Exeter FSTF

Chemical Process Description for Bioremediation

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1. INTRODUCTION

Bioremediation, involving microbial breakdown of contaminants, is an effective remedial process for soils impacted with organic compounds. This document describes illustrative (bio)chemical reactions involved within the bioremediation processes to be carried out at the Exeter Fixed Soil Treatment Facility.

Commonly, soil contamination to which bioremediation is effectively applied derives from the petroleum range fuels including diesel, kerosene and light fuel oils, as well as solvents and organic cleaning and lubrication fluids. These contaminants contain a broad spectrum of hydrocarbon compounds, varying in molecular weight, shape and degrees of substitution, aromatisation and halogenation, such as aliphatic compounds, aromatic compounds consisting of PAHs, chlorinated aromatics and nitro aromatics. These varying physical and chemical properties lead to a variety of breakdown products, stages and pathways during the bioremediation process. Some of the more commonly encountered pathways are illustrated within this document. Occasionally, bioremediation can be applied to inorganic contaminants such as mercury or arsenic where combination with organic ligands can lead to volatilisation or immobilisation of these elements. It is not proposed to apply bioremediation to inorganic contaminants at the Exeter Facility.

Bioremediation is underlain by a wide variety of enzyme driven biochemical processes, mediated by bacteria, fungi, yeasts and related microorganisms that are native to and often already present in the soils to be accepted at the facility. This process can be enhanced through the addition of suitable macro or micronutrients, enzyme precursors and growth stimulators to the incoming materials, such as the branded products "Seapower" and "Bio8" proposed for regular use. In some instances, it may be desirable to supplement the naturally occurring microbiota with strains or mixed communities of self-selected microbes that have been cultured from other polluted environments. These augmentations can increase the rate of treatment or the effectiveness against more recalcitrant molecules, such as long chain aliphatics and high molecular weight PAHs. The Bio8 product range also includes freeze-dried cultures of these naturally occurring microorganisms which are readily revived and multiplied on addition to the nutrient suspension to be applied. This has proven to be

highly effective over several years of application at other UK Remediation facilities. Where the chemical analysis of soils to be treated indicates a positive benefit, the Bio8 augmentation culture will be applied.

Although anaerobic bioremediation will be used from time to time for treatment of particular functionally substituted organics such as chlorinated or nitrated aromatics, the vast majority of the bioremediation propose will be aerobic, with the target organic molecular bonds acting as energy source and oxygen as the terminal electron acceptor for microbial energy metabolism. The main principle in aerobic bioremediation is oxidation through the action of oxygenase enzymes, followed by various peripheral metabolic reactions.

Following the initial enzyme mediated oxidation pathways resulting in energy respiration (TCA Cycle), biosynthesis of biomass is initiated, leading to cell growth and multiplication of the microbes active within the soils, which will increase the total remediation rate and increase the contact surface area in the soil. Once the compounds have been sufficiently broken down by enzyme action, the compounds can then enter the TCA (Krebs) Cycle as Acetyl CoA where they are further broken down through respiration resulting in water, carbon dioxide and energy for the microbes (National Research Council, 1993). In cometabolic bioremediation, apparently unresponsive molecules such as heavier PAHs or highly chlorinated compounds, can be biodegraded inside reactions initiated by the metabolism of more easily degraded contaminants or supplemented nutrients.

There are a number of limiting factors which affect the bioremediation process such as temperature and nutrient availability, as well as the availability of terminal electron acceptors, typically oxygen. Optimum temperature for biodegradation in soils is generally between 30-40°C, although locally adapted consortia may have maximum metabolic rates at temperatures well below this. Enzyme cytochrome P450 monooxygenase, a significant monooxygenase enzyme, is reported to have an optimum temperature range of 25°C-40°C (Singh et al., 2012). Not only do the metabolic processes within the microorganisms occur at a higher rate between these temperatures, but the solubility and therefore microbial uptake of hydrocarbons also increases with temperature. This is also due to microbial cell membranes becoming

more fluid at higher temperature, allowing easier incorporation of hydrocarbons into the cell. However, above these temperatures, the rate of biodegradation decreases due to the die-off of the microorganisms because of denaturing of the enzymes involved within the metabolic processes.

2. PRE-ACCEPTANCE / PRE-TREATMENT LIMITS

Before material is accepted into the Exeter Treatment Facility it must be tested for potential contaminants in order to accept the waste. Along with the chemical analysis, the origin must accompany it in order to accept the waste under the correct waste code and to enter it into the most suitable treatment stream.

There are several limiting factors which affect the bioremediation process that cannot be controlled as process variables within the engineered bioremediation. These will therefore constitute limiting conditions on the pre-acceptance assessment of candidate soils for treatment at the Exeter Treatment Facility. The current permitted metals and TPH upper limits for bioremediation processes are:

TPH – 20,000 mg/kg max

Heavy metals – 1000 mg/kg for each individual metal

The concentrations of many metals and other toxic non-target contaminants such as free and complex cyanides, within soils play an important role in limiting the effectiveness of bioremediation. Metals such as Calcium, Chromium, Cobalt, Copper, Iron, Magnesium, Sodium, Potassium, Nickle and Zinc are micronutrients to many microbes, involved in redox processes within cells and forming active regions within enzymes important within bioremediation. However, the concentration of these metals must be carefully assessed, if concentrations are too high, then this can negatively affect the cells within the microbes, resulting in death. Certain heavy metals; mercury, gold, silver, cadmium, lead and aluminium have no biological role within the cells and therefore are ecotoxic to soil life. Hg^{2+}, Cd^{2+} and Ag^{2+} attack and bond to the sulfhydryl group on enzymes essential to microbial degradation of contaminants, displacing the previously attached divalent metal, rendering the enzyme incapable of performance (Olaniran et al., 2013). Therefore, upper thresholds of these metals are set much lower than for those that do participate in essential biochemical reactions.

In order to ensure optimum microbial activity, the pH must be between 6 and 9, which is believed to be the ideal pH range of soil for microbial activity (Singh et al., 2012). This will also be tested during the pre-acceptance laboratory analysis. If pH is not within this range, it will lead to low levels of microbial degradation. The pH can also affect the

oxidative state of metals within the soils and therefore alter metal solubility, impacting microbial uptake of electron acceptors and increasing the solubility of toxic heavy metals, negatively impacting the rate of microbial activity. This can be altered within pre-treatment with the addition of lime to increase the pH and the addition of ammonium sulphate (agricultural fertiliser) to lower the pH if needed.

The soil environment is manipulated in order to provide the optimum conditions, principally by the addition of heat to increase the rate of biochemical reaction, by adjustment of pH to enzymatically favourable range and through manipulation of the soil redox environment. The rate of microbial degradation is most commonly increased by increasing the rate of oxygen supply to the soil pore spaces where active microbial metabolism takes place. Control of soil moisture content is another factor that can positively or negatively influence the rate and ultimate end points of bioremediation. The use of engineered biopiles with monitoring of treatment batch temperature, redox potential and pore moisture allows good process control of these parameters.

Water supports much of the metabolic processes within the contaminated soils, therefore, there must be sufficient soil moisture. This can be tested for, pre-treatment, and amended where appropriate. Soil moisture should usually be above 15-20% at the beginning of the bioremediation process. If moisture of potential treatment batches is assessed at below 15% in pre-acceptance laboratory results, or during acceptance confirmatory checks, then water can be added accordingly. If moisture is above 40%, then this can fill soil pore spaces and limit oxygen availability, reducing the rate of biodegradation of contaminants. Any need for further drying will be identified during the preacceptance and pre-treatment stages. Overly wet material will be dried through the introduction of drainage, by forced air drying within the biopile, by application of low temperature heat, wither in the biopile or as a pretreatment step, as elsewhere described, or by the addition of lime where raising the pH would also be desirable for optimising microbial activity.

Where pre-treatment testing indicates that the indigenous microbiota may be starting from a low metabolic base, or where particular well adapted strains or functional enzymes are found to be largely absent (e.g. in the case of recent spills of heavy oils, or for reductive dichlorination of halo-alkenes) then augmentation of the natural

microbial content of the soil may be considered. In addition to the application of the Bio8 augmentation culture, UK registered augmentation cultures such as "Surge" ® and "Bio-Dechlor" ® will be used where pre-acceptance characterisation and pretreatment tests indicate this is appropriate.

3. BIOREMEDIATION PROCESS PATHWAYS FOR TYPICAL CONTAMINANT GROUPS

3.1 Mono-aromatics

Mono-aromatic compounds, such as phenols and BTEX are widely fully biodegraded through microbial activity into TCA cycle constituents for microbial respiration. The recalcitrance of these compounds varies with varying structure. There are five pathways through two processes to degrade monoaromatics, through ring hydroxylation pathways or alkyl substitution processes in order to form dihydroxyl compounds such as catechols which can then be further degraded. Toluene is the most readily degraded BTEX compound, followed by p-xylene, m-xylene, benzene and ethylbenzene, therefore recalcitrance increases as alkyl substitution increases. This rule does not apply to benzene, as it is not alkylated, it can only undergo breakdown through ring hydroxylation and therefore it is not as readily degraded as toluene (Cao et al., 2009).

In the ring hydroxylation process dioxygenases enzymes attack the aromatic ring to produce a dihydroxyl compound while monooxygenases enzymes attack the aromatic ring to produce arene oxides, unstable intermediates, which then instantly convert into phenols. These monooxygenases carry out another monooxygenation on these phenols to convert them to dihydroxyl compounds, such as catechols to undergo ring fission in order to produce intermediates for the TCA cycle (Cao et al., 2009).

Alkyl substitution oxidation pathway involve the oxidation of the alkyl group on the monoaromatic compound to produce dihydroxyl compounds. These dihydroxyl compounds then undergo ring fission to produce TCA cycle intermediates for microbial respiration.

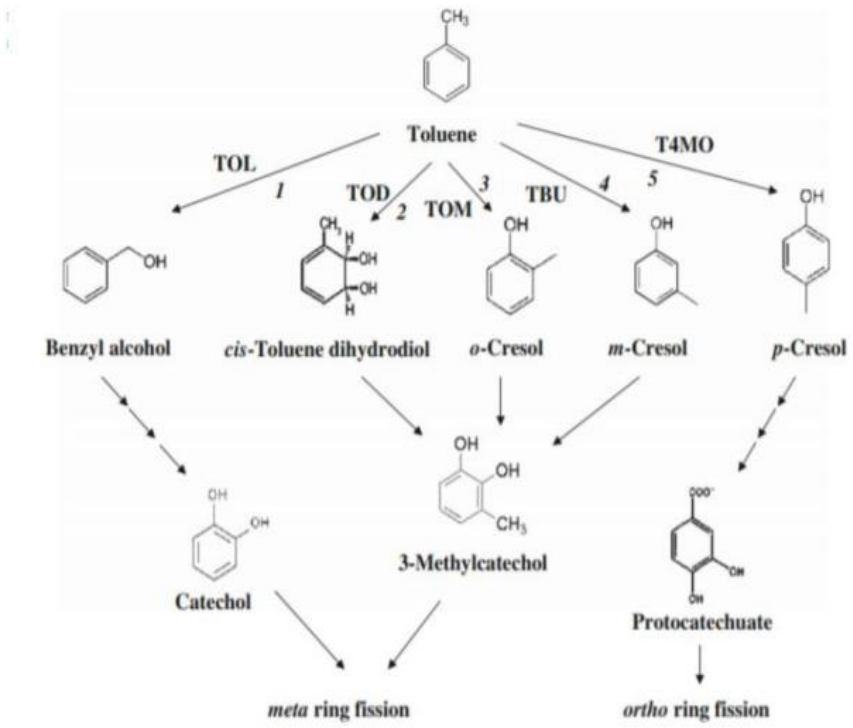

Figure 1: Illustrative pathways of microbial mediated breakdown of toluene to meta and ortho ring fission intermediates which are further broken down to TCA Cycle reagents for microbial respiration. These ortho and meta fission pathways are shown in figure 3 (Cao *et al.*, 2009).

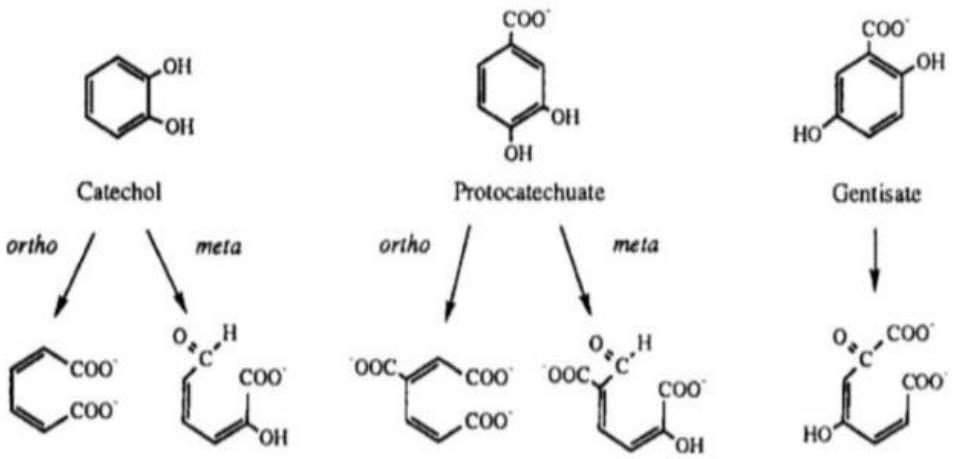

Figure 2: Dihydroxyl intermediates which result from the breakdown of aromatic compounds which can then be cleaved to form TCA Cycle reagents, as shown in Figure 3 (Cao *et al.*, 2009).

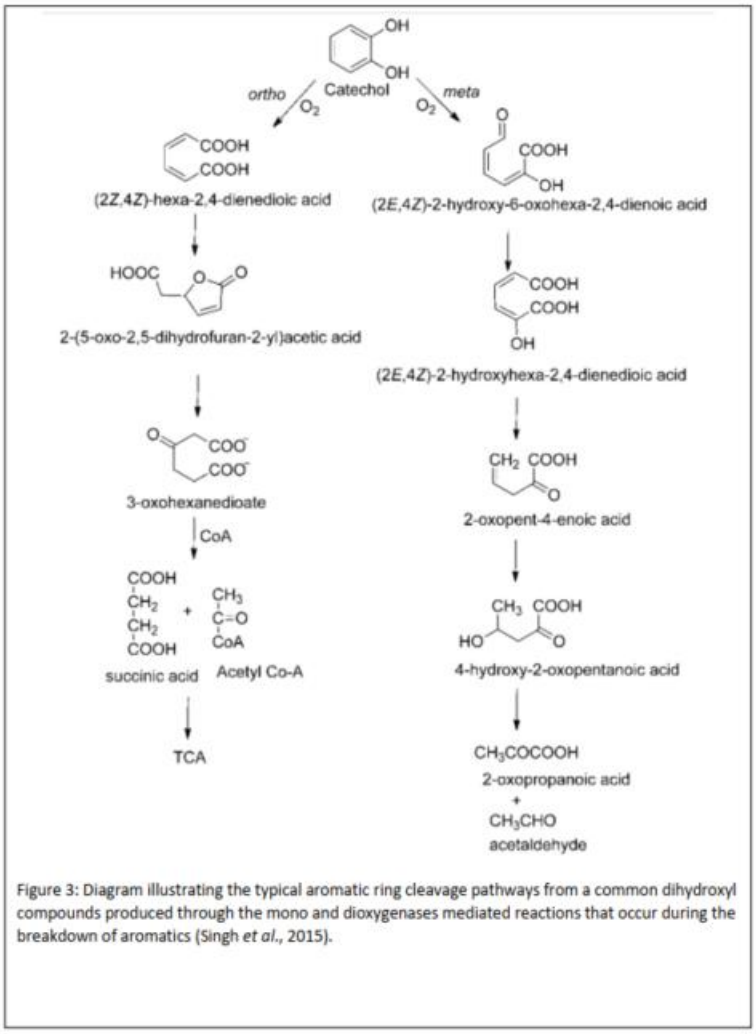

Figure 3: Diagram illustrating the typical aromatic ring cleavage pathways from a common dihydroxyl compounds produced through the mono and dioxygenases mediated reactions that occur during the breakdown of aromatics (Singh *et al.*, 2015).

3.2 Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are organic molecules consisting of two or more benzene rings. Common PAHs within the environment typically comprise of 2-7 benzene rings ranging from naphthalene (C10H8) to a 7 ringed coronene (C24H12). As molecular weight increases, polarity and solubility decrease, making microbial uptake increasingly difficult. Therefore bioavailability, biodegradation capability and rate decreases with the increasing number of benzene rings. These higher molecular weight compounds tend to be recalcitrant, with much slower rates of breakdown and lower percentage reduction end-points achievable under standard conditions (Manoli, and Samara, 1999). High molecular weight PAHs can still effectively be biodegraded by cometabolic processes, although at much slower rates than for more labile molecules. They can also be chemically oxidised, breaking them down to more labile molecules, thus increasing their bioavailability and suitability for general bioremediation. Chemical

oxidation can be used as the first step in a treatment train to include subsequent bioremediation, this will be evaluated during pre-acceptance of materials to Wheal Jane and is envisaged as one of the main applications of chemically mediated oxidation to be used.

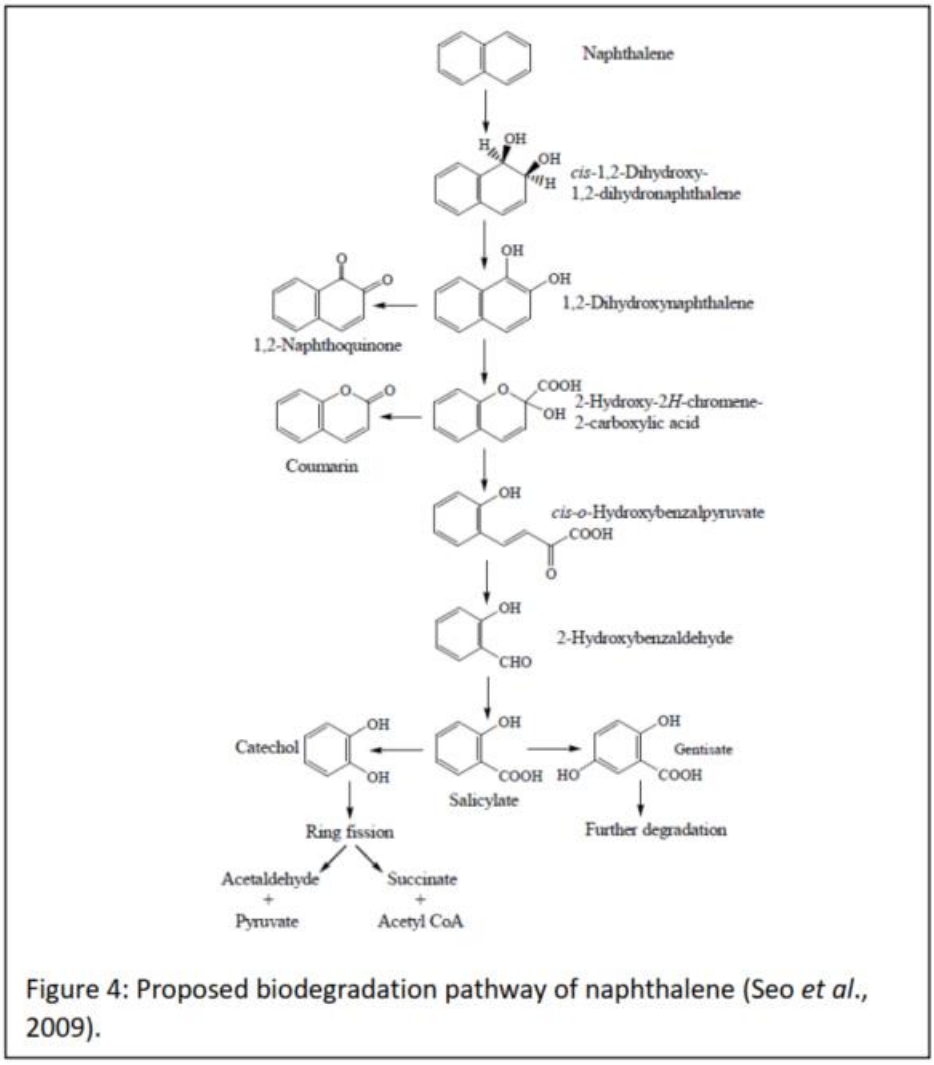

Figure 4: Proposed biodegradation pathway of naphthalene (Seo *et al.*, 2009).

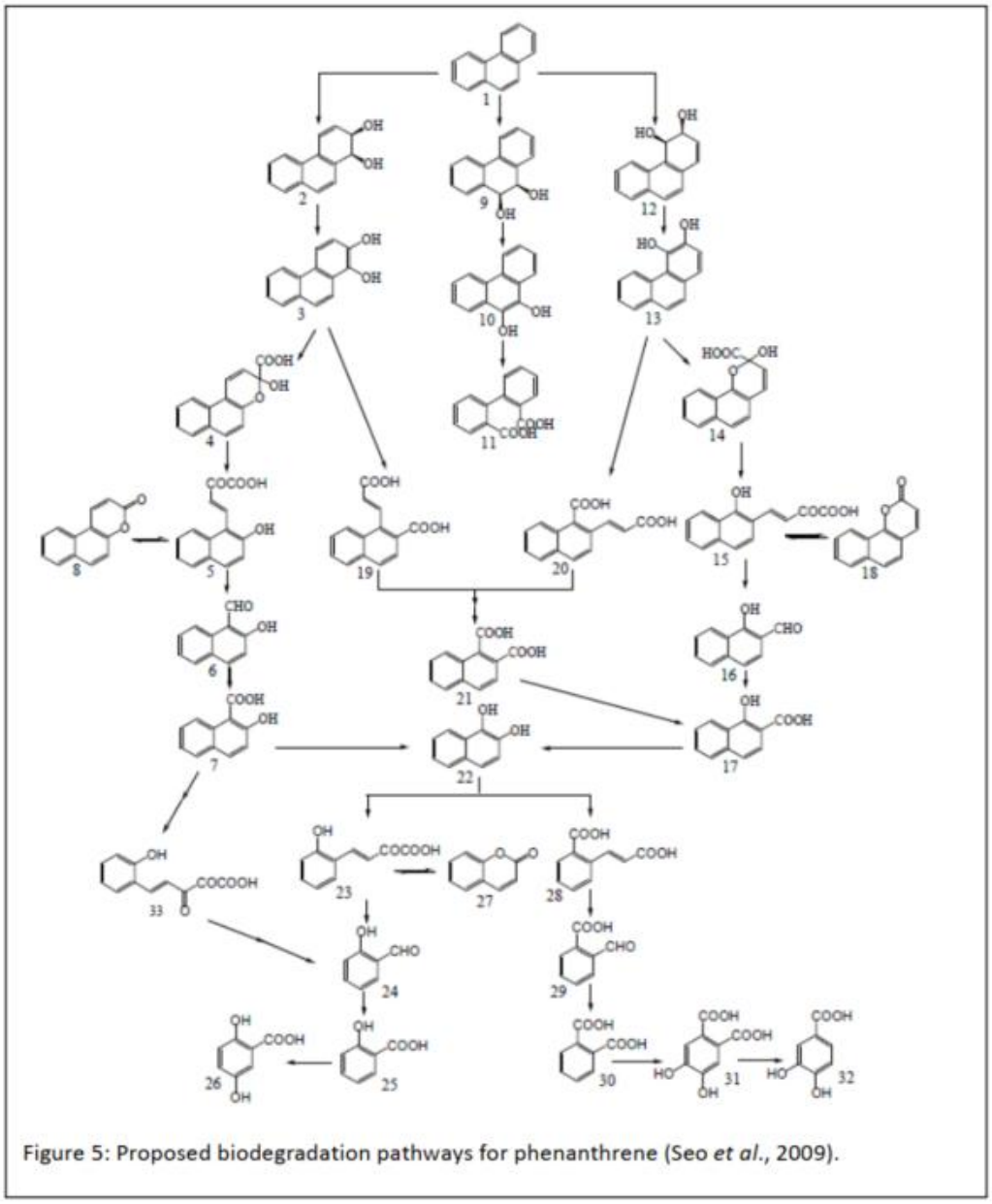

Figure 5: Proposed biodegradation pathways for phenanthrene (Seo *et al.*, 2009).

PAHs microbially degrade most effectively under aerobic conditions as anaerobic conditions are not energetically favourable, and so therefore if they occur under anaerobic conditions the process is very slow. Depending on number of aromatic rings, the more potential pathways of degradation there are, as illustrated in figures 4, 5 and 6. However, usually most of these pathways undergo oxygenation through reactions catalysed by either mono or di- oxygenase. These enzymes either attack the benzene rings by adding one or two hydroxyl groups. Naphthalene dioxygenase is a particularly useful bacterial enzyme for oxidising bi and tri cyclic PAHs (Peng et al., 2008). The varying pathways of microbial breakdown depends on the target carbons/target dissociated double bonds leading to a variety of different breakdown pathways and

breakdown products. For example, fluorene begins with three possible pathways as the initial oxygenation of fluorene by a dioxygenase mediated oxidation at the 1,2 or the 3,4 position or by monooxygenase at the C9 position (Brzeszcz and Kaszycki, 2018) as shown in figure 6.

These compounds are then rearomatized by dehydrogenases to form dihydroxyl aromatic intermediates (eg. catechol and protatechate). Two more enzymes, intradiol dioxygenases and extradiol dioxygenases then cleave the aromatic ring of these dihydroxy intermediates by the addition of an oxygen. In the ortho-cleavage pathway, the oxygen attacks the bond between the two hydroxyl groups, in the meta-cleavage pathway the oxygen attacks the bond adjacent to one of the two hydroxyl groups (Cao et al., 2009).

The subsequent meta and ortho cleavage pathways lead to the formation of TCA cycle constituents for respiration (Acetyl CoA) from the dihydroxyl intermediate eg. catechol is shown in figure 3.

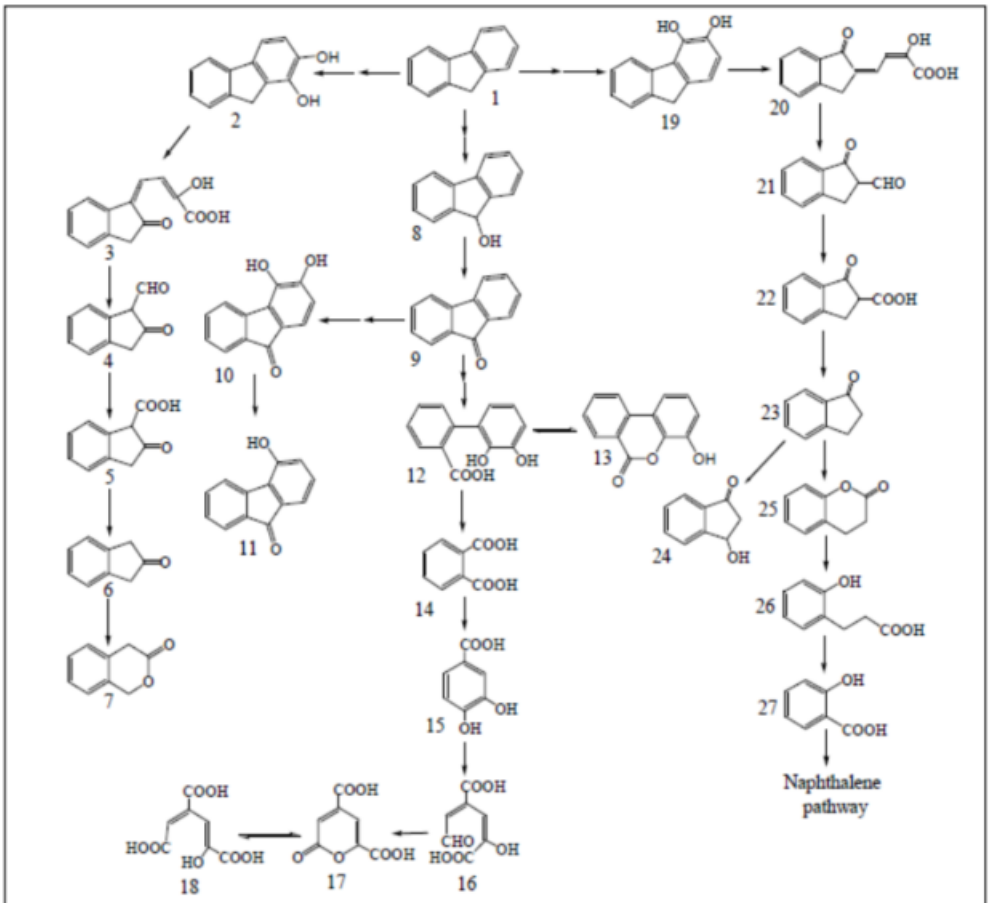

Figure 6: Proposed biodegradation pathways for fluorene (Seo *et al.*, 2009).

3.3 Aliphatic Hydrocarbons

3.3.1 Alkanes

Alkanes make up to 50% of crude oil depending on the source and so are very common constituents of hydrocarbon contamination encountered at soil treatment facilities. Alkanes are saturated hydrocarbons, classified as linear n-alkanes, cyclic cycloalkanes and branched alkanes.

Aliphatic hydrocarbons generally have low reactivity due to the lack of functional groups, apolarity and therefore low water solubility thus low bioavailability. However, alkanes are widely microbially degraded compound. Lower molecular weight n-alkanes are most easily degraded but, chains up to C44 can be broken down microbially. Most commonly, the first step within the degradation process is the oxidation of the aliphatic

compound by monooxygenases enzymes. The low reactivity of the alkane is overcome by the monooxygenase by producing a highly reactive oxygen ion. This oxygen then oxidises the alkane at the methyl group to a primary alcohol which is then transformed into an aldehyde then into a fatty acid. This acid compound either then gets converted into CO2 through respiration within the TCA Cycle or assimilated into cell biomass through biosynthesis. (chapter 17, microbial degradation of alkanes, Singh, 2011).

Terminal degradation

R-CH3 > R-CH2-OH > R-CHO > R-COOH

n-alkane > primary alcohol > aldehyde > fatty acid

Subterminal degradation

R-CH3 > R2-CH-OH > R2-CHO > R-O-CHO-R' > R-CH2-OH > R-CHO > R-COOH

n-alkane > secondary alcohol > methyl ketone > acetyl ester > primary alcohol + Acetate (into TCA

Cycle) > aldehyde > fatty acid

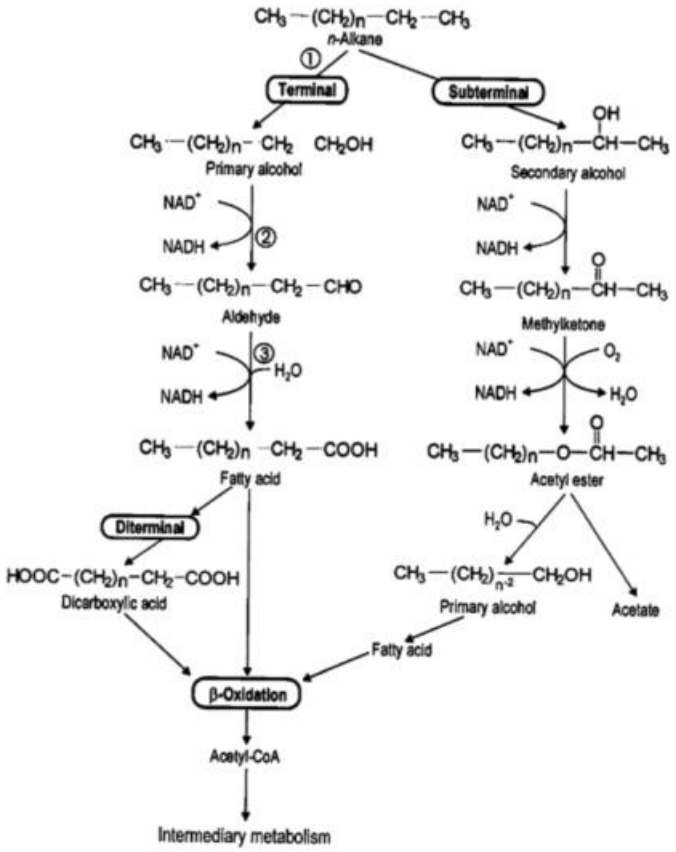

Figure 7: Schematic showing the typical breakdown pathways of a typical n-alkane (Singh *et al.*, 2012)

3.3.2 Alkenes

Alkenes, unsaturated hydrocarbon chains, are microbially degraded by the attack of the double bond at the unsaturated end of the chain or by the a similar mechanism to n-alkanes, through oxidation of the saturated end of the chain in order ultimately to produce fatty acids for respiration and biosynthesis (Abbasian et al., 2015).

3.3.3 Cycloaliphatics

Cycloaliphatics make up a large proportion of petroleum products. Cycloaliphatic compounds, such as cyclohexane, are six carbon alkane chains bound at each end to form a cyclic shape. These are primarily used as non-polar solvents, due to their a-polarity. These compounds differ from aromatic compounds as they are cyclic compounds formed from a saturated carbon chain, rather than aromatic compounds where each of the carbons are theoretically joined by double bonds but form a delocalised central aromatic ring. Cycloaliphatics are readily degraded within

bioremediation. This process is initiated by the conversion of the cycloaliphatic to an alcohol or ketone by monooxygenase enzymes then into a fatty acid through hydrolysis, Eg. Cyclohexane:

Cyclohexane > cyclohexanol > cyclohexanone > ε-caproactone + H2O > adipic acid

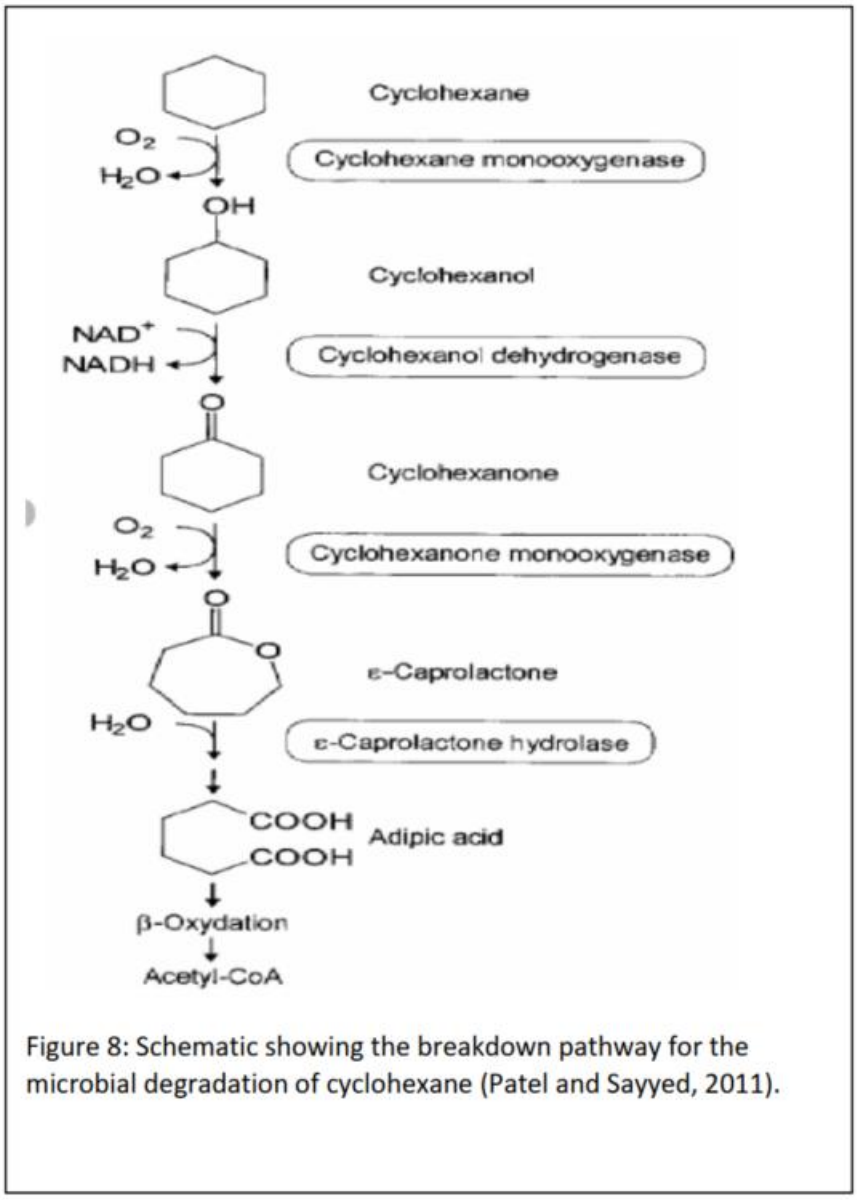

Figure 8: Schematic showing the breakdown pathway for the microbial degradation of cyclohexane (Patel and Sayyed, 2011).

3.4 Chlorinated Compounds

Reductive dehalogenation is an important means of biodegradation for many halogenated organic compounds, such as organochlorine pesticides, alkyl solvents and aryl halides. These compounds make up most of the persistent organic pollutants.

Reductive dehalogenation is currently the only known pathway of bioremediating PCBs, PCP and PCE. Dehalogenation is a process which renders compounds less toxic and therefore more readily biodegraded microbially through further peripheral pathways outlined earlier within this report. Reductive dehalogenation occurs under anaerobic conditions; however, it is known to occur under aerobic conditions for certain highly halogenated compounds.

This is carried out by 2 processes, hydrogenolysis, which involves the replacement of the halogen substitute by a hydrogen atom and the second is vicinal reduction, the removal of 2 halogen substitutes from adjacent carbons with the formation as an additional bond between them. Both processes involve an electron donor, and so is biologically catalysed. The replaced halogen atoms are released as free halide anions (Mohn and Tiedje, 1992). These resulting alkanes and alkenes are then broken down in the process as described previously:

Alkyl hydrogenolysis

$$Cl\text{-}CH_2\text{-}CH_2\text{-}Cl + H^+ > CH_3\text{-}CH_2\text{-}Cl + HCl$$

Vicinal reduction

$$Cl\text{-}CH_2\text{-}CH_2\text{-}Cl + 2e^- > CH_2{=}CH_2 + 2Cl^-$$

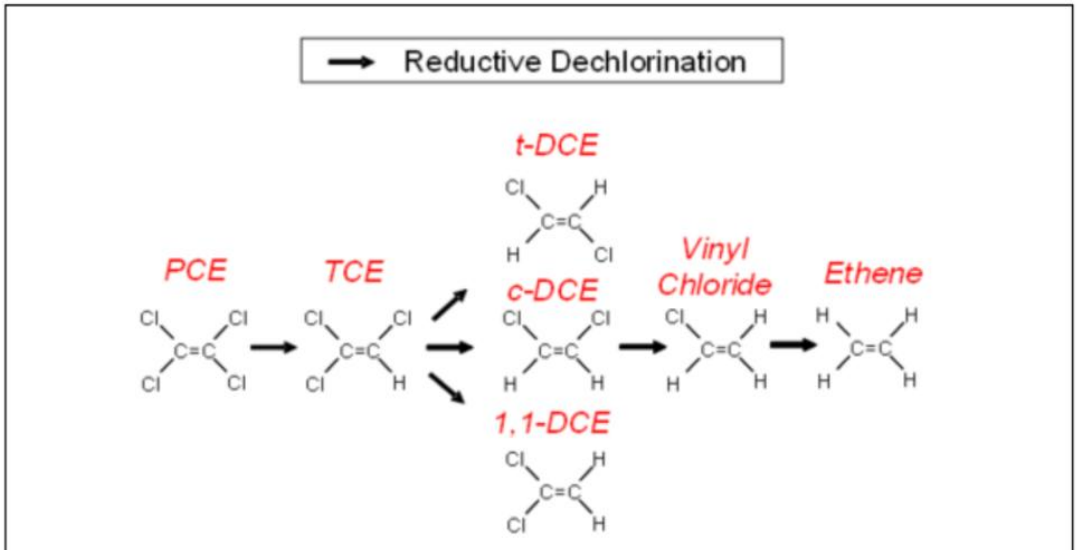

Figure 9: The reductive dichlorination of PCE.

3.5 Halogenated Aromatics

The recalcitrance of organics increases with the level of halogenation. Chlorinated aromatic compounds are very common within the environment due to their wide use as pesticides. Halogenated aromatics follow two pathways in order to remove the halogen group with a H+ or a OHsubstitution. These processes occur under anaerobic conditions, much like the degradation of nonaromatic halogenated compounds. Reductive dehalogenation can occur under aerobic conditions, however, this is rare, and so it is important to maintain an anoxic environment when treating soils high in chlorinated organic compounds (Mohn and Tiedje, 1992).

Aerobic conditions do not usually result in microbial degradation as the release of chlorine interferes with many dioxygenase enzymes that initiate the breakdown of aromatic rings. Many microbes have dehalogenase enzymes that facilitate the removal of chlorine from the aromatic rings (Copley, 1997). Once the ring has been dehalogenated these aromatic rings can then be readily degraded in the pathways outlined earlier in this report.

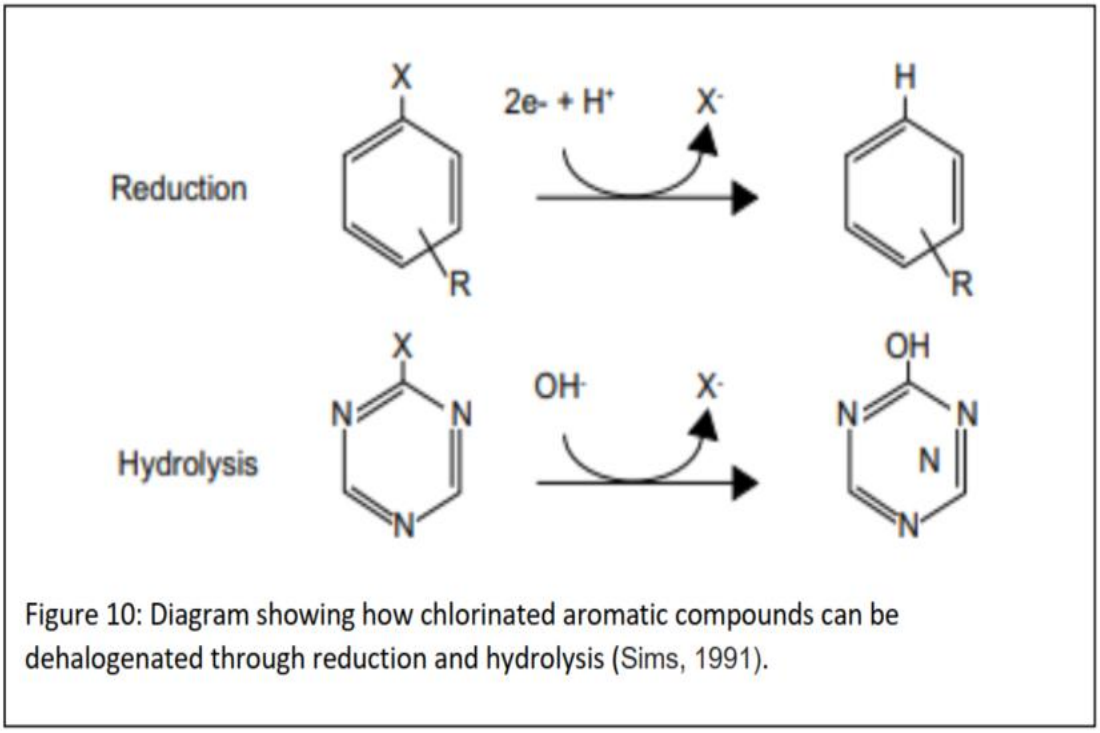

Figure 10: Diagram showing how chlorinated aromatic compounds can be dehalogenated through reduction and hydrolysis (Sims, 1991).

3.6 Nitroaromatics

Nitroaromatic compounds are compounds formed from at least one nitro group attached to an aromatic ring, such as nitrobenzene, nitrophenols and nitrotoluenes. Nitroaromatic compounds can be degraded both anaerobically and aerobically in order to remove the nitro group so that the aromatic ring can undergo microbial breakdown via either the ortho or meta cleavage pathway as shown in figure 3. Most nitroaromatics with multiply nitro groups are broken down via anaerobic processes and aromatics with lesser degrees of nitro substitution are more likely degraded through aerobic microbial activity.

3.6.1 Anaerobic Biodegradation of Nitroaromatics

Within the anaerobic degradation of nitro aromatics, the nitro group is reduced into either hydroxyl amines or through the reduction facilitated by nitro reductase enzymes. However, due to the typical breakdown pathways of aromatic rings, it is very rare for the complete mineralisation of nitroaromatics to be catalysed by one group of enzymes, as the most favourable breakdown pathway for benzene rings is through aerobic degradation (Singh et al., 2015). A process of anaerobic desubstitution, followed by aerobic mineralisation is therefore often selected.

In anaerobic conditions, nitrogen can be used as the electron acceptor, via nitrate reducing bacteria, these bacteria can switch from aerobic and anaerobic respiration. Methanogens cannot directly transform these complex compounds; however, they can facilitate the denitrification of the rings.

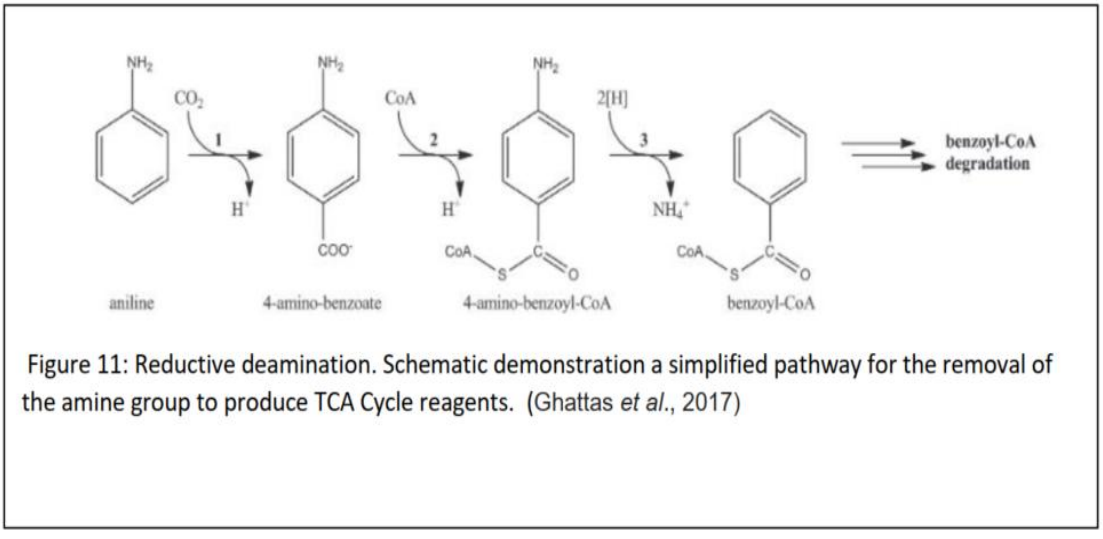

Figure 11: Reductive deamination. Schematic demonstration a simplified pathway for the removal of the amine group to produce TCA Cycle reagents. (Ghattas *et al.*, 2017)

3.6.2 Aerobic Biodegradation of Nitroaromatics

Nitroaromatics are degraded aerobically through oxidation via either a mono or di-oxygenase enzyme, or they are partially reduced via a nitro reductase enzyme mediated reaction.

Through partial reduction of nitrobenzene, nitrobenzene is reduced to hydroxyl aminobenzene by reductase enzymes. This hydroxyl aminobenzene then undergoes intramolecular transfer of the hydroxyl group to form an amino phenol which can then undergo meta-cleavage, breaking the benzene ring to form aminomuconic semialdehyde. This intermediate is then immediately oxidised to aminomuconate which goes on to be deaminated and then decarboxylated. However, depending on the bacteria, the order in which the compound is deaminated and decarboxylated can vary (Ju and Parales, 2010). These breakdown products are then hydrolysed and cleaved, resulting in TCA Cycle reagents.

Alternately, nitrobenzene can be fully oxidised, via mono or di oxygenase to substitute out the nitro group on the benzene ring, leading to the formation of catechol, which can then be microbially degraded following the ortho or meta cleavage pathways.

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