Enhanced biodegradation of PAHs in historically contaminated soil by *M. gilvum* inoculated biochar

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ABSTRACT: The inoculation of rice straw biochar with PAH-degrading *Mycobacterium gilvum* (1.27×10^{11}±1.24×10^{10} cell g^{-1}), and the subsequent amendment of this composite material to PAHs contaminated (677 mg kg^{-1}) coke plant soil, was conducted in order to investigate if would enhance PAHs biodegradation in soils. The microbe-biochar composite showed superior degradation capacity for phenanthrene, fluoranthene and pyrene. Phenanthrene loss in the microbe-biochar composite, free cell alone and biochar alone treatments was, respectively, 62.6±3.2%, 47.3±4.1% and non-significant (P>0.05); whereas for fluoranthene loss it was 52.1±2.3%; non-significant (P>0.05) and non-significant (P>0.05); and for pyrene loss it was 62.1±0.9%; 19.7±6.5% and 13.5±2.8%. It was hypothesized that the improved remediation was underpinned by i) biochar enhanced mass transfer of PAHs from the soil to the carbonaceous biochar “sink”, and ii) the subsequent degradation of the PAHs by the immobilized *M. gilvum*. To test this mechanism, a surfactant (Brij 30; 20 mg g^{-1} soil), was added to impede PAHs mass transfer to biochar and sorption. The surfactant increased solution phase PAH concentrations and significantly (P<0.05) reduced PAH degradation in the biochar immobilized *M. gilvum* treatments; indicating the enhanced degradation occurred between the immobilized *M. gilvum* and biochar sorbed PAHs.

Keywords: PAHs, Biochar, Microbe immobilization, Soil, Degradation
Abbreviations

PAHs\textsuperscript{1}, AC\textsuperscript{2}, BC\textsuperscript{3}

HA\textsuperscript{4}, DOC\textsuperscript{5}, CP soil\textsuperscript{6}

BET\textsuperscript{2}, *M. gilvum*\textsuperscript{8}, MM\textsuperscript{9}

LB\textsuperscript{10}, SEM\textsuperscript{11}, qPCR\textsuperscript{12}

CPD\textsuperscript{13}, EPS\textsuperscript{14}, DLVO\textsuperscript{15}

CSH\textsuperscript{16}, PHE\textsuperscript{17}, FLA\textsuperscript{18}, PYR\textsuperscript{19}

\textsuperscript{1} Polycyclic aromatic hydrocarbons
\textsuperscript{2} Activated carbon
\textsuperscript{3} Biochar
\textsuperscript{4} Humic acids
\textsuperscript{5} Dissolved organic carbon
\textsuperscript{6} Beijing coking plant soil
\textsuperscript{7} Brunauer-Emmett-Teller
\textsuperscript{8} *Mycobacterium gilvum*
\textsuperscript{9} Minimal medium
\textsuperscript{10} Lysogeny Broth
\textsuperscript{11} Scanning electron microscopy
\textsuperscript{12} Real time quantitative PCR
\textsuperscript{13} Critical-point drying
\textsuperscript{14} Extracellular polymeric substances
\textsuperscript{15} Derjaguin–Landau–Verwey–Overbeek theory
\textsuperscript{16} Cell surface hydrophobicity
\textsuperscript{17} Phenanthrene
\textsuperscript{18} Fluoranthene
\textsuperscript{19} Pyrene
1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are carcinogenic and mutagenic compounds (Mastrangelo, Fadda et al. 1996, Goldman, Enewold et al. 2001). Their ubiquitous occurrence (Samanta, Singh et al. 2002), intrinsic toxicity (Mastrangelo, Fadda et al. 1996, Goldman, Enewold et al. 2001) and bioaccumulation potential (Baussant, Sanni et al. 2001) raise significant concerns for human and ecosystem health, wherever PAHs are present at elevated concentrations, such as those found in urban or industrial soils (Wagrowski and Hites 1996, Bakker, Casado et al. 2000, Tang, Tang et al. 2005).

Developing cost-effective methods to clean up PAHs from contaminated land remains a technological challenge (Gan, Lau et al. 2009). One approach is to lower the bioavailability of PAHs through the introduction of sorbent amendments, to strongly bind the PAHs, and thereby reduce exposure and associated risks (Ghosh, Luthy et al. 2011). The most extensively studied amending agents for such purposes, being a sorption "sink" for PAHs, are carbonaceous materials such as activated carbon (AC) and biochars (Beesley, Moreno-Jiménez et al. 2011, Hale, Hanley et al. 2011, Lehmann and Joseph 2012, Oleszczuk, Hale et al. 2012). Such carbonaceous materials have been observed to sorb PAHs up to 10-1000 times stronger (per unit mass) than other types of soil organic carbon (Accardi-Dey and Gschwend 2003, Cornelissen and Gustafsson 2004). When a strong sorbent, like biochar or AC, is introduced to a contaminated soil, the contaminants are transferred from the aqueous phase and weaker sorption (fast-desorption) sites of the contaminated soil to the strong sorption site of the added sorbent (Werner, Ghosh et al. 2006, Rhodes, Carlin et al. 2008, Cho, Werner et al. 2012, Oleszczuk, Hale et al. 2012, Liu, Chen et al. 2015). As an example, the uptake of polychlorinated biphenyls in passive samplers decreased up to 73% with amendment
of 3.7 % of AC after 5 years (Cho, Werner et al. 2012). Though AC can sorb PAHs more effectively than biochars (Cornelissen, Breedveld et al. 2006, Gomez-Eyles, Yupanqui et al. 2013), biochars offer other advantageous traits, such as lower cost, the ability to be made from organic waste materials, and reduced (or even negative) CO₂ emissions associated with their production (Lehmann 2007, Lehmann and Joseph 2015).

One significant disadvantage of sorbent amendments is that they generally lead to reduced biodegradation of PAHs because the sorbed PAHs have a reduced microbial bioaccessibility and cannot, therefore, be degraded (Rhodes, Carlin et al. 2008, Rhodes, McAllister et al. 2010, Cho, Werner et al. 2012, Arp, Lundstedt et al. 2014). For example, the mineralization extent of phenanthrene (PHE) decreased by up to 50% with amendment of 5% BC (Rhodes, Carlin et al. 2008); whereas for AC amendment, it declined from 87.2% (in absence of AC) to 0.4% (5% AC) (Rhodes, McAllister et al. 2010). This is potentially problematic as many regulatory standards, and site-specific remediation targets, are based on total concentrations, and not bioavailable concentrations (Latawiec, Swindell et al. 2010, Ortega-Calvo, Harmsen et al. 2015).

Therefore, establishing technologies that both lower total soil concentration (via biodegradation) and bioavailability through sorption and entrapment would be advantageous.

These conflicting goals might be reconciled through the impregnation of sorbent amendments with PAH-degrading microorganisms (Wick, Springael et al. 2001, Wick, De Munain et al. 2002, Uyttebroek, Ortega-Calvo et al. 2006). Through such an approach the mass transfer of PAHs from contaminated soil to the degrading microbial community might be enhanced, and in addition there would be an enrichment of degrading bacteria and biofilm formation on the strong sorbing materials (Bastiaens, Springael et al. 2000, Wick, Springael et al. 2001, Uyttebroek, Ortega-Calvo et al. 2006),
which could provide to stimulate the biodegradation of PAHs (Liu, Chen et al. 2015).

Supporting this reasoning, additions of humic acids (HA) (Smith, Thullner et al. 2009), model synthetic amberlite sorbents (Uyttebroek, Ortega-Calvo et al. 2006), clay (Ortega-Calvo and Saiz-Jimenez 1998), and dissolved organic carbon (DOC) (Haftka, Parsons et al. 2008) have been shown to promote both PAH sorption and degradation. Particularly, a 4.8- and 9.5-folds increase in PHE degradation rates was respectively observed when in the presence of HA (Smith, Thullner et al. 2009) and model synthetic amberlite sorbents (Uyttebroek, Ortega-Calvo et al. 2006). It is important in this context to note that biochars, with high surface area and porosity, make good candidates for microbial habitats (Saito 1990, Pietikäinen, Kiikkilä et al. 2000, Hale, Luth et al. 2014). Microbe-AC/biochar composites have been employed in wastewater treatment process, to provide protection to microbes, and/or to increase contaminant removal performance (Morsen and Rehm 1990, Song, Edwards et al. 2006, Lin, Donghui et al. 2010). For example, a 2-times higher pyridine-degradation rate constant (0.12 mg g\text{carrier}^{-1} h^{-1}) was observed when Paracoccus sp. strain KT-5 was pre-immobilized on bamboo-carbon (Lin, Donghui et al. 2010).

Elevated metabolic activities resulting from biochar amendment to soil have also been observed (Steinbeiss, Gleixner et al. 2009), but studies focus on the application of microbe-biochar composite in the remediation of contaminated soils are rare. Therefore there is a research gap regarding the effectivity of such approaches. In order to evaluate the potential for bacteria-inoculated biochar to remediate PAH contaminated soil, we investigated the influence of pre-immobilizing the actinobacteria cells, of M. gilvum, on biochar, and quantified its ability to sorb and biodegrade PAHs in a real-world, historically contaminated soil.
2. Materials and methods

2.1. Reagents and Chemicals

Hexane, cyclohexane, acetone, and dichloromethane (all HPLC-spectro grade) were purchased from Tedia (TC, USA). Internal standard hexamethylbenzene (99% purity) was acquired from Dr·Ehrenstorfer (Augsburg, Germany). The surrogate standard mix was acquired from AccuStandard (CT, USA), and contained naphthalene-$d_{10}$, acenaphthene-$d_{10}$, phenanthrene-$d_{10}$, chrysene-$d_{12}$ and perylene-$d_{12}$ (4 mg ml$^{-1}$). A standard solution of 16 US EPA PAHs, and the non-ionic surfactant Brij 30 [CH$_3$(CH$_2$)$_{10}$CH$_2$(OCH$_2$CH$_2$)$_n$OH, were obtained from Sigma-Aldrich (St. Louis, MO).

2.2. Soil

A real-world, PAH contaminated soil was collected from a former Coking Plant (CP soil) (N 39°51′ 0.42″, E 116°31′ 38.83″) in Beijing, China. The soil samples were stored in dark glass containers at -80°C until use. The total concentrations of 16 US EPA PAHs in this soil, indicating heavy contamination, were 677 mg kg$^{-1}$. Physical and chemical properties of CP soil include: pH 8.37, total organic matter 10%, total N 0.231%, total C 12.92%, total S 0.593%, clay 2.39%, silt 15.4%, and sand 82.2% (see the Supporting Information (SI) for quantification details).

2.3. Biochar

Rice straw (*Oryza Sativa*), sewage sludge, and pig manure were applied as raw stocks to produce separate biochars by pyrolysis at 500°C (see the SI for more information about selection of pyrolysis temperature) for 4 h in a muffle furnace under limited oxygen conditions. Characteristics of the produced biochars were analyzed (see the SI
Rice straw derived biochar was ultimately selected to study as the inoculum carrier, as the largest specific surface area (68.1 m² g⁻¹), pore volume (0.17 cm³ g⁻¹) and surface basic groups (0.172 mmol g⁻¹) were observed in this biochar (Table S1 & S2, SI), indicating a higher bacterial adsorption capability (Krisdhasima, Vinaraphong et al. 1993, Hale, Luth et al. 2015). Moreover, rice straw is a practical biomass-waste feedstock for biochar production (Wu, Yang et al. 2012).

Other physical and chemical properties of this biochar were: pH 10.14, total N 1.73%, total C 48.6%, ash content 29.3%. The total concentration of 16 US EPA PAHs was 4.35 mg kg⁻¹ (see the SI for quantification details).

**2.4. Bacteria strain, isolation, culture conditions, and preparation of cell suspensions**

*Mycobacteria* have been reported to be adept degraders of gasoline components and sorbed PAHs (Kim, Kweon et al. 2010). As an indigenous bacteria in the tested soil, *M. gilvum* was employed. *M. gilvum* was isolated from the CP soil via a classical shaken liquid medium enrichment method as described elsewhere (Bastiaens, Springael et al. 2000). *M. gilvum* was confirmed, in preliminary tests, to be capable of degrading naphthalene, fluorene, phenanthrene, anthracene, fluoranthene (FLU), and pyrene (PYR) effectively in minimal medium (MM) solutions (described in the SI Table S3) (see the SI Figure S2). *M. gilvum* isolate was maintained using pyrene as the sole carbon and energy source on MM agar plates. For the preparation of cell suspensions, one loop of isolate was picked up and inoculated into a liquid Lysogeny Broth (LB, no PAHs were added). After 1-week incubation on a rotary shaker at 30°C, 180 rpm, cell growth approached a steady state (OD₆₀₀=1.6) and cells were then harvested. The cell culture was centrifuged at 3500 r min⁻¹ for 10 min, the supernatant was discarded and sterilized
fresh LB was added to re-suspend the cells; thereby, the cell suspension was condensed and prepared for further inoculations.

### 2.5. Immobilization of *M. gilvum* cells on biochar

To immobilize *M. gilvum* cells on rice straw biochar, the biochar was firstly grounded by a wood roller in valve bag and then passed through a 2-mm sieve, and 0.25 g biochar (dry weight) was then soaked with fresh LB (1:20, w/v) in 50 ml-flasks. Subsequently, the flasks were closed with Teflon-lined stoppers and sterilized twice at 121°C for 30 min. Cell suspensions were introduced to the flasks, after they had cooled, with each flask receiving 2.5 ml of condensed cell suspension, containing $8.45 \times 10^{10}$ cells (confirmed by plate counting). The flask contents were then incubated on a rotary shaker at 30°C, 80 rpm for 48 h. The mixtures were separated with a 75-µm sieve and rinsed with de-ionized water thrice to remove the planktonic cells. Obtained *M. gilvum*-composite should be collected and stored at 4°C if immediate inoculation into soil is not possible (Lin, Wu et al. 2015). All operations were performed under strict aseptic conditions.

The accumulated biomass of *M. gilvum* on rice straw biochar was evaluated by real-time PCR assays. To test the immobilization durability, biochar inoculated with *M. gilvum* cells, following 48-h of culturing, was reintroduced to fresh LB and then incubated on a rotary shaker (30°C, 180 rpm min⁻¹) for 30 days. Samples incubated for 4, 8, 12, 16, 18, 22, 26, and 30 day periods were collected, with three flasks being sacrificed at each sampling event. Parts of the samples were used for scanning electron microscopy (SEM) imaging, and the remainder was stored at -80 °C until DNA extraction.
2.6. SEM Observation

Biochars inoculated with *M. gilvum* cells were imaged using SEM. Samples were prepared by chemical fixation and critical-point drying (CPD) (Karcz, Bernas et al. 2012). Briefly, samples were fixed in 2.5% glutaraldehyde in 0.1 M PB buffer (pH 7.2) for 2 h. The samples were then rinsed by 0.1 M PB buffer twice, and dehydration was carried out in a graded ethanol/water series of 30, 50, 70, 90, and 100%, at 20 min for each concentration. Dehydrated samples were then dried to the critical-point with carbon dioxide in a Pelco CPD2 apparatus. A 5-nm gold film was sputter-coated on the samples. Images were then recorded by SEM (Hitachi S4800) operated at 5 kV.

2.7. Biodegradation

A batch biodegradation experiment was conducted in 150-ml flasks. CP soil (5 grams dry weight) and 30 ml MM solutions were mixed and incubated for 72 hours to revive the soil microbes. Four different treatments were then prepared. These were: (i) raw CP soil only (the control), (ii) 5 g of raw CP soil inoculated with 2.5 ml of condensed *M. gilvum* cell suspension (circa $1.69 \times 10^{10}$ CFU/g dw soil), (iii) 5 g of raw CP soil with 0.25 g dry weight of sterile rice straw biochar (at a ratio of $0.05 \text{g}_{\text{dw biochar}}/\text{g}_{\text{dw soil}}$), and (iv) 5 g of raw CP soil added with 0.25 g dry weight of biochar inoculated with $1.27 \times 10^{11}$ cells/g dw biochar, resulting in a cell density in the CP soil of $6.43 \times 10^9$ cells/g dw soil.

Unavoidably, less *M. gilvum* cells were introduced in this treatment compared with direct inoculation of free cells (due to incomplete transfer of *M. gilvum* cells from the loading solution onto the biochar). Ultimately, all flasks were supplemented with more MM solutions to obtain a final volume of 50 ml (final soil to water ratio was 1:10, w/v).

For each treatment, independent biodegradation assays were performed in triplicate, and all flasks were randomly placed on a rotary shaker (180 rpm) in the dark at 30°C,
for 18 days. Sterile MM solution was supplemented every 2 days to keep the suspensions at a fixed volume. Slurries with a volume of 10 ml were taken from all treatments for DNA extraction at day 0 and day 18.

In order to explore the hypothesized mechanism, that biochar would promote mass transfer of PAHs to the biochar and the immobilized cells would then degrade the transferred PAHs, a further set of flasks were prepared. These were identical to those described above but to each flask the anionic surfactant Brij 30 was added (20 mg g\(^{-1}\) soil; thereby achieving an experimental concentration of 2 g L\(^{-1}\)). Brij 30 has demonstrated high PAH solubilizing ability that results in a considerable reduction of PAH sorption to biochars (53%) (Ahn, Kim et al. 2008).

2.8. DNA Extraction and Real-Time PCR

DNA extraction was achieved using FastDNA SPIN kit for soil (MoBio Laboraories, Carlsbad, CA, USA), in accordance with the manufacturer's instructions. FastDNA SPIN kits have previously been used in DNA extraction from biochar amended soil (Quilliam, Marsden et al. 2012, Leite, Balieiro et al. 2014). Real-time quantitative TaqMan PCR assays were conducted, targeting *Mycobacterium nidA*. Primer and probe sets target conserved regions determined from a multiple alignment of *nidA* obtained from several PAH-degrading *Mycobacterium* (DeBruyn, Chewning et al. 2007). The TaqMan probe sequences were 5′ -FAMTCCTACCCGTCGCCGGTACA-BHQ1, forward and reverse primer sequences were 5′ -TTCCCGAGTACGAGGGATAC and 5′ -TCACGTGATGAACGACAAA, respectively.

Quantitative PCR assays were performed using a real-time quantitative PCR detection system (Roche 480, Roche, Indianapolis, IN, US., Light Cycler FastStart DNA Master Hybridization Probes (Roche) was used for qPCR reactions. Reaction
conditions were as follows: 50°C for 5 min, 95°C for 15 min, then 40 cycles of
denaturing at 94°C for 30 s, annealing at 56°C for 30 s, lengthening at 72°C for 30 s.
For each sample, independent quantitative assays were carried out in triplicate. In every
amplification reaction, 10-fold diluted standard plasmid containing *nidA* genes were
amplified with the primers to obtain standard curves, and negative controls were also
included. Efficiencies of amplification were 90% to 110% for all samples.

### 2.9. Analysis of PAHs

PAHs in the soil phase and aqueous phases were analyzed as follows. Biodegradation
assay flasks were allowed to settle for 1 h, then the supernatant solution was pipetted
out and stored in 80-ml K-D tubes. The remaining soils were freeze-dried, and the 16
US EPA PAHs were analyzed using a method previously reported for quantifying PAHs
in biochars and biochar amended soils ([Fabbri, Rombolà et al. 2013](#)) (see the SI).

Analysis of aqueous PAHs in samples without Brij 30 amendment was carried out
following a method described elsewhere ([Ahn, Werner et al. 2008](#)). Briefly, aluminum
sulfate was added to the K-D tubes to flocculate suspended solids, and then
hydrochloric acid was added to adjust the pH to 7. The K-D tubes were capped and then
shaken by hand to enhance flocculation for 3 min, and subsequently centrifuged for 15
min at 2000 rpm twice to remove flocs. The clear supernatant was transferred to 250-
ml size screw-capped flasks, hexane (15 ml) was added to each flask, and the flasks
were then shaken on a rotary shaker at 180 rpm for 30 min. The extracts were then
separated using separatory funnels. Extraction of each batch of supernatant was
performed three times to ensure full PAH recovery. Subsequent up-concentration and
clean-up steps of the extracts are described in the SI.

The presence of Brij 30 in the aqueous phase of the surfactant dosed treatments
precluded back extraction of the aqueous supernatant into hexane (as the surfactant causes demulsification of the mixture), and so, in these treatments, aluminum sulfate was added and the samples were shaken and centrifuged (as described above) to obtain a clear supernatant. Thereafter, PAHs were quantified using high-performance liquid chromatography (HPLC) with direct injection of the clear supernatant (Zhu and Aitken 2010).

2.10. Statistical analysis

Microsoft Excel software (version 2013) was used for data processing. SPSS (version 22.0) was used to verify normality of data with Shapiro-Wilk’s test. Means were then compared using one-way ANOVA followed by either the LSD test or Dunnett’s T3 test, depending on whether equal variances were or were not assumed, respectively, to compare differences between multiple groups.

3. Results

3.1. Immobilization of M. gilvum on Biochar

The presence of M. gilvum cells on rice straw biochars after the initial 48-h culturing or 18-day of incubation in LB after culturing was imaged using SEM, alongside images of the sterile biochar and free M. gilvum cells (Figure 1). After 48-h of culturing, the adhesion of M. gilvum cells on rice straw biochar was observed (Figure 1C). Cells were observed to frequently colonize the surface and pore entrances of the rice straw biochar. As evident from Figure 1C, the cells appear grouped together as cell aggregates, additionally extracellular polymeric substances (EPS) were observed. After 18 days, the EPS was further pronounced, as observed in Figure 1E and Figure 1F, and formed
network-like structures extending over and in between the *M. gilvum* cells (Figure 1F).

**Figure 1.** SEM images of samples collected after 48 h (A–C) and 18 days (D–F). (A) Surface structure of sterile rice straw biochar (×5000). (B) Free *M. gilvum* cells with rough and raised cell walls (×15000). (C) Cell aggregates of *M. gilvum* isolate attached on rice straw biochar after two-day culturing, and the net-like extracellular polymeric substances (EPS) formed around the cells (×5000). (D) Surface of biochar soaked in LB for 18 days without bacteria inoculation (×5000). (E) Biochar inoculated with *M. gilvum* cells (×5000). (F) Same as E but showing biofilm arranged in mesh-like aggregates, and EPS visible as network-like structures extending over and in between the *M. gilvum* cells (×6000).

The accumulated biomass of *M. gilvum* isolate on biochars was assessed by quantitative PCR assays (Figure 2). The initial amount of *nidA* copies after 48-h inoculation was $1.27 \times 10^{11} \pm 1.24 \times 10^{10}$ copies/g$_{dw}$ biochar. Afterwards, the *nidA* copies on biochars varied during the incubation period from $2.7 \times 10^{10} \pm 9.26 \times 10^9$ to $1.1 \times 10^{11} \pm 5.3 \times 10^9$ copies/g$_{dw}$ biochar (respectively, representing a 5-folds decreased to a 10-folds increase). A dynamic fluctuation of *nidA* copies was evident by a sharp decrease in the first eight days, followed by a rapid increase over the next 10 days. Ultimately, a stationary biomass (approximated $7.0 \times 10^{10} \pm 1.5 \times 10^9$ copies/g$_{dw}$ biochar) was maintained at 18 days. This combination of SEM imaging and qPCR assays revealed that the immobilization of *M. gilvum* cells on rice straw biochar were effective and durable in LB.
Figure 2. *NidA* copies on biochar for 30 days of incubation

### 3.2. Biodegradation of PAHs

A preliminary test using *M. gilvum* cells inoculated biochar indicated 98% removal of pyrene within 5 days when 0.5 g of inoculated biochar was placed in a 50 ml MM solution with 50 mg L\(^{-1}\) of pyrene (Figure S3, SI). It is anticipated that the presence of soil and other PAHs may slow down the removal rate compared to a single PAH. The residual PAHs in CP soil with the different treatments mentioned above were analyzed after 18 days (Table S4, SI). Recoveries of the 6 surrogate standards ranged from 78% to 99% for all samples (i.e. for acenaphthene-\(d_{10}\), phenanthrene-\(d_{10}\) and chrysene-\(d_{12}\), they were 78±4%, 88±2%, 86±5% in soils without any treatment, and 74±3%, 89±7%, 82±2% in soils with 5% biochar amendment, respectively). No significant difference in recoveries between raw soil and soil with 5% of biochar amendment was observed (\(P=0.257, 0.906, 0.319\) for acenaphthene-\(d_{10}\), phenanthrene-\(d_{10}\) and chrysene-\(d_{12}\) respectively). No corrections for recovery were made. Residual PAH concentrations in the different treatments established after 18 days are reported in Table S4 (SI).

PHE, FLA, and PYR concentrations were observed to decrease most extensively (Table S4). A preliminary experiment with PAHs in solution with *M. gilvum* cells
(Figure S2) showed marked decreases in these three PAHs, as well as naphthalene, fluorene and anthracene, but not other PAHs. Naphthalene, fluorene and anthracene were degraded to a lesser extent in CP soil in comparison to PHE, PLA and PYR (Table S4). The difference in the extent of degradation for the different PAHs are suggested to relate to lower bioaccessibility of naphthalene, fluorene and anthracene in the CP soil as a consequence of native bacteria degrading these compounds in the field. This reasoning is supported by the relatively low concentrations of these three PAHs (naphthalene at 0.6%, fluorene at 1.0% and anthracene at 2.5% of the total 16 US EPA PAHs concentrations (Table S4)). By contrast, PHE, FLA and PYR, respectively, contributed 11.2, 16.2 and 18.3% of the total 16 US EPA PAHs to the CP soil. Given the greater prevalence and marked reduction in their concentrations, further discussion in this study is directed towards PHE, FLA and PYR.

The loss of PHE, FLA, and PYR in the control treatments was minimal over 18 days (< 4.0%±3.5%) (Figure 3A); while biochar amendment resulted in a small amount of PHE, FLA, and PYR loss compared to the control, of 7.2%, 2.8% and 13.2%, respectively. In contrast, the rice straw biochar inoculated with M. gilvum cells exhibited the highest removal ability. Specifically, losses of PHE, FLA, and PYR, within 18 days, were 62.6±3.2%, 52.1±2.3%, and 62.1±0.9%, respectively, or compared to the control, 58.6%, 49.9%, and 61.6%, respectively. In comparison, free M. gilvum cells resulted in 43.3%, 4.1%, and 19.2% losses of PHE, FLA, and PYR when compared to the control. Thus, biochar inoculated with M. gilvum cells resulted in 15.5% (PHE), 45.5% (FLA), and 42.6% (PYR) more degradation than just the free M. gilvum cells, at 18 days of incubation.

Changes in PHE, FLA, and PYR concentrations in the presence of 20 mg g⁻¹ Brij 30 are presented in Figure 3B and Table S4. The surfactant, on its own, or in the presence
of the biochar or free cells amendment, enhanced biodegradation of PHE, FLA and PYR compared to the treatments without surfactant. However, for the inoculated biochar amendments, the degradation assays with surfactant showed reduced biodegradation compared to assays without surfactant (Figure 3). Specifically, for treatments containing inoculated biochar, the degradation for PHE, FLA, and PYR, decreased in the presence of surfactant by 10.5%, 13.3%, and 23.6%, respectively, when compared to the surfactant-free treatments amended with the inoculated biochar. Thus, while the surfactant enhanced biodegradation in the presence of the free microbes; presumably on account of improved soil to solution mass transfer of PAHs (making them more bioavailable), the surfactant was effective in impeding onward mass transfer of PAHs to the biochar and their subsequent degradation by the *M. gilvum* cells.
**Figure 3.** Residual phenanthrene (PHE), fluoranthene (FLA), and pyrene (PYR) concentrations in coke plant soil in the absence of Brij 30 (panel A) and presence of Brij 30 (panel B) while incubated in a mixed-media solution, comparing the initial concentration (CP-0d), with concentrations after 18 days following natural removal with no amendment (CP-18d), biochar-only amendment (CP-BC-18d), *M. gilvum* free cells amendment (CP-*M. gilvum*-18d), and biochar containing immobilized *M. gilvum* amendment (CP-BC-*M. gilvium*-18d) inoculation. Error bars represent the standard deviation (n=3). Different letters indicate the mean difference is significant between treatments at the 0.05 level.

3.3. Abundance Monitoring of *M. gilvum* in CP Soil with Different Treatments

The change in *M. gilvum* cell numbers in the CP soil with different treatments at day 0 and day 18 were monitored by Taqman PCR assays, targeting the PAH-degrading *mycobacteria* nidA dioxygenase gene. The indigenous nidA copies in CP soil was 2.5x10^7±3.4x10^6 copies/g dw soil, and the initial inoculation of free and biochar-immobilized cells increased nidA copies to 1.5x10^10±1.8x10^9 and 4.9x10^9±1x10^8 copies/g dw soil (i.e. by factors of 622 and 288) respectively (Figure 4). Evidently, fewer *M. gilvum* cells were introduced to the treatments via the inoculated biochar amendment than the free cell addition, due to incomplete immobilization or biomass loss during the inoculation process. Figure 4 shows that, after 18-days of incubation, the nidA copies in the CP, Brij 30, and biochar amended soil was approximately 1.7 to 3.6 greater than those observed on day 0, but no significant difference in nidA copies among these treatments (P>0.05) was observed. However, in the case of free-cell and biochar-inoculated *M. gilvum* treatments, nidA copies all decreased at day 18 to about 60% of the day 0 numbers (down to 5.9x10^9±5.7x10^8, 1.9x10^9±6.8x10^8 copies/g dw soil, respectively). At day 18, nidA copies on the inoculate-biochar amended soils remained lower than the free cell inoculations (P<0.01). The presence of 20 mg g^-1 Brij 30 had little influence on nidA copies for each of these two treatments (both P>0.05 for free cell treatment and inoculated-biochar treatment).
**Figure 4.** NidA copies in CP soil with different treatments at day 0 and day 18, including natural removal with no amendments (CP-0d and CP-18d), biochar-only amendment (CP-BC-0d and CP-BC-18d), *M. gilvum* free cells amendment (CP-M. gilvum-0d and CP-M. gilvum-18d), and biochar containing immobilized *M. gilvum* amendment (CP-BC-M. gilvum-0d and CP-BC-M. gilvum-18d). Different lower-case letters indicate the mean is significantly different between treatments at the 0.05 level.

### 4. Discussion

#### 4.1. Immobilization of *M. gilvum* on biochar

SEM images and *nidA* copies presented in Figures 1 and 2 highlight the potential for biochar to provide a suitable habitat for microbial colonization (Saito 1990, Pietikäinen, Kiikkilä et al. 2000, Thies and Rillig 2009). Previous authors have proposed that two stages are involved in the immobilization of microbes on biochar (Klein and Ziehr 1990); the initial stage being adsorption of microbes onto biochars, which could be interpreted by the Derjaguin–Landau–Verwey–Overbeek (DLVO) theory of colloid stability (Hermansson 1999), and the second stage being biofilm formation. The accumulated biomass on biochar has been previously related to specific intrinsic properties of biochar, which vary significantly based on the preparation temperature and feedstock of biochar (Tang, Zhu et al. 2013). In particular, pore size distribution, pore volume, surface area and surface properties (hydrophobicity, metallic oxides and functional groups) of chars, are critical parameters that affect the ability of biochar to
serve as carrier for introducing bacteria to soils (Messing and Oppermann 1979, Rivera - Utrilla, Bautista - Toledo et al. 2001, Yamamoto, Nakakoshi et al. 2001, Upadhyayula, Deng et al. 2009, Hale, Luth et al. 2015). Messing et. al found that pore size distribution governed the optimum loading of bacteria, and that the maximum accumulation of stable biomass occurred when the pore diameters were in the range of one to five times the major dimension of the bacteria (Messing and Oppermann 1979).

The existence of such pores within the rice straw biochar was evident in the SEM images (Figure S1(A)). The microbial adsorption capacity of chars has also been commonly observed to increase with the specific surface area, surface hydrophobicity and the amount of macropores (Krisdhasima, McGuire et al. 1992, Krisdhasima, Vinaraphong et al. 1993, Rivera - Utrilla, Bautista - Toledo et al. 2001). Metallic oxides and oxygen functional groups on the surface of chars are excellent adsorbents of microbes and thereby will increase the accumulation biomass (Rivera - Utrilla, Bautista - Toledo et al. 2001, Upadhyayula, Deng et al. 2009). Modification of AC with cations of Fe$^{3+}$, Ca$^{2+}$, Mg$^{2+}$, respectively, has been found to lead to 87.8%, 54.7% and 24.8% increases in the microbial biomass accumulation (Rivera - Utrilla, Bautista - Toledo et al. 2001). While the development of inoculant after incorporation into soil is more closely associated with the physical features of biochar, including surface area, pore opening diameter and water-filled pore spaces, which might play a significant role in protecting pre-immobilized colonies from predation (Hale, Luth et al. 2015).

On the other hand, microbes in themselves are expected to play a role in cell immobilization on biochar, particularly the surface hydrophobicity (CSH) of cells, as hydrophobic bacteria are favorably attached to abiotic/hydrophobic surfaces (Krasowska and Sigler 2014). Genus of Mycobacterium has been reported to have extremely hydrophobic cell envelops (Hartmans, de Bont et al. 2006). Overall,
hydrophobic areas of the right pore size therefore likely serve as centers for clustering
of *M. gilvum* cells and biofilm, and the large surface area and pore volume (BET surface
68.06 m\(^2\) g\(^{-1}\), pore volume 0.17 cm\(^3\) g\(^{-1}\)) support the potential for successful *M. gilvum*
cell immobilization (Bastiaens, Springael et al. 2000, Uyttebroek, Breugelmans et al.
2006).

### 4.2. PAH degradation after immobilization of *M. gilvum* cells on biochar

PAH degradation depends on environmental conditions, the number and type of
microorganisms, as well as the properties and chemical structure of the compound being
degraded (Haritash and Kaushik 2009). The lower *nidA* copies detected in the biochar-
inoculated cell treatment compared to the free cell treatment, which underwent more
biodegradation in CP soil, indicates that increased cell numbers alone is not the driver
of increased PAH biodegradation (Figures 3 & 4). The aqueous concentrations of PHE,
FLA, and PYR after 18 days (Figure S4, panel A, SI) were similar in all treatments
(without surfactant). This indicates that neither the presence of biochar nor *M. gilvum*
cells significantly affected the aqueous concentration of PAHs (based on the
quantification method used).

Other researchers have reported increased PAH biodegradation is related to the
increased presence of certain sorbing matrices (Ortega-Calvo and Saiz-Jimenez 1998,
Uyttebroek, Ortega-Calvo et al. 2006, Mayer, Fernqvist et al. 2007, Smith, Thullner et
al. 2009). Uyttebroek and co-workers observed the final degradation extent of PHE in
porous synthetic amberlite sorbent (IRC50) (53-62%) was significantly higher than in
absence of IRC50 (18-52%) for all tested *Mycobacterium* strains; these results were
interpreted as preferential degradation of PHE sorbed to IRC50 (Uyttebroek, Ortega-
Calvo et al. 2006). Furthermore, in the presence of IRC50, the maximum PHE
mineralization rate (1.1-1.9 ng ml\(^{-1}\) h\(^{-1}\)) for all tested *Mycobacteria* were significantly higher than the initial abiotic desorption rate (0.2 ng ml\(^{-1}\) h\(^{-1}\)), suggesting that the bacterial utilized sorbed PHE with a higher rate than can be explained by abiotic desorption. Smith et al. observed sorption to humic acids increased degradation rates of PHE by factors up to 4.8 while leaving the water-dissolved PAH concentrations unchanged; proposing that additional humic acids-mediated transport was the responsible mechanism for enhanced PHE degradation (*Smith, Thullner et al. 2009*).

Ortega-Calvo and co-workers detected a shortened biodegradation acclimation phase and higher PHE mineralization rates in the presence of 100 μg ml\(^{-1}\) humic acid and 10 g L\(^{-1}\) clay, both separately and in combination. They proposed that the enrichment of PHE and degrading cells on these soil components resulted in the improved utilization of PHE, and thus the total degradation was increased (*Ortega-Calvo and Saiz-Jimenez 1998*). These studies all indicate that both dissolved and solid sorbing matrices can facilitate PAH degradation by assisting in PAH mass transfer to degrading cells in a sorbent-amended system. We propose, in our system, that biochar may act in a similar way, playing a role in the supply of PAHs to sorbed *M. gilvum* cells.

Reduced PAH degradation by immobilized bacteria, in the presence of surfactant Brij 30 (Figure 3), further supports this mechanism. The toxicity of Brij 30 to *M. gilvum* cells can be excluded as the reason of reduced PAH loss, because i) in our study increased biodegradation was exhibited in other parallel treatments in the presence of Brij 30 (Figure 3), without notable changes in *nidA* copies; and ii) Brij 30 exhibits low microbial toxicity below 1.5 g L\(^{-1}\) (*Kim, Park et al. 2001*). Several studies have reported that organic contaminant adsorption onto ACs, and especially biochars, would be considerably restrained by surfactants (*Ahn, Kim et al. 2008, Han, Liu et al. 2013*). Ahn et al. found that the amount of PHE adsorbed onto AC and biochar substantially
decreased in the presence of Brij 30, likely due to the high solubilizing ability of the Brij 30 surfactant (Ahn, Kim et al. 2008). Aqueous concentrations for PHE, FLA, and PYR in the Brij 30 amended system after 18 days, were generally greater by a factor of 8.3, 6.1 and 4.5, respectively (SI-Figure S4B, Table S5). Thus, the lower biodegradation observed in the Brij 30 systems is likely due to less sorption to both biochar and M. gilvum cells.

4.3. Effective biodegradation of biochar-sorbed PAHs

Biodegradation of sorbed PAHs has been reported by various pathways, in particular: (1) high-affinity uptake systems of degrading cells (Wick, Springael et al. 2001, Wick, De Munain et al. 2002), (2) adhesion/biofilm formation on sorbed-PAHs (Wick, De Munain et al. 2002, Johnsen and Karlson 2004, Uyttebroek, Breugelmans et al. 2006), and (3) biosurfactant excretion (Deziel, Paquette et al. 1996, Willumsen and Karlson 1996).

*M. gilvum* is one of the most effective PAH-degrading *Mycobacteria* (Kim, Kweon et al. 2010). This genus has been reported to have a high specific substrate affinity towards PAHs, well adapted to degrade sorbed PAHs (Guerin and Boyd 1992, Bastiaens, Springael et al. 2000, Derz, Kliner et al. 2004, Hartmans, de Bont et al. 2006, Uyttebroek, Ortega-Calvo et al. 2006). *Mycobacteria* have complex and extremely hydrophobic rigid cell envelopes, rich in mycolic acids (Hartmans, de Bont et al. 2006), which are important for the interaction with or uptake of hydrophobic compounds (Rijnaarts, Norde et al. 1992). Particularly, several studies reported that the mycolic acid wall monolayer in *Mycobacteria* forms a hydrophobic surface, which may enhance the specific substrate efficiency of PAHs (Bastiaens, Springael et al. 2000, Wick, De Munain et al. 2002, Wick, Pasche et al. 2003). Concentrations of PAHs could be
effectively reduced at the *M. gilvum* cells surface; hence, creating a steep concentration gradient between cell surface and biochar-binding PAHs, which could lead to continuously uptake-driven desorption. In the case of biochar-inoculated with *M. gilvum* cells, biochar acts as an effective sink, to increase PAH flux more than just the freely suspended *M. gilvum* cells.

*M. gilvum* cells may also experience advantages associated with sorbed-PAHs as a substrate (Wick, De Munain et al. 2002, Uyttebroek, Breugelmans et al. 2006). Our durability test data showed appreciable *M. gilvum* biomass was steadily maintained on rice straw biochar (approximately $7.0 \times 10^{10}$ copies/g dw biochar) on the 18th day after immobilization (Figure 2). The enrichment of PAHs on the biochar, as a substrate, not only increases contact opportunity with PAHs, it also increases concentration gradients between PAHs sorbed to biochar surfaces and cell-surfaces across short distances, as was observed elsewhere for 3-chlorodibenzofuran (Harms and Zehnder 1995). Mayer et al reported that the direct contact between a digesting gut and sediment matrix resulted in a ~230 times increase in the PHE mass transfer coefficient (Mayer, Fernqvist et al. 2007), indicating that efficient contact between *M. gilvum* cells and PAHs on biochar would be beneficial to the PAH mineralization.

An additional explanation to account for the quicker degradation kinetics is biofilm formation on biochar. Wick et al. reported the attachment and biofilm formation of *Mycobacterium* sp. LB501T on solid anthracene surfaces using SEM (Wick, Ruiz et al. 2002). In our study, biofilm flocs and EPS on the surface of biochar immobilized *M. gilvum* cells were commonly recorded during SEM imaging (Figure 1 (E) & (F)). Biofilm and EPS formation are conducive to the mass transfer or substrate uptake of PAHs, and the biofilm from the *Mycobacterium* is likely unique to other cell-interface
process occurring in the CP soil. On the other hand, biochar in itself can harbor both hydrophilic and hydrophobic functional groups (SI, Table S2), particularly, comparably higher surface basic (hydrophobic) groups in rice straw biochar would be advantageous for the adsorption of hydrophobic organic contaminants. Thus, several plausible mechanisms may explain why *M. gilvum* cells immobilized on biochar degraded PAHs more rapidly than free *M. gilvum* cells.

5. Conclusions

Indigenous PAH-degrading microbes (*M. gilvum*) were immobilized on rice straw biochar, with high abundance and durability. The *M. gilvum*-biochar composite showed superior degradation capacity for phenanthrene, fluoranthene and pyrene, with a 62.6±3.2%, 52.1±2.3% and 62.1±0.9% of removal from the historically contaminated soil, respectively. It was proposed that the improved remediation was attributed to the targeted degradation of biochar sorbed PAHs. Biochar in itself was a carbonaceous ‘sink’ both for pollutants and degrading cells. Such simultaneous enrichment provided a means to reduce pollutants and degrading microbes being spatial isolated from one and other. The proposed mechanism was further supported by observing a significantly decrease in the degradation of PAHs in a biochar-*M. gilvum* composite system when the surfactant Brij 30 was added to impede the PAH mass transfer to biochar. Further research to investigate the delivery of PAHs towards the biochar surfaces could provide better understanding of the underlying mechanisms of this enhanced PAH-degradation soil system. Whether similar results would occur in the field as observed in this lab-study is uncertain, and would need to be investigated as a follow-up study.
Development of inoculant on biochar is also expected to be investigated for a better understanding of biochar-microbe interactions.

**Supporting Information.**

Additional methods, data tables and figures can be founded in the Supporting Information.

**Acknowledgment**

This research was financially supported by the National Natural Science Foundation of China (Grant No. 41271324) and the National High-Tech R&D Program of China (863 Program) (Grant No. 2013AA06A211).
References


Supporting Information
Enhanced biodegradation of PAHs in historically contaminated soil by *M. gilvum* inoculated biochar cells

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Number of pages: 12
Number of tables: 5
Number of figures: 4

**S1. Supporting Methodology**

**pH** was measured in a suspension of soil/biochar and 0.01 M CaCl₂ (1:2.5).
Total C, N and S in soil, biochar were measured by dry combustion using a macroelementor (VarioMax CNS, Germany). Soil (400 mg) and biochar (300 mg) samples were processed using combustion temperature (H1) 1140°C, post combustion (H2) 800°C and reduction (H3) 850°C, while He flow was 680 ml/min as mentioned in the operational manual. Soil particle size was measured using the Mastersizer 2000 (Malvern Instruments Ltd, UK)

Soil organic matter (SOM) was determined by oxidation with potassium dichromate–titration with FeSO₄.

Soil particle size distribution was measured using the micro-pipette method. (Miller and Miller 1987)

Selection of biochar pyrolysis temperature. Rice straw was pyrolized at 300°C, 400°C, 500°C, 600°C to produce separate biochars. Characterization of these biochars showed that their specific surface area, pore volume and mesopore volume increased with an increase of pyrolysis temperature. Specifically, the specific surface area of biochar produced at 300°C, 400°C, 500°C, 600°C was 9.45, 11.36, 68.06 and 76.14 m² g⁻¹, respectively; whereas for pore volume, it was 0.0114, 0.0159, 0.1732 and 0.1862 cm³ g⁻¹, respectively; and for the mesopore volume, it was 6.4, 9.5 15.7 and 17.3 m² g⁻¹, respectively. However, biochar yield decreased substantially with an increase of pyrolysis temperature, with a yield of 42.23%, 35.52%, 34.23% and 25.94%, respectively. Higher specific surface area, pore volume and mesopore volume are advantageous for bacterial adsorption. Considering these fetures of biochar prepared at 500°C and 600°C were similar, ultimately we selected pyrolysis temperature of 500°C.
for a higher yield of biochar.

The Brunauer-Emmett-Teller method of multiple points was employed for surface area calculation, while four data points, with relative pressures of 0.05 to 0.3, were used to construct the monolayer adsorption capacity.

Ash content was determined by ASTM proximate analysis method for biochars (ASTM D1762-84, reapproved 2007). Detailedly, ash is determined as the residue after burning to constant weight at 750°C.

The titration method proposed by Boehm (Boehm 2002) was used to estimate the number of oxygenated surface groups. Rice straw biochar (0.5 g) was respectively added with 25 ml of the following solutions: 0.1 M HCl, 0.1M NaOH, 0.1 M Na₂CO₃ and 0.1 M NaHCO₃ in 50-ml centrifuge tube. All solutions were then incubated on a rotary shaker at 200 r min⁻¹ for 30 min. After that solutions were equilibrated in a constant temperature incubator at 25°C for 24 h. After the equilibration process, samples were filtered through the polycarbonate filter. A volume of 10 ml of each solution obtained by the filtration process was transferred into 150-ml flasks and diluted with 40-ml distilled water. A drop (200 μl) of the indicator (phenothalin/methyl red) was added to each of the test flask which were subsequently stirred at the vortex. Then the all prepared solutions were used to the acid–base titration by 0.1 M NaOH or 0.1 M HCl.

PAH analysis in CP soil. PAHs in CP soil with different treatments were extracted using a previously established method for quantifying the 16 US EPA PAHs in biochars and biochar amended soils. (Fabbri, Rombolà et al. 2013) Briefly, 1 g dw soil was extracted in a
cellulose thimble in 200 ml of acetone:cyclohexane (1:1, v/v) in a Soxhlet for 48 h. Twenty-five μl of surrogate standard mix at a concentration of 400 μg ml\(^{-1}\) were added prior to extraction. Soxhlet extracts were concentrated to approximately 1 ml by rotary vacuum evaporation at 39°C. Then 15 ml of hexane were added and further concentrated to 1 ml to accomplish the exchange of solvent before clean-up, following the US EPA Method 3630C, and thereafter exchanged back to hexane and diluted by a factor of 100 (to quantify the abundant PAHs in the CP soil). Fifty μl of internal standard hexamethylbenzene (10 μg ml\(^{-1}\) in acetone) were added before analysis by gas chromatography-mass spectroscopy.

____________________________
# S2. Supplementary Data Tables

## Table S1 Characteristics of biochar derived from different feedstocks

<table>
<thead>
<tr>
<th>Biochar</th>
<th>Elemental analysis (%)</th>
<th>Ash content (%)</th>
<th>pH</th>
<th>BET SSA (m²·g⁻¹)</th>
<th>Pore volume (cm³·g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rice straw biochar</td>
<td>48.6 1.7</td>
<td>29.3</td>
<td>10.14</td>
<td>68.06</td>
<td>0.173</td>
</tr>
<tr>
<td>Sewage sludge biochar</td>
<td>27.1 3.4</td>
<td>70.4</td>
<td>10.00</td>
<td>5.42</td>
<td>0.014</td>
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<tr>
<td>Pig manure biochar</td>
<td>63.9 4.9</td>
<td>70.4</td>
<td>9.52</td>
<td>10.48</td>
<td>0.020</td>
</tr>
</tbody>
</table>

## Table S2 Results of Boehm titrations of different biochars

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<thead>
<tr>
<th>Biochar</th>
<th>Acidic surface functions (mmol g⁻¹)</th>
<th>Basic groups (mmol g⁻¹)</th>
<th>Total surface oxides (mmol g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Carboxyl groups</td>
<td>Lactones</td>
<td>Hydroxyl group of phenolic character</td>
</tr>
<tr>
<td>Rice straw biochar</td>
<td>0.167</td>
<td>0.156</td>
<td>0.228</td>
</tr>
<tr>
<td>Sewage sludge biochar</td>
<td>0.272</td>
<td>0.172</td>
<td>0.611</td>
</tr>
<tr>
<td>Pig manure biochar</td>
<td>0.161</td>
<td>0.056</td>
<td>0.278</td>
</tr>
</tbody>
</table>
Table S3 Formula of minimal medium (MM) solutions and plate

One liter MM solution contained $K_2HPO_4$ (6.0 g), $KH_2PO_4$ (5.5 g), $Na_2SO_4$ (2.0 g), KCL (2 g), 1 ml of trace element solution.

**Trace element solution:**

<table>
<thead>
<tr>
<th>Chemical Formula</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrilotriacetic acid</td>
<td>1.500 g</td>
</tr>
<tr>
<td>MgSO$_4$×7 H$_2$O</td>
<td>3.000 g</td>
</tr>
<tr>
<td>MnSO$_4$× H$_2$O</td>
<td>0.500 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>1.000 g</td>
</tr>
<tr>
<td>FeSO$_4$×7 H$_2$O</td>
<td>0.100 g</td>
</tr>
<tr>
<td>CoSO$_4$×7 H$_2$O</td>
<td>0.018 g</td>
</tr>
<tr>
<td>CaCl$_2$×2 H$_2$O</td>
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</tr>
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<td>ZnSO$_4$×7 H$_2$O</td>
<td>0.180 g</td>
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<tr>
<td>CuSO$_4$×5 H$_2$O</td>
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<tr>
<td>KAl(SO$_4$)$_2$×12 H$_2$O</td>
<td>0.020 g</td>
</tr>
<tr>
<td>H$_3$BO$_3$× H$_2$O</td>
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<tr>
<td>Na$_2$MoO$_4$×2 H$_2$O</td>
<td>0.010 g</td>
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<td>NiCl$_2$×6 H$_2$O</td>
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<tr>
<td>Na$_2$SeO$_3$×5 H$_2$O</td>
<td>0.300 mg</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000.000 ml</td>
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</table>

Extra 15 g of agar was added in one liter MM solution during the preparation of MM plate.
Table S4 Residual PAH in CP soil with different treatments for 18 days

<table>
<thead>
<tr>
<th>PAH</th>
<th>CP-0d (mg kg⁻¹)</th>
<th>without Brij 30</th>
<th>residual PAH (mg kg⁻¹)</th>
<th>2 mg g⁻¹ Brij 30</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>CP-18d</td>
<td>CP-BC-18d</td>
<td>CP-M. <em>gilvum</em> -18d</td>
<td>CP-BC-M.<em>gilvum</em> -18d</td>
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<tr>
<td>Naphthalene</td>
<td>4.01±0.39</td>
<td>3.84±0.53</td>
<td>4.07±0.33</td>
<td>4.07±0.33</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>2.42±1.47</td>
<td>4.48±0.74</td>
</tr>
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<td>Acenaphthylene</td>
<td>9.81±0.60</td>
<td>9.28±0.34</td>
<td>9.90±0.83</td>
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<td></td>
<td></td>
<td></td>
<td>8.90±0.63</td>
<td>9.58±0.28</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>2.69±1.05</td>
<td>2.94±0.87</td>
<td>2.26±0.18</td>
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<td></td>
<td></td>
<td></td>
<td>1.91±0.85</td>
<td>2.86±0.69</td>
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<tr>
<td>Fluorene</td>
<td>6.67±1.87</td>
<td>6.23±1.74</td>
<td>6.37±0.51</td>
<td>6.35±0.51</td>
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<td>4.93±1.31</td>
<td>7.00±0.50</td>
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<td>Phenanthrene</td>
<td>73.74±3.75</td>
<td>70.81±6.59</td>
<td>65.89±5.46</td>
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<td>27.59±17.33</td>
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<td>Anthracene</td>
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<td>11.13±2.73</td>
<td>15.09±1.54</td>
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<td>Fluoranthene</td>
<td>108.34±6.09</td>
<td>105.94±1.31</td>
<td>105.97±2.82</td>
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<td>51.91±2.51</td>
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<td>Pyrene</td>
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<td>Dibenz(a,h)anthracen</td>
<td>19.40±0.11</td>
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<td>Σ 16 PAHs</td>
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### Table S5 Aqueous PAH insuspensions separated from soils with different treatments for 18 days

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<tr>
<th>PAH</th>
<th>without Brij 30</th>
<th>Aqueous PAH (μg L⁻¹)</th>
<th>2 g L⁻¹ Brij 30</th>
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<tr>
<td>Naphthalene</td>
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<td>Acenaphthylene</td>
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<td>20±2.4</td>
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<td>Fluorene</td>
<td>14±2.9</td>
<td>12.2±1.6</td>
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<td>Phenanthrene</td>
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<td>358.8±24.6</td>
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<td>Fluoranthen</td>
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<td>35.1±3.1</td>
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<td>Pyrene</td>
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<td>Indeno(1,2,3-cd)pyrene</td>
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<td>Dibeno(a,h)anthracene</td>
<td>13.6±2.3</td>
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<td>Benzo(g,h,i)perylene</td>
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<td>Σ 16 PAHs</td>
<td>652.9±35.1</td>
<td>567.36±40.6</td>
<td>634.6±29.7</td>
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</tbody>
</table>
S3. Supplementary Figures

Figure S1. SEM image of biochar derived from different feedstocks: (A), rice straw biochar (×2000); (2) sewage sludge biochar (×2000); and (3) pig manure biochar (×2000). As evident in Figure S1, the surface structure of three biochars were varied greatly: porous structure was commonly observed in rice straw biochar; whereas on sewage sludge and pig manure biochar, it was faultage and bulk aggregates. The existence of suitable pores within the rice straw biochar was evident in the SEM image of Figure S1 (A), indicating that rice straw biochar was more suitable carrier for microbial immobilization than sewage sludge and pig manure biochar.
Figure S2. Biodegradation of 9 PAHs by *M. gilvum* in MM solutions. *M. gilvum* was confirmed to be capable of degrading naphthalene (NAP), fluorene (FLU), phenanthrene (PHE), anthracene (ANT), pyrene (PYR), and fluoranthene (FLA). Benzo(a)pyrene (BAP) was partially degraded. No significant difference in acenaphthylene (AYL) and acenaphthene (ACE) concentrations was observed for 8-day incubation (P=0.964, 1.000 for AYL and ACE respectively), indicating *M. gilvum* was not able to use both AYL and ACE as solo sources of carbon and energy. These results agree with other reports. (Kim, Park et al. 2001)
**Figure S3.** Removal of pyrene in minimal medium solution with different treatments for 5 days. Removal of pyrene was significantly increased in the presence of biochar immobilized with *M. gilvum* cells (P<0.05), regardless of biochar particle size.

Abbreviations: *M. gilvum*—free *Mycobacterium gilvum*, BC<sub>D1-D3</sub>*M. gilvum*—*Mycobacterium gilvum* cells immobilized on biochar with particle size ranging from 0.25mm-2mm (D1), 0.15mm~0.25mm (D2), and < 0.15mm (D3) respectively. Sample numbers n = 3, error bar = 1 standard deviation. Different lower-case letters indicate the mean difference is significant between treatments at the 0.05 level.
Figure S4. Aqueous PAH concentration in suspensions separated from soils with different treatments for 18 days. Panel A: Treatments without Brij 30 amendment. Panel B: treatments with 2 mg g\textsuperscript{-1} soil\textsuperscript{-1} Brij 30. Abbreviations: CP—raw CP soil only; CP-BC—CP soil with sterile rice straw biochar. CP-\textit{M. gilvum}—CP soil with free \textit{Mycobacterium gilvum} cells inoculation. CP-BC-\textit{M. gilvum}—CP soil with biochar impregnated \textit{M. gilvum} amendment

Extended Discussion of Figure S4

In Figure S4A, similar water-dissolved PAH concentrations were detected in solutions separated from soils with four different treatments. Mean porewater solution concentrations of PHE, FLA, and PYR were 371.33±8.38, 38.79±6.13, 76.91±6.39 µg L\textsuperscript{-1}. Those values were a little higher than concentrations of porewater solutions of PAHs reported in analogous studies (Beesley, Moreno-Jiménez et al. 2010). This may be attributed the presence of dissolved organic carbon (DOC), such as originating from extracellular polymeric substances, increases the number of dissolved sorption sites for PAHs, enhancing the fraction of PAHs into the aqueous phase (and lowering apparent \textit{K}_D values based on total aqueous concentration).
S4. References to the Supplementary Information Section


