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Bioremediation of biosolids with *Phanerochaete chrysosporium* culture filtrates enhances the degradation of polycyclic aromatic hydrocarbons (PAHs)

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ABSTRACT

The supplementation of agricultural soils with dewatered sewage sludge represents a technical solution not only to the disposal of the large quantities of biosolids generated daily, but also a potential means of increasing soil fertility and productivity. However, the presence of organic pollutants such as polycyclic aromatic hydrocarbons (PAHs) found in biosolids limits their application to agricultural soils. The application of *Phanerochaete chrysosporium,* its lignocelluloytic cell-free extract, a commercial preparation of the laccase enzyme for the enhanced removal of three PAHs (naphthalene, phenanthrene and pyrene) at two different concentrations (1 and 10 mg g^{-1} biosolids) from a biosolid sample was assessed in terms of both PAH degradation and their impact on the microbial community of the biosolids. The addition of *P. chrysosporium* biomass, a commercial laccase preparation, a *P. chrysosporium* cell-free extract at low PAH concentrations (1 mg g^{-1} biosolids) and high PAH concentrations (10 mg g^{-1} biosolids) resulted in a significant increase ($P < 0.05$) in PAHs degradation when compared with the control (natural attenuation). *P. chrysosporium* cell-free extract showed the highest degradation impact with an average of 80%. The results suggest that this treatment could be commercially used to allow the potential application of biosolids to agricultural soil. Importantly, no obvious effect on the microbial diversity (bacteria and fungi) from PAH-contaminated biosolids was observed. Therefore, the addition of cell-free culture filtrates and bioaugmentation remediation strategies were shown to be effective as an environmentally benign treatment for the removal of PAHs associated with biosolids with potential for large-scale application.

1. Introduction

Dewatered sewage sludge, or “biosolids” are the organic solids derived from municipal and industrial wastewater treatment processes. The sludge contains organic matter, micronutrients and essential plant nutrients (such as nitrogen, phosphorus, potassium, calcium and sulfur) that have a positive influence on soil fertility, soil microbial activity and crop productivity (Al-Dhumri et al., 2013; Clarke, 2008; During and Gath, 2002). Biosolids are produced and stored in large volumes; on average, ninety grams of biosolids (dry weight basis) are produced per capita daily after primary, secondary and tertiary wastewater treatments (Clarke, 2008; Oleszczuk et al., 2012). The US and the European Union report an annual production of 8 and 5.6 million tonnes of dry biosolids from wastewater treatment plants respectively (Clarke, 2008; Oleszczuk et al., 2012). In 2010, the Australian and New Zealand Biosolids Partnership reported that Australian biosolids production ranged between 330,000 and 360,000 dry tonnes annually and this number is increasing steadily due to increasing population (Pritchard et al., 2010). The end-use of Australian biosolids varies from one state to another; however in total 59% is applied to agricultural soil while 20% remains stockpiled, only 10% is used for landscaping and rehabilitation (Pritchard et al., 2010).

Australian agricultural soils are generally considered as infertile, acidic, light-textured with poor physical characteristics and with low organic matter content (McLaughlin et al., 2007). As a result of intensive agricultural practice over the last 200 years, the organic matter content of Australian agricultural soils has been depleted; the addition of biosolids to these soils represents a potentially sustainable approach

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to increasing soil fertility and productivity (Lazzari et al., 2000; Pritchard et al., 2010). The addition of biosolids to Australian soils and the subsequent impact on crop productivity and soil properties has therefore been the subject of many studies, with the focus on understanding the risks posed to public health and the surrounding environment (McLaughlin et al., 2007; Pritchard et al., 2010). Research has confirmed that the physical properties of soil amended with biosolids (including water holding capacity, water penetration, aggregate stability, bulk density and porosity) improved significantly due to the positive effect of organic matter and nutrients (Clarke, 2008).

However, a key issue remains regarding the utilization of Australian biosolids in agriculture. Biosolids not only contain beneficial substances such as organic matter and nutrients, but also potentially toxic chemicals (e.g., heavy metals), pathogens and persistent organic pollutants (POPs, such as polycyclic aromatic hydrocarbons, PAHs) (Clarke, 2008; During and Gath, 2002; Pritchard et al., 2010).

As a result of the prevalence of POPs such as PAHs, the Victoria Environment Protection Authority (EPA-Australia) regulates biosolids according to the concentration of contaminants – C1 (least contaminated), C2 or C3 (highly contaminated) and the required level of treatment T1 (most processed), T2, T3 or T4 (least processed). For instance, historic stockpiles (C3) – are unsuitable for land use as they contain heavy-metals and high concentrations of contaminants discharged over many years by heavy industry in Melbourne's west, while biosolids production (C2) – resulting from cleaner sewage discharges in recent years are treated to a T1 grade and are suitable for forestry and farming applications. The biosolids used in the current project were classified as C2 (Al-Dhumri et al., 2013). The potential adverse effects of biosolids in Melbourne, Australia, has resulted in the requirement for stockpiling management of the biosolids at an annual cost of $AUD 300 per dry tonne, which equates to approximately $100 M per year nationally (Pritchard et al., 2010).

Contreras-Ramos et al. (2008, 2009) reported that although the biostimulation of pristine land with biosolids introduces additional nutrients and increases the diversity of the indigenous microorganisms; PAH removal must be carried out prior to any commercial applications (Contreras-Ramos et al., 2009, 2008). The high cost of removing PAHs from PAH-contaminated sites using traditional means (such as adsorption, volatilization, photolysis, and chemical degradation) has led to a significant bottleneck in its application to agricultural soils (Haritash and Kaushik, 2009). Increasing concern regarding the economic feasibility of cleaning biosolids polluted with PAHs for soil/land application by conventional physico-chemical remediation strategies has necessitated the need to explore biological means as a cost effective and applicable technology (Shahsavari et al., 2015).

PAHs are considered one of the main groups of organic contaminants found in sewage sludge (Haritash and Kaushik, 2009; Oleszczuk et al., 2012). PAHs comprise the largest component of POPs and their concentration in biosolids is increasing, generating concern not only to the wastewater treatment industry but also to agricultural industries. Naphthalene, phenanthrene and pyrene are three of the most abundant PAHs in polluted environments (Sack et al., 1997). PAH concentrations in biosolids vary from trace values (600 mg g^{-1} biosolids) up to 1% (10,000 mg g^{-1} biosolids). Research is urgently required to improve the PAH degradation rates during treatment, thereby providing biosolids that are safe and environmentally benign. A potential approach for POPs such as PAHs is to assess the potential for biodegradation. To date the degradation of organic pollutants present in biosolids has rarely been studied.

Unlike traditional physical and chemical degradation technologies which are expensive and not applicable on a large scale, microbial degradation represents a potential large scale degradation process for biosolids contaminated with PAHs (Haritash and Kaushik, 2009). The microbial degradation of PAHs in soil is well studied and one particular group of microorganisms, the white-rot fungi (e.g., *Phanerochaete chrysosporium*) has been shown to be a key PAH-degrading organism in the environment due to their lignin-degrading enzymes such as laccase. The white-rot fungi show non-specific activity against PAHs and *P. chrysosporium* is able not only to degrade large number of PAH fractions but also has the capability to secrete a cocktail of ligninolytic enzymes which act synergistically against a battery of lignin-related and PAH compounds (Anastasi et al., 2009; Chandra et al., 2016). The aim of this work was to assess the potential application of *P. chrysosporium*, its lignocelluloytic cell-free extract and commercially available laccase for the removal of three PAHs (naphthalene, phenanthrene and pyrene) at two concentrations (1 and 10 mg g^{-1} biosolids) from Australian biosolids in terms of both PAH degradation and the impact on the biosolids microbial community. The novelty of this work is the assessment not just of fungal biomass, but also cell free culture filtrate as well as commercial enzymes to degrade PAHs spiked in biosolids. The use of a cell free culture filtrate offers the potential of a simple and economic amendment to biosolids for the removal of POPs such as PAHs.

2. Materials and methods

2.1. Biosolids collection

Two types of biosolid samples (solid granules and slurry) were obtained from a rural Victorian wastewater treatment plant. The dried biosolids were ground under sterile conditions (in a pestle and mortar) and characterised in terms of elemental analysis using X-ray fluorescence spectroscopy (XRF; Bruker S4 Pioneer) and Scanning Electron Microscope/Energy Dispersive Using X-Ray (SEM-EDX; Philips XL30) available at Royal Melbourne Institute of Technology (RMIT, Melbourne, Australia).

2.2. Elemental analyses

Elemental analyses of the ground biosolids was undertaken semi-quantitatively using X-ray fluorescence spectroscopy (XRF) and (SEM/EDX) according to previous studies (Dos Anjos et al., 2000; Karathanasis and Hajek, 1996; Woods et al., 2014). For XRF, samples were pressed into a sample holder to ensure a flat examination surface; for SEM/EDX analyses about 10 mg of the fine biosolids sample was compressed onto an aluminium SEM pin-stub (12.6 mm) on a fixed-carbon tape and the samples coated with carbon under 7.5 V for 3 s. Analysis was conducted in triplicate under low vacuum (0.5 Torr), high pressure (30 kV), with a working distance of 10 mm and 200× magnification (Woods et al., 2014).

2.3. Polycyclic aromatic hydrocarbon (PAH) contamination

Three contaminants: naphthalene, phenanthrene and pyrene (Supplementary Table 1) were purchased from Sigma-Aldrich (Sydney, Australia) as pure sources to spike the biosolids samples. The chemicals belong to the group of polycyclic aromatic hydrocarbons (PAHs). The PAHs used in this experiment were a combination of equal concentrations of naphthalene, phenanthrene and pyrene to give two concentrations (based on prior testing for optimal concentrations) (Lee et al., 2015; Rivera-Espinoza and Dendooven, 2007; Ting et al., 2011; Wang et al., 2009), 1 mg g^{-1} (1000 ppm) and 10 mg g^{-1} (10000 ppm). The combined concentration at lower level 1000 ppm (1 mg g^{-1}) of the three PAHs was 333.3 μg g^{-1} of each PAH. While at higher level 10000 ppm (10 mg g^{-1}) of the three PAHs was 33.33 mg g^{-1} of each PAH. The PAHs were dissolved in hexane, added and mixed with the biosolids and the hexane allowed to evaporate in the fume hood.

2.4. Enzyme, cell-free extract and culture of Phanerochaete chrysosporium

P. chrysosporium (ATCC 24725) was kindly donated by Professor David Catcheside (Flinders University, South Australia, Australia). *P. chrysosporium* was cultured on Potato Dextrose Broth (PDB) by

transferring 1 square cm plugs (grown on Potato Dextrose Agar) into each flask (250 ml) containing (100 ml) PDB and incubated for 6 days at 28–30 °C at 150 rpm. A cell-free extract of *P. chrysosporium* was prepared by inoculating the fungus (0.7 g l^{-1} DW) into Bushnell and Haas (BH) medium (Sigma-Aldrich; Sydney, Australia) containing sterilised ball-milled rice straw as the main carbon source followed by incubation for 6 days at 28–30 °C at 150 rpm for the effectiveness of crude enzyme production (cell-free extract) and for the enhancement of hydrolyzing and oxidative enzymes (such as laccase). Thus, *P. chrysosporium* was initially cultured on BH-agar medium followed by transferring 1 square cm plugs (grown on BH-Agar) into each flask containing BH-liquid medium where rice straw was used as the sole carbon and energy source. Bushnell Haas (BH) mineral salts medium containing 0.2 g l^{-1} $MgSO_4$-$7H_2O$, 0.02 g l^{-1} $CaCl_2$-$2H_2O$, 1.0 g l^{-1} KH_2PO_4, 1.0 g l^{-1} $(NH4)_2HPO_4$, 1.0 g l^{-1} $(NH4)_2SO_4$, 0.05 g l^{-1} $FeCl_3$-$6H_2O$; pH 7.0 was prepared as a selective media without carbon source and sterilized at 121 °C for 15 min. This medium was amended with ball-milled rice straw (0.5% w/v) as sole carbon and energy source (Bushnell and Haas, 1941; Taha et al., 2015). The cell-free extract was centrifuged at 4 °C at 4700*g* for 15 min and the supernatant was filtered (0.22 µm) and stored at −20 °C until further use. Laccase activity was measured on the *P. chrysosporium*'s cell free extract using ABTS substrate after 6 days of incubation. Commercial laccase was purchased from Sigma-Aldrich (Sydney, Australia) and was dissolved in Sodium Citrate Buffer (0.05 M, pH 4.8) to achieve a final concentration of 50 U ml^{-1}.

2.5. Biosolids-PAHs biodegradation experiments

Four different bioremediation methods were applied to assess the potential for PAH degradation within the biosolids sample: natural attenuation, two amendment treatments and bioaugmentation; each 500 ml sterilised flask contained 100 g of both liquid (slurry) and 100 g dry biosolids spiked with the PAH mixture (naphthalene, phenanthrene and pyrene) at the desired contamination level (1 mg g^{-1}) and (10 mg g^{-1}). Natural attenuation treatments were carried out by adding sterile water (50 ml). Bioaugmentation treatments were conducted by adding *P. chrysosporium* biomass (50 ml, with an inoculation rate of 0.7 g l^{-1}; DW). Amendment experiments included two types of amendment treatments i. the addition of *P. chrysosporium* cell free extract (50 ml at 50 U ml^{-1}) and ii. the addition of cell free laccase (50 ml at 50 U ml^{-1}). Three replicates were used for each treatment so a total of 24 flasks were used. Following amendment, flasks were incubated for 14 d at 28–30 °C and samples taken regularly under aseptic conditions for analysis in terms of microbial community and PAH concentrations.

2.6. DNA extraction and PCR-density gradient gel electrophoresis GGE for microbial community assessment

Genomic DNA was extracted using a MO-BIO PowerSoil DNA Isolation Kit according to the manufacturer's guidelines (Carlsbad, CA, USA). The extracted DNA (2 µl) was kept at −20 °C and used as a template for bacterial and fungal PCR amplification. Universal bacterial primers with GC clamp 341F-GC (CGCCCGCCGCGCCCCGCG-CCCGTCCCGCCGCCCCCGCCCGCCTACGGGAGGCAGCAG) and 518R (ATTACCGCGGCTGCTGG) were used for the amplification of bacterial species, and universal fungal primers with GC clamp ITS1F-GC (CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGCTTGGT-CATTTAGAGGAAGTAA) and ITS2R (GCTGCGTTCTTCATCGATGC) were used for the amplification of fungal species. The above underlined region (40-base) represents a DGGE-required GC-clamp added to the front of forward primers (Muyzer et al., 1993; Sheppard et al., 2011). Universal primers (bacterial and fungi) without the GC-clamp were used for the identification of dominant microbial species from DGGE gels. The PCR reaction mixture (50 µl) was composed of 27.75–29.75 µl sterile water, 10 µl of GoTaq flexi buffer (10×), 3–5 µl of $MgCl_2$ (25 mM), 1 µl of dNTPs mixture (2 mM), 2 µl of each primer (10 pmol μl^{-1}) and 0.25 µl of Taq DNA polymerase enzyme (5 U μl^{-1}). For each sample, DNA template (2 µl) was added to the PCR mastermix (48 µl). The main bacterial thermocycling programme for 341F-GC and 518R primers was one cycle of 5 min at 95 °C; 31 cycles of 30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C and a final extension at 72 °C for 10 min. The profile program for fungal amplifications using ITS1F-GC and ITS2R was 1 cycle of 5 min at 95 °C; 35 cycles of 45 s at 94 °C, 45 s at 58 °C and 45 s at 72 °C and a final extension at 72 °C for 10 min. All PCR reactions were performed in a thermocycler (BioRad, Australia).

Denaturing Gradient Gel Electrophoresis (DGGE) analysis was carried out using the Universal Mutation Detection System (BioRad) with a 9% urea-formamide denaturant gradient polyacrylamide gel. Polymerisation of gels was catalysed by the addition 10% ammonium persulfate solution and *N,N,N′,N′*-tetramethylethylenediamine (TEMED) before pouring into the gradient maker. The denaturant gradient ranged over 40–65% for both bacterial and fungal communities. The gel was loaded with bacterial and fungal PCR amplicons (15 µl) in a 40–65% gradient gel and run for 16 h at 60 °C and 60 V in 1× TAE buffer for microbial community analysis (at day 0, 7 and 14) using DNA-PCR-DGGE for both bacterial and fungal communities (Shahsavari, 2013). After electrophoresis, the DGGE gel was silver stained before the gel was scanned, photographed and saved as TIFF images with an Epson V700 scanner. Cluster analysis was performed using UPGMA with Phoretix 1D. The total richness was estimated by the number of DGGE bands for each treatment. The Shannon diversity index was calculated using the formula $H' = -\Sigma\, p_i \mathrm{L}\mathrm{N}p_i$ (Girvan et al., 2003).

2.7. Identification of dominant PAHs microbial degraders

Four and five major DGGE bands, representing the most dominant PAHs degraders were selected from the bacterial and fungal DGGE gels, respectively. Dominant bands from DGGE gels were sliced out and aseptically placed into 1.5 ml tubes containing 0.5 ml sterilized water for extraction of the DNA from each band. The tubes were held at 37 °C overnight for complete DNA diffusion and elution. After centrifugation, further amplification of the DNA was conducted using the universal bacterial and fungal primers without a GC-clamp described above. The resulting PCR products were checked in 1.5% agarose gel and purified using a GENECLEAN turbo kit (MP Biomedicals; Australia) and quantified using a Nanodrop Lite Spectrophotometer (Thermo Scientific, Australia). After optimizing, the concentration and the purity of purified PCR samples (forward and reverse primer products) were sent to the Australia Genome Research Facility. The retrieved sequences were analysed using a sequencer (Version 5.0, USA) and blasted using National Centre for Biotechnology Information. The overall similarities of bacterial and fungal sequences were determined (Table 2).

2.8. PAH extraction and GC–MS analyses

The PAHs were extracted from the biosolids samples following development and validation of the extraction method. For quality assurance/control (QA/QC) the method was validated with three standards. The linearity of the validated method was evaluated by analysis of working standard solutions extracted from both acid-washed sand and biosolids at five different concentrations (n = 3). Concentrations of PAHs were monitored over time in all treatments. For extraction, one gram of each sample (in triplicate) was added to 20 ml hexane (GC grade, Sigma) and the tubes vortexed for 15 min. The tubes were then sonicated for 5 min at 16 kilohertz (kHz) and then centrifuged at 4700*g* for 5 min (Lau et al., 2010). An aliquot (100 µl) was transferred into a fresh tube containing hexane (19.9 ml) for dilution. Finally, an aliquot (1.5 ml) was transferred into a clean GC vial and analysed by GC–MS. From each vial, 1 µl was injected at 55 °C and 13.03 psi onto the Agilent column (19091S-433UI; with HP–5 ms Ultra Inert). The Agilent column

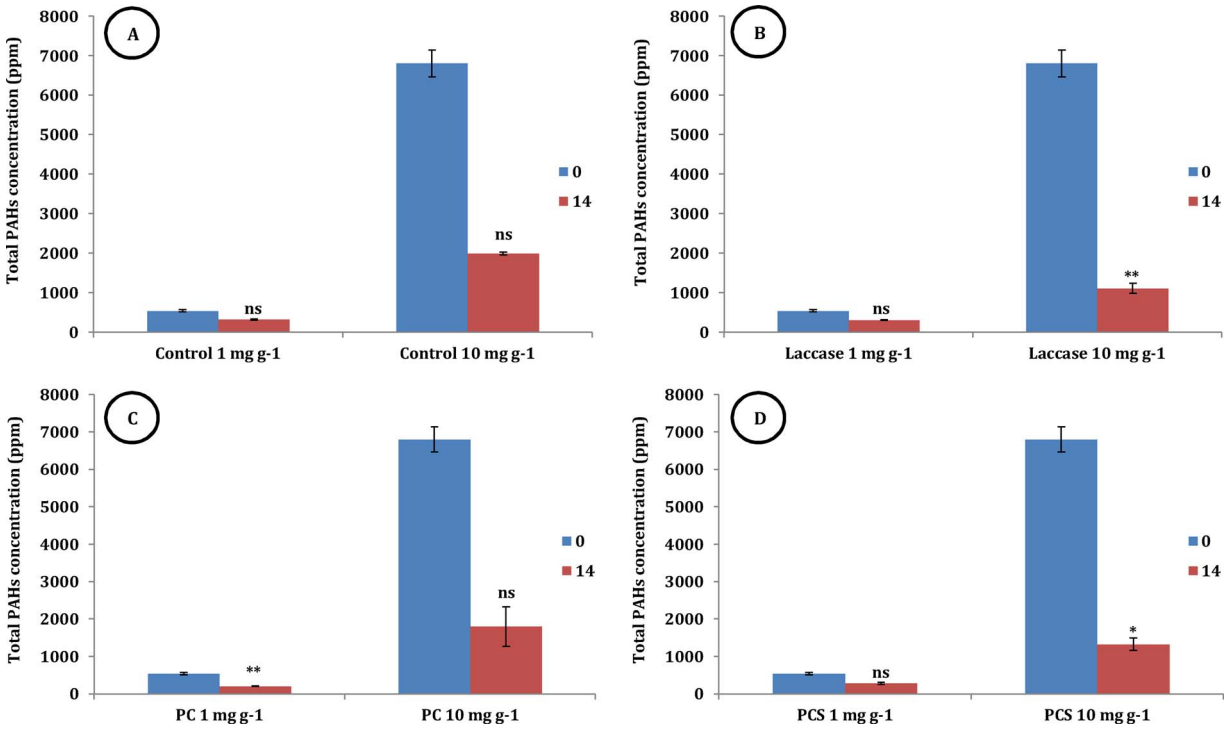

Fig. 1. Impact of natural attenuation (control) (A), amendment with laccase at 50 U l^{-1} (B), bioaugmentation with P. chrysosporium (C) and amendment using *P. chrysosporium* cell-free extract (D) on the removal of PAHs (naphthalene, phenanthrene and pyrene) at 1 mg g^{-1} (1000 ppm) and 10 mg g^{-1} (10,000 ppm) from biosolids. ns = not significant, * and ** indicate significant in P value 0.05 and P value 0.01 respectively among different treatments (red bars). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

parameters were 30 m × 250 µm × 0.25 µm and the injection flow rate was adjusted to 1.5 ml min^{-1}. Helium and nitrogen were used as quench and collision gases at 2.25 and 1.5 ml min^{-1}, respectively. The GC-oven was heated from 55 °C up to 325 °C (25 °C min^{-1}) and each sample allowed to run for 17 min.

2.9. Data analysis

T test and one way analysis of variance (ANOVA) were used to determine the statistical difference in the PAH concentrations in all treatments using GraphPad Prism (version 6.0) to determine the significant difference among of all treatments ($P < 0.05$).

3. Results and discussion

3.1. The bioremediation of PAHs

Biosolids represent a renewable source of organic matter and nutrients, and our elemental analysis of the biosolids used in this study (using XRF and EDX-SEM) confirms their suitability as potential agricultural amendments. Phosphorus (P), calcium (Ca), silicon (Si), aluminium (Al) and iron (Fe) were present in relatively high concentrations (Supplementary Fig. 1). Extensive studies have confirmed the beneficial application of using biosolids not only to improve soil fertility and crop productivity but also significant economic and environmental improvements in the forestry sector (such as pine plantations) were observed compared with utilising inorganic fertilisers (During and Gath, 2002). The physical properties of the soil amended with biosolids improved significantly due to the positive effect of increasing organic matter, nutrients and microbial populations. Therefore, the idea of supplementing poor agricultural soil with a sustainable supply of clean biosolids that are rich in organic and inorganic fertilizers is well established (Clarke, 2008; During and Gath, 2002).

Before collecting biosolid samples for extracting PAHs, approximately 90% of the selected PAHs were recovered when extracted from both biosolids and acid-washed sand, confirming and ensuring the applicability of the validated extraction method for the quality control of GC–MS analyses (data not shown). The effect of natural attenuation (which acted as a control), amendment treatments (either through the addition of commercial laccase or *P. chrysosporium* cell-free extract) and bioaugmentation (through the addition of *P. chrysosporium* biomass) on PAH reduction at two PAH concentrations of 1 mg g^{-1} (1000 ppm) and 10 mg g^{-1} (10,000 ppm) was monitored in the contaminated biosolids at 0 and after 14 days (Fig. 1). The measured PAHs at time 0 confirmed the exact initial starting concentrations as (1 mg g^{-1}) 41, 1000, 577 ppm and (10 mg g^{-1}) 2695, 9604 and 8103 ppm respectively across all incubations for naphthalene, phenanthrene and pyrene respectively. The relatively low concentration of naphthalene suggests significant initial volatilization due to its high vapor pressure (36 PL/Pa) when compared to phenanthrene (0.107 PL/Pa) and pyrene (0.0119 PL/Pa) (Mackay et al., 2006).

All bioremediation treatments, including the cell free culture filtrate showed a reduction in PAH concentrations over the 14 d experiment at both concentrations. At the lower PAHs-concentration (1 mg g^{-1}), bioaugmentation treatment using *P. chrysosporium* biomass resulted in significantly greater PAH degradation from the initial concentration 539 ppm to 204 ppm corresponding to approximately 62% PAHs degradation compared to the natural attenuation's reduction from initial concentration 539 ppm to 320 ppm corresponding to approximately 40% reduction. Other amendment treatments using laccase and *P. chrysosporium* cell-free extract showed only a slightly greater level of PAH degradation than the natural attenuation treatments. When commercial laccase was applied, it resulted in a slight PAH-degradation from initial concentration 539 ppm to 300 ppm corresponding to approximately 44% when compared to the natural attenuation treatment while in the case of using *P. chrysosporium* cell-free extract, the initial concentration reduced from 539 ppm and reached approximately 280 ppm corresponding to approximately 48% reduction (Fig. 1).

Table 1
Biodegradation of the three PAHs (naphthalene, phenanthrene and pyrene at equal concentrations to give total concentrations of 10 mg g^{-1} by the different treatments of natural attenuation (control), laccase, P. chrysogenum biomass (PC) and P. chrysogenum cell-free extract (PCS)over 14 days. (mean ± SD, n = 3).

PAHs concentration	Time (Day)	Degradation of PAHs (%)			
(10 mg g^{-1})		Control	Laccase	PC	PCS
Naphthalene	0	0.00	0.00	0.00	0.00
	7	94.35	97.11	95.92	96.96
	14	96.39	**97.50**	97.26	97.18
Phenanthrene	0	0.00	0.00	0.00	0.00
	7	24.93	46.39	48.55	60.49
	14	71.15	**87.41**	80.67	82.94
Pyrene	0	0.00	0.00	0.00	0.00
	7	45.04	70.11	57.80	72.23
	14	61.83	**74.81**	58.56	72.99

Interestingly, at the higher PAHs concentration (10 mg g^{-1}), using commercial laccase exhibited significantly greater PAH degradation from the initial PAHs concentration 6800 ppm to 1106 ppm reaching approximately 84% PAH degradation, while when *P. chrysosporium* cell-free extract was applied, the PAHs concentration reduced by 81% from 6800 ppm to 1322 ppm. However, in the natural attenuation and bioaugmentation treatments, the percentage of PAH degradation reduced from the initial concentration 6800 ppm and reached to 1987 ppm and 1795 ppm corresponding to 70 and 74% reduction respectively (Fig. 1). Overall, the addition of *P. chrysosporium* biomass at low PAH concentrations and the addition of laccase and *P. chrysosporium* cell-free extract at high PAH concentration resulted in a significant increase in PAH degradation compared with the natural attenuation treatments. The addition of fungal biomass at low PAH concentrations increased the PAH degradation because at those low level, toxicity related to PAHS is low and as a result, *P. chrysosporium* was able to survive while the addition of the cell free extract and laccasse increased the degradation at higher concentrations because the PAH compounds in high concentration may be more toxic for *P. chrysosporium*. Therefore, the potential application of the amendment and bioaugmentation remediation strategies was shown to be effective for the biodegradation of PAHs from biosolids with potential for application on a commercial scale.

3.2. PAH biodegradation patterns

In general, the removal pattern of the individual PAHs varied significantly from one treatment to another and depended on the chemical structure of PAHs (Table 1 and Supplementary Table 1). Overall, naphthalene (the simplest PAH tested) was the most biodegradable PAH using all treatments except natural attenuation (97% maximum degradation) compared to both phenanthrene (87%) and pyrene (74%); this pattern has also been previously described (Mackay et al., 2006). The lower levels of pyrene degradation seen in this work may be due to its complicated and recalcitrant chemical structure ($C_{16}H_{10}$, four fused rings) when compared to naphthalene and phenanthrene (Supplementary Table 1). In a comparative study of the biodegradation rate of both phenanthrene and pyrene by different wood-decaying fungi, Sack et al. (1997) found that phenanthrene was degraded at a faster rate than pyrene. The authors reported that both phenanthrene and pyrene were distinguished by their water solubility and ionization potential ((IP), the energy required to form radicals). Pyrene solubility (0.14 mg/l) was approximately 10 times lower than phenanthrene (1.3 mg/l) while the IP for phenanthrene (8.19 eV) was slightly higher than for pyrene (7.50 eV) (Sack et al., 1997). They reported that after 63 days incubation of liquid culture, 9.0 and 1.9% of the phenanthrene and pyrene, respectively, was degraded by different wood-decaying fungi. In a recent study conducted by Lee et al. (2014) found that 11 white-rot fungi species not only exhibited a high tolerance against to PAHs, but also their ligninolytic enzymes were involved in the biodegradation of the four PAHs: phenanthrene, anthracene, fluoranthene and pyrene at 30 mg l^{-1} (Lee et al., 2014). The same trend was observed by (Jacques et al., 2008). Our biodegradation values of phenanthrene and pyrene were comparable to those previously reported (Jacques et al., 2008; Lee et al., 2014; Sack et al., 1997). Overall, comparing all treatments naphthalene was degraded to a similar level across all treatments (Table 1) while phenanthrene and pyrene degradation were greatest in the biosolids amended with laccase (87% and 75%), compared with only 71% and 62% for the control treatments respectively.

3.3. Microbial community analysis

The microbial diversity of the biosolids exposed to the different treatments was examined using PCR-denaturing gradient gel electrophoresis (PCR-DGGE) at both PAH concentrations levels at selected times. These times (days 0, 7 and 14) were selected based on the observed changes in the rate of PAH degradation. There were some changes in microbial diversity as shown in the dendrograms: however, both the Shannon diversity index and richness were not significantly different among the treatments (data not shown). The resultant dendrograms (Figs. 2 and 3) show that there was no obvious effect on the bacterial or the fungal diversity for the biosolids contaminated with PAHs at both concentrations, nor were there any obvious differences in the structure of the bacterial and fungal communities between the natural attenuation, amendments and bioaugmentation treatments. The microbial communities did however change with time; similarity values were in the range 65–85% for both bacteria and fungi (Figs. 2 and 3). The results suggest that amendments or bioaugmentation treatments

Table 2
Closest relatives to the predominant microbial species identified from PCR-DGGE gels from the PAH-contaminated biosolids.

Band No.	Name	Phylum	Accession Number	Similarity (%)	Query Cover (%)
Bacterial species					
1	*Bacillus cereus*	Firmicutes	GU222440.1	100	99
2	*Arthrobacter rhombi*	Actinobacteria	KC128947.1	99	98
3	*Microbacterium oxydans*	Actinobacteria	HQ425663.2	97	97
4	*Lysinibacillus sphaericus*	Firmicutes	KP347685.1	99	100
5	*Streptomyces cinereus*	Actinobacteria	KR063215.1	98	100
6	*Sporosarcina aquimarina*	Firmicutes	KM036087.1	99	100
Fungal species					
1	*Phanerochaete chrysosporium*	Basidiomycota	KP135092.1	100	100
2	*Mucor circinelloides*	Mucoromycotina	EU484195.1	99	100
3	*Penicillium rubens*	Ascomycota	KP329843.1	100	100
4	*Penicillium madriti*	Ascomycota	KP329774.1	98	97
5	*Trichosporon guehoae*	Basidiomycota	KF990133.1	100	95
6	*Nectria haematococca*	Ascomycota	KJ528884.1	99	95

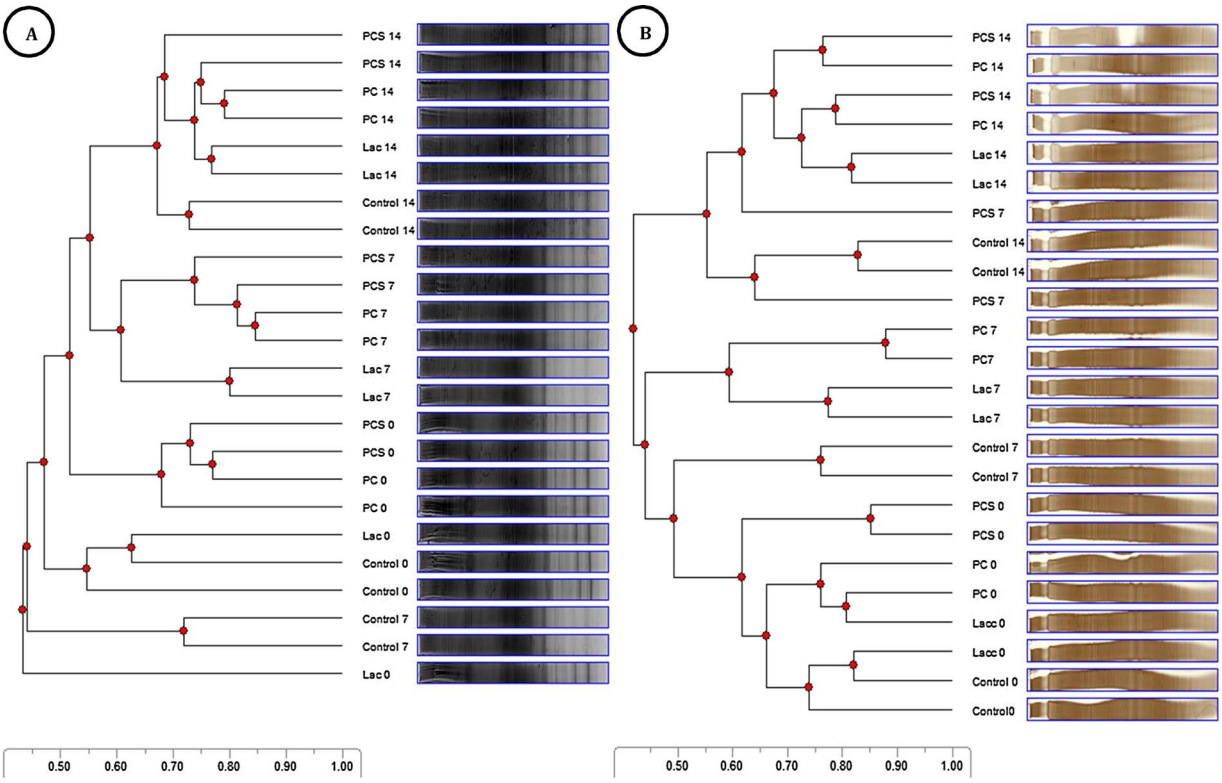

Fig. 2. Dendrogram of bacterial community from biosolids at 0, 7 and 14 days of all treatments (control, laccase, *P. chrysosporium* and *P. chrysosporium* cell-free extract) at (A) low (1 mg g^{-1}, 1000 ppm) and (B) high (10 mg g^{-1}, 10,000 ppm) PAH concentration.

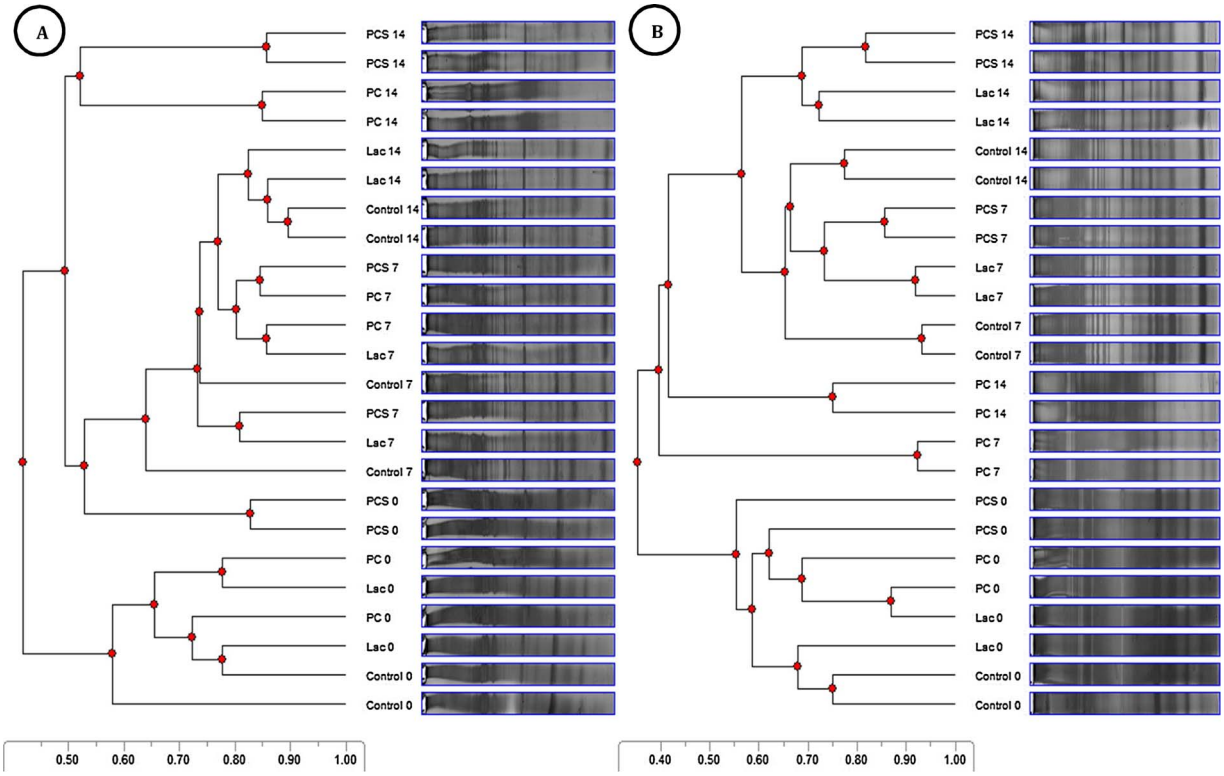

Fig. 3. Dendrogram of fungal community from biosolids at 0, 7 and 14 days of all treatments (control, laccase, *P. chrysosporium* and *P. chrysosporium* cell-free extract) at (A) low (1 mg g^{-1}, (1000 ppm)) and (B) high (10 mg g^{-1}, (10,000 ppm)) PAH concentration.

which resulted in increased PAH degradation did not appear to change the structure of the indigenous microbial community. This is important as treatment should not affect the degradation of other organic contaminants within the wastewater.

3.4. Identification of potential PAH-degrading microbes

Microbial community profiles at the selected time points did, however, show some dominant bacteria and fungi for all treatments at both PAH concentrations; these bands, because of their dominance during treatment, may represent PAH degraders. Selected dominant species (6 bacteria and 6 fungi) from bacterial and fungal PCR-DGGE gels were extracted, amplified using 16S rRNA primers without GC clamp, sequenced and identified (Table 2).

Previous studies have found that dominant species played an active role in the biodegradation of PAHs at high concentrations of PAH (Jacques et al., 2008). Most of the microbial species identified were previously isolated from a crude oil-contaminated soil or petrochemical sludge landfarming site and were exploited effectively as a polycyclic aromatic hydrocarbon–degrading microbial consortium (Jacques et al., 2007; Küce et al., 2015). Our results confirm that the native microbial community in the wastewater/biosolids contains potential PAH degraders. However, addition of live fungal biomass or amendment with fungal extract or free cell laccase increases the degradation suggesting that the addition of key fungal oxidative enzymes may carry out non-specific attack on the PAHs, enabling the indigenous microbial community to degrade the contaminants more readily. White-rot fungi are able to utilize PAHs through the action of ligninolytic enzymes such as lignin peroxidase, manganese peroxidase and laccase. The white-rot fungi use this enzyme to degrade lignin but the enzyme is also capable of degrading other aromatic structures such as PAHs.

One of the dominant fungal bands detected in the bioaugmentation treatments was identified as *P. chrysosporium* (Table 2), confirming the success of the bioaugmentation; this dominant band was absent in the natural attenuation and both amendment treatments. *Mucor circinelloides* and *Penicillium* sp. were also identified as dominant fungal species (Table 2). These fungal species have previously been isolated and identified from sewage sludge and phylogenetic analysis showed that these genera are dominant species in biosolids (Frąc et al., 2014; More et al., 2010). The occurrence of the other dominant fungal species such as *Penicillium rubens*, *Penicillium madriti* and *Trichosporon guehoae* has also been reported (Frąc et al., 2014; More et al., 2010; Sack et al., 1997). Previous studies reported that *Penicillium* species (*Penicillium rubens* and *Penicillium madriti*) found in sewage sludge were present inaround 50% of all studied fungal communities (Table 2) (Frąc et al., 2014; Lee et al., 2014; More et al., 2010).

Jacques et al. (2008) successfully isolated potential PAH degraders (such as *Bacillus cereus* and *Microbacterium* sp.) from a PAH-contaminated site with the metabolic ability to degrade different concentrations (250, 500 and 1000 mg kg^{-1}) of PAHs. A further study reported more efficient PAH-degrading microbial consortia that also contained *Microbacterium* species (Jacques et al., 2009). In fact two *Microbacterium* species: *Microbacterium oleivorans* and *Microbacterium hydrocarbonoxydans* were described as Gram positive novel oil degrading bacteria in 2005 (Schippers et al., 2005) and previously another *Microbacterium* strain with the ability to degrade PAHs was also found in polluted Greek soils (Zhang et al., 2004).

The strains *B. cereus* and *Microbacterium* sp. were identified in this study as dominant species in the biosolids (Table 2). *Arthrobacter rhombi* was also a dominant bacterium and has been classified as a halotolerant PAH degrader in Russia (Plotnikova et al., 2011). Moreover *Sporosarcina aquimarina* was recently isolated from a petroleum contaminated soil in Mexico and its ability as a plant growth promoting bacteria was also reported (Cruz-Morales et al., 2016). Previously this strain had been detected in coastal mangrove pioneer plants (sona Janarthine et al., 2011). *Lysinibacillus sphaericus* was reported for the first time as a PAH degrader in a study involving the rhizosphere of four crops in Chinese contaminated fields (Ma et al., 2010). Therefore, the strains described above have great biotechnological potential. The findings from sequencing of the predominate bands also suggest that the biosolids microbial communities for the degradation of PAHs become robust and stable since the early days of the treatment and it may explain why the addition of treatments did not change the communities dramatically.

4. Conclusions

PAHs have the ability to remain in biosolids for long periods and limit their use in land application. *P. chrysosporium* and its extracellular enzymes can be exploited for the biodegradation of PAHs which are associated with biosolids to enable their use for soil amendment and to ensure environmental protection. All treatments tested: bioaugmentation, both amendments and natural attenuation, led to a high percentage of PAH removal from the biosolids at both concentrations tested (1 and 10 mg g^{-1} biosolids). Bioremediation provides a mechanism whereby the large volumes of biosolids generated daily can be treated to remove PAHs and thus enable their utilization for land application. Moreover, the use of cell free culture filtrates in particular represents a novel, effective and economic technology for PAH removal from biosolids.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.apsoil.2017.11.002.

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