Testing Primed White Rot Fungi for Bioremediation of Petroleum Hydrocarbon Contaminated Soil.

&

Bioremediation Options Plan for Napo Concession Area in Ecuador.

By

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Abstract

Bioremediation has gained traction for its sustainable principles. Although, advancements in effectiveness are still needed to enable widespread application. This research has two major components. First, priming fungi could prove to be a useful tool to increase efficiency of white-rot fungi when used to bioremediate petroleum hydrocarbons contaminated soil. This study evaluated *T. versicolor* colonized in two substrates to test this theory. TPH was extracted from the soils using hexane shaking method, and measured on a CG-MS. The study results were not conclusive, and more research should be conducted to determine if priming white-rot fungi can increase the effectiveness of degradation of TPH in contaminated soils. Second, historical and unethical oil production in Ecuador has left an environmental and human health disaster. The goal of this study was to produce a high-level bioremediation plan that can be used and amended for site specific applications in Ecuador.

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List of Acronyms

CP	Control Primed
CUP	Control Un-Primed
D	Diesel
LC	Liquid culture
P	Primed
PAH	Polycyclic Aromatic
PHC	Petroleum Hydrocarbons
ST	Straw
TPH	Total Petroleum Hydrocarbons
UP	Un-primed
BTEX	Benzene, toluene, ethylbenzene and xylene
PCB’s	Polychlorinated biphenyl

Chapter 1. Introduction to Petroleum Products and Soil Reclamation

1.1 Petroleum Products in the Environment

Petroleum products such as, gasoline, diesel, and engine oil are produced from crude oil and are made up of petroleum hydrocarbons (PHC's) (CCME 2001). They can form thousands of compounds that will differ, depending on source, soil type, degree of processing (crude, refined, or blended) and weathering. Petroleum hydrocarbons are known toxicants with base building blocks composed of carbon, hydrogen, and small amounts of nitrogen, sulfur, and oxygen.

Petroleum hydrocarbons are toxic to humans and biota because they contain organic compound such as polycyclic aromatic hydrocarbons (PAH's), benzene, toluene, ethylbenzene, and xylene (BTEX's) (WHO 2008), as well as, heavy metals, detergents, and surfactants (Sandvik and Wong 2016). Crude oil and its constituents enter the human body through three primary routes: skin absorption, ingestion of food and drink, and inhalation of oil on dust or soot particles (Bagnoud 1994). Routes of exposure can either be acute, such as inhalation at a gas station, or chronic. Chronic exposures, such as ingesting contaminated drinking water, has been linked to cancer, decreased birthweight, miscarriages, decreased immunological function, and impaired renal or liver functioning (UDAPT 2013; WHO 2008).

Environmental contamination from petroleum hydrocarbons is relatively common in aquatic and terrestrial environments (WHO 2008). Aquatic environments can be contaminated via tanker and ship accidents, and off coast extraction, as well as near shore spills which migrate into streams, rivers, or oceans. Petroleum products are released into the soil through many vectors, ranging from small underground leaking storage tanks to pipeline leaks, and often results from negligent oil production practices (Rhodes 2014).

When released to the environment, the composition of a petroleum product changes (Sandvik and Wong 2016). When left in place, it is typically broken down (i.e., weathered), which may change the ability of the contaminant to move from or throughout

the environment. Although some petroleum constituents biodegrade readily (e.g., volatile organic compounds), weathered petroleum is generally immobile and remains in place. Labile constituents which biodegrade rapidly and volatilize quickly are of lesser concern than weathered petroleum (Sandvik and Wong 2016).

One widely accepted assessment of sites contaminated by petroleum hydrocarbon is analysing soils for total petroleum hydrocarbons (TPH) (CCME 2001). Total Petroleum Hydrocarbons represents the total mass of hydrocarbons rather than the identification of individual components. Hydrocarbons with similar physical and chemical properties can be assigned to a specific equivalent carbon range, known as fractions.

1.2 Environmental Standards for Petroleum Hydrocarbon in Soil

Once a petroleum product has been released into the environment, government regulations often impose remediation policies (CCME 2001). These policies state that the spill must not only be contained, but that the environment must also be remediated. For example, Canada has the *Canadian-wide standards for petroleum hydrocarbons in soil and subsoil*. This policy states the allowable limit for the four land use classes: agricultural, residential/ parkland, commercial, and industrial.

The Canadian Council Ministry of Environment (CCEM) (2008) outlines that the allowable limits for agricultural land with fine grained soils is 201 mg/kg TPH or if the contaminant is close to potable water the limit is 170 mg/kg TPH. However, standards are not universally recognized at the global scale, and each country may set their own allowable limits. In fact, the discrepancy between countries' allowable limits for TPH may differ dramatically. For instance, the United States (US) has an allowable limit of 400 mg/kg TPH, and Ecuador has an allowable limit of 1000 mg/kg TPH (Beltman n.d.). Soil testing is necessary when determining how best to remediate an area and to determine if levels have reached acceptable limits after remediation.

1 .3 Cost-Benefit of Implementing Bioremediation Strategies

Financial clean-up costs and environmental impacts are important factors when evaluating the efficacy of remediation methods. Current soil remediation such as incineration, solvent extraction, indirect thermal, soil venting, soil washing, and landfills are: costly, environmentally unsustainable, energetically expensive, and technologically complex (Stamets 2005, pg. 91), In addition, implementing these remediation strategies in remote environments, such as the Arctic, is often not feasible due to lack of accessible infrastructure.

Large companies and industrial environmental consultancies will undoubtedly benefit from more cost-effective remediation methods. However, there are situations where a particular cleanup onus may not always be clear (e.g., non-point source contamination; (US-EPA 2017). Moreover, there are many small-scale spills on private land which may not be feasible to use industrial remediation strategies (Healing City Soils n.d).

In addition, social and environmental injustices are still prevalent across Canada and the globe (Haluza-Delay 2007). For instance, Ecuador has over 900 contaminated sites due to unethical petrochemical disposal methods deployed by Chevron (formerly Texaco) between 1967 and 1992 (UDAPT, n.d), and beyond. This is an exemplary case of all benefits being captured by a few individuals, the military, and corporate CEO's, while the general urban and rural populations, as well as indigenous groups, have suffered declining health, and witnessed their homelands be inundated by toxic effluent (Bagnoud 1994). Ecuador, Chevron, and Petroecuador all deny responsibility for this environmental disaster, while the local inhabitants of the region, and the environment, continue to suffer (Gropper, Personal Communication, August 2018).

Biological technologies (hereafter, bioremediation) are being explored as cost-effective, safe, natural, low maintenance, fast, and low-tech methods for restoring degraded landscapes. Both *in-situ* and *ex-situ* bioremediation methods can be applied for the purpose of remediating water and soil (Kumari et al. 2018; Rhodes 2014).

Bioremediation is the use of plants, microorganisms, or fungi to clean-up contaminated soil or effluent.

The general objective of my research is to contribute to the development of low-tech, low-cost bioremediation strategies to remediate PHC contaminated soils with the use of fungi, plants, and bacteria. I approach this through a mycoremediation experiment, as well as by evaluating bioremediation options for contaminated sites in Ecuador.

Chapter 2. Mycoremediation Research

2.1 Introduction

Mycoremediation is the use of fungi to degrade, hyperaccumulate, or bioconvert pollutants into less toxic components (Adenipekun & Lawal 2012). Comprised of mushrooms, molds, mildews, smuts, rusts, chytrids, and yeasts, fungi are a diverse group of eukaryotic single celled or multinucleate organisms that associate with living hosts or persist in a free-living form, and derive their sustenance via absorptive nutrition (i.e., by enzymatically decomposing organic material, and re-absorbing nutrients from their growth medium or substrate). Fungal research has shown that mushrooms and other fungi can degrade a variety of organic pollutants such as polycyclic aromatic hydrocarbons (PAH's), polychlorinated bisphenols (PCB's), and dioxins (Adenipekun & Lawal 2012, Zitte et al. 2012, Kumar 2018), while others can mineralize or hyperaccumulate heavy metals into the fruiting body or vegetative mycelium. Given their potential to degrade organic pollutants, my project focuses on fungal bioremediation of petroleum hydrocarbon (PHC's) contaminated soils.

Several different white-rot fungi, such as *Trametes versicolor, Pleurotus ostreatus*, *P. tuber-regium*, *Panerochaete chrysosporium* and *Lentinus squarrosulus,* have been used in the bioremediation of polluted soil (Adenipekun and Omoruyi 2008). In particular, *Pleurotus* spp., and *Trametes versicolor* have been shown to be excellent candidates for *in-situ* remediation of hydrocarbon contaminated soils because of their nutritional mode, stress-resistant traits, and their robust mycelial structures (Adedokun 2015, Stamets 2005, pp. 113).

One landmark study compared the efficiency of four on-site bioremediation treatments (bacteria treatment, enhanced bacteria treatment, mycelium treatment, and control) on aged petroleum contaminated soils. The study determined that mycelial treatment was highly effective, even after as little as nine weeks after inoculation (Thomas et al. 1998). Due to the importance of documenting findings through inexpensive and easily accessible metrics, as well as funding constraints, the study used biological indicators as evidence, i.e. plant colonization on the mounds. The bacterial bioremediation treatments and untreated controls still stank of petroleum, and there was

no plant colonization. Whereas, the mycelial treatment showed qualitative improvement. For instance, other wild fungal taxa grew, and even fruited, on these previously contaminated mounds. The absence of a petroleum odor indicated that the mycelial treatment may be more effective at remediating the polluted mound, than were either the bacterial treatments or the untreated controls.

2.2 Mushroom Remediation Mechanism

The key to understanding the mushroom remediation mechanism is understanding the mushroom ecology. There are four basic categories of mushrooms: parasitic, endophytic, mycorrhizal, and saprophytic (Stamets 2005, pp. 23-34); all of which occupy an ecological niche. The mushroom life cycle begins with a germinating spore and develops into filamentous strands called hyphae (Zitte et al. 2012). Two compatible hyphae connect to reproduce. This is the beginning of a unique strain that grows and branches to form a mycelial mat. Once the mycelia have colonized an area, or there are environmental triggers, such as a change in seasonal temperature, the fungus begins to put its energy into forming sporocarps, or fruiting bodies, as illustrated by an emerging pileus (i.e., mushroom cap). Primordia, or 'pinheads' are the earliest stage of mushroom development; the mushrooms continue to develop until mature and are morphologically capable of releasing their spores. Each time two hyphae mate, they create a new genetically distinct individual (i.e., ecotype or individual strain) with their own genetic information and phenotypic characteristics.

Saprotrophic fungi are the engines of nutrient cycling, as their premier function in forests are as decomposers. Many saprotrophic fungi are basidiomycetes. Saprotrophic fungi can be categorized into primary, secondary, and tertiary decomposers (Stamets 2005, pp. 21). These fungi use a variety of substrates, produce numerous types of fruiting structures, and support numerous ecosystem services. For example, preventing erosion via aggregating soils, which may foster ecological restoration or support site-specific environmental conditions (Stamets 2005, pp. 21). In addition, mycelial networks can draw water and nutrients throughout the substrate.

Saprotrophic fungi are incredibly diverse and contain sugar fungi, white-rot fungi, and brown-rot fungi. Brown-rot fungi begin by breaking down cellulose while white-rot

fungi mainly decompose lignin in woody materials and leave a residual white bleached appearance from their exudation of peroxidases (Stamets 2005, pp. 19). They decompose organic matter via exuding digestive enzymes from their hyphae, or mycelium (the root like structure of the fungus). Recent research suggested that white-rot fungi are the only group of organisms capable of completely mineralizing lignin (Anderson and Juday 2013).

Their ability to mineralize lignin makes them the focal functional group for fungal bioremediation of soils contaminated with organic pollutants. Mycelium secretes three main enzymes: laccases, manganese peroxidase, and lignin peroxidase to extracellularly digest their food source: organic matter and woody debris (Durr 2016). Laccase, secreted by white-rot fungi, is needed to break down lignin and other complex organic compounds. Since these enzymes are non-specific, consequently they can break down and mineralize complex organic contaminants (i.e. hydrocarbon bonds) into smaller and less-toxic molecular components.

2.3 Culturing Mycelial Mass for Bioremediation using White-Rot Fungi

Culturing mycelium involves starting with a small volume of spawn and then expanding it (Stamets 2000). Spawn refers to a substrate that has been inoculated with fungal cultures. Spawn can be generated from pure culture (liquid culture or in-vitro), or spawn can be produced from mycelia growing on a variety of substrates, including sawdust or woodchip, grains, wooden dowels, or straw. Liquid spawn is water, or nutrient media, enriched with liquid spores or a mycelia slurry. In-vitro culture is pure culture growing on petri dishes. Sawdust spawn and woodchip spawn are made from a variety of wooden materials; hardwoods are particularly effective substrates for fungi. Straw spawn is pasteurized spawn inoculated with mycelia for mushroom growth and proliferation (Stamets 2000).

Remediation of contaminated soils using white-rot fungi will require large volumes of organic matter to host the introduced mycelial cultures and mushroom spawn. Mushroom mycelia may not persist in contaminated soils without an extra carbon source (Durr 2016). Fortunately, mushrooms can use many different substrates carbon

sources, including. straw, corn cobs, coffee grounds, brewery wastes, nut casings, pulp and paper-waste, wood-waste, and more (Stamets 2005).

2.4 Mushroom Priming

Recent research suggested that fungi respond to stress by phenotypic plasticity, acclimation, and by adaptation (Romero-Olivares et al. 2017). Likewise, when a fungal culture is introduced to a foreign contaminant, it will either tolerate this substance or use it as a food source, or else the fungus will perish.

Priming a mushroom culture involves introducing a small concentration of a contaminant to the culture (Darwish 2013). If the mushroom culture is tolerant or has the genetic ability to digest the contaminant and use it as a food source, then it will begin to recognize the contaminant as edible. Priming mushroom cultures might enhance their effectiveness at degrading organic contaminants such as petroleum hydrocarbons (Cotter 2014).

2.5 *Trametes versicolor* (Turkey Tails) Species Profile

My work focused on *Trametes versicolor*, which is a saprotrophic white-rot fungus, belonging to the subphylum basidiomycota (Linkoff 1981). This fungal species is a primary decomposer, which is globally distributed, ubiquitous in ecosystems worldwide, and are potentially the ideal model mushrooms for bioremediation of hydrocarbon contaminated soils, because it is pervasive across the globe, and is relatively easy to cultivate.

Turkey tail are shelf-like mushrooms. Overlapping, small, leathery, thin stalkless caps with many multi-coloured bands (Linkoff 1981). They can be found fruiting from deciduous wood or from wounds on conifer trees. The most common species is *Trametes versicolor*. *Trametes versicolor* fruits from May-December, and their caps are ~ 2.5 to 10 (cm) wide, with silky, hairy, or velvety zones alternating with smooth zones. The flesh of *T. versicolor* is 1-2 mm thick. *Trametes versicolor* has a white spore print. -

Turkey tails can be cloned by collecting and wrapping the fruiting body on materials made of woody pulp, such as wet paper towels (Cotter 2014). Fruiting can take

from 8-12 months for outdoor inoculation depending on temperature and inoculation rate. They prefer to fruit on wood logs with sawdust spawn and have also been successfully grown on fresh cedar woodchips. For indoor cultivation, it is best to use sterilized or super pasteurized sawdust supplemented with wheat or rice bran at 5 percent dry weight. Indoor inoculation to fruiting ranges between 25-35 days. Turkey tail mushrooms have shown potential for bioremediation of soil contaminated with organic compounds. Turkey tails have also been found growing in Ecuador in open waste pits, which are highly contaminated with petroleum hydrocarbons (L. Gropper, personal communication, August 6, 2018).

2.6 Mycoremediation Knowledge Gaps

Extensive research needs to be conducted on mushroom remediation of contaminated soils before these technologies can be used on a large scale. Inherently, mycoremediation projects vary greatly due to site-specific environmental conditions, project scale, and contaminant variability. Although mycoremediation is not new, it is not often employed as a bioremediation technique due to the paucity of awareness on mycoremediation methods and the limitations associated with scaling up these technologies to large volumes of contaminated media (Kumar 2017). Therefore, it would be immensely valuable to develop remediation methods and to document best management practices (BMP's) for conducting mycoremediation at both small and large project scales.

Using mushrooms for remediating pollution *in-situ* would be beneficial because they are natural, safe, low-maintenance, reusable, and cost-effective, and they also proliferate quickly. In addition, by using a consortium of different fungi, it may be possible to reclaim soils contaminated with several different pollutants at the same site (Kumar 2017).

In many bench-scale experiments, as well as a few pilot-scale experiments, studies have demonstrated that mushrooms can be used as a fast, effective, and economic way to remediate soil contaminated with hydrocarbons (Adenipekun et al. 2011, Rhodes 2014). However, more research is needed before bioremediation using mushrooms can be implemented as a commonly employed technique.

I identified several knowledge gaps, which should be addressed with future research. First, *in-situ* mycoremediation site design and implementation methods must be documented and evaluated. Second, feasibility studies must be conducted for *in-situ* mushroom remediation methods, including cost and time comparisons with traditional remediation methods. Third, comparisons of mushroom species will provide valuable information of the efficiency of fungal bioremediation. Fourth, enzyme collection, preservation, and application methods for maintaining the viability of fungal enzymes for bioremediation. The aforementioned research will inform the development of best management (BMPs) for mycoremediation.

This research aims to determine the most effective method to degrade PHC's in soil through a bench-scale experiment using fungi. The experiment investigates application methods of fungal enzymes using treatments comparing the efficacy of un-primed fungi, and primed fungi, to degrade diesel.

2.7 Research Question, Goal, & Objective

Will primed mushroom be more effective at degrading petroleum hydrocarbons than un-primed mushroom cultures in controlled conditions?

H_O: There will be no significant difference between the effectiveness of treatments for degrading petroleum hydrocarbons, comparing between treatments with un-primed mushroom cultures, and primed cultures under controlled conditions.

H_A: There will be a significant difference between the effectiveness of un-primed mushroom cultures, and primed cultures under controlled conditions. I predict that the primed mushroom treatment will be more effective than the un-primed mushroom treatment at degrading petroleum hydrocarbons because the enzymes secreted by the mycelium will be tailored specifically for the degradation of the contaminant.

Goal: Contribute to developing effective mycoremediation strategies for soils contaminated with petroleum hydrocarbons.

Objective: Determine if priming *Trametes versicolor* increases effectiveness at degrading petroleum hydrocarbons in contaminated soil.

Chapter 3. Methods

The aim of this mycoremediation experiment is to determine if priming the fungi will increase the effectiveness to degrade diesel in soil. *Trametes versicolor* has been cultured on straw (experiment 1) and liquid culture (LC) (experiment 2) in two separate experiments.

3.1 Experiment 1: Effectiveness of Biodegradation using Primed and Un-Prime Mycelium Cultured on Straw Substrate

Experimental Design

The experimental design is a complete randomized design and is the same for mycoremediation experiments 1 and 2. The treatments for the experiments are primed mushrooms and non-primed mushrooms applied to contaminated soils, with three levels of controls, primed mushrooms added to clean soil, non-primed mushrooms added to clean soil, and contaminated soil with no mushroom treatment (Figure 1).

Each treatment was contained in 250 ml mason jars, replicated five times, and randomly deployed. Mason jars have been chosen because they are relatively inexpensive, widely available, and glass is inert; therefore, they will not interact with hydrocarbons in the soil. The soil was precured from Davidsons Farm, the soil characteristics are 50% manure, 40% screened topsoil, 10% washed sand.

Three subsamples were taken from each 250-ml mason jar for experiments 1 and 2. Soil testing has been done at British Columbia Institute of Technology using a Gas Chromatograph Mass Spectrometer (GC-MS).

Primed (P) and un-primed (UP) mushroom treatments were applied at 20% by weight (100 g of topsoil to 20 g of inoculum). The inoculation rates for mycoremediation of petroleum hydrocarbons has ranged from 5%-65% (Maddela et al. 2017, Stamets 2006). Mushroom inoculums were prepared using grain, then hay, then transferred to straw (see descriptions in the following subsections). Diesel was applied at 1% by volume to UP and P treatments. This amount of diesel was chosen because the *T.*

versicolor was able to thrive in concentrations of 1% diesel during the priming process. Five replicates of each treatment were prepared. However, there was not enough viable primed inoculum for five replicates of Control Primed (CP), thus the CP treatment only had four replicates (14 replicates total). For experiment 1, the "ST" on the treatment label is an abbreviation for "straw" substrate. For example. e.g. CP-ST is an abbreviation from control primed straw substrate. The treatment additions were as followed:

- Diesel (D), 1.0 ml of diesel was added to the soil.
- Control Un-primed (CUP-ST), 20 g of un-primed *Trametes versicolor* bulk substrate was added to the soil
- Control Primed (CP-ST), 20 g of primed *Trametes versicolor* bulk substrate was added to the soil, see subsection priming hay culture found below.
- Un-primed (UP-ST), 1.0 ml of diesel and 20 g of un-primed *Trametes versicolor* straw bulk substrate was added to the soil.
- Primed (P-ST), 1.0 ml of diesel and 20 g of primed *Trametes versicolor* bulk substrate was added to the soil.

Each jar was hand mixed after the addition of fungi and/or diesel with a spatula for 1 minute. Experiment 1 was set out on September 27th, 2019 and remained at ambient temperature (approx. 23^{o}C) in a dark cupboard until January 23rd (188 days).

Inoculum Preparation Grain Spawn & Priming Process Solid Media

Grain spawn was created using liquid culture (LC) mycelium from Mycoboutique in Montreal, Quebec. The grain spawn was stored in the fridge (4 ^{o}C) until use (9 months later). At this point second generation spawn was created for this experiment (figure 12). The grain spawn was then transferred to prepared organic hay substrate and pasteurized at 75 ^{o}C for 1 hour (figure 13). Then drained and cooled following the methods of McCoy (2013), see Appendix D for detailed methods.

Ten 250-ml mason jars were filled with 80% by volume prepared organic hay, and 20% by volume grain spawn (appendix D). The grain spawn was mixed into the hay by hand for 1 minute per jar. To prime the fungi 0.5% diesel by volume (1 ml) was added to five of 250-ml mason jars. The diesel and grain where mixed simultaneously into the

hay substrate for 1 minute each. The other five diesel free jars were used as control cultures. The jars were kept at room temperature in a dark cupboard undisturbed.

After 14 days the mycelium had completely colonized the hay. Thus, the second transfer was carried out. The same methods were used as the first transfer except the spawn to hay ratio was increased to 50% by volume, the replicates were increased to 10, and the diesel volume was increased to 1% (1.25-ml per jar).

After a subsequent 14 days the mycelium had completely colonized the hay. Thus, the third transfer was carried out with a 50% hay to spawn ratio by volume, 10 replicated per treatment and diesel concentration increased from 1-2.5% (2.5-ml per jar).

After 14 days the controls and the diesel-spiked hay cultures had very little growth. Thus, they were left undisturbed for another three weeks. After five weeks there was still little growth, and many smelled of mold. At this point the strongest cultures from the controls and the primed cultures were transferred off hay to pasteurized organic straw pellets and Aspen shavings with a 25%/75% inoculum to substrate ratio. Eight control replicates were created for each substrate, Aspen clean, straw clean, aspen with diesel, straw with diesel (four on 1% and four on 2.5%).

At the end of the priming process the cultures were inspected to qualitatively assess growth (smell and sight). The straw cultures seemed to be slightly stronger in comparison to the aspen cultures. Hence the straw cultures were used for the final experiment.

3.2 Experiment 2: Effectiveness of Biodegradation using Primed and Un-Prime Mycelium Cultured in Liquid Media

Experimental Design

The experimental design for experiment 1 and 2 were almost very similar with four differences. First, the primed and un-primed *Trametes versicolor* treatments were applied at 20% by volume from liquid culture (LC) to diesel contaminated soil by weight. The LC was mixed into the soil for 1 minute per Mason jar. Second, five replicates of each treatment were prepared, except there was only enough viable control primed LC

for three replicates (13 replicated total). Third, 2% diesel was applied the UP and P treatments. The treatments are as followed:

- Diesel (D), 2.0 ml of diesel was added to the soil.
- Control Un-primed (CUP), 20 ml of *Trametes versicolor* LC was added to the soil
- Control primed (CP), 20 ml of *Trametes versicolor* LC was added to the soil.
- Un-primed (UP), 2.0 ml of diesel and 20 ml of *Trametes versicolor* LC was added to the soil.
- Primed (P), 2.0 ml of diesel and 20 ml of trained *Trametes versicolor* LC was added to the soil.

Fourth, three subsamples from each 250 ml mason jar were taken at time 0 and time 1. Where time 0 was within 36 hours of the addition of diesel and fungi cultures (following the same procedure as described for experiment 1). the jars were sampled again 36 days later using the same methods (time1).

Innoculum Preparation and Priming Liquid Culture

A liquid culture syringe was sourced from Mushroom Canada. The LC was expanded with a sucrose glucose medium which was filtered and sterilized. 2-ml of the LC syringe was added per 450-ml of LC medium (appendix E) (Cotter 2013). The LC was swirled for 1 minute per jar then left in at room temperature undisturbed for 4 days. After the four days of rest each jar was swirled daily for 1 minute to reoxygenate the media (McCoy 2013).

The primed cultures were prepared with the same methods as described above, except for the addition of diesel (Maltz, personal communication, May 2019). The LC with the diesel was swirled for 1 minutes to homogenize and oxygenate the liquid (five replicates). LC was primed through three transfers 0.5% 1%, and 2%. Each priming transfer the non primed cultured were also transferred into new LC media. This was to ensure that the mycelium for primed and un-primed mushrooms were the same generation when applied to the final experiment.

Three fungal species *Pleurotus djamore*, *Pleurotus ostreatus*, and *Trametes versicolor* were primed. However, the *Pleurotus* spp. were contaminated during the priming process and could not be used in the final experiment.

3.3 Experiment 1. & 2. Baseline Characterization of Soil

Soil used in experiment 1 & 2 was analyzed for basic nutrients and soil quality by the Plant Science Lab. Nutrient NO_3-N, SO_4-S were extracted with ammonium acetate solution, potassium, and phosphate were extracted using Mehlich" Soil quality parameters that were measured were, percent organic matter content (OM), Acidity (pH), and electrical conductivity (EC). The chemical characteristics of the soil used include: The pH in the soil in 7.8. Moisture 33%. Low organic matter 4.3%, N 76 ppm, P1 489, K 1225. Toxic EC 3.375.

3.4 Experiment 1. & 2. Soil Analyses for TPH

To determine the level of degradation of petroleum hydrocarbon, soil samples from each treatment in experiment 1 and 2 were analysed. The amount of biodegradation of petroleum hydrocarbon, specifically fraction 2 (C10-C16) and 3 (C16-C34), were done using the GC-MS (CCME 2008). Three 10-g subsamples from each 250 ml mason jar were taken as described in the hexane extraction method for experiment 1 and 2. Experiment 2 soil samples were taken at time 0 (within 48 hours of the addition diesel and fungi culture on December 16, 2019). Then again 36 days later at time 1 (January 21st). Soil testing for diesel abundance was been done at British Columbia Institute of Technology, Chemistry Department, using a Gas Chromatograph-Mass Spectrometer (GC-MS) on December 18, January 21, and February 4th. The GC-MS conditions were based on established methods described in CCME (2008).

3.5 Experiment 1. & 2. GC-MS Calibration Procedure

The samples were prepared in hexanes using the hexane shaking method (Karppenin et al. 2017). The GC-MS was calibrated according to the CCME Reference Method for the Canada-wide Standard for Petroleum Hydrocarbon in Soil-Tier 1 Method (2008). Sample Analysis was done for the F2 (C16) and F3 (C34) Hydrocarbon Fraction range as representative fractions for TPH. Four-point calibration curve was used to integrate the area under the chromatograph between retention times.

Calculations:

Calculations of average response factor (RF_{avg}) is used to calculate the hydrocarbon in each of the range

F2

$$\text{C10-C16 hydrocarbons (mg/kg)} = \frac{A_{c10\text{-}c16} * Vol * F}{Rf_{avg} * Wd}$$

F3

$$\text{C16-C34hydrocarbons (mg/kg)} = \frac{A_{c16\text{-}c34} * Vol * F}{Rf_{avg} * Wd}$$

Where:

AreaC10-C16 = The integration of all area counts from the apex of the C10 peak to the apex of the nC16 peak.

AreaC16-C34 = The integration of all area counts from the apex of the C16 peak to the apex of the nC34 peak.

F = Dilution factor applied to bring the samples and standards into appropriate peak height range.

RF_{avg} = response factor calculated above

W_d = Dry weight of samples taken (g)

The results could not be statistically analyzed. Concentrations were converted using the CCME (2008) calibration curve but were not consistent and did not make logical sense. When comparing the chromatograms empirically for an indication of the problem, the chromatograms seemed to correspond with the strange concentration results. Therefore, statistical analysis was not done. See appendix G for sample calculations. The planned statistical analysis was a one-way analysis of variance (ANOVA).

Chapter 4. Results

4.1 Gas Chromatograph Analysis

However, when reviewing the chromatograms to compare them to the converted concentrations to see if there was a mathematical error the chromatograms seemed to correspond with the strange concentration results. Therefore, statistical analysis was not done. See appendix G for sample conversion calculations

The chromatograms discussed in the results represent one sub-sample (1 of 14 experiment 1 & 1-13 experiment 2) per treatment. All the chromatograms were visually compared, and one was chosen that was representative of the treatment trend. All chromatograms for experiment 1 can be found in appendix A and all chromatograms for experiment 2 can be found in appendix B.

4.2 Experiment 1: Effectiveness of Biodegradation using Primed and Un-Prime Mycelium Cultured on Straw Substrate

The chromatogram results showed that the control primed (CP-ST) and the non-primed (CUP-ST) fungal treatments had no diesel (Figure 2 & 3). The replicates and subsamples were all very similar to each other, a little bit of variation is normal (Soulsbury, personal communication, February 2020).

The chromatogram results for the diesel treatment (D-ST) showed a graph profile that is indicative of detected diesel (Figure 4). It showed the F2 range (minute 6-13) has very little diesel. However, F3 hydrocarbon fractions were present (the peaks at approximately minute 13 to 19). The highest peak had a response signal of 15 000.

Overall un-primed straw (UP-ST) treatments had lower response signals than the primed straw (P-ST) treatments after four months, 10 000 and 22 500, respectively (Figure 6 & 5). Primed straw (P-ST) replicates and subsamples were consistant and were very similar to each other. UP-ST and subsampled were also consistent with each other.

4.3 Experiments 2: Effectiveness of Biodegradation using Primed and Un-Prime Mycelium Cultured in Liquid Media

The chromatogram profile for the control primed (CP) treatment indicated that diesel was present, even though no diesel was added to the soil. The highest peak for the CP treatments at time 0 had a response signal of approximately 110 000. After 36 days the highest peak had a response signal of approximately 130 000 (Figure 7).

The overall chromatogram profile for the control un-primed (CUP) treatment was not representative of the presence of diesel. Although some of the peaks from minute 4-8 appear to be quite high (Figure 8).

The response signal for the diesel treatment (D) upon preparation (time 0) is higher than after 36 days (time 1), and was reduced from 1 400 000 to 160 000 at the highest peaks. The response signal at peak 18 had the highest reduction from 1 400 000 to 80 000 over 36 days, which is a reduction of 89%. Peak 18 is an unknown hydrocarbon compound, probably a biodiesel (Figure 9) (Soulsbury, personal communication April, 2020)

The chromatograms indicated that un-primed (UP) treatments showed a consistent drop in response signal. The highest response signal at time 0 was peak 18 with 1 000 000, and time 1 was at approximately 300 000. Other than the response signal decrease at minute 18, the overall profile decreased from 600 000 to 140 000, which is a relative reduction of 76.7 % (Figure 10).

Primed (P) treatments of *T. versicolor* at time 0 had a response signal of 1 000 000. Remaining consistent with the trend the peak at minute 18 has been reduced drastically at time 1 to 550 000. The overall profile remained relatively unchanged with response signal of approximately 550 000 (Figure 11).

Chapter 5. Discussion

5.1 Experiment 1. Effectiveness of Biodegradation using Primed and Un-Prime Mycelium Cultured on Straw Substrate

The diesel treatment samples (D-ST) are relatively consistent with each other. This indicates that the method used to extract the diesel from the contaminated soil was also consistent. Unexpectedly, the D-ST samples have very little diesel compounds remaining in the F2 fraction range. This could mean that the F2 range was volatilized during the 188-day experiment period (CCME 2008). The control primed (CP-ST) and un-primed (UP-ST) treatments as expected had no diesel.

Unexpectedly, the P-ST samples had consistently more diesel than the UP-ST samples and the D-ST. The results from the chromatograms response signal indicates that priming the fungi was unsuccessful and that it might have damaged the viability of the fungi. Experiment limitations and possible experimental errors from weaken fungi Cultures, and/ or possible diesel extraction error.

The fungi cultures could have been weakened from the use of hay as a priming and bulking substrate could have negatively affected the growth of the fungi. Wheat seed heads maintain enzymes that are potentially toxic for white-rot fungi (Malts, Personal Communication, September 2019). The first and the second transfer the fungi colonized the hay as expected, within two weeks. The cultures appeared to be strong because the mycelial growth was thick and had completely colonized the substrate (figure 16). However, the *t. versicolor* did not respond well to the third transfer. After five weeks with very little growth the cultures were transferred onto ground straw and aspen shavings. These two substrates were used because *T. versicolor* has been known to have rigorous growth on both substrates, especially hardwoods (Cotter 2014, page 350). Unfortunately, the cultures did not recover well on either substrate. Most of the replicates smelled of mold and mycelial growth was limited.

In this case, I hypothesized that the cultures were not strong enough to outcompete other soil microbes even though the substrates had been pasteurized. The pasteurization process basically gives the intended culture a heads up but is not the

same as a sterile media (Cotter 2014). It has been recommended by Cotter (2014) to use a nutrient supplement of 5% dry weight wheat or rice bran and sterilized sawdust.

The following limitation applies to experiment 1 and 2. Replicates and subsamples for each treatment were similar without too much variation, meaning the diesel extraction method was at least consistent. The hexane shaking method was adapted from Karppinen et al. (2017). However, in this method the soil samples were shaken at 200 rpm overnight on a Barnstead-Labline rotator (Thermo Scientific, Lenexa, KS). However, budget would not allow for the purchase of a Barnstead-Labline rotator. Thus, I used Mandel Scientific GFL-3011 shaker because it was available. The motion was a slow circular swirling, whereas the Barnstead-Labline rotator motion is vigorous and back and forth. Thus, it is possible that the shaking motion was not vigorous enough and therefore extraction was not complete.

5.2 Experiment 2. Effectiveness of Biodegradation using Primed and Un-Prime Mycelium Cultured in Liquid Media

The CUP results were as expected, no diesel was detected. However, abundance of diesel for CP appears to be higher at Time 1 rather than at Time 0. This result could be due to a range of errors in the methods. Instrumental error on the GC-MS, procedural error during extraction, or human error throughout weighing the soils for extraction. In addition, there should not be diesel in the CP treatments. This could be a result of an experimental design flaw. Extra diesel could have been introduced because of the method used during the priming process.

Most studies have found that fungi do not completely mineralize PHC's. Even the same strain will degrade PHC's at varying rates which are dependent on many environmental variables, such as soil characteristics, moisture, temperature, microbial community, and pollutant concentration (Al-Hawash et al. 2018). For example, one study exposed pure cultures of *Pleurotus ostreatus* to 20-ml, 40-ml, and 60-ml of crude oil. Then the concentration was measured after four weeks. Diesel was reduced 95%, 87%, and 85%, respectively (Zitte et al. 2012). Thus, there could have been a relatively high concentration of diesel in the P treatment before it was added to the diesel spiked soil.

In hindsight, a better approach may have been to mimic selective enrichment strategies. Selective enrichment is the process of taking a sample of the microbial community directly from a PHC contaminated site (Kulkarni 2014). Then the PHC degrading microbes are isolated and purified (Al-Hawash et al. 2018; Wan et al. 2016). Future mycoremediation studies that focus on priming saprophytic fungi could use spores instead of already germinated strains. The spores could be introduced directly to diesel contaminated substrate (Stamets 2005). The idea here is to use a similar process to selective enrichment. When using spores instead of already established strains the cultures most suitable to the contaminated environment will establish. In essence, the strain will be “Primed” for the contaminant, then clones and cousins of these individuals can be propagated and used to answer my original question, “will primed fungi be more efficient at degrading diesel than un-primed strains?” This method would eliminate the possibility of accidently introducing diesel into the final experiment.

Diesel Treatment

Overall, the diesel only decreased marginally. Natural attenuation is a well documented effect (Speight and El-Gendy 2017). Microbes in the soil could be slowly breaking down the diesel constituents. Natural attenuation is sometimes considered as a remediation strategy for oil spills (Azubuike et al. 2016). However, often the process is not viable as autochthonous communities without bioaugmentation are ineffective at degrading PHC’s in an appropriate time frame, i.e. < 5 year (Adenipekun et al. 2012). It is possible that one or some of the soil microbes in the soil have an affinity for the peak at minute 18. This peak is in the F3 fraction range which has low volatility, and therefore has likely not volatilised (CCME 2008).

UP treatments demonstrated a consistent drop in diesel concentrations between time 0 and time 1 (36 days later). Especially the 18-minute peak which was markedly and consistently lower at time 1. However, the diesel treatments also demonstrated a similar pattern and had a higher reduction in diesel abundance. Primed treatments responded the worst. The overall profile did not change between Time 1 and Time 0 and the response signal remained at approximately 550 000. The peak at minute 18 did have a drastic reduction in the response signal, 55%. The un-primed and primed treatment results indicated an inhibitory effect to the degradation of PHC’s rather than a beneficial effect. One possible explanation is that the microbial community in the soil was not

cohesive and instead of acting symbiotically they were antagonistic. Lladó et al. (2013) similarly, found that soil colonization by white rot fungi *T. versicolor* and *Lentinus tigrinus* strains was clearly hampered by an active native soil microbiota in PAH degradation industrial polluted soil.

5.3 Overall Experiment 1. and 2.

Substrate nutrients and quality can have a large effect on fungal growth and enzyme activity of heterotrophic bacteria (Maddela et al. 2016). In general fungus used for bioremediation has been observed to grow best in substrates that have a pH between 6.5-7.5 (Nyer et al. 2001). However, each fungal species has optimum growing acidity ranges. As an example, two species *Lentinus crinitus* and *Psilocybe castanella* are two tropical basidiomycetes that had highest growth at a pH of 4.5 and highest laccase activity of at 3.5 for *L. crinitus* and 4.5 for *P. castranella* (Neto et al. 2009). One study demonstrated that laccase activity in *T. versicolor* is highest at 50°C between pH 4.5–5.5 (Litwińska, et al., 2019). However, Han et al. (2005) found that *T. versicolor* exhibits high enzyme activity over broad pH and temperature ranges, with optimum activity at pH 3.0 and a temperature of 50°C.

In this case, the soil analysis showed that pH was 7.8. It is also likely that the pH was increased with the addition of diesel (Maddela et al. 2017), this could have had an impact on the survival of the fungi. Contrastingly, *L. crinitus* and *P. castranella* were able to modify the pH of the growth substrate until it reached the optimal conditions (Luiz et al. 2009). This study only had the resources to test the soil one time for nutrients and quality. Future research should address the effects *of T. versicolor* on neutral-alkaline substrates to see if they also can manipulate the pH of the growth media.

5. 4 Conclusions and Future Consideration

The results for experiment 1 and 2 were inconclusive. This does not mean that priming fungi is ineffective. The priming method was ineffective but also inefficient because it took many months to prime the fungi. However, priming using different methods could still prove to be effective. For example, inoculating contaminated substrate with fungal spores to ensure germination of strains most suitable for the

contaminant in question. In addition, concentrated enzyme application is a new line of research that could reduce the volume of organic matter used in mycoremediation of soils.

An unintended result of my thesis included the colonisation of the polyfill air filter, see appendix G for more information. Polyfill is an artificial material that is a polymer (LiberTexts 2019, accessed May 2020). A polymer can be made into plastics and other material. This unintended result demonstrates the powerful possibilities of using *T. versicolor* to degrade complex hydrocarbon compounds and synthetic materials.

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Figures

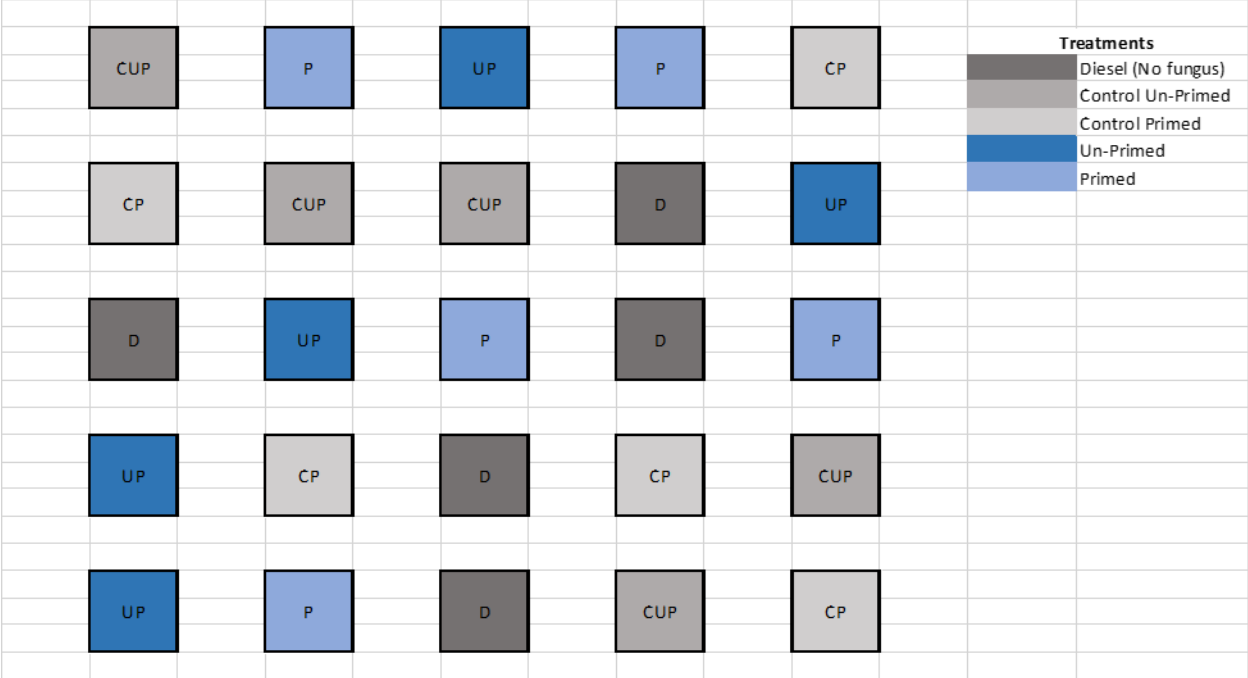

Figure 1. Schematic of a complete randomized design which was used for experiment 1 and 2. Treatments are as followed: D= Diesel (no fungus), CUP= Control un-primed (no diesel), CP=Control primed (no diesel), UP=Un-primed (with diesel), P=Primed (with diesel). Trametes versicolor was used for experiment 1.and 2. Straw bulk substrate was used for experiment 1 and liquid culture was used for experiment 2.

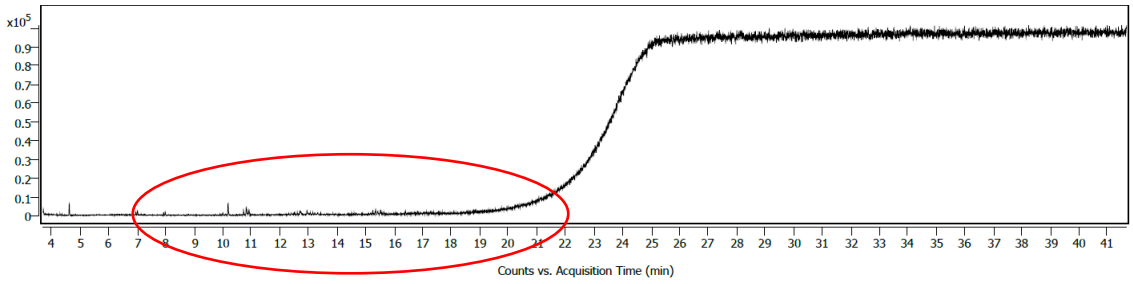

Figure 2. GC-MS chromatogram of control primed T. versicolor treatment after being applied for 4 months. Retention time is on the x-axis of the graph and the response signal is on the y-axis.

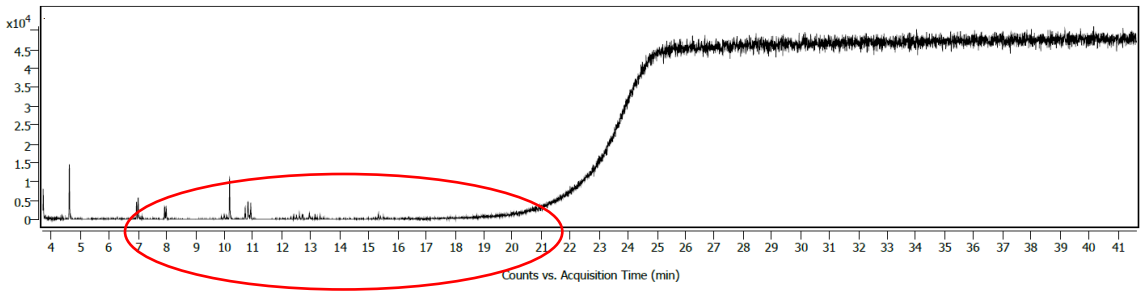

Figure 3. GC-MS chromatogram of Control Un-Primed T. versicolor treatment after being applied for 4 months. Retention time is on the x-axis of the graph and the response signal is on the y-axis.

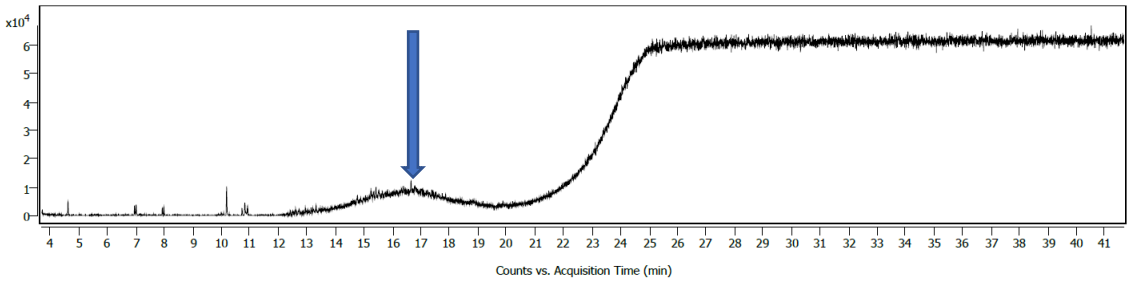

Figure 4. GC-MS chromatogram of the diesel 1% (no fungus) treatment after being applied for 4 months. Retention time is on the x-axis of the graph and the response signal is on the y-axis. The response signal at the highest peak is approximately 15 000.

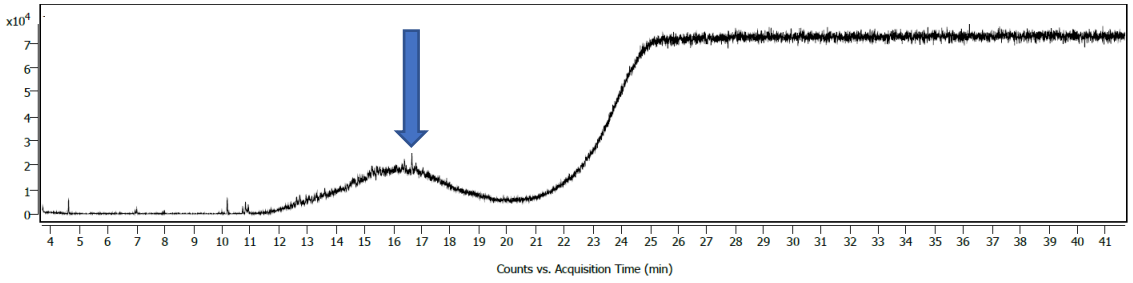

Figure 5. GC-MS chromatogram of the primed T. versicolor treatment after being applied for 4 months. Retention time is on the x-axis of the graph and the response signal is on the y-axis. The response signal at highest peak is approximately 22 500

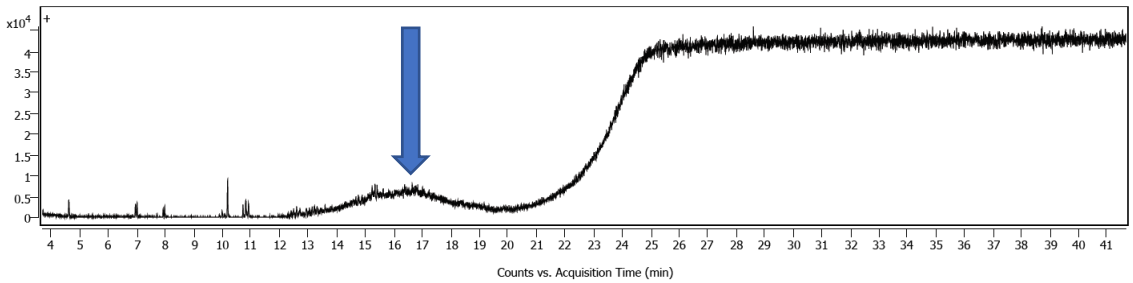

Figure 6. Figure 6. GC-MS chromatogram of the un-primed T. versicolor treatment after being applied for 4 months. Retention time is on the x-axis of the graph and the response signal is on the y-axis. The response signal at highest peak is approximately 10 000.

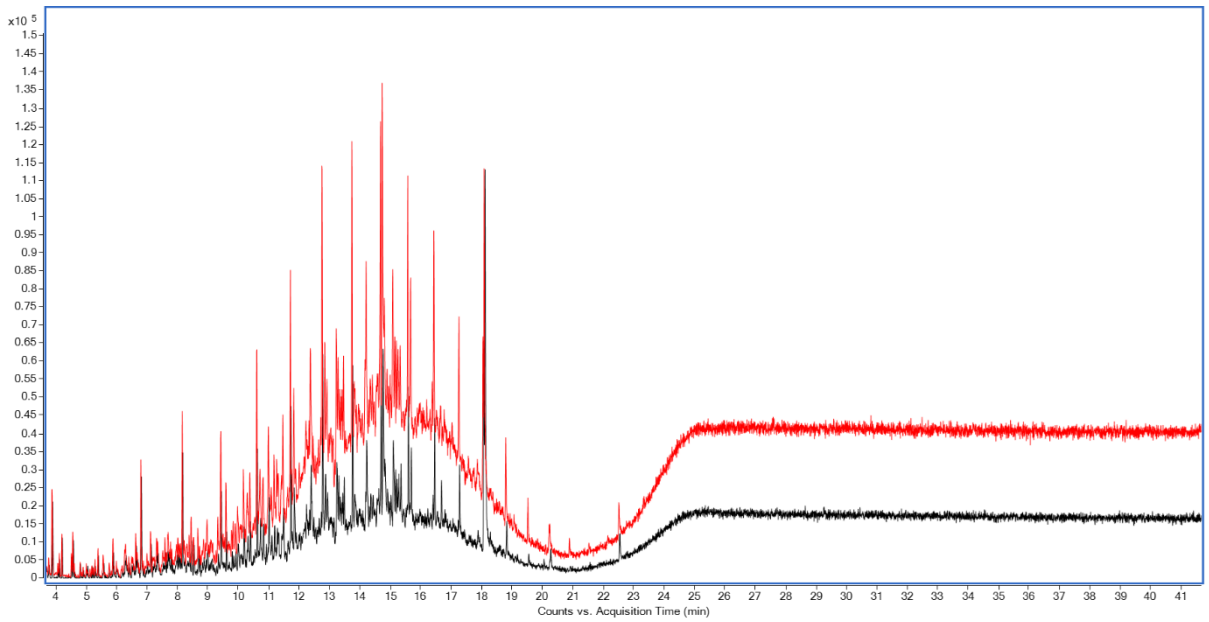

Figure 7. GC-MS chromatograms comparison of time 0 (black line) and time 1 (red line) for the control primed (no diesel) liquid culture T. versicolor treatment Retention time is on the x-axis of the graph and the response signal is on the y-axis. The response signal at the highest peak for time 0 is 135 000. The response signal at the highest peak for time 1 is 115 000

F

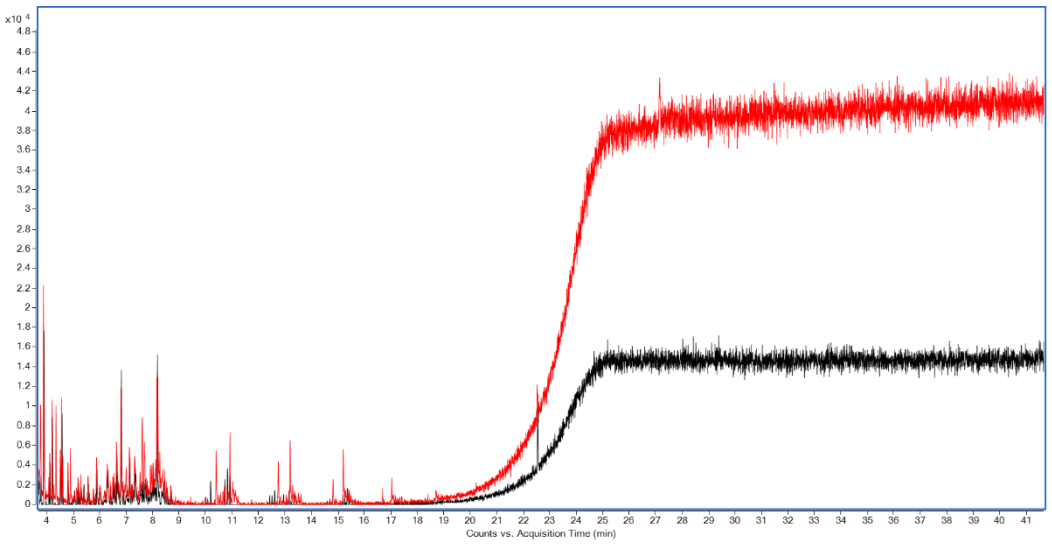

Figure 8. GC-MS chromatograms comparison of time 0 (black line) and time 1 (red line) for the control un- primed (no diesel) liquid culture T. versicolor treatment. Retention time is on the x-axis of the graph and the response signal is on the y-axis. No diesel detected.

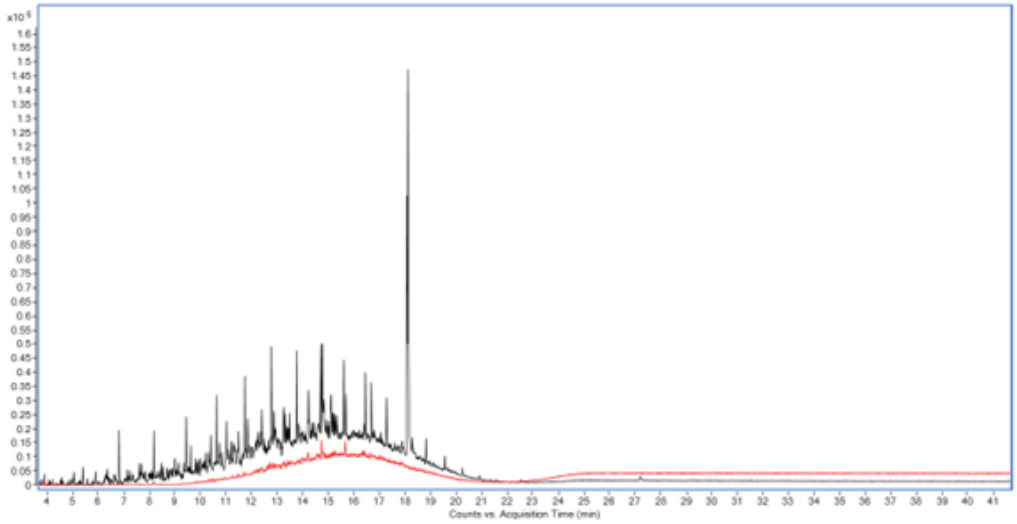

Figure 9. GC-MS chromatograms comparison of time 0 (black line) and time 1 (red line) for the control diesel 2% treatment. Retention time is on the x-axis of the graph and the response signal is on the y-axis. The reponse signal at the highest peak for time 0 is 1 500 000. The response signal at the highest peak for time 1 is 150 000

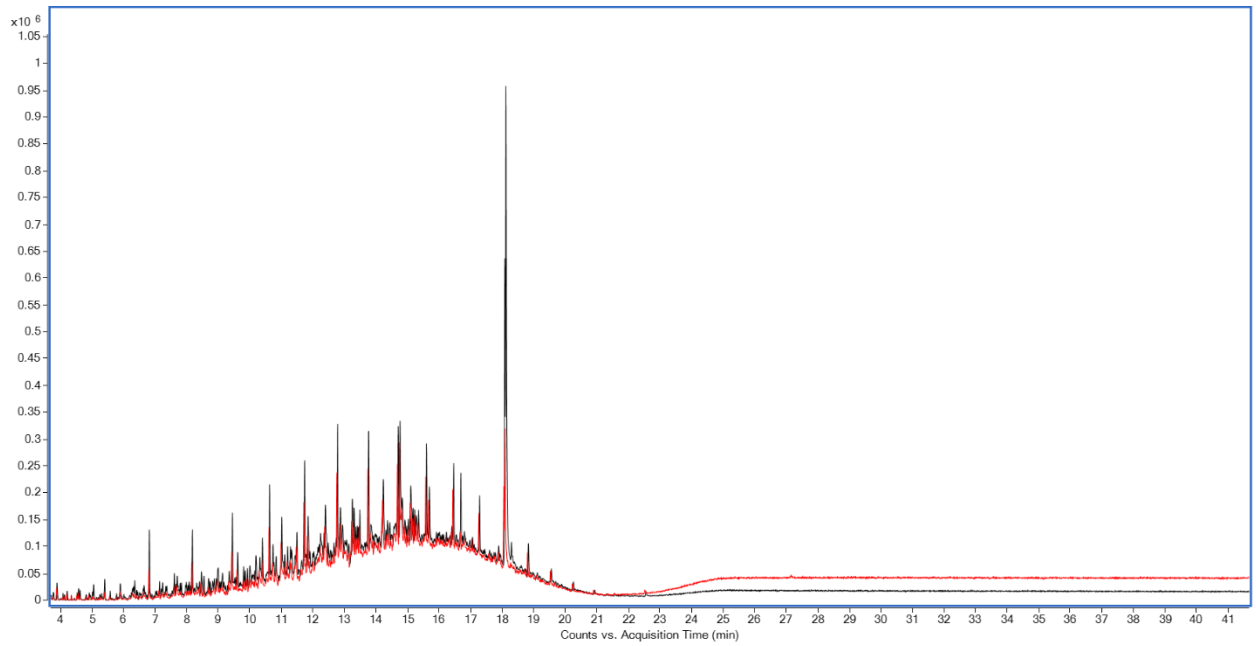

Figure 10. GC-MS chromatograms comparison of time 0 (black line) and time 1 (red line) for the un- primed liquid culture T. versicolor treatment with the addition of 2% diesel. *Retention time is* on the x-axis of the graph and the response signal is on the y-axis. The response signal at time 0 for the highest peak is 950 000. The response signal at time 1 for the highest peak is 350 000

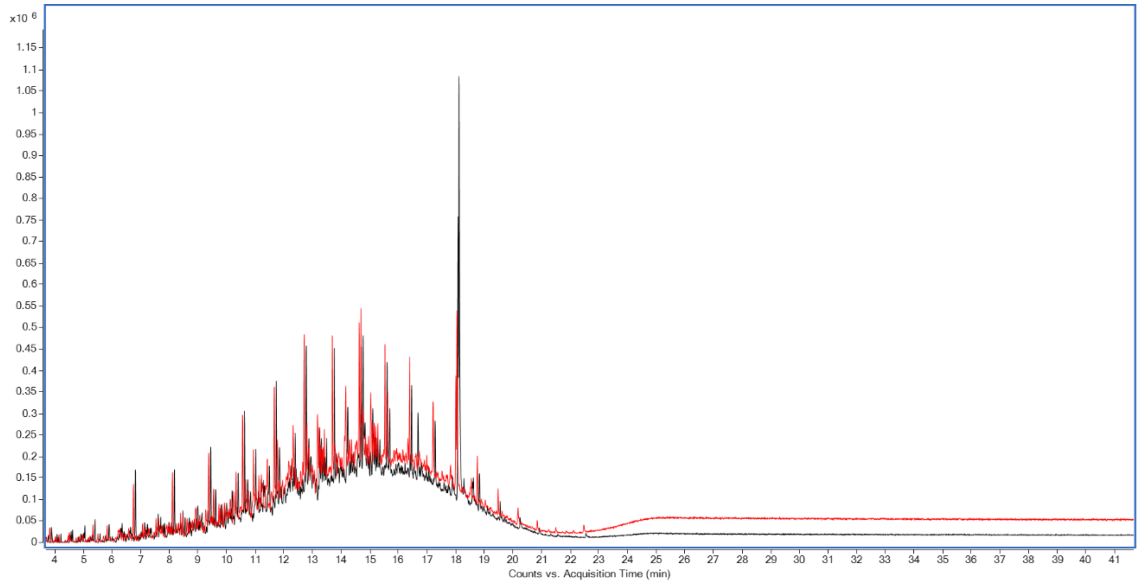

Figure 11. GC-MS chromatograms comparison of time 0 (black line) and time 1 (red line) for the primed liquid culture T. versicolor treatment with the addition of 2% diesel. Retention time is on the x-axis of the graph and the response signal is on the y-axis. The response signal at time o for the highest peak is 1 100 000. The relative response signal at time1 for the highest peak is 550 000.

Project Photos

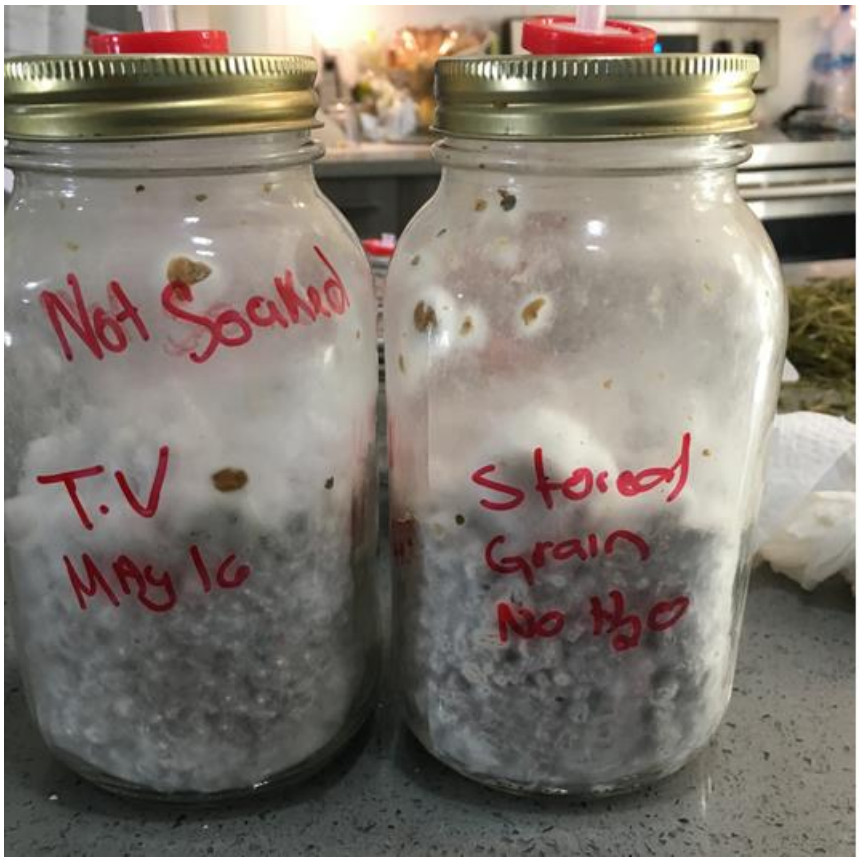

*Figure 12. T. versicolor g*rain spawn was stored in the fridge at 4°C for nine months without water.

Figure 13. Pasteurized and chopped hay in preparation for inoculation with grain inoculum, May 27, 2019

Figure 14. Mason jars with lids with fitted with 0.22-micron air filters.

Figure 15. Colonized hay (T. versicolor) during the priming process at 1 percent diesel, June 10th, 2019.

Figure 16. Overview of prepared aspen shavings, grain colonized with Trametes versicolor, and liquid culture medium, September 16, 2019.

Figure 17. Liquid culture medium prepared with three methods. The jar on the left is made with glucose and dextrose. The middle jar is made with light malt extract and dextrose (not filtered). The right jar is made with light malt extract, dextrose and it was filtered. In addition, a pebble was put in the bottom to easily break up mycelium throughout the colonization process, September 2019

Figure 18. Ground pelletized wheat straw, for inoculation with T. versicolor hay substrate, September 3, 2019.

Figure 19. Mycelium growing mainly on the top surface of liquid culture during the priming process with 0.5% diesel, after 14 days.

Figure 20. Comparison of mycelial growth in liquid media, no diesel on the left and diesel of on the right.

Chapter 6 Napo Concession Area Bioremediation Option Plan

6.1 Introduction to Chevron Oil Contamination, Ecuador

Ecuador is one of the most diverse countries on the planet, hosting thousands of plant and animals species (Morrone 2000). The Orient region of the country to the east of the Andes mountains is the most species rich area in Ecuador and is predominated by tropical rainforest. This area is home to 500 000 people, many of whom rely of the land for subsistence.

Oil extraction has been prevalent in the Orient since 1967 when Texaco, now known as Chevron, started extracting crude oil. Texaco leased an area they called “Napo Concession” which contains 16 oil fields (Bagnoud 1994). The petroleum extraction process produces drilling wastes which are a by-product (Kimerling 1991). The drilling waste contained a mixture of highly toxic materials such as drilling muds (used as lubricants and sealants), petroleum, natural gas, and formation water from deep below the earth's surface, containing hydrocarbons, heavy metals and high concentrations of salts. The oil operation produced 1.7 billion barrels of oil, 489 million barrels of formation water, and more than 355 trillion feet of cubic gas (Kimerling 1991).

Until 1992, Texaco continued to dispose of the drilling waste by digging and filling unlined surface pits (Kimerling 1991). Texaco was negligent and did not use the (then) current technologies for environmental and social welfare. At the time (1967-1992), it was known that water-based mud instead of oil-based drilling muds should be used. Technology also allowed for the re-injection of produced waters deep into the ground instead of dumping in unlined pits. General housekeeping was also mainly ignored such as proper maintenance and monitoring of shipping pipelines and production facilities, and negligent spill prevention and response measures (Bagnoud 1994).

6.2 Remediation

Remediation is a branch of ecological restoration that is specific to cleaning-up contaminated soil, water, or air to mitigate unacceptable risks to human health and the

environment (Nathanail et al. 2017). The common practices with traditional remediation are dig and dump, bury, pump, and burn (Smith 2019). However, remediation has its own environmental footprint which can have unintended impacts on local, regional, and global scales (Martino et al. 2016). Negative ecological impacts that remediation can have are air pollution (GHG emissions, and air-born particulate matter); soil (erosion, nutrient depletion, soil bank depletion when landfilled, and geochemical changes); and water (changes in hydro cycles, and extensive water use) (Gibson and Illaszewicz 2009). For example, contaminated soil excavation uses fuel for heavy machinery and transportation of the soil off-site. Thus, greenhouse gases are released, which negatively effects the global climate.

Remediation of PHC can be done a multitude of ways (Villacís et al. 2016). Treatments may destroy or alter the contaminant, extract or separate the contaminate from the substrate, or immobilize it. Each site should be thoroughly surveyed and site-specific remediation strategies selected. Multiple strategies may be used on one site depending on the complexity. Soil properties such as permeability, contaminant properties, cost to remediation, legal framework, and stakeholder opinions are some of the factors that determine the final remediation plan (Villacís et al. 2016).

Many researchers have found bioremediation strategies to be the most viable in this situation for the following reasons: cost, scale of the devastation, and lack of technologically advanced infrastructure (Malts, personal communication, Maddella et al. 2015, Evans, Personal Communication, and Merchan-Rivera, 2017). In addition, key stakeholders (Amisacho Research Station) agrees that bioremediation strategies should be implemented because they are considered sustainable when compared to conventional strategies. Bioremediation is often considered *sustainable* because the methods are centered on recycling waste substrates, transforming and biodegrading contaminants, and reusing materials when possible. However, there is more to consider in terms of sustainability.Several collaborative initiatives in the UK and US have developed frameworks for “sustainable remediation” (SR). The idea is to shift the focus of remedial practices to a holistic viewpoint.

Remediation approaches that have undergone a sustainability assessment in conjunction with framework guidelines are considered SR (Smith and Kerrison 2013). The foundation of SR “the three pillars of sustainability” is a holistic model applied to

society which encompasses environmental sustainability, economic sustainability and social sustainability (Rizzo et al. 2016). SR frameworks have set broad indicator categories for each pillar, which is supposed to be used to evaluate how a remedial project plan meets each category on a scale (table 1). For example, on a scale ranging from excellent to good to poor a project plan might rate *excellent* in the “impact on air quality” category, might take *good* human health and safety measures, but have *poor* community involvement.

This document aims to evaluate in detail the bioremediation potential for the Napo Concession. This research will contribute to the development of a detailed sustainable bioremediation option plan for the terrestrial landscapes and wetlands of the Napo Concession Area. Although this report focuses on the suitability of bioremediation as a remedial strategy it is important to frame remediation plans using the sustainable remediation model to uphold best practices moving forward.

6.3 Goals and Objectives

Goal: Identify suitable bioremediation options in collaboration with Amisacho Research Station to remediate soils in habitats that have been contaminated by petroleum hydrocarbons in the Napo Concession area in Ecuador.

Objective 1: Identify the possible habitat types and associated habitat conditions that could be found in the Napo Concession Area by reviewing peer reviewed journals, academic texts, and topographic maps.

Objective 2: Compile types of chemical contaminants, concentrations, and disturbance types caused by legacy oils and gas exploitation throughout the Napo Concession by reviewing site reports, scientific journals, and through personal communications.

Objective 3: Develop a bioremediation feasibility decision tree that will outline best bioremediation options for each disturbance type in the Napo Concession

6.4. Study Site

Ecuador has four major regions, Coastal Plane, Highlands, Amazonia, and The Galapagos Islands (Moreno & Bernal 2018). The concession area lies in Amazonia. Its northern boundary is in the province of Sucumbíos in northeastern Ecuador. The concession area is approximately rectangular shaped and travels southbound for approximately 113 km and passes through four provinces in Amazonia Ecuador: Sucumbíos. Napo, Orellana, and Pastaza. The concession contains multiple oil fields, they are named as followed: Lago Agrio, Parahuacu, Dureno, Tacapi, Guanta, Shushufindi, Sacha, Yulebra, Coca, Yuca, Yuca sur, Culebra, Auca, Aura sur, Rumyacu, and Conocaco, (figure 21).

The Napo Concession contains 350 well sites that each have 2-5 associated drilling waste pits. There are 913 drilling waste pits that are highly toxic (See section 9.2.1 for contaminant constituents) (Gropper, personal communication, 2018). The contamination has affected the terrestrial landscape, wetlands, surface streams and ground water. Most of the concession falls within the Napo Moist Forest Ecoregion. Vegetative communities are forests and grasslands (see section 9.1 for details).

6.5. Historical Environmental Site Conditions

The Napo Concession contains two relief forms, the Peri-Andean Amazonia reliefs and the Peri-Andean Hills (García-Sánchez et al. 2018). The Peri-Andean reliefs occur at 300 - 900 meters above sea level. They have a mean temperature of 22 ºC, and an average rainfall of 4500 mm per year. The Peri-Andean Hills region is part of the Amazon Basin 600-250 meters above sea level. and is characterized by undulating hills and plains. Peri-Andean mean annual temperature is 23 °C, and mean annual precipitation range is from 2500 up to 4500 mm (García-Sánchez et al. 2018).

The soils of the concession area that fall within the Peri-Andean reliefs follow the horizon sequence Ap/Bw1/Bw2 (Sanchez et al. 2018). These are moderately drained soils with high clay content creating conditions for frequent temporary flooding in flat areas. The soils have a low base saturation (<35%) and low CEC (5–10 cmols/kg^{-1}), medium to low organic matter content, moderately acidic to extremely highly acidic (pH <

4.5). These are unfertile soils that promote the presence of Al toxicity and high leaching of cations (Si, Na+, K+, Ca2+, and Mg2+) (Sanchez et al. 2018).

The Peri- Andean Hills are dissected sedimentary banks. The substrates are completely weather rocks and soft boulders which leave behind clay (Sanchez et al. 2018). The plains developed over sand boulders. The soils of the Peri-Andean hills generally have the horizon sequence A/B/C/R. They are highly acidic (< 4.5) red soils that have undergone high Si leaching. Two main types of soils are present, they are classified as Typic or Oxic Dystrudepts. These soils are desaturated (1–20% base saturation) with high clay and Al content (Sanchez et al. 2018).

The lower part of the Peri-Andean region is the alluvial environment which is peppered with swamps along side the streams (Sanchez et al. 2018). The soils are classified in the great group Endoaquepts. The area is prone to extensive and persistent flooding and the soil is saturated most of the year. The horizon sequence Ap/Bg/Cg is a representative profile for this landscape. The water table appears often at a few centimeters below the surface. Soil pH is moderately acid (5.5), CEC is <10 $cmol/kg^{-1}$ and base saturation is <35% (Sanchez et al. 2018).

The streams and smaller tributaries within the concession flow east from La Sierra and ultimately drain into the Amazon River which is the main water tributary of the Amazon rainforest. The Napo River and the Aguarico River are hydrological features within the concession area.

In the Oriente there are a variety of aquifers and based on the soils, lithology and hydrogeology units, the physical properties include a medium permeability with discontinuous shallow aquifers (US Army Corps of Engineers 1998). Sand has been found as a common deposit and subsurface material in the Concession Area.

The Napo Concession area lies within the bioregion Amazonia and the ecoregion Napo Moist Forest. This region is floristically diverse and complex. The main canopy layer averages 25-30 meters and forms a dark closed canopy forests. The sub-canopy is often predominated by palms and light tolerant samplings waiting for gap disturbance to emerge. The understory layer contains small trees and shrubs that have developed

morpohological adaptations to survive in low light conditions. Emerging trees reach up far above the canopy up to 50-60 meters high.

The Napo Moist Forest ecoregion hosts some of the highest biodiversity on the planet; 219 species of mammals, 649 species of birds and 96 reptiles have been found in the Yasuni Biosphere Reserve (Sears et al. 2018).

6.6 Methods

The habitat types, the contaminant constituents and the disturbance types have been described by reviewing legal reports, topographic maps, peer reviewed journals and personal communication with Lexie Gropper (founder of Amisacho Research Station), and fungal expert Dr. Mia Maltz.

The bioremediation tree has been built based on a literature review of bioremediation techniques which allowed me to identify the most important factors that affect the efficacy of bioremediation techniques (Appendix J). In addittion to the few sources that have studied conditions within the Napo Concesssion. They are as followed: EPA 1994, Maddela et al. (2015 [a] [b]), and Merchan-Rivera 2017.

6.7. Results

6.7.1 Habitat Types and Landscape Conditions

Within the Napo Concession boundary there are five major classes of forest. Within the concession boundary exist five forest complexes, they are as followed: Evergreen forest in the western peninsula of the Amazon, flood forest of the flood plain of white-water rivers of western Amazon, flooded forest and lacustrine-riparian vegetation of black waters of the Amazon, Swamp palm forest of the floodplain of the western Amazon.

CES408.523 Evergreen forest in the western peninsula of the Amazon (Bosque Siempreverde de la Penillanura del Oeste de la Amazonia)

Landscape dynamics and Soils

The penillanura forests landscape is predominated by rounded hills, flat high terraces usually found at an altitude between 150-300m and small valleys between them (Ministerio del Ambiente del Ecuador 2012). The soils vary in composition with high fertility in areas near the Andes and poor, sandy acidic soil found in certain areas of the lower Güeppí river basin (Cerón and Reyes 2003).

Vegetation

The Penillanura forest is the most diverse habitat of the Amazon and vegetation communities vary greatly (Báez et al. 2010). They are non-flooded evergreen forests with a high closed canopy of 25-35 m, multi-stratified, and emergent trees that can reach over 40 m tall. The canopy trees have straight shafts with diameters between 0.8 and more than 1.2 m (Ministerio del Ambiente del Ecuador 2012). The undergrowth is usually more open than the canopy and it is composed of terraces. Areas with high sand content in the soil have a different vegetation community composition. They are characterized by the abundance of tree species with diameters at chest height less than 20 cm and the predominance of tree stems smaller than 10 cm (Ministerio del Ambiente del Ecuador 2012).

CES408.532 Flood forest of the flood plain of white-water rivers of the west of the Amazon (Bosque Inundable de la Llanura Aluvial de r¡os de Aguas Blancas del Oeste de la Amazonia)

This habitat types occures at < 350 masl. The land cover is classified as woody wetland. The general relief of the lowlands are mesorelieve terraces and plains. The habitat can be found in the following provinces: North-Western Amazon, Sectors: Cuyabeno-Güeppí, Napo-Curaray and Tigre-Pastaza

Landscape Dynamics and Soils

The duration and gradients of the flood determine the dynamics of these vegetation communities. The floodplain complex vegetation communities of white-water rivers are loaded with sediments (Ministerio del Ambiente del Ecuador 2012). This dynamic is highly variable and includes the frequent changes to channel meanders, banks, and islets. The communities that are farther from the rivers or located on higher

terraces flood daily or weekly and remain inundated up to three months per annum. They are generally on the outer edge of the meander curve (Báez et al. 2010).

Areas near the banks of white-water rivers with low banks or complexes of naturally occurring depressions and dikes are flooded for long periods of time (Báez et al. 2010). These complexes include the multi-stratified highland communities on riverbanks, islands that are formed in the flooding processes and that are moderately drained, as well as the vegetation of the permanently flooded or saturated depressions and the successional complexes formed by the intense fluvial dynamics. They are usually found on the internal banks of the meanders (Ministerio del Ambiente del Ecuador 2012).

Floodplain plant communities of sediment derived from the Andean rivers are medium to high forests, and are semi-open to dense (Báez et al. 2010). They have 3 to 4 strata that are predominated with trees, palms, and vines. Areas that are predominantly palms and vines are representative of the first stages of succession, they are distributed in the margins that are periodically destroyed during large floods and at the same time annually receive deposits of sandy-muddy sediments dragged by the river.

The soils are relatively rich and vary from loamy loam to clay sand, with an imperfect to good drainage level (Ministerio del Ambiente del Ecuador 2012). The gradient of pH, nutrient content, conductivity and suspended material vary between rivers originating in the Andes of northern Ecuador and those in the south and center and there is a fairly high range of variation from the upper, middle course of these rivers.

Vegetation

The diversity of species is relatively low when compared to their mainland counterpart. At the structural level, the forests show a lot of variation in the density or abundance of individuals per hectare although in general they are less dense forests than the forests in land-based ecosystems. The number of individuals can vary from 400-600 individuals per hectare with an average basal area is between 20 and 35.5 m^2, (Balslev et al.. 1987, Nebel et al. 2001, Báez et al. 2010). Juvenile individuals in these ecosystems are very sensitive to flood regimes if they are in the lower areas of the flood

plain causing high mortality rates (Wittmann et al. 2003) (Ministerio del Ambiente del Ecuador 2012).

Moving away from the white-water riverbanks towards mainland Amazonia (Ministerio del Ambiente del Ecuador 2012). The vegetation communities include herbaceous communities of the beaches, grass-predominated formations in the backwaters, shrub communities, riparian reeds, and middle and open successional forests. Succession forests occupy the most remote and relatively stable parts of the flood plain. The beaches can be sandy or muddy, with varying floristic composition depending on the substrate and hydrodynamics. The undergrowth is thin to slightly dense with predominance of *heliconias, marantáceas* and *piperáceas*. Depressions or channels, if permanently flooded, present herbaceous aquatic vegetation.

CES408.536 Flooded forest and lacustrine-riparian vegetation of black waters of the Amazon

This habitat occurs at < 350 masl and the land cover is classified as flooded low lands and lake forest. The fluvial dynamics are annual floods. This habitat can be found in the following provinces: North-Western Amazon, Sectors: Cuyabeno-Güeppí, Napo-Curaray.

Landscape Dynamics

The system develops on flooding lands with slightly depressed reliefs, of erosional origin due to river dynamics (Ministerio del Ambiente del Ecuador 2012). The soils are mainly clays which allows for water to stagnate. The black water drains with high concentrations of tannic substances and humic and fulvic acids resulting from the decomposition of the organic matter that covers the sand and clays of the adjacent lands. The soils in the Cuyabeno-Lagartococha lake complex are distinguished by a mixture of sandy-clayey soils from quaternary alluviums (Ministerio del Ambiente del Ecuador 2012).

Vegetation

The forest canopy reaches 20 m or less in some areas (Ministerio del Ambiente del Ecuador 2012). The average basal area for this type of forest is between 35 and

39.54 m^2 (Silman et al. 2001). There are areas where forests vegetation is very dense and other areas where trees are very distant from each other. The density of individuals vary between between 450 and 570 stems per hectare (Silman et al. 2001).

The communities are distinguished because the species of trees that are adapted to the aquatic environment have tortuous trunks, very lenticular barks, and in general the foliage is scleromorphic and the species possess seeds with floats (Ministerio del Ambiente del Ecuador 2012). Trees adapted to the banks develop arched branches defining foliage that looks like an umbrella.

CES408.538 Swamp palm forest of the floodplain of the western Amazon (Bosque inundado de palmas de la llanura aluvial de la Amazonía)

In Ecuador this habitat type occupies the eastern portion of the Yasuni National Park towards the border with Peru and in the interfluvium between the Napo and Aguarico river (Ministerio del Ambiente del Ecuador 2012). This habitat occurs at 350 (south of the country 800-1200 masl). The landcover is classified and flooded forest and: *Mauritia flexuosa* palm is dominant. This habitat can be found in the following provinces: North-Western Amazon, Sectors: Cuyabeno-Güeppí, Napo-Curaray, Tiger-Pastaza

Landscape dynamics and soils

This system is permanently flooded and *Mauritia Flexuosa* is a predominant feature, in some cases it forms monospecific stands (Ministerio del Ambiente del Ecuador 2012). The species are adapted to the flooded hydromorphic terrain of slightly depressed and swampy plains. This habitat type occurpies large areas especially in the northern central part of the Ecuadorian Amazon. The accumulation of water occurs due to runoff from the rains of the adjacent lands, the slow drainage of black water rivers, and water seepage from the main riverbeds. The system also occurs around permanent bodies of water, where it is flooded with black or mixed water, and rainwater (Ministerio del Ambiente del Ecuador 2012). The soils are mainly silty clay with abundance of humus (Ministerio del Ambiente del Ecuador 2012).

Vegetation

The abundance of *Mauritia flexuosa* palm ranges from about 100 to 500 individuals/ha (Ministerio del Ambiente del Ecuador 2012). The canopy trees reach 25 to 30 m high, with emerging trees from 35 to 40 m high. The diameter at breast height is generally from 30 to 50 cm. To the south of Ecuador, the canopy is lower and reaches up to 15 m. The undergrowth is composed mainly by seedlings of the palm. The herbaceous stratum is predominated by *Marantáceas, cyclanthaceas, zingiberaceas* and ferns (Ministerio del Ambiente del Ecuador 2012).

In southern Ecuador it represents small permanently flooded forests where Mauritia flexuosa is the predominant species and reaches a maximum height of 15 m (Ministerio del Ambiente del Ecuador 2012). There are other tree species, all adapted to flooded hydromorphic lands in depressions that occupy small areas (around 5 ha), especially in the Nangaritza canton.

The forest structure is made up of three to four strata with presence of stipulated and cespitose palms. There may be a few other tree species, vines, and epiphyte dicots (Ministerio del Ambiente del Ecuador 2012). Vegetation adapts to develop hydrophilic structures to tolerate high water saturation. The *Mauritia flexuosa* individuals develop modified or pneumatophores roots, with negative geotropism, the other species develop wading roots and plenty of lenticels in the barks.

CES408.550 Whitewater riparian succession vegetation complex of the Amazon (Complejo de vegetación sucesional riparia de aguas blancas de la Amazonía)

This habitat type is classified as woody wetland and is predominated by thickets, grassland and savana type vegetation.

Landscape Dynamics and Soils

This habitat is representative of riparian communities of the first stages of succession. The habitat is frequently destroyed during large annual flood which bring deposits of sandy-muddy sediments (Báez et al. 2012). Moving away for the riverbanks towards land throughout this habitat the following vegetation communities are found: herbaceous communities composed of annual vegetation growing on the new beach

deposits, graminoid canals of the backwaters or lower areas with slow currents, shrub communities, riparian reeds and middle and open successional forests. Successive riverine forests occupy the most remote and relatively stable parts of the beaches. The beaches can be sandy or muddy, varying the detail of the floristic composition depending on the substrate and hydrodynamics (Báez et al. 2012).

Vegetation

The areas closest to the river and sandbars have a strip of primary succession predominated by *Gynerium sagittatum* (*Poaceae)* and *Tessaria integrifolia* (*Asteraceae*) (Báez et al. 2012). When these banks age and stabilize secondary successional specie typical of the Amazonian plains begin to arrive, such as several *Cecropia* species (*Cecropiaceae*) and *Triplaris americana* (*Polygonaceae*).

6.7.2 Types of chemical contaminants, concentrations and disturbance types caused by legacy oils and gas exploitation throughout the Napo Concession

Contaminant Constituents

The contaminant is weathered crude oil which contains TPH, PAH's (benzo (a) pyrene and benzo (a) anthracene, pyrene, naphthalene), BTEX, phenols, heavy metals (zinc, barium, chromium IV, lead, sulphur, copper, and salts (UDAPT 2013).

UDAPT (2013) reported in the unpublished document that total petroleum hydrocarbons exceed the national standard of 1000 mg/ kg for residential soils, at 91 percent of the sites. The highest concentration of TPH found in the soil surface outside the waste pits was 333,262 ppm The highest TPH concentration found within the waste pits was 900,000 ppm PAH's benzo (*a*) pyrene and benzo (*a*) anthracene exceeded Ecuadorian standards in more than half of the sites (UDAPT 2013). Pyrene exceeded Ecuadorian standards in 90 percent of the sites; and the naphthalene exceeded Ecuadorian standards in 82 percent of the sites.

Garcia-Ruiz (2017) tested two waste pits within the Charapa field at 309 masl in the province of Sucumbios and found TPH concentration inside the ponds and

surrounding soil was above 5000 mg/kg in Pond 1 and 2 and was 1200 mg/kg in the surrounding soil.

Disturbance Types

The sites have been cleared of vegetation and upper soil layer. A drilling pad was built to extract the crude oil. The drilling activities created contaminated residue material called drilling mud which are solids that are found in the drilling stream. Each site contains 2-5 waste pit treatment pools, the drilling residue is mixed with chemical products to create a stabilized, homogenous mixture (Scholten et al. 2000).

There are currently over 900 waste pits that continue to pollute by leaching out into the environment as the pits degrade or overflow with rainwater (Gropper, personal communication 2018). The waste pits are fitted with an overflow pipe which most always is diverted straight into nearby streams and rivers thereby contaminating critical drinking sources. The waste pits are either filled with drilling mud, formation water, or a hardpan (Gropper, personal communication 2018). In addition, there are many micro sites that are polluted from various oil and gas production activities and old unkempt oil pipes that have leaked.

Some of the drilling mud waste pits are covered by a layer of decomposed organic matter and have been recolonized by native plants. Garcés-Ruiz et al. (2017) found a 10-cm layer of organic matter and the following tree species within the pits: *Ficus insipida* (wild fig*), Ficus americana* (West Indian laurel fig or Jamaican cherry fig), *Hieronyma alchorneoides* (mascarey) and *Croton lechleri* (dragon's blood). The following herbaceous species are found within the pits: *Dimerocostus strobilaceus* (sour cane), *Carludovica palmata* (Panama hat plant), *Heliconia chartacea* and *Araceae* spp. (i.e., *Euterpe precatoria* and Mart species), *Miconia* spp., *D. strobilaceus* (sour cane) *Costus* spp. (sour cane, and ginger like species), *C. palmata, H. chartacea,* and *Marantaceae* spp.

In the surrounding soils tree species found were *Ficus, Croton lechleri* and *Sapium glandulosum* while herbaceous species found were *Costus scaber, Carludovica palmata, Heliconia chartacea* and *Araceae* spp. which were only predominant beside pasture and cassava, banana cacao crop plantations (Garcés-Ruiz et al. 2017).

6.7.3 Bioremediation Feasibility Tree

The bioremediation feasibility tree has been split into two trees, unsaturated contaminated soil (Figure 22) and saturated conditions (Figure 23). The saturated conditions bioremediation feasibility tree lump in all waste-pits and waste-pit overflow discharge.

The feasibility trees are meant to allow a site assessor to move through the tree by answering “yes” or “no” dependent on the site in question. After evaluating the soil conditions and contaminant characteristics the assessor arrives at feasible bioremediation technique(s). They must then develop a site plan. The final enhanced bioremediation category indicates whether enhancement is feasible.

6.8 Discussion

It has been 60 years since Texaco began oil extraction within the Napo Concession (Bagnoud 1994). Oil extraction in this area has left behind severe environmental and human health consequences (Bagnoud 1994; WHO 2008). Consequences include health issues related to oil exploitation such as high cancer rates, loss of clean aerable land, and emotional traumas from lost lives, livelihoods, and loss of spiritual connections to the land (Bagnoud 1994). Yet there have been very few attempts to remediate the area (UDAPT 2013).

Although this report focuses on the environmental pillar within a *sustainable remediation* framework and to a lesser extent the economic pillar, the social pillar is equally important. The social context of remediation is often neglected (CL:AIRE. 2011). If ignored remediation activities have the potential to have negative social impacts inadvertently exacerbating the problem. Meaningful stakeholder engagement should be highlighted because local stakeholders perspectice, resources, and lifestyles can vary dramatically.

6.8.1 Habitat Types and Habitat Conditions

Habitat types will have a major effect on site-specific remediation plans. It will dictate which bioremediation technique is feasible to use. The main driver that

determines habitat type is soil conditions such as pH, saturation, texture, and nutrients (Villacís et al. 2016). For example the saturated conditions of the flooded black waters forests have clay soils with low permeability. Thus, we can expect to have limited options for sites found within these habitat types. Further, bacteria, fungi, and plants require the right conditions to thrive. Any plants used in phytoremediation efforts will need to be fully adapted to saturated conditions (Table 3). Plants have the high variability with the conditions; thus, plants must be assessed individually to determine optimal conditions.

6.8.2 Contaminant Constituents & Disturbance Types

Petroleum contamination changes the soil conditions such as permeability of water and oil, waterholding capacity, soil temperatures (Wang et al. 2013). Total petroleum hydrocarbons (TPH) and heavy metal concentration are likely affecting autochthonous microbial communities. Concentration ≥ 50 000 ppm for TPH and 2500 ppm for heavy metals have been found at some of the sites, bioremediation should still be considered (EPA 1994). In this case, the content of cadmium, water-holding capacity, and microbial populations have been adversely affected in test soil compared to the control (Maddela et al. 2015). This finding is consistent with multiple studies that found crude oil coats soil particles, blocks soil pores, and reduces the permeability of water and air (Khamehchiyan, Hossein Charkhabi, and Tajik 2007; Kisic et al. 2009; Wang et al. 2013) and thus affecting the soil water content.

Discharge of crude-oil waste increased the soil pH to neutral (pH 7.34) from acidic (pH 5.12): (Maddela et al. 2015). Crude oil contamination in soil has been found to be linked with the accumulation of exchangeable base (such as Ca^{2+}, Na^{+}) and a reduction in exchangeable acidity and effective cation exchange capacity (ECEC) (Kisic et al. 2009). It is theorized that these mechanisms underpin the increase of pH values in the crude oil polluted soil.

Despite an increase in pH to neutral nearly 100 times fewer heterotrophic bacteria and fungi were observed in the test soil when compared with the control sites (Maddela et al. 2015). Mesocosm studies can be performed to test site specific bioremediation strategies that will create the conditions where TPH degrading microbes can survive. Species of plants and microbes that have been found in the Napo

Concession sludge waste pits and are excellent contenders for bioremediation applications (Table 4).

Each disturbance type (i.e., unsaturated soils, saturated soils, waste pit dilling muds, waste pit formation water, and hardpan) have varying condition and must be treated via different techniques. The waste pits are either filled with drilling mud, formation water, or they have a hardpan layer. The hardpan is the result of burning the sludge waste (Lexie Groppper, personal communications, 2018). This was a negligent effort to remediate some of the drilling mud waste pits. In addition, there are many micro sites that are polluted from various oil and gas production activities and old unkempt oil pipes that have leaked.

6.8.3 Bioremediation Feasibility Tree

Nine bioremediation options have been evaluated for this report. They are: phytoremediation, bioventing, biosparging, bioslurping, biopiles, landfarming, treatment wetlands, bioreactors and natural attenuation. However, only the first seven have been deemed feasible. Bioreactors are not suitable because they will not be able to accommodate the volume of soil that needs to be remediated, approximately 3,788,000 m^3 (EPA 1994, UDAPT n.d.). However, using a bioreactor to determine site-specific optimal conditions during pilot testing could be highly valuable (EPA 1994).

The Napo Concession remediation will be extremely complex due in part by the sheer number of sites. Moreover, each site that contains waste pits will have many conditions that are derived from geographic location, soil conditions, and toxicity of the contaminant and the disturbance type (s). Due to the array of varying conditions it is likely that most of the technologies would need to be used in some capacity for the remediation of the Napo Concession sites. For example, saturated sites must be treated differently than unsaturated sites, and only treatment wetlands, biosparging and bioslurping are appropriate technologies to use (EPA 1994). Each technique has advantages and limitations (Tables 5 & 6). Enhancement is recommended for each of the bioremediation techniques. The only exception is for the hardpan waste pits where the best option for remediation might be capping.

The bioremediation feasibility trees are meant to be used as a tool for site assessors to determine if bioremediation is appropriate for that specific site and to determine what unique constraints that a particular site may have. Constraints such as space, saturated soil conditions, or equipment availability may push one technique over another to the forefront.

Unsaturated sites

Biopiles, windrows, bioventing, landfarming, and phytoremediation are feasible options for the remediation of unsaturated soils. One key characteristic of petroleum-contaminated soils is their poor aeration which affects microbe processes and is often the limiting factor (Alrumman et al. 2015). For this reason, bioremediation techniques for PHC degradation are centered around oxygenating the soil. Each bioremediation option can be enhanced by selecting a consortium of autochthonous microbes or specialized TPH degrading microbes can be cultured and incorporated into the site plan (Maddela et al. 2015; Yanto and Tachibana 2014).

Saturated Sites

Biosparging is essentially the only biological treatment available for saturated soil and waste pits filled with drilling muds. Dissolved iron plays an important role in determining the effectiveness of biosparging and should not exceed 10 mg/L (EPA 1994). High concentrations of dissolved ferrous iron can render air sparging wells useless. If ferrous iron is exposed to oxygen under saturated conditions, then it is oxidized to ferric iron and precipitates out and will fill soil pore space decreasing permeability. Precipitation of iron oxide near sparging is prevalent surrounding the well screens where oxygen content is the highest. If the site is not free of an impermeable layer, the intrinsic permeability is higher than k-9 then a non-biological remediation technique may be required (EPA 1994).

Treatment wetlands have been split into A and B (see figure 6). Overflow discharge should be diverted from emptying directly in the streams and rivers to specialized variable flow treatment wetland (raingarden). Treatment wetland B (formation water waste pits) could have experimental bioremediation techniques built into the existing waste pits. Hardpan site will need further investigation, no information that I know of has been described in the literature. It may be necessary to reclaim the

land by hard capping and converting the land into community led projects such as raised community gardens.

Ex-situ

The ex-situ techniques are more versatile than *in-situ* because the soil structure, texture, pH, moisture content, nutrients, and microbial communities can be manipulated to create optimal conditions for biodegradation (Bharagava et al. 2017). However, high clay content in the soils can still affect bioremediation by decreasing oxygen infiltration thus increasing prolonging the bioremediation process (EPA 1994). *Ex-situ* techniques should seriously be considered as they generally have shorter bioremediation time frames.

In-situ

In-situ is preferred (if possible) by many practitioners because soil does not need to be excavated, and importantly the risk to remediation technicians are lowered because there is no need for frequent turning and handling of large volumes of soil (EPA 1994). Further, the cost is generally lower. However, soil and contaminant constraints may make in-situ remediation not feasible. Intrinsic permeability is commonly the limiting factor for bioventing, biosparging, and bioslurping (EPA 1994). TPH and heavy metal concentrations is also a major limiting factor because ≥ 50 000 ppm (TPH)/ 2500 ppm (heavy metals) will have significant effects on the survival rate of the microorganisms. However. microbial inoculums and nutrients additions can be injected into the treatment zone through injection well sites.

Phytoremediation will require careful selection of plant species but would restore some function to the landscape. Restoration of the vegetation cover generally leads to improvements in soil properties as has been previously demonstrated in Ecuador and other South American regions (Villacís et al. 2016). After a few years of revegetation, organic matter increases, and biological activity is stimulated restoring the functions of the soil. Moreover, these enhanced soil properties and the plants growing in them can neutralize or stabilize the soil contaminants, potentially rendering them unavailable to other organisms (Azubuike, Chikere, and Okpokwasili 2016). Although, Phytoremediation in general can only be used for low TPH concentrations, and plants will possess variable tolerances to heavy metal contamination (Darwish 2013, pp 178).

Plants that have developed a symbiotic relationship with arbuscular mycorrhizal fungi (AMF) may increase their ability to survive in harsh toxic conditions. AMF are soil inhabitants forming associations with plant species. They play key roles in soil processes (e.g., soil structure, biogeochemical cycles) (help plants to acquire nutrients in exchange for carbohydrates and protect them from biotic and abiotic stresses (Garcés-Ruiz et al. 2017, Plouznikoff et al. 2016). Some of the weathered crude oil ponds are between 30 to 40 years old;however, despite the intensive soil contamination,these have been graduallyrecolonized naturally by plants (Garcés-Ruiz et al. 2017). This study demonstrated roots from several native plants species were highly colonized and diverse communities of AMF belonging to Glomus, Rhizophagus, Archaeospora and Acaulospora were associated with *C. scaber*, *E. precaria* and *C. palmata*. Further, it demonstrated the need for more studies in relation to AMF species identification and their ecological role. Seventy-four percent of operational taxanomic units could not be attributed to an existing AMF species (Garcés-Ruiz et al. 2017).

Enhancement is recommended for each technique since the weathered crude is recalcitrant and TPH concentration remain high after 60 years (Maddela et al. 2017). Enhancement best management practices are continually evolving. It used to be that isolating one effective TPH bacteria or fungi and creating essentially a monoculture was seen as best practice. However, as the understanding of microbial communities is increasing, there is a larger focus on developing effective TPH degrading consortiums (Maddela, et al. 2015; Yanto and Tachibana 2014).

Four microorganisms (*B. cereus*, *B. thuringiensis*, *Geomyces pannorum*, and *Geomyces* sp.) were isolated from crude oil-contaminated soil collected from the Napo Concession Area. Individually, the isolates were able to degrade crude oil at varying rates from 49.71% to 77.34%. However, co-culturing the microorganisms resulted in an increased degradation rate to 79.9 % of the crude oil in 30 days. (Maddela et al. 2015)

The bioremeditors at have been documented in the Napo Concession Area only scratches the surface of potential effective bioremediatiors and is not by any means a place to stop researching (Table 7). Only a handful of studies to the best of my knowledge, have focused on using plants and microbes to remediate the Amazon and evidently, much more work will be required..

6.8.4 Experimental Bioremediation Designs

The most important factor is to mitigate human and environmental risk. Which requires containing and stabilizing the contaminated media, then bio-transforming, or removal of the contaminant through biological or physical means.

In Ecuador, permissible toxicant limits are regulated by two regulatory documents: Reglamento Sustitutivo del Reglamento Ambiental para las Operaciones Hidrocarburíferas en el Ecuador (RAOHE, Decreto No. 1215); and, Texto Unificado de Legislación Secundaria del Ministerio del Ambiente2 (TULSMA), under The Law of Environmental Management. (Merchán-Rivera 2017). The current permissible levels are illustrated in tables 2 and 3. and are based on human health risk. The permissible levels differ depending on land use. The permissible limits will help guide remediation targets.

Treatment Wetland A

Treatment wetland A is designed as a biofiltration wetland (raingarden) which will filter the polluted runoff before it is discharged into nearby water resources. Raingardens are vertical-flow shallow-constructed wetlands that are planted with native vegetation that are tolerant to wet conditions and tolerant to pollutants. The raingarden should be able to withstand some variability in flow rates.

Raingarden Design Specifics:

- The depth of the raingarden will change the waterholding capacity. The ponding depth can vary between 6-12 inches. Shallow depression must be ≥ 10% of waste pit area. Big and deep depressions will allow for large volumes of water. The depth should be 0.9 or more meters with a level bottom.
- Soil drainage must be >0.1 inches per hour.
- The raingarden soil mix can be a mixture of compost and native soils or screened sand and compost mixture: 65% native soils, 35% compost, or 60% screened sand and 40% compost well mixed. Compost can be enhanced with spent mycelium.

- The use of symbiotic mycorrhizal fungi will ncrease plant resilience, and using a consortium of bacteria and fungi will help to degrade PHC's.
- Inflow swales and exflow swale should be installed to slow down the flow of water and encourage sedimentation.
- It is important to incorperate planting Zones in the raingardens. For example, desgin the sides with fast spreading for sides and top drought tolerant plants, zone 1. The interior should have plants that are tolerant to wet conditions but can also survive in a droubt (zone 2).
- Mulch after the raingarden has been planted to prevents erosions and this also helps to keep weed maintenance down.
- Maintain dense coverage of plants especially in Zone 2 of the rain garden. If plants die replace with plant species that did well.
- The rain garden could be multiple rain gardens connected by rock swales to slow down water and increase the amount of pollutants removed from the raingardens.
- The rock or vegetated swales are feature help to filter the water through sedimentation, infiltration, and increases the chemical and biological contact time in the soils. Research has shown that swales reduce the total suspended solids by an average of 72% (Fletcher and Deletic 2009).

Treatment Wetland B

Treatment wetland B is designed as a floating treatment wetland, it is designed to use the existing drilling mud waste pits or the formation water waste pits. This type of treatment wetland can accommodate highly variable flow rates (Tondera et al. 2018). Artificial vegetated rafts are installed throughout the pond. The rafts are anchored to the substrate to avoid drifting. The rafts are a biological enhancement treatment that should be used in conjunction with another remedial technique.

The plant roots are essential for the efficacy of floating treatment wetlands (Tondera et al. 2018). They extend into the water and in doing so, they mechanically slow the flow of water. The root as well as the biofilm attached to the root produce

organic exudates, extracellular polymeric substances and humic compounds that promote floc formation that may enhance settling of fine particulates (Borne et al. 2015). In addition, plant detritus can biosorbe and uptake metals and organic compounds.

The performance of floating treatment wetlands varies considerably and is still an experimental treatment (Tondera et al. 2018). Important factors to consider are plant species and their tolerance to heavy metals and hydrocarbons. Plant island distribution and growth, and maintenance e.g. harvesting of the plants must occure overtime to remove potential toxins that have been uptaken by the plants.

6.9 Conclusions

Environmental sustainability is achieved when ecological integrity is maintained, and ecosystems are kept in balance while natural resources are used by humans at a rate where they can replenish themselves (University of Alberta 2010). Economic sustainability is when people and communities can maintain their independence and have access to financial and other resources that they require to meet their needs, such as secure sources of livelihood. Finally, social sustainability is the enforcement of universal human rights, and basic necessities are attainable by all people. Individuals must have access to enough resources to keep their families healthy and secure. Further, communities have just leaders who ensure that personal, labour and cultural rights are respected and all people are protected from discrimination (University of Alberta 2010) The three pillars of sustainability have accosicated indictors categories have been developed by SuRF-UK to standardize the evaluation of environmental sustainable remediation plans (Table 1) (CLAIR 2011).

The environmental and human health crisis in the Napo Concession remains stagnant after 60 years. Unfortunately, not much can be done until the contaminated properties are untied from litigation procedures. This high-level bioremediation option plan has been done with the guidance of Amisacho Research Station and researcher Dr. Mia Maltz who are important stakeholders of the Napo Concession. The bioremediation feasibility trees should be used as a tool to assess individual sites upon developing site specific remediation plans. In most cases bioremediation is a feasible option with the addition to enhanced microbial consortiums. However, techniques should be paired with site conditions. In addition, it is important to develop a remediation plan under a

sustainable remediation framework that heavily involves participatory stakeholder engagement.

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Tables

Table 1.SuRF-UK sustainable remediation indicator categories (adapted from CL:AIRE 2011).

Environmental	Social	Economic
Air	Human health and safety	Direct economic and cost benefit
Soil and ground conditions	Ethics and equality	Indirect Economic and cost benefit
Ground water and surface water	Neighborhood and locality	Employment and employment capital
Ecology	Community and Community involvement	Induced economic cost and benefit
Natural resources and waste	Uncertainty and evidence	Project lifespan and flexibility

Table 2. Ecuadorian standards; permissible levels of mud and drilling wastes when disposal area has no impermeable barrier.

Parameter	**ROAHE 1215**
pH (-)	6-8
Conductivity	4000
TPH (mg/L)	1.0
PAH (mg/L)	0.003
Cadmium (mg/L)	0.05
Total Chromium (mg/L)	1.0
Vanadium (mg/L)	0.2
Barium (mg/L)	5.0

Table 3 Ecuadorian Standards, permissible contaminant levels for soil and sediments.

Parameter	RAOHE 1215	TULSMA			
		Residential	Commercial	Industrial	Agricultural
TPH (mg/kg)	2500	230	620	620	150
Benzene (mg/kg)	none	0.08	5	5	0.03
Ethylbenzene (mg/kg)	none	0.1	20	20	0.1
Styrene (mg/kg)	none	5	50	50	0.1
Tolulene (mg/kg)	none	0.37	0.8	0.8	0.08
Xylene (mg/kg)	none	2.4	11	20	0.1
PAH (mg/kg)	2	none	none	none	none
Anthracene (mg/kg)	none	none	none	100	0.1
Benz(a)anthracene (mg/kg)	none	1	1	10	0.1
Benz(a)pyrene (mg/kg)	none	0.7	10	0.7	0.1
Benz(b)fluoranthene (mg/kg)	none	1	0.7	10	0.1
Benz(k)fluoranthene (mg/kg)	none	1	10	10	0.1
Dibenz(a,h)anthracene (mg/kg)	none	1	10	10	0.1
Indeno(I,2,3-cd)pyrene	none	1	10	10	0.1
Fluroranthene (mg/kg)	none	none	10	100	0.1
Naphthalene (mg/kg)	none	0.6	none	22	0.1
Pyrene (mg/kg)	none	10	22	100	0.1
chrysene (mg/kg)	none	none	none	100	0.1
Phenanthryene (mg/kg)	none	5	50	50	none
Cadmium (mg/kg)	2	4	10	10	2
nickle (mg/kg)	50	100	100	50	50
Lead (mg/kg)	100	140	150	150	60
Conductivity (µS/cm)	none	200	400	400	200
pH (-)	none	6.0-8.0	6.0-8.0	6.0-8.0	6.0-8.0
Barium (mg/kg)	none	500	2000	2000	750
Copper (mg/kg)	none	63	91	91	63
Total Chromium (mg/kg)	none	64	87	87	65
Vanadio (mg/kg)	none	130	130	130	130

Table 4. Optimal conditions for bacterial remediation, mycoremediation, and phytoremediation.

	Bacterial Remediation	**Mycoremediation**	**Phytoremediation**
pH	6.5-7.5	5.0-7.5	Varies per species
Oxygen	> 1 % Aerobic	> 1 % Aerobic	Varies per species
Substrate moisture	30-35%	30-65%	Varies per species
Carbon/Nitrogen/Phosphorus	100/10/1 to 100/10/0.5	100/10/1 to 100/10/0.5	Varies per species
Temperature	30º C	25º C	Varies per species
Total Petroleum Hydrocarbons Concentration	Low-high	Low-high	Low-medium
Heavy Metal Concentration	Low	Variable	Varies per species

Table 4. Strengths and limitations of ex-situ bioremediation techniques (adapted from Azubuike et al 2016).

Bioremediation Techniques	Strengths	Limitations
Biopiles	Can Remediate wide variety of soils, sand to clay Proven track record up to 95% effective TPH removal Technically simple Applicable for course to fine soils Short remediation time	Heavy machinery for earthworks Power needed for even aeration Need some space for piles
Windrows	6-month trial outcompeted biopiles, >95% TPH removal Technically simple Short remediation time	Heavy machinery needed for earth works Constant turning of the soil Best for finer soils Needs space for windrows Higher health risk for workers
Landfarming	Can Remediate wide variety of soils, sand to clay Very effective Technically simple In-situ or ex-situ	Needs lots of space Heavy machinery needed for earth works Periodic tilling of the soil Promotes toxic volatiles
Bioreactors	Very effective up to 97% Can be done in slurry phase or solid Good to study optimal conditions for bioremediation	Heavy machinery needed to excavate soils Technologically advance technology Cannot process high volumes of contaminates substrate
Natural attenuation	No equipment or technical expertise needed	Highly variable results Time frame often unreasonably long Long-term monitoring is required

Table 5.Strengths and limitations of in-situ bioremediation techniques

Bioslurping	Used for LNAPL Can convert system into bioventing after LNAPL has been removed Minimal site disturbance	Should not be used in areas with fluctuating water table Should not be used in low permeable soils Heavy machinery for installation Skilled personnel
Biosparging	Proven effective track record, up to 99% Can easily be combined with bioventing system	Heavy machinery for equipment Power for aeration Skilled personnel Bring in treatment system Not applicable for soils with low permeability and non-uniform soils, unconfined aquifers
Bioventing	Proven effective track record <95% TPH removal Relatively shot cleanup time	Heavy machinery for installation Skilled personnel Not applicable for soils with low permeability and high clay content Bring in treatment system Power needed for aeration
Biofilters	Can be simple or complex system Highly customizable	Heavy machinery for earthworks
Phytoremediation	Simple technique no skilled personnel needed Plants, soil prep, and maintenance are main costs	Limited data on phytoremediators Low-medium concentrations

Table 6. THP degrading Bacteria, fungi and TPH tolerant plants found in the Napo Concession sludge waste pits.

Class	Species	Source
Bacterial Remediators	Bacillius cereus	Madella et al. 2015
	Bacillius. thuringiensis	Madella et al. 2015
Filamentus fungi	Geomyces sp.	Madella et al. 2015
	Geomycetes pannorum	Madella et al. 2015
White Rot Fungi	Ganoderma sp.	Gropper 2018, personal communication
	Trametes sp.	Gropper 2018, personal communication
AMF	Acaulospora spp.	Garcés-Ruiz et al. 2017
	Glomus spp.	Garcés-Ruiz et al. 2017
	Rhizophagus spp.	Garcés-Ruiz et al. 2017
Known phytoremediators of Napo Concession	Carludovica palmata	Garcés-Ruiz et al. 2017
	Costus lima	Garcés-Ruiz et al. 2017
	Costus pulverulentus	Garcés-Ruiz et al. 2017
	Costus scaber	Garcés-Ruiz et al. 2017
	Euterpe precatoria	Garcés-Ruiz et al. 2017
	Flemingia macrophylla	Villacis et al. 2016
	Geonoma cf. deversa	Garcés-Ruiz et al. 2017
	Myrica. aff. fallax	Villacis et al. 2016
	Monotagma sp.	Garcés-Ruiz et al. 2017
	Platymiscium pinnatum	Villacis et al. 2016
	Piptadenia pteroclada	Villacis et al. 2016
	Polybotrya sp	Garcés-Ruiz et al. 2017

Class	Species	Source
	Zygia. longifolia	Villacis et al. 2016
Tree species found in waste pits and surrounding soils	Croton lechleri	Garcés-Ruiz et al. 2017
	Ficus cf. americana	Garcés-Ruiz et al. 2017
	Ficus insipida	Garcés-Ruiz et al. 2017
	Hieronyma alchorneoides	Garcés-Ruiz et al. 2017
	Sapium glandulosum	Garcés-Ruiz et al. 2017
Herbacous species found in waste pits and surrounding soil	Araceae spp.	Garcés-Ruiz et al. 2017
	Carludovica palmata	Garcés-Ruiz et al. 2017
	Costus spp.	Garcés-Ruiz et al. 2017
	Dimerocostus strobilaceus	Garcés-Ruiz et al. 2017
	Heliconia chartacea	Garcés-Ruiz et al. 2017
	Marantaceae	Garcés-Ruiz et al. 2017
	Miconica spp.	Garcés-Ruiz et al. 2017

Figures

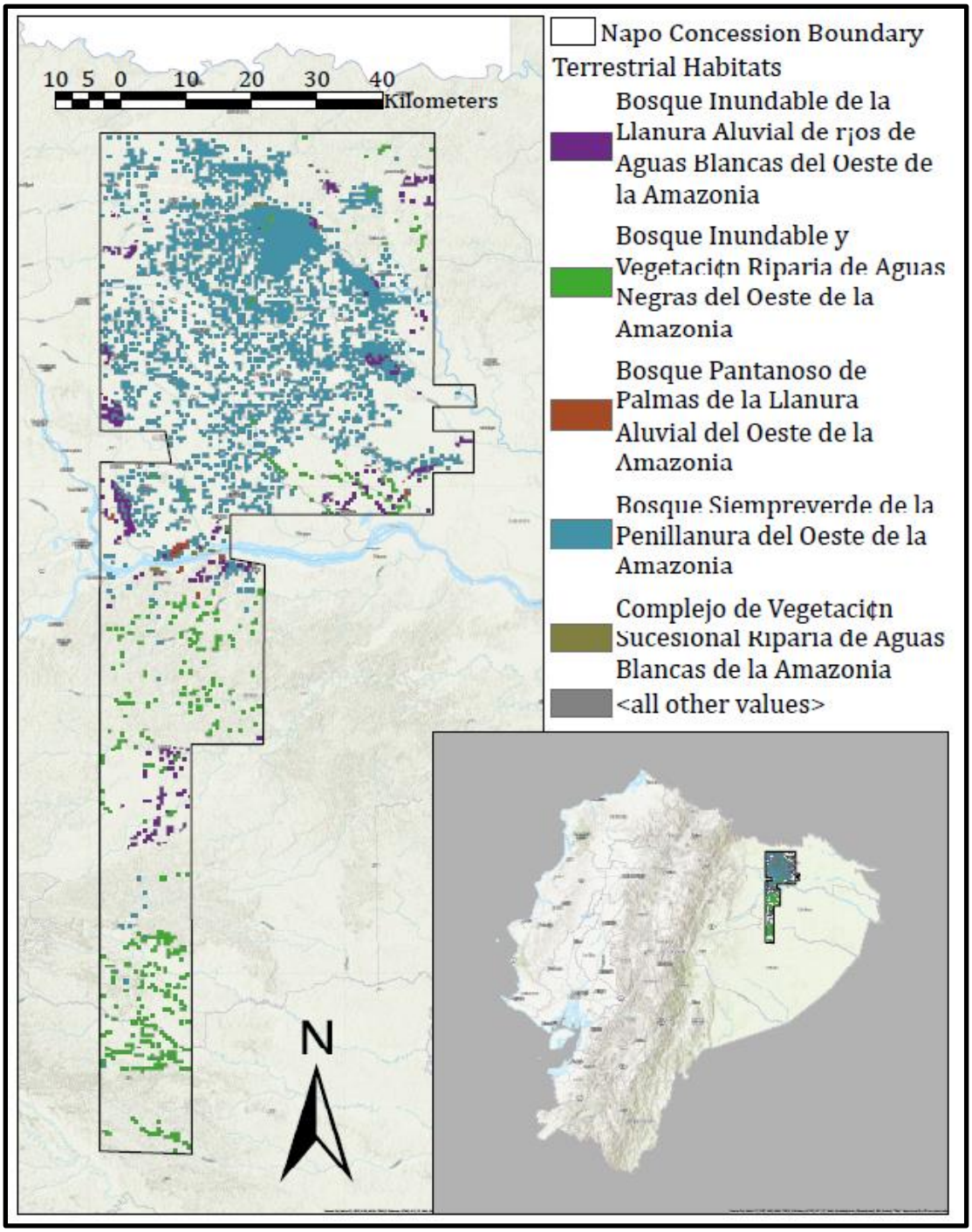

Figure 21. The habitat complexes within the Napo Concession boundary. (Hines, 2020 ArcMap Pro)

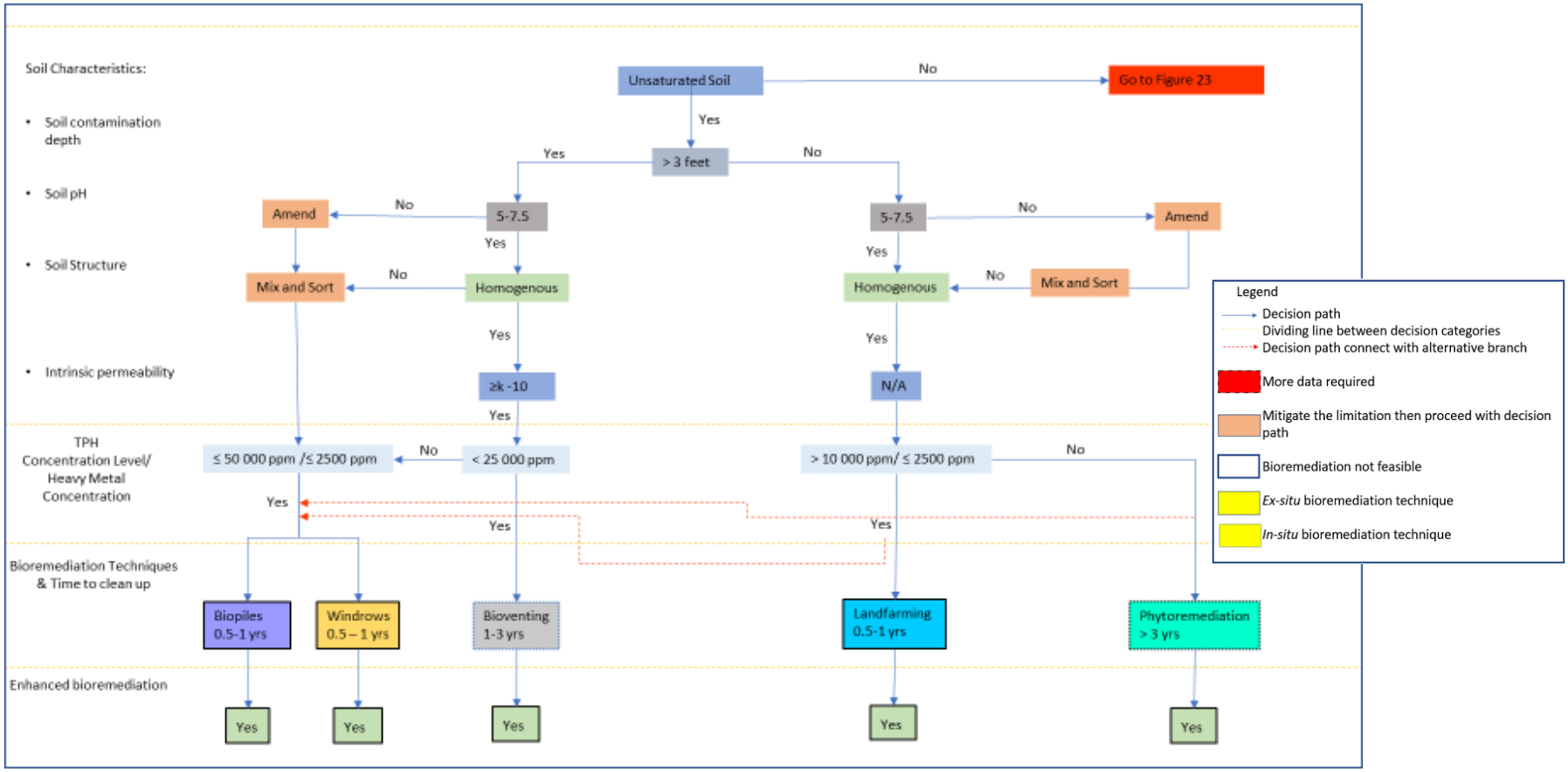

Figure 22. Bioremediation feasibility tree for contaminated un-saturated soil in the Napo Concession. The yellow dotted line indicates larger decision categories, they are soil characteristics, contaminant concentrations, bioremediation techniques, and enhanced bioremediation. Red dotted arrow line indicates that it is also feasible to use the alternate branch of bioremediation techniques. Source EPA 1994, Maddela et al 2015 [1] [2], Merchan-Rivera 2017.185

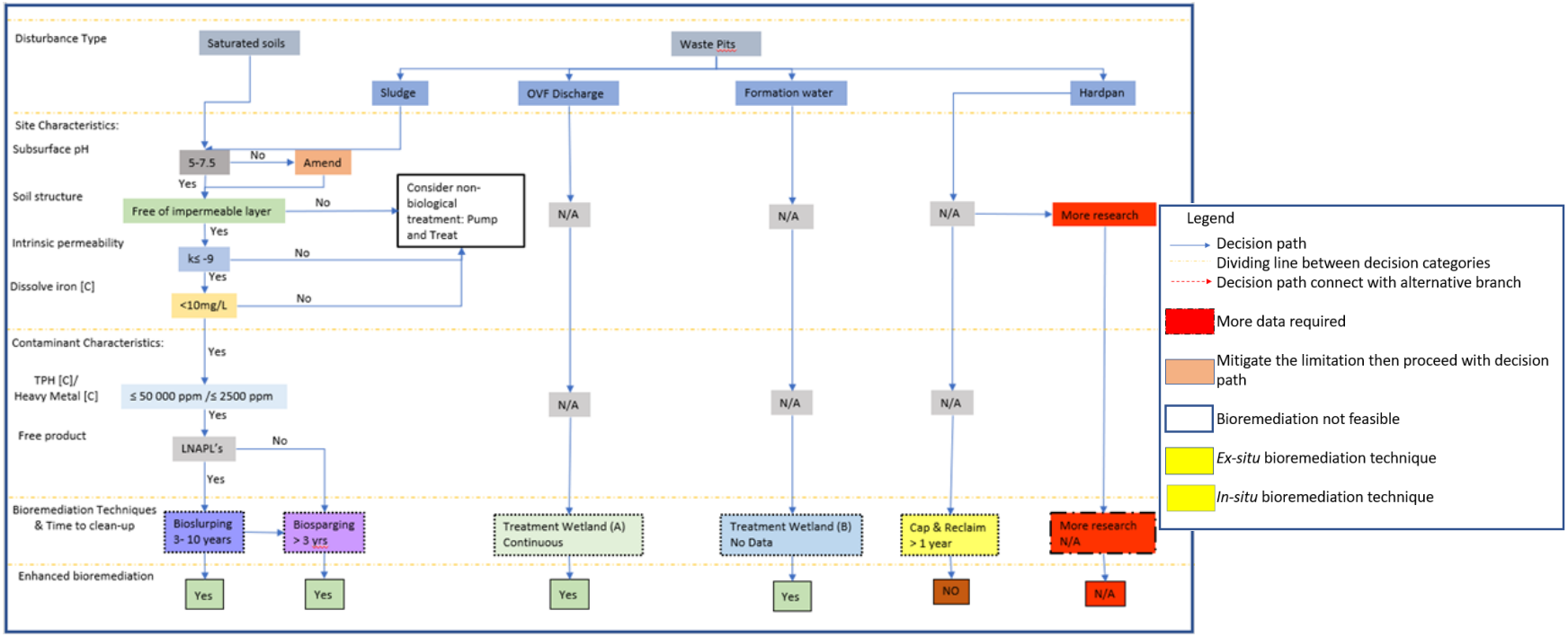

Figure 23. Bioremediation feasibility tree for contaminated saturated soil and petroleum production waste pits in the Napo Concession. The tree is sectioned into disturbance type, site characteristics, contaminant characteristics, bioremediation techniques, and enhanced bioremediation.

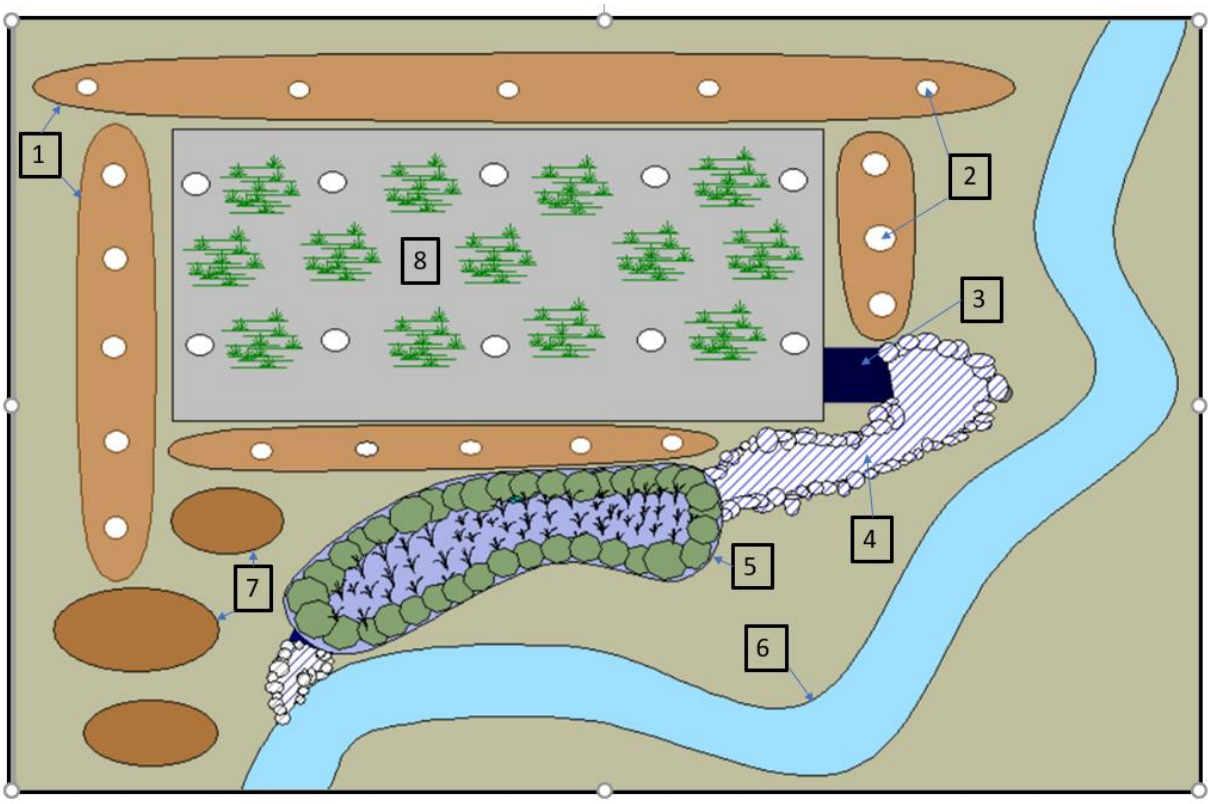

Figure 24. Representation of an experimental bioremediation site design which includes, a variety of remediation techniques including biopiles, and windrows.

Legend

1. Biopiles, in the form of windrows
2. Installed piping for aeration (with or without blower)
3. Outflow pipe
4. Rock or vegetated swale
5. Treatment Wetland A, vertical flow raingarden
6. Nearby surface stream
7. Windrow composting
8. Treatment Wetland B, floating treatment wetland with installed biosparging unit.

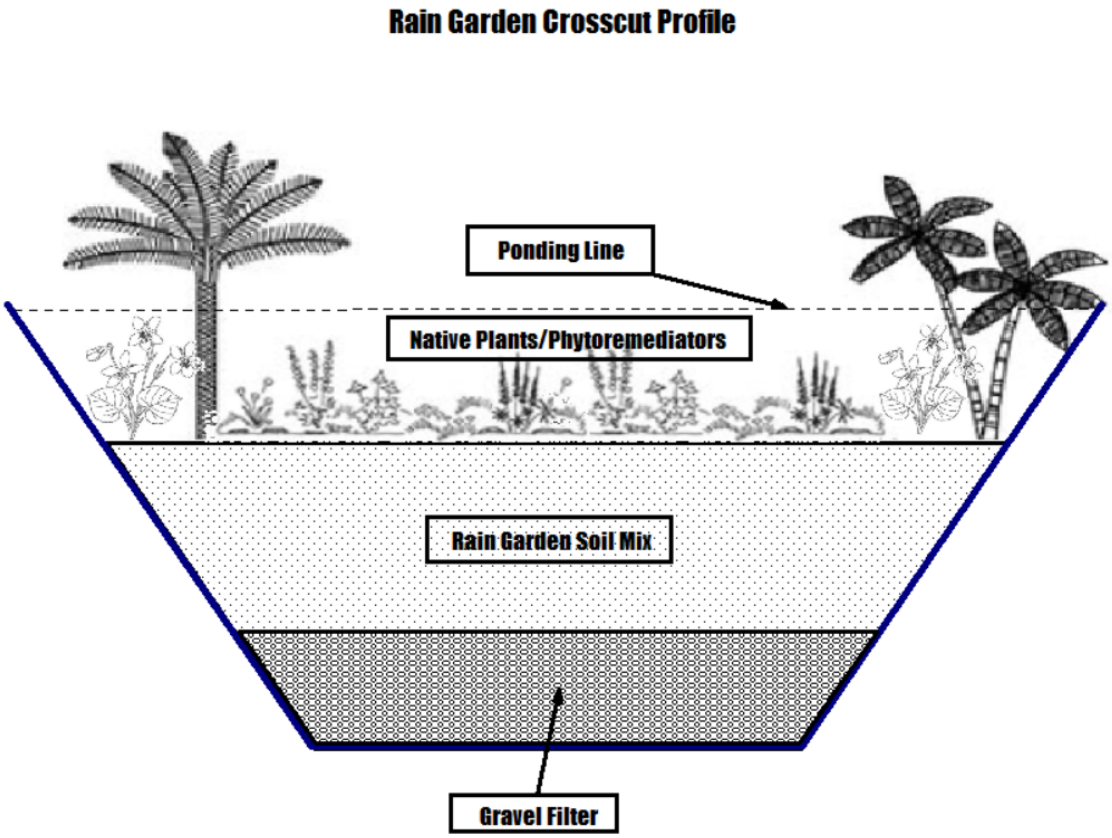

Figure 25. Cross section of Treatment Wetland A. It is a vertical flow raingarden with a gravel filter bottom, a specialized raingarden soil mix planted with native plants. These plants must be tolerant to fluctuating water levels, and contaminant characteristics.

Appendix A- Chromatograms, Effectiveness of biodegradation using trained and untrained mushrooms cultured on straw medium

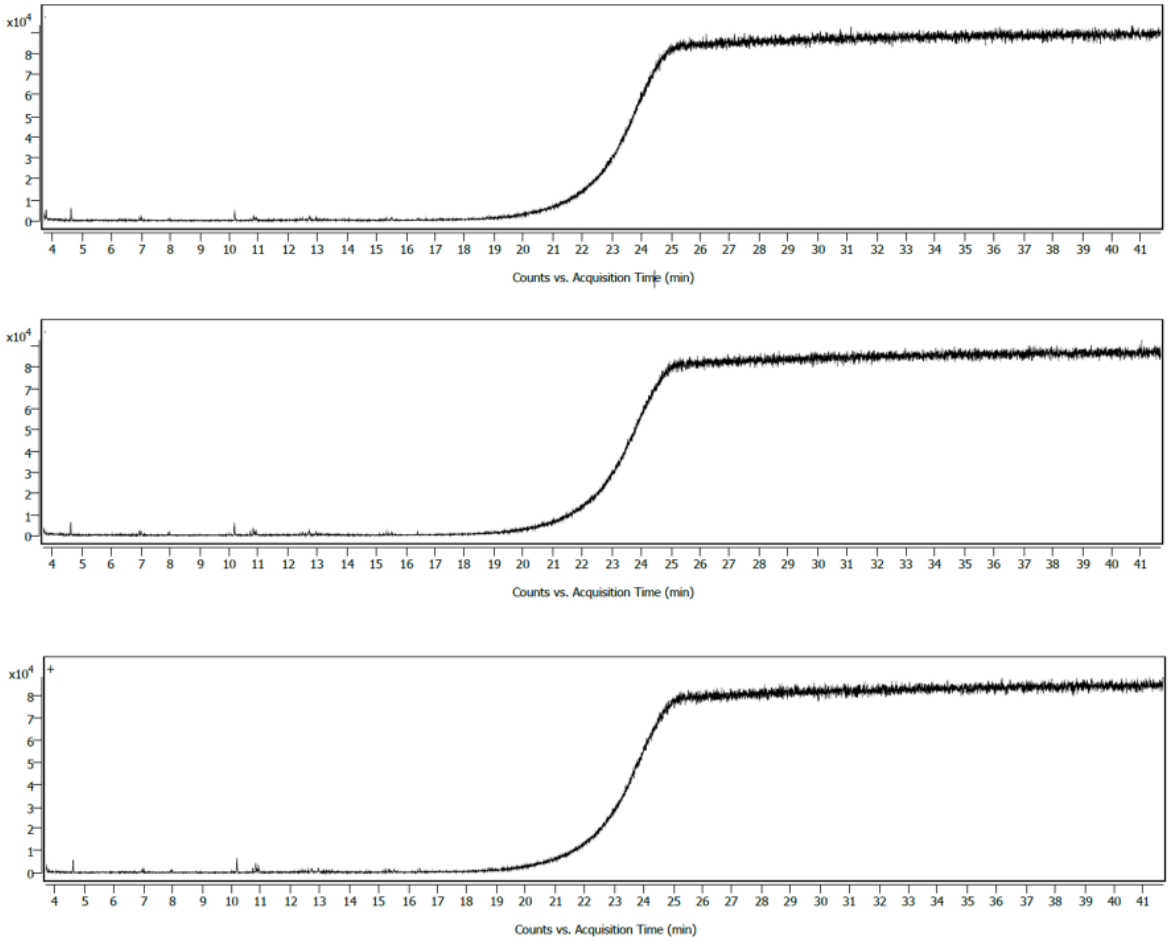

Figure A-1. Comparison of chromatograms control primed 2 (a, b, c) after 188 days. Retention time is on the x axis and response signal is on the y access.

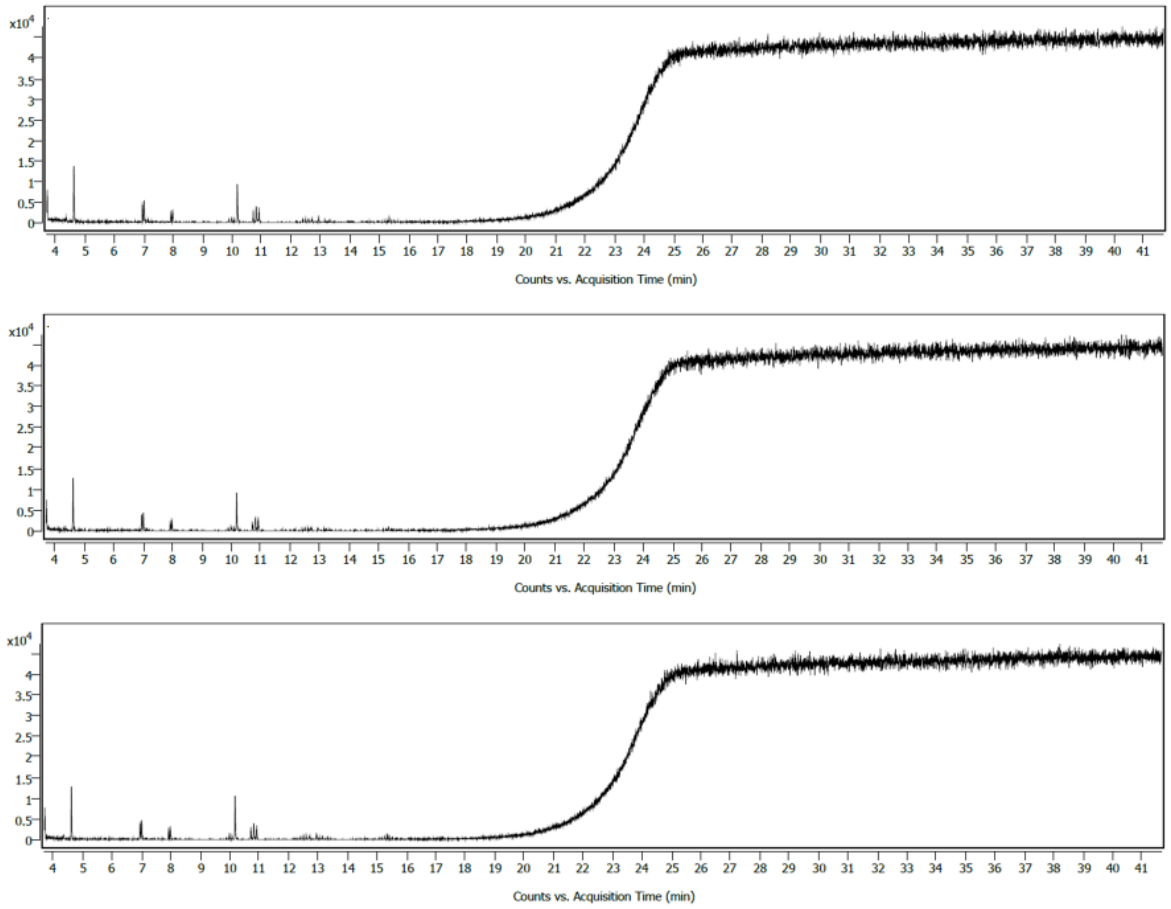

Figure A-2. Comparison of chromatograms control un-Primed 3 (a, b, c) after 188 days. Retention time is on the x-axis of the graph and the response signal is on the y-axis.

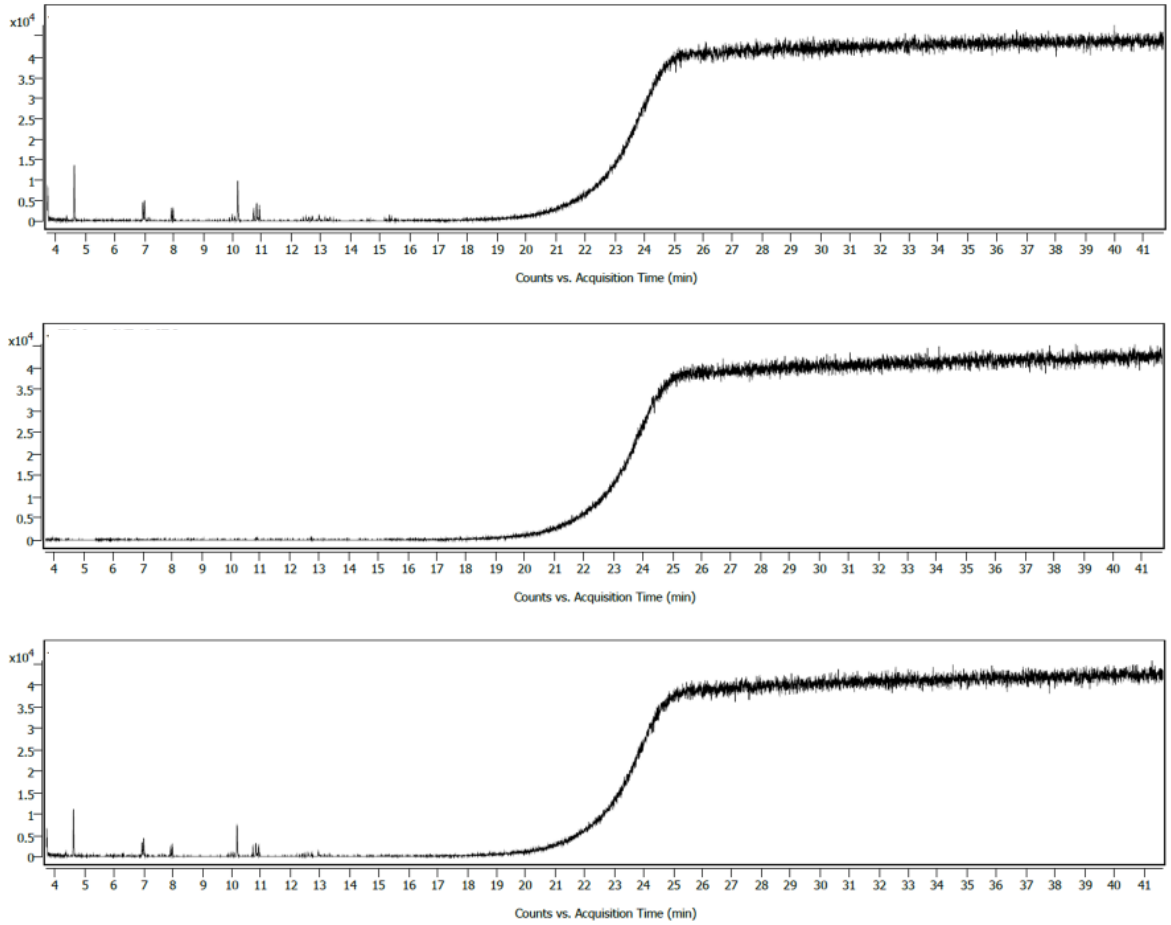

Figure A-3. Comparison of control un-primed 4 (a, b, c). Retention time is on the x axis and response signal is on the y -axis.

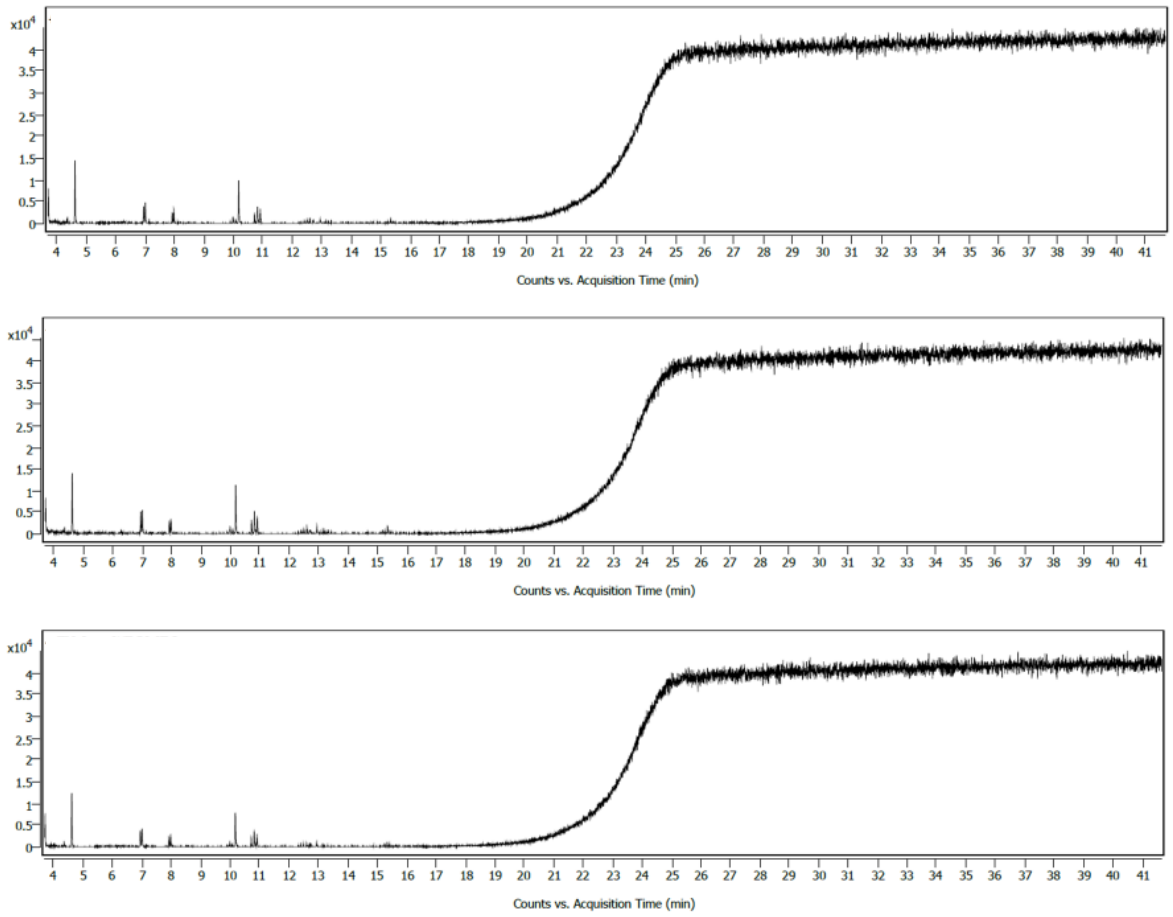

Figure A-4. Comparison of chromatograms control un-primed 5 (a, b, c) after 188 days. Retention time is on the x-axis of the graph and the response signal is on the y-axis.

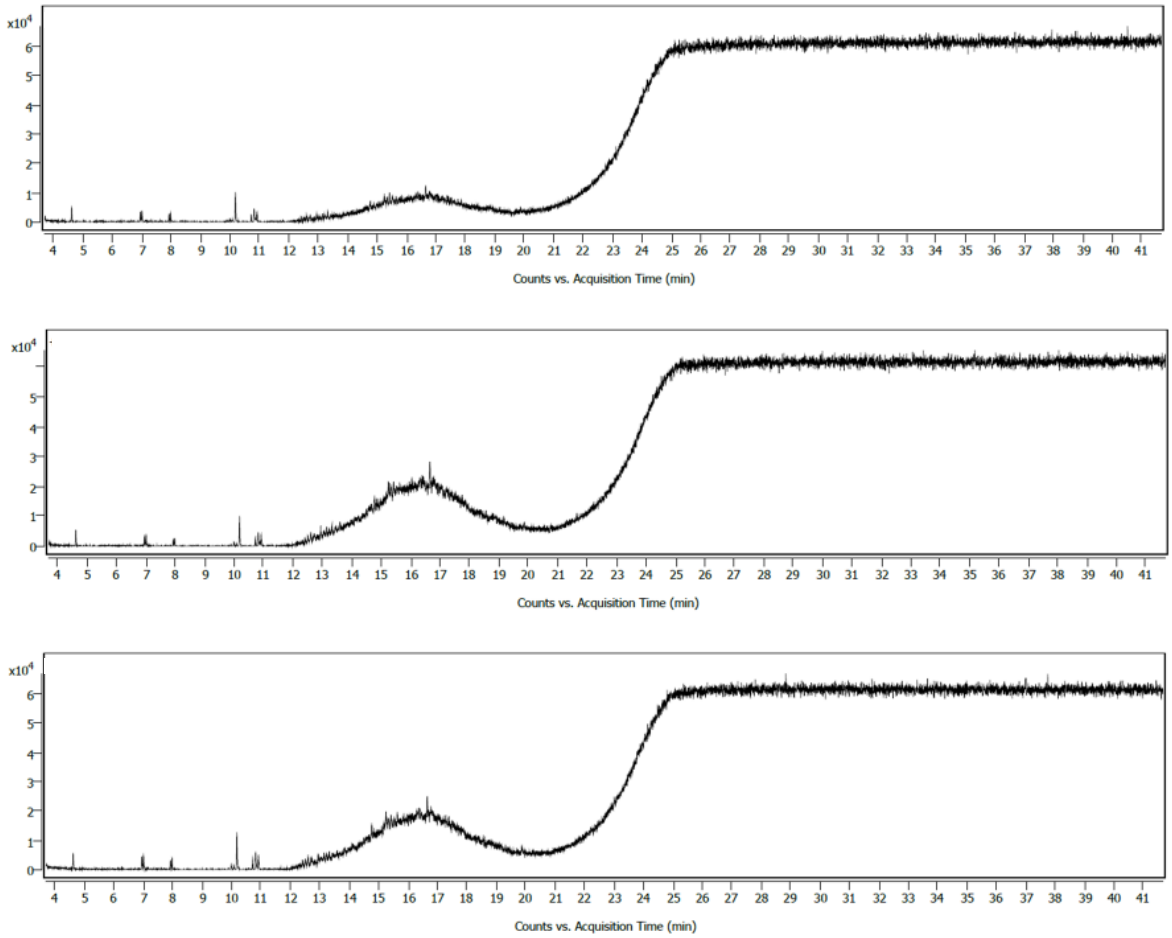

Figure A-6. Chromatogram comparison of diesel 1 (a, b, c), after 188 days. Retention time is on the x-axis of the graph and the response signal is on the y-axis.

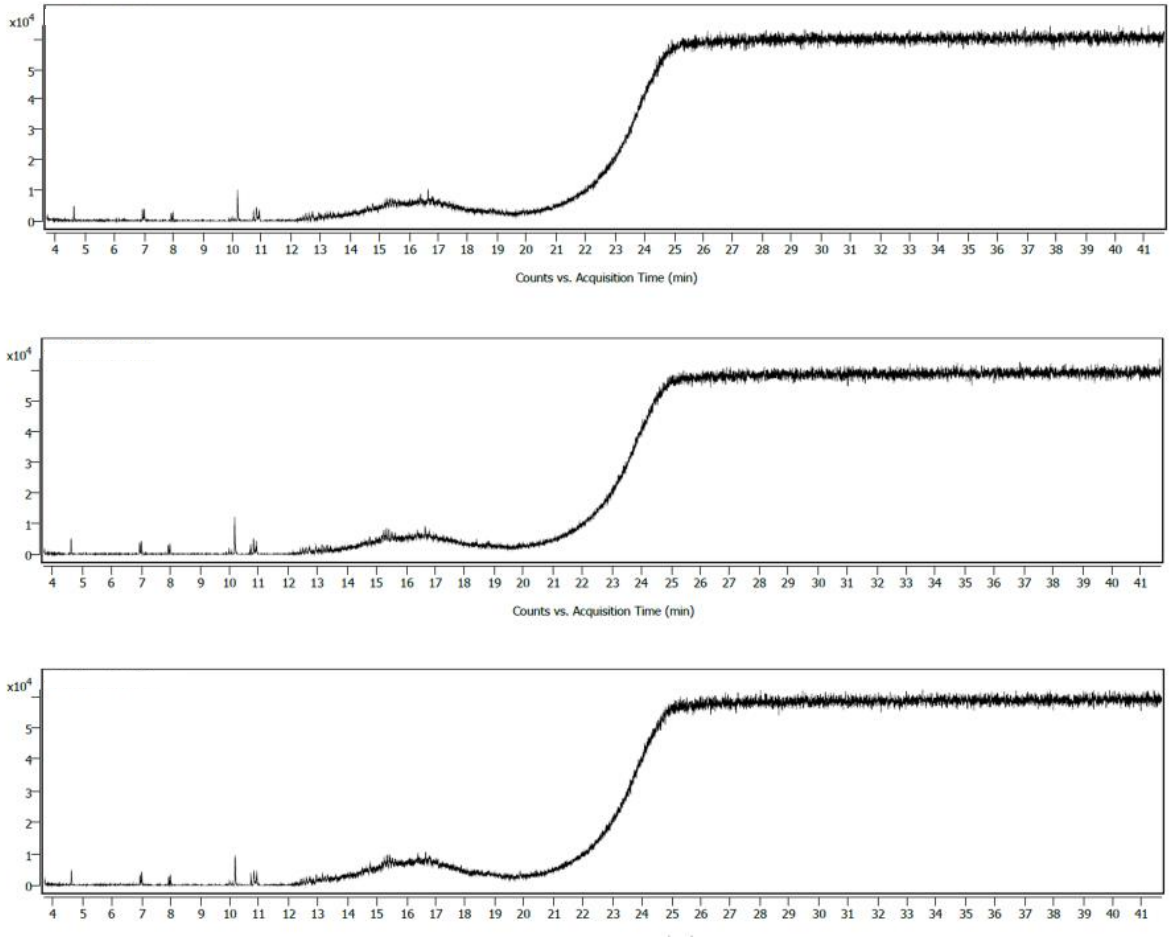

Figure A-7. Chromatogram comparison of diesel 2 (a, b, c), after 188 days. Retention time is on the x-axis of the graph and the response signal is on the y-axis.

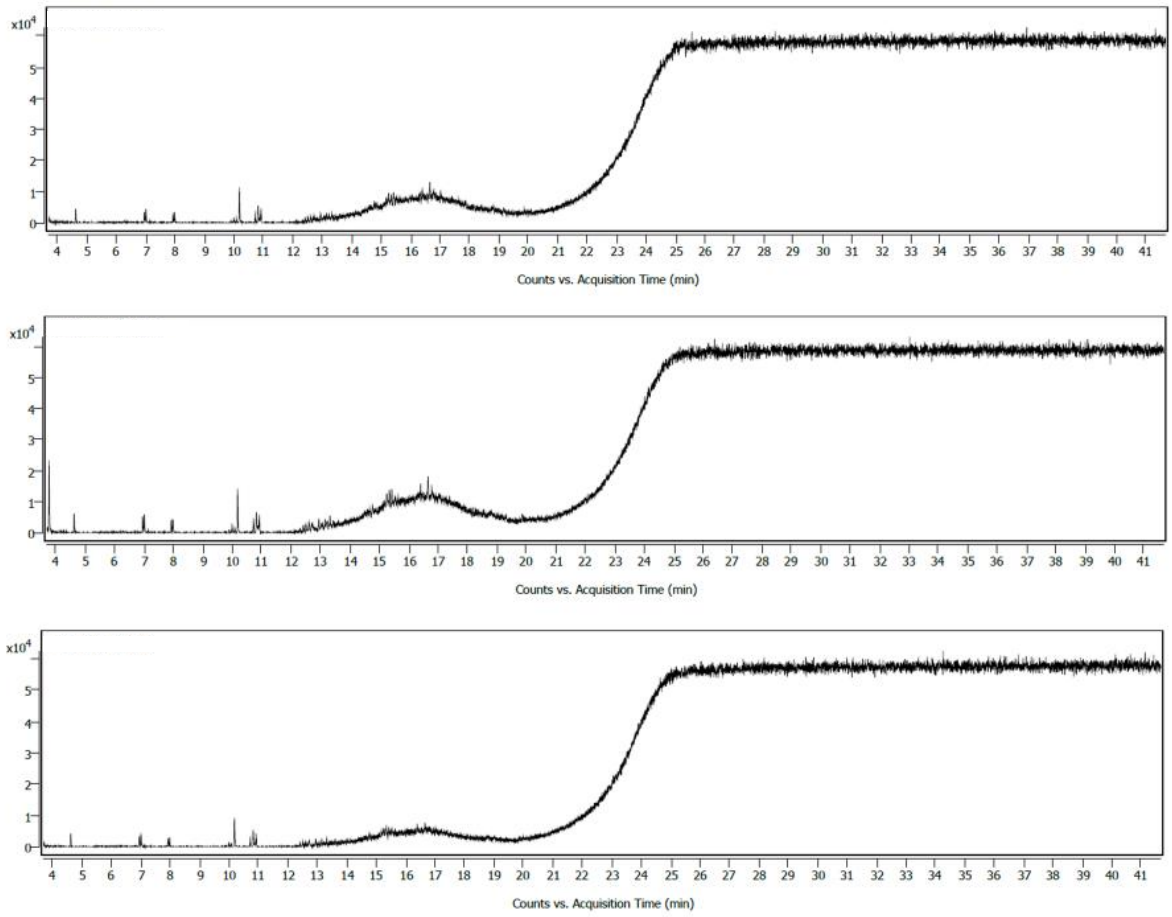

Figure A-8. Chromatogram comparison of diesel 3 (a, b, c), after 188 days. Retention time is on the x-axis and response signal is on the y-axis.

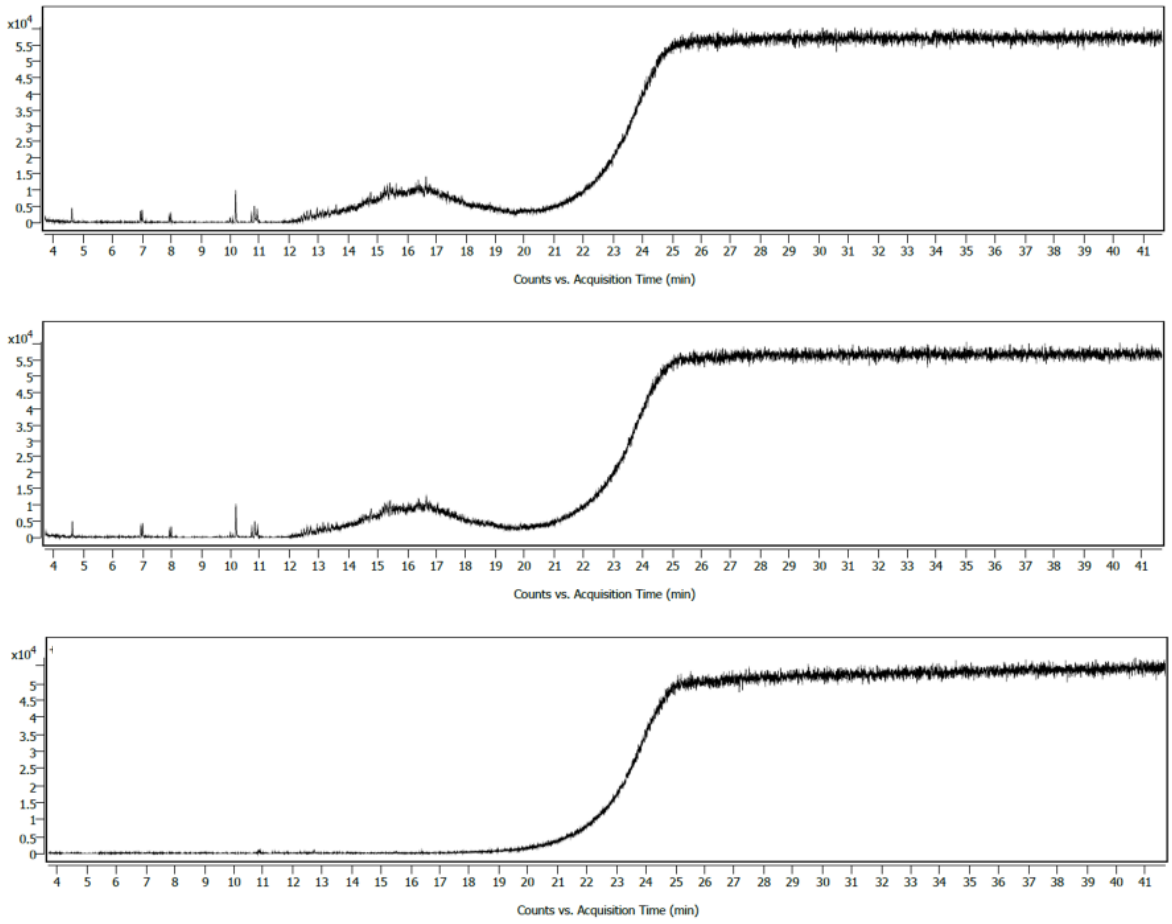

Figure A-9. Chromatogram comparison of diesel 4 (a, b, c), after 188 days. Retention time is on the x-axis of the graph and the response signal is on the y-axis.

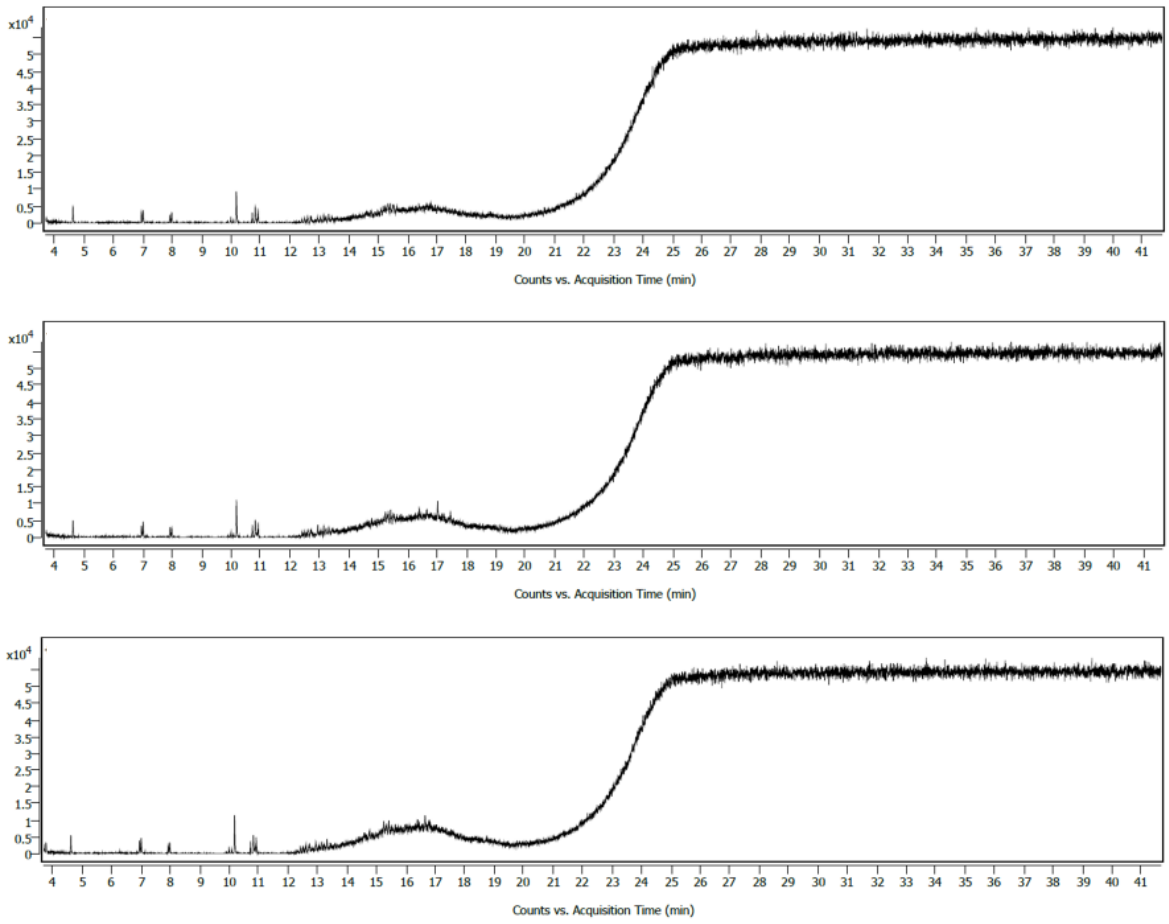

Figure A-10. Chromatogram comparison of diesel 5 (a, b, c), after 188 days. Retention time is on the x-axis of the graph and the response signal is on the y-axis.

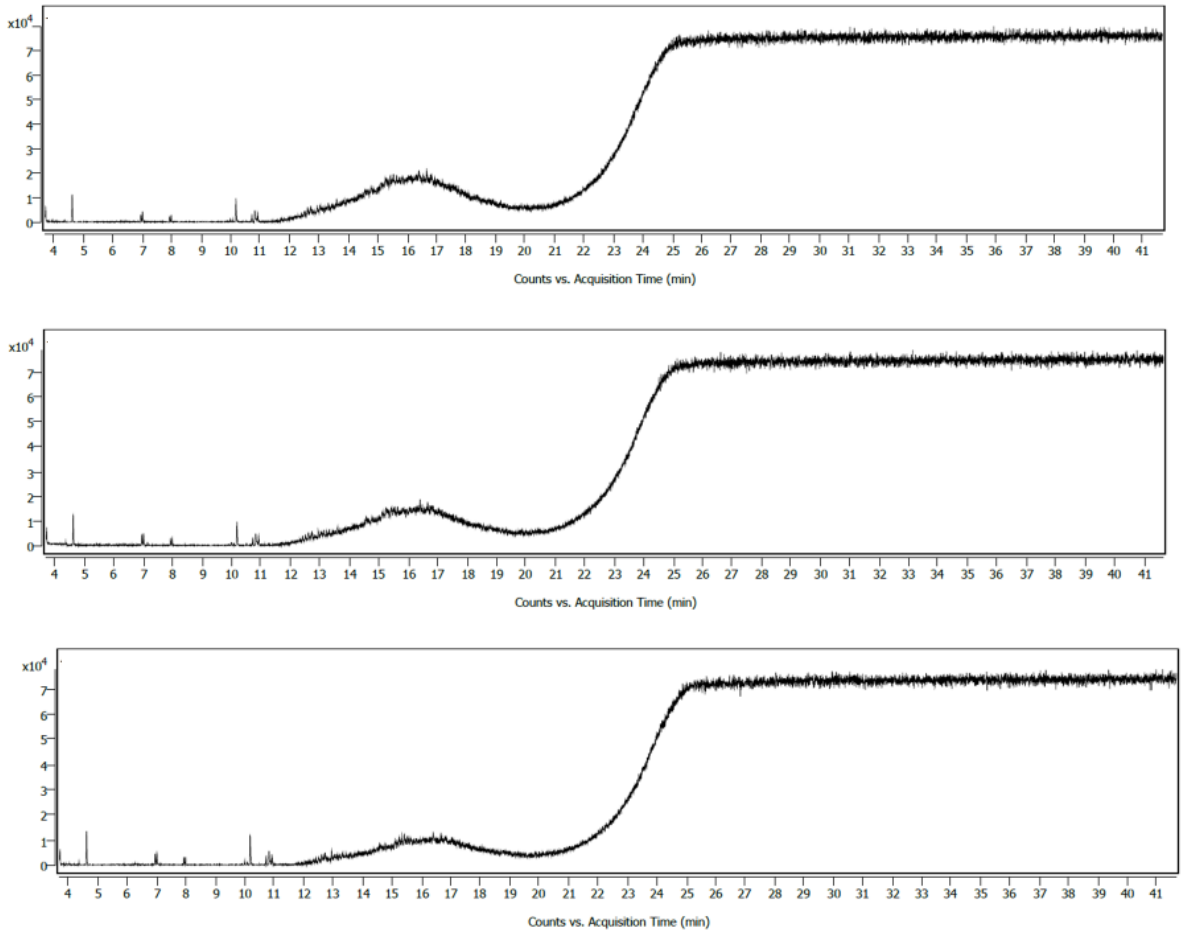

Figure A-11. Chromatogram comparison of primed T. versicolor 1 (a, b, c), after 188 days. Retention time is on the x-axis of the graph and the response signal is on the y-axis.

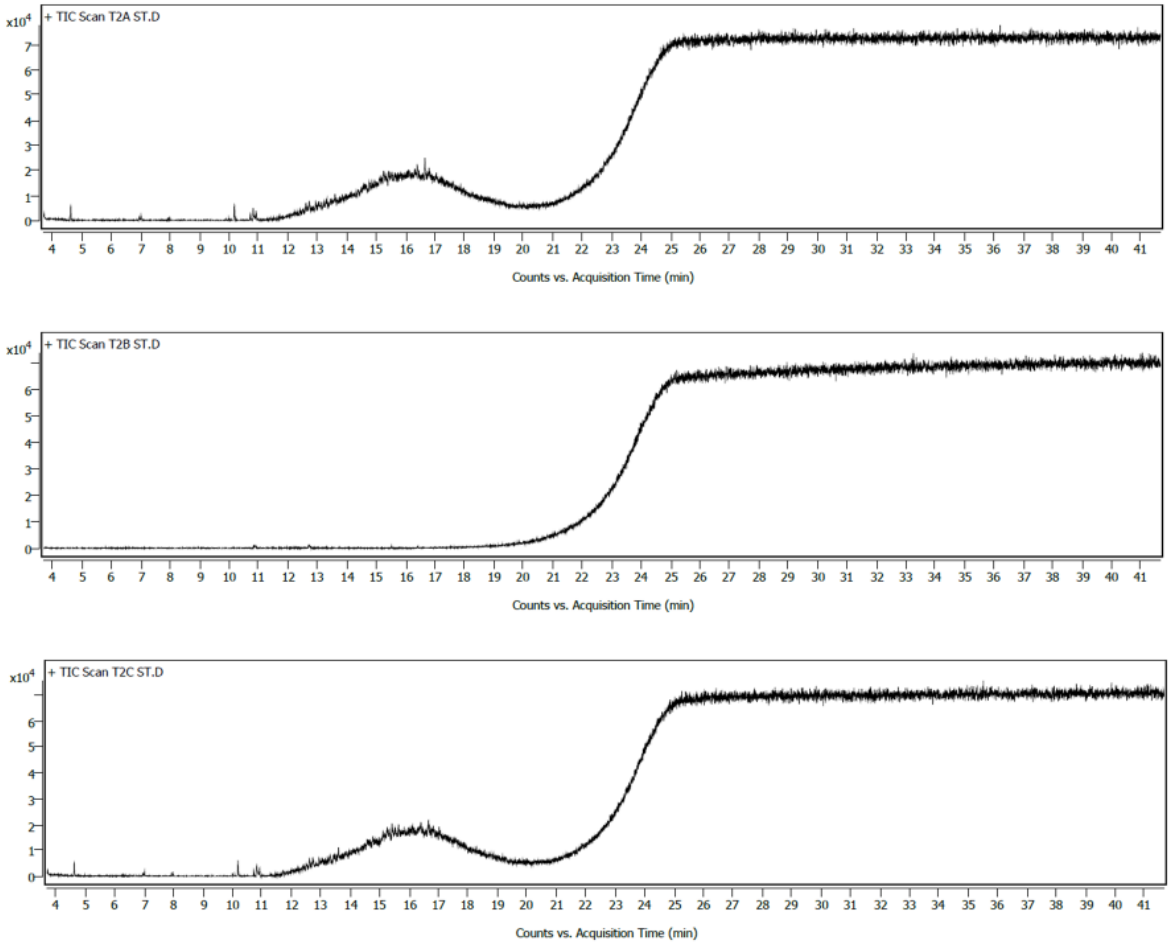

Figure A-12. Chromatogram comparison of primed T. versicolor Primed (a, b, c), after 188 days. Retention time is on the x-axis of the graph and the response signal is on the y-axis.

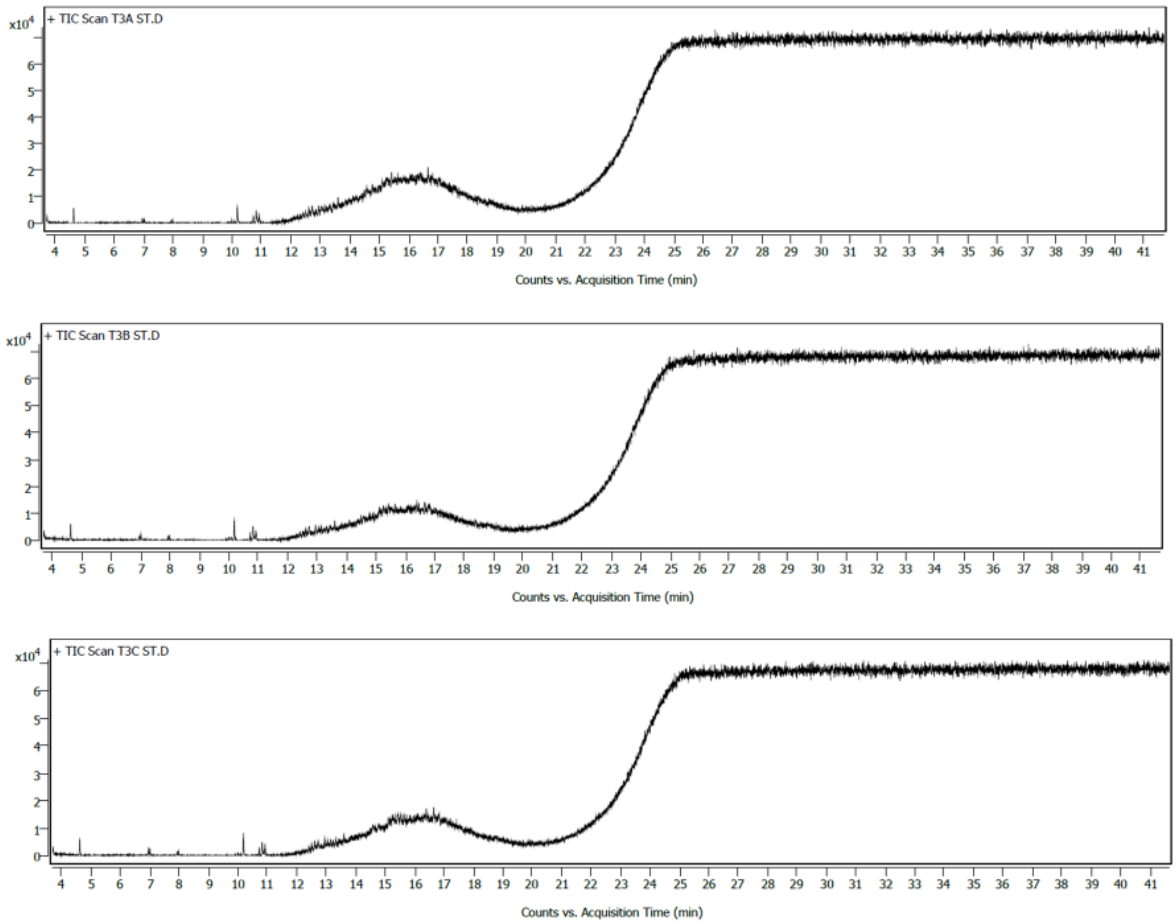

Figure A-13. Chromatogram comparison of primed T. versicolor 3 (a, b, c), after 188 days. Retention time is on the x-axis of the graph and the response signal is on the y-axis.

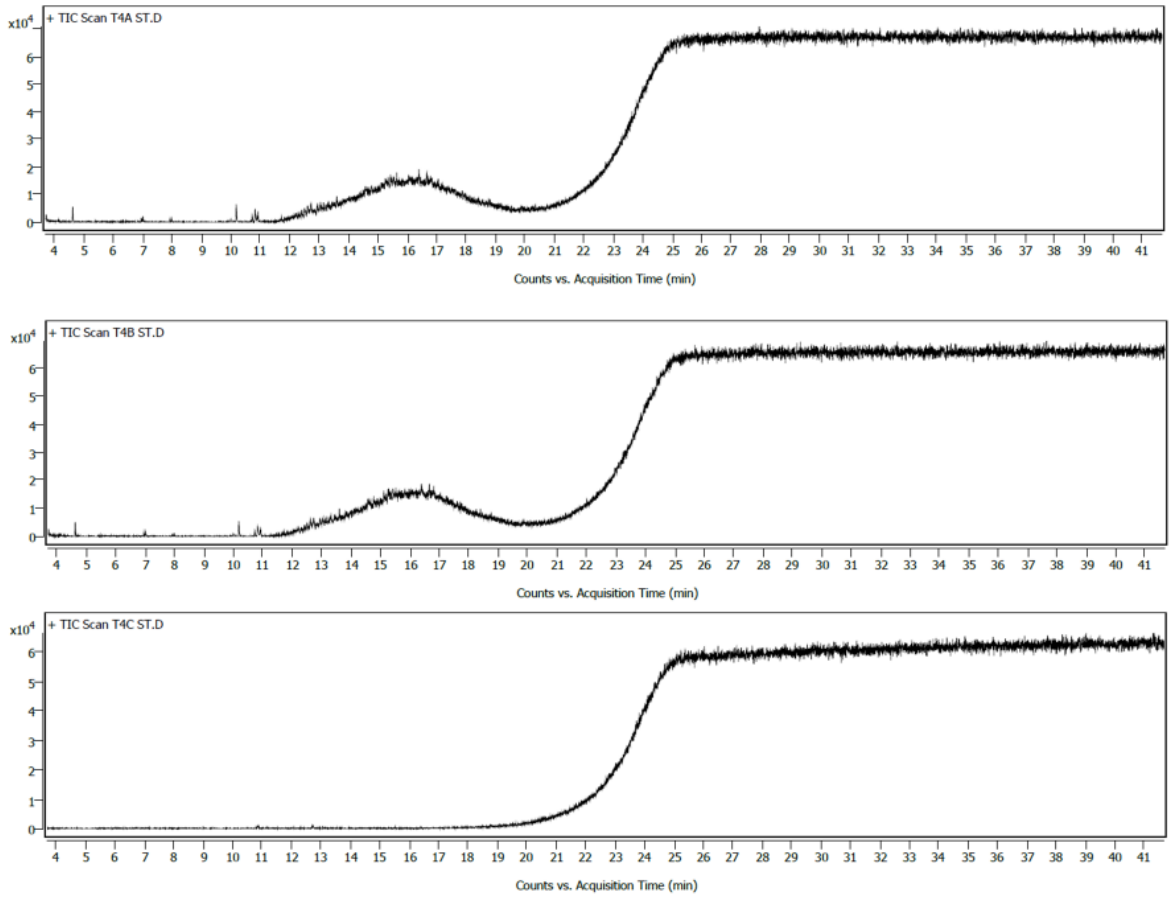

Figure A-14. Chromatogram comparison of primed T. versicolor 4 (a, b, c), after 188 days. Retention time is on the - axis and response signal is on the y-axis

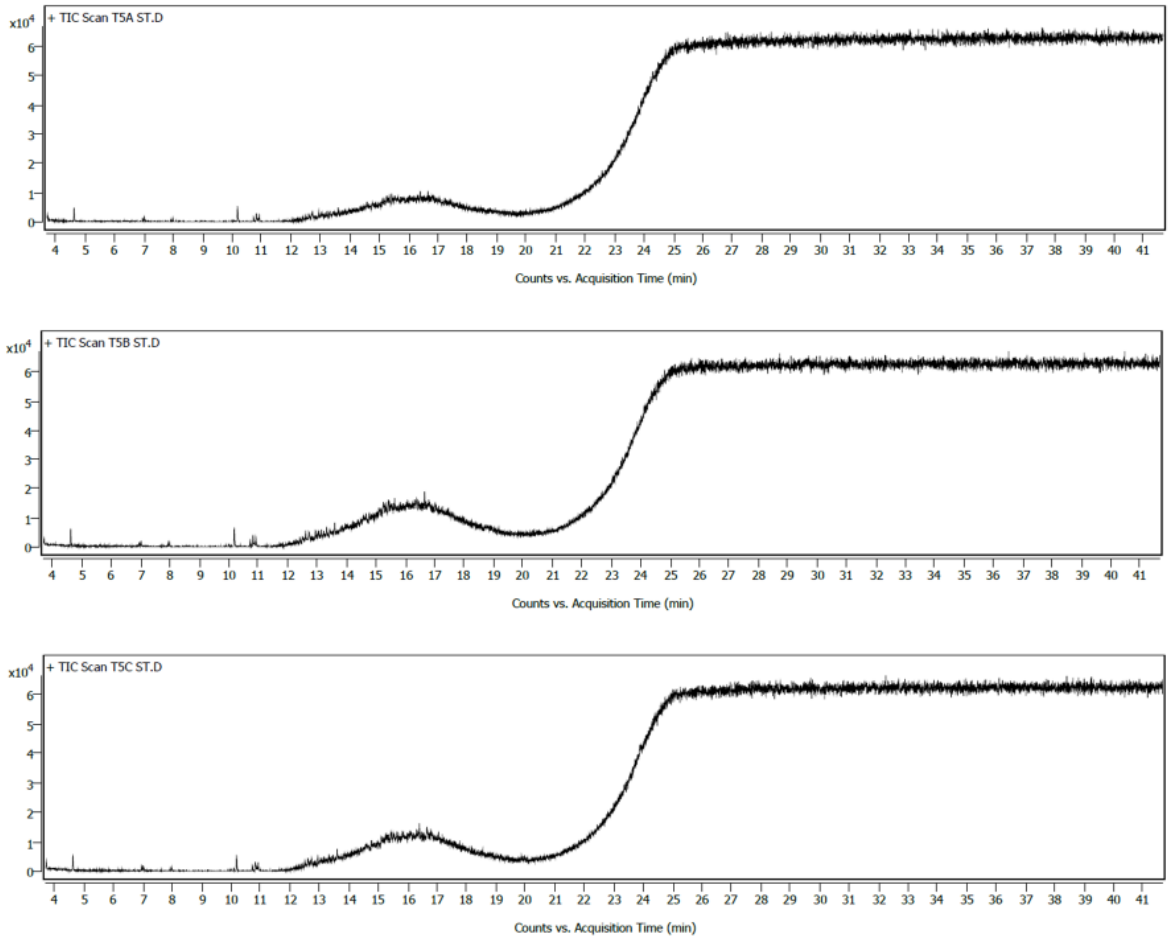

Figure A-15. Chromatogram comparison of primed T. versicolor 5 (a, b, c), after 188 days. Retention time is on the x-axis of the graph and the response signal is on the y-axis.

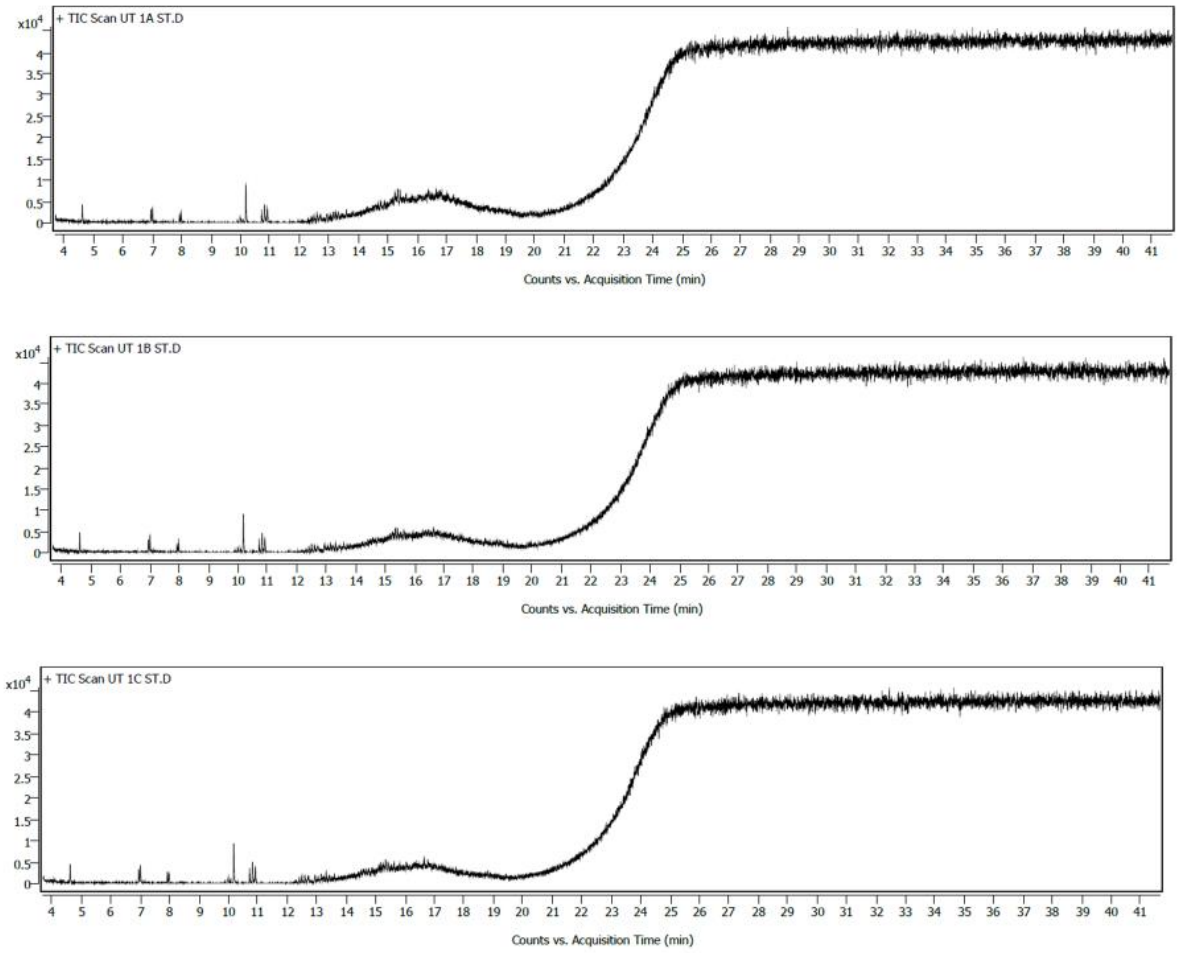

Figure A-16. Chromatogram comparison of Un-primed T. versicolor 1 (a, b, c), after 188 days. Retention time is on the x-axis of the graph and the response signal is on the y-axis.

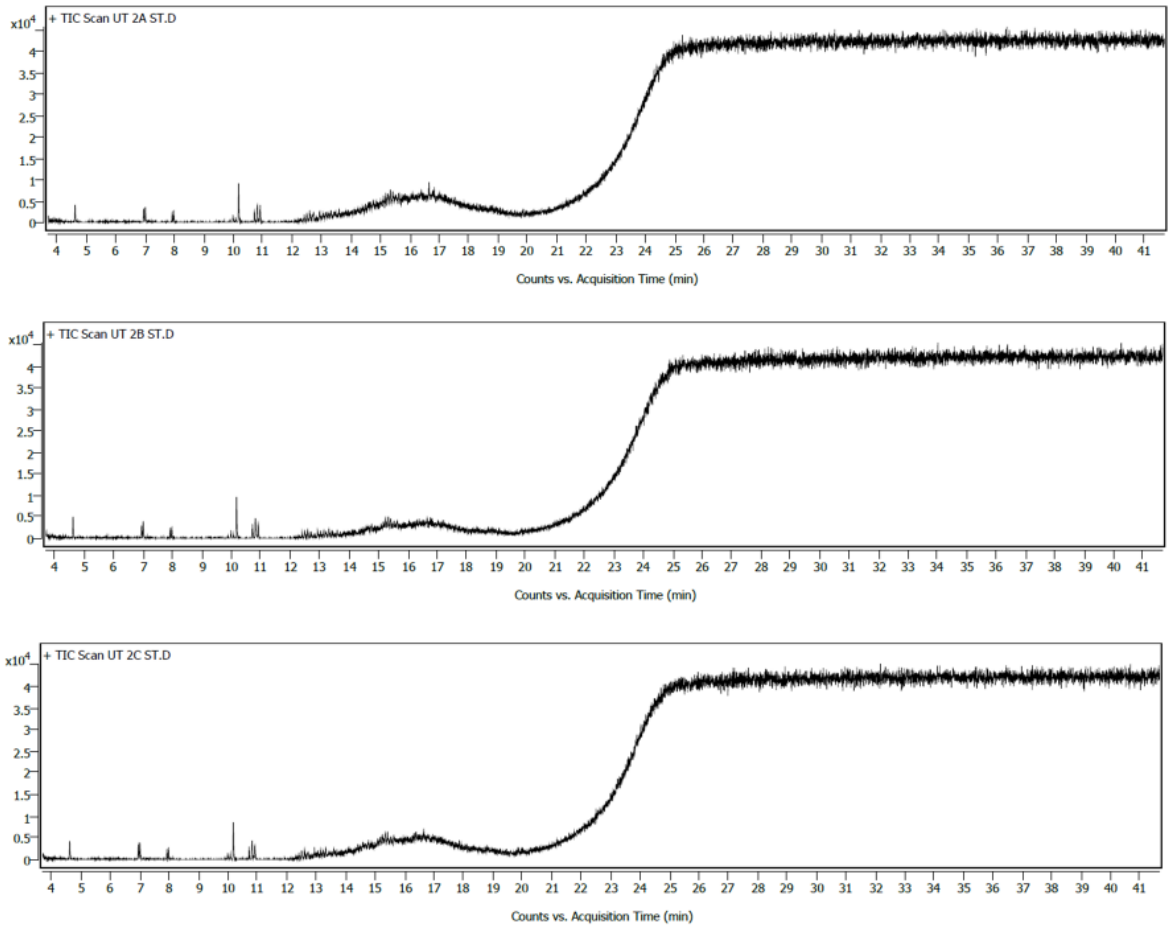

Figure A-17. Chromatogram comparison of un-primed T. versicolor 2 (a, b, c), after 188 days. Retention time is on the x-axis of the graph and the response signal is on the y-axis.

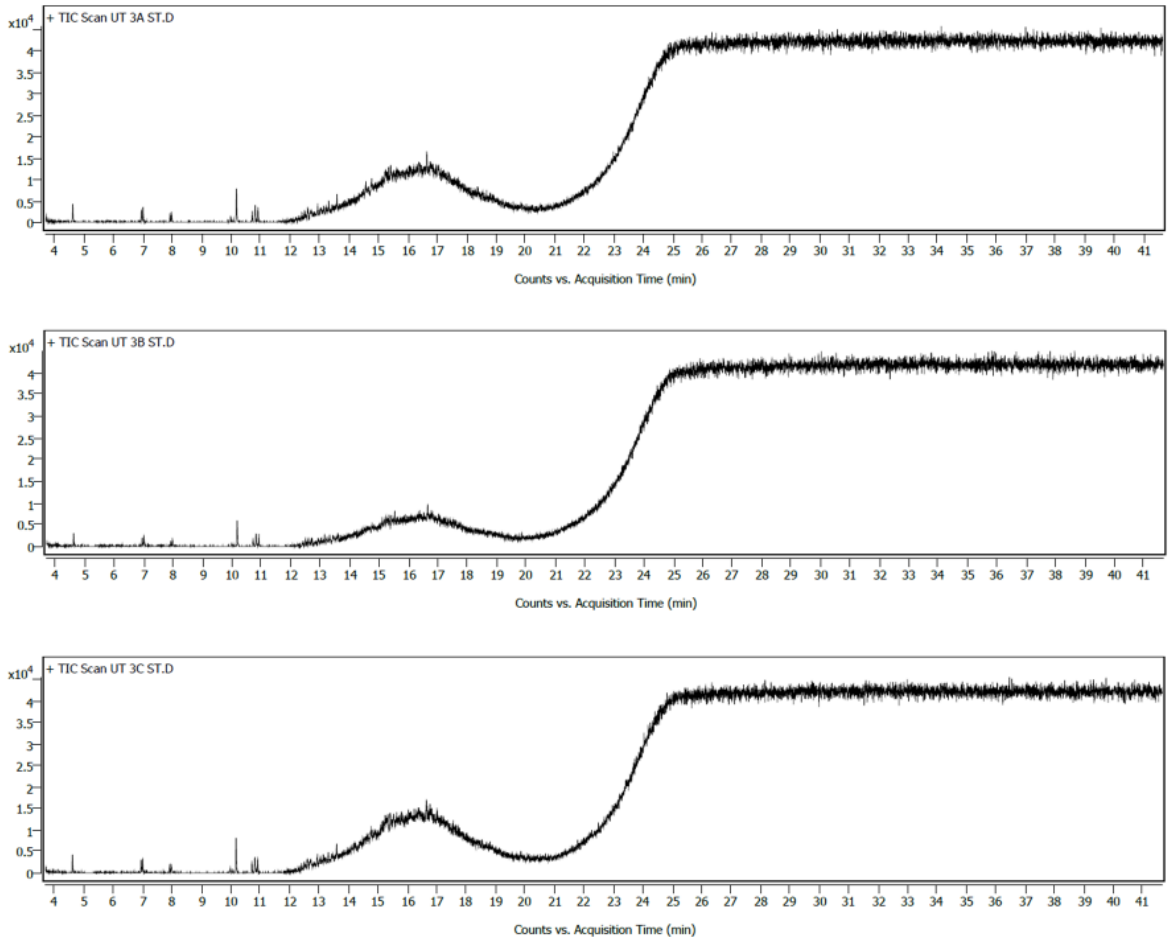

Figure A-18. Chromatogram comparison of Un-primed T. versicolor 3 (a, b, c), after 188 days. Retention time is on the x-axis of the graph and the response signal is on the y-axis.

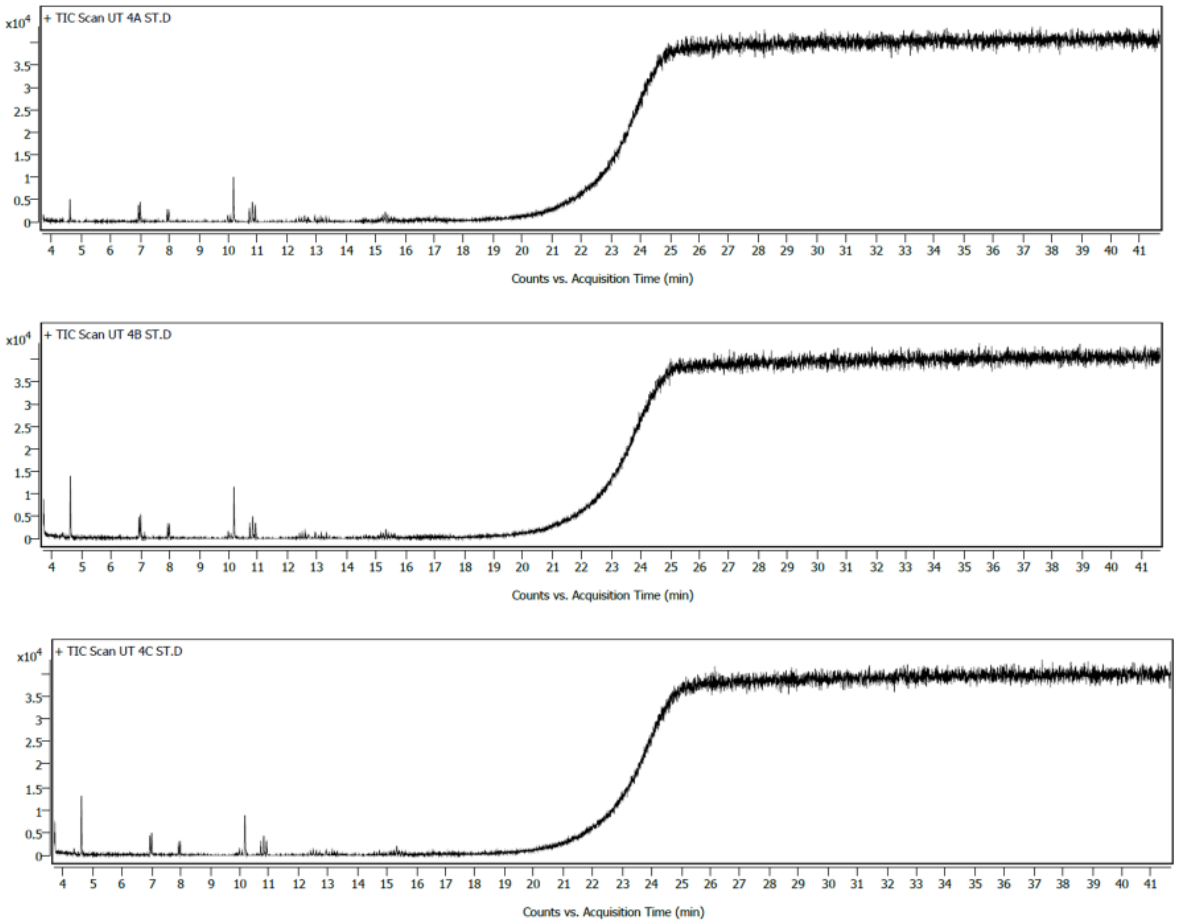

Figure A-19. Chromatogram comparison of un-primed T. versicolor 4 (a, b, c), after 188 days. Retention time is on the x-axis of the graph and the response signal is on the y-axis.

Appendix B- Chromatograms Effectiveness of biodegradation using trained and untrained mushrooms cultured in liquid culture

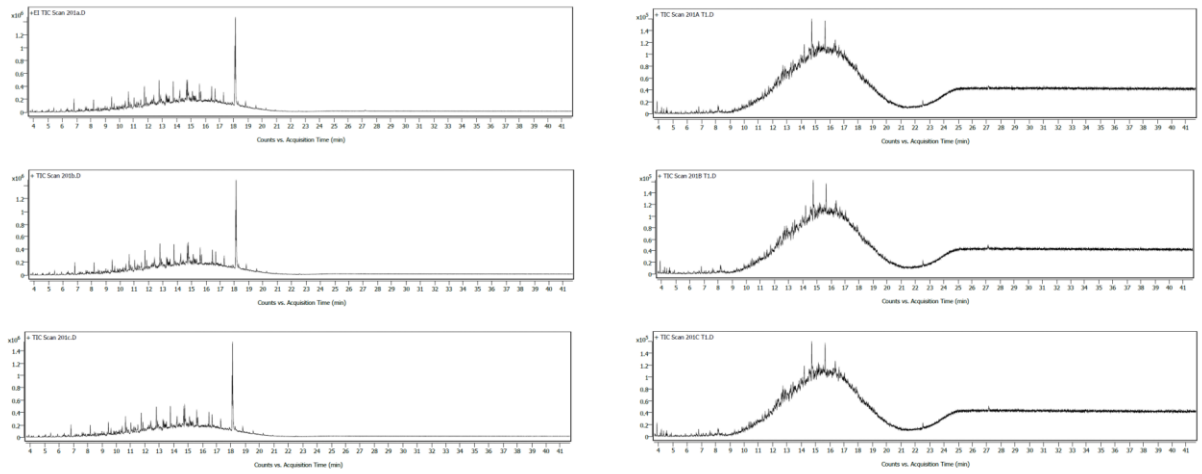

Figure B-1. Comparison of chromatograms diesel 1 (a, b, c), time 0 (left) and time 1 (right. Retention time is on the x-axis of the graph and the response signal is on the y-axis.

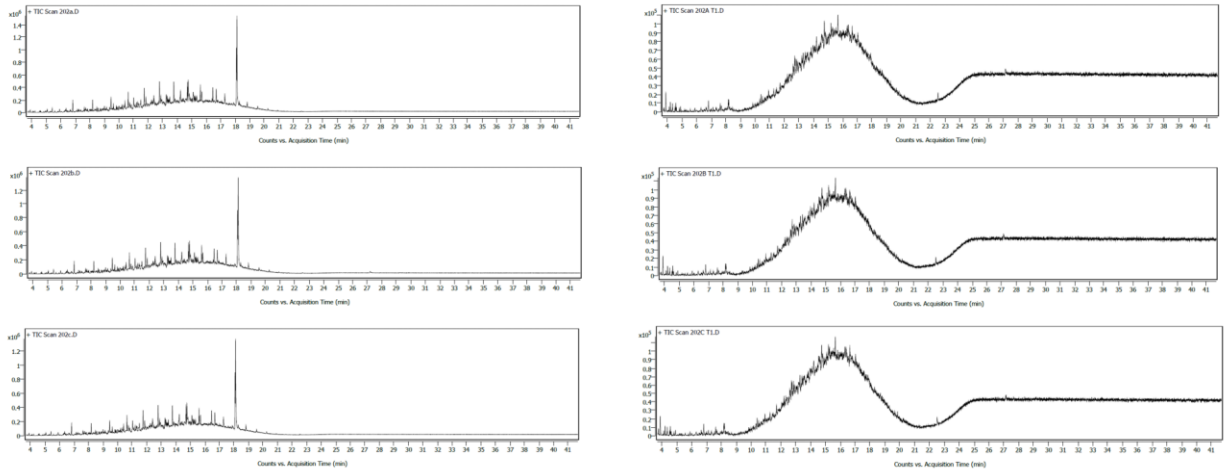

Figure B-2. Comparison of chromatograms diesel 2 (a, b, c), time 0 (left) and time 1 (right). Retention time is on the x-axis of the graph and the response signal is on the y-axis.

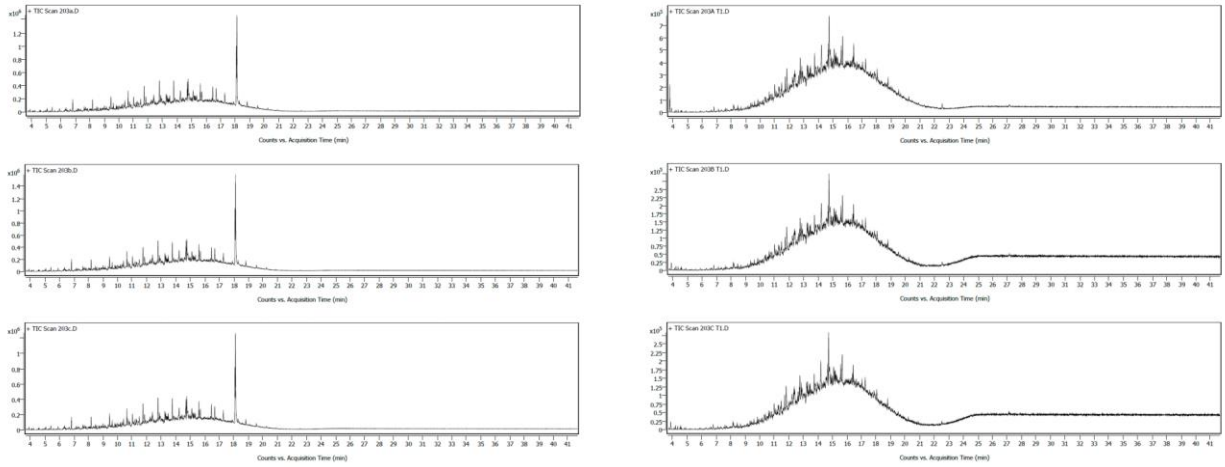

Figure B-3. Comparison of chromatograms diesel 3 (a, b, c), Time 0 (left) and Time 1 (right). Retention time is on the x-axis of the graph and the response signal is on the y-axis.

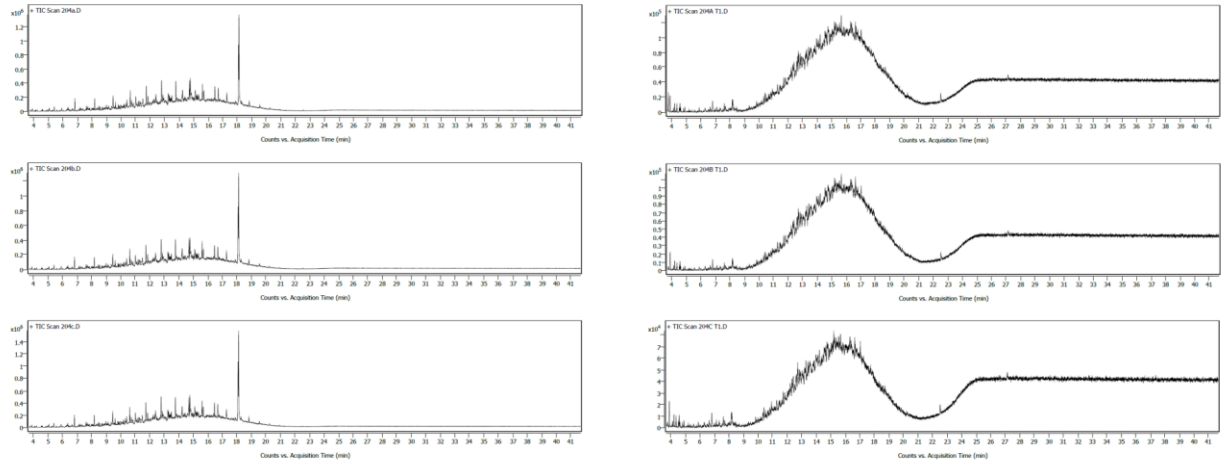

Figure B-4. Comparison of chromatograms diesel 4 (a, b, c), time 0 (left) and time 1 (right). Retention time is on the x-axis of the graph and the response signal is on the y-axis.

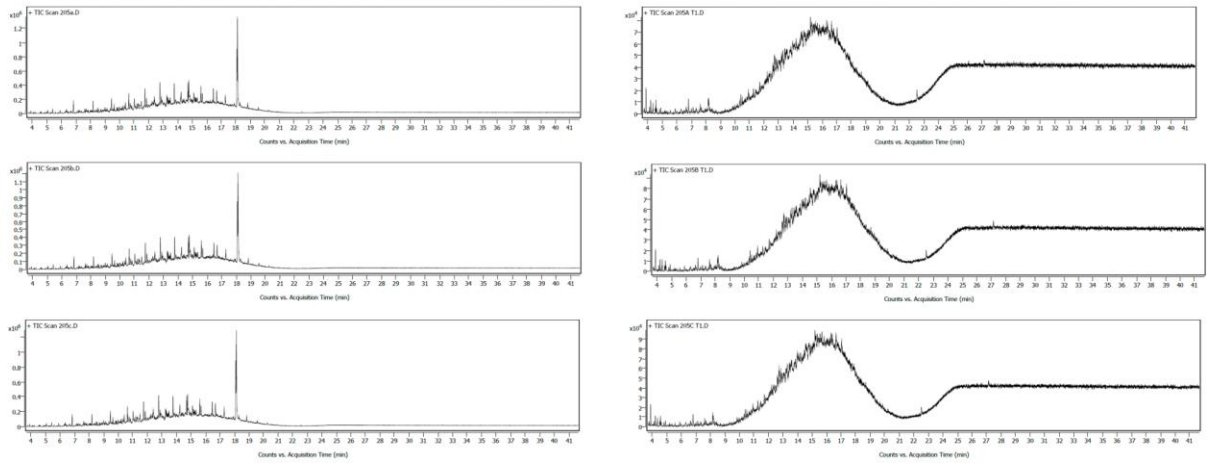

Figure B-5. Comparison of chromatograms diesel 5 (a, b, c), time 0 (left) and time 1 (right). Retention time is on the x-axis of the graph and the response signal is on the y-axis.

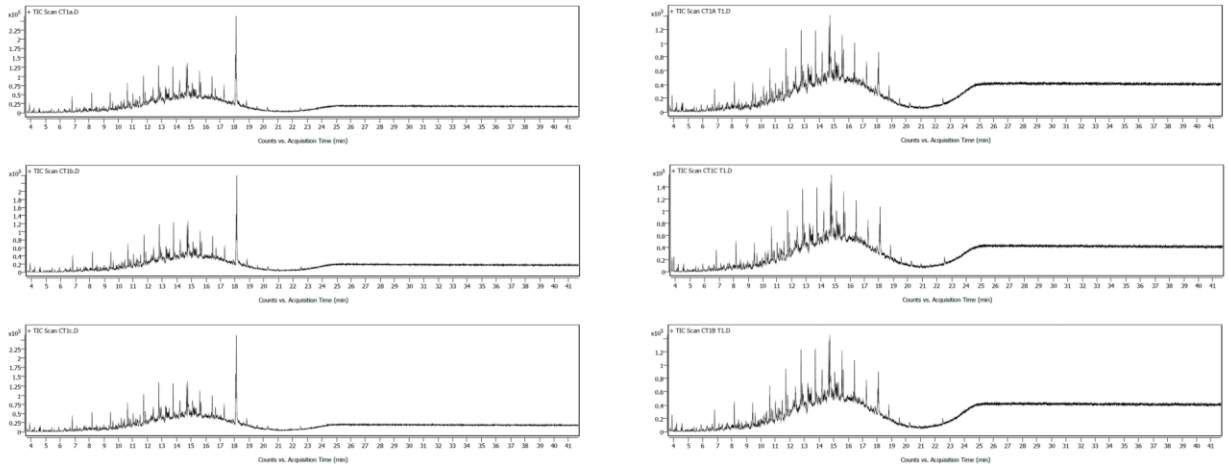

Figure B-6. Comparison of chromatograms control primed 1 (a, b, c), time 0 (left) and time 1 (right. Retention time is on the x-axis of the graph and the response signal is on the y-axis.

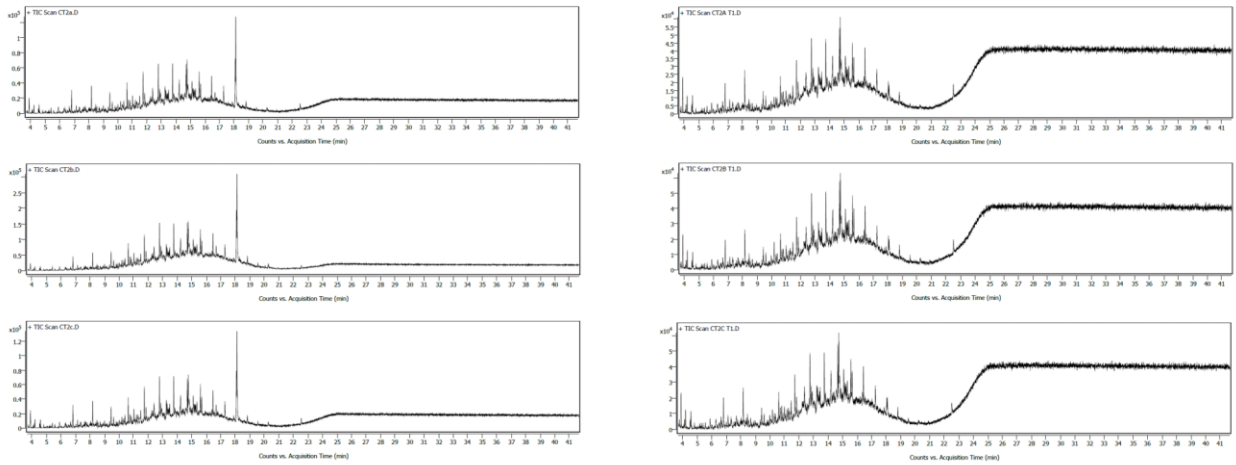

Figure B-7. Comparison of chromatograms control primed 2 (a, b, c), time 0 (left) and time 1 (right). Retention time is on the x-axis of the graph and the response signal is on the y-axis.

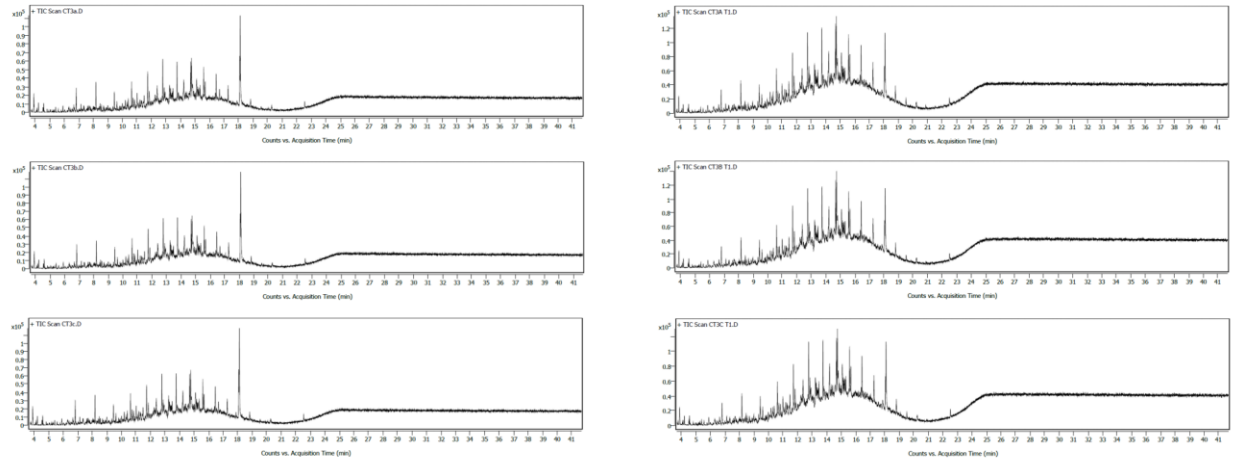

Figure B-8. Comparison of chromatograms control primed 3 (a, b, c), time 0 (left) and time 1 (right). Retention time is on the x-axis of the graph and the response signal is on the y-axis

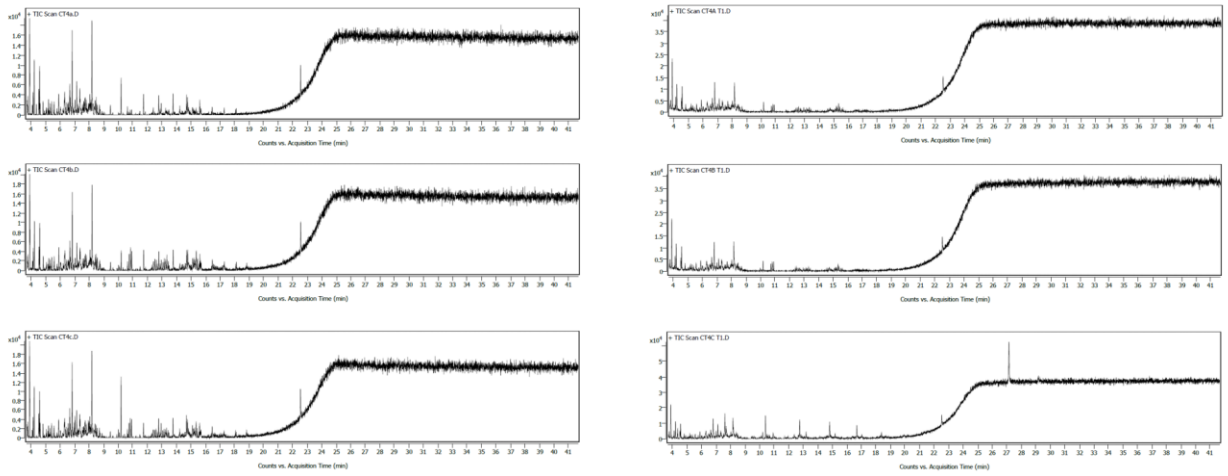

Figure B-9. Comparison of chromatograms control primed 4 (a, b, c), time 0 (left) and time 1 (right. Retention time is on the x-axis of the graph and the response signal is on the y-axis.

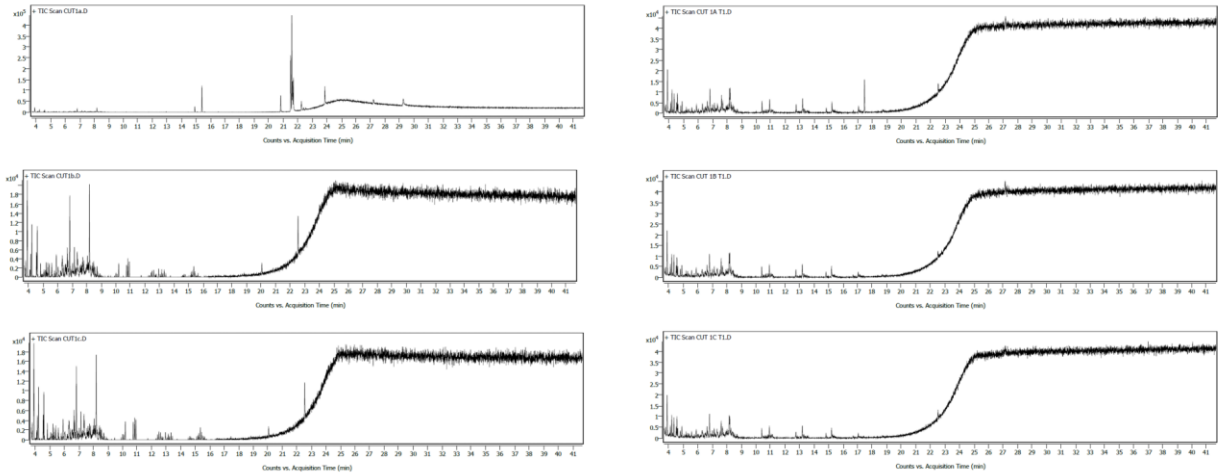

Figure B-10. Comparison of chromatograms control un-primed 1 (a, b, c), time 0 (left) and time 1 (right). Retention time is on the x-axis of the graph and the response signal is on the y-axis.

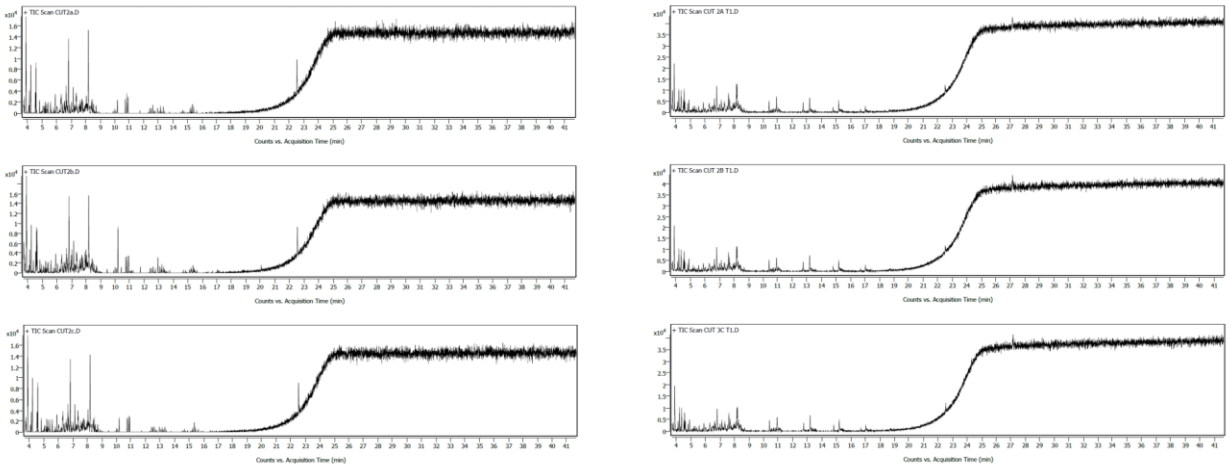

Figure B-11. Comparison of chromatograms control un-primed 2 (a, b, c), time 0 (left) and time 1 (right). Retention time is on the x-axis of the graph and the response signal is on the y-axis.

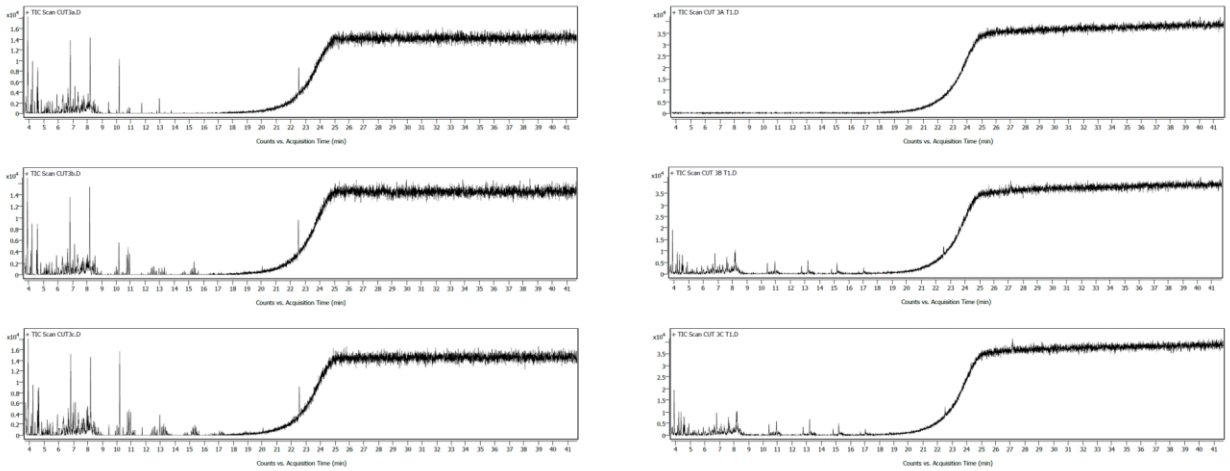

Figure B-12. Comparison of chromatograms control un-primed 3 (a, b, c), time 0 (left) and time 1 (right. Retention time is on the x-axis of the graph and the response signal is on the y-axis.

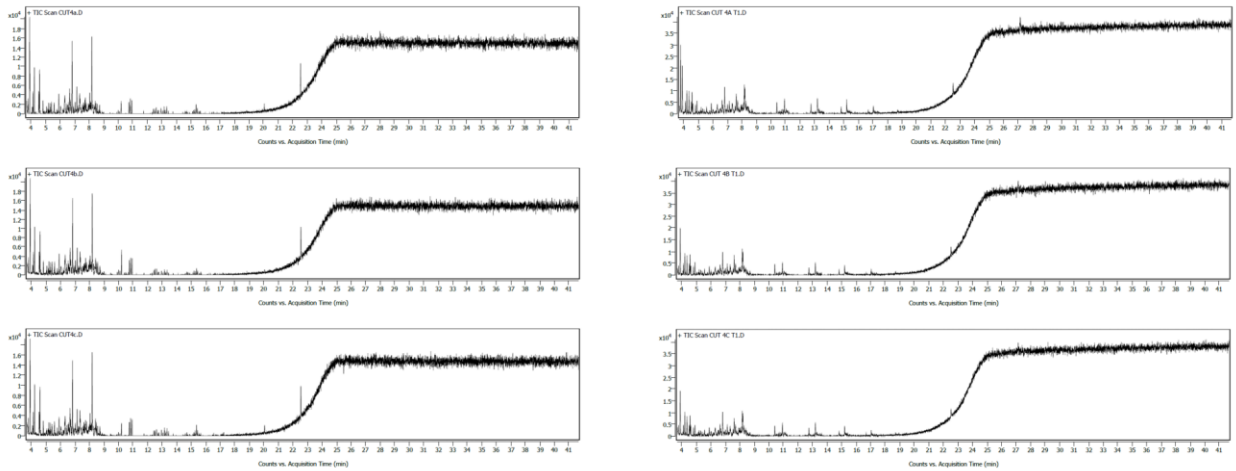

Figure B-13. Comparison of chromatograms control un-primed 4 (a, b, c), time 0 (left) and time 1 (right). Retention time is on the x axis and response signal is on the y axis.

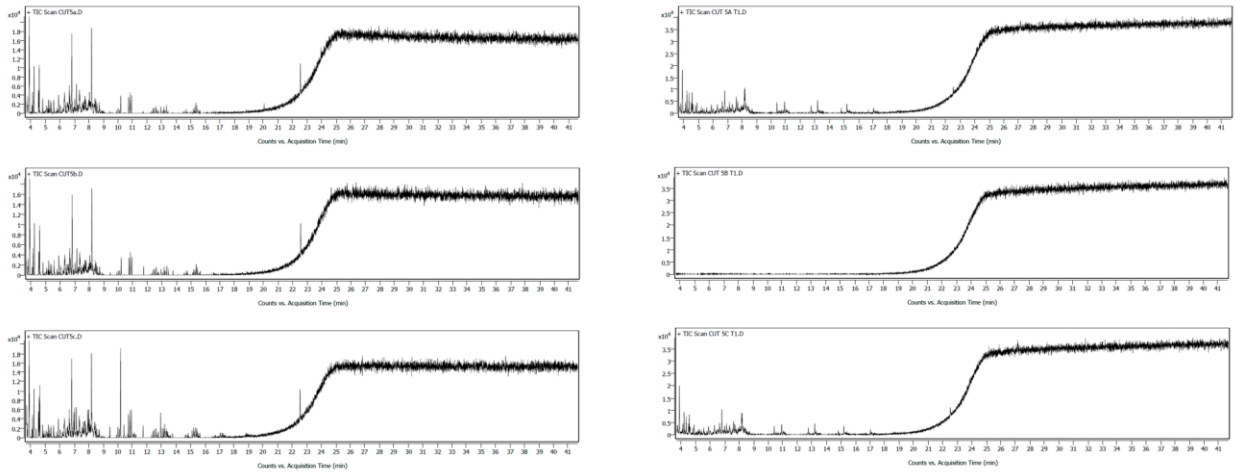

Figure B-14. Comparison of chromatograms control un-primed 5 (a, b, c), time 0 (left) and time 1 (right. Retention time is on the x-axis of the graph and the response signal is on the y-axis.

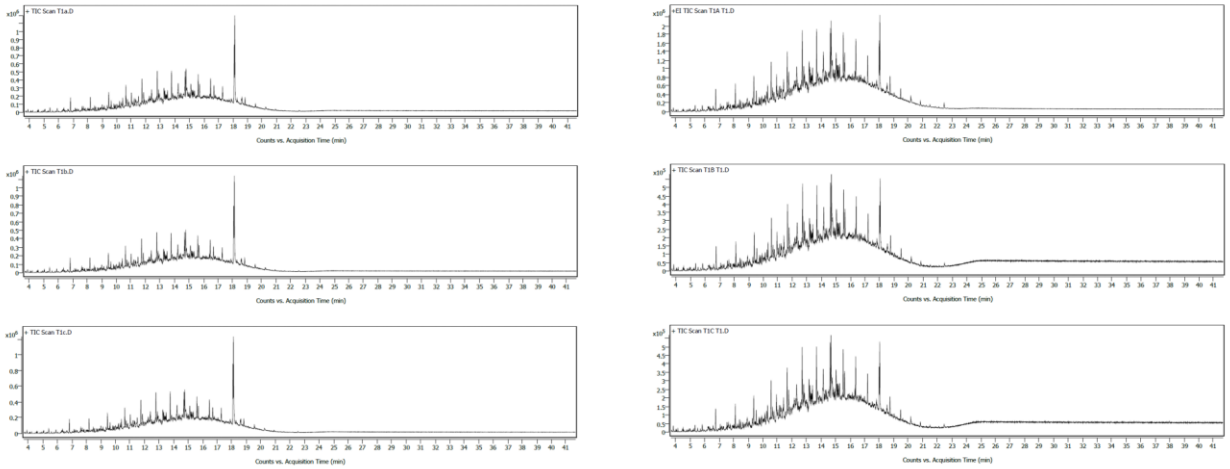

Figure B-15. Comparison of chromatograms primed 1 (a, b, c), time 0 (left) and time 1 (right). Retention time is on the x-axis and response signal is on the y-axis.

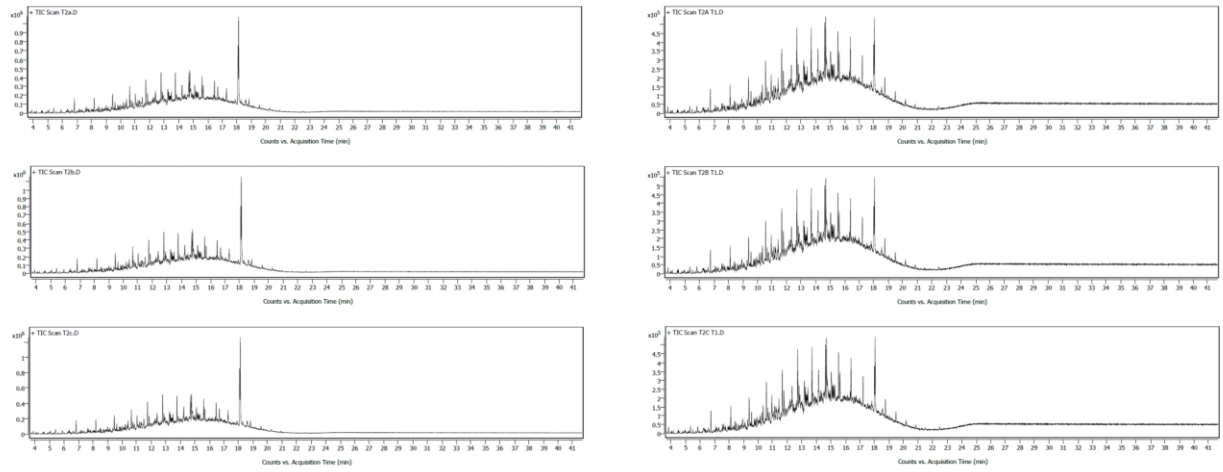

Figure B-16. Comparison of chromatograms primed 2 (a, b, c), time 0 (left) and time 1 (right). Retention time is on the x-axis and response signal is on the y-axis.

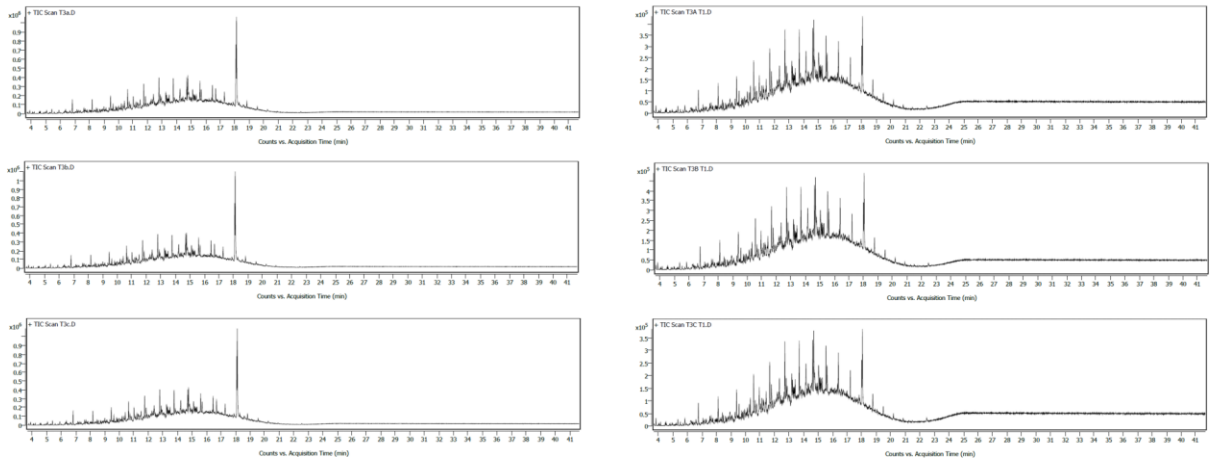

Figure B-17. Comparison of chromatograms primed 3 (a, b, c), time 0 (left) and time 1 (right). Retention time is on the x-axis and response signal is on the y-axis.

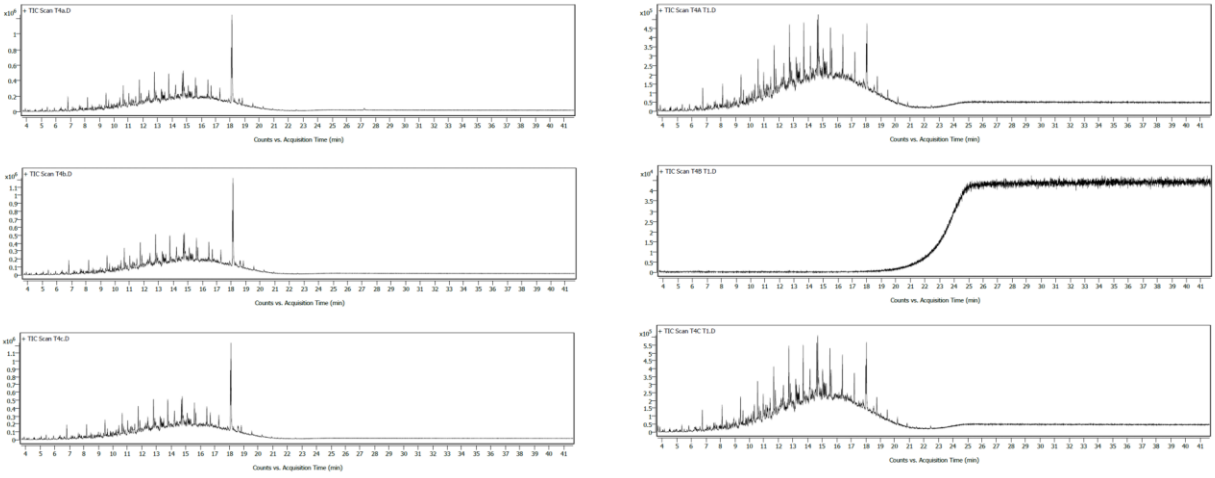

Figure B-18. Comparison of chromatograms primed 4 (a, b, c), time 0 (left) and time 1 (right). Retention time is on the x-axis of the graph and the response signal is on the y-axis.

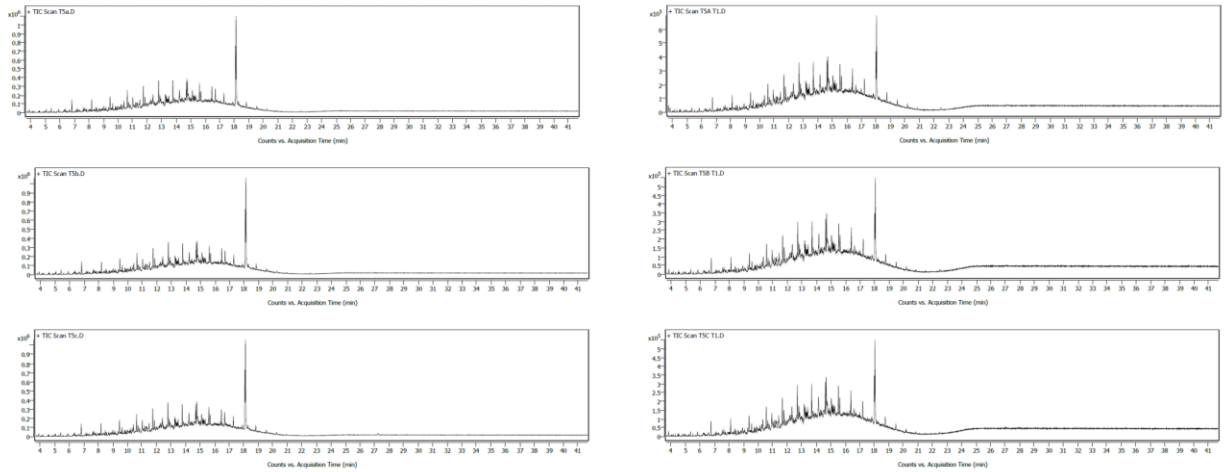

Figure B-19. Comparison of chromatograms primed 5 (a, b, c), time 0 (left) and time 1 (right). Retention time is on the x-axis of the graph and the response signal is on the y-axis.

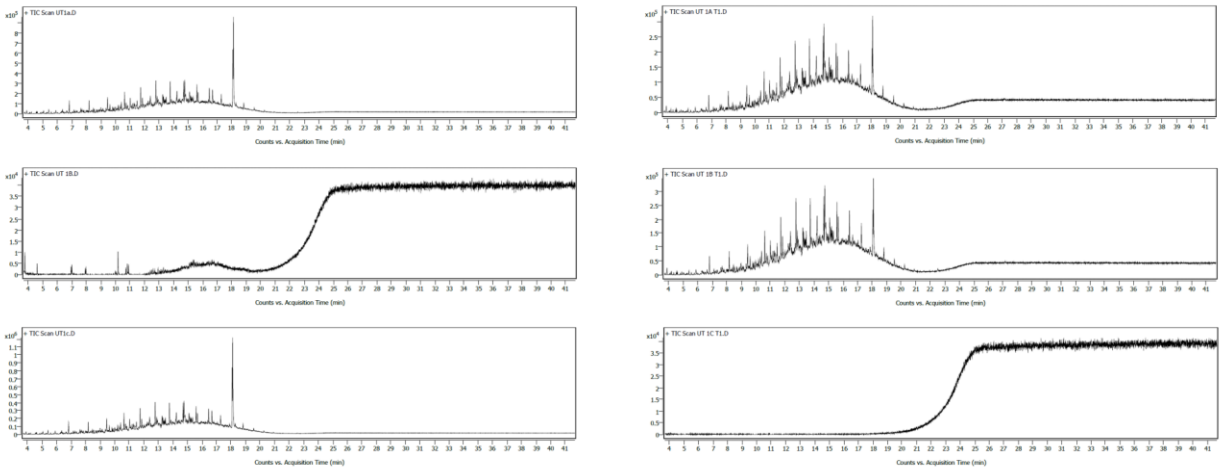

Figure B-20. Comparison of chromatograms un-primed 1 (a, b, c), time 0 (left) and time 1 (right). Retention time is on the x-axis of the graph and the response signal is on the y-axis.

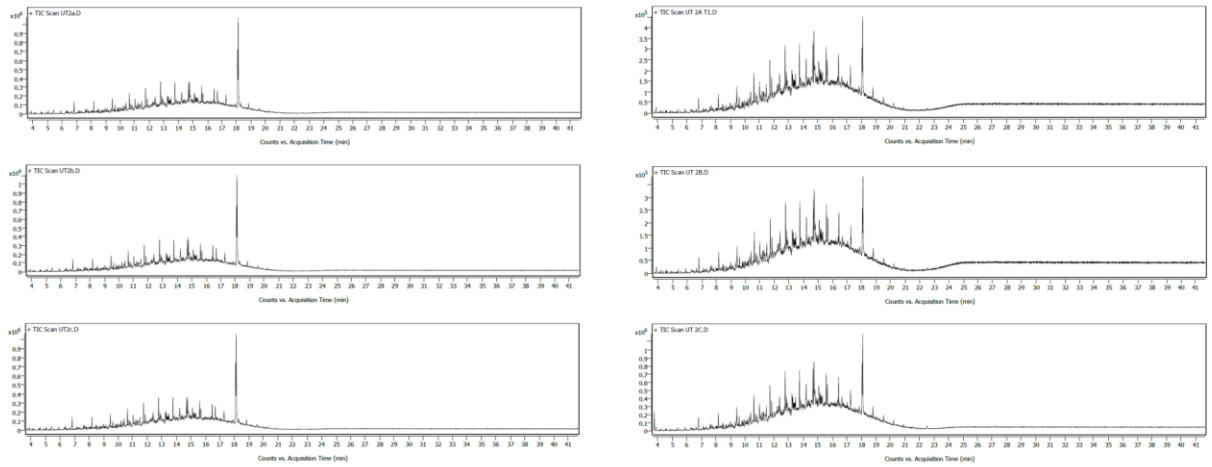

Figure B-21. Comparison of chromatograms un-primed 2 (a, b, c), time 0 (left) and time 1 (right). Retention time is on the x-axis of the graph and the response signal is on the y-axis.

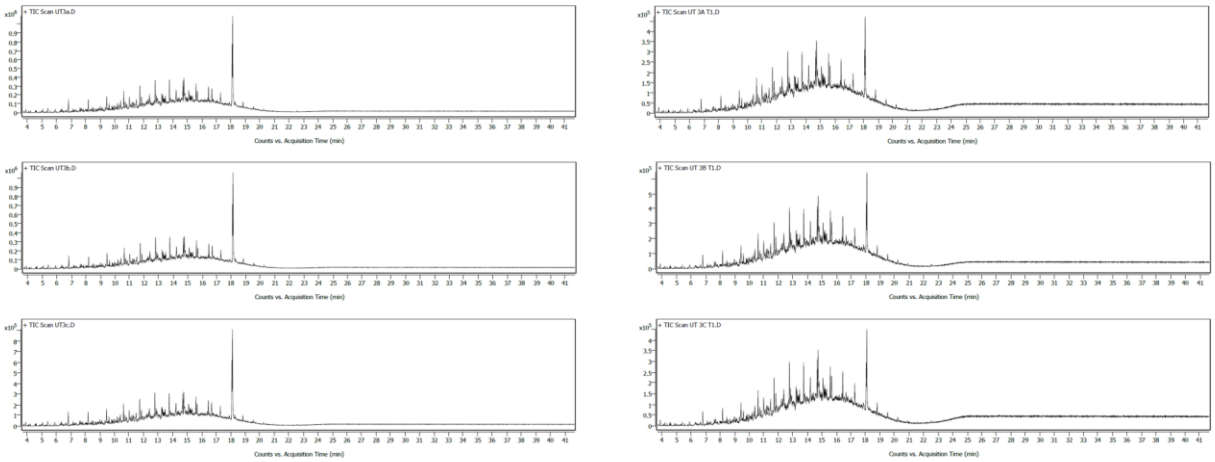

Figure B-22. Comparison of chromatograms un-primed 3 (a, b, c), time 0 (left) and time 1 (right). Retention time is on the x-axis of the graph and the response signal is on the y-axis.

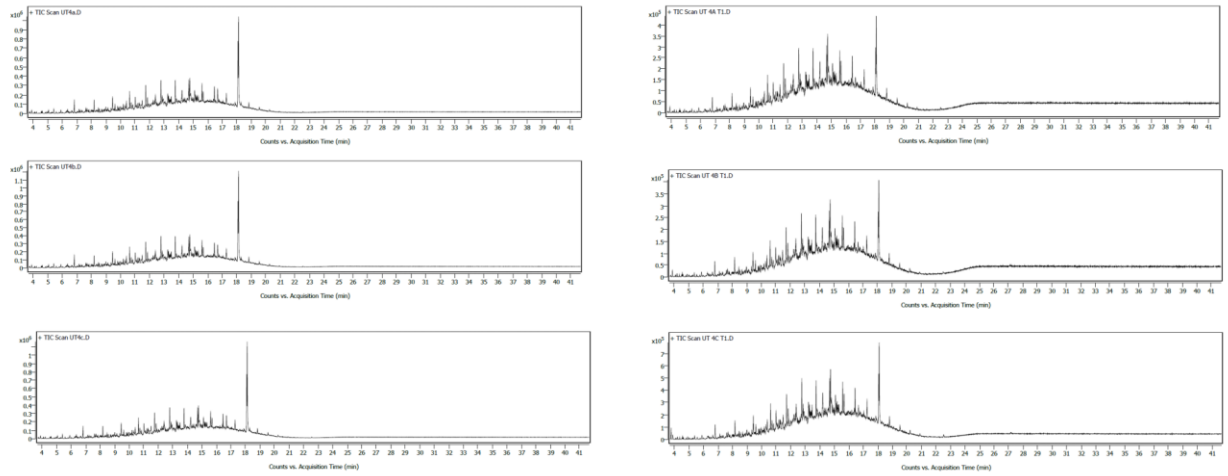

Figure B-23. Comparison of chromatograms un-primed 4 (a, b, c), time 0 (left) and time 1 (right. Retention time is on the x-axis of the graph and the response signal is on the y-axis.

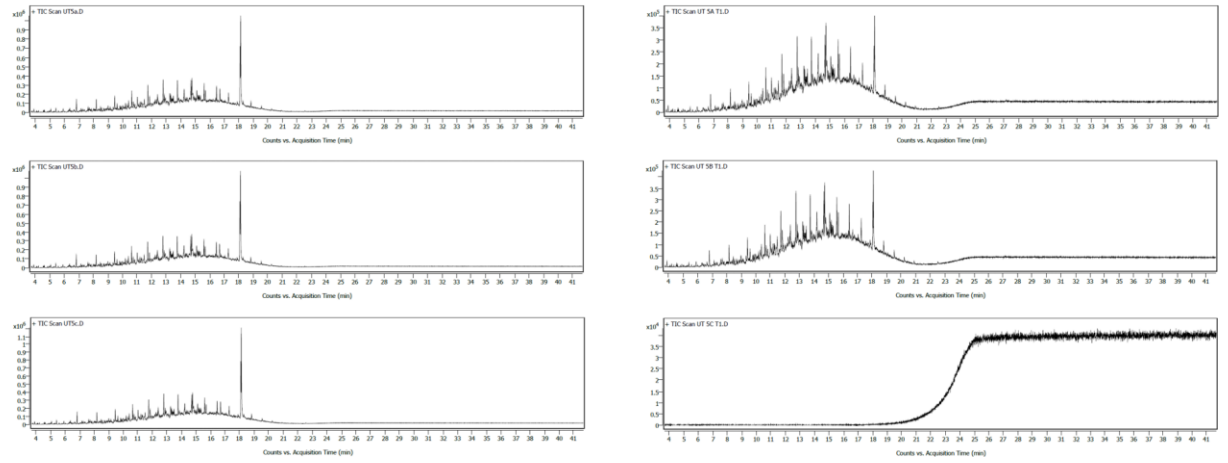

Figure B-24. Comparison of chromatograms un-primed 5 (a, b, c), time 0 (left) and time 1 (right). Retention time is on the x-axis of the graph and the response signal is on the y-axis.

Appendix C- Grain Preparation

Making Grain Spawn, G1 (Cotter 2014): Pre-cooking Method

Supplies/Equipment

- Per 1- L Jar 200 g dry weight organic grains
- 1 g gypsum
- 1-L Mason jars & Lids amended (filter and injections ports)
- Pressure cooker
- Stove
- 70% isopropyl alcohol
- 1 liquid Syringe (e.g. *Pleurotus ostreatus, Trametes versicolor*)
- 1 Alcohol lamp or flow hood
- 1 Lighter
- 1 Particulate mask
- Paper towel
- Scale
- Scoop for grains
- Strainer
- Spoon
- Nitrile gloves

Instructions

The following was prepared on September 10, 2018. The method is adapted from Cotter (2014). Mills organic faro grains was rinsed with cold water until water ran clear. The grains were covered with non-chlorinated water brought them to a boil. They were promptly removed

from heat and left for 5 minutes, until the grains were al-dente. Grains were slimy, so I rinsed with cold water and then pored on boiling water again to allow for some of the moisture to evaporate off.

Seven sterilized 1-L mason jars were filled with 350 g (wet weight) of cooked grains and 2 g of gypsum. The mason jars were shaken until the gypsum thoroughly mixed into the grains until. The lids were Closed the amended lids (with syringe filters and injections ports. Then sterilized in pressure cooker at 15 psi for 1 hour. The grains were cooled overnight before inoculation.

On September 11, 2018 I used semi-sterile conditions to inoculate the prepared grains with T. versicolor liquid culture (LC). The LC was purchased from MycoBoutique in Montreal, QC. Clean cloths, mask and gloved were worn throughout the experiment. The working bench and all equipment were sterilized with 70% isopropyl alcohol.

The inoculation was done in a sterilized plastic box tipped on its side to shield against air born microbes that could come in on the sides and from above. An alcohol lamp was used to sterilize the immediate working area. The LC syringe was sterilized by holding the needle in the alcohol lamp flame until it was red hot. The needle tip was cooled with an alcohol swab and injected the four 1-L mason jars with 2ml of *Trametes versicolor* liquid culture into the prepared grains. Sterilization of the syringe needle was done before the inoculation of all jars. The inoculated mason jars were shaken to jars to distribute inoculum and then stored in optimal temperature range of 65-75 F (18-24 C).

Let inoculated grain sat undisturbed until it has been completely colonized (approximately 21 days). After the first week the grain was broken up by taping the mason jars to redistribute mycelium growth.

Transferring Grain to Grain (G2):

Supplies/equipment

- 10 mason jars with amended lids (filters)
- Inoculated 1-L mason jars *T. versicolor*
- Inoculated 1-L mason jars with *P. ostreatus*

The Rest of supplies are the same as above

Instructions

Step 1: Follow steps 1-9 to sterilize additional grains. Each colonized 1-L grain jars can inoculate 10 more 1-L jars (x10).

5 1-L mason jars inoculated with *Pleuroteus ostreatus* = 50 1-L mason jars *P. ostreatus*

Best to transfer grain in a sanitized flow hood station, can be done in glovebox.

Appendix D- Hay and Straw Bulk Substrate Preparation

Supplies/ Equipment

Straw (20% by volume)

- Inoculum
- Large pot
- Water
- Compost Thermometer

Method

All methods were the same for hay and straw bulk substrate, except the hay was cut with scissors until the pieces were approximately 2 inches in length or less. The straw used was pelleted wheat straw then was ground in a coffee grinder until powder. The substrates were moistened until 35-60% moisture. 50-g wet weight of substrate was put into 16 250 ml mason jars fitted with lids that had polyfill air filter.

The substrate was pasteurized by keeping the internal temperature between 140-160 F (60-76°C) for 1 hour. The substrate was cooled before inoculating with grain or hay inoculum. The grain inoculum was mixed in at 20% by volume and mixed by shaking the jars fo1 minute each.

Priming Process

The same method was used to begin the priming process except 0.5% diesel by volume (1 ml) was added to five other 250 ml mason jars. The diesel and the grain where mixed simultaneously into the hay substrate for 1 minute each. The jars were kept at room temperature in a dark cupboard undisturbed.

Appendix E- Liquid Culture Medium Preparation

Source: Cotter 2013.

Equipment

- Pressure cooker
- 1-L mason jars
- Amended Lids
- Coffee Fillers

Recipe (1 liter)

- Non-chlorinated water
- Dextrose 2 Tbs
- Light malt extract 2 Tbs

The non-chlorinated water was brought to a boil then removed from heat. The dextrose and light malt extract were added to the water and stirred until dissolved. The liquid was filtered with non- dyed natural coffee filters (doubled up) to remove sediment. 500-ml (a bit less due to evaporation) was added add to each 1- L mason jar fitted with amended lids (air filter patch and self healing injection port. The liquid media sterilised by was pressure cooking at 15 Psi for 20 minutes. It was cooled completely before inoculation.

Appendix F- GC-MS Specifications

The instrument used was an Agilent Gas Chromatograph-Mass Spectrometer (GC-MS) consisting of a 7890A GC-MS attached to a 5975C GC-MS. The GC-MS column used was a non-polar HP5-MS column (Agilent part #19091S-433). The GC-MS conditions were based on established methods (CCME 2008). The main details are listed below:

- Sample injection volume 1 ul,
- Inlet Temperature – 250°C. Inlet Liner: split type with glass wool (Agilent part #5183-4647)
- GC-MS Mode: Split Injection with a split ratio of 25:1.
- Gas flow rate 1.2 ml/min. Constant Flow Program
- Oven Temperature – Initial Temperature 60 °C, Initial Hold Time 2 minutes. Rate of temperature increase 12 °C/min. Final Temperature 300 °C. Final Hold Time 10 min.
- Transfer Line Temperature 280 °C
- Mass Spectrometer conditions : Source Temperature 230 °C, Quadrupole Temperature 150 °C
- Acquisition Mode: Scan. Solvent delay 3.6 minutes
- Mass (m/z) Range Acquired : 50 to 500

Appendix G- Sample Calculations for Diesel Concentrations

Straw	$A_{C10-C16}$	$A_{C16-C34}$	RF_{AVG}	$C_{C10-C16}$	$C_{C16-C34}$		F2	F3
CT1a	125313	20343624	4047.9757	31	5026		31	4884
CT1b	127778	20012051	4047.9757	32	4944			
CT1c	119187	18959539	4047.9757	29	4684			
CT2a	89116	17839960	4047.9757	22	4407		27	4289
CT2b	128580	17305670	4047.9757	32	4275			
CT2c	115356	16938789	4047.9757	28	4185			
CT3a	6532	16693064	4047.9757	2	4124		13	4040
CT3b	78231	16358188	4047.9757	19	4041			
CT3c	73212	16004630	4047.9757	18	3954			
CT4a	271027	15888548	4047.9757	67	3925		57	3864
CT4b	194685	15672230	4047.9757	48	3872			
CT4c	226171	15368309	4047.9757	56	3797			
CT5a	190324	8116149	4047.9757	47	2005		59	1997
CT5b	211233	7994051	4047.9757	52	1975			
CT5c	312314	8145964	4047.9757	77	2012			
CUT1a	89066	9542279	4047.9757	22	2357		20	2298
CUT1b	76675	9256714	4047.9757	19	2287			
CUT1c	74055	9110583	4047.9757	18	2251			

Total Averge	**F2**	**F3**
CT	37	3815
CUT	18	2140
D	52	3499
T	110	3438
UT	24	2397

Appendix H- Colonization of Polyfill

Throughout the experiment *Trametes versicolor* mycelium was propagated on Aspen shavings in large autoclavable bags fitted with a polyfill filter (see Figure H-1). These cultures did not end up being used for the purposes of my experiment. However, the bags where left undisturbed in a cardboard box for from August 29, 2019 until March 2020. Once, I removed the bags from the box it was discovered that four out of five fungal grown up to the polyfill filters. It appears that they had started to consume the polyfill filters. However, more testing would have to be done to see if they started to break down and consume the polyfill or simply weave throughout it. This is very exciting, because depending on the results this could demonstrates the ability of *T. versicolor* to be used for the breakdown of synthetic materials.

Figure H-1 T. versicolor protruding through the polyfill air filter after 7 months undisturbed in a sealed cardboard box.

Figure H-2. Close up of the how T. versicolor colonization of the polyfill air-filter, possibly consuming the material.

Figure H-3. Cross section close-up of T. versicolor having colonized a polyfill air filter.

Appendix I- Cost of Bioremediation and Soil Volume for the Napo Concession Area

The Napo Concession Area sites are complex, widespread, and numerous which makes it very difficult to determine the actual costs of remediation. It has been estimated that one oil well site has an area of 691,000 m^2, and 80% of these pools contain soils with TPH concentrations greater than 1,000 ppm, therefore it is estimated that 80% of the waste pits requires remediation (Evens 2019). The estimates for the contaminated surface area outside the pools is approximately 316,000 m^2. Thus, the total area to be remediated would be 947,000 m2 (SUOLO). Total volume of soil that needs to be remediated is 3,788,000 m^3 (SUOLO). Corresponding with these soil volume calculation it has been estimated that the total cost for the remediation of contaminated soils using mycoremediation enhanced bioremediation is $ 1,852,000,000. This estimation was made by Larry Evens when proposing a mycoremediation action plan.

The following described the cost driver for each technique which will greatly vary depending on site characteristics, and the price of good and services in the given area.

Cost driver In-situ Bioremediation Techniques

Bioventing

Initial equipment capital, cost increases with site size due to blower size, frequency of well site installation and well depth (EPA 2002). The permeability of the soils will affect how much energy is used to blow air through the soil matrix. An off-gas capture and treatment system may be needed and would significantly increase costs.

Biospsarging/Bioslurping

The cost driver for biosparging are similar to bioventing energy costs will differ due to a different air injection rates (EPA 2002). Bioslurping is essentially the same as biosparging accept costs will be higher because of the extra step and installation of the high-powered vacuum to slurp up the free product.

Cost Driver Ex-situ Bioremediation Techniques

Biopiles and Windrow Composting

Factors that contribute to the cost of biopiles and windrow composting are the excavation construction of the structures (EPA 2002). Soil characteristics, soils with low porosity may require bulking agents to increase the airflow through the compost pile. Soils must be homogenous therefore screening may be required to remove large rocks and debris. Other costs include turning the soil, nutrient amendments, irrigation, lime (pH) to maintain optional conditions for microbes. Further, control measures may be needed for volatile constituents, dust, or odor emissions (EPA 2002)

Cost drivers of Landfarming

The major costs involved with landfarming are like composting methods (EPA 2002). The soil needs to be screened to homogenize and remove large rocks and debris. Silty/clayey soils with high moisture contents are difficult to aerate and require more extensive and more frequent tilling compared to soils with high permeability. The contaminant type and degradability impact the treatment duration (EPA 2002). Additionally, irrigation and nutrients amendments may be required. Volatile or dust emissions may require control measures.

Bioreactors

The main costs are the bioreactor technology and excavating and transporting the soils (EPA 2002). In addition, the bioreactor will require maintenance and repairs from wear and tear. Further, post-treatment dewatering, secondary wastewater treatment, and solids disposal may be required which significantly increasing overall treatment costs. The soil characteristics and heteroginaity will significantly affect treatment costs for example, sizing of materials prior to putting them into the reactor can be difficult and expensive, and clayey soils can be difficult to work with in a bioreactor (EPA 2002).

This technology is not cost effective (EPA 2002). It requires transport of the contaminated soil to the bioreactor which must be a suitable size to for the volume of soils. It can be effective small scale, but large volumes are to expense to transport and treat. In addition, the bioreactors themselves are expensive pieces of equipment (EPA 2002). Bioreactors can be interesting tools to investigate optimal conditions by accurately controlling certain variable which will help researchers understand limiting environmental factors.

Appendix J- Bioremediation Literature Review

Introduction

Remediation is a branch of ecological restoration that is specific to cleaning-up contaminated soil, water, or air to mitigate unacceptable risks to human health and the environment (Nathanail et al. 2017). Across the globe there are potentially millions of contaminated sites that pose undue risk. Throughout Europe there is an estimated 340 000 contaminated sites that will most likely require remediation (EEA 2014). The United States Environmental Protection Agency (US EPA) estimates are similar to those of the European Environmental Agency and 350 000 sites will need to be remediated by 2034 (US EPA 2004). In Ecuador, oil exploitation threatens the health of the local people, as well as the flora and fauna . Legacy oil production practices have led to vast crude-oil contamination of soils, ground water, and surface water. The people of the Ecuadorian Amazon are seeking relief as their health, livelihood, and homes are at risk (Harvard School of Public Health 1994).

Bioremediation is often viewed as a sustainable remediation strategy, because is environmentally friendly, and cost-effective remediation strategy when compared to traditional remediation (Azubuike, Chikere, and Okpokwasili 2016). Further, bioremediation techniques allow for community led grass-roots initiatives. It has been successfully used in the degradation of petroleum hydrocarbons in many studies, and at various scales (laboratory/mesocosm/field trials) and has also been successfully implemented in full remediation initiatives using well developed techniques(Agamuthu, Tan, and Fauziah 2013; Helmy, Laksmono, and Kardena 2015; Maddel, Masabanda, and Leiva-Mora 2015).

The objective of this literature review is to identify the functional mechanisms of key bioremediation techniques, as well as to review studies that focus on bioremediation of the Napo Concession so that appropriate bioremediation of petroleum hydrocarbons strategies for the Napo Concession can be identified.

Methods

The Simon Fraser University Library catalog was used to search for relevant references. The first search “Bioremediation in Ecuador” turned up 79 references. The peer reviewed journals titles were scanned for bioremediation of petroleum contaminated soil. The search was

then broadened to “Bioremediation in the Amazon of petroleum contaminated soils” this search turned 66 peer review articles and 25 books. Then a general search of “bioremediation was done”, thousands of scientific articles and many books were available to review. The search was narrowed to include only references published between 2009-2019.

Results and Discussion

Bioremediation Mechanisms

Bioremediation is the use of biotic organisms to remediate an area through destruction or transformation of a toxicant (Agamuthu, Tan, and Fauziah 2013). There are a number of in-situ and ex-situ bioremediation techniques each one is designed to create optimal conditions for biological organisms to thrive so that they can degrade, volatilize, or accumulate toxicants (Nyer et al 2001 pg. 264). There are two strategies, the use of microorganism (bioremediation) or plants (phytoremediation). Since the term bioremediation is used synonymously with microbial remediation (which can be confusing) I will herby be referring to microbial remediation as the use of microorganisms in remediation. Microbial remediation can be further subdivided into mycoremediation which is the use of fungi as the main remediator and bacterial remediation (the use of bacteria).

Most bioremediation techniques are focused on either stimulating autochthonous microbial activity or introducing microbial communities, this is known as enhancement (Azubuike, Chikere, and Okpokwasili 2016). Whereas, with phytoremediation the central organisms are plants. However, the important remedial mechanism is often plant-microbial symbiosis.

Microbial Remediation

Microbial remediation uses microorganisms to degrade organic compounds, bind heavy metals so they are less bioavailable, or transform them into inert forms (Darwish 2013 pg. 35). Microorganisms include bacteria, fungi, algae, protozoa, and metazoa. (Nyer et al. 2001 pg. 261). In general, the microorganisms responsible for degradation organic toxicants use them as a food source for energy, growth, and reproduction (Darwish 2013 pg. 35). Alternatively, they metabolize them with a food source. Enzymes are the key mechanism concerning the effectiveness of microbial remediation.

Enzymes are produced internally for bacteria and externally by fungi (Nyer et al. 2001 pg. 263). The mechanism works as followed: Enzymes are induced, respiration occurs, organic compounds are cleaved releasing energy. During degradation, the organics are converted to simpler organic compounds while deriving energy (Hara and Uchiyama 2013, pg. 117). Ultimately, the organic compounds are degraded to the point of mineralization which results in carbon dioxide or methane, inorganic ions, and water.

A microorganism growth cycle can be divided into several phases called the lag phase, exponential phase, stationary phase, and death phase (Nyer et al. 2001 pg. 271). The death phase occurs when organic carbon (food) usually becomes limiting and the microorganism population can no longer be sustained (Nyer et al. 2001 pg. 271). The role of the bioremediator is the to design biological systems that maintain a high growth rate until the environmental pollutant has been degraded or modified enough to meet project goals.

Bacterial Remediation

Bacteria are tiny single celled organisms and their size ranges from < 2µm to 20 µm. They preform important ecosystem services such as, improving soil structure by binding soil particles creating microaggregates (Ingham, 2009) . Improved soil structure increases water infiltration and increases water holding capacity of the soil.

Bacteria are classified by their physiological activity (Hoorman 2019). Heterotrophs are the most important bacteria for the degradation of petroleum hydrocarbon (PHC) contaminated soil. Heterotrophs get their carbon and energy from complex organic substances/organic matter, decaying roots and plant residues. They obtain their nitrogen from nitrates and ammonia compounds (proteins) present in soil and other nutrients from soil or from the decomposing organic matter (Hoorman 2019). Autotrophs synthesize their food from atmospheric CO^2 as the carbon source and sunlight as the energy source.

Most bacteria are called aerobes and require oxygen for their enzymatic processes which oxidize organic compounds (Hoorman 2019). Thus, the soil rhizosphere hosts the highest rates and diversity of bacteria and other microorganisms. This area is a highly productive because plant roots aerate soils (Inham 2009).

There are approximately 70 genera of known oil-degrading microorganisms, including bacteria such as *Achromobacter, Acinetobacter, Actinomyces, Bacillus, Burkholderia,*

Exiguobacterium, Klebsiella. Microbacterium, Nocardia, Pseudomonas, Spirillum, Streptomyces and Vibrio, and fungi such as Allescheria, Aspergillus, Candida, Debaryomyces, Mucor, Penicillium, Saccharomyces and Trichoderma (Maddela et al 2015). However, no one bacteria is capable of mineralizing all petroleum hydrocarbon fractions (Yanto and Tachibana 2014). The activity of microorganisms and their enzymes is also determined by the properties of hydrocarbons, such as their degree of solubility in the soil solution and the amount of benzene rings in molecules of different hydrocarbons (Smreczak and Maliszewska-Kordybach 2003). Hydrocarbons with two, three, and four rings (e.g., naphthalene, phenanthrene, anthracene, and pyrene) are generally susceptible to microbial decomposition. Hydrocarbons with more than four benzene rings are strongly adsorbed and thus poorly bioavailable (Klamerus-Iwan et al. 2015)

Three distinct types of bacterial responses to diesel in a consortium were observed by (Lisiecki et al. 2013). Members of *Alcaligenes* and *Sphingobacterium* were not affected by the type of fuel while abundance of Citrobacter was increased as the amount of biodiesel increased, and *Achromobacter, Comamonas, Pseudomonas* and *Variovorax* were suppressed by higher levels of biodiesel.

In order to understand the functional mechanisms of each bioremediation technology we must first understand the fundamental conditions needed for healthy microbial communities and plant growth in the context of the bioremediation of petroleum hydrocarbons. The main factors that affect microbial remediation effectiveness are as followed:

- Water

Saturated conditions create anoxic conditions and favor microorganisms that are less beneficial in terms of bioremediation (Nyer et al. 2001 pg. 272)

- pH

General optimum bacteria pH is 6.5 - 7.5 The most important factor with pH is to not allow major shifts in pH during remediation (Nyer et al. 2001 pg. 272).

- Temperature

No single microorganism will grow over this entire range. Bacteria are frequently divided into three broad groups: thermophiles, which grow at temperatures above 55ºC; mesophiles,

which grow in the midrange temperature of 20º to 45ºC; and psychrophiles, which grow well at 0ºC (Nyer et al. 2001 pg. 272).

- Oxygen.

Generally, an oxygen atmosphere in soil of less than one percent will change the predominant respiration reaction from aerobic to anaerobic (Paul and Clark 1989). In aqueous environments, oxygen concentration less than approximately 0.5 to 1.0 mg/L can switch metabolism from aerobic to anaerobic (Nyer et al. 2001. pg. 274).

- Nutrition.

Microorganism growth requires assimilatory reactions where the organism gathers carbon (C), nitrogen (N), phosphorous (P), and micronutrients. The ideal C/N/P ratio to maintain or accelerate biodegradation in soil is approximately 100/10/1. Micronutrients commonly required by microorganisms include sulfur, calcium, iron, zinc, copper, cobalt, manganese, and molybdenum. Often N & P are limiting nutrients may need to be added (Nyer et al. 2001 pg. 275). -

- Heavy Metals

Heavy metals and halogens can disrupt cellular activity by interfering with protein function (Nyer et al. 2001 pg. 276). Oxidizing and reducing environments influence the mobilization and immobilization of metals. (Nyer et al. 2001 pg. 277)

Microbial Bioremediation Techniques

Bioremediation techniques can be divided into ex-situ and in-situ (Azubuike, Chikere, and Okpokwasili 2016). Ex-situ bioremediation techniques are as followed: Biopiles, windrows, bioreactors, and landfarming. In-situ techniques are natural attenuation, bioslurping, bioventing, biosparging, phytoremediation, and constructed wetlands sometimes referred to as biofilters.

Ex-situ Techniques

- Biopiles

Biopile composting is used for the remediation of unsaturated soil (Azubuike, Chikere, and Okpokwasili 2016). The basic components are aeration, irrigation, nutrient amendments, and leachate collection system. Sometimes heat can be added to stimulate bacteria. Aeration is created within the mounds by the installation of perforated pipes which are placed so that air can be injected by blowers into the soil (Azubuike, Chikere, and Okpokwasili 2016). The aeration, irrigation and nutrients are incorporated into the soil and optimal conditions are maintained to stimulate the growth and reproduction of heterotrophic bacteria. In some cases, aeration will decrease contaminant concentrations through volatilization. Sand to clay soils have been used with successful trials.

Key to successful remediation are initial sorting and mixing of the biopiles, applying appropriate soil amendments and nutrient ratios, maintaining appropriate moisture levels and even distribution of air (Azubuike, Chikere, and Okpokwasili 2016). This technique has been used successfully at drastically reducing PHC's up to 95%, it is cost-effective, technically simple, and can be used in relatively limited spaces. However, limitations include needing for heavy machinery needed for excavation, power needed for proper even aeration, treatment space needed (Azubuike et al 2016).

Time: 3 months- 1 year

•Windrows

Windrow composting is used to remediate unsaturated soil which is excavated and formed into long shaped piles (Azubuike et al 2016)). Principle components are water, nutrient additions, and constant turning of the soils. Bulking agents and organic nutrients are added to the soils which are turned regularly, daily for maximum biodegradation rates. Increased microbial actions raises temperatures within the windows. Thermophilic conditions (54 to 65 °C) must be maintained to properly compost soil contaminated with hazardous organic contaminants (Azubuike, Chikere, and Okpokwasili 2016).

This technique has been more successful at removing PHC's than biopiles (Coulon et al. 2010). In a six-month field-trial, windrows outcompeted biopiles, and where more successful at degrading bunker fuels in heavy textured soils. Further, nutrient addition and bioaugmentation of the windrows led to a two- times higher degradation rate of the aliphatic fraction, although aromatic fractions showed limited enhancement. Success has been correlated to soil type and

coarser textured soils may be more treatable by biopiles. Limitations of window treatments are the need to turn the soils throughout the treatment period. As well, windrows may not be suitable for substances with toxic volatiles, and may increase the risk to human health, and constant labour. Moisture, and temperature must be monitored closely (Azubuike, Chikere, and Okpokwasili 2016).

Time: 0.5 – 1 year

•Bioreactor

Bioreactor composting is done with vessel that can treat contaminated soil or water (Azubuike, Chikere, and Okpokwasili 2016). The vessel accurately controls pH, moisture, and temperature for optimal microbial conditions. Soil can be treated in a solid state or slurry. Slurry states have been more efficient at degrading PHC's compared to solid phase. (Azubuike, Chikere, and Okpokwasili 2016) used a stir tank bioreactor to treat crude oil polluted sediment and degraded 82% of the crude oil with an initial concentration 19 ppm, 97% crude with an initial concentration of 3.1 ppm

Time: 0.5- 1 year

•Landfarming

Contaminated soil is spread on the surface in an even layer and tilled for aeration (Azubuike, Chikere, and Okpokwasili 2016). As with the other bioremediation techniques landfarming involved, aeration, nutrient additions, and irrigation. Landfarming the simplest and most cost-effect of the bioremediation techniques and can be done in-situ or ex-situ dependent of the depth of contamination. If the contamination is < 1m then remediation may be able to occur without excavation. However, > 1.7 m below the surface and excavation is necessary (Azubuike, Chikere, and Okpokwasili 2016). In some cases, landfarming as been done relatively successfully even without the addition of nutrients. For example, >80 % degradation of diesel was achieved by rototilling for aeration at a remote Canadian Arctic location over a 3-year study period (Paudyn et al 2008). A major limitation with this technique is space, large flat areas are needed to spread-out large volume of contaminated soil (Azubuike, Chikere, and Okpokwasili 2016). In addition, this technique should not be used for toxic volatiles as this technique promotes volatilization.

Time: 0.5- 1 year

In-Situ Techniques

In situ techniques are named so because treatment happens in place without the excavation of soils (Azubuike, Chikere, and Okpokwasili 2016). Cost are reduced because soil excavation, transport and storage are not needed. However, in-situ techniques generally require more on-site infrastructure.

•Natural attenuation

Natural attenuation implies that if the site is left undisturbed microbial remediation and weathering (volatilization) will occur without mitigation (Azubuike, Chikere, and Okpokwasili 2016). It is important to consider, but often will not be effective in an appropriate timeframe.

Time: Highly variable

•Bioslurping

Bioslurping combines bioventing, soil vapor extraction, and vacuum-enhanced pumping (Azubuike, Chikere, and Okpokwasili 2016). This technique is designed for free products, such as light non-aqueous phase liquids (LNAPL's). The vacuum slurps up the free products which at the surface separates from water and air. after the removal of the LNAPL's the system can be converted into a bioventing system to finish remediation.

This technique cannot be used effectively in areas with fluctuating water table depths and is not suitable for soil with low permeability (Azubuike, Chikere, and Okpokwasili 2016). Excessive soil moisture limits air permeability, and decreased air transfer rates therefore decreasing microbial activity.

•Biosparging

Biosparging is like bioventing although it injects air into saturated soils and uses higher air flow rates (Azubuike, Chikere, and Okpokwasili 2016). There are two main factors that affect the effectiveness soil permeability (determines pollutant bioavailability to microorganisms), and pollutant biodegradability. Major limitation is predicting the direction of airflow.

Time: > 3 years

•Bioventing

Controlled air or oxygen is blown into the vadose (unsaturated) zone in contaminated soil along with nutrients and irrigation to increase microbial activity (Azubuike, Chikere, and Okpokwasili 2016). Bioventing has been particularly effective with the light weighted PHC's. The main factor with bioventing is air delivery and injections rates. The most important factors are even distribution of low-pressure air. This can be achieved through small injection rods set 0.5m apart (Azubuike, Chikere, and Okpokwasili 2016). High injections rates and frequent injection intervals does not result in higher degradation or transformation rates and may even cause soil channeling. This will reduce efficiency by creating an air path that direct air away from the contaminated area.

Time: 1-3 years

•Phytoremediation

Phytoremediation is the use of plants to remediate organic compounds and heavy metals through six different mechanisms which are (1) phytoextraction, (2) phytodegradation, (3)phytovolatilization, (4) rhizodegradation, (5)rhizofiltration, and (6)phytostabilization (Darwish 2013 pg. 70).

1. Phytoextraction is when plants called hyperaccumulators take up contaminants i.e. heavy metals, metalloids or radionuclides through their roots and store them in their stems and leaves.

2. Phytodegradation is when plants release enzymes that break down organic compounds through the metabolic process photosynthetic oxidation and reduction. Water and nutrients are taken up by the plant, and carbon dioxide, oxygen, water, and photosynthates are released to the environment. (Nyer et al. 2001 pg. 393)

3. Phytovolatilization is when plants take up volatile contaminants and release them through transpiration. Depending on the contaminant it can be released in the original form or transformed into a less toxic state and then released into the atmosphere (Darwish 2013 pg. 70).

4. Rhizodegradation is when there is a microbe-plant association (Nyer et al. 2001 pg. 393). The microbes break down or transform the contaminants; certain plants create environments that facilitate this process. The processes occurring within the rhizosphere are integral to phytoremediation. Plants supply oxygen to the soil and release exudates, which include sugars, alcohols, amino acids, and enzymes. The exudates and enzymes enhance microbial growth.

The overall effect of the plant-microbe growth is an increase in microbial biomass by up to an order of magnitude or more, compared with microbial populations in the bulk soil. The microbes subsequently promote degradation and co-metabolism of organics (Schnoor et al. 1995). The fate of organics and inorganics in the rhizosphere, and the corresponding tendency of these constituents to be taken up by plants, can be predicted using the logarithm of the octanol-water partition coefficient (KOW) of the particular constituent (Nyer et al. 2001 pg. 406).

5. Phytofiltration is when plants can filter water by adsorbing contaminants to their roots and tissue (Darwish 2013 pg. 70).

6. Phytostabilization also known as green capping, is when plants can immobilize contaminants by adsorbing then to their roots and releasing exudates that transform the contaminant into a less toxic state. Green capping also reduces contaminant spread through erosion and leaching (Darwish 2013 pg. 70).

Advantages and Disadvantages of Phytoremediation

Phytoremediation is a low tech in-situ, and low-cost remediation technique. Cost estimates range from 33.3 percent to 10 percent of traditional remedial techniques (Nyer et al. 2001 pg. 396). However, Phytoremediation is a long-term remedial technology at most sites, with treatment times taking on average from 3-5 years. Often phytoremediation will be used in combination with other bioremediation techniques this is because plants are susceptible to phytotoxicity. For example, the presence of high concentrations of salts, low or high soil pH, lack of nitrogen, etc., can be phytotoxic. Phytoremediation is generally appropriate for mild to medium contamination (Nyer et al. 2001 pg. 396).

Important bioremediation engineering considerations are as followed:

1. Fully characterized site conditions including geology, agronomic condition, and contaminating characterization.

2. Careful selection of fungi, bacteria, and plant alongside potting studies or greenhouse studies and pilot testing are recommended prior to the full-scale implementation of projects (Nyer et al. 2001 Pg. 430). Some factors that should be considered when selecting the plant species include water requirements and availability, tolerance to agronomic conditions, disease and insect resistance, acclimation, and use of native versus non-native species. (Nyer et al. 2001 pg 431).

3. Clearly defined remedial objectives. As with any technology, the remedial objectives, including the cleanup goals and site end use, need to be clearly defined.

4. Known fate of contaminants. The fate of contaminants needs to be understood and factored into the program for each site (Nyer et al. 2001 pg 431).

6. Monitoring and maintenance is key to the success of bioremediation projects. This step must not be overlooked, and a solid monitoring and maintenance plan is essential. Monitoring and maintenance requirements include watering, fertilization, and insecticide applications. If plants have been used as a biological treatment and wildlife predation is possible, fencing or other access barriers to wildlife should be considered (Nyer et al. 2001 pg. 431).

Napo Concession Studies

Six peer-reviewed journals were found specific to the Napo Concession petroleum contamination. The research studies that have been done begin to explore autochthonous (indigenous) microorganism and plants that have the potential for effective bioremediation for this area.

- Site characteristics

Soils in this area are typically acidic, and have low nutrient levels and high aluminum contents (Villacís et al. 2016); these singularities lead to rapid soil erosion and infertility after the vegetation is removed from the oil fields (Nichols et al. 2001).

- Contaminated Substrates

Discharge of crude-oil waste increased the soil pH to neutral (pH 7.34) from acidic (pH 5.12 (Maddela et al 2015). The content of cadmium, water-holding capacity, and microbial populations had been adversely affected in test soil compared to the control. Nearly 100 times fewer heterotrophic bacteria and fungi were observed in the test soil than in the control samples.

- Microbial remediation

Maddela as the primary author on four of the papers isolated petroleum degrading microorganism found in crude oil contaminated soils in the Napo Concession. In these studies Maddela et al (2015[1], 2015[2], 2016, 2017) took the readers through a sequential set of bioremediation studies.

Maddela et al 2015 first began this line of research by conducting a study where the primary objective was to isolate alkane-degrading microbes (Maddela 2015). The authors isolated two PHC eating bacterial cultures (*Bacillius cereus* & *B. thuringiensis*) and two PHC eating fungi culture (*Geomyces pannorum* and *Geomyces sp*.) from contaminated substrates found in the Lago Agrio oil fieid.

Laboratory assays were done to determine the effectiveness of the four microbes by using Erlenmeyer flasks containing 20 mL of liquid minimal salts medium plus 1% of diesel oil the only carbon source (Maddela et al 2015[1]).

The results for this study found that PHC's were not completely mineralized, they were partially degraded n-alkane peaks from C10–C18. (Maddela et al 2015[1]). The experiment ran for 30 days and the reduction rate of diesel-oil for isolate-1 and -2 of fungi and bacteria were 77.34, 68.55, 62.62 and 49.71, respectively. Fungi reduction rates were significantly ($p < 0.05$) higher compared to the bacteria. (Maddela et al 2015[1]). When comparing the effectiveness of the two TPH-degrading *Geomyces spp*. after 30 days of incubation in culture medium the TPH degradation was 77.3 % and 68.6 % for diesel fuel and 43.4 % and 24.0 % for crude oil with isolates 1 and 2, respectively. This not only indicates the complexity of the crude oil over diesel fuel, but also the greater efficiency of isolate-1 to degrade petroleum hydrocarbons.

Increasingly, research is demonstrating that co-culturing or using a consortium of microbes is more efficient than single species enhancement. Thus, the authors mixed the two

fungi isolates. Results of the mixed culture increased the degradation rate to 79.9 % of the crude oil in 30 days. As a secondary quantitative tool Meddela et al (2015) used percent germination of cow pea seeds to assess the substrate toxicity. The results corroborated PHC reduction rates and germination of cow pea seeds significantly increased from 20 % to 100 % in bioremediated soil.

Maddela et al. (2016) investigated the efficiency of using the PHC-degrading microbes, in solid phase (SOP) soil and slurry phase (SLP) soil . SOP only nutrients were added and the soil bed was aerated mechanically at set intervals. In the SLP water was added to enhance the physical mixing. Research has shown that SLP bioremediation is much faster than many other bioremediation techniques (Eziuzor and Okpokwasili 2013).

In this study the authors used the consortium of the four isolated microorganisms and compared the efficiency of SOP and SLP. Results were significant, the percent removal of TPHs from crude oil-treated soil after 30 d in SOP was 79.47 % and SLP was 87.77%. SLP is more suitable for treating heterogenous and low permeable soils (like Ecuadorian soil). However not feasible because it would require the use of bioreactors.

Lastly Maddela et al. (2017). Conducted a field scale study to determine the TPH degrading potential of the four microbes previously isolated from the Napo Concession contaminated soils. 9 wooden boxes were filled with soil and amended with NPK fertilizer (1% w/w), dry cow dung (1:10 w/w), glucose solution (250 mg L-1), and sand 10%. Plus 5% (w/w) heavy crude oil (collected from Tarapoa Block, Petroamazonas, EP) of 21.4 API gravity then mixed once per week for aeration . Three treatments were compared TC0 (soil amendments and 5% crude incubated for 0 days), TC90 (soil amendments and 5% crude incubated for 90 days), and TM90 (soil amendments, 5% crude, and microbial consortium enhancement incubated for 90 days) The three treatment were included in triplicates and TM90 was enhanced with the same four microbial species (*Bacillius cereus, B. thuringiensis, G. pannorum, Geomyces sp*.).

The ecotoxicity of crude oil residues contained in soil samples of three treatments was also tested by determining seed germination and plant growth of cowpea (Vigna unguiculata). Percent seed germination, germination time, plant height and number of branches per plant in all the treatments were recorded (Maddela et al. 2017)

Throughout the research Maddela et al. (2015[1], 2015[2], 2016, 2017) found some key pieces of information concerning the substrate conditions that have significant effects on microbial growth.

First, the nutrient status (N–P–K) of soil has direct impacts on microbial activity and biodegradation (Maddela et al. 2015). Nitrogen and phosphorus are necessary for cellular metabolism. Sporulation rates for isolate-1 and 2 in a mineral salt medium containing diesel fuel and 4 g/L of N–P–K was 46.2 % and 75.2 %, respectively. However, rates declined at higher concentrations of N–P–K. The same was noticed with crude oil; however, isolate-2 tolerated up to 6 g/L N–P–K. several authors have also reported the negative effect of high N–P–K levels on the hydrocarbon biodegradation, specifically on the aromatics (Margensin and Schinner 2001, Maddela et al. 2015).

•Second, The effects of pH on growth of fungi in mineral salt medium containing either diesel fuel or crude oil were also studied. Both isolates exhibited optimum sporulation rates at pH 5, irrespective of the substrate. For instance, with diesel fuel, rates for isolate-1 and -2 at pH 5 were 208 and 158 times that in control soil, respectively. However, these values were significantly ($p>0.05$) lower for crude oil. For both substrates' sporulation was negatively affected below or above pH 5. Similarly, Kiran et al. (2008) found that *Phanerochete* degradation increase as pH increased from 5-7 but was negatively affected above pH 7. Each fungus has an optimal pH range.

Third, comparison of both bacterial and fungal populations in TC0 soil sample with those of TC90 soil sample clearly indicates that there was no change in total heterotrophic bacteria and total fungi. However, populations of TPH-degrading bacteria and fungi in TC90 soil sample increased significantly during 90-day incubation, suggesting that the indigenous soil microorganisms capable of utilizing crude oil as a source of carbon and energy. 'zymogenous' population alone proliferated while 'autochthonous' soil populations were suppressed.

•The degradation of TPHs in crude oil by the indigenous soil microorganisms was marginal (13.66%). But, biodegradation of TPHs was significantly rapid (87.45%) when the same amended soil was inoculated with mixed populations of the selected strains of both bacteria and fungi. The significant removal of TPHs from crude oil was confirmed following ecotoxicity studies involving *A. salina* and *V. unguiculata* as the test organisms.

Phytoremediation

1, Villacis et al. (2016). Studied the Selection of forest species for the rehabilitation of disturbed soils in oil fields in the Ecuadorian Amazon. The goal of the study was to produce a list of species recommended for their use in restoration projects of oil fields in the Amazon Basin based on their abilities to survive, grow, and amend soils affected by petroleum extraction activities

A variety of native and non-native plants were planted in -situ field plots. Two years after reforestation TPH significantly decreased up to 22% in mud and drill cutting cells plots. Uncontaminated but compacted soil substrates from oil-platforms showed an overall improvement in their physicochemical characteristics.

Result indicated that the plant species *F. macrophylla, M. aff. fallax, P. pteroclada, P. pinnatum, and Z. longifolia* exhibited the best survival and growth across sites and would be suitable species to be used for the potential rehabilitation of oil-field sites in Ecuadorian Amazon. all but one of these species are native, and the species studied have a wide geographical distribution throughout the Amazon Basin.

2. Garces-Ruiz (2017) studies Arbuscular Mycorrhizal Fungal (AMF) Community Composition in *Carludovica palmata, Costus scaber* and *Euterpe precatoria* from Weathered Oil Ponds in the Ecuadorian Amazon. The aim of this study was to analyze AMF root colonization in different plants species colonizing the ponds and surrounding soil to determine the AMF community from three specific plants (*Carludovica palmata, Costus scaber and Euterpe precatoria*) present across the sites.

The analysis of plant species in the ponds as well in the surrounding soil, demonstrated the systematic presence of AMF in roots with levels of colonization above 50% in each species analyzed. Root colonization was observed for the first time in E. precatoria, C. scaber, C. palmata. AMF observed were *Monotagma sp.1, Polybotrya sp.2, Geonoma cf. deversa, Costus pulverulentus, Costus lima, and Polybotrya sp.1*. Archaeospora was detected in 22% of the total number of OTUs. This family was present in the three sites and associated with all plant species analyzed. However, all the sequences were represented by unknown *Archaeospora spp.*•

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Appendix K- Species List By Habitat Types

CES408.523 Evergreen forest in the western peninsula of the Amazon. Bosque Siempreverde de la Penillanura del Oeste de la Amazonia

Representative Trees: *Inga yacoana, Inga tocacheana, Parkia velutina, Swartzia sp., Dialium guianense, Bauhinia brachycalyx, Guarea sp., Sagotia racemosa, Pourouma bicolor, Perebea guianensis, Sorocea muriculata, Rinorea viridifolia, Rinorea apiculata, Mabea speciosa, Miconia sp., Lindackeria paludosa, Tetrathylacium macrophyllum, Lunania parviflora, Oxandra mediocris, Licania octandra, Capparis alone, Grias neuberthii, Gustavia hexapetala, Eschweilera coriacea, Eschweilera tessmannii, Ocotea costulata, Protium sp., Iryanthera sp., Virola sp.,Pouteria lucumifolia, Ocotea aciphylla, Pausandra trianae, Eugenia feijoi, Mouriri oligantha, Calyptranthes sp.,Palicourea sp., Psychotria flaviflora, Neea floribunda. Palms: Iriartea deltoidea, Socratea exorrhiza, Astrocaryum murumuru, Astrocaryum chambira, Phytelephas tenuicaulis, Geonoma sp., Geonoma maxima, Geonoma macrostachys, Geonoma stricta, Bactris hirta. Shrubs: Faramea salicifolia, Psychotria callithrix, Psychotria zevallosii, Miconia sp., Pentagonia parvifolia, Urera baccifera, Abuta grandifolia, Piper reticulatum* (Báez et al 2010).

The following species are characteristic: *Micrandra spruceana, Eschweilera amara, Eschweileraspp., Clathrotropis macrocarpa, Otoba parvifolia, Hevea spp., Ocotea aff. Bofo, Licania aff. incana, Symphoniamicrophylla, Theobroma grandiflorum, Brosimum utile, Cariniana micrantha, Oenocarpus bataua, Iriartea deltoidea, Virola duckei, Otoba glycicarpa, Otoba parvifolia, Parkia spp., Simarouba amara, Dussia tessmannii, Hymenaea oblongifolia, Cedrelinga cateniformis, Ceiba pentandra, Chorisia insignis. In Peru Trees: Eschweilera tessmannii, Eschweilera coriacea, Eschweilera gigantea, Eschweilera itayensis, Eschweilera albiflora, Eschweilera rufifolia, Cariniana decandra, Nealchornea yapurensis, Guarea macrophylla, Guarea kunthiana, Guarea pubescens, Guarea pyriformis, Guarea pterorhachis, Guarea carinata, Guarea grandifolia, Guarea guidonia, Guarea silvatica, Leonia glycycarpa, Iryanthera paraensis, Iryanthera laevis. Palm trees: Astrocaryum murumuru, Astrocaryum chambira, Lepidocaryum tenue, Euterpe precatoria, Phytelephas tenuicaulis, Phytelephas macrocarpa, Attalea butyracea* (Báez et al 2010).

CES408.532 Flood forest of the flood plain of white-water rivers of the west of the Amazon (Bosque Inundable de la Llanura Aluvial de r¡os de Aguas Blancas del Oeste de la Amazonia)

The predominant families in this system are *Arecaceae, Moraceae, Fabaceae s.l., Bombacaceae, Myristicaceae, Rubiaceae, Meliaceae, Euphorbiaceae and Lecythidaceae* (Balslev et al. 1987, Nebel et al. 2001) (Ministerio del Ambiente del Ecuador 2012). The areas closest to the river and sandbars have a strip of primary succession predominated by *Gynerium sagittatum (Poaceae)* and *Tessaria integrifolia (Asteraceae)* (Ministerio del Ambiente del Ecuador 2012). When these banks age and stabilize, other succession species typical of the Amazonian plain appear as several species of *Cecropia (Cecropiaceae)* and *Triplaris americana (Polygonaceae)*.

Diagnostic species: Acacia glomerosa, Aegiphila integrifolia, Attalea butyracea, Calathea sp. , Calycophyllum spruceanum, Castilla ulei, Ceiba pentandra, Ceiba samauma, Ceiba samauma, Clarisia biflora, Couroupita guianensis, Couroupita guianensis, Eucharis morei, Ficus insipida, Grias neuberthii, Guarea guidonia, Guarea kunthiana margaphiana, Inga kathiana, Inga kunathiana oerstediana, I. punctata., Inga splendens, Leonia crassa, Leonia crassa Perebea guianensis, Myriocarpa stipitata, Palicourea spp., Psidium acutangulum, Quararibea witii, Sapium laurifolium, Schizolobium parahyba, Sloanea grandialogaumia, Steraculumumum, Sloaculia apeiaculia, Apnea Trophis racemosa, Trichilia laxipaniculata, Urera caracasana, Virola calophylla, Virola surinamensis, Zygia juruana, Z. Longifolia ,. In succession complexes it is common to observe: Acalypha diversifolia Cecropia engleriana, C. ficifolia, C. Membranacea, Cordia alliodora, Gynerium sagitatum, Heliconia episcopalis, H. marginata, H. rostrata, Tessaria integrifolia (Ministerio del Ambiente del Ecuador 2012).

Other species*: Attalea sp., Euterpe precatoria, Iriartea deltoidea, Socratea exorrhiza, Cordia nodosa, Jacaratia sp., Rinorea flavescens, Combretum laxum, Paullinia reticulata, Rourea cuspidata, Echinodorus sp., Aphelandra sp., Costus sp., Calathea sp., Heliconia sp (Ministerio del Ambiente del Ecuador 2012).*

CES408.536 Flooded forest and lacustrine-riparian vegetation of black waters of the Amazon

Representative species are *Astrocaryum jauari, Bactris concinna var. concinna, B. maraja, Cecropia latiloba, Coussapoa Trinervia, Croton cuneatus, Crudia glaberrima, Duroia petiolaris, Eschweilera parvifolia, Genipa spruceana, Hirtella elongata, Hydrochorea corymbosa, Inga ruiziana, Inga stenoptera, Iryanthera tessmannii, Leonia racemosa, Licania appeal it var. apetala, Luehea cymulosa, Lueheopsis hoehnei, Macrolobium acaciifolium, Macrolobium microcalyx, Mauritiella armata, Mezilaurus itauba, Myrciaria dubia, Oxandra euneura, Parkia balslevii, Pourouma cucura, Pouteria laevigata, Pouteria multiflora, Pseudobombax munguba, Pterocarpus amazonum, Rourea camptoneura, Securidaca divaricata, Symmeria paniculata, Terminalia dichotoma, Trichilia pachypoda, Triplaris weigeltiana are also frequent the species that make up the so-called Gramalotes Hymenachne donacifolia, Salvinia auriculate* (Ministerio del Ambiente del Ecuador 2012).

Other representative species of this system in the herbaceous stratum are: *Heliconia hirsuta, Urospatha sagitifolia* (Ministerio del Ambiente del Ecuador 2012).

CES408.538 Swamp palm forest of the floodplain of the western Amazon (Bosque inundado de palmas de la llanura aluvial de la Amazonía)

Representative species are *Apeiba aspera, A. tibourbou, Astrocaryum urostachys, Attalea butyracea, Attalea maripa, Buchenavia amazonia, Cecropia putumayonis, Coussapoa trinervia, C. longepedunculata, Croton tessmannii, Euterpe precatoria, Ficus spp. ssp., Heliconia juruana, Hieronyma alchorneoides, Iriartea deltoidea, Isertia rosea, Macrolobium angustifolium, Manilkara inundata, Mauritia flexuosa, Mauritiella armata, Mollia lepidota, Oenocarpus bataua, Parkia nitida, Pterocarpa amazonum. , Socratea exhorriza, Symphonia globulifera, Tabernaemontana siphilitica, Virola calophylla, Virola surinamensis*

CES408.550 Whitewater riparian succession vegetation complex of the Amazon (Complejo de vegetación sucesional riparia de aguas blancas de la Amazonía)

The following species are diagnostic for this ecosystem: Gynerium sagittatum, Alchornea castaneifolia, Tessaria integrifolia, Salix humboldtiana, Ochroma pyramidale, Cecropia concolor, Cecropia membranecea, Cecropia latiloba, Cecropia peltata, Ficus insipida, Senna reticulata, Erythrina poeppigiana, Triplaris americana, Inga marginata, Croton draconoides, Calycophyllum spruceanum, Kyllinga pumila, Alchornea castaneifolia, Cassia sp., Mimosa sp., Pseudobombax munguba ("punga"), Montrichardia arborescens, Cyperus spp., Paspalum repens, Echinochloa

polystachya, Paspalum fasciculatum, Oryza grandiglumis, Hymenachne amplexicaulis, Calliandra angustifolia, Adenaria floribunda, Ludwigia decurrens, Fimbristylis littoralis (Báez et al 2012).