The Potential of *Medicago sativa* for Microbial-Enhanced Phytoremediation of Diesel Fuel Contaminated Sites*

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Abstract

Oil spillage, a major source of diesel fuel contamination, is the most persistent environmental menace resulting from oil and gas operations. Diesel spills are difficult to remediate because they have less volatile and less biodegradable characteristics compared to petrol (gasoline) spills. (Kuo et al., 2012; Silva-Castro et al., 2015). Traditional solutions for remediation such as excavation and off-site treatments are expensive and usually impractical because of the amount of soil involved.

One of the emerging strategies, categorised as "phytoremediation", is the use of plants to extract and stabilise contaminants (Pilon-Smits and Freeman, 2006; Weyens et al., 2009). While this is an interesting strategy, the slow growth rate and low metabolic activity of natural attenuation limits its effectiveness (Azubuike et al., 2016). Thus, microbial-enhanced phytoremediation (an aspect of geomicrobiology) as a new technology is gaining growing attention.

In line with this, my research examined through a series of pot experiments the potential of *Medicago sativa* to withstand hydrocarbon toxicity while degrading diesel fuel hydrocarbons through the actions of associated plant growth-promoting rhizobacteria. The growth of *Medicago sativa* under different concentrations of diesel fuel was monitored during a 60-day period. Relative growth rate (RGR) and total biomass were calculated to understand the plant's ability to withstand phytotoxicity. To better understand the effect of diesel fuel on microbial colonization and plant growth, scanning electron microscopy (SEM) was used to examine nodule development.

The results show that diesel fuel initially slowed the growth of *Medicago sativa*. However, the development of nodulation and its colonization by rhizobacteria significantly enhanced the plant's growth, with relative growth rates in contaminated soils exceeding that of control within the first 30 days for 5 g/Kg and 50 days for 10 g/Kg diesel fuel concentrations. In addition, diesel fuel at both concentrations significantly enhanced the rhizosphere microbial density (as revealed by SEM micrographs) and total biomass production. This is a strong indication of the plant's potential for microbial-mediated phytoremediation. We hope that this will eventually become the panacea for diesel fuel contaminated sites.

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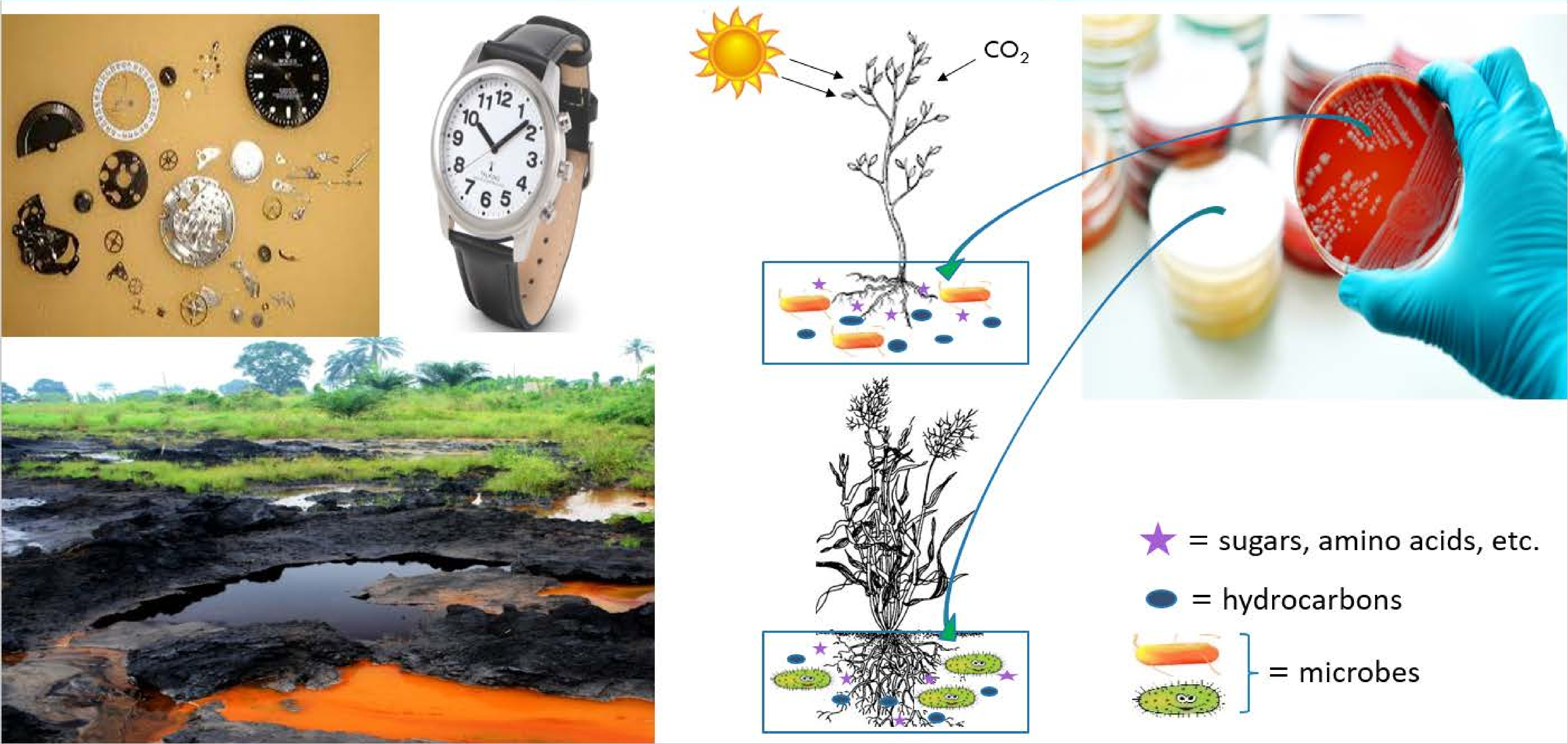
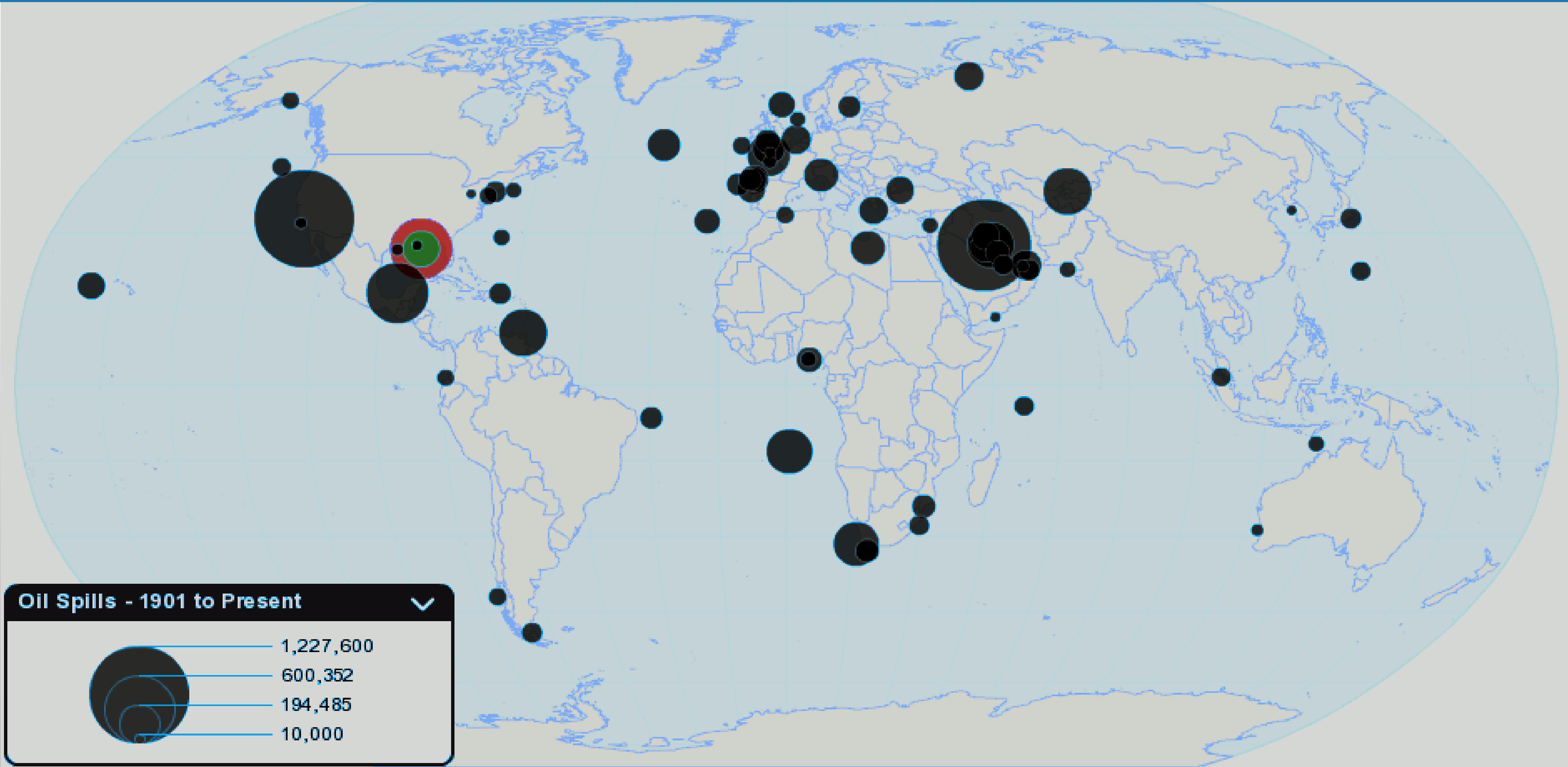
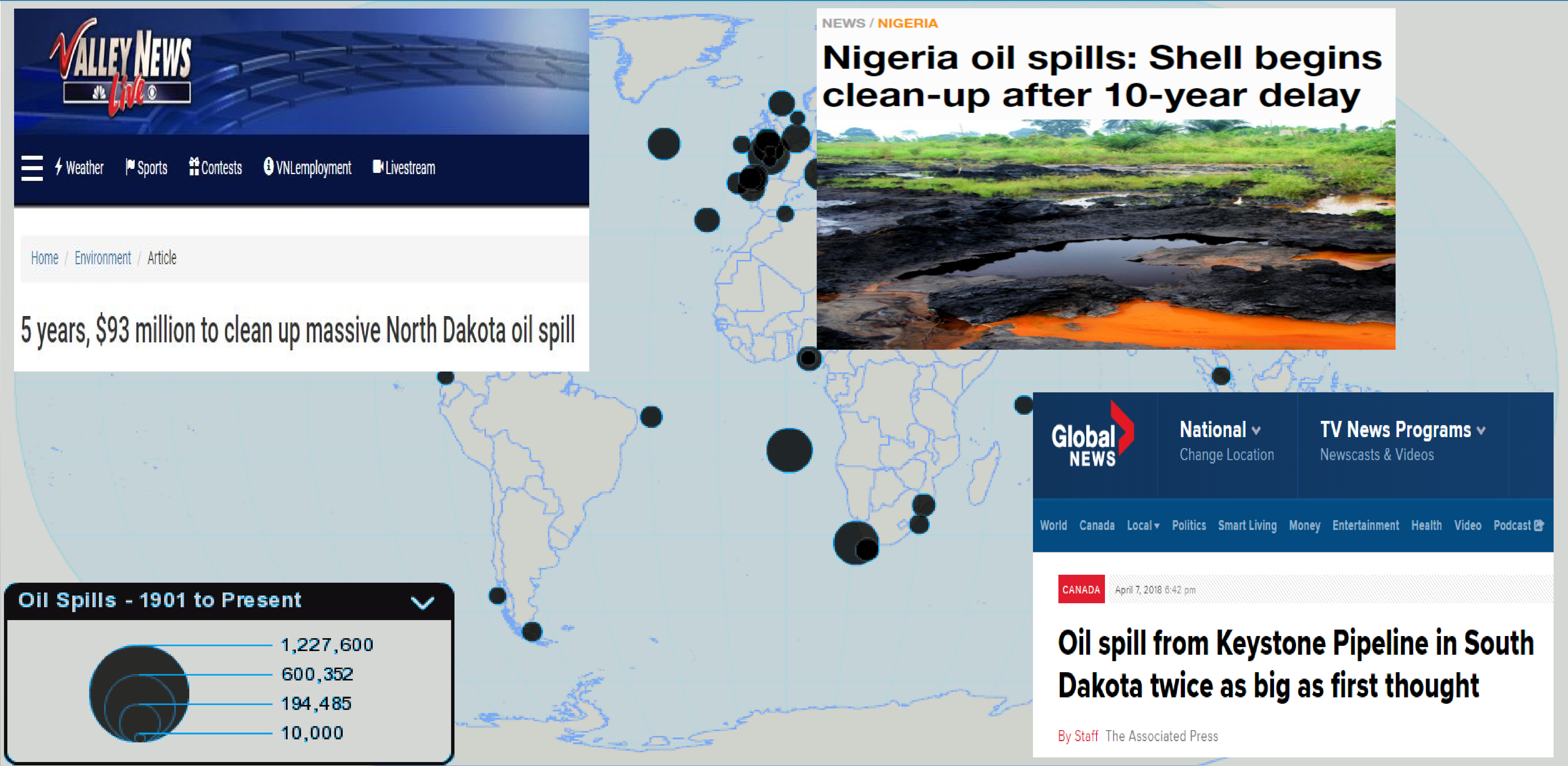
"The Whole is Greater than the Sum of its Parts": Building the Biological Team for Oil Spill Remediation



Petroleum Spills – The Menace We All Face!

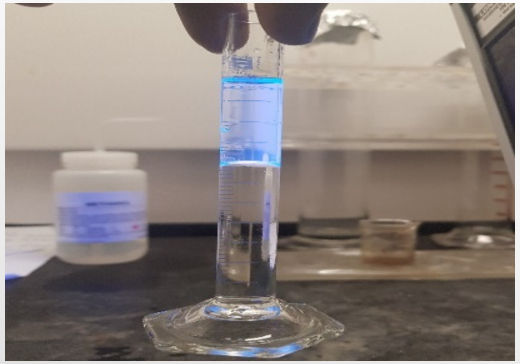


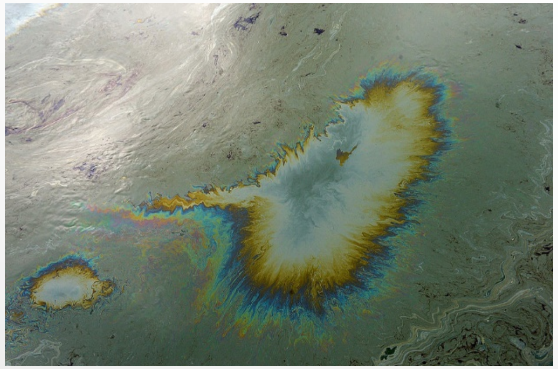
Petroleum Spills – The Menace We All Face!



Statement of the Problem

- Petroleum hydrocarbons especially diesel fuel components are highly hydrophobic

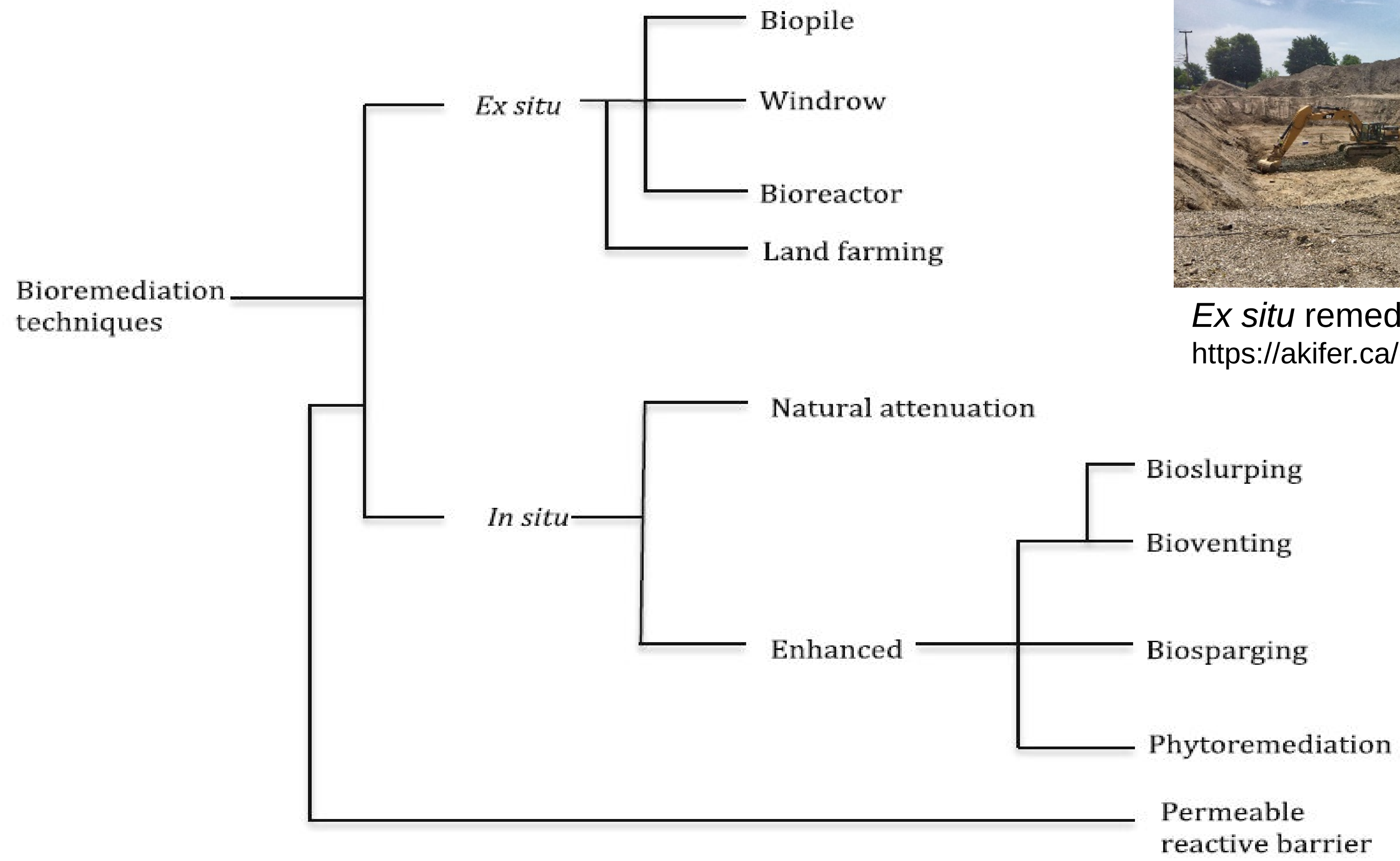




- Traditional methods of remediation are very expensive and environmentally unfriendly

- Natural attenuation exhibits slow metabolic activity

What Has Been Done?

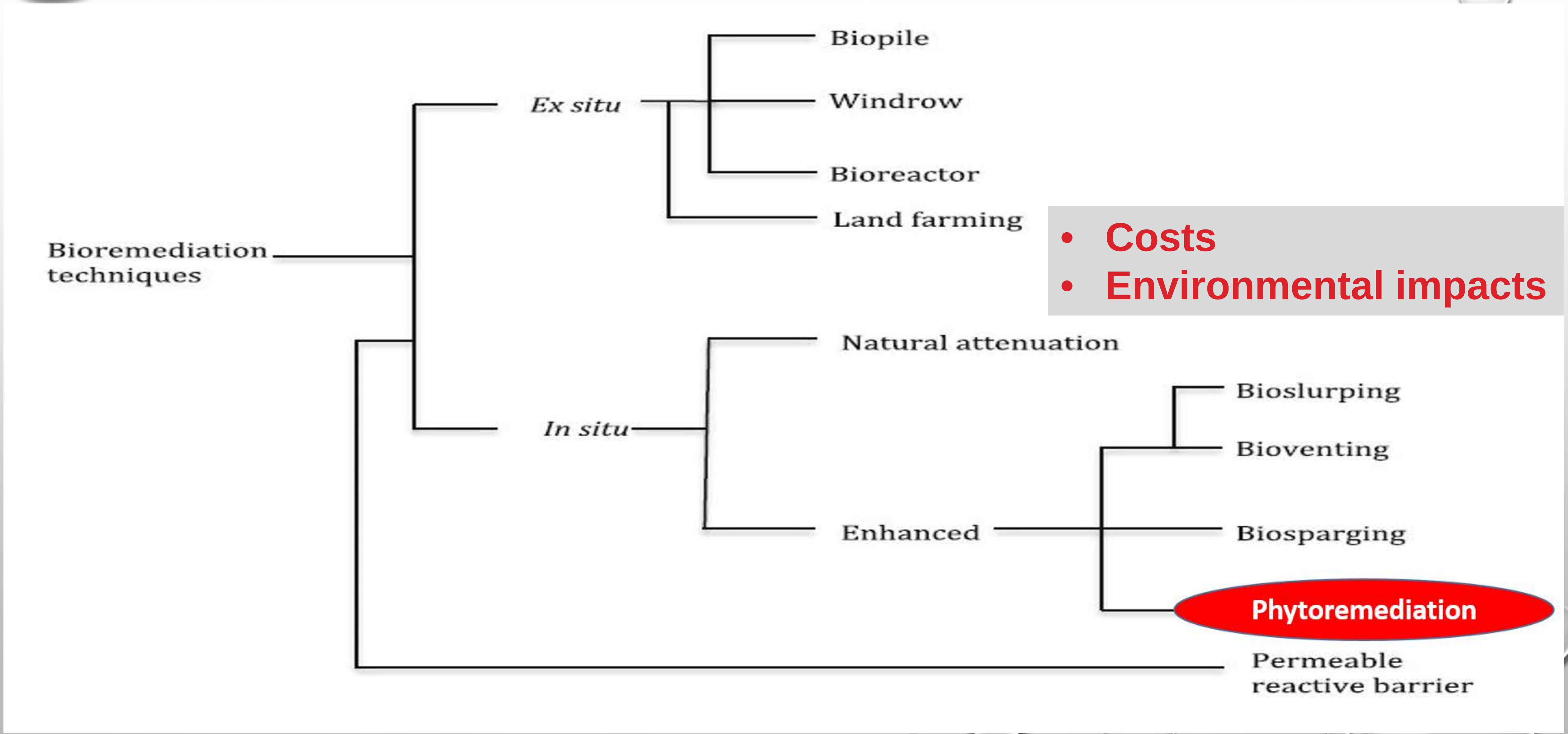


Ex situ remediation techniques at Sainte-Marie and Papineauville
https://akifer.ca/en/environmental-remediation

Dennis, 2016. NMED 2016 Strategic Plan

A review of bioremediation techniques. (Azubuike, et al., 2016. *World J Microbiol Biotechnol.* 32:180).

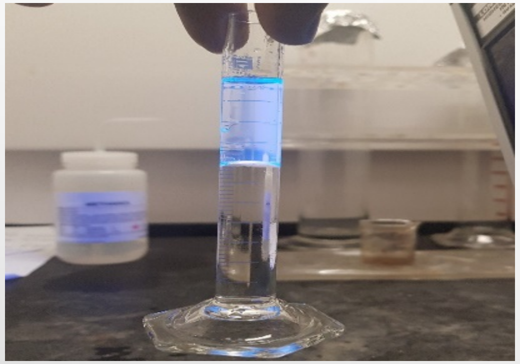
Remediation Techniques

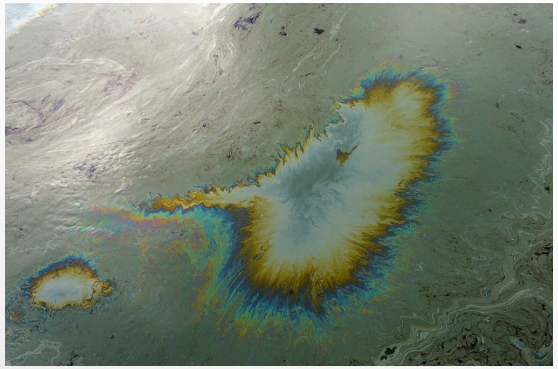


Azubuike, et al., 2016. *World J Microbiol Biotechnol.* 32:180.

Statement of the Problem

- Diesel spills are less biodegradable compared to petrol spills





- Traditional methods of remediation are very expensive and environmentally unfriendly

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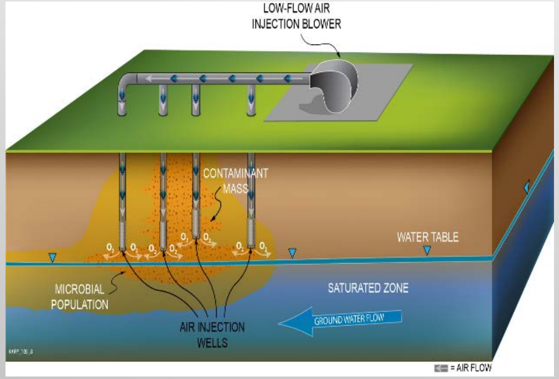


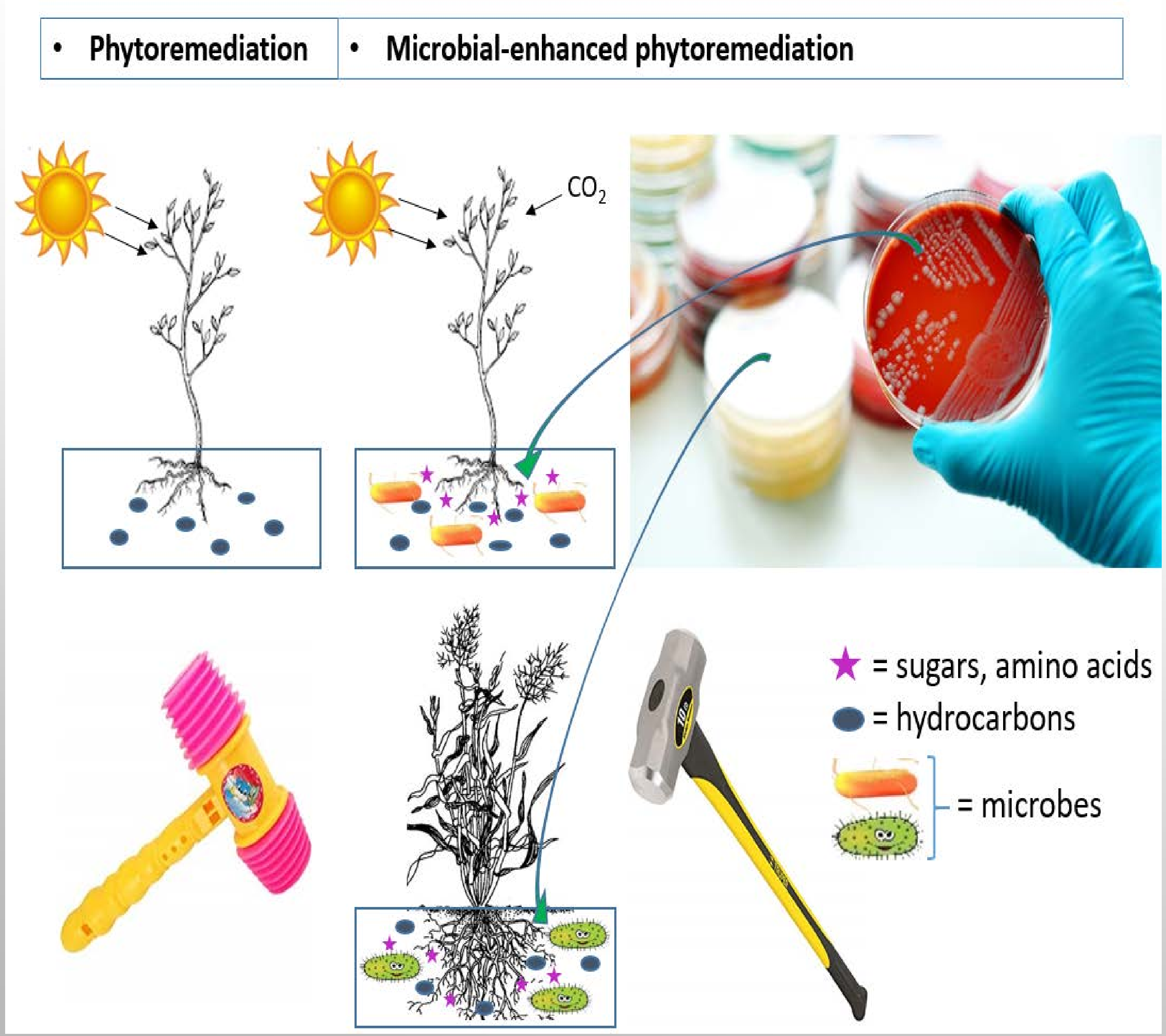
Dennis, 2016. NMED 2016 Strategic Plan

- Natural attenuation exhibits slow metabolic activity

- Diesel fuels are phytotoxic to many plants and this limits the effectiveness of phytoremediation

Cocksfoot plants grown in 0 g, 25 g and 50 g diesel/Kg soil (Adam and Duncan, 2001)

Way Out?



➢ Microbial-enhanced phytoremediation

To identify culturable and most effective microbial symbionts to enhance phytoremediation

Main Goal

To develop the right plant-microbe symbionts for enhanced rhizoremediation of diesel fuel contaminated sites

Research Outline

Leaching Experiment

Project 1

Phytotoxicity Experiment

Project 2

Microbial Genomics

Project 3

Effectiveness of PGPR-enhanced phytoremediation

Project 4

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Gottingen Genomics Laboratory, University of Gottingen

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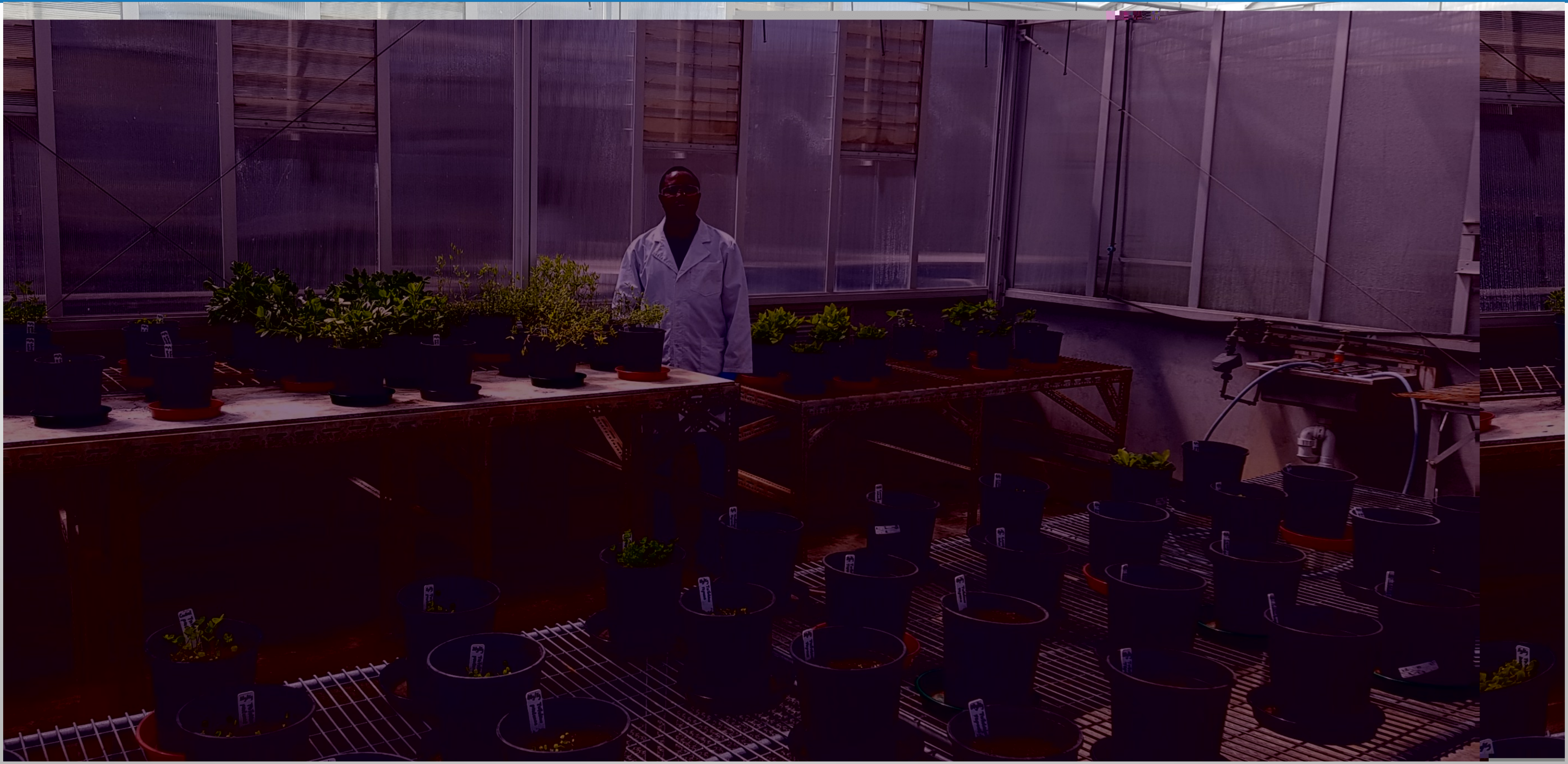
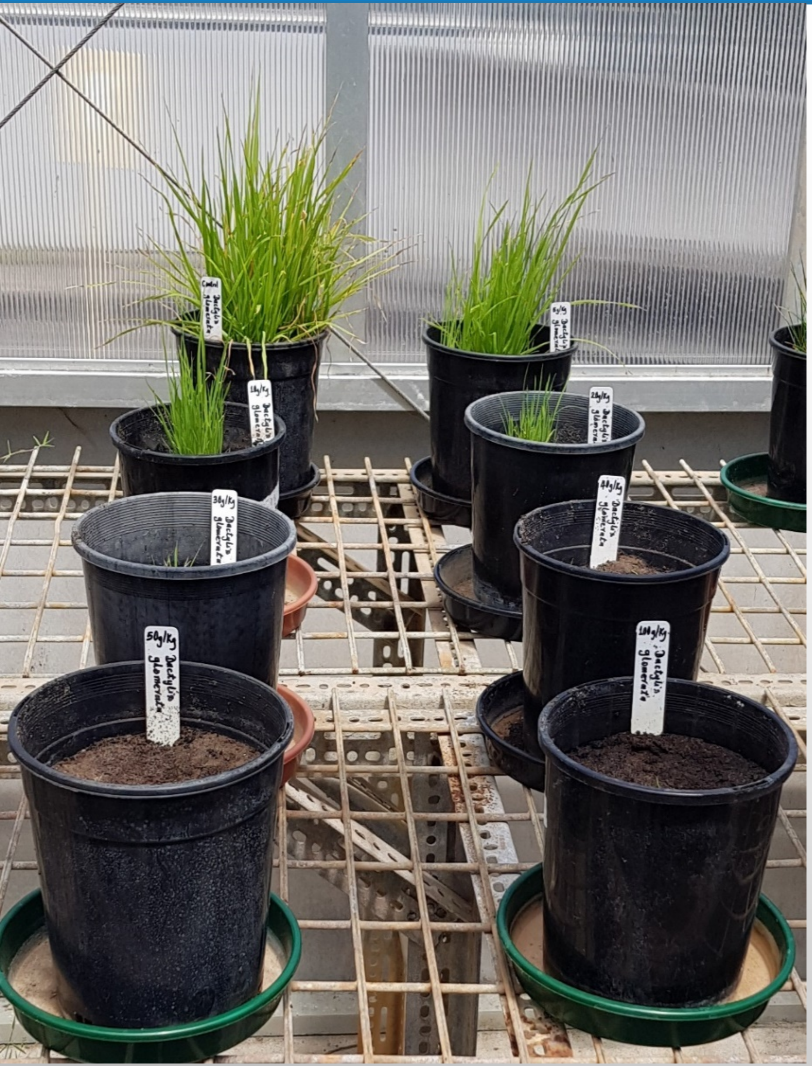
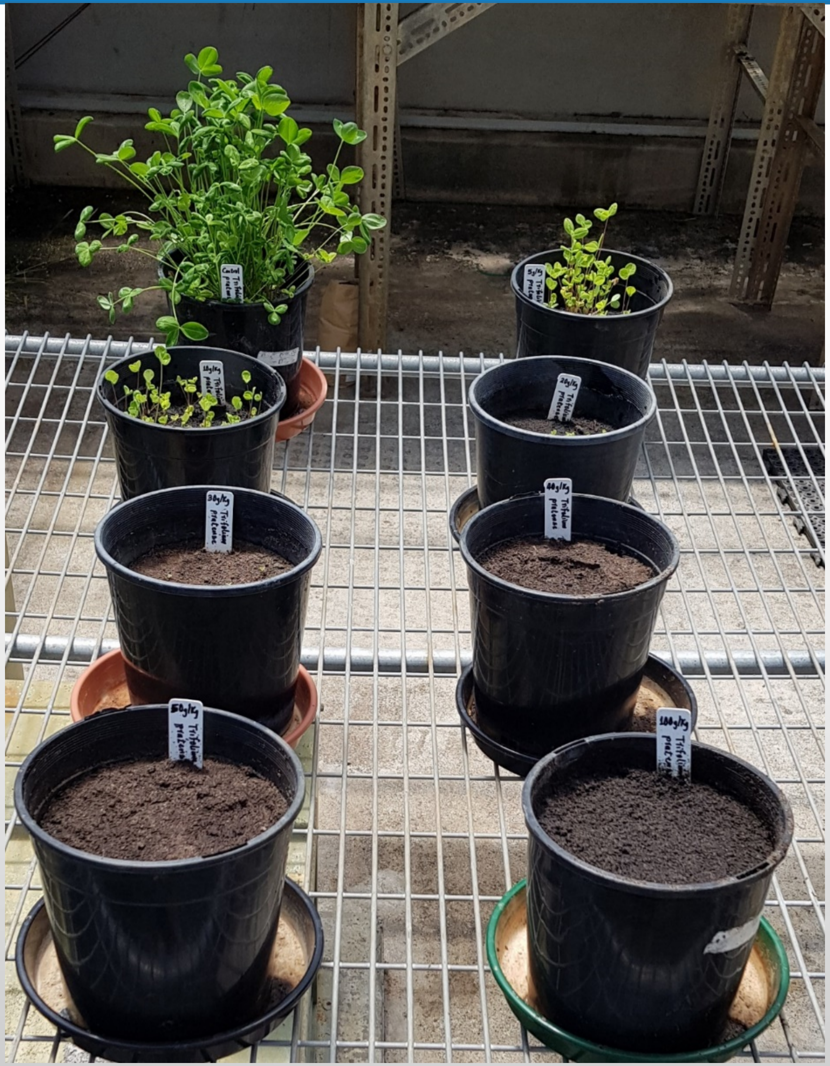
Phytotoxicity Experiment



Project 2: Results



Dactylis glomerata (cocksfoot grass)



Trifolium pratense (red clover)

- Diesel fuel hydrocarbons impacted on germination and growth of plant species
- Decreasing biomass production with increasing diesel fuel concentrations

Project 2: Results

Vicia faba

Vicia faba

Vigna unguiculata

- Diesel fuel hydrocarbons alters C:N:H ratio leading to chlorosis (nitrogen deficiency) in the absence of nitrogen-fixing rhizobacteria

Project 2: Results

Medicago sativa (3 weeks)

Medicago sativa (8 weeks)

- Initial slow growth rate
- Subsequent enhanced growth rate in diesel soil (hormesis)

Statistical Analysis: From Greenhouse to Models

The log-logistic model

The *classic* four parameter log-logistic model :

$$f(\text{dose}) = c + \frac{d-c}{1+\exp[b\{\log(\text{dose}) - \log(e)\}]} = c + \frac{d-c}{1+(\text{dose}/e)^b}$$

Recall interpretation of the parameters:

c - lower limit, d - upper limit, e - ED50, b - proportional to the slope in ED50

3-parameter log-logistic model

$$f(x; b, d, e) = \frac{d}{1+\exp(b(\log(x) - \log(e)))}$$

Cedergreen-Ritz-Streibig model for describing hormesis

$$f(x) = c + \frac{d - c + f exp(-1/(x^{\alpha}))}{1 + exp(b(log(x) - log(e)))}$$

f - rate of growth stimulation at doses close to 0

Biological Experiments

- *At very high doses (concentration of contaminants), all test organisms die.*
- *Therefore, lower asymptote, c = 0*
  - ➡ *3-parameter log-logistic model*
  - ➡ *Cedergreen-Ritz-Streibig CRS.4a model*

Fitting Dose-Response Models

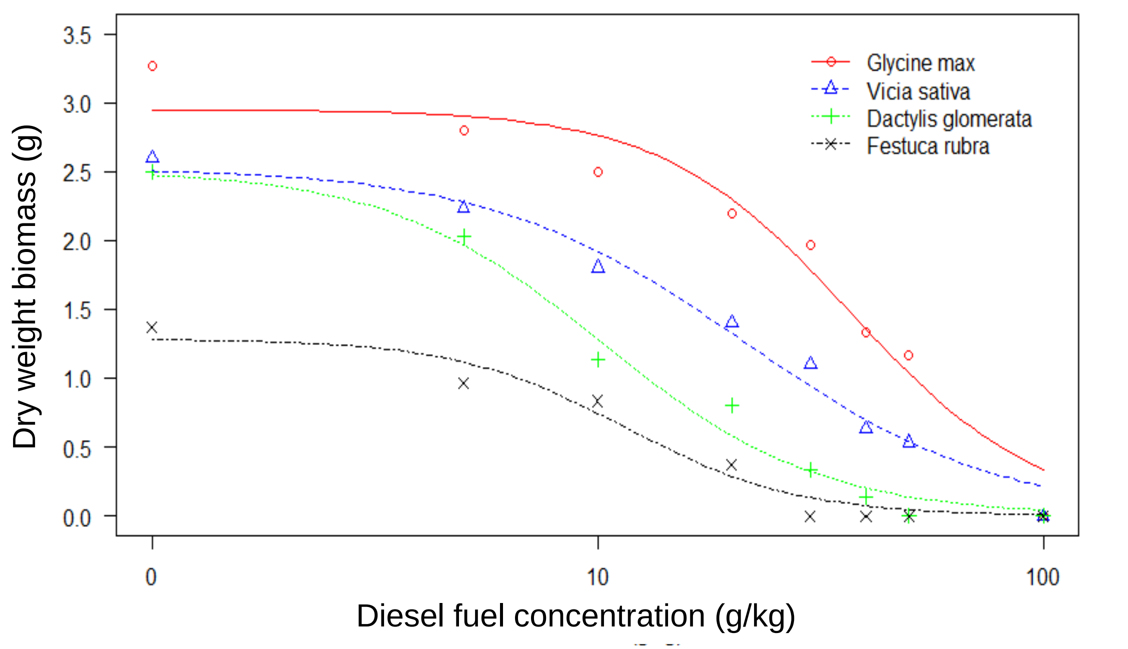
\# Fitting a 3-parameter log-logistic model:

```
Plant.Biomass.LL.3 <- drm(biomass ~ conc, specie, data = Plant.Biomass, fct = LL.3())
```

\# Plotting the fitted model:

```
plot(Plant.Biomass.LL.3, broken = FALSE, col=c("red", "blue", "green", "black"),
   xlab="concentration(g/kg)", ylab="plant biomass(g)")
```

p<0.001



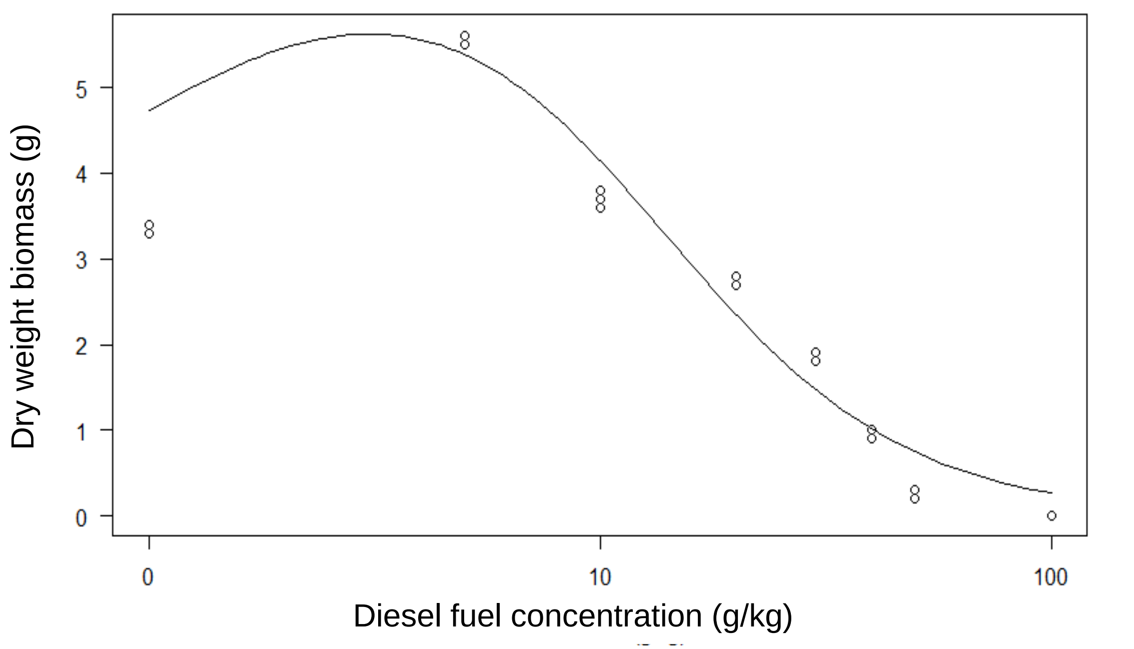
\# Fitting Cedergreen-Ritz-Streibig model:

```
Msativa.crsm1 <- drm(biomass ~ conc, data = Msativa.Biomass, fct=CRS.4a())
```

\# Plotting the fitted model:

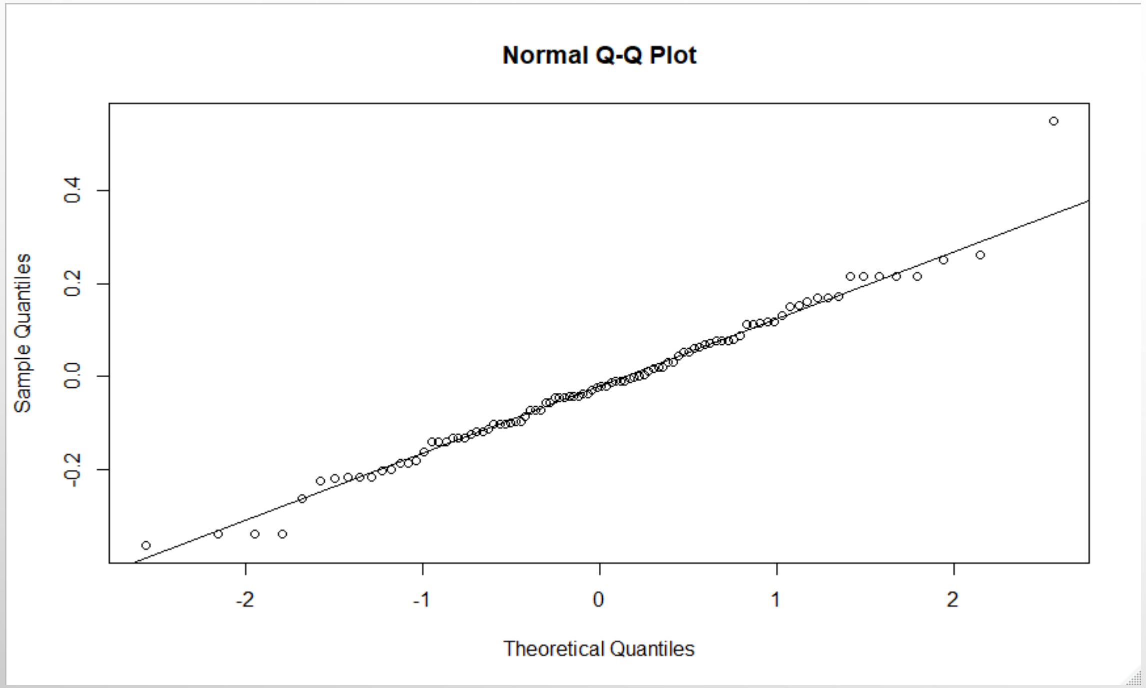
```
plot(Msativa.crsm1, broken = FALSE, type = "all",
   xlab="concentration(g/kg)", ylab="plant biomass(g)")
```

p<0.01



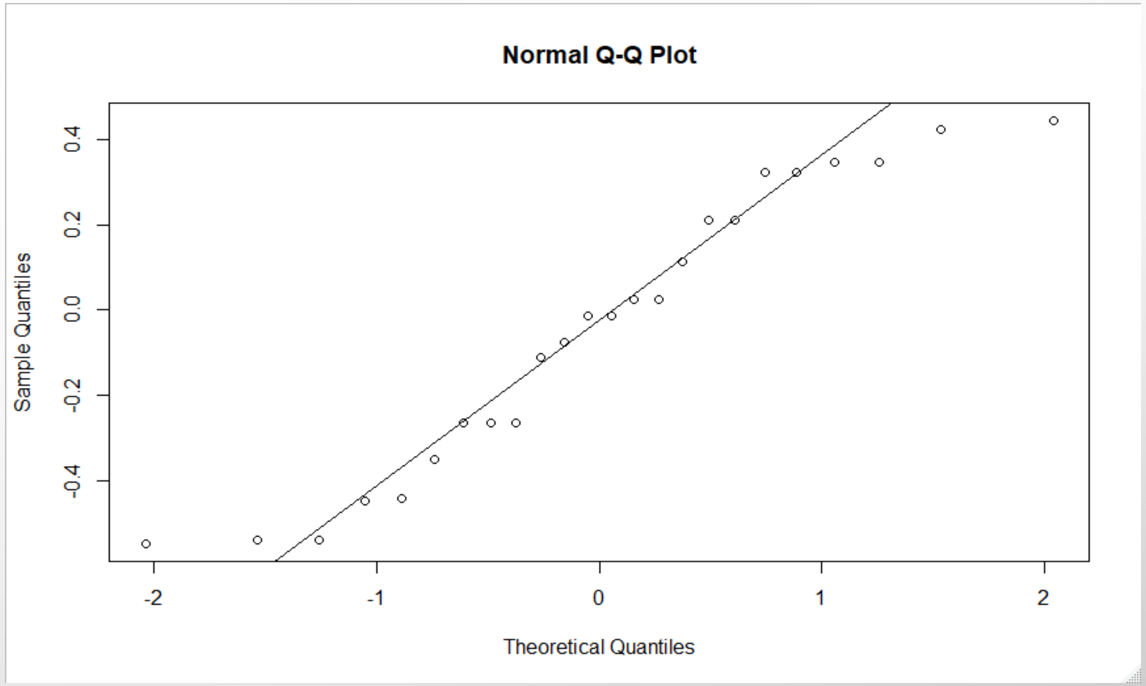
Models Diagnostics and Effective Doses

3-parameter log-logistic model:



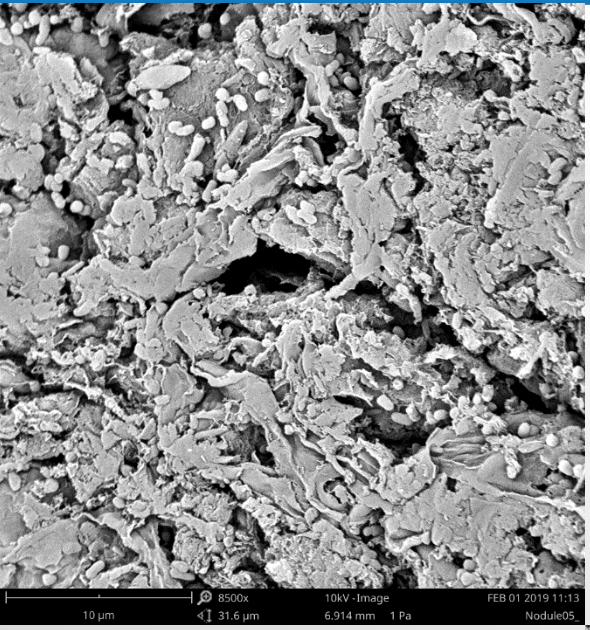

```
> ED(Plant.Biomass.LL.3,10)

Estimated effective doses

                          Estimate Std. Error
e:Dactylis glomerata:10    3.00487    0.46351
e:Festuca rubra:10         4.40357    1.61036
e:Glycine max:10          12.66799    2.01480
e:Vicia sativa:10          5.07367    1.08953
```

Cedergreen-Ritz-Streibig model:

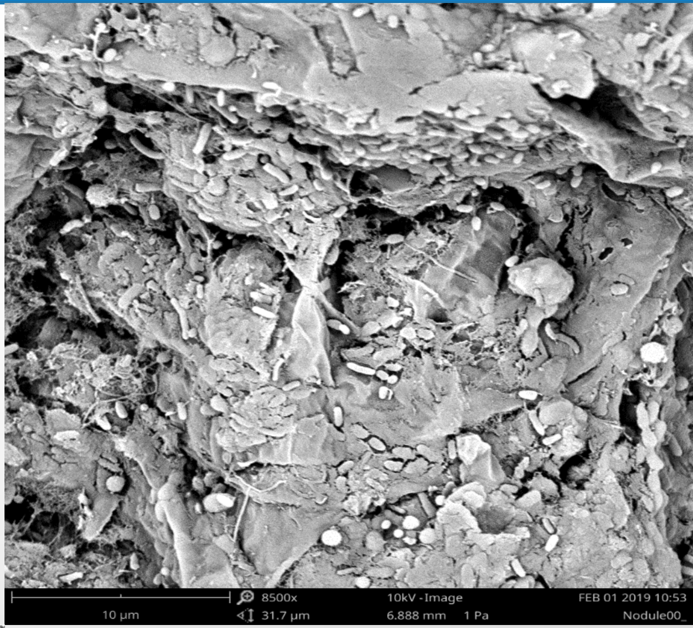


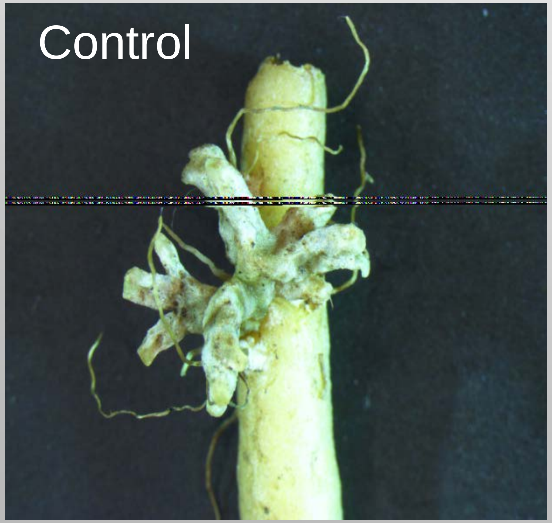

```
> ED(Msativa.crsm1,10)

Estimated effective doses

       Estimate Std. Error
e:1:10  15.3299     1.4709
```

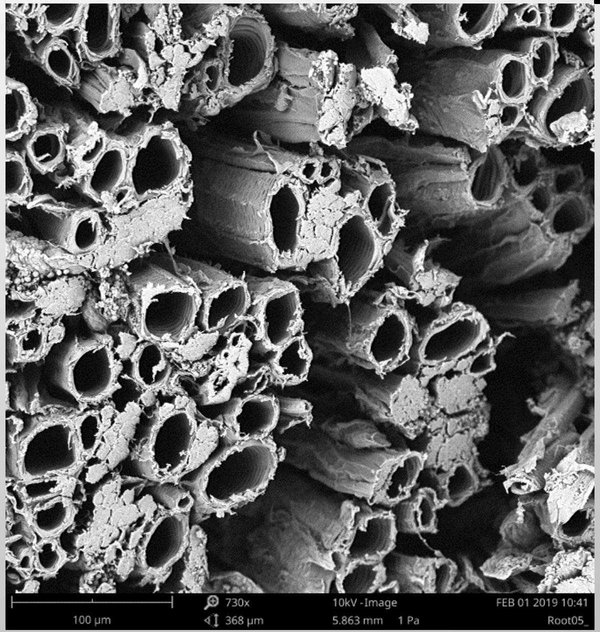
Light and Electron Microscopy



- Enhanced nodule development
- Enhanced colonization of the roots by plant growth-promoting rhizobacteria
- Interestingly, root tissues of *M. sativa* are undamaged by diesel fuel contamination

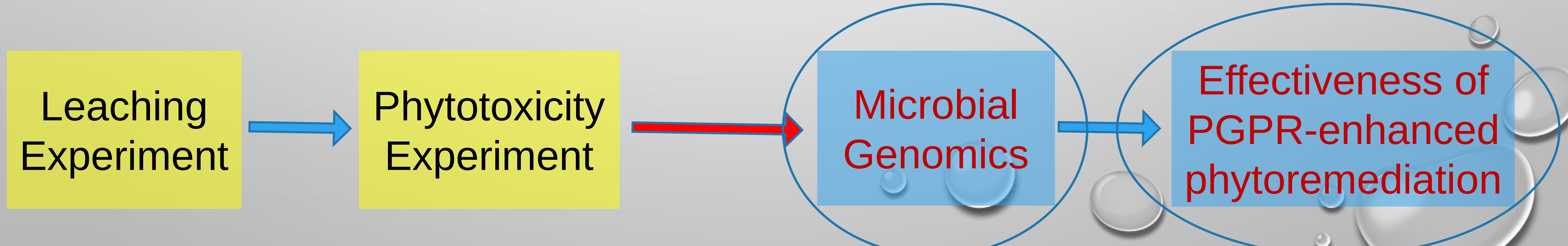






Summary

COMPLETED	NEXT
• Diesel fuel exerted hormetic influence on *Medicago sativa* with enhanced biomass production, nodule development, and microbial colonization.	• Isolation, characterisation and culturing of microbial symbionts of *Medicago sativa*. • Molecular analysis of degradation products.



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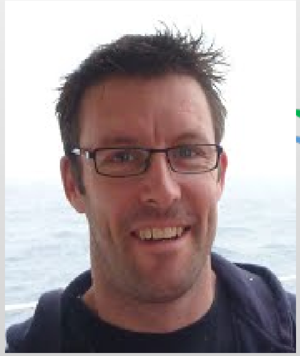
Director, MQ Marine Research Centre.



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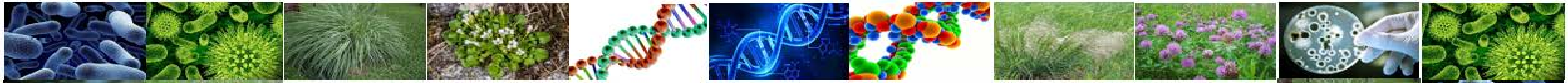

Thank You for Listening