A review of germination and early growth as a proxy for plant fitness under petrogenic contamination — knowledge gaps and recommendations

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ABSTRACT

Germination—an important stage in the life cycle of plants—is susceptible to the presence of soil contaminants. Since the early 1990s, the use of germination tests to screen multiple plant species to select candidates for phytoremediation has received much attention. This is due to its inexpensive methodology and fast assessment relative to greenhouse or field growth studies. Surprisingly, no comprehensive synthesis is available of these studies in the scientific literature. As more plant species are added to phytoremediation databases, it is important to encapsulate the knowledge thus far and revise protocols. In this review, we have summarised previously-documented effects of petroleum hydrocarbons on germination and seedling growth. The methods and materials of previous studies are presented in tabulated form. Common practice includes the use of cellulose acetate filter paper, plastic Petri dishes, and low numbers of seeds and replicates. A general bias was observed for the screening of cultivated crops as opposed to native species, even though the latter may be better suited to site conditions. The relevance of germination studies as important ecotoxicological tools is highlighted with the proposed use of root imaging software. Screening of novel plant species, particularly natives, is recommended with selection focussed on (i) species phylogeny, (ii) plant morphological and functional traits, and (iii) tolerance towards harsh environmental stresses. Recommendations for standardised protocols for germination and early growth monitoring are made in order to improve the robustness of statistical modelling and species selection in future phytoremediation evaluations and field programs.

KEYWORDS: Bioindicators, ecotoxicological assessment, PAHs, phytoremediation, seed germination, selection criteria

Capsule: Germination studies are important to rule out inefficient plant species from phytoremediation experiments and prove useful in assessing site ecotoxicological status.

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1. Introduction

Anthropogenic releases of petrochemicals contaminate terrestrial ecosystems with high-molecular-weight organic compounds of a carcinogenic and/or mutagenic nature (Banks and Schultz 2005; Corgié et al. 2004; Dong and Lee 2009; Maila and Cloete 2002). Spillage or loss of containment events commonly occur as a result of activities associated with the extraction, production and transportation of oil for both manufacturing industries and agricultural operations. Subsequent clean-up expenses for contaminated sites can run into billions of dollars. For example, in the USA, the largest producer of oil, it is estimated that the clean-up of all current hydrocarbon-contaminated sites will cost approximately US\$1 trillion (Kuiper et al. 2004; Stroud et al. 2007). In soils ‘aged’ with hydrocarbon contaminants, the residence time of recalcitrant fractions and their breakdown products can be more than decades. For example, Rezek et al. (2008) experimented with remediation of soil contaminated as long ago as World War II (1939–1945) and found that even after 50+ years, the concentrations of most polycyclic aromatic hydrocarbons (PAHs) were still high and they had not degraded significantly after 18 months of vegetation or fertilisation amendments. Such long-term persistence presents a high risk of contaminants leaching into groundwater and their potential bioaccumulation in the food chain; this is widely discussed in the literature (Collins et al. 2006; Fujikawa et al. 1993; Gao and Zhu 2004; McCready et al. 2000; Meador et al. 1995; Siddiqui and Adams 2002; Simonich and Hites 1995). As a result, the United States Environmental Protection Agency (USEPA) has registered 16 such PAHs as hazardous priority contaminants (Lamichhane et al. 2016).

As physical methods of remediation such as incineration and thermal desorption are unacceptable both on an environmental and economical basis (Henner et al. 1999; Joner and Leyval 2001; Smith et al. 2006), biologically-assisted remediation, i.e. remediation by soil microorganisms in the absence of plants, has been successfully employed. Traditionally, bioremediation techniques have been studied in conjunction with fertiliser supplements to ensure C:N:P ratios favour high microbial activity (Atlas 1991; Dibble and Bartha 1979; Walworth and Reynolds 1995). In general, a molar ratio of 100:10:1 is recommended and used in most studies (Leys et al. 2005). However, hydrocarbon spills also cause ecological disturbance of the soil indigenous micro-biota (Megharaj et al. 2000) and fertiliser additions rarely aid recuperation by the microbial biota to the pre-spill state, as it is mostly hydrocarbon degrader communities that persist. Indeed, evidence suggests that increased use of fertilisers can cause ammonia or nitrite toxicity to microorganisms (Tibbett et al. 2011).

In the past two decades, phytoremediation (the use of plants to degrade contaminants of all types, e.g. organic, heavy metals) as a ‘green liver’ concept has been extensively investigated in relation to the degradation of hydrocarbon contaminants (Sandermann 1994). Phytoremediation garners high public acceptance due to its apparently sustainable and ecologically-holistic approach. Moreover, numerous studies have reported greater degradation of contaminants in the presence of plants than in microbe-only controls (Aprill and Sims 1990; Binet et al. 2000; Gaskin and Bentham 2010; Günther et al. 1996; Meng et al. 2011; Miya and Firestone 2001; Peng et al. 2009; Reilley et al. 1996). However, understanding the underlying mechanisms involved in the breakdown phases of a natural phyto-remediator system is slow owing to the complexity of the multiple mechanisms that plants can employ to remove the hydrocarbons (Table 1). It is also important to point out that some studies have reported that the growth of test plants in hydrocarbon-contaminated soil failed to catalyse efficient degradation compared with unplanted controls (e.g. Fang et al. 2001; Ferro et al. 1997; Olson et al. 2007b; Watkins et al. 1994). Plant species may differ in their tolerance of hydrocarbon contaminants; therefore, it is essential to screen more plant species for hydrocarbon tolerance to identify the most tolerant species and those best-suited to specific situations as there is huge variability in soil type, contaminant history, climatic conditions and nutritional status of contaminated sites. This may increase the likelihood of selecting species effective in causing biodegradation.

Table 1 Mechanisms of phytoremediation

Types	Definition ¹
<u>In planta</u>	
<i>Phytoextraction</i>	Ability of plants to uptake contaminants from the subsurface and translocate to leaves or other parts (metals, inorganics compounds only)
<i>Phytovolatilisation</i>	Diffusion of a volatile contaminant or its degradation product from plant parts (volatile forms of organic and inorganic compounds)
<i>Phytodegradation</i>	Breakdown of contaminants inside the plant after uptake (organics of appropriate hydrophobic range, inorganic compounds)
<i>Rhizofiltration</i>	Removal of the contaminant from the dissolved phase and thereafter accumulation in the root system (inorganic compounds only)
<u>Ex planta</u>	
<i>Phytostabilisation</i>	Reduced contaminant mobility due to plant-associated processes; physical (from wind and water erosion) and chemical (adsorption or precipitation of contaminant on plant roots or soil) (metals and inorganics only)
<i>Phytohydraulics</i>	Use of phreatophytic trees and plants to transpire large volumes of water to limit leaching of the contaminant from the vadose zone after precipitation (wide range of contaminants)
<u>Interface</u>	
<i>Rhizodegradation</i>	Breakdown of contaminants within the plant root zone, i.e. the rhizosphere (mostly organic chemicals)

¹ As defined in USEPA 2000; Gerhardt et al. 2009; Germida et al. 2002; Kamath et al. 2004; Pilon-Smits 2005; Susarla et al. 2002; Wenzel 2009.

Screening of plant species for hydrocarbon tolerance, based on growth characteristics, requires months of plant development (Marques et al. 2010); maintenance of a large number of species in a replicated experiment over this time is expensive (Kirk et al. 2002). Associated procedures such as solvent extraction of contaminated soils and gas chromatography-mass spectrometry (GCMS) analyses of the extracts add further to the costs. To fast-track screening and reduce operational expenses, many authors have supported the use of germination screening tests (Maila and Cloete 2002; Smith et al. 2006). Also, the most effective approach to establishing vegetation on disturbed lands, such as contaminated sites, is direct seeding, as it ensures better survival and is cheaper than alternative transplanting techniques (Merritt and Dixon 2011; Palmerlee and Young 2010). Even so, high germination rates are desirable to reduce seed costs. To our knowledge, this review is the first attempt to consolidate the present knowledge on seed germination and early growth in hydrocarbon-contaminated soils. Phytoremediation databases such as PhytoPet (Frick et al. 1999), Clean-up Information (USEPA Clu-in), and the Kansas State and North Dakota University databases (KSU 2012; NDSU 2012) are being developed

by enlisting species with high potential to enhance biodegradation. Most of these species grow naturally on contaminated sites and are, therefore, added to the list. Efficient use of these databases for future field trials will require extensive testing at germination and other plant growth stages. Therefore, it is important to address the knowledge gaps to improve the current germination protocols. In this review, we discuss the effect of contaminant characteristics on the physiological and morphological aspects associated with seed germination and the significance of germination tests as a tool for ecotoxicological assessment. For the purpose of formulating lists of prospective candidates to screen, selection criteria are compiled based on insights into beneficial plant attributes known to stimulate biodegradation. Lastly, we summarise the methodology of most of the previous germination trials, illustrate the key results with examples (from the literature and one of our studies) and propose recommendations for improved germination protocols.

2. Nature and fate of petrogenic contaminants in soil and their effect on germination

Petroleum (or crude oil) hydrocarbons (PHCs) can be broadly classified as (1) aliphatics (alkanes, alkenes, alkynes and cycloalkanes) and (2) aromatics (monoaromatics and polycyclic aromatic hydrocarbons, PAHs). The aliphatics contain chain(s) of carbon atoms, and the aromatics have one or more benzene rings (hexagonal arrangement of carbon atoms with alternate double bonds) bonded together. In the natural environment, although incomplete combustion of coal, wood and organic matter produces PAHs (Alkio et al. 2005; Samanta et al. 2002), the main source of contamination with these compounds is crude oil and its derivatives. Crude oil is a mixture of aliphatics, aromatics, resin and asphaltene (Colwell et al. 1977). It is separated into light, intermediate and heavy distillates by the process of fractional distillation (Table 2), where the mixture is heated and its fractions (vapours), based on their boiling points, condense back onto vertical trays placed along the length of the fractionation column.

Table 2 Products of fractional distillation of crude oil and their constituents

Distillate type	No. of carbon atoms	Boiling point range	Constituents (References)
Light (gasoline-range organics, GROs) e.g. solvents, petrol, kerosene, jet fuel	C ₄ –C ₁₂	<200°C	40–70% alkanes and cycloalkanes, <10% alkenes, 20–50% aromatics (*BTEX and 2-ring PAHs) (USEPA 2000)
Intermediate (diesel-range organics, DROs) e.g. diesel, heating oil	C ₈ –C ₂₆	200–300°C	60–90% alkanes and cycloalkanes, <5% alkenes, 10–30% aromatics (2–4-ring PAHs) (Trapp et al. 2001; USEPA 2000)
Heavy (mineral oil range organics, MOROs) e.g. lubricating oil, fuel oil, grease, waxes	C ₁₅ –C ₅₀	300–450°C	Aromatics (4–6-ring PAHs), alkyl-PAHs (ATSDR 1999)

*BTEX (benzene, toluene, ethylbenzene and xylene); PAHs (polycyclic aromatic hydrocarbons)

In most experimental studies, soil is spiked with crude oil or diesel (fresh contamination) to evaluate the effect of an incremental increase in contaminant concentration on germination and emergence, and sometimes on the subsequent survival (Table 3). Other fuel types such as petrol, kerosene and transformer oil are much less studied (Sharonova and Breus 2012). The highest tested contamination levels were documented by Udo and Fayemi (1975) who observed a 100% and 50% reduction in germination at 10.6% and 4.2% crude oil, respectively, in freshly-contaminated soils. Usually, >0.5% contamination is reportedly enough to produce a significant reduction in seed emergence (Gaskin et al. 2008). Here, it is important to mention that in some studies the terms ‘germination’ and ‘emergence’ have been used interchangeably. However, they represent distinct phases in life-stage transitions of a developing plant, so it is important not to compare germination results with those of emergence. Generally, germination is defined as the unequivocal appearance of the radicle (i.e. primary root) outside of the fruit/seed following incubation under suitable temperature, light and moisture conditions, whereas emergence is a post-germination early seedling stage understood as the protrusion of the shoot/plumule or cotyledons out of the growth medium in the field (Bewley et al. 2006).

Most screening studies are specific-to-site(s) with one or similar source(s) of contamination. Therefore, it is uncommon in the literature to find comparisons among more than one contaminant type (Table 3). Reports on germination studies aimed at assessing individual constituents or pure compound(s) are limited and rather uninformative for applying to field situations across varying conditions and sites. However, these targeted assessments are useful to differentiate the effects of a single compound which are generally difficult to observe with the use of a composite mixture. On a broader scale, useful inferences can be drawn on the individual effects of volatile, aliphatic, aromatic and polar constituents by comparing different contaminants of inherently-dissimilar compositions (see studies cited in Table 3).

In one example of a comparative study, Henner et al. (1999) demonstrated a marked negative effect of volatile and hydrophilic aromatic compounds (identified as benzene, toluene, xylene isomers, styrene, indene and naphthalene) on the germination of several species by using Petri dish evaluations of various treatments that included: open versus closed lids, pure PAHs versus composite-contaminated soil, contaminated soil versus contaminated soil leachate, and freshly excavated versus aged vegetated soils. However, the germination assays versus field observations results were inconsistent. Previously, Chaineau et al. (1997) distilled fuel oil into light (nC_9 – nC_{14}) and heavy fractions ($>nC_{15}$) and found that the light fraction was 20 times more toxic than the heavy fraction. A recent study by MacKinnon and Duncan (2013) showed volatile branched cyclohexanes of diesel cause detrimental effects on germination and emergence. They hypothesised that the phytotoxicity of diesel in the early stages is short-lived as germination improves with time. Therefore, volatiles are likely to be largely responsible. Plice (1948) conjectured that the improvement in plant growth in successive seasons occurred due to the escape of volatile fractions.

In a pot experiment, Shahsavari et al. (2013) found no significant difference in the emergence of the 11 species that were tested in 1% diesel-engine or crude oil-contaminated soils (10,000 mg HC kg⁻¹). Contrary to these results, we undertook a study assessing the emergence potential of a selection of Australian native species compared against a suite of exotics (Fig. 1), where emergence in crude oil was far greater than in diesel-contaminated soils. Viability and optimal germination temperature were first assessed to determine the most suitable test conditions. After this, seeds of all species were assessed in a 1.5%, 3% and 6% contamination with crude oil or diesel in aluminium foil dishes (15 × 9 × 7 cm) containing 600 g of contaminated or control white sand. We found that 1.5% diesel was as lethal as 3% crude oil in some cases. Diesel oil is a complex mixture of aliphatic (80–90%) and aromatic (20–10%) hydrocarbons

(Ogbo et al. 2009; Serrano et al. 2009). As mentioned earlier, the higher concentration of volatile and aliphatic compounds in diesel was likely responsible for its greater toxicity (Henner et al. 1999) and probably caused variation in the germination response of nearby replicates.

In some instances, aged or low levels of fresh contamination in soils results in more germination (Reed and Glick 2005). This phenomenon is known as hormesis (Calabrese 2005; Kirk et al. 2002; Ma et al. 2010) and has been observed in most studies for at least one tested species (e.g. Kirk et al. 2002; Salanitro et al. 1997; Shahsavari et al. 2013; *Rodanthe chlorocephala* in Fig. 1). Hormesis results from the plant response to a slight level of stress optimising its physiological processes to give a head start to the developing embryo and often results in higher germination counts. Hormesis extends beyond seed germination, by enhancing crop growth (Carr 1919; Harper 1939) and early flowering (Vwioko and Fashemi 2005) in oil-contaminated soils. Reed and Glick (2005) suggested that at low concentrations, hydrocarbons may play a similar role as phytohormones, such as auxins. However, other processes may also be important including (i) oil contamination killing microbes thereby enhancing the amounts of soil nutrients, ii) the presence of compounds in oil that promote plant growth, and (iii) biological nitrogen fixation which may increase with the presence of oil in the soil (Baker 1971). Recently, some studies have documented the hormesis effect of benzo(α)-pyrene (a common PAH) on germination (Chigbo and Batty 2013; Diao et al. 2011), while others have found no adverse impact on seedling emergence (Ren et al. 1996; Sverdrup et al. 2007).

Fig. 1 Emergence of seeds (normalised to uncontaminated control as 100%, blue line) in white sand spiked with crude oil (red bars) or diesel (green bars); and grown at optimum temperatures; 20/10°C (*) or 35/20°C. In parentheses, family names: P—Poaceae; F—Fabaceae, Am—Amaranthaceae, As—Asteraceae, A—Apiaceae and B—Brassicaceae. One exotic species is placed in each row (right side). (Means±SE, n=3, each replicate contains >50 seeds with viability >70%).

In comparison to freshly-contaminated soils, most seeds germinate better in soils rested with contamination ('aged') before the addition of seeds. Adam and Duncan (2002) proposed that the loss of volatiles alone is not always responsible for this improvement and that reduced bioavailability also plays a significant role. As the spill of hydrocarbons in the soil matures with time, some compounds adsorb to organic matter and colloids in the soil; hence, only a small portion of the total remains bioavailable (Tao et al. 2009). Bioavailability can be defined as the fraction of contaminant present that can be readily assimilated or transformed by living organisms, commonly microorganisms (Cofield et al. 2008). While whole product toxicity is of prime interest, the byproducts formed by the natural weathering of the parent compounds can induce plant stress. For instance, Ren et al. (1996) studied the effects of photo-induced PAHs (anthracene, benzo(a)pyrene and fluoranthene) on the germination and early seedling development of *Brassica napus*. They found that, on exposure to sunlight, PAHs produce compounds that are more toxic and water soluble than parent compounds, with root fresh

weight (but not germination percentage, frequency, root numbers or root size) negatively affected by the photo-modified compounds, which suggests poor root-water retention. This result may explain why many species which germinate well in oil-contaminated environments do not subsequently survive well (Besalatpour et al. 2008; Chaîneau et al. 1997; Henner et al. 1999).

In practical scenarios, before final abandonment, sites may be contaminated sporadically for long periods due to their proximity to a contamination source such as machinery. Therefore, a challenge in the reclamation of such sites is the mixed effects of fresh and aged contamination caused by contaminants of varying composition and the overall heterogeneity caused by landscape features such as soil type (discussed in section 7.5).

3. Methodological considerations with germination assays

There is a lack of consensus among the results of germination studies, even when similar plant species are used. Other than the presence of organic matter and hydrocarbon weathering, these differences are mostly attributed to the use of different germination methodologies (Reynoso-Cuevas et al. 2008). To evaluate the germination potential of seeds in oil, germination assays have used a range of growing mediums and containers. The use of aged or freshly-contaminated field soil is most common (Table 3), although some authors have also used sterile sand (Chaîneau et al. 1997) and *in vitro* gels (Reynoso-Cuevas et al. 2008). In many cases, filter paper has also been used as a test medium (e.g. MacKinnon and Duncan 2013; Graj et al. 2013). However, cellulose, the main component of filter papers, sorbs hydrophobic organic compounds (Lamichhane et al. 2016) and may reduce their bioavailability in a preferential manner. Earlier, Wang (1993) criticised the use of filter papers for reducing the toxicities of metal cations, silver and cadmium. However, phenol, also a test substrate, showed no difference in germination among various materials that used filter paper, growth pouches and Petri dish seed trays. Petri dishes, unlike pots, are convenient to use for germination tests because they offer several benefits such as less shelf space and material (contaminant, fertiliser, etc.), the ability to test multiple plant species simultaneously, and transparency for light penetration. Petri dishes are made of polystyrene, a polymer inert to weak acids and bases. However, recently, Rochman et al. (2013) conducted three-monthly samplings for one year of two areas in San Diego Bay and tested 25 PAHs. They found that the pellets of polystyrene sorbed 8–200 times higher concentrations from time zero, when compared with other common polymers, namely polyethylene terephthalate (PET), high-density polyethylene (HDPE),

polyvinyl chloride (PVC), low-density polyethylene (LDPE) and polypropylene (PP). Differences in results are also reported depending on whether Petri dishes remained open or closed during germination assays, presumably because most oil types have a significant amount of volatile compounds. Henner et al. (1999) reported a 50% decline in seed germination in closed Petri dishes due to volatiles in tar at 150 mg kg^{-1} (0.015%). Because these conditions are not experienced by germinating seeds in field or glasshouse environments, we suggest that closed Petri dishes should not be used where high concentrations of volatiles are present. Also, those made from polystyrene should be used cautiously, more for the purpose of pure compound studies than composite.

4. Changes in seed physiology and seedling morphology – processes at microscsles

Often the main goal of a germination assay is to select one or a few species, from many possible options, for use in the bioremediation of a specific site and contaminant type (Marques et al. 2010). Species are ranked based only on germination counts or rates and, sometimes, aspects of early seedling performance such as above- or below-ground dry weight or length. This focus on plant selection is one reason why the mechanisms for differential sensitivity of plant species to organic contaminants remain poorly researched. There are many common symptoms observed during seed germination and seedling growth in oil-contaminated soils, which are summarised in Figure 2. We now discuss three widely-used explanations for these symptoms—oxidative stress, water and nutrient constraints, and penetrative damage.

Fig. 2 Common symptoms and limitations for seeds germinating in hydrocarbon-contaminated soil during successive developmental stages (A – Imbibition, B – Germination, C – Emergence and D – Seedling).

4.1 Oxidative stress

Insufficient aeration in soil occurs when the air-filled pore space decreases due to the spillage of hydrocarbons (De Jong 1980; Gudim and Syrratt 1975; Rowell 1977). This can lead to oxidative stress, which is defined as the disturbance of the cellular redox state under various environmental stresses including the lack of oxygen (Mittler et al. 2004). Generally, in response to oxidative stress, plants produce high concentrations of reactive oxygen species (ROS) in their tissues. ROS are chemically-reactive compounds containing oxygen such as superoxide, peroxide, hydroxyl radical and singlet oxygen, and high concentrations of these cause cell damage (Liu et al. 2009). Several PAHs and their metabolites such as phenanthrene, fluoranthene, 3,4-benzopyrene and 1,2-benzacenaphthene can bioaccumulate in plant tissue and are believed to produce ROS (Kolb and Harms 2000; Liu et al. 2009). Analyses of hydrogen peroxide concentration, cellular DNA damage and enzyme activities have been used to study the cell's response to oxidative stress in response to high salt, drought and heavy metals. Under stressed conditions, hydrogen peroxide, stored in peroxisomes as a normal byproduct of metabolic pathways, diffuses out due to a loss of membrane integrity and damage

to cell metabolism (Chen et al. 2013, references therein). Histological studies using diaminobenzidine staining of *Acorus tatarinowii* leaf samples showed an increase in hydrogen peroxide accumulation with increased diesel stress (Chen et al. 2013). Alkio et al. (2005) also found that phenanthrene-induced production of hydrogen peroxide was linked to the processes involved in cell death in shoots of *Arabidopsis thaliana*. Various authors have agreed that the formation of a persistent hydrophobic organic film on the surface of seeds growing in contaminated soil impedes gaseous exchange in the embryo, eventually killing it (Adam and Duncan 2002; Baker 1970; Chaîneau et al. 1997). Increased microbial activity in the presence of oil as an additional carbon source is another possible cause of oxygen depletion around an oil-treated seed (Amakiri and Onofeghara 1984; Gudín and Syrratt 1975; Udo and Fayemi 1975).

4.2 Water, nutritional and energy constraints

Contamination with hydrocarbons also affects the ability of soil to provide moisture and nutrients to plants (Reilley et al. 1996). The availability of both is critical during germination and seedling development. Due to the hydrophobic nature of oil, water spreads unevenly in oil-contaminated soils, leading to water deficiency in some areas (Merckl et al. 2005). Moreover, the presence of hydrocarbons may impede the ability of roots to explore soil for water. Once a seed has germinated under normal conditions, early root development occurs primarily due to cell expansion as opposed to cell division (Taiz et al. 2015). Ren et al. (1996) found that PAH toxicity led to the production of shorter roots in seedlings and inhibited root fresh weight accumulation. They suggested that PAHs can cause mechanical disruption to the fragile cellular membranes of new roots and thereby diminish their capacity to retain water. Cell expansion processes may also be impeded in contaminated soils due to disruption of hormone action (auxins) or cell organelle metabolism (Maila and Cloete 2002). A close look at root tips of four-day-old *Vigna unguiculata* seedlings germinated in crude oil-contaminated soil revealed a significant reduction in cell size and mitotic activity (Achuba 2006). Bona et al. (2011) also found that the expansion of cotyledons following emergence was slower in diesel-contaminated soil.

Closely related to water deficit are the nutritional constraints experienced by young seedlings in contaminated environments. The presence of oil in the soil can cause a significant reduction in plant-available forms of phosphorus, potassium (Dimitrow and Markow, 2000) and nitrate

(Baldwin 1922; Murphy 1929), and an increase in the concentrations of elements, such as manganese and sulfur, which can be toxic to plants (McGill and Rowell 1977).

In addition to reducing water and nutrient availability, oil contamination may affect plant growth through negative impacts on plant carbon exchange. Early experiments that investigated the herbicidal effects of oils on plant leaf surfaces found decreased respiration and photosynthesis in oil-contaminated plants (Baker 1970 and references therein). In addition, Achuba (2006) recorded reduced activity for two starch-degrading enzymes, total amylase and starch phosphorylase, in the cotyledons of seeds germinated in the presence of crude oil, which suggests inaccessibility of native reserves for energy production. However, compared to mangroves and algae (Naidoo et al. 2010), there are no reports on the effects of oil-contaminated soils on respiration and photosynthesis in terrestrial plants.

4.3 Penetrative damage

Depending on the soil characteristics, the aforementioned negative effect of oil contamination on seed germination and seedling emergence caused from reduced levels of oxygen, moisture and nutrients could be overcome by selecting plant species adapted to germinate in similar conditions. However, the most potent damage to seeds is that of a penetrative nature, i.e. movement of a pollutant into the seed embryo. As the wetting capacity (hydrophilicity) and penetrating power of a dry seed are high, the embryo can die if in contact with oil due to the penetration of oil components through the seed coat. Oil can enter seeds via the micropylar end (dicots), the coleorhizal end (monocots) or even through injured tissue, with the rate of penetration affected by the integrity and hardness of the seed coat (Amakiri and Onofeghara 1984). Adam and Duncan (2002) used relatively easily-penetrable *Linum usitatissimum* seeds that were pre-soaked in diesel for a week; after two weeks, 70% had germinated compared with 97% in the controls. In contrast, after eight months soaking in crude oil, seeds of *Capsicum frutescens* remained 100% viable, but the germination lag phase increased three-fold compared to the controls (Amakiri and Onofeghara 1984). The difference between species may be due selective permeability of the seed coats (Wierzbicka and Obidzińska 1998) which plays an important role in protecting the embryo from external hazards. Klock (1984) argued that seed resistance to phytotoxic properties of oils should be attributed to cell wall structure. Seed size may play an important role since large seeds generally have thicker seed coats than small seeds (Fenner 1985). Robson et al. (2004a) investigated the abundance of eight species on a contaminated site and categorised them into four groups based on seed mass. Contaminated

plots had significantly more seeds in the second highest seed mass group (0.1–9.9 mg) than the uncontaminated plots. The authors speculated that due to low fertility at the contaminated site (C:N ratio 27:1), the large seeds were able to draw upon their reserves. There is no information available on the effect of seed constituents such as soluble carbohydrates, lipids or proteins on hydrocarbon penetration or resistance. Several studies have pointed out that the intensity of translocation of hydrophilic organics into the plant biomass is closely correlated with the octanol-water coefficient, K_{ow} (Schneunert et al. 1994; Simonich and Hites 1995; Trapp et al. 1990). We assume that the penetration of hydrophilic organics such as naphthalenes into the seed is correlated with K_{ow} , as is the case with the rest of the plant.

5. Germination as an ecotoxicological indicator

Although some reports have mentioned the lack of sensitivity of germination tests in predicting the subsequent growth of a species in contaminated environments, many researchers consider that these assays are a promising tool for assessing the ecotoxicity of contaminated soils (Banks and Schultz 2005; Kirk et al. 2002; Maila and Cloete 2005; Wang et al. 2001). The plant species recommended by various agencies for this purpose are listed in Table 4. Germination bioassays have been adopted to evaluate the phytotoxic effects of heavy metals (Chang et al. 1992), herbicides (Boutin et al. 2004) and explosives (Frische 2003; Gunderson et al. 1997). The use of a germination ecotoxicological assessment for petrogenic contaminants is fairly new. Soil phytotoxicity consistently affects germination dynamics (Sharonova and Breus 2012) and could, therefore, be used to build a predictive germination tool for future ecotoxicological assessments. A common method for estimating ecotoxicity is based on measuring the total concentration of contaminants after extraction with organic solvents. It tends to overestimate contaminant bioavailability (Cofield et al. 2008; Tang et al. 2002) and so is of little use for predicting ecotoxicity (Banks and Schultz 2005; Baud-Grasset et al. 1993). In this regard, owing to their simple but repeatable methodology, low cost, moderate sensitivity to contaminants and potential for use *in situ* and *ex situ*, germination assays may be useful bioindicators for estimating ecotoxicity (Maila and Cleote 2005; Siciliano et al. 1997). Additionally, the seeds required for such assays can be purchased in bulk, have low maintenance costs, lose viability slowly when stored correctly and, therefore, can be used for repeated testing, as may be required to track the impact of remediation over time (Wang et al. 2001). Suggested species for this purpose based on their stability and high reproducibility are *Lepidium sativum* (Maila and Cleote 2002), *Cucumis sativus* (Wang et al. 2001), *Vicia faba* (Malallah et al. 1996) and *Lactuca sativa* (Banks and Schultz 2005).

Table 4 Plant species recommended by environmental regulatory agencies for use in ecotoxicity assessments of land contaminated with organic contaminants (adapted from Wang and Freemark 1995).

Agency/report year	Species required
FDA 1987	<i>Phaseolus vulgaris</i> , <i>Brassica oleracea</i> , <i>Daucus carota</i> , <i>Zea mays</i> , <i>Cucumis sativus</i> , <i>Lactuca sativa</i> , <i>Avena sativa</i> , <i>Lolium perenne</i> , <i>Glycine max</i> , <i>Solanum lycopersicum</i> , <i>Triticum aestivum</i>
OECD 1984	<i>Vigna radiata</i> , <i>B. rapa</i> subsp. <i>pekinensis</i> , <i>Lepidium sativum</i> , <i>Trigonella foenum-graecum</i> , <i>L. sativa</i> , <i>Brassica nigra</i> , <i>A. sativa</i> , <i>Raphanus sativus</i> , <i>Trifolium pratense</i> , <i>Oryza sativa</i> , <i>Lo. perenne</i> , <i>Sorghum bicolor</i> , <i>B. rapa</i> subsp. <i>rapa</i> , <i>Vicia sativa</i> , <i>T. aestivum</i>
USEPA 1985	<i>L. sativa</i> , <i>D. carota</i> , <i>Allium cepa</i> , <i>Z. mays</i> , <i>C. sativus</i> , <i>L. sativa</i> , <i>A. sativa</i> , <i>Lo. perenne</i> , <i>G. max</i> , <i>S. lycopersicum</i>
ISO 2012	<i>A. sativa</i> (compulsory), <i>B. rapa</i> or <i>B. rapa</i> spp. <i>rapa</i> (compulsory), <i>Z. mays</i> , <i>S. bicolor</i> , <i>Saccharum officinarum</i> (optional)

Note: FDA (Food and Drug Administration, Washington DC), OECD (Organisation for Economic Cooperation and Development, Paris), USEPA (U.S. Environmental Protection Agency) and ISO (International Standardization Organization).

The primary disadvantage of using seed germination as a bioindicator assay of ecotoxicity is the plant intra- and inter species differential response to organic contaminants. Njoku et al. (2008) compared four accessions of *Glycine max* (soybean) and *Lycopersicon esculentum* (tomato) and found in each case only one or two accessions were suitable for germination and growth in crude oil contaminated soil treatments. In another experiment, they found that EC₅₀ (effective concentration that caused 50% decrease in germination) values for 10 accessions of *Sorghum bicolor* differed in the range of 5-19% (Njoku et al. 2011). Siciliano et al. (1997) tested the bioindicator potential of 17 prairie grass species using a seed germination assay and found a 12-fold difference in their response towards 2-chlorobenzoic acid, an intermediate in the degradative pathway of polychlorinated aromatics. They noted the similarity between their results and those of the meta-analysis of Fletcher et al. (1990) who found an 11-fold difference among species in their response to herbicides. The data used by the latter were from the PHYTOTOX database, which consists of the response of plant species to organic contaminants

and herbicides and, at the time of analysis, was a collation of 230 published studies using 151 species from 43 families. Although the mode of action of herbicides is different to other environmental contaminants, in the absence of substantial bio-assay literature on other organic contaminants, a correlative generalisation of results for extrapolation to non-standard species or test conditions is valid (Clark et al. 2004). Other reports found large differences among potential bioindicator plant species in their response to polychlorinated biphenyls (Meier et al. 1997), PAHs (Sayles et al. 1999) and heavy metals such as lead (Chang et al. 1997). In addition to the variability among species, another reason impeding the widespread use of germination bioassays is their lack of sensitivity compared with other commonly-used assays which use earthworms, bacteria, algae and enzymes. The placement of seed germination assays in a hierarchy of sensitivity with respect to other bioassays is controversial as it may vary with the contaminant. For example, the decreasing order of sensitivity towards heavy metals was algae > daphnids > bacteria > plant seeds > earthworms, while for herbicides it was plant seeds >> algae=bacteria > daphnids >> earthworms (Miller et al. 1985; Thomas et al. 1986). Later, Dorn et al. (1998) showed that for three types of crude oil (light, medium and heavy) the order was earthworms > microtox solid-phase >> plant growth > seed germination, with earthworms 77 times more sensitive than seed germination. The influence of soil properties such as organic matter, clay, pH and temperature in such experiments has not been studied, making it difficult to differentiate plant effects from physical effects of contaminant and soil (Sayles et al. 1999). Therefore, it can be concluded that the results of seed germination bioassays for various contaminants, especially those with significantly different physical and chemical properties, may not be the most useful choice as a bioindicator for species in a particular scenario. Specific research is required on the toxicity of petrogenic contaminants under the relevant soil properties.

The suitability of a bioindicator plant species will depend on various factors such as soil, climate and species growth attributes. However, the most important prerequisite is that the measurement of its parameters (e.g. growth, emergence) must give interpretable response curves across a range of environmental parameters (Adema and Henzen 1989; Hund and Traunspurger 1994). Usually, this information is graphed as dose-dependent logarithmic response curves to calculate regression equations. The middle part of the curve is highly sensitive to contaminant concentration (Klassen and Eaton 1991) and using a bioindicator with an appropriate response range can help to predict toxicity levels (Siciliano et al. 1997). In the context of petrogenic hydrocarbons or their metabolites, only a few plant measurements (also

known as ‘endpoints’) have been used to plot response curves. These are root elongation (Baud-Grasset et al. 1993; Wang et al. 2001, 2002), shoot length (Reynoso-Cuevas et al. 2008), shoot biomass (Salanitro et al. 1997) and, most commonly, percent emergence (Baud-Grasset et al. 1993; Siciliano et al. 1997; Wang et al. 2001). The endpoints can be broadly classified as reproductive (seed production, seed viability, germination capacity, thousand-grain weight) or vegetative (shoot/root length and biomass) and the sensitivity of each is compound specific (Clark et al. 2004). Studies also include $EC_{25\text{or}50}$ (effective concentration to cause 25 or 50% inhibition) values for plant species. One challenge with the use of laboratory tests is that they may not be ecologically meaningful as, in the field, the contaminant is likely to degrade at a different rate than in the laboratory (Wang and Freemark 1995); faster rates may result in the release of toxic metabolites (Chaineau et al. 1995; Phillips et al. 2000).

When considering the use of higher plants as opposed to other test organisms such as earthworms to assess ecotoxicity, it is logistically difficult to test multiple species with multiple petrogenic contaminants under multiple soil characteristics for the desired correlations. Nevertheless, higher plants have been advocated for toxicity assessments due to their ecological relevance in the food webs, and with a particular focus on germination and early growth for the reasons mentioned previously. More research is needed to determine the antagonistic, synergistic or additive nature of the metabolite-contaminant relationship so that proper corrections can be made to bioindicator calibration curves (Siciliano et al. 1997). Figure 3 shows the effect of increasing diesel concentrations on root growth in *B. napus*. Note that parameters such as root length cannot provide linear trends; however, with the help of imaging software like WinRhizo, more insightful measurements such as root surface area, average root diameter, the proportion of the root system in root diameter classes and the number of root tips and forks can be made.

Fig. 3 The effect of increasing diesel contamination on shoot and root growth of *Brassica napus* grown in white sand for four weeks at 25°C. Inset shows WinRhizo colour classification of root diameters as red, yellow and green for root diameters of <0.5, 0.5–1.0 and 1.0–1.5 mm, respectively. Values are means ± s.e., n=3.

6. Screening plant species for industrial and agricultural land use

While industrial areas are more prone to oil spills (Maddock 2015), transporting oil in pipelines poses a huge contamination risk for agricultural areas (Corbin 2013). Most phytoremediation projects prefer to grow native plants on contaminated sites because of their innate adaptability to climate and soil conditions (Gerhardt et al. 2009; Kirk et al. 2002; Ogbo, 2009; Reynoso-Cuevas 2008). However, due to their complex germination requirements—such as species-specific temperature requirements, innate dormancy mechanisms and low seed viability—native species may be difficult to establish (Farley et al. 2013; Merritt et al. 2007). Also, some species take a long time to germinate, which may not be a successful strategy in freshly-contaminated soils as it may provide time for penetrative damage to the seed. Seeds of most cultivated plants, on the other hand, generally do not have primary dormancy and germinate rapidly. Correlation between germination time and penetrative damage may explain the results

of Sharonova and Breus (2012) who tested 50 plant species and found that cultivated plants tolerated hydrocarbon contamination better than natives. It is also important to note that cultivated plants that have been hybridised to produce large seeds and high relative growth rates (i.e. *Zea mays*, *Triticum aestivum*, *Glycine max*) may respond differently to hydrocarbon contamination than those grown for fodder (i.e. *Melilotus officinalis*) (Elias and Chadwick 1979). Moreover, as per the recommendations of environmental organisations, readily-available agronomic species have often been used for phytoremediation because they are good bioindicators of toxicity for various types of contaminants, predominantly herbicides and pesticides (Table 4). Assessment of the most elaborate database, PHYTOTOX (currently more than 3500 published studies), showed that little to no data exist for species from grassland, desert, coniferous or other biomes outside the northern temperate zone and that the data are heavily biased for agronomic types (Clark et al. 2004). There are too few reports on plant responses to petrogenic hydrocarbons in this database to draw broad conclusive trends. Also, the relevance of these research outcomes to countries such as Australia is questionable, due to the significant differences in climate, soil type, endemic flora and environmental regulation criteria (Michael et al. 2007). Moreover, using cultivated plants for remote locations such as mining sites is not feasible because basic requirements such as water and fertiliser supplements may not be easily met. Considering that the relative suitability of native and cultivated plant species for revegetation strongly depends on the requirements of the contaminated site, more species need to be screened for phytoremediation suitability.

7. Selection criteria – plant attributes and challenges

Literature suggests that germination and the subsequent survival of a particular species in contaminated soil are independent and may not correlate with the ability of the species to initiate biodegradation (Besaltpour et al. 2008; Smith et al. 2006). Therefore, it is important to formulate selection criteria based on plant mechanisms that are known to maximise the biodegradation of TPH and PAH contamination in soils. The best approach may be a combination of the factors outlined below.

7.1 Species phylogeny

Plant species vary in their tolerance to contaminants. Indeed, significant differences have been found among varieties/cultivars of the same species. For example, Graj et al. (2013) noticed that a *B. napus* variety with high (40–60%) erucic acid content in seed was better than a low (2%) erucic acid variety in terms of germination index, root elongation and biomass production

under contaminated conditions. Our results (Figure 1) show that species of Poaceae had the lowest germination when sown in diesel and high concentrations of crude oil compared with species from other families. Whether such differences can be explained by phylogeny was the topic of investigation in some recent meta-analyses. Collins and Willey (2009) ran a residual maximum likelihood analysis (REML) on data derived from ten studies incorporating 75 species. The shoot and root dry weight data were converted to a tolerance index (TI) with reference to controls with no contamination as $TI = 1$. They also ran separate models to account for root and shoot uptake of organic contaminants by converting the data to RCF (root concentration factor) and SCF (shoot concentration factor). Notably, Asterales and Brassicales were the most tolerant orders, with order and genus levels accounting for 54% of the 78%, respectively, of the variation accounted for by the model. Further, monocots were more sensitive than eudicots and the order Poales (consists of family Poaceae) was one of the most sensitive orders. This suggests that many grass species, although desirable for their high root density, may be more difficult to establish on contaminated sites than other clades. The species with the most tolerance to contamination were *Helianthus annuus*, *L. sativa* and *Salvia officinalis*. *Daucus carota* was mentioned in the study above and elsewhere (Adam and Duncan 2002) as tolerant of organic chemicals as herbicides; it belongs to the order Apiales under the same superorder, Asterid 2, as Asterales. Consistent with this, we found that emergence of *Rhodanthe chlorocephala* (Asterales) was the highest of all tested species at 1.5% diesel contamination (Fig. 1).

A more targeted meta-analysis was done by Ma et al. (2010) on data from 41 studies which measured the biodegradation of PAHs in the rhizosphere. In total, 668 comparisons of 51 species were made; the families Alliaceae and Amaranthaceae exhibited the highest biodegradation. The authors suggested that because plants in these families released aromatic compounds into the rhizosphere, they were likely to stimulate degraders more effectively. These compounds, known as plant-derived biosurfactants, increase the bioavailability (lability) of PAHs for microbial breakdown, causing enhanced biodegradation. For example, artificial and natural saponins from Chinese soapberry (*Sapindus mukorossi*) increased phenanthrene desorption in lab-based incubation experiments (Zhou et al. 2011, 2013) and the application of cyclodextrins from maize (*Z. mays*) caused tetrachloroethene degradation (Adani et al. 2010). The occurrence of some aromatic compounds in plants such as flavonoids, terpenes and alkaloids, serves an important role in plant defence mechanisms (Wink 1988). Naturally, seeds of such plants germinate in soils enriched with the litter of similar plant(s) and, therefore, may

possess specialised strategies to limit damage from hydrocarbons or enhance biodegradation of hydrocarbons via microbiological pathways.

The above examples suggest that phylogeny may be a useful way to identify species with bioremediation potential. Table 5 summarises survey reports of species present at contaminated sites; note that Poaceae, Asteraceae and Amaranthaceae are the dominant plant families. As discussed earlier (and evident in Figure 1), while Poaceae may be sensitive at germination and/or subsequent growth stages, and possibly more so in freshly-contaminated soils, it may emerge later on a contaminated site due to successional changes after species from more tolerant families are established. It is plausible that the effective species in the initial stages of bioremediation, when aliphatic compounds are high in concentration, do not remain beneficial at later stages when aromatic recalcitrant compounds persist. The evolutionary basis for why plants of some families/genera are more tolerant than others has not been studied using molecular techniques and reports on the differences in germination response to PAHs on plant families are elusive. More research on these aspects would benefit future screening studies.

Table 5 Plant families that grow at sites contaminated with petrogenic hydrocarbons

Site survey	Plant families
Anoliefo et al. 2006	(*11 – Poaceae), (8 – Asteraceae), (5 – Eupobiaceae), (4 – Amaranthaceae, Caesalpiniaceae, Cyperaceae), (2 – Malvaceae, Piperaceae, Portulacaceae, Rubiaceae, Solanaceae, Urtinaceae), (1 – Araceae, Asystasiae, Bromeliaceae, Bignoniaceae, Crassulaceae, Capparaceae, Chlorideae, Caricaceae, , Fabaceae, Ficeae, Lamiaceae, Loganiceae, Musaceae, Nyctaginacea, Spermacoceae, Vernoniaceae)
Henner et al. 1999	(6 – Asteraceae), (4 – Poaceae), (3 – Brassicaceae), (2 – Amaranthaceae, Crassulaceae, Onagraceae, Solanaceae), (1 – Apiaceae, Boraginaceae, Betulaceae, Caryophyllaceae, Hypericaceae, Loganiaceae, Papaveraceae, Ranunculaceae, Rosaceae, Verbasceae, Salicaceae)
Olson and Fletcher 2000	(15 – Asteraceae), (13 – Poaceae), (3 – Verbenaceae), (2 – Amaranthaceae, Fabaceae), (1 – Brassicaceae, Campanulaceae, Capriloliaceae, Cucurbitaceae, Cyperaceae, Euphorbiaceae, Geranlaceae, Moraceae, Phytolaccaceae, Ranunculaceae Rubiaceae, Solanaceae, Typhaceae, Ulmaceae, Urhcaceae, Vitaceae)
Gudin and Syrratt 1975	Poaceae, Fabaceae, Asteraceae, others (surveyed by % cover)
Robson et al. 2004b	(10 – Poaceae), (4 – Asteraceae), (1 – Amaranthaceae, Cyperaceae, Caprifoliaceae, Rosaceae)

*Number of plant species recorded from a particular family

7.2 Plant morphological traits

Microbial populations in the rhizosphere can be several orders of magnitude higher (10^2 – 10^4) than bulk soil (Hinsinger et al. 2005; Macek et al., 2000; Siciliano and Germida 1998) as the rhizosphere provides stimulating conditions for soil microbes (discussed later, *iii* and *iv*). The study by Aprill and Sims (1990) is often cited to illustrate that a high root surface area is a key factor for boosting plant–microbe interactions. However, this may be oversimplified and other root architecture traits, such as those associated with efficient nutrient foraging, could play a more decisive role. Both plant access to nutrients and remediation of hydrocarbons by rhizosphere microbial populations will increase with the proportion of soil volume that is in the rhizosphere. The volume of rhizosphere soil can be considered as a cylinder and calculated simply as a function of total root length (height) and root hair length (radius), i.e. $\pi r^2 h$ (Haling et al. 2016; Yang et al. 2015). The best approach for a plant to maximise the volume of rhizosphere soil, in terms of the energy required for root production, is production of a long length of thin roots with long root hairs (Haling et al. 2016; Yang et al. 2015). The extensive fibrous root systems produced by members of the Poaceae are an example of such root systems and in experimentally-contaminated soils species of Poaceae often have the highest remediation potential (Gaskin and Bentham 2010; Gunther et al. 1996; Olson et al. 2007a; Reilley et al. 1996). A desirable attribute discussed by many authors is a high rate of shoot and root biomass production (Adam and Duncan 2002; Gaskin et al. 2008). In addition to the fast production of a large volume of rhizosphere soil, rapid root development will increase water uptake for evapotranspiration and nutrient acquisition and, for some species, enable the translocation of derivatives of parent-compound degradation from the roots to aboveground parts thus reducing the overall amount in the soil via *in planta* mechanisms (Wang et al. 2012).

Insoluble organic compounds are also incorporated into the plant transpiration stream (Kamath et al. 2004; Licht and Isebrands 2005). In this regard, various tree species such as poplars (*Populus* sp.), mulberry (*Morus* sp.) and willow (*Salix* sp.) have been successfully trialled for use in contaminated soils (Kamath et al. 2004). Mature poplar trees have an impressive transpiration capacity of 200–1000 litres of water per day (Pilon-Smits 2005). Tree roots have been reported to reach depths of up to 15 m (Pilon-Smits 2005) which is, obviously, useful when remediating contamination from beneath surface layers as the roots of herbaceous species typically only reach 100 cm (annuals) to 200 cm (perennials) depth (Bell et al. 2006). As previously mentioned, the presence of hydrocarbon contaminants often deters plant growth, with the roots more severely affected than shoots. Despite this, important root characteristics

related to fitness and survival such as total root length, average diameter, root hair length and frequency, and root to shoot ratio have not been well investigated in regards to their effects on biodegradation. In a recent study by Hernández-Ortega et al. (2014), roots of 10-day-old *Medicago sativa* seedlings lost their tetrarch vascular arrangement when the number of metaxylem vessels increased under diesel stress. As a result, root hydraulic conductivity declined significantly. Interpretations such as this can be useful in selecting species that perform well post-emergence in contaminated environments. It is, however, important to remember that these characteristics vary depending on environmental factors such as water availability, nutrients, soil physical properties and temperature. Also, the overall response to these physical factors, and the growth and development of roots, is largely governed by the genetic make-up of the plant species or variety (Hutchison et al. 2003).

7.3 Fabaceae – for prospective candidates

To deduce mechanistic pathways, numerous studies have attempted to explain successful phytoremediation outcomes in the light of the functional traits of the plants used. Root nodulation by members of Fabaceae (legumes) is a notable example. In contaminated-soil environments, the ability to fix nitrogen is considered highly advantageous because the carbon to nitrogen ratio becomes imbalanced due to the presence of hydrocarbons (Lee et al. 2008; Palmroth et al. 2002; Parrish et al. 2005). Under natural conditions, Fabaceae plants are often the pioneering species at disturbed sites (Gudin and Syrratt 1975), but have mostly been used as comparative plants (Hall et al. 2011). Microorganisms of genera such as *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Azospirillum*, *Azotobacter* and *Frankia* can make symbiotic associations (nodules) with plant roots and can fix atmospheric nitrogen as ammonia (Graham and Vance 2000). Fabaceae, the third largest terrestrial plant family, consists of around 19,000 species (Giller 2001). Of the limited number studied so far, more than 90% of two of the three subfamilies (Papilionoideae, Caesalpinioideae and Mimosoideae) form nodules (Giller 2001). Since the advent of phytotechnologies, only a few legume species have been tested; however, the most widely studied are from *Medicago* and *Trifolium* genera and these are effective at bioremediation (Figure 4). Nichols et al. (1997) found that for every gram of rhizosphere soil, the counts of microbial degrader populations for inoculated *Medicago sativa* were four times more than those for the grass *Poa alpina*, when grown in soil spiked with a mixture of synthetic compounds found in crude oil. Similar reports have been discussed in a review by Hall et al. (2011). Hall et al. (2011) support the use of Fabaceae for hydrocarbon bioremediation because they can improve: (i) soil structure and aeration by tap root penetration, (ii) soil N status by

biological fixation, (iii) plant access to P by forming symbiotic associations with arbuscular mycorrhizal fungi (AMF), and (iv) contaminant bioavailability by releasing biosurfactants and other carbonaceous compounds from roots. It should, however, be noted that legumes may depend heavily on AMF for P uptake as the hyphae explore soil outside the rhizosphere (Jakobsen et al. 1992). Most grass species can also be colonised by AMF (O'Connor et al. 2001), but may not rely so heavily on the fungi for accessing P due to their fibrous root system (see *ii*).

7.4 Root exudates – common and specialised

Many recent studies in the area of rhizoremediation and rhizosphere biology have focussed on the range of substances exuded by roots, such as sugars, amino acids, proteins, mucilage and organic acids. Known as the rhizosphere effect, it is speculated that these exudates are responsible for selecting specific *type(s)* and possibly quantitative *numbers* of microbial communities to proliferate in the vicinity of the plant roots (Kirk et al. 2005; Joner et al. 2002; Macek et al. 2000). About 30–60% of photosynthates are transferred to the roots, 10–20% of which can be released in rhizodeposition (Marschner 1995). One of the most abundant exudates are the carboxylic acids. Since they have low molecular weights and presumably provide a labile source of carbon for microbes, they have been hypothesised to play a key role in stimulating biodegradation rates (Louvel et al. 2011; Martin et al. 2014; Rentz et al. 2005). With an increase in phytoremediation candidates in the future, such interactions need to be investigated at the greenhouse and field scale. The feasibility of screening seedlings for exudation rate is, however, an area that requires investigation. The amount of rhizosphere carboxylates may differ with seedling age, and not in a consistent manner among species (Suriyagoda et al. 2012). Moreover, exudation may be greatly influenced by biotic and abiotic factors such as presence of mycorrhizal fungi, soil type and water and nutrient availability (Ryan et al. 2014; Suriyagoda et al. 2012).

A review by Singer et al. (2003) presented an interesting case for the use of plant produced/exuded secondary metabolites for biodegradation. The authors postulated that due to the similarities in the chemical structure of hydrocarbons (branched or aromatics) and naturally-occurring plant metabolites such as flavonoids, terpenoids, and coumarins, the latter can be used to induce the genes responsible for the production of degenerative enzymes. Considering the reported heterogeneity of degradative pathways among microbial systems (Bogan et al. 2001), this new area of research would have significant benefit if the relevant

species were trialled in screening experiments. In the literature, studies on the effects of plant-derived extracellular enzymes such as nitroreductases, dehalogenases, laccases and dehydrogenases are limited but have shown promise in both laboratory and field experiments (Wenzel 2009). For example, nitroreductase and laccase enzymes break down TNT (2,4,6-trinitrotoluene), dehalogenase aids the removal of chlorine from TCE (trichloroethylene), and peroxidases degrade phenols and phosphatases can cleave phosphate groups from organophosphate pesticides (Schnoor et al. 1995; Susarla et al. 2002). Again, the analogy between the chemical structures of these compounds and that of petrogenic contaminants may be applicable for enhancing biodegradation mechanistically.

Fig. 4 A representation of the plant species (grasses, trees (underlined), legumes and forbs) used for rhizoremediation from the early 1990s to 2013. The value in parentheses is the number of times a species has been grown in studies involving an individual contaminant type or comparative studies containing two or three contaminant types – TPHs (total petroleum hydrocarbons), BTEXs (benzene toluene, ethylbenzene, xylene) and PAHs (polycyclic aromatic hydrocarbons) (adapted information; for common names and reference studies see Appendix 1 of Cook and Hesterberg (2013)).

7.5 Tolerance towards harsh environmental stresses

As discussed earlier, the presence of hydrophobic petrogenic contaminants creates low permeability and infiltration of water into the soil. As a consequence, patches of water saturation and artificial drought can occur simultaneously in the soil profile. Roots of plants that are tolerant to such variable soil conditions are essential for creating macropores in the subsurface layers. Macropores not only facilitate the movement of water and nutrients but also improve aeration and the availability of oxygen to stimulate microbial activity, thus increasing biodegradation (Hutchinson et al. 2001). Plant roots can also oxidise the rhizosphere directly. In a review by Shimp et al. (1993), specific strategies are discussed about how trees and plants that are flood-tolerant or adapted to wetlands, transfer more oxygen into the rhizosphere, stimulating the biodegradation of organic contaminants. They suggested that flood tolerance is an inverse function of alcohol dehydrogenase enzyme activity. However, morphological adaptations to the low oxygen conditions under waterlogging may also aid survival in contaminated soils. For example, the formation of adventitious roots, an unsuberized epidermis and the degeneration of the Casparian strip, and the formation of lenticles (e.g. *Salix alba*) to release into air toxic metabolites such as ethanol, acetaldehyde and ethylene (Colmer and Flowers 2008; Shimp et al. 1993). As much as $0.5 \text{ mol O}_2 \text{ m}^{-2}$ soil surface per day is reportedly released by roots (references in Shimp et al. 1993). Thus, it is possible that plant adaptations to the low oxygen conditions caused by waterlogging may provide an advantage for growth under hydrocarbon contamination, but only if the plant is otherwise well-adapted to the growing conditions.

The identification of plant species which have biodegradation potential and can tolerate moisture limitations will be important, especially for semi-arid and arid areas such as in Western Australia and Persian Gulf countries. Water is essential for microbial growth and mobility, and to alleviate nutrient limitation or toxicity to plants at high water potentials (Walworth et al. 1997). Across different soils, a water-filled porosity between 20 and 80% has increased hydrocarbon biodegradation rates (Tibbett et al. 2011). Moreover, contaminated sites are frequently associated with high levels of salinity, exhibiting electrical conductivities above 4 dS m^{-1} (Hutchinson et al. 2003; Robson et al. 2004b). Soils with conductivities above 2 dS m^{-1} are considered salt-affected; therefore, the compilation of a list of salt-resistant plant species would assist proper site management (Hutchinson et al. 2003). Ultimately, soil effects and topography regulate the abiotic stress faced by plants. Properties such as bulk density, soil

texture, hydraulic conductivity and the level of the water table should also be considered before making decisions on which species should be trialled (Hutchinson et al. 2003). For a particular site, useful information on relevant plant species can be obtained from local experts such as seed suppliers (Hutchinson et al. 2003) and surveys of indigenous vegetation accrued as a result of ecological succession (Olson and Fletcher 2000; Robson 2003). Most non-agricultural contaminated sites are low-nutrient systems, and such records will help to target species that are well adapted to particular site conditions (Table 5). Therefore, the selection of native species is likely to be advantageous.

8. Recommendations – a prototype of improved germination protocols

Many authors agree that determination of the most efficient plant species for biodegradation is the most important step in the field of phytoremediation (Graj et al. 2013; Macek et al. 2000; Yateem et al. 2007). It is, therefore, necessary to compile known and future research outcomes to improve screening experiments. In this regard, a major problem is the lack of standardised protocols, as the results of most experiments cannot be generalised or usefully compared due to the complexity generated by factors such as methodology, environment, soils and plant species. To address this issue, below is a list which could enable better control of treatment conditions.

- a. When reporting results, authors must be clear about plant life stages, especially regarding germination versus emergence which are two distinct events (James et al. 2013) and will produce different results when comparing species, contaminants and experimental approaches (e.g. Petri dish versus pot studies).
- b. The categorisation of contamination levels done by Gilyazov and Gaisin (2003) is useful. Other studies have used the same scale to divide contamination into four levels—low (0.5, 1 and 2 wt. %), medium (3 and 5 wt. %), high (10 wt. %) and very high (15 wt. %)—and applied the same as parameters for modelling differences in plant species (Potashev et al. 2014; Sharonova and Breus 2012). Conforming to this categorisation would help future inter/intra-studies modelling assignments.
- c. The use of filter papers should be avoided. Other substances in soil such as humic acids, organic matter and clay particles can also sorb PAHs. The use of washed white sand as a growth medium can reduce these confounding effects in different types of soils and is easily sterilised to remove microbial communities.
- d. The use of plastic Petri dishes should also be avoided.

- e. Petri dishes (plastic or glass) have a limited depth which means that large seeds may not be fully covered with the contaminated medium. The use of deeper containers would likely result in less variation in germination and emergence. Also, trials could be extended by a few weeks to allow assessment of seedling survival and the collection of more plant growth data.
- f. Proper ventilation arrangements should be in place when using open Petri dishes. High-molecular-weight pure petrogenic compounds such as anthracene, phenanthrene and pyrene are less volatile at room temperature and can, therefore, be used in closed Petri dishes.
- g. Some studies use few seeds per replicate and/or few replicates. It is advisable to use a minimum of 25 seeds and four replicates (Martyn et al. 2009). For smaller-seeded species, more seeds per replicate can be used (e.g. 75–100 per replicate).
- h. Seed viability is a concern, especially with native seeds. Various tests such as tetrazolium staining and floatation can be used to separate non-viable seeds *a priori*. More recently, X-ray analysis has provided an alternative viability assessment option and is a rapid and non-destructive method applicable to a wide range of native species (Erickson and Merritt 2016). Species naturalised or native to a site evolve based on natural climatic conditions and thus inculcate dormancy mechanisms which break only upon survival of precise conditions to ensure species survival.
- i. Remediation of hydrocarbon-contaminated soils is not usually achieved within one growing season. Native plants that require minimal maintenance (e.g. fertilisers, pruning, watering) should be considered. The low-nutrient status of most contaminated sites may enhance plant requirements for the establishment of a sustainable vegetation cover (Harris et al. 1996). Continual plant growth depends on various factors such as competition, abiotic/biotic seed dispersal processes and seasonal drought cycles along with other management-related issues. Many of these problems can be addressed by selecting the correct plant species. For predicting the trajectory of successional outcomes following the restoration of degraded areas, James et al. (2013) proposed the use of a system-based approach which means that, in hierarchical models, some ecological processes should be given more importance than others depending on the specific requirements of a site (system).
- j. Easy to measure physiological indexes such as leaf and root area have been largely ignored. These, coupled with other physiological parameters such as photosynthetic ability and root/leaf respiration using equipment such as LiCOR, can yield important results for unravelling the mechanisms that ensure better plant survival and possibly, germination.

- k. We suggest that root architectural traits that confer a large volume of rhizosphere soil, i.e., long thin roots with long root hairs, may be indicative of a high bioremediation potential and could be easily used to screen seedlings.

9. Conclusions

At the time of dormancy release, germination is controlled by multiple, often interacting, abiotic and biotic factors, which are influenced by the presence of hydrocarbons. The complication ensued by the differences among contaminants, their bioavailability and ecotoxicity at varying levels, produces variation in the response among plant species, which is also highly dependent on phylogenetic relationships at a species, genus and family level. In the literature, there is a generalised trend of screening species that belong to Poaceae and Fabaceae only. Our review of hydrocarbon contamination studies is not conclusive as to whether the species from these two families germinate better than those from other families. Whether germination results can be used as an added criterion for plant selection is questionable, as some plants may germinate well, but suffer toxicity post-emergence or be unsuited to growth conditions in the field. Nonetheless, germination studies are beneficial for deciding which species can be ruled out from further research. The standardisation of germination and emergence protocols will enable stronger inferences from meta-analyses and models, and will help to assess phylogenetically closely-related plant species.

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Table 3 Summary of experimental studies of seed germination and emergence under petrogenic contamination. (F—freshly spiked, A—aged contamination; concentration values in % unless otherwise stated)

Study	Contaminant	Concentration	Growing medium	Container	Days	No. of species	Rep.	Seeds/rep.	Temperature	Parameters
Achuba 2006	crude oil (F)	0.25, 0.5, 1, 2	sandy soil	plastic bag	12	1	20	5	–	germ. %; amylase and phosphorylase activity; sugar, amino acids, proteins and chlorophyll content; root meristematic mitotic activity
Adam and Duncan 2002	a. diesel (F)	2.5, 5	sandy soil (compost)	petri dish	14	25	2	25 or 100	22°C	germ. %
	b. diesel (F)	2.5, 5	as above	petri dish	42	8	2	25 or 100	8°C	as above
	c. diesel (A)	2.5, 5	as above	petri dish	14	5	2	25 or 100	22°C	as above
	d. seeds soaked in diesel for 7 d		as above	petri dish	14	1	2	40	22°C	as above, tetrazolium test for seed viability
Adenipekun et al. 2008	spent oil (F)	0.2, 0.4, 0.6, 0.8, 1, 2, 3	top soil	black polythene bag	7	1	3	–	20/34°C	plant height, stem girth, leaf number, area and chlorophyll content, biomass, plant moisture content
Amakiri and Onofeghara 1984	crude oil (F)	Seeds soaked in oil for variable periods	filter paper	petri dish	variable	3	4	25	27°C	germ. rate, germ. %, tetrazolium test for seed viability
Besalatpour et al. 2013	PAHs in landfill soil (A)	4, 7	soil	plastic pot	14	7	3	–	22°C or 37°C	germ. % and rate; shoot length, total biomass
Bona et al. 2011	diesel (F)	9 (treatments made by timed ageing)	soil	plastic tray	20	1	5	50	38/9°C	germ. %, total height, fresh and dry biomass, ecophyll biomass and leaf area
Bossert and Bartha 1985	oily sludge	8.4	sandy loam	polypropylene trays	11	2	1	100	ambient	emergence, after 25 d – shoot height and dry weight
Chaîneau et al. 1997	a. fuel oil and dearomatised fuel oil (F)	various, 0.1 to 20	sand	glass petri dish	8	7	–	100	22°C	germ. rate
	b. fuel oil; light and heavy fraction (F)	1, 2, 4, 6, 8, 10	sand	glass petri dish	8	1	–	100	22°C	as above

Study	Contaminant	Concentration	Growing medium	Container	Days	No. of species	Rep.	Seeds/rep.	Temperature	Parameters
Chen et al. 2013	diesel (F)	0.2, 0.4, 0.8, 1	sand	petri dish	30	1	3	30	28°C	germ. % and rate; root length, diameter and surface area; shoot H ₂ O ₂ accumulation; biomass DNA damage
Dorn and Salanitro 2000	a. light crude oil (F)	5	a. low organic carbon soil	petri dish	21	3	–	100	–	germ. %, average dry weight per plant
	b. medium crude oil (F)		b. high organic carbon soil	petri dish	21	3	–	100	–	as above
	c. heavy crude oil (F)									
	d. Two soils (A)									
Gaskin et al. 2008	diesel-engine oil mix (60:40) (F)	0.5, 1	mine site soil	pots	60	9	4	50	20–30°C	seedling emergence %
Ghanem et al. 2010	pyrene (F)	50, 100 (mg kg ⁻¹)	soil	plastic petri dish	14	3	4	7	ambient	germ. %, shoot and root length
Graj et al. 2013	diesel (F)	0.15, 0.3, 0.6, 1.2, 2.4	soil, filter paper on top	Phytotoxkit	6	4	3	–	22°C	germ. rate, root length, dry biomass plus bioaugmentation treatments
Henner et al. 1999	a. six pure PAHs	20 (mg kg ⁻¹)	soil; filter paper	glass petri dish	10	1(soil); 8	3	75–200	21°C dark	germ.%, after 60 d – height
	b. leachate of contaminated soil	–	filter paper	glass petri dish	10	–	3	75–200	21°C dark	as above
	c. viscous tar	14, 30, 50, 75, 150 (mg kg ⁻¹)	agricultural soil	glass petri dish (open/closed)	10	2	3	75–200	21°C dark	as above
	d. industrial soil (F and A)	3250 (mg kg ⁻¹)	industrial soil	glass petri dish	10	17	–	75–200	21°C dark	as above
Issoufi et al. 2006	crude oil(F); artificially weathered by heating	1, 5, 10	silt loam	conical cells with perforated base, suspended in a container	28	6	5	5	24/18°C	emergence %, seedling height, shoot and root dry weight
Korade and Fulekar 2009	anthracene (F)	10, 25, 50, 75, 100 (mg kg ⁻¹)	soil	petri dish	7	1	3	10	26/23°C	germ. %
Kulakow et al. 2000	Contaminated sediments (A)	na	air-dried sediments	pots	14	29	4	50–100	10/28°C	seedling emergence; after 60 d – height, aboveground biomass, root biomass,

Study	Contaminant	Concentration	Growing medium	Container	Days	No. of species	Rep.	Seeds/rep.	Temperature	Parameters
										root/shoot ratio, mean root diameter, root length density, and root-surface-area density
MacKinnon and Duncan 2013	a. diesel (F)	2.5, 5	sandy soil (compost)	petri dish	14	3	2	25	22°C	germ. %
	b. 4 cycloalkanes (F)	0.1, 1, 5 (mg L ⁻¹)	filter paper	petri dish	14	2	3	15	22°C	germ. %, shoot length
Maila and Cloete 2002	a. 5 pure compounds (F)	50, 150, 300, 500, 1000 (mg kg ⁻¹)	a. sandy loam	trays; polystyrene petri dish	3	1	3	50	ambient	germ. %, seedling weight
	b. industrial soil (A)	1.2 (mg kg ⁻¹)	b. PHC industrial soil	polystyrene petri dish	3	1	3	50	ambient	as above
		control	c. non PHC industrial soil	polystyrene petri dish	3	1	3	50	ambient	as above
		control	d. white playpen sand	polystyrene petri dish	3	1	3	50	ambient	as above
Marques et al. 2010	light crude oil (F)	0.05, 0.5, 2, 4, 6	sterilised fine sand	HDPE box	35	6	4	200	25°C	germ. %, germ. rate, germ. period, shoot and root biomass
Mezzari et al. 2011	diesel (F)	0.5, 1, 2	soil	plastic pot	21	3	3	–	–	germ. %, subsequent growth, biomass, soil bacteria quantification
Murphy 1929	crude oil (F)	~7.5, 2.5, 1.25, 0.25	soil	Tin container buried in ground	40	1	2	25	–	germ. %
Noori et al. 2014	crude oil (F)	2.5, 5, 7.5, 10	sandy loam compost (+/- inoculum)	plastic pot	112	1	3	5	30/5°C	root and shoot length, dry weight, root to shoot ratio, mycorrhizal colonization %, spore abundance per g soil
Ogbo 2009	diesel (F)	1, 2, 3, 4, 5	filter paper	petri dish	3	4	5	10	–	germ. %, radicle and plumule length
Oyedeji et al. 2012	crude oil (F)	0.5, 1, 1.5, 2 (ml kg ⁻¹)	loamy soil	planting bag	21	1	5	3	–	emerg. %, co-efficient of germination velocity, seedling height and girth
Ren et al. 1996	a. anthracene b. benzo[α]pyrene	various, 0–10 (mg L ⁻¹)	30 ml dimethyl sulfoxide	seed tray of a large polystyrene petri dish	6	1	9	12	–	Root and shoot growth, chlorosis

Study	Contaminant	Concentration	Growing medium	Container	Days	No. of species	Rep.	Seeds/rep.	Temperature	Parameters
	c. fluoranthene		test solution							
Saterbak et al. 1999	a. crude oil (A) – 7 sites b. lubricating oil (A) – 1 site	2–7 dilutions of contaminated soils	soil	plastic petri dish	4	4	3	10	23°C dark	germ. %, root length
Shahsavari et al. 2013	a. diesel-engine oil (F) b. crude oil (F)	1	sandy soil	plastic pot	28	11	3	5–25	27–30°C	germ. %, shoot and root length and dry biomass
Sharifi et al. 2007	engine oil (F)	1	sandy soil	plastic pot	28	11	3	5–25	27–30°C	as above
Sharonova and Breus 2012	engine oil (F)	2.5, 5, 7.5, 10	artificial sandy loam	plastic pot	14	6	3	20	27–30°C	germ. %, shoot length and dry biomass
Sharonova and Breus 2012	kerosene (F)	1, 2, 3, 5, 10, 15	leached chernozem soil	petri dish	14	50	–	10–25	26°C dark	germ. %
Siddiqui and Adams 2002	diesel (F) (aged 24 weekly increments)	0.64, 1.6, 4, 13.6	a. soil A (±N and P)	petri dish	7	1	3	100	22°C	germ. %
		0.4, 1, 1.5, 2, 3, 4, 5, 6	b. soil B (±N and P)	petri dish	7	1	3	100	22°C	as above
			c. soil A	petri dish	14	1	1	100	22°C	as above
Smith et al. 2006	a. 7 PAHs mix (F) b. coal tar (F) c. coking plant soil (A)	0.1 0.16 0.5	sandy soil sandy soil sandy clay loam	pots pots pots	14 14 14	7 7 7	4 4 4	0.2 g (each species)	40/10°C	germ. %, at day 84 – foliage dry weight
Smreczak and Kordybach 2003	PAHs mix (fluorene, pyrene, anthracene, chrysene)	1, 10, 100 (mg kg ⁻¹)	a. light loamy soil b. light loamy soil + compost	glass petri dish	7	4	4	8-10	20°C dark	germ. %, root length
Udo and Fayemi 1975	crude oil (F)	1.1, 2.1, 4.2, 6.4, 8.5, 10.6	soil	plastic pot	7	1	3	4	–	germ. %, total biomass, nutrient uptake