Published in final edited form as:

Remediation (N Y). 2016 ; 26(2): 59–72. doi:10.1002/rem.21458.

A New Perspective on Sustainable Soil Remediation—Case Study Suggests Novel Fungal Genera Could Facilitate *in situ* Biodegradation of Hazardous Contaminants

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Abstract

Deciding upon a cost effective and sustainable method to address soil pollution is a challenge for many remedial project managers. High pressure to quickly achieve cleanup goals pushes for energy-intensive remedies that rapidly address the contaminants of concern with established technologies, often leaving little room for research and development especially for slower treatment technologies, such as bioremediation, for the more heavily polluted sites. In the present case study, new genomic approaches have been leveraged to assess fungal biostimulation potential in soils polluted with particularly persistent hydrophobic contaminants. This new approach provides insights into the genetic functions available at a given site in a way never before possible. In particular, this article presents a case study where next generation sequencing (NGS) has been used to categorize fungi in soils from the Atlantic Wood Industries Superfund site in Portsmouth, Virginia. Data suggest that original attempts to harness fungi for bioremediation may have focused on fungal genera poorly suited to survive under heavily polluted site conditions, and that more targeted approaches relying on native indigenous fungi which are better equipped to survive under site specific conditions may be more appropriate.

INTRODUCTION

Sites polluted with hydrophobic contaminants, such as heavy polycyclic aromatic hydrocarbons (PAHs), are challenging to remedy sustainably because these contaminants sorb strongly onto soils. The location of the present case study, Atlantic Wood Industries Superfund site (AWI) has PAHs as its main contaminants of concern. In particular, high molecular weight PAHs are of concern because they are highly recalcitrant in soil and contain known carcinogenic constituents such as benzo[a]pyrene, indeno(1,2,3-c,d)pyrene, and benz[a]anthracene. Similar to other sites, attempts to minimize risk at AWI have focused on excavation and *in situ* stabilization because remediation options that are both sustainable and degrade the contaminants of interest are limited. Thus, it is clear that more sustainable remediation options are needed as excavation and *in situ* stabilization are expensive, draw heavily on resources, and have a large carbon footprint.

Bioremediation is a sustainable alternative to physico-chemical treatment which has been relied upon since the late 1980s (Sloan, 1987). Inherently sustainable because it harnesses natural biological processes to degrade pollutants, bioremediation has grown more favorable in recent years since awareness has spread about the need to minimize remedy carbon footprint (Forum, 2009). Fungal degradation processes have been of interest for over 30 years since they were discovered to cause decay in wooden utility poles despite their creosote treatment (Morrell and Zabel, 1985). The ability of fungi to grow and thrive in environments with mixtures of the toxic, hydrophobic PAHs in creosote motivated further interest into the metabolism of these fungi as a possible remedy for contaminated soil

treatment. A series of studies on white rot fungi showed that fungal metabolism relied on extracellular enzymes secreted outside the cell. These extracellular enzymes have evolved to be highly nonspecific in order to break down the different bonds holding lignin and cellulose together in wood, but these same enzymes have been shown to also degrade other contaminants with similar chemical structures including creosote, pesticides, munitions, and chlorinated solvents (Cameron *et al.*, 2000). Further studies showed that fungal processes could improve soil structure and that fungi may be better suited to challenging site conditions including, for example, lower nutrient and pH conditions (Miles and Chang, 1997). Furthermore, site conditions where nutrients are unevenly distributed or scarce may be better suited for fungi than bacteria because fungi have strategies to face those scenarios such as forming mycelia that conduct oxygen in their interior, requiring less nitrogen, and sporulating in times of extreme scarcity (Dowson *et al.*, 1988; Tuisel *et al.*, 1990; Mancera-López *et al.*, 2008; Leitao *et al.*, 2011). In addition, more inclusive studies on microbes showed degradation synergies between fungi and bacterial degraders where fungi initiated degradation and then bacteria carried it forward (Lade *et al.*, 2012). Fungi with hydrophilic filaments have been seen to conduct bacterial degraders throughout the network, aiding in dispersal and resulting in better biodegradation than in the absence of their filaments (Kohlmeier *et al.*, 2005; Warmink *et al.*, 2011). Thus, involving fungal processes in a bioremediation strategy have the potential to lead to sustainable treatment of otherwise recalcitrant pollutants in soils.

All of these studies motivated the development of fungal bioaugmentation strategies to introduce these well-known fungi to soils polluted with recalcitrant compounds wherein the fungi would be added along with materials to help them colonize the soil. However, when strategies using this field-scale fungal bioaugmentation approach proved disappointing, remedies involving fungi dropped in popularity. Bioremediation failures were difficult to directly study in the time that preceded the genomics era because one could not assess the soils for full fungal or bacterial colonization. However, it was thought that the native microbial communities might have had some role to play in these failures. To answer questions about the role of native microbiota, fungi were examined in sterile and nonsterile soils. Fungi were seen to exhibit different behavior in sterile soils as compared to nonsterile soils and it was concluded that the fungi that failed did not have adequate ability to compete with the native microbes for a space in the soil's ecology (Andersson *et al.*, 2000). It became clear that a new strategy involving the site's native fungi in a more active role was needed (Harms *et al.*, 2011). In this article, the authors present a discussion on fungal biostimulation, propose a framework wherein native fungi are centrally involved in remediation, and provide a case study wherein the genomic framework method was applied.

Background

A strategy focusing on a site's indigenous fungi must first categorize the site's fungi and assess them for bioremediation potential before fungal biostimulation can proceed. In a worst case scenario, conditions may exist where some sites are too heavily polluted to allow fungi to survive. Recent advances employing next generation sequencing (NGS) have revolutionized the study of microbiomes and made it possible to scan the entire soil's DNA to provide information about: 1) the identity of microbes present at a given site; 2) the

genetic capacities the microbes carry; and, 3) the specific microbial functions which are actively being used. These three pieces of information about a site's native microbes can provide a more thorough explanation of the site microbial ecology. In the past, NGS has been leveraged in water pollution scenarios to explain why microbial mats remove pollutants from wastewater (Akyon *et al.*, 2015). NGS has also been used to explain why specific bioremediation strategies failed, by tracking microbial function over a specific timescale. In previous studies carried out prior to NGS advances, signs pointed to the introduced fungi not being competitive enough in their new environment. Here, the framework proposed leverages NGS to examine biostimulation strategies focusing on already established indigenous fungi as opposed to bioaugmentation (i.e., addition of exogenous fungi to the soils).

The likelihood is high that fungi found at the site beyond the classic white rot fungi will have adapted an ability to degrade the site pollutants. In the time since early interest in white rot fungi, many other fungi have been identified which are capable of degrading a range of pollutants, raising questions about the degree of shared degradation ability in fungi. The kingdom Fungi is broken down into phyla that are further divided into classes, orders, families, genera, and species. The fungi that garnered much attention in the 1980s (i.e., white rot fungi) belong to the phylum Basidiomycota. However, Harms *et al.* later summarized findings from studies on fungi inhabiting different areas of the environment and discussed how the degradation capacity spread well beyond Basidiomycota into Ascomycota, Glomeromycota, Chytridiomycota, Mucoromycotina, and other phyla.

In particular, many common fungi have been identified in soils which belong to the Ascomycota phylum. Using culture-based techniques, fungal species capable of degrading soil contaminants have been identified and further characterized. For example, the ascomycete *Lasiodiplodia theobromae* was cultured from a soil contaminated by the Beijing Coking Plant in China that could degrade benzo[a]pyrene, pyrene, and phenanthrene using its laccases and lignin peroxidases (Wang *et al.*, 2014). *Trichoderma asperellum*, another member of the Ascomycota phylum, was cultured from a heavy crude oil-contaminated soil and found to degrade benzo[a]pyrene, pyrene, and phenanthrene using laccases and peroxidases (Zafra *et al.*, 2015). Ye and coauthors cultured yet another fungus within the Ascomycota phylum, *Aspergillus fumigatus*, that could degrade anthracene present in contaminated soil near a gas station (Ye *et al.*, 2011).

Although degradation mechanisms have not been studied extensively throughout the kingdom Fungi, it is clear that various fungi have evolved different enzyme systems to suit specific niche environments. Even within the wood rotting fungi, a variety of enzymes are used by each genus in degrading pollutants (Barr and Aust, 1994; Rivera-Hoyos *et al.*, 2013). Mechanisms of action resulting in contaminant degradation range from enzyme attack and resulting oxidation outside the cell to uptake of the contaminant into the cell where it becomes accessible to intracellular enzymes for degradation (Barr and Aust, 1994). Current literature suggests that various strategies will be used to degrade contaminants in different fungi, though it is generally accepted that degradation ability is common throughout the kingdom Fungi.

Purpose

To some, it may seem that categorizing indigenous fungi does not contribute much to the ultimate remedy because the different strains may use different enzymes. However, it should be noted that the value in categorizing indigenous fungi lies in its power to inform meaningful fungal biostimulation. Despite the different enzyme systems that white rot fungi use to degrade pollutants, all their enzymes can be stimulated by wood substrate addition because it is known that they thrive in the presence of wood. Thus, site amendments can be recommended to stimulate site-specific fungal growth and overall contaminant degradation. For example, fungi that have evolved chitin-degrading enzymes may be stimulated more by chitin additions than by wood additions. The same could be said about fungi that have evolved closely with plants, which may benefit more from xylan-rich substrates. Furthermore, species-level differences in metabolism may be less important if site fungi can co-metabolically degrade the contaminant in response to stimulation by the same substrate. As we continue to develop a better understanding of the enzyme systems associated with specific groups of fungi and the substrates which stimulate those enzymes, fungal biostimulation strategies can be devised. However, the first step towards this goal is identifying and characterizing the indigenous fungal population at a given site using NGS.

MATERIALS AND METHODS

In this article, we present the result of efforts to categorize fungi using NGS applied to severely contaminated soil in order to inform biostimulation strategies as shown in Exhibit 1. In contrast to the fungal bioaugmentation approach of years past, this framework centers around fungal biostimulation. The framework in Exhibit 1 answers the question of whether there are fungi inhabiting the soils by collecting samples from different levels of pollution then leveraging NGS to identify and categorize fungi already established in the soils. Results from the NGS effort feed into the second step wherein the list of categorized fungi inhabiting each contaminated soil is cross-referenced with fungal literature where fungi with demonstrated bioremediation ability have been characterized. Because the NGS effort results in a list of closest-related fungal categories down to the genus, a further literature review and laboratory studies can then be used to determine if degradation is feasible either as a metabolic or cometabolic process. This stage requires verification of degradation using a laboratory-scale bioreactor. Finally, the results can be used to inform a biostimulation strategy that encourages growth *in situ* based on the types of indigenous fungi present.

NGS Efforts

Samples were collected from the Atlantic Woods Industries Superfund Site in Portsmouth, Virginia and subjected to DNA extraction for NGS analysis. To this end, first, Mo BIO's PowerSoil Powerlyzer DNA extraction kit was used to extract DNA from the samples (MO BIO Laboratories, Inc., Carlsbad, California). The suggested protocol was followed with two modifications. The recommended mass to start with was increased to 0.3-0.4 grams (g). Also, in order to get a more thorough separation between the PAH and the DNA, between 400-450 microliters (μL) of phenol-chloroform was added before the bead beating step. Then bead beating lysis was performed on a minibeadbeater (Biospec Products, Bartlesville, Oklahoma) for 20 seconds. Next, polymerase chain reaction (PCR) was used to amplify

genes informative for fungal characterization. The ribosomal large sub-unit (LSU) was amplified using primers LR3 and LR0R linked with adapter sequences for Illumina MiSeq (Illumina, Inc., San Diego, California) (Lundberg *et al.*, 2013). DNA was subjected to 25-30 cycles of LSU amplification with these primers depending on the sample and how difficult amplification proved. The PCR protocol involved initial denaturation at 95 °C for 10 minutes followed by 25-30 cycles of 1 minute at 95 °C, 1 minute at 55.3 °C, and 90 seconds at 72 °C, with a final extension of 10 minutes at 72 °C. Ribosomal amplicon sequencing was performed on the Illumina MiSeq platform, 250 bp paired-end sequencing. The resulting sequences were processed using CutAdapt, USEARCH, and QIIME (Bittinger *et al.*, 2010; Martin, 2012; Edgar and Flyvbjerg, 2015). Once the sequences were processed for length and quality, fungi were categorized into their respective phyla, classes, orders, families, and genera using the Ribosomal Database Project's Classifier LSU training set 11 (Wang *et al.*, 2007). Once fungi were identified, they were cross-referenced with relevant literature to highlight fungal genera that contain species shown to be involved in degradation. These were termed "candidate" genera.

Chemical Analysis

A total of 34 PAHs were analyzed in the same samples used for the NGS analysis using ultrasonication extraction in hexane:acetone (1:1) followed by silica gel cleanup then measured via gas chromatography combined with electron impact mass spectrometry based on a method published in Clark *et al.* (Clark *et al.*, 2013).

CASE STUDY-ATLANTIC WOOD INDUSTRIES SUPERFUND SITE

Background

To validate the framework presented in Exhibit 1, the described method was applied to several samples collected from AWI. The main contaminants at AWI are PAHs that had originated from historic operations and improper disposal of creosote when AWI operated a creosote wood treatment plant on the site. AWI is composed of approximately 48 acres of industrialized waterfront which extends into the sediments of the Elizabeth River. The sediments in this area have PAH concentrations which are orders of magnitude greater than background levels (Clark *et al.*, 2013). In fact, this waterfront property has some of the highest PAH concentrations in the world (Di Giulio and Clark, 2015).

Soils with high contamination were excavated temporarily on site before they were contained fully as part of the selected remedy. For the present study, approximately 50 g was collected from the surface of four excavated soil and wood chip piles generated during the excavation. These piles were produced during an excavation of a sewer junction which was conveying site contaminants to the Elizabeth River and, thus, these samples were expected to have high PAH concentrations. Creosote was visible in the bottom of the pit post-excavation and the piles appeared to have a range of creosote concentrations based on visual inspection. In total, samples were collected from three soils of different visible creosote pollutant levels, and one woodchip pile, consisting of woodchips roughly 100 mm x 50 mm x 20 mm in size, which had less visible creosote but still caused glove-staining upon collection, suggesting significant creosote contamination. In addition, clean soil was sampled from an

uncontaminated area near the site as a control. Samples were kept at 4 °C until DNA extraction. This proof of concept case study measured fungal communities in the clean soil as well as the woodchip and three differentially polluted excavated soil piles.

Results

Exhibit 2 shows the average PAH concentration measured at each site. The average consists of 34 PAHs that range in molecular weight from the two-ringed naphthalene to the six-ringed dibenzo(a,l)pyrene. The full list of PAHs includes: 1-methylnaphthalene, 2,6-dimethylnaphthalene, naphthalene, acenaphthene, acenaphthylene, carbazole, dibenzofuran, dibenzothiophene, fluorene, 1-methylphenanthrene, 2-methylphenanthrene, anthracene, phenanthrene, retene, 1,2-benzofluorene, 3,4-benzofluorene, fluoranthene, 1,2-benzanthracene, benzo(c)phenanthrene, chrysene, pyrene, 3-methylcholanthrene, benzo(a)fluoranthene, benzo(b,k,f)fluoranthene, benzo(a)pyrene, benzo(b)chrysene, benzo(e)pyrene, dibenzo(a,j)anthracene, dibenzo(a,h)anthracene, perylene, picene, indeno(1,2,3,c,d)pyrene, benzo(g,h,i)perylene, and dibenzo(a,l)pyrene. The lowest contaminated soil (Sample 1), had a total PAH concentration of 180 ± 0.023 micrograms per gram ($\mu\text{g/g}$; Exhibit 2). The total PAH concentration in Sample 2 was 974 ± 0.074 $\mu\text{g/g}$. Sample 3, the woodchip deposit, had a total PAH concentration of $5,854 \pm 1.473$ $\mu\text{g/g}$. The most contaminated soil was Sample 4 with a total PAH concentration of $18,407 \pm 0.825$ $\mu\text{g/g}$.

Using NGS, we detected 99 different taxa from the media sampled. The phylum-level classification is shown in Exhibit 3. It should be noted that because these data focus on DNA, they only provide a snapshot of the fungi present and not their activity. As may be expected, there is relatively high diversity in the control soil and Sample 1 (the least contaminated soil). The control soil is dominated by members of Ascomycota (shown in dark grey), with members of Basidiomycota (in diagonal stripes) second most prevalent. Ascomycota are also seen here to dominate the fungal community in Samples 2 and 3. Sample 1 is split equally between Ascomycota and unclassified fungi (in light grey) with a smaller amount of Chytridiomycota (in white) and Basidiomycota. The fungi from Sample 4 fall mostly within Basidiomycota with secondary prevalence of Ascomycota. The phylum-level grouping is informative because it suggests that fungal biostimulation strategies which target members of Basidiomycota may not match up with the most prevalent members of the fungal community, at least based on the fungi detected in AWT's contaminated media sampled. In fact, this analysis suggests that Ascomycota may be a better phylum to target with a biostimulation strategy in the majority of these samples.

Exhibit 4 shows how the fungal genera grouped into different groups of “candidates” warranting further attention varied across the contaminated media. This exhibit describes the results of grouping fungal genera together based on a literature review of species demonstrating degradation capacity. The literature review behind Exhibit 4 is of importance because it identifies conditions that produced the results shown, conditions that can inform biostimulation strategies. It is important to note that the fungi not classified as candidates have not yet appeared in bioremediation or functional studies and, thus, have not yet been characterized in terms of bioremediation capacity. Thus, the proportion of these does not

imply that they cannot degrade contaminants, but rather that they have not been looked at through a bioremediation lens yet.

These data show that the candidate genera fraction out of the total population range from a minimum of 20 percent in Sample 1 to a maximum of 100 percent in Sample 2. In the more polluted samples, Samples 3 and 4, approximately 60 and 30 percent of the total fungal genera were found to be candidates, respectively. These results were somewhat surprising, as it may be unexpected to find any microorganisms at the extremely high PAH concentrations found in Samples 3 and 4 which had contamination levels on the order of milligrams per gram (mg/g). Some fungal genera detected in AWI samples were assumed to have degradation capacity because they contain species known as agents of PAH degradation. These groups are represented in black in Exhibit 4 (Ravelet *et al.*, 2000; Potin *et al.*, 2004; Cerniglia and Sutherland, 2010; Wang *et al.*, 2014; Zafra *et al.*, 2015). This fraction only represented 5 percent of the control soil which is expected because of the low PAH concentration. The fraction increased to approximately 15 percent of Sample 1, and then reached nearly 70 percent of Sample 2's fungal community. Again, these results are encouraging as they suggest that the presence of the contaminants enriches for potential fungal genera capable of breaking down the contaminants. Sample 3 also had many fungal genera containing agents of PAH degradation, at just over 40 percent of the total fungal community. In Sample 4, this fraction returned to around 5 percent. Based on these data, it either appears that the known PAH degrading fungal genera are incapable of surviving beyond a certain threshold, or that there is the possibility that previously uncharacterized fungi outcompete at the higher concentration.

Other fungal species previously shown to degrade contaminants other than PAHs were also detected at AWI (Daechul and Hyun, 2008; Cosgrove *et al.*, 2010; de Oliveira *et al.*, 2013; de Souza Pereira Silva *et al.*, 2015). This suggests that these genera may also be able to degrade PAHs since they were enriched under high contamination concentrations. The fraction of non-PAH degrading fungi is shown in dark grey in Exhibit 4 and comprises 35 percent of the control soil and 5 percent of the lesser polluted Sample 1. Sample 2 contained a larger fraction (~25 percent of the total) while Sample 3 had less than 5 percent. The majority of the candidate genera within Sample 4 were in this category, however, at 20 percent of the total. This analysis suggests that the fungi shown to be agents of non-PAH degradation should be examined for PAH degradation, since they are so prevalent in the presence of high PAH concentration.

Because fungal enzymes have been shown to have substrate promiscuity, enzyme production was also used as a proxy for characterizing likely degradation capacity. The analysis behind Exhibit 4 revealed fungi capable of producing such enzymes. Fungal genera producing polyphenol oxidases and laccases, which are known to degrade PAHs, are shown in light grey in the exhibit, while enzymes of lesser degradation certainty are shown in white with a black outline (Cameron *et al.*, 2000). The fraction of fungal genera that produce extracellular enzymes known to degrade PAHs is low across all the sampled communities, comprising less than 5 percent of all samples and the control, except for Sample 4, which had about 10 percent (Verdin *et al.*, 2004; Yang *et al.*, 2005; Qasemian *et al.*, 2012; Barbi *et al.*, 2014; Zafra *et al.*, 2015). Fungal genera that have been shown to contain species producing

enzymes yet unstudied in a bioremediation context are only present in the control soil and Sample 3, at 10 percent each (Fenice *et al.*, 1997; Bojsen *et al.*, 1999; Kang *et al.*, 2004; Yu *et al.*, 2004; Zhao *et al.*, 2013). This analysis suggests that biodegradation studies may need to target other enzymes with known substrate promiscuity besides lignin peroxidase, manganese peroxidase, and laccase.

CONCLUSION

This case study suggests that fungi are present and that biostimulation may be possible in highly contaminated soils. Here, NGS was shown to be a tool capable of tremendous insight into the contaminated soil ecology. Specifically, NGS was demonstrated to be useful as a first pass identification tool to characterize fungi present in polluted soils. NGS data revealed that the polluted media was suitable for more than Basidiomycota, and that the soils can be comprised entirely of fungal genera with observed degradation capacity. It was surprising that a high prevalence of the well-studied Basidiomycota was not reflected in all of the contaminated media sampled. Rather, the fungal communities were more composed of a blend of Ascomycota and Basidiomycota. This case study suggests that existing strategies developed to stimulate members of Basidiomycota may be insufficient for remediation sites such as AWI. There is a clear need to also develop ascomycete targeting strategies to take advantage of established fungi that have shown to be capable of degradation. Ultimately, the case study provides evidence to suggest usual fungal bioremediation strategies may not target the soil fungi that have established themselves in polluted soils.

Moving forward, it would serve the remediation community to use NGS more often. As the proposed framework becomes applied to more polluted sites, more fungi can be identified that can survive under immense environmental stress. This will improve remedy sustainability by harnessing fungi more often in sites with recalcitrant soil pollutants. Future work should also incorporate a functional level analysis (RNA) to infer what functions are possible under the site's specific conditions.

ACKNOWLEDGMENTS

We wish to thank the High Throughput Sequencing Facility at the University of North Carolina-Chapel Hill for the sequencing involved in this work. Additionally, we would like to thank Marc Guterman, Randy Sturgeon, and Joe Alfano, site personnel at Atlantic Wood Industries Superfund site for help with logistics and site access. Also, we wish to thank Lauren Redfern, Dan Brown, Jordan Kozal, Nishad Jayasandara, Bryan Clark, and Josh Osterberg for helping collect samples in the field. Katherine Davis should also be recognized for contributions to chemical analyses involved in this work. Funding supported this research through the National Institute of Environmental Health Sciences' Superfund Research Program grant *P42-ES010356*.

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Exhibit 1.
Proposed fungal bioremediation framework

Exhibit 2.

Total PAH concentration measured in Atlantic Wood Industries contaminated media; numbers shown represent the average concentration $\pm$ one standard deviation ($n=6$)

Exhibit 3.

Fungi inhabiting control and polluted media categorized at the phylum-level

Exhibit 4.
Fungi categorized at the genus level and grouped by type of potential demonstrated