Fungi consortia \textit{in situ} biodegradation of xenobiotic, military shooting range, Kachia, Kaduna, Nigeria

Abstract

A major limitation of the white-rot fungus is its sensitivity during biodegradation of mixed matrix explosive pollutants and the scale of Kachia military shooting since 1967, Nigeria. The amplified 16S rRNA gene of each microbial isolate was processed for sequencing and characterization with GenBank database. Fungal species heavy metal reduction in increasing order of \textit{Aspergillus niger} > \textit{Trametes versicolor} > \textit{Rhizopus spp} > \textit{Phanorochate chrysosporium} > \textit{Penicillium} spp were identified. The total explosive contents shows a significant difference for all locations in both dry and wet seasons (P<0.05) using Anova test. Microbial fungi consortium (MFC) bioremediate heavy metal significantly at 61.7% relative to isolated fungi species because of the lateral gene transfer/co-metabolism, where \textit{Trametes versicolor} and \textit{Aspergillus niger} act as gene mediators. MFC growth in 1% mineral salt medium munitions was significantly than fungal species isolate. Deploying Myco Bio-Augmentation / Phytoremediation/Biosimulation (Myco B-P-B) techniques to optimize the RDX and HMX characterized by a higher Nitrogen/Carbon ratio since fungi lack the beta-glucuronidase (GUS) gene to utilize carbon source directly. Pollutants bio-stimulation will enhances co-metabolism by MFC. Plant detoxification capabilities can be improved using fungi genes laccases and cytochrome P450 monoxygenase expressed effectively in plants using protoplast fusion.

Keywords: PC3R technology, explosives, enzymes, heavy metals, white rot fungi, peroxidase co-metabolism, biofertilizer, bioremediation, entophytes, phytoremediation, tobacco, xenobiotic myco B-P-B techniques and microbial fungi consortium

Introduction

Xenobiotic waste streams constituents from explosives military shooting ranges operations globally results in the soil contamination are nitroaromatics and Nitra mines including TNT (2,4,6 trinitrotoluene), RDX (Hexahydro-1,3,5-trinitro-1,3,5-Triazine), HMX (Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine), Picric Acid (2,4,6-trinitrophenol) Tetryl (Methyl-2,4,6 trinitro-phenyltrimramine), PETN (Pentaerythritol tetrarinate), TATB (Triaminitro-trienobenzene). The impurities and degradation products from military shooting ranges includes: 2,4-DNT (2,4-dinitrotoluene), 2,6-DNT (2,6 dinitrotoluene), 4A-2,6-DNT (4-amino-2,6-dinitrotoluene), 2A-4, 6-DNT(2-amino-4,6-dinitrotoluene), TNB (1,3,5-trinitrobenzene), DNB (1,3 dinitrobenzene), NB (Nitrobenzene), Picramic Acid (2-amino-4,6-dinitrophenol) was reported.\textsuperscript{1-9} Microbial biodegradation using microbes is optimized when interact within their niche, under the most favourable conditions.\textsuperscript{80-279} Microbial bioremediation consortium techniques that occurs simultaneously during explosive biodegradation are: bio-stimulation, bio-augmentation, bio-accumulation was reported;\textsuperscript{136,280-372} bio-sorption\textsuperscript{136} the use of biofilms.\textsuperscript{318,320}

Pollutants bioremediation involves a number of species of microbes having different mechanisms depending upon the environmental factors and the nature of chemicals\textsuperscript{40} either to degrade or to remove the toxic contaminants from the environment. Elevated ambient atmospheric temperature (a climate change factor) may have impacts on the microbial activity;\textsuperscript{218} especially on the upper layers of soils. Explosives which are sufficiently sensitive to be detonated by a local hotspot or shock are called primary explosives (lead azide and lead styphnate) secondary (TNT, RDX) explosives less sensitive materials, which require an explosive shock wave for detonation can use primary explosives for detonation.\textsuperscript{117} Soil contamination by energetics at manufacturing sites, conflict areas, and military ranges is an international concern. Energetic compounds may enter
the soil environment via numerous avenues including the following:

i. Ammunition production facilities, for example, wastewater lagoons, filtration pits;
ii. Packing or warehouse facilities;
iii. Waste disposal and destruction facilities, for example, open dumps, burn pits, incinerators;
iv. Weapons firing ranges; and
v. Weapon impact areas.

RDX is reportedly the most important military high explosive currently in use, being about 40% more powerful than TNT. Approximately 85% of all landmines contain TNT. At elevated CO₂, microbes’ exhibit increased ability to decompose soil organic matter where soil microbial community plays an important role in cycling of carbon. The microbial biodegradation processes, the challenges are:

a. Physicochemical characteristics of environment or the abiotic factors,
b. Biological factors or biotic factors
c. Climatic conditions whereby physicochemical and climatic conditions are among the major factors affecting the metabolic rates in microbes.

A fluo-spot approach, which can be employed for in situ detection of trace amount of explosives. The fluorescence molecule used is 4-(dicyanomethylene)-2-methyl-6-(4-dimethylaminostyryl)-4H-pyran) (DCM). DCM is featured by its high fluorescence quantum yield (15% in solid state, measured over the DCM deposited on a polytetra-fluoroethylene film) and unique electronic push-pull structure. The sensor reaction mechanism push-pull structure is a widely used design strategy for organic fluorescent molecules, as both the fluorescence efficiency and wavelength are very sensitive to the position and ability of the electron withdrawing or donating groups, which in turn can be changed by reacting with the analyses species (Figure 1).

**Figure 1** (a) Molecular structures of the fluorescence sensor, DCM, and the explosive compounds, RDX, HMX and PETN. (b) UV photolysis of RDX and PETN produces NO₂⁻ and NO₃⁻ respectively; these reactive species can then be captured by DCM to produce the adduct DCM-NO₂, leading to complete fluorescence quenching of DCM. (c) Scheme of the fluo-spot detection method adapted.

### Fungi biodegradation of TNT, RDX and HMX

Explosives environmental impacts on humans shows that RDX affects the central nervous system, and classified as class C carcinogens. HMX exists as α (orthorhombic), β (monoclinic), γ (monoclinic) and δ (hexagonal) forms, of which ‘β’ form is the least sensitive to impact and the most stable. HMX affects rats, aquatic organisms, bacteria, earthworm reproduction test and classified as Class D carcinogenic. White rot fungi are able to transform or mineralize TNT using nonspecific extracellular enzyme systems such as lignin peroxidase, manganese peroxidase, laccase. The findings asserted that micromycetous fungi like Alternaria spp., Aspergillus terreus, Fusarium spp., Mucor mucedo, Penicillium spp., Rhizoctonia spp are able to transform TNT but do not mineralize it. Agaricus aestivalis, Agrocybe praecox and Clitocybea beanorae transform TNT, with degree of mineralization ranging from 5 to 15% reported. The white rot fungus P. chrysosporium degrade TNT with complete transformation 10 - 40% mineralization was reported. In a study reported that Phanerochaete chrysosporium degraded RDX where it removed RDX (62 mg/L) from liquid medium containing glycerol as the main carbon source, Figures 2&3. Approximately 53% of the molecule was mineralized, and the major by product (62%) of the degradation was N₂O. HMX was observed to be reduced from 61±20 mg/kg to 18±7 mg/kg during 62 days of incubation using P. chrysosporium. Under nitrogen-limiting conditions Phanerochaete chrysosporium mineralized HMX was reported. After 25 days of incubation, 97% of HMX was removed with reduction with the accumulation of 4-nitro-2,4-diazabutanal was reported that the presence of glucose enhanced the degradation of HMX in marine sediment from a military UXO disposal and in which after 50 days, the HMX concentration in the aqueous phase (1.2 mg/L) was reduced by 50%. The disappearance of HMX was accompanied by the formation of a mononitroso derivative.

**Figure 2** Proposed biodegradation pathways for hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). Path a Reduction of RDX to nitres' derivatives before ring cleavage. Path b Reduction of RDX to 1,3,5-triamino-1,3,5-triazine. Path c Reduction of RDX via Aspergillus niger nitrate oxidoreductase enzyme. Dashed arrows Multiple pathway steps based on hypothetical intermediates, which are not shown. Dashed plus dotted arrows hypothetical enzyme reactions.

### Xenobiotic biodegradation treatments construct

Explosives biodegradation occurs in sequential incomplete process, potentially leading to the accumulation of toxic metabolites that can be further introduced into the food chain. This necessitate the convergence of bioremediation, phytoremediation and applied ecology xenobiotic degradation protocol called PC3R technology (Pollution Construct, Remediation, Restoration and Reuse) reported. Pollution construct (xenobiotic bioavailability in terrestrial environments for biodegradation) techniques (in situ or ex situ) Figure 4 soils/water contaminants for pesticides, herbicides, hydrocarbon, produced water, munitions, heavy metals and others for Remediation via microbial, phytoremediation and mineralization. Restoration ecosystems for bioremediation.

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zero pollutants footprints for sustainable re-generative Reuse of post treated soils site bio stimulated for bioenergy crops (rice, soy bean or maize) and tobacco cultivation converted to biofuel production (ethanol). Rhizosphere remediation (plant-microbes interactions) by application for microbial biofertilizer (bacteria, fungi, etc.) to improves crop plant growth, soil ecosystem and biodiversity of the post-treated site.

**Figure 3** Principal methods used by fungi to degrade organic chemicals. Although fungi primarily co-metabolize organic pollutants, they do grow volatile organic62, 271, 169, 249, 229 via pollutant attack may occur extracellularly or intracellularly adapted.\(^{147}\)

**Figure 4** Xenobiotic biodegradation treatments construct called PC3R Technology adapted from Brannon and Myers 15 and illustrated in Figures 13, 14, 15 and 16 respectively below.

**PC3R technology**

**Pollution construct**

Laboratory studies reports that, most nitro-substituted explosives were found to be toxic for all classes of organisms, including bacteria, algae, plants, invertebrates, and mammals.\(^{156, 198, 288, 344}\) TNT and RDX are listed as ‘priority pollutants’ and ‘possible human carcinogens’ by the U.S. Environmental Protection Agency (EPA) reported.\(^{198, 141}\) Phytoremediation, or the use of plants for cleaning up pollution metabolizing toxic pesticides.\(^{194, 308}\) Environmental pollutants compounds may enter the environment during their production (wastewater lagoons), disposal (burn pits), storage, or usage (dispersed or unexploded ordnance), hydrocarbon and agriculture resulting in contamination of groundwater, surface water, marine, and terrestrial environments, and abiotic and biotic processes will influence the fate of explosive compounds.\(^{303, 172}\) Physico-chemical properties of the compounds impacts on rate and extent of transport and transformation with environmental and biological factors including the presence and/or absence of explosives-degrading microorganisms Biodegradation pollutants construct solution become a requirement for the sustainable management of xenobiotic with converged technologies, Figure 4. Pollutants fate of compounds driven by: a) Processes that influence transport dissolution, volatilization adsorption b). Processes that influence transformation, photolysis, hydrolysis, reduction and biodegradation. Manufacture of explosives, testing and firing on military ranges, and decommission of ammunition stocks have generated toxic wastes, leading to large-scale contamination of soils and groundwater.\(^{146}\)

**Microbial remediation**

Microbial bioremediation using microorganisms for the treatment of explosive-contaminated sites and range of contamination now the ‘top ten technologies for improving human health.’\(^{35}\) Different classes of microorganisms, including bacteria and fungi, are capable of transforming energetic pollutants, which can be used as energy, carbon, and/or nitrogen sources reported by scholars.\(^{146, 130, 367, 276}\) White-rot fungi that secrete powerful peroxidases that mineralization of TNT.\(^{346}\) *P. chrysosporium* is not a good candidate for bioremediation of TNT-contaminated sites containing a high concentration of explosives because of its high sensitivity to contaminants reported showed that the lignin peroxidase activity of *P. chrysosporium* is inhibited by the TNT intermediate hydroxylamino-dinitrotoluene. This is the prerequisite for the role of fungi consortium in xenobiotic bioremediation, Figure 3.

**Phytoremediation**

Transgenic plants exhibiting biodegradation capabilities of microorganisms bring the promise of an efficient and environmentally friendly technology for cleaning-up polluted soils.\(^{368}\) Phytoremediation of recalcitrant pollutants involve uptake, translocation, transformation and compartmentalization and sometimes mineralization.\(^{238}\) Plant roots secrete metabolites, which stimulate the growth of microorganisms in the rhizosphere, which in turn can degrade and mineralize the organic compounds. Plants lack xenobiotic degradative capabilities unlike bacteria of fungi. Therefore, introduction of genes for degradation of recalcitrant environmental pollutants from microorganisms or other eukaryotes will further enhance their ability to degrade/mineralize recalcitrant environmental pollutants. The *MnP* gene\(^{351}\) or the *laccase* gene from white-rot fungi has already been introduced into tobacco plants was reported (Figures 5–18).\(^{303}\) Hirai et al.,\(^{311}\) reported that laccase cDNA (scL) from white-rot fungus *Schizophyllum* was used as a model ligninolytic enzyme, efficient expression of scL and removal of a recalcitrant compound in transgenic tobacco plant by decreasing the CpgGnucleotide motif content in order to develop phytoremediation with a ligninolytic enzyme-producing transgenic plant. Phytoremediation merit over other remediation options includes: low installation and maintenance costs; and zero impact on the environment with carbon sequestration and biofuel production.\(^{353}\) Figure 16.

Plants lack the catabolic enzymes necessary to achieve full mineralization of organic molecules, potentially resulting in the accumulation of toxic metabolites.\(^{354}\) Microbes and mammals are
heterotrophic organisms that possess the enzymatic machinery necessary to achieve a complete mineralization of organic molecules, complementing the metabolic capabilities of plants. The detection of oxidation and reduction metabolites, as well as large molecular weight products and non-extractable fractions suggests that plants metabolize explosives according to the ‘green liver’ model. Like the liver of mammals, detoxification by plants typically involves three different phases: activation of the initial toxic compound (phase I), conjugation with a molecule of plant origin (phase II), and sequestration in plant organelles or structures (phase III). Plants via roots system have developed diverse detoxification mechanisms and have for long been recognized as capable of metabolizing organic compounds. Nitro-substituted explosives are efficiently taken up, Figure 13 and partially metabolized inside plant tissues.

Additionally, fungi can find application in phytoremediation if their potential genes of enzymes like peroxidases, laccases are expressed effectively in plants. Extensive reports on biofertilizer have revealed their capacity to provide nutrients to plants and consequently enhance crop yield. The goal of using such organic matter are twofold since it not only improves the quality and fertility of degraded soils but also simultaneously eliminates organic waste in a rational and environmentally friendly manner. Sustainable practices with the addition of biofertilizer could be a useful tool for maintaining and even increasing the organic matter content for respiration in agricultural soils, thus preserving and improving soil fertility.

![Figure 5](Image URL) Map of Kachia within Kaduna state map, Nigeria showing study area (Nigerian Army Shooting Artillery; NASA Range).

Source: Geography Department NDA, Kaduna, Nigeria.

![Figure 6](Image URL) Percentage of Soil texture in various locations, Kachia, Nigeria. Location (1) - zero metre, L1 200m Location 1 200 metre, Loc 1, 400 Location1 400 metre, Loc 20m - locations (2). 0 metre, Loc 2- Location (2).

![Figure 7](Image URL) Microbial consortium fungi bioremediation of heavy metal.

**Restoration biofertilizer**

Molasses is a very effective carbon source that enhances the TNT degradation rate significantly over other carbon sources and compost. Additionally, fungi can find application in phytoremediation if their potential genes of enzymes like peroxidases, laccases are expressed effectively in plants. Extensive reports on biofertilizer have revealed their capacity to provide nutrients to plants and consequently enhance crop yield. The goal of using such organic matter are twofold since it not only improves the quality and fertility of degraded soils but also simultaneously eliminates organic waste in a rational and environmentally friendly manner. Sustainable practices with the addition of biofertilizer could be a useful tool for maintaining and even increasing the organic matter content for respiration in agricultural soils, thus preserving and improving soil fertility.

![Figure 8](Image URL) Growth Rate of (O.D Values) of Fungi in 1% Explosive Mineral Salt Medium broth.

*Means on the same column having different superscripts are significantly different (P<0.05) according to Duncan Multiple Range Test.

![Figure 9](Image URL) Mean seasonal variation of physico-chemical parameters of composite samples of four shooting range locations.

![Figure 10](Image URL) Shows the explosives fungal consortium isolates > Aspergillus niger > Trametes versicolor > Rhizopus spp > Penicillium chrysogenum > Phanorocnthe chrysoporium as good mycoremediation potential for xenobiotic (Plate 1).

Microbial communities contribute to fundamental processes that provide stability and productivity of agro-ecosystems.

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affects ecosystem stability by regulating plant diversity, aboveground net primary production, and species asynchrony. The rhizosphere, which is the narrow zone of soil surrounding plant roots, can comprise up to 1011 microbial cells per gram of root and above 30,000 prokaryotic species that in general, improve plant productivity. The collective genome of rhizosphere microbial community enveloping plant roots is larger compared to that of plants and is referred as microbiome whose interactions determine crop health in natural agro-ecosystem by providing numerous services to crop plants viz., organic matter decomposition, nutrient acquisition, water absorption, nutrient recycling, weed control and bio-control.

**Figure 11** Ribbon representation of the X-Ray crystallographic structure of *Trametes versicolor* laccase adapted from Viswanath et al., and www.chem.ox.ac.uk/icl/faagroup/fuelcell.html.

The detection of TNT metabolites, such as aminodinitrotoluene and amino-dinitrobenzoate, suggests that plants can carry out both reductive and oxidative transformation of nitroaromatic compounds. Identified RDX transformation products include reduction derivatives, such as hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine,

**Figure 12** The catalytic cycle of laccinyltoxin peroxidases and laccases differ in their oxidizing substrate (X), their target reducing substrates/mediators (A) and their electron acceptor metal co-factors (coloured circles). The peroxidases (LiP, DyP, MnP and VP) react with hydrogen peroxide to form oxoferryl intermediates (red and yellow circles), while laccases contain a four-copper active site that reduces oxygen to water to gain oxidative potential, adapted from Fisher et al.

**Reuse and mineralization**

However, unlike bacteria and mammals, plants are autotrophic organisms that lack the enzymatic machinery necessary for efficiently metabolizing organic compounds, often resulting in slow and incomplete remediation performance. This led to the idea to modify plants genetically by the introduction of bacterial or fungi for complete xenobiotic biodegradations and mineralization.

**Figure 13** Illuminating the deconstruction of complex pollutants substrates requires many different types of enzymes and diverse chemical reactions within pollutants matrix simultaneously with resilience to environmental fluctuations by deploying microbial and phytoremediation techniques as affirmed by scholars.

**Figure 14** Green liver model for the metabolism of xenobiotic in plants and adapted from Burken et al.

**Figure 15** Schematic representation of production of hybrid plant via protoplast fusion. Representative scheme of functional diversification and classification of fungal CYP systems, adapted from Durairaj et al., reports. Categorization of the functional properties of Class II CYP systems based on the primary metabolism, secondary metabolism and xenobiotic detoxification was adapted and ribbon representation of the X-Ray crystallographic structure of *Trametes versicolor* laccase modified. www.chem.ox.ac.uk/icl/faagroup/fuelcell.html.

The detection of TNT metabolites, such as aminodinitrotoluene and amino-dinitrobenzoate, suggests that plants can carry out both reductive and oxidative transformation of nitroaromatic compounds. Identified RDX transformation products include reduction derivatives, such as hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine,
and a range of breakdown products, such as 4-nitro-2,4-diaza-butanal, formaldehyde, and nitrous oxide. Another important barrier to field application of transgenic plants for bioremediation arises from the true or perceived risk of horizontal gene transfer to related wild or cultivated plants. Therefore, it is likely that the next generation of transgenic plants will involve systems preventing such a transfer, for instance by the introduction of transgenes into chloroplastic DNA or the use of conditional lethality genes.

Two genes, one (encoding PETN reductase) and nfsl (encoding nitroreductase), under the control of a constitutive promotor provided the transgenic plants with increased tolerance to TNT at concentrations that severely affected the development of wild type plants. Research scholars reported that, N. tabacum is able to extract metals only from the upper part of the contaminated layer (0.2 m) unlike Z. mays (maize) and S. viminalis which could extract metals from depths up to 0.75 m. Consequently, tobacco is suitable only for the phytoextraction of areas where the contaminated soil does not have a great depth.

Figure 16 Potentially more cost-effective for in situ bioremediation, is the transfer of genes encoding microbial aerobic degradation pathways into transgenic plants tobacco (Nicotiana tabacum) will optimize the environmental risk to mineralization unidentified products during biodegradation, adapted Mo et al.238

French et al., designed the first transgenic plants specifically engineered for the phytoremediation of organic compounds by Doty tobacco plants (Nicotiana tabacum) were modified by the introduction of a bacterial pentaerythritol tetranitrate (PETN) reductase, an enzyme involved in the degradation of nitrate esters and nitroaromatic explosives, and derived from an Enterobacter cloacae previously isolated from an explosive-contaminated soil. Meagher report the mineralisation of TNT by native plants is inefficient and generally incomplete.

To engineer plant tolerance to TNT, two bacterial enzymes (PETN reductase and nitroreductase), able to reduce TNT into less harmful compounds, were over expressed in N. tabacum cv. ‘Xanthi’. The two genes, one (encoding PETN reductase) and nfsl (encoding nitroreductase), under the control of a constitutive promotor provided the transgenic plants with increased tolerance to TNT at concentrations that severely affected the development of wild type plants. Research scholars reported that, N. tabacum is able to extract metals only from the upper part of the contaminated layer (0.2 m) unlike Z. mays (maize) and S. viminalis which could extract metals from depths up to 0.75 m. Consequently, tobacco is suitable only for the phytoextraction of areas where the contaminated soil does not have a great depth.

Figure 17 Using this genetic engineering, transgenic plants in which ligninolytic enzymes are expressed will be useful tool for phytoremediation of recalcitrant environmental pollutants adapted from Olcín-Hernández et al.249,255

Rationale

Jimenez et al.168 and Maruthamuthu et al.215 applied selected microbial consortia, over single strains, for such biotechnological purposes presents important advantages. One of them is that microbial consortia are able to perform complex tasks that single strains. The reason is that the deconstruction of complex pollutants substrates requires many different types of enzymes and diverse chemical reactions, all at the same time or at short time intervals within pollutants matrix. Biodegradation is in phases, resilience to environmental fluctuations (resistance in variation substrate composition) and resistance to microbial invasion play major roles.111,224 White rot fungi can grow effectively under water stress conditions where no plant growth occurs.23 To optimize remediation approaches the development of genomes of specific white rot fungi like P. chrysosporium genome will result to the elucidation of specific cytochrome P450 monoxygenases, which may be differentially expressed in the presence of xenobiotic compounds. However, identification of specific interactions between microbes in a complex consortium is a difficult task, as it is highly likely that different interactions happen simultaneously.218 Application of synthetic microbial consortia could be a solution to this problem, as such consortia are usually composed of few well-identified organisms ex situ inoculant. At times, those species do not co-inhabit the same environment, but their metabolic activities may be combined.163 Determination of nutrient utilization, metabolite production, exchange of metabolites, production of signal molecules,
spatial distribution, genome sequence and population dynamics are fundamental to enhance our understanding of the interactions, (Figures 4&16).

The knowledge could be applied to extend the stability of the microbial consortia and the process efficiency. The sheer scale of contamination of military shooting range in hectares, means that while conventional remediation techniques may not be suitable, phytoremediation could be successfully applied to both remediate existing sites and to contain pollution from future use. RDX is readily taken up by plant roots and trans located to the above-ground tissues, but the in planta. Figure 14 (construct) degradation of RDX is slow in soil, yet the RDX likely to be released back into the ground following leaf abscission. The detoxification of RDX by endogenous plant enzymes involves the reduction to hexahydro-1-nitroso -3, 5-dinitro-1,3, 5-triazine and hydroxy-hydro-1,3-nitroso-5-nitro-1,3, 5-triazine, with nitrous oxide and nitric oxide-2,4-diazabutetal also detectable Photolytic degradation has also been reported. The logKow values are 0.90 for RDX and 1.86 for TNT and bioaccumulation has been reported for TNT transformation intermediates. By transferring microbial genes relaying xenobiotic-catabolism into selected plant species, detoxification, or, ideally, mineralization of organic pollutants can be achieved. Both RDX and TNT are toxic, organic pollutants which are calcitrant to degradation in the environment. Additionally, fungi can find application in phytoremediation if their potential genes of enzymes like peroxidases, laccases are expressed effectively in plants. However, in toxic metal removal applications, it is important to ensure that the growing cells can maintain a constant removal capacity after multiple bioaccumulation–desorption cycles, and a suitable method is required to optimize the essential operating conditions. The situation demands a multi-prong approach including strain isolation, cell development and process development in order to make the ultimate process technically and economically viable. A. niger could remove significant quantities of Cu and Pb from growth media but was less resistant against Cr. Multispecies consortia are considered more efficient over the monospecies culture due to greater resistance against environmental fluctuations and the metabolic relations among the member strains and the capabilities of such co-aggregates. Five different enzymes, lignin peroxidase, manganese peroxidase, laccase, cellulose and hemicellulase, were believed to be the most important catalysts in biodegrading process, and they always worked synergistically. Instead of depending upon single species, a better approach could be towards designing a consortium of strains having high metal biosorption, bioaccumulation and bioprecipitation capacities. The positive interaction between constituent species may also facilitate the survival of sensitive strains. 

**Limitation of fungi biodegradation**

Most of the studies dealing with microbial metal remediation via growing cells describe the biphasic uptake of metals, that is, initial rapid phase of biosorption followed by slower, metabolism-dependent active uptake of metals. However, in toxic metal removal applications, it is important to ensure that the growing cells can maintain a constant removal capacity after multiple bioaccumulation –desorption cycles, and a suitable method is required to optimize the essential operating conditions. The situation demands a multi-prong approach including strain isolation, cell development and process development in order to make the ultimate process technically and economically viable, (Figures 3&4). A. niger could remove significant quantities of Cu and Pb from growth media but was less resistant against Cr. However, until whole pollutant mineralisation, the use of different toxicity tests are needed to ensure the safety of the by-products formed and the rationale for PC3R technology. Whole genome sequence analysis can reveal the capability of fungi for multiple metabolic adaptations owing to diversified enzyme functions such as cytochrome P450 monoxygenase. Phytochelatin synthase (PCS) is an enzyme catalyzing the biosynthesis of phytochelatin from glutathione which protects cells against the toxic effects of non-essential heavy metals. The genome analysis of fungi is helpful in tracing such genes like pcs and studying their evolutionary aspects.

A major limitation of the white-rot fungus is its sensitivity to biological process operations. The fungus does not grow well in a suspended cell system and enzyme induction is negatively affected by mixing actions and the ability of the fungus to effectively attach itself to a fixed medium is poor. The majority of the research on fungal performance has been conducted on autoclaved soil or on synthetic media. Although, the results show that the white rot fungus efficiently and successfully degrades highly toxic complex organic pollutants under these conditions, the results may not be as significant when they are grown under natural environmental conditions with variable native soil organims, temperature, moisture and pH. One problem with field application deals with the strict growth conditions required for most white rot fungi. For example, P. chrysosporium has a high temperature requirement (30-37°C) for growth and ligninolytic enzyme production and like many white rot fungi, it has low competitive capabilities in the environment. Selection of fungal species with a competitive capability from nature and genetic engineering are needed for the future. The disadvantages of transforming filamentous fungi include changes in post-transcriptional treatment of recombinant proteins, resulting in low activity, defects in the morphology, low frequencies of transformation. Probably, the development of techniques of genome editing such as CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats) in fungi could solve these problems in a near future to optimize bioremediation process.

**Material and method**

**Study site**

The study was conducted in the permanent military shooting/ training range located at 5km east of Kachia town in Kaduna state, north central Nigeria. The range was established in 1965 and it covers an area of about 24.95 square kilometre that lies between longitudes 9°N and 70°N 58’ E, with an elevation of 732 meter above sea level and the topography is undulating and the vegetation is Guinea Savannah, Nigeria (Figure 5, Table 1). The area where the munitions /explosive are fired (the impact areas) is a valley consisting of about four large rocks, where the fired munitions/explosives land and explode during military training. Five military exercises involving the deployment of explosives are carried out annually by the Nigerian Defence Academy (NDA) Kaduna, Nigerian Air force (NAF) Kaduna, Nigerian Army School of Infantry (NASI) Jaji, Armed Forces Command.

**Sampling points**

Four sampling points selected for the study are locations 1, 2, 3 and Table 1, locations 1 and 2 are the two smallest rocks closest to the road While locations 3 and 4 are much larger rocks heavily impacted by munitions/explosives between locations 1 and 2 is a flat ground where rain runoffs flow through to the stream in this site [map with global positioning system (GPS) coordinates, Figure 5. Locations 1 and 2 approximately 200 metres from the Plateau that is where the
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Small arms are fired such as FN, Kalashnikov, Greenad, GPMG, SMG and Pistols. The soil in locations 1 and 2 is made up of 50% silt and a flat ground with shrubs and drainage that flow through to the farm lands near the sites. Location 3 is approximately 9000metres away from the Plateau lies between 90°53' and 44°71" N and 70°53' 17.87° E. The impact area of locations 3 and 4 are mainly largely rocks containing high concentration of explosive due to the extensive use of bombardment by the artillery weapons, 155 mm mortarizer, and other heavy weapons while location 4 is ahead of location 3 is about 10,000 metres from the Plateau top where heavy weapons are fired too. The distance between locations 3 and 4 was covered with various shrubs and two major streams (Figure 5).

Table 1: Explosives sites analyses of wet and dry seasons, Kachia, Nigeria

| NS | Explosives | Wet Season µg kg⁻¹ | Dry Season µg kg⁻¹ |
|----|------------|-------------------|-------------------|
| 1  | TNT        | 0.12–26.6         | 0.11–10.29        |
| 2  | HMX        | 1.43–68.19        | 0.39–35.67        |
| 3  | RDX        | 0.38–15.33        | 0.49–68.19        |
| 4  | PETN       | 1.17–7.12         | 0.39–4.38         |

Sampling technique and soil treatment

Soil sampling: Sampling collected both dry and wet season. Four locations located within NASA (Table 1) shooting/training range Kachia were eamed as sampling sites for this study using soil iron auger. Where 10 grams of soil sample (0-30cm in depth) with diameter of 9cm were collected from 3 different points within a location and harmonized to form a composite sample at various locations of the sites. All samples taken 2015 (June-August) and 2016 (February-March) were sieved using a 63 (106 m) mesh size laboratory sieve and then stored in black labelled polythene bags until for analyses. Samples for microbial analyses were kept in a cool box (site sampling) refrigerated with ice pack to retain the original microbial activities.

Soil sample pre-treatment

Sampling points were treated in the laboratory before digestion as executed. 10 grams of the soil sample was weighed into a clean dried beaker and put into an oven at about 100°C for one hour. The soil sample was then ground in a porcelain mortar with pestle and sieved through 250 µg mesh size to obtain a homogenous sample. The soil sample was stored in sterilized polythene bags, label and kept for next stage of pre-treatment. This procedure was repeated for all the collected soil samples. Preparation of media were used for analysing heavy metal and explosives content for soil samples.

Fungi species characterization

Preparation of media: Nutrient Agar - Nutrient agar (Antec /USA) by dissolving 28g of the Agar in 1 litre of distilled water in a conical flask. The conical flasks with the media were autoclave at 121°C at 15 psig pressure (121°C) for 15 minutes and cooled to 40°C before pouring or dispensing if a sterile petri dishes. MacConkey - MacConkey agar was prepared by dissolving 49.53g of dehydrated medium in 1000 ml distilled water in a conical flask. It was heated to dissolve the medium completely. The medium was sterilized by autoclavating at 15 lbs pressure (121°C) for 15 minutes and cooled to 45-50°C, mixed well before pouring into sterile petri plates. Dextrose Agar (PDA).16S ribosomal RNA and Polymerase Chain Reaction (PCR) for Amplification of Catabolic Genes Sequencing of the 16S ribosomal RNA (rRNA) gene and PCR based approaches. The concept of comparing gene sequences from microbial communities revolutionized microbial ecology.

Serial dilution

The sterile dilution blanks were marked in the following manner. 100 ml dilution blank was 102 ml and 9 ml tubes sequentially were 103, 104, 10-6 one gram of soil sample was weighed from each sample located and added to the 12-2 dilution blank and vigorously shaken for at least one minute with the cap securely tightened. All the 10-2 dilution was allowed to sit for a short period. The 1ml from this dilution was aseptically transferred to the 10-3 dilution was again transferred to the 10-4 dilution. The procedure was done to 10-5 and 10-6. A flask of nutrient agar from the 45°C water bath was especially poured into each petri plates for that set. 15ml was poured enough to cover the bottom of the plate and mixed with the one ml inoculum in the plate. Each set was gently swirled on the bench so that the inoculum gets thoroughly mixed with the agar. All the plates were allowed to stand without moving so that the agar solidified and set completely. The plates were inverted and stacked into pipette carriages and placed in the incubator or at room temperature until after 48 hours the same procedures were applied for Macconkey agar and Potato Dextrose Agar (PDA). 16S ribosomal RNA and Polymerase Chain Reaction (PCR) for Amplification of Catabolic Genes Sequencing of the 16S ribosomal RNA (rRNA) gene which is a universal marker for bacteria. Today mycological researches are also very keen for Deoxyribonucleic acid (DNA) barcoding of fungal species. Examples of marker genes are present in multiple copies and contain conserved coding (small subunit, large subunit, LSU) as well as variable non-coding parts (internal transcribed spacers, ITS). Thus, they are useful to distinguish taxa at many different levels can be introduced for fungus taxonomical studies.

Subsequently, a suite of molecular methods was developed that employ rRNA sequences referred to as rRNA. Medlin et al., first described amplification of 16S-like rRNA from algae, fungi, and protozoa, and reports using 16S rRNA of bacteria and other eukaryotes soon followed. Bacterial diversity can be distinguished with 16S rRNA gene which is a universal marker for bacteria. Today mycological researches are also very keen for Deoxyribonucleic acid (DNA) barcoding of fungal species. Examples of marker genes are present in multiple copies and contain conserved coding (small subunit, large subunit, LSU) as well as variable non-coding parts (internal transcribed spacers, ITS). Thus, they are useful to distinguish taxa at many different levels can be introduced for fungus taxonomical studies.

Sequence-based classification and identification

Principle: Under the International Code of Nomenclature of Prokaryotes (ICNP) 16S rRNA gene sequences are required for the description of new species, with a defined cut-off of 97% similarity between species. The connection of this DNA-based observation to its source organisms was made when researchers obtained rRNA gene sequences from previously cultivated fungi that matched the environmental DNA clade. One of the first such efforts

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was the Ribosomal Database Project (RDP)\textsuperscript{50,121,122} which initially consisted of nuclear small subunit (SSU) rRNA gene sequences from Archaea, Bacteria, and Eukarya. Recent additions to RDP include new databases for fungal ITS and large subunit (LSU) rRNA genes.\textsuperscript{72} The fungal LSU rRNA gene\textsuperscript{200} and ITS\textsuperscript{270}

The entirely on analyses of PCR-amplified nuclear rRNA genes, particularly the internal transcribed spacer (ITS) region, and they draw on comprehensive taxonomic and functional databases. These are the so-called “dark taxa”\textsuperscript{286} that reside in the International Nucleotide Sequence Database Collaboration (INSDC), with its three partners: Gene Bank at the National Centre for Biotechnology Information (NCBI). This is incorporated in a broader effort to add type material identifiers to the NCBI Taxonomy database, which allows use of BLAST and other tools to search multiple databases for reliable entries that are specifically tied to type material.\textsuperscript{107} PCR consist of an exponential amplification of DNA fragment and the principle is based on the mechanism of DNA replication \textit{in vivo}, double stranded DNA is denatured to single stranded DNA, each single strand DNA is anneal by the forward and reverse primers of known sequence and elongated using tag DNA polymerase to produce copies of DNA template time for analysis as reported.\textsuperscript{180} The amplified 16S rRNA gene of each isolates were further characterized using gel electrophoresis. The amplified 16S rRNA gene of each microbial isolate was processed for sequencing and characterization.

The sequencing Kit (Applied Biosystems) with the Fungi Species Isolation of Genomic DNA. The genomic DNA of each fungus with observed remediation capabilities was isolated. 1ml of each fungal culture was pelleted by centrifuging at 12,000 rpm for 2 minutes; the pellet was treated with lysis solution and proteinases K and incubated at 60°C for 30 minutes. DNA was extracted from each fungus precipitated with isopropanol by centrifuging at 10,000 rpm for 10 minutes, washed with 1 ml of a 70% (v/v) ethanol solution and dissolved in 0.1 ml of a T.E buffer. The purity and quantity of (DNA) of each sample was examined using UV absorption spectrum and agarose gel (1%) electrophoresis as described by scholars.\textsuperscript{174,347} Fungi species genes are present in multiple copies and contain conserved coding (small subunit, SSU) and large subunit, LSU) as well as variable non-coding parts (internal transcribed spacers, ITS),

Table 2 Fungi isolates implicated in biotransformation and mineralization. Kachia, Kaduna, Nigeria

| Fungi isolates species | Explosives | Conditions | References |
|------------------------|------------|------------|------------|
| \textit{Rhizopus spp}  | TNT        | TNT disappearance in malt extract broth | Otaiku\&Alhaji\textsuperscript{223} |
| \textit{P. chrysosporium spp} | TNT | Electrophilic attack by microbial oxygenases | Rylott\&Bruce\textsuperscript{277} |
|                        |            | Mineralization to CO\textsubscript{2} by mycelium and 90% removal of TNT | Fernando et al.\textsuperscript{13}, Rh et al.\textsuperscript{280} Sublette et al.\textsuperscript{240} |
|                        |            | Mineralization to CO\textsubscript{2} by spores | Spiker et al.\textsuperscript{229} Donnelly et al.\textsuperscript{25} |
|                        |            | Mineralization to CO\textsubscript{2} by mycelium | Michels and Gottschalk\textsuperscript{232} Huang and Zhou\textsuperscript{135} |
|                        |            | Mineralization correlated with appearance of peroxidase activity | Sublette et al.\textsuperscript{240} Stahl\&At\textsuperscript{234,337} |
|                        |            | Mineralization to CO\textsubscript{2} in soil-corn cub cultures and stable | Fernando and Aust\textsuperscript{11} Yinon and Zitrin\textsuperscript{403} |
| Compost microbes      | TNT        | Biotransformation by Thermophile microorganisms | Kaplan and Kalpan\textsuperscript{129}, Isbister et al.\textsuperscript{142} |
|                        |            | Transformed ring-[14C]-labeled TNT, humification reactions | Binks et al.\textsuperscript{11} William et al.\textsuperscript{276} |
| \textit{P. chrysosporium spp} | RDX | Biotransformation under nitrate-reducing | Freedman\&Sutherland\textsuperscript{144}, Alc et al.\textsuperscript{8} |

Citation: Otaiku AA, Alhaji AI. Fungi consortia in situ biodegradation of xenobiotic, military shooting range, Kachia, Kaduna, Nigeria. J Appl Biotechnol Bioeng. 2020;7(6):246–274. DOI: 10.15406/jabb.2020.07.00241
Table continued...

| Fungi isolates species | Explosives | Conditions | References |
|------------------------|------------|------------|------------|
| White rot fungus       | RDX        | Coagulation in contaminated water by nitrate reductase | Sullivan et al., 143; Bhushan et al., 24 |
|                        | TNT        | Treated TNT in wastewater and achieved 99% degradation | Huang and Zhou, 151; Bennett, 25 |
|                        | TNT        | First step was degradation to OHADNT and ADNT, Second step was to DANT including HMX and RDX | Axtel et al., 4; Stahl&Aust, 137 |
| Aspergillus niger spp  | HMX        | Sequential denitration chemical substitution | Urbanski, 131; Kaplan, 177 |
|                        | PETN       | Mono-nitrated pentaerythritol to un-nitrated pentaerythritol products | Williams and Bruce, 45 |
| Mineralization         | PETN       | Biodegraded by sequential denitration to pentaerythritol | Binks et al., 31; Bait & Aust; de Boer et al., 65 |
| P. chrysosporium spp   | PETN       | Nitroglycerin, or glycerol trinitrate, degraded co-metabolism by sequential removal of nitro groups | Wendt, et al., 36; Barr and Aust; Stahl et al., 238 |
| White rot fungus       | Explosives | Dissolution rates: TNT > HMX > RDX > PETN | Townsend and Myers, 32; Brannon and Pennington, 46 |
|                        | PETN       | Degraders glycerol trinitrate and isosorbide dinitrate : nitrate esters | White & Snape, 32 |

Results and discussion

Physio-chemical properties of the military firing ranges

The temperatures of 22-34°C during the dry season and 27.16°C during the wet season were recorded respectively, Table 4. The values obtained fell within the acceptable Federal Ministry of Environment (FMENV), Nigeria limit of <40. Similarly the pH of 6-8 has been suggested to be appropriate for microbial bioremediation. In the current investigation, the soil pH range for all the soil samples falls well within the range suggested by US-EPA and range of 27-35°C similar to the report of Jenkins et al., in characterization of explosives contamination at military firing ranges with soils characterization of silt, clay and loamy, (Table 3&Figure 6).

The increased temperature obtained during the dry season can be attributed to the high concentration of explosives due to the frequent exercises by the personnel of Armed forces of Nigeria, Table 4 (Appendix 2). Compared with the wet season that the temperature is less as a result of rain washing away heavy metals and explosives drown the streams and soil porosity, Figure 6. The high value of electric conductivity (EC) recorded during the wet season is in agreement with the finding of Usman et al., on their physico-chemical of the soils samples within the Federal ministry of environment, Nigeria (FMENV) and World Health Organization (WHO) permitted limits of 500/µS/cm and Penington et al., within the value of 2000 mg/L which was contrary to the finding values were within 2400 -5900 mg/L and is in agreement with Usman et al., the high values of Total Dissolve Solid (TDS) would be due to the deposit of organic and inorganic matters necessitated by the military activities.

It was revealed that RDX had the highest concentration of 68.19 µg/kg in dry season compared to wet season that was not very significant, Table 1 using the Anova test for total explosive contents shows a significant difference for all locations in both dry and wet seasons (P<0.05). In zero metre of the objective TNT, RDX and HMX were consistently found at the highest values of 49.39 mg/kg, 68.19 mg/kg and 35.67 mg/kg, respectively (location 4). An impact area and beyond within the hand grenade range, a 105 mm howitzer firing point and the heavy artillery and mortar range that had been analysed by GC-ECD. TNT concentrations ranged from 0.11 to 49.39 mg/kg in dry season compared to wet season that was not very significant, Table 1 using the Anova test for total explosive contents shows a significant difference for all locations in both dry and wet seasons (P<0.05). In zero metre of the objective TNT, RDX and HMX were consistently found at the highest values of 49.39 mg/kg, 68.19 mg/kg and 35.67 mg/kg, respectively (location 4).

Myco-remediation of heavy metals: microbial fungi consortium (MFC)

Fungi role as primary and secondary decomposers in these classic “cycles” of nature, and spent fungal biomass from industrial
fermentations is an available resource for the concentration of heavy metal contamination. Fungi myco-remediation mechanism are:

(i) The target compound is used as a carbon source

(ii) The target compound is enzymatically attacked but is not used as a carbon source (co-metabolism)

(iii) The target compound is not metabolized at all but is taken up and concentrated within the organism (bioaccumulation).

Although fungi participate in all three strategies, they are often more proficient at co-metabolism and bioaccumulation than at using xenobiotic as sole carbon sources. “Co-metabolism refers to any oxidation of substances without utilization of the energy derived from the oxidation to support microbial growth.” The phenomenon has also been called “co-oxidation” and or “gratuitous” “fortuitous” metabolism. Many biochemists dislike the term. Nevertheless, it has become well entrenched in the literature. A second meaning of co-metabolism is to describe the degradation of a given compound by the combined efforts of several organisms pooling their biochemical resources for mutual efforts, Figures 7. Fungal isolates showed very good activity in the reduction of heavy metals when observed. Lead concentration was reduced by Aspergillus niger to 20%, Rhizopus to 31.5%, Penicillium spp to 25%, Trametes versicolor to 54.5%, P. chrysoporium to 57% and Microbial fungi consortium (MFC) to 61.7%. Cadmium was reduced by A. niger to 16.5%, Rhizopus spp to 17.5%, Penicillium spp to 15.3%, T. versicolor to 24.8%, P. chrysoporium to 20.5% and MFC to 100%. Zinc was reduced by A. niger to 32.49%, Rhizopus spp. to 38.8%, Penicillium spp to 34.2%, T. versicolor to 32.8%, P. chrysoporium to 37.85% and MFC to 39%

Co-balt was reduced by A. niger to 100%, Rhizopus spp to 19.7%, Penicillium spp 21.8%, T. versicolor to 100% and MFC to 100%. Copper was reduced by to 22.1%, Rhizopus spp to 6.5%, Penicillium spp 23.19%, T. versicolor to 34.5%, P. chrysoporium to 32.8% and MFC to 39%. Manganese was reduced by A. niger to 60.4%, Rhizopus spp to 7%, Penicillium spp to 16%, T. versicolor to 61.6%, P. chrysoporium to 63.3% and MFC to 68.2%. Nickel was reduced by A. niger to 17.1%, Rhizopus spp to 8%, Penicillium spp to 18.6%, T. versicolor to 26.8%, P. chrysoporium to 28.90% and MFC to 31.3%. Chromium was reduced by A. niger to 14.8%, Rhizopus spp to 5%, Penicillium spp to 10%, T. versicolor to 43.2%, P. chrysoporium to 41.7% and MFC to 45.5% while. Arsenic was reduced by A. niger to 13.3%, Rhizopus spp to 14%, Penicillium spp to 19.4%, T. versicolor to 19.8%, P. chrysoporium to 19.8% and MFC to 20.1%

The best fungal isolates for heavy metal reduction in increasing order were Aspergillus niger > Phanorochete chrysoporium >Trametes versicolor > Rhizopus spp > Penicillium spp while, in the overall, the mixed culture consortium are the highest percentage reduction of each heavy metal analysed in this study as a result of co-metabolism and bioaccumulation as reported by Dagley. Anova test for total explosive contents shows a significant difference for all locations in both dry and wet seasons (P<0.05), Appendix 1. Fungi pattern of growth and development is less predictable with fixed into developmental pathways and affirmed by the report of Robson et al. who reported that the life cycle of a fungus is unpredictable and flexible. The growth phases demonstrated by the fungi identified in this study show that the pattern of growth (Figure 8) displayed must have responded to the fixed nutrients of mineral salt medium supplemented with 1% muntions. The stages of growth are typically of any organism growing in a fixed quantity of nutrient. Every single isolate of fungus grew rapidly in the beginning that is, the exponential phase. This could be attributed to their previous association with muntions/explosives contaminated environment (Figure 10) and impacts of soil matrix on fungi physiology, Figure 11.

This is in conformity with Diaz & Massol-Deya, discovered that most of the fungal species were able to grow efficiently and appear concurrently in the environment as novel growth and energy substrate. Therefore, these fungi have a potential to degrade xenobiotic compounds (Figure 9). The growth pattern of fungus had different maximum growth peaks. This could be as a result of exhaustion of nutrients and release of toxic materials into the medium (transformation of waters explosives) and there is a need for improved biodegradation techniques with zero pollutants footprint (Figure 4). The growth pattern of fungi in 1% muntions/explosives and minimal salt broth is represented Figure 8.

Appendix 1

Table 3 Mean seasonal variation of physcio-chemical parameters of composite samples of four firing rae locations (12 different metres)

| Parameters                  | Unit  | Wet  | Diy  | Mean |
|-----------------------------|-------|------|------|------|
| Temperature                 | OC or OF | 27.16 | 22.71 | 24.94 |
| PH                          | EC µs/cm | 6.07  | 2.31  | 4.19  |
| Electric Conductivity (EC)  | EC µs/cm | 3011  | 312   | 1661.5 |
| Total Dissolved solutes (TDS)| (mg/L) | 6.13  | 347.7 | 347.7 |
| Soil Organic Carbon (SOC)   | %     | 5.11  | 5.76  | 5.44  |
| Available phosphorus        | (mg/kg)| 4.7   | 4.9   | 4.8   |
| Total Nitrogen              | %     | 9.32  | 9.72  | 9.52  |
| Sodium conc                 | ppm   | 498   | 863   | 680.5 |
| Potassium conc (ppm)        | ppm   | 169   | 162   | 165.5 |
| Sulphur conc                | ppm   | 560   | 724   | 642   |
| Carbonate (CO3-2)           | mg/100g | 0.22  | 0.35  | 0.285 |
| Bicarbonate (ITCO3)         | mg/100g | 1.11  | 1.68  | 1.4   |
| Manganese                   | mg/kg | 35.87 | 59.56 | 35.87 |
| Moisture                    | %     | 59.56 | 51.12 | 55.34 |
| Water holding capacity      | %     | 56027 | 69.39 | 62.83 |

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### Appendix 2

#### Table 4 Mean seasonal variation of total explosives concentration in various locations of NDA shooting range Kachia, Kaduna state µg/ kg, Nigeria

| Season | Distance Meter (M) | Sites | Depth (Cm) | TNT µg Kg⁻¹ | HMX µg Kg⁻¹ | RDX µg Kg⁻¹ | PETN µg Kg⁻¹ |
|--------|-------------------|-------|------------|-------------|------------|-------------|--------------|
| Wet    | Zero M            | L1    | 0-30       | 2.96        | 10.39      | 0.38        | BDL          |
| Wet    | Zero M            | L2    | 0-30       | 2.1         | 7.12       | BDL         | BDL          |
| Wet    | Zero M            | L3    | 0-30       | 26.6        | 29.6       | 15.33       | BDL          |
| Wet    | Zero M            | IA    | 0-30       | 1.34        | 2.21       | 7.42        | 2.07         |

| Season | Distance Meter (M) | Sites | Depth (Cm) | TNT µg Kg⁻¹ | HMX µg Kg⁻¹ | RDX µg Kg⁻¹ | PETN µg Kg⁻¹ |
|--------|-------------------|-------|------------|-------------|------------|-------------|--------------|
| Wet    | 200 M             | L1    | 0-30       | 0.12        | 1.43       | 0.449       | 0.17         |
| Wet    | 200 M             | L2    | 0-30       | BDL         | 6.31       | BDL         | BDL          |
| Wet    | 200 M             | L3    | 0-30       | BDL         | BDL        | 19.9        | BDL          |
| Wet    | 200 M             | IA    | 0-30       | 0.19        | BDL        | BDL         | 1.39         |

| Season | Distance Meter (M) | Sites | Depth (Cm) | TNT µg Kg⁻¹ | HMX µg Kg⁻¹ | RDX µg Kg⁻¹ | PETN µg Kg⁻¹ |
|--------|-------------------|-------|------------|-------------|------------|-------------|--------------|
| Wet    | 400 M             | L1    | 0-30       | BDL         | 12.4       | 0.67        | 1.15         |
| Wet    | 400 M             | L2    | 0-30       | BDL         | 6.11       | BDL         | 7.12         |
| Wet    | 400 M             | L3    | 0-30       | 0.39        | BDL        | 11.8        | BDL          |
| Wet    | 400 M             | IA    | 0-30       | BDL         | BDL        | 12.73       | 0.71         |

| Season | Distance Meter (M) | Sites | Depth (Cm) | TNT µg Kg⁻¹ | HMX µg Kg⁻¹ | RDX µg Kg⁻¹ | PETN µg Kg⁻¹ |
|--------|-------------------|-------|------------|-------------|------------|-------------|--------------|
| Dry    | Zero M            | L1    | 0-30       | 10.29       | 17.6       | 16.13       | 3            |
| Dry    | Zero M            | L2    | 0-30       | 1.14        | 19.88      | 22.61       | 0.39         |
| Dry    | Zero M            | U     | 0-30       | 16.31       | 33.07      | 23.14       | BDL          |
| Dry    | Zero M            | IA    | 0-30       | 49.39       | 35.67      | 68.19       | 1.6          |

| Season | Distance Meter (M) | Sites | Depth (Cm) | TNT µg Kg⁻¹ | HMX µg Kg⁻¹ | RDX µg Kg⁻¹ | PETN µg Kg⁻¹ |
|--------|-------------------|-------|------------|-------------|------------|-------------|--------------|
| Dry    | 200 M             | L1    | 0-30       | 0.11        | 1.6        | 15.11       | BDL          |
| Dry    | 200 M             | L2    | 0-30       | 0.13        | 13.31      | 2.69        | BDL          |
| Dry    | 200 M             | U     | 0-30       | 2.05        | BDL        | BDL         | BDL          |
| Dry    | 200 M             | IA    | 0-30       | 1.79        | 2.97       | 1.08        | BDL          |

| Season | Distance Meter (M) | Sites | Depth (Cm) | TNT µg Kg⁻¹ | HMX µg Kg⁻¹ | RDX µg Kg⁻¹ | PETN µg Kg⁻¹ |
|--------|-------------------|-------|------------|-------------|------------|-------------|--------------|
| Dry    | 400 M             | L1    | 0-30       | BDL         | 0.39       | BDL         | BDL          |
| Dry    | 400 M             | L2    | 0-30       | 2.16        | BDL        | BDL         | BDL          |
| Dry    | 400 M             | U     | 0-30       | 0.15        | BDL        | 2.38        | BDL          |
| Dry    | 400 M             | IA    | 0-30       | BDL         | 0.69       | 0.49        | 4.38         |

**N.B:** TNT, 2, 4, 6 – trinitrotoluene; RDX, royal demolition explosive; – 1, 3, 5 trinitrotoluene, HMX, 1, 3, 5 trinitro 1, 3, 5 – trinitrobenzene high melting explosion; BDL; below detectable level

Similarly, Gunasekaran et al. identified *Rhizopus spp*, *Aspergillus niger*, *T. versicolor* and *P. chrysoporium* as good mycoremediation potential for xenobiologic pollutants (Plate 1). The fungal consortium performs better than the single culture isolates of the fungi species which may be as result of a synergistic interaction of the fungal isolates. Similarly, Okerentugba & Ezeronye reported that a single microbe does not possess the enzymatic capability to degrade all or even most of the organic compounds in a polluted soil but mixed microbial communities possess powerful biodegradable potential because the genetic information of more than one organism is necessary to degrade the complex mixtures of organic compounds present in contaminated areas.

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Fungi consortia in situ biodegradation of xenobiotic, military shooting range, Kachia, Kaduna, Nigeria

Fungi species (explosives by turbidometry) - the growth potential

Fungal celluloses provide a good example of the contrasting growth of a single enzymatic capability, example in the non-specificity of the white rot fungi is ideally suited to treating low concentrations of mixed wastes in a nutrient deficient habitat, complete pathways of degradation are more likely to occur through the combined effects of many organisms biochemistry. These include fungal amylases, glucoamylases, lipases, pectinases, and proteases17,12 and secondary products like lipA and lipB.44 Penicillium janthinellum degrade high molecular weight polycyclic aromatic hydrocarbons more efficiently than does either microorganism alone.9 The Figures 8 and 10 shows the optical density reading of biodegrading activity of each fungal isolate on 1% explosive mineral salt medium (MSM) broth (Basal salt medium). It was revealed that Aspergillus niger had a maximum growth peak at 9.004 on the 30th day as affirmed by similar work of Svecova et al.44 Rhizopus spp had maximum growth peak at optical density 8.000 - 8.500 on the 25th, 35th and 40th respectively, Penicillium spp had the lowest growth peak at optical density 2.500 on the 30th day while the maximum was 6.010 on the 10th day. The maximum growth peak of Trametes versicolor was at 7.800 on the 20th day and the lowest growth peak was at 1.500 on the 10th day (Plate 1).

Phanorochaete chrysoporium had the maximum growth peak at 6.000 on the 20th day while the lowest for P. chrysoponion was at 1.000 on the 10th day as a results of the impacts of lignin peroxidases genes [lipA, lipB, lipC, lipD, lipE, lipF, lipG, lipH, lipI and lip J] as reported affirmed by scholar’s reports.71,48 Aspergillus niger had the best ability to degrade explosive while Penicillium spp had the least ability. The analysis of variance result shows that there is a significant difference in the overall growth pattern of fungi in MSM broth supplemented with 1% v/v explosive (P<0.05). The overall result also indicates that the growth rate increased significantly from the zero hour to the 5th day and beyond. Means on the same column having different superscripts are significantly different (P<0.05) according to Duncan multiple range test.

Furthermore, Fritsche et al.118 demonstrated that manganese peroxidase and lignin peroxidase from P. chrysoporium and T. versicolor have the ability to oxidize or reduce cyclic nitromines. This result is in agreement with Shererata & Hawai214 similar work revealed that P. chrysoporium, T. versicolor, or Aspergillus niger and Rhizopus spp, are capable of utilizing heavy metals (Figure 7) and TNT, RDX and HMX. Also, in this study, fungi pattern of growth and development is less predictable. Fungi were not fixed into developmental pathways. The life cycle of a fungus is unpredictable and flexible because the mechanism of action is co-metabolism and bioaccumulation as confirmed by Dagley.23 Complete transformation of TNT and 10 - 40% mineralization were discovered in Phanorochaete chrysoporium, a white rot fungi was reported.214,48 P. chrysoporium, a white rot fungus, was used in the degradation of RDX. It removed RDX (62 mg/L) from liquid medium containing glycerol as the main carbon source. Approximately, 53% of the molecule was mineralized, and the major by product (62%) of the degradation was N.O. P. chrysoporium mineralized HMX under nitrogen-limiting conditions. After 25 days of incubation, 97% of HMX was removed via reduction with the accumulation of 4-nitro-2,4-diazabutanal. Zhao et al.,199 reported that, the presence of glucose enhanced the degradation of HMX in marine sediment from a military UXO disposal and in which after 50 days, the HMX concentration in the aqueous phase (1.2mg/L) was reduced by 50%. The disappearance of HMX was accompanied by the formation of a mononitroso derivative.

MFC biodegradation significance

Fungal biodegradation mechanisms life cycle is unpredictable and flexible with intermediate bioremediation products which showed formation of MNX and ring cleavage and the subsequent formation of methylene dinitramine (MEDINA) by the white-rot fungus P. chrysoporium with RDX14 because the mechanism of action is co-metabolism and bioaccumulation as confirmed by Dagley.22 Nutrient availability is one important factor that plays a significant role in the rate of explosives biodegradation. Compared to other common pollutants, explosives, particularly RDX and HMX, are characterized by a higher N/C ratio. Although they may theoretically serve as sources of both carbon and nitrogen, only nitrogen is used by some organisms, and hence, another carbon source must be added and see Figure 4. Since denitrification is frequently carried out by strains that are capable of utilizing the explosive compound as a nitrogen source, a negative response is often observed in microcosm experiments when external nitrogen sources (either ammonium or nitrate) are added, as these nitrogen-containing compounds may compete with degradation of the explosives for nitrogen.30,33,73,291, 243

There is a need for the development of ex-situ bio-augmentation products for the biodegradation of xenobiotic to assist mineralization because the additional nitrogen supports microbial growth without competing with the explosive compounds for metabolism of RDX as reported by scholars291,321,379 and TNT41 nitrogen-bearing compounds are also potential inhibitors of enzyme expression, as presented by Nejdat et al.,291 who showed that ammonium and nitrite repress fungi lignin peroxidase. The mechanism by which the fungi mineralize the explosive is not known, but preliminary information about the process has been reported230,231 and mineralization by microbial consortium affirmed in similar work by Young et al.404,405 employed a consortium from horse manure to successfully biodegrade RDX. Biodegradation rates are also influenced by the type of electron acceptors and electron donors. For example, the degradation of RDX, HMX and TNT was shown to be enhanced by additional hydrogen or hydrogen-producing electron donors under anarobic conditions 1, 2 and the degradation of HMX was enhanced by adding mixed electron acceptors to anaerobic microbial consortia.24 Redox potential is another important factor that not only plays an important role in dictating the mechanism of degradation, but also influences the actual biotic degradation rate. Biodegradation is generally enhanced under reduced conditions, and in saturated soils.272, 283,129

Laccase reaction mechanism

Laccases are of particular interest with regard to potential applications, because of their capabilities to oxidize a wide range of environmentally dangerous substrates. Greater attention on laccase, an eco-friendly enzyme and a green catalyst in recent past is generating information that appeared in a number of reviews.17,321,388 Some soil ascomycete species from the genera Aspergillus, Curvularia and Penicillium,19 Trametes versicolor,290,232 are some examples of basidiomycetes that produce laccases.178 Laccases from different sources displays a wide range of redox potentials. The T1 site of laccase of T. versicolor shows a high redox potential of 780-800mV267 Figure 10 Expression of laccase in some fungi is regulated by nitrogen-containing compounds at molecular level in T. versicolor was examined.11 There was a direct correlation between concentration of nitrogen nutrient in growth medium and the level of lcc expression by T. versicolor. Copper is also often a strong inducer of laccase gene transcription, and this has been suggested to

Citation: Otaiku AA, Alhaji AI. Fungi consortia in situ biodegradation of xenobiotic, military shooting range, Kachia, Kaduna, Nigeria. J Appl Biotechnol Bioeng. 2020;7(6):246-274. DOI: 10.15406/jabb.2020.07.00241
be related to a defense mechanism against oxidative stress caused by free copper ions.\textsuperscript{75,86,108,119,204, 257,327} In addition to copper, other metal ions such as Mg$^{2+}$, Cd$^{2+}$ or Hg$^{2+}$ can stimulate laccase expression.\textsuperscript{119,206,327} Figure 9 shows the explosives fun-ga-l consortium isolates impacts on biodegradation of munitions explosive because of laccase. The importance of adequate copper concentration for proper laccase folding was further corroborated by studies in which two genes related to copper-trafficking in \textit{T. versicolor} were over-expressed in \textit{S. cerevisiae} expressing \textit{T. versicolor} lcc3 gene; the heterologous laccase production by \textit{S. cerevisiae} was improved up to 20-fold.\textsuperscript{101} Figure 11. The effect was suggested to result from more efficient transport of copper to the Golgi compartment.\textsuperscript{86} Laccase biocatalyst protein engineering potentials in the development of robust, active and tailor made enzymes. In addition to substrate oxidation, laccase can also immobilize soil pollutants by oxidation, coupling to soil humic substances - a process analogous to humic acid synthesis in soils\textsuperscript{39} and an active biocatalyst for biofertilizer.\textsuperscript{295}

Also, engineered to improve the efficiency of particular bioremediation processes like the \textit{T. versicolor} in \textit{Aspergillus niger} and mediators to attack non-phenolics. Laccases are multi-copper oxidases produced by fungi, bacteria, plants and even insects that catalyse the one-electron oxidation of four equivalents of reducing substrate, with the corresponding four-electron reduction of atmospheric oxygen to water.\textsuperscript{97,98, 311} Figure 12. Fungal laccases are known to have higher redox potentials than bacterial laccases and are produced by a wide range of fungi. Biological role and significance of laccases in lignin degradation remains unclear like \textit{P. chrysosporium} contains no laccase homologs in its genome, yet depolymerize lignin\textsuperscript{101,106,218} and see Figure 8. The application of selected microbial consortia, over single strains, for such biotechnological purposes presents advantages to perform complex tasks that single strains.\textsuperscript{115} The reason is that the deconstruction of complex substrates requires many different types of enzymes and diverse chemical reactions within pollutants matrix simultaneously with resilience to environmental fluctuations was reported\textsuperscript{206,211} and illustrated by Figure 12 (microbial and phytoremediation construct).

Application of synthetic microbial consortia could be a solution to this problem, as such consortia are usually composed of few well-identified organisms. Sometimes, those species do not co-inhabit the same environment, but their activities may be combined.\textsuperscript{103} Figure 13.

**Phytoremediation with laccase gene**

Phytoremediation for removal of recalcitrant environmental pollutants involve uptake, translocation, transformation and compartmentalization and sometimes mineralization (Figure 16).\textsuperscript{208} Plant roots also secrete metabolites, which stimulate the growth of micro-organisms in the rhizosphere, which in turn can degrade and mineralize the organic compounds. Although plants have several advantages over Fungi as candidates for bioremediation, they lack xenobiotic degradative capabilities unlike fungi. Hence, introduction of genes for degradation of recalcitrant environmental pollutants from microorganisms or other eukaryotes will further enhance their ability to degrade/mineralize recalcitrant environmental pollutants.

Microorganisms’ gene sequences are rich in CpG dinucleotides and have highly skewed codon usages, both of which are particularly unfavorable to efficient expression in plants.\textsuperscript{204} The \textit{MnP} gene\textsuperscript{61} or the \textit{laccase} gene\textsuperscript{281} from white-rot fungi has already been introduced into tobacco plants. Hirai et al.,\textsuperscript{131} reported laccase cDNA (scL) from white-rot fungus \textit{Schizophyllum commune} was used as a model ligninolytic enzyme to obtain efficient expression of \textit{scL} and removal of a recalcitrant compound in transgenic tobacco plant by decreasing the CpG dinucleotide motif content in order to develop phytoremediation with a ligninolytic enzyme-producing transgenic plant and a construct model in Figure 12.

**Findings**

**Gene transfer into the fungi and myco-remediation**

**HGT and adaptation to different environments:** Horizontal gene transfer (HGT), or lateral gene transfer, describes the transmission of genetic material between organisms, specifically across species boundaries.\textsuperscript{11,91,180,251} The term species is of course a difficult concept to apply to asexual microbes.\textsuperscript{71,90,19} HGT represents an important factor in shaping the genomes of prokaryotes and has provided a key source of evolutionary innovations.\textsuperscript{164,251} Several routes for transfer have been identified including gene transfer agents, transduction, transformation and conjugation.\textsuperscript{290,350} Fungi theoretically lack the beta-glucuronidase (GUS) gene and therefore the metabolic capacity to utilize glucuronides as a carbon source. Wenzl et al.,\textsuperscript{305} screened for fungi in vertebrate urine using culture enrichment with selection for fungi with glucuronide metabolic capabilities. Using this approach they identified GUS genes in \textit{Penicillium canescens} and \textit{Scopulariopsis spp}. Subsequent, phylogenetic analysis demonstrated that these genes, along with GUS genes of \textit{Aspergillus} and \textit{Gibberella}, were derived by HGT from bacteria,\textsuperscript{103,38} again demonstrating cases of HGT which have expanded the metabolism of fungi and enabled them to adapt to new environments. The implication for the study Figure 10 shows the explosives fungal consortium isolates biodegradability in increasing order > \textit{Aspergillus niger} > \textit{Trametes Versicolor} > \textit{Rhizopus spp} > \textit{Phanorochete chrysoporum} > \textit{Penicillium spp}. identified. Similarly to the Gunasekaran et al.,\textsuperscript{135} reported that, \textit{Rhizopus spp}, \textit{Aspergillus niger}, \textit{Trametes versicolor} and \textit{P. chrysosporium} as good mycoremediation potential for xenobiotic (Plate 1). The fungi-to-fungi transfers identified encompass five gene clusters representing a total of 53 individual gene phylogenies and seven gene cluster transfer events.\textsuperscript{184, 261,324,325,326} Perhaps the most striking example of a gene cluster transfer was the transfer of a 23-gene cluster from the \textit{Aspergillus} lineage to \textit{Podospora}.\textsuperscript{325} The majority of gene transfers between fungi (53 of 66 genes) were located in gene clusters, providing direct support for the hypothesis that gene clustering has played a role in facilitating gene transfer in fungi.\textsuperscript{100} Functional annotation of the 323 HGTs demonstrates that transfer has played a role in reconfiguring the core nutrient metabolism of many fungi, added to osmoprotic capacity of fungi, and allowed fungi to colonize ‘new’ environments. Mechanisms for gene transfer in fungi remain unclear, but the patterns of transfer (originating from bacteria and fungi) provide circumstantial evidence that supports two previously hypothesized routes of transfer: inter-domain transformation and conjugation-like transfer (for genes of bacterial origin) and anastomosis of cells (for those of fungal) reported.\textsuperscript{281}

**Surface sequestration**

The fungi hyphal surface that is in direct contact with the environment consists of a cell wall, a cell surface component that is composed typically of chitin, 1, 3-β- and 1,6-β-glucan, and mannan along with different proteins and pigments (that is, melanin). Although, such interactions include physical adsorption, complexation, coordination, or chelation\textsuperscript{4,106} Figure 17, which do not depend on intracellular metabolism, their effects on the cellular metabolism remains unclear.\textsuperscript{311} The surface sequestration depends on three factors:

a) Intracellular wall composition and the distribution of the metal binding groups,\textsuperscript{266}

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b) Several physicochemical factors that include primarily the environmental variables (temperature, pH, ionic strength, etc.) and
c) The pollutants on centraions and their chemical behaviour in the environment.

Biodegradation with extracellular enzymes with plethora of nonspecific extracellular enzymes are synthesized by fungi that can convert the complex extracellular growth substrates into simpler molecules, which can subsequently be absorbed into their cells as nutrients. The enzymes that are most beneficial Laccase gene transcription and biosynthesis, for example depends on a wide array of environmental cues, which include metals ions (e.g., copper (II) and iron (II)) and several aromatic compounds in the environment. In Figure 8, microbial fungi consortium (MFC) to 61.7% bioremedeate heavy metal better than the isolated fungi species because the lateral gene transfer with where T. versicolor in Aspergillus niger are HGT mediators and similar to Bolitin et al., report.

**Intracellular degradation**

Fungal cells also possess the ability to catalyze internalized pollutants using their intracellular enzymes. One of the best studied enzyme systems used for this purpose comprises a set of mixed function oxidases called cytochrome P450s, which demonstrate the ability to catalyze a wide range of biodegradation reactions that involve epoxidation, hydroxylation, deamination, nitro reduction, dehalogenation, and desulfuration reactions. Example of mycoremediation involving cytochrome P450 systems include biodegradation of kerosene and pharmaceutical waste products, of mycoremediation involving cytochrome P450 systems include biodegradation of kerosene and pharmaceutical waste products, and affirmed by scholar Burken et al., Phyto
eremediation with protoplast fusion from micro-organisms, Figures 13 and 14. Most of the microorganisms’ enzyme activities could not be detected in microorganisms’ gene-introducing transgenic plants, and most studies in these transgenic plants have used Western blot analysis to detect these target proteins. Protoplasts have a negative charge on the surface that helps to repulse surrounding protoplasts. They need to be in close contact prior to fusion, which can be achieved physically (by mechanical pressing with micropipette, by centrifugation) or chemically. Protoplast fusion consists of three steps: agglutination, the fusion of membranes in small localized places and forming of bridges among protoplasts, rounding of fused protoplasts. Protoplast fusion requires approaching, adhesion and joining of two different types of protoplasts. Approaching of the protoplasts is determined by many electrostatic forces arising from the potential on the cell surface. The result of the interaction is either fusion or complete failure.

**Kachia pollutants ecosystem restoration**

Vegetation is not only an aid to ecosystem restoration, it is a key indicator of munitions xenobiotic. Plant species that are present on a site, as well as their quantities and condition, describe a watershed’s health and resilience. Kachia (Figure 19, Appendix 3) lies in the more southern Guinea savannah zone, Nigeria. Semi-arid zones of northern with soil fertility management systems by smallholders that combine organic and inorganic inputs to sustain agricultural production. Adopting best-fit legume technologies in cropping systems will contribute to improved soil fertility through biological N₂-fixation and thereby increasing productivity of agricultural fields. Legume crop residue provides high quality fodder to feed livestock. Groundnut and soybean (Glycine max) variety TGx 1448-2E are widely grown by farmers that are common legumes and cultivate ginger for income generation and bar for food as well as income and the application of biofertilizer will help bio-remediate the residual pollutants within the ecosystems. Therefore, the use of the less efficient wild type tobacco plants could be combined with profit making operations, such as bioenergy cultivated (Figure 18) using PGPRs Biosurfactants presents in OTA1 AG² I nocula in the biofertilizer (https://www.youtube.com/ watch?v=Vi_OpgVeFeg biofertilizer) from bio-waste in anaerobic digester inoculated with beneficial microbes that exhibit differing metabolic capabilities.

**Appendix 3**

**Agro-ecological of Kachia, Kaduna state, Nigeria**

![Figure 19 Agro-ecological of Kachia (B), Kaduna state, Nigeria. Source: Foli, Samson (2012).](https://example.com/figure19)

Kachia ecosystem restoration protocol is illuminated in Figure 15 where ‘Green liver model’ for the metabolism of xenobiotic in plants intracellular degradation role of fungi gene in planate and affirmed by scholar Burken et al., Phyto
eremediation with protoplast fusion from micro-organisms, Figures 13 and 14. Most of the microorganisms’ enzyme activities could not be detected in microorganisms’ gene-introducing transgenic plants, and most studies in these transgenic plants have used Western blot analysis to detect these target proteins. Protoplasts have a negative charge on the surface that helps to repulse surrounding protoplasts. They need to be in close contact prior to fusion, which can be achieved physically (by mechanical pressing with micropipette, by centrifugation) or chemically. Protoplast fusion consists of three steps: agglutination, the fusion of membranes in small localized places and forming of bridges among protoplasts, rounding of fused protoplasts. Protoplast fusion requires approaching, adhesion and joining of two different types of protoplasts. Approaching of the protoplasts is determined by many electrostatic forces arising from the potential on the cell surface. The result of the interaction is either fusion or complete failure.

**Protoplast fusion**

Somatic hybridization is an important tool of plant breeding and crop improvement by the production of inter-specific and inter-generic hybrids. The technique involves the fusion of protoplasts of two different genomes followed by the selection of desired somatic hybrid cells and regeneration of hybrid plants. Protoplast fusion provides an efficient mean of ‘gene transfer’ with desired trait from one species to another and has an increasing impact on crop improvement. Somatic hybrids were produced by fusion of protoplasts from rice and barley using electro-fusion treatment for salt tolerance. In vitro fusion of protoplast opens a way of developing unique hybrid plants by overcoming the barriers of sexual incompatibility. The technique has been applicable in horticultural industry to create new hybrids with increased fruit yield and better resistance to diseases. Kachia pollutants ecosystem restoration for biodiversity of the shooting ranges is illustrated with Figure 16 xenobiotic myco/phyto
eremediation.
Green liver model

Phytoremediation is the term used to describe those methodologies that use living higher organisms, which include green vegetation, plants, aquatic plants, trees and grasses, to remove toxic compounds. This technology has the advantage of in situ treatment of contaminated soils, sediments, groundwater, surface water and external atmosphere.261,271,311

To some extent, molecular biology approaches have already been used to evaluate phyto-remediation and reveal elimination of toxicity from contaminated sites.26,58,192,295 However, these sequences of microorganisms’ gene, which might be introduced into plants, are rich in Cpg dinucleotides and have highly skewed codon usages, both of which are particularly unfavorable to efficient expression in plants.294 Practically, these Mnp gene - or laccase gene-introducing transgenic plants have produced very low amounts of these enzymes.166,238 The mechanisms of degradation of xenobiotic by plants (Figure 14). Prior to the introduction of xenobiotic to plant cells, they must be taken-up through the roots. Research studies reports predictive relationships between the uptake rate of a compound and its physical-chemical properties.152,256 Enzymatic transformation of xenobiotic by plants follows the ‘green-liver-model’ Figure 14 and involves three steps. First, the foreign compounds taken up by plants are transformed by enzymes such as cytochrome P450, carboxylesterases, and peroxidase.253 Secondly, the transformed xenobiotic are conjugated with D-glucose, glutathione, or amino acids108 by enzymes such as glutathione S-transferases, glucosyltransferases and malonyltransferases, resulting in either soluble or insoluble products. The third step is storage and compartmentation; the soluble compounds are stored in vacuoles or as cell-wall materials by further processing, and the insoluble compounds are generally assumed to be stored in the cell wall (Figure 14).270 The transformed products of TNT are further conjugated and sequestered in plant cells. Over 80% of the TNT label was associated with plant biomass, suggesting that the labelled carbon from TNT was sequestered in the plant tissues.124 The exemplar protocol for transformation and mineralization of TNT,114 RDX290 and HMX, in Figures 15 and 16 the xenobiotic biodegradation schematic. RDX biotransformation occurs in a variety of environments from surface and subsurface soils cold marine sediments169 and several microorganisms have been shown to co-metabolize both RDX and HMX.311 RDX and HMX has generally been thought to be recalcitrant to aerobic biodegradation114 and requires alternative biodegradation method like white rot of wood.362 Enzymatic transformation of xenobiotic by plants follows the ‘green-liver-model’ Figure 14 and involves three steps. First, the foreign compounds taken up by plants are transformed by enzymes such as cytochrome P450, carboxylesterases, and peroxidase.253 Secondly, the transformed xenobiotic are conjugated with D-glucose, glutathione, or amino acids108 by enzymes such as glutathione S-transferases, glucosyltransferases and malonyltransferases, resulting in either soluble or insoluble products. The third step is storage and compartmentation; the soluble compounds are stored in vacuoles or as cell-wall materials by further processing, and the insoluble compounds are generally assumed to be stored in the cell wall (Figure 14).270 The transformed products of TNT are further conjugated and sequestered in plant cells. Over 80% of the TNT label was associated with plant biomass, suggesting that the labelled carbon from TNT was sequestered in the plant tissues.124 The exemplar protocol for transformation and mineralization of TNT,114 RDX290 and HMX, in Figures 15 and 16 the xenobiotic biodegradation schematic. RDX biotransformation occurs in a variety of environments from surface and subsurface soils cold marine sediments169 and several microorganisms have been shown to co-metabolize both RDX and HMX.311 RDX and HMX has generally been thought to be recalcitrant to aerobic biodegradation114 and requires alternative biodegradation method like the Figure 15 biodegradations protocol affirmed by similar research report by Wang et al.191 reported that, RDX is degraded via sequential reduction of the nitro functional groups followed by abiotiriging-cleaveage.

Xenobiotic myco bio-augmentation/ phytoremediation/ biosimulation (xenobiotic Myco B-P-B) techniques

The mechanisms by which TNT and its metabolites affect RDX and HMX degradation appear to lie in their cytotoxicity to most of the microorganisms present in the explosives - degrading culture reported.302 TNT and its degradation products are adsorbed to the upper soil layer, whereas RDX and HMX are not, being transported instead with the infiltrating water affirmed and reported108,402 that the potential for RDX biodegradation in the upper 4m is extremely high. The Kachia biodegradation and restoration of the military shooting range will requires xenobioc myco /phytoremediation construct, Figure 16. Mycoremediation is a type of bioremediation using fungi, including WRF, for that purpose and it refers to their possibilities as microorganisms of degrading a great number of recalcitrant pollutants and transforming industrial wastes into products (Figure 16).314

Some of the most widely investigated fungal species capable of synthesising LiP are P. anerochaete chrysosporium, Trametes versicolor; Trichoderma reesei, Aspergillus niger, Phlebia radiata, Pleurotus ostreatus, Pleurotus sueror-caju.105 Biotransformation involving enzymes does not cause accumulation of toxic by-products, and the enzymes are digested after the completion of the process by the microorganisms dwelling in contaminated environments. Secondly, increasing the bioavailability of contaminants is more easily achieved than in the case of using whole cells.231 Laccase (benzenediol, oxygen oxidoreductases, EC 1.10.3.2) is one of the few lignin-degrading enzymes that have been extensively studied since 18th century. RDX degradation has also been examined in constructed wetlands.322 Both submerged and emergent plants were shown to take-up pollutants accumulated in sites of active growth. Biotransformation of RDX to unidentified products was also observed. Mineralization was very low (less than 5%) and production of volatile organics negligible. The nature of the chloromethane cycle17 transformation products remains unclear by Figure 16 xenobiotic protocol will optimize the environmental risk. Recently, laccases were reported from eukaryotes e.g. fungi, plants and insects.311 This makes laccases highly interesting for a wide variety of processes, such as textile dye decolouration, pulp bleaching, effluent detoxification, biosensors and bioremediation.78 Laccase is involved in the pigmentation process of fungal spores, the regeneration of tobacco protoplasts (Figure 13), as fungal virulence factors, and in lignification of cell walls and delignification during white rot of wood.220 Trametes versicolor is one of the most efficient laccase-producing fungi. Laccases are also produced in conjunction with LiP, Mnp, or both. Laccases from marine fungi effectively mineralized 14C-(ring)-labelled synthetic lignin to14CO2.270 Laccase genes several strains of Trametes versicolor.310

Plants deal with organic explosives, such as RDX and TNT in three phases as depicted in Figure 14 and reported by scholars.207,270,297 Phase I (transformation).The contaminant is metabolized into a more soluble and less toxic intermediate products by several reactions, such as oxidation, reduction, or hydrolysis. The oxidative metabolism of explosive compounds is generally mediated by cytochrome P450 mono-oxygenase in plants. In plants, cytochrome P450 forms a largest group of plant protein which plays an important role in degradation of explosives.276 Phase II (conjugation)-In conjugation between organic contaminant and endogenous hydrophile molecules, such as D-glucose, glutathione, or amino acids, soluble or insoluble substances are produced to be subsequently sequestered in different cellular compartments of the plant for storage.307,409 Conjugation also enhances metabolic activity which is further catalysed by glycosyl-, malonyl-,and glutathione S-transferases. Phase III (compartmentation), (Figures 15&18).

Cytochrome P450-mediated phytoremediation

Cytochrome CYP superfamily of enzymes exist in all biological domains and their presence predates the emergence of oxygen-metabolizing life form.310 Cytochrome P450 (CYP) monooxygenases, the ubiquitous enzymes with catalytic versatility, substrate diversity and atypical kinetics are one of the most fascinating targets for biocatalysis and play diverse roles in biotechnology, medicine and bioremediation.51,120,155 Being a ubiquitous organism, fungi inhabitant’s diverse ecological niches, adapts to various sources of carbon and nitrogen for their survival and metabolism.130 Comprehensive biochemical analysis of molecular mechanisms showed that fungal adaption are often facilitated by CYP monooxygenases.130 Fungal CYPs play an essential role in their adaptations to ecological niches

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due to their diverse roles in the production of metabolites critical for pathogenesis, detoxification of xenobiotic and exploitation of substrates (Figure 2). To date, a vast number of CYPs have been identified in >2500 fungal species, and these are classified into approximately 400 CYP families (namely CYP51-CYP69, CYP501-CYP699, and CYP5001-CYP6999).

Under aerobic conditions, RDX is highly persistent in soil and groundwater; it is much less susceptible than TNT to biotransformation, is less strongly adsorbed to soil, and undergoes much less immobilization. Some notable examples of the fungal primary metabolism are housekeeping functions such as ergosterol biosynthesis, meiotic spore-wall biogenesis, and n-alkane hydroxylation, whereas fungal secondary metabolism deals with the biosynthesis of hormones, mycotoxins, and the like (Figure 15) reported by Creină and Petrič. Fungal CYPs are also capable of detoxifying and degrading various xenobiotic compounds encountered in their environments, such as polycyclic aromatic hydrocarbons (PAHs), phenolic compounds, and other toxic environmental pollutants. Remarkably, the transcriptional regulation of CYP-dependent metabolic pathways can be alternatively induced or up-regulated by defined high or low nitrogen conditions, as observed in P. chrysosporium, C. versicolor, P. placenta, and A. oryzae (Figure 17).

Considering the extraordinary potential of CYPs, several reviews have addressed their limitations and have focused on tackling these challenges to promote CYPs as robust (Figure 15) biocatalysts. Fungal CYPs also inherently possess the same limitations; and recent advancements has sought to overcome the major hurdles posed by fungal CYPs. Enzyme engineering through mutagenesis is one of the tools to modify fungal CYPs as sustainable catalysts by solving issues concerning low expression levels and poor activity. For example, the oxidizing activity of the P. chrysosporium PAH oxidase (CYP5136A3) was significantly improved by a rational designing approach through site-directed mutagenesis. Artificial CYP-CPR fusion constructs termed “Molecular lego” are a competent strategy for comparative analysis of differential redox partners to find the optimal redox system (Figure 16). In addition, improved intracellular electron recycling can be obtained through cofactor regeneration by co-expressing CYPs along with glucose/formate dehydrogenase or aldehyde reductase (Figure 15).

Biofertilizer-soil amendments

The use of biofertilizer amendments such as compost, biochar, biofertilizer and molasses to ‘kick-start’ microbial degradation has been well studied for many xenobiotic reported by Wu et al. and Otoku et al. But few studies have yet investigated the effect of amendments for phytoremediation on explosives. A laboratory study showed that the application of molasses to open burn/detonation areas enabled Guinea Grass (Panicum maximum) to remediate RDX, with plant and microbial communities’ fully remediating soil contaminated with 1.3 mg/kg RDX within the 15-week study. When biofertilizer applied as soil inoculants, they multiply and participate in nutrient cycling and benefit crop productivity.

Depending on the family of N. tabacum 30-40% of the seed is in average oil and is composed of linoleic acid (71.63%), oleic acid (13.46%) and palmitic acid (8.72%). Although tobacco seed oil is a new renewable alternative diesel engine fuel. Tobacco is a plant, which fulfills all the characteristics for a suitable phytoremediation plant and combined with the production of bioenergy it could become one of the main plants used in the field of phytoremediation. Tobacco is a plant, which fulfills all the characteristics for a suitable phytoremediation plant and combined with the production of bioenergy. Niche fungi consortium research on diversity and distributions will enhance the re-construction of the Kachia biogeography spatial scale relevant to ecology and evolution. The convergence of biogeography and ecology is a relatively recent phenomenon species sorting and niche partitioning; local/regional saturation; and diversification of regional biotas analyzes organism-environment relations through change over space and time, and often includes human-biota interactions (Figure 13).

Site monitoring

The significant cost factor of site remediation is monitoring of contaminant levels, and this is particularly difficult given the large scale, and inaccessibility of military sites. Novel studies have quantified the response to explosives of plant species native to military ranges in temperate regions. By measuring multiple variables representing morphological and physiological responses, such studies identified vegetation responses that could potentially be used to determine levels of TNT and RDX contamination. Hyper spectral imaging technologies are also being developed to remotely detect vegetation response using aerial drones as suitable carriers to record contamination levels on surface in accessible sites.

Myco B-P-B techniques–exemplar

Despite several studies, bioremediation of TNT is still considered challenging. Majority of studies have focused on discovering microorganisms that could be used to degrade pollutants was reported by Ayoub et al. and No Ivak et al. research reported the effect of bioremediation methods and combinations (biostimulation, bioaugmentation, phytoremediation) on the degradation of TNT in soil. Biostimulation approaches add nutrients or electron acceptor/donors to the polluted environment to enhance the bioremediation of pollutants like TNT. Biostimulation can also be used in combination with bio-augmentation to improve the survival and catalytic activity of introduced microorganisms.

Phytoremediation

Phytoremediation utilizes green plants to treat contaminated soil or water and has been successfully applied to remediation of different pollutants, including TNT. Plants release root exudates and enzymes that stimulate microbial activity and contaminant degradation in the rhizosphere, enabling the application of rhizoremediation, which is considered particularly effective for the treatment of contaminated soil (Figure 16). Using 16S rRNA gene full-length sequencing (BCCM/LMG, Belgium), the predominant bacterial strains in the inoculum were determined to be members of Pseudomonas and Stenotrophomonas genus.

The research indicates that all treatments used resulted in decreased concentrations of TNT in soil. Similar to previous reports, TNT and its aminodervatives 2ADNT and 4ADNT were the only compounds detectable by HPLC; all of these were reduced to a considerable degree of the initial concentrations. The isomeric aminodervatives (2ADNT and 4ADNT) are formed during microbial transformation of TNT; these compounds exhibit less toxicity than TNT and bind more tightly to soil clay particles and organic matter, thereby reducing bioavailability as well as toxicity in soil.

Of the treatments used, the combined bioaugmentation - biostimulation approach coupled with plant (especially rye) cultivation was the most effective.
Fungi consortia in situ biodegradation of xenobiotic, military shooting range, Kachia, Kaduna, Nigeria

The effective treatment resulting in the lowest final TNT concentrations. This corresponds well to other more recent results with similar TNT contamination rates.\textsuperscript{385} 

Future work

The Figures 14–16 xenobiotic myco/phytoremediation construct are an integrated approach to metabolic engineering has been termed inverse metabolic engineering, where first a desired phenotype is formulated, then the necessary factors to create it are determined, finally these are assembled in the host (microbial/Phyto) as affirmed by Bailey et al.\textsuperscript{18} as a construct for xenobiotic biodegradation model called PC3R technology. Finally, incremental genetic changes in vivo and input the results of each change back into the model. The technological and methodological tools now exist to accomplish PC3R technology where \textit{Aspergillus} as exemplar as gene mediator. Further work on PC3R technology with respect to xenobiotic biodegradation will require the solution mathematical modelling of metabolic pathway networks; the theory is well developed at present\textsuperscript{340,380} and comprises several complementary approaches to metabolic modelling, e.g., stoichiometric and carbon flux models (metabolic flux analysis)\textsuperscript{239,271} kinetic models (metabolic control analysis and morphologically structured models).\textsuperscript{213,245,252} The development of remediation approaches is the determination of the genomes of specific white rot fungi. For example, research into the \textit{P. chrysosporium} genome has resulted in the elucidation of specific cytochrome P450 monooxygenases, which may be differentially expressed in the presence of xenobiotic compounds.\textsuperscript{400}

In the future, efforts should be made to develop strategies to improve the tolerance, uptake, and yperaccumulation of heavy metals/metalloids using genomic and metabolic engineering approaches. Pathways that control the uptake, detoxification, transport from root to shoot tissues and translocation and hyper-accumulation in the aboveground storage tissues can be engineered using gene-stacking approaches. Genome editing strategies can be designed using TALENs (transcription activator like effectors nuclease) technology or the powerful CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats) system to produce microbes/plants for bio/phytoremediation purposes (Figures 17&18). Recently, efficient and successful CRISPR/Cas9- mediated targeted mutagenesis has been reported in \textit{Populus} plants.\textsuperscript{106}

Conclusion

There is a need for the development of \textit{ex-situ} bio-augmentation products for the biodegradation of xenobiotic to assist mineralization because the additional nitrogen supports microbial growth without competing with the explosive compounds for metabolism of RDX as reported by scholars\textsuperscript{291,329,370} and TNT\textsuperscript{41} nitrogen-bearing compounds are also potential inhibitors of enzyme expression, as presented by Nejidat \textit{et al.},\textsuperscript{241} who showed that ammonium and nitrite repress fungi lignin peroxidase. Extracellular oxidoreductases. A unique characteristic of fungi is their ability to attack organic compounds using a range of extracellular oxidoreductases with relatively nonspecific activities. These enzymes have probably evolved to support fungal growth on recalcitrant substrates of random structure such as lignocellulose substrates that are not accessible for most bacteria through the depolymerisation and removal of lignin and its humic substance derivatives.\textsuperscript{6,134,209} Hence, these enzymes give fungi an ecological advantage over bacteria in particular decomposition niches.\textsuperscript{6} Genetic engineering of peroxidases to improve their catalytic properties allows enhancement of the $H_2O_2$ resistance of the enzymes, or the structural modification of already commercially available enzymes to enable the oxidation of other compounds with high redox potentials.\textsuperscript{366} Radical reactions may also covalently bind metabolites (for example, of TNT) to soil organic matter.\textsuperscript{144,248} Cytochrome P450 monooxygenases\textsuperscript{60} also contribute to the catabolic versatility of fungi lacking extracellular oxidoreductases.

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Conflicts of interest

The authors declare that there was no conflict of interest.

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