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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