proteobacteria (39.04 ± 0.81%) > Actinobacteria (27.27 ± 0.49%) > Firmicutes (22.14 ± 0.46%)) in the presence of AB1% (Fig. 3) ($P < 0.05$). In contrast, ABP induced a significant increase in Actinobacteria that shifted microbial abundance to Actinobacteria (38.45 ± 0.53%) > Proteobacteria (32.45 ± 0.76%) > Firmicutes (17.82 ± 0.85%) ($P < 0.05$).

When soil was amended with 5% clay products, the relative abundance of microbial groups was as follows (high to low): Actinobacteria > Proteobacteria > Firmicutes for B and AB whereas
Dance and habitat sharing of OTUs relate their distance in regard to treatments applied (Fig. 6B). For example, soil amended with AB5% was different compared with its application at 1%, whereas both ABP1% and ABP5% remained close to unamended (control) and the parent clay (B)-amended soils (Fig. 6B).

Discussion

Effect of clay amendments on respiratory activity and microbial profile

During PAH biodegradation in soil, native microorganism profiles may undergo changes within the community structure as a consequence of the availability of carbon source over time, competition, etc. (Viñas et al., 2005). However, changes may be further influenced as a result of changes in physicochemical properties arising from soil amendments applied to enhance bioremediation (Jung et al., 2014; Al-Kindi and Abed, 2016). The application of clay amendments to contaminated soil may alter microbial community structure (Cébron et al., 2015), thereby influencing metabolic activity of soil microbial cells (as detailed in Fig. 2).

Whilst changes in microbial community profiles as a result of organoclay amendments has rarely been studied (Abbate et al., 2009; Abbate et al., 2013), there is a desire to study potential ecotoxicity caused by these materials due to their potential application for remediation purposes (Lazzara et al., 2018). In our previous studies, the synthesized modified organoclay product (ABP) was identified as growth supportive for native heterotrophic bacteria in uncontaminated soil (Biswas et al., 2015) and for Mycobacterium gilvum in an aqueous suspension containing phenanthrene (Mandal et al., 2016). Those studies measured bacterial numbers and soil enzyme activity highlighting that microbial enzyme function might be impacted by conventional organoclay (e.g. AB) due to bacterial exposure to the cationic surfactant (e.g. Arquad®) from the clay surfaces and pores (Sarkar et al., 2013). Arquad® or related surfactants comprised quaternary ammonium compounds, containing nitrogen cations (N⁺) supported by four species of alkyl or aryl groups; they are the primary concern for toxicity to soil native microorganism (Li and Brownawell, 2010). Conceivably, these functional groups could exert toxicological impact on soil microorganisms (Reeve and Fallowfield, 2017) and inhibit biodegradation of hydrocarbon compounds (Ugochukwu et al., 2014). However, the modified form of that organoclay (ABP) provided a congenial microhabitat for soil microorganism potentially through (i) anchoring of the exposed sites of the cationic surfactant through non-toxic fatty acid molecules and (ii) creating a more hydrophobic clay mineral surface to facilitate solid surface-supported microbial biofilms (Singh et al., 2006; Leglize et al., 2008). In the present study, we aimed to characterize microbial
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Fig. 4. Venn diagram of OTUs (n = 3 replicates) in PAH/Cd co-contaminated soil amended with 1% bentonite (B), Arquad™-modified bentonite (AB), palmitic acid-modified AB (ABP) or without clay amendments (NC). The percentage value was derived from the occurrence of OTUs corresponding to phylum within each pie chart.

Groups exposed to such organoclays and assess their health in clay-amended soil. A significant finding of the study was that ABP amendment to PAH–Cd contaminated soil-reduced microbial (e.g. bacteria and archaea) OTU numbers but not the major potential PAH-degrading microbial groups (Figs 4 and 5).
comparative diversity index of microbial OTUs between amended soils further supports the biocompatibility of ABP over AB (Fig. 6). Extraction of gDNA at the end of the incubation period (day 70) from clay-amended soil also indicated that ABP may support producing more DNA mass than raw bentonite. It is worth noting that the
DNA extraction kit used in this study (see experimental section) extracts DNA from variety of organisms including bacteria, fungi and algae. Therefore, although it is partially relevance, our previous report on changes in bacterial colony forming unit (CFU) from the same soil and its amendments supports the present result (Biswas et al., 2018) (Fig. S2).

Implications for the remediation of PAH/Cd co-contaminated soil

Material-based amendments to suppress metal toxicity may be an important approach for the successful application of bioremediation in co-contaminated soil. Kuppusamy et al. (2016) reported that metal-tolerant and PAH-degrading Gram-negative bacteria such as those belonging to Proteobacteria were abundant in contaminated soil. In connection to the bioavailability of Cd reported in our previous study (Biswas et al., 2018), we found that Proteobacteria were proportionally more abundant in the presence of Cd compared with the other two major groups, Actinobacteria and Firmicutes (Fig. 3 and Fig. S3). Indeed, Cd bioavailability in soil could be a significant determinant for the extent of metal toxicity on microorganisms (Thavamani et al., 2015), and therefore, amendments that can bind Cd and other toxic metals may be useful to reduce microbial community impacts.

The soil used in this study contained a mixture of PAHs and Cd, where Proteobacteria, Firmicutes, Actinobacteria and Chloroflexi were the major bacteria (Fig. 3). Cadmium addition (and associated nitrate) to PAH-contaminated soil may impact the microbial community structure (Lopez-Fernandez et al., 2018; Luo et al., 2019), and however, these impacts are likely to be seen across all soil treatments as it was added shortly after the inclusion of clay materials. Using co-contaminated soil (PAHs/metals such as Pb, Cr etc. in soil collected from coking plant wasteland, France; soil pH ~ 6.72–7.59, total organic carbon ~ 5.9%), Bourceret et al. (2016) reported that Proteobacteria, Actinobacteria and Bacteroidetes were dominant, whilst Acidobacteria was low in abundance. In this study, we also observed a relationship between Proteobacteria and Actinobacteria, although the abundance of each varied depending on the amendment applied. Reportedly, these bacterial groups are key degrader of PAHs in various types of oil-contaminated soil and sludge (Bastiaens et al., 2000; Vinas et al., 2005; Isaac et al., 2015).

Effect of clay loading on microbial profiles and respiratory activity

Microbial relative abundance profiles changed by the addition of clay and modified clays to PAH/Cd-contaminated soil but this depended on amendment loading. For example, at a low dose (1%), ABP-amended soil had a high number of Actinobacteria and low count of Proteobacteria, which was opposite, whilst the dose was 5% of the same material (Fig. 3). The presence of a high abundance of PAH-degrading Proteobacteria even at high applications (5%) of ABP may suggest that reportedly ‘toxic organoclays’ such as AB could be modified to reduce the toxicity of surfactant functional groups before application. Although surfactants are biodegradable in

![Fig. 6. Diversity index (A) and UPGMA distant matrix (B) of unamended (control) and clay-amended soils. NC = No clay (control), B = bentonite (raw clay), AB = Arquad-bentonite (organoclay) and ABP = Arquad-palmitic acid bentonite (modified organoclay). Loading rates (e.g. 1% or 5%) are expressed with the clay products, such as B1% is the expression of 1% of B loading. In the UPGMA, similarity matrix = Bray–Curtis, Bootstrapping Number = 1000. The number of OTUs was obtained as the mean of 3 replicates, and the bar on the ‘A’ graph shows SD of means. The different letters on each bar represent a significant difference among all treatments including loading rates at a 95% level of confidence using Duncan post hoc analysis.](image)
soil under aerobic conditions, cationic surfactants as quaternary ammonium compound (OAC) might be strong biocidal effect (Ying, 2006). Degradation or toxicity of such QAC depends on factors including alkyl chain length, position of quaternary ammonium in the carbon chain and loading concentration of surfactant per unit soil environment (Zhang et al., 2015). Since AB was originally synthesized with a surfactant loading equivalent to 100% of bentonite cationic exchange capacity (Biswas et al., 2016), it is expected that the surfactant was protected in the bentonite interlayer through cation exchange without significant leaching of surfactant molecules (Li et al., 2003; Biswas et al., 2019). The present study did not assess either the leaching potential of surfactants and palmitic acid molecules that were grafted into clays or biodegradation of them. This may warrant a further study. However, controlling similar conditions, the loading concentration of AB and ABP was comparable in both metabolic activity and microbial abundance where any potential exposure of surfactant was mitigated by palmitic acid in ABP. In this perspective, materials modified with surfactants or fatty acid might have the role player instead of unbound modifying compounds. For example, both ABP1%- and ABP5%-amended soil provided habitats for microorganisms that were similar to control (NC) and raw clay-mixed soil (see the distant matrix, Fig. 6B).

The redox-based fluorescence stain marker, used to profile metabolic activities (active vs. inactive cells), revealed that with increasing modified clay load, CTC stain-based metabolic rates were reduced, in particular for the cationic surfactant-modified bentonite (AB) (Fig. 2). This is in contrast to the stimulatory effect on soil DNA concentration (Fig. 1), particularly for soil amended with the tailored organoclay (ABP). The effect was even more pronounced as clay amendment loading was increased (e.g. 5%). This contrasting phenomenon could be linked to the dormancy of soil native microorganism (Jones and Lennon, 2010) or carbon source-specific microbial variability (Garland and Mills, 1991), which implies that (i) particular microbial groups utilized ABP-amended soil to grow favourably but remained inactive specifically in the reduction-based metabolism of CTC or similar compounds and/or (ii) the cultivable microbial strains that grew well in the agar medium did not actively participate in the biodegradation of target compounds (Fig. S2).

Conclusions and future implications

In a field soil contaminated with PAHs and Cd, clay and modified clay amendments altered 16S rRNA microbial profiles mainly among commonly reported PAH-degraders (Actinobacteria, Proteobacteria, Firmicutes and Chloroflexi). Even at high dose (5%), the presence of Proteobacteria and Actinobacteria in soil amended with clay or modified clay may be advantageous for PAH biodegradation (Fuentes et al., 2016). However, as revealed by redox-fluorescence staining, metabolically active microbial cells were not as abundant in soil amended with modified organoclay compared with raw clay-amended soil. Reducing the amendment load may improve microbial metabolic activity and minimize impacts such as reduced diversity. This is highly important in order to ensure the biocompatibility of modified clays for environmental remediation (Biswas et al., 2019). A limitation of this study is that only 16S rRNA was used for the assessment of bacterial diversity, whilst the identification of functional genes responsible for PAH degradation or metal tolerance may provide greater insight in (organo)-clay–microbial interactions and biodegradation impact (Liang et al., 2019). Apart from bacteria and archaea, fungal community members are another significant group that may contribute to PAH degradation in co-contaminated soil (Liu et al., 2017). Further investigations considering these aspects and other environmental conditions, such as contrasting soil types and leaching of modifying agents from clays, would be beneficial to determine the impact of clay and modified clay addition of native soil microbial functionality.

Experimental procedures

Clay-amended soil preparation and microcosm setup

A loamy sand soil (pH = 6.4) with minimal clay content (4%) was received in mid-2015 and stored at 4°C. Originally, it was collected from a mine site in South Australia (34.48 S, 138.37 E) (Juhasz et al., 2014). The soil, containing a low fraction of clay (clay = 4%, silt = 8%, and sand = 88%, organic carbon = 2.1%), was chosen to minimize the effect of endemic clay content in order to assess the effect of clay addition into the soil. The soil was originally contaminated with PAH (ΣPAHs = ~ 917 mg kg⁻¹ soil). Briefly, soil (25 g) was mixed with clay products at (i) 1% and (ii) 5% w/w and agitated on an end-over-end shaker for 5 days. During this stage, soil was maintained at 30% water holding capacity. Three clay products were used as soil amendments; detailed characterization of bentonite (designated B) and its modified products, Arquad°-bentonite (AB) and Arquad°-palmitic acid-treated bentonite (ABP) are described elsewhere (Biswas et al., 2015, 2016; Mandal et al., 2016) (Fig. 7).

Soil without clay addition served as the control treatment, named ‘NC’. Following clay addition, Cd(NO₃)₂ (150 mg Cd kg⁻¹ soil) (> 99% purity, Chem-supply, Australia) was added to the soil to effect a PAH/Cd co-contaminated soil. Cadmium was spiked into amended soil and mixed for an additional 24 h after which the moisture content was increased and maintained at 60% WHC.
throughout the incubation period (70 days during the end of 2016). We utilized an incubation timeframe of 70 days based on the previous report where we stated that over this incubation period soil native bacteria has the potential to utilize available PAHs as a carbon and energy source fractions of PAHs variably depending on soil amendments; for example, \( \sum \)PAHs became \(~ 402 \text{ mg kg}^{-1} \text{ soil} \) in the control soil (Biswas et al., 2018). Soil sub-samples were withdrawn from microcosms in triplicate in order to conduct various analyses as detailed in the following sections.

### Soil DNA concentration in clay-amended soil

Following 70 days incubation, gDNA was extracted from soil samples (250 mg in triplicate) using the PowerLyzer\textsuperscript{®} PowerSoil\textsuperscript{®} DNA isolation kit (Mo Bio Laboratories, Inc., California, USA). Extracted DNA was quantified using fluorometer (Qubit\textsuperscript{®} 2.0, Invitrogen, Life Technologies\textsuperscript{TM}, California, USA) and expressed as \( \mu \text{g g}^{-1} \text{ soil} \).

### Assessment of respiration-active microbial cells using Flow Cytometry

**Microbial cell isolation and CTC + staining.** The metabolically functioning prokaryotic community was assessed using BacLight\textsuperscript{™} RedoxSensor\textsuperscript{™} Vitality kits (Molecular Probes\textsuperscript{TM}, Invitrogen, California, USA) following Whiteley et al. (2003). At the end of the incubation period (70 days), cells were isolated from soil (1 g) in duplicate. In brief, soil was added to sterile centrifuge tubes containing five sterile 5 mm-sized glass beads and 4 ml phosphate buffer saline (PBS, pH = 7.4). Tubes were then shaken horizontally at 300 rpm to disperse cells from the soil matrix. After 1 h, samples were centrifuged (750 \( \times \) g for 6 min at 4°C) to obtain microbial suspensions. Since fine soil particles and clay minerals are in the same size range of microbial cells, further separation with a gradient medium was necessary to obtain soil-free cells (Amalfitano and Fazi, 2008). Supernatant (1.5 ml) were transferred onto 2 ml Eppendorf tubes containing non-ionic density gradient medium Histodenz\textsuperscript{™} (400 \( \mu \)l of a 1.3 g ml\(^{-1}\)) (Sánchez-Andrea et al., 2012). Tubes were centrifuged at 14 000 \( g \) for 30 min, and the Histodenz\textsuperscript{™} PBS interface containing microbial cells (500 \( \mu \)l) was carefully withdrawn, transferred into another tube and diluted twofold with sterile 0.2 \( \mu \)m-filtered PBS. Cell suspensions were stained with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) at a final concentration of 5 mM. Briefly, a stock of CTC (25 mM) was prepared in 0.2 \( \mu \)m-filtered water. Fresh cell suspensions (1000 \( \mu \)l) were mixed with 250 \( \mu \)l of the CTC stock to obtain a 5 mM CTC final concentration. Mixtures were then incubated in the dark for 2 h at room temperature (\(~ 23^\circ\text{C}\)). To stop the reaction and to increase staining contrast, 1% paraformaldehyde (w/v) was added to suspensions. Controls were included to distinguish positive and null staining, including (i) sterile PBS with CTC stain and (ii) cell suspension without CTC addition.

**Flow cytometry of CTC-stained samples.** Samples were assessed using a FACSCanto flow cytometer (BD Bioscience, California, USA). Microbial cells were gated on their light scatter properties, and CTC positivity was measured in the PerCp channel equipped with a 670 longpass filter at 488 nm.

**Soil microbial 16S rRNA diversity profiling**

**PCR and sequencing of amplicons.** Following DNA extraction, PCR amplification and sequencing was performed by the Australian Genome Research Facility. 16S rRNA (V3–V4) amplicons were generated, and PCR was performed. The primers and PCR cycle conditions are presented in the SI 4. The resulting amplicons were sequenced on Illumina MiSeq (San Diego, CA, USA) with 2 \( \times \) 300 base pairs paired-end chemistry.

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Sequencing assembling, diversity analysis and statistical interpretation. Paired-end reads were assembled by aligning the forward and reverse reads using PEAR (v0.9.5) (Zhang et al., 2014) followed by trimming of primers using seqtk (v1.0). Trimmed sequences were processed using Quantitative Insights into Microbial Ecology (QIIME 1.8) (Caporaso et al., 2010), usearch (v7.1.1090) (Edgar, 2010; Edgar et al., 2011) and uparse (Edgar, 2013) software. Throughout this process, quality filtering, removing full-length duplicate sequences and sorting by abundance were performed. Singleton or unique reads in the data set were discarded. Sequences were clustered followed by chimera filtered using ‘rdp_gold’ database as the reference.

To obtain the number of reads in each OTU, reads were mapped back to OTUs with a minimum identity of 97%. Using qiime, taxonomy was assigned using the SILVA r132 database (Quast et al., 2012). These OTUs were sorted into available taxa, and the taxonomic level ‘phylum’ was used to produce relative abundance chart. The OTUs were taken to construct (i) distant matrix with unweighted pair group method with arithmetic mean (UPGMA) and similarity index ‘Bray–Curtis’ (Bootstrap N: 1000 using past3 software) and (ii) Venn diagram to identify the shared and distinct taxa and Simpson’s index for diversity analysis (using ‘VennDiagram’ and ‘Vegan’ package in R, respectively) (Chen, 2018; Oksanen et al., 2019).

Analysis of variance (ANOVA) and post hoc analysis with Duncan’s multiple range test at 95% confidence level (P < 0.05) were applied to compare the mean of treatments by the use of MB SPSS Statistics 24 (New York, USA).

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Conflict of interest

None declared.

Data availability statement

Quality-filtered sequences have been submitted to NCBI archive and can be found under the Bioproject ID: PRJNA369284 and submission ID: SUB2358511.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Relative abundance of microorganisms (Bacteria and Archaea) which appeared to be less than 2% at phylum level after taxa annotation in SILVA database (r132). See experimental section in the main text for details.

Fig. S2. Bacterial growth (colony forming unit) (Left Y-axis with Line) and microbial DNA mass (Right Y-axis with Bar) in clay-amended long-term PAH/Cd-contaminated soil. The CFU data have been replotted from our previous paper (Biswas et al., 2018) with the permission of Elsevier® 2018. See experimental section in the main text for details.

Fig. S3. The bioavailability of Cd in soil. The detail method of this experiment is found elsewhere (Biswas et al., 2018). The figure has been reused with the permission of Elsevier® 2018. The data presented in the inset table is the value for the day 70 only, extracted from the above figure. B, bentonite; AB, Arquad®-modified bentonite, ABP, palmitic acid-modified AB.

Section SI4: PCR and sequencing of amplicons.