Mauritius Research and Innovation Council

INNOVATION FOR TECHNOLOGY

DOES USING CORN STEEP LIQUOR AND TRICHODERMA HARZANIUM IN COMBINATION SIGNIFICANTLY SPEED UP THE BIOREMEDIATION AND RECOVERY OF MANGROVES POST-WAKASHIO?

Final Report

November 2022

Mauritius Research and Innovation Council

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This report is based on work supported by the Mauritius Research and Innovation Council under award number MRC/SBR-P05. Any opinions, findings, recommendations and conclusions expressed herein are the author's and do not necessarily reflect those of the Council.

1. INTRODUCTION

Bioremediation of oil contaminated soil is a necessity for Mauritius in the wake of the Wakashio disaster. In this research project, we aim to assess the effectiveness of a novel technique to enhance bioremediation, using the promising results found with the Trichoderma harzanium fungus in helping plants grow in oil contaminated media, the success of corn steeped liquor in promoting growth of bacteria that also degrade hydrocarbons in soil, and combining these two techniques to enhance the action of these two biological promoters. The aim of the project is to demonstrate that the combination of Trichoderma harzianum fungi and corn steep liquor enhances degradation of hydrocarbons from oil polluted soil from the south coast better than each of these bioremediation agents on their own, and apply these findings in controlled laboratory conditions on mangrove seedlings. The project was approved and contract signed on the 28/01/2021 between Genius Dr Ltd and the MRIC. The project leader set things into motion as soon as the confirmation of project approval was obtained.

2. OBJECTIVES

No.	***Objective***	***Date achieved***
1.	***Evaluate hydrocarbon degradation in the four experimental designs at various intervals.***	***03/06/2021***
2.	***Evaluate best CSL-TsTh mixture concentration for most effective results.***	***20/07/2021***
3.	***Assess response of mangrove seedling growth to bioremediation agent in laboratory conditions.***	***16/11/2021***
4.	***Develop protocol for in situ application of bioremediation agent.***	***31/07/2022***

3. WORK COMPLETED AND DELIVERABLES

Chronology

Date	Activity
01/02/21	Zoom meeting with team members to plan team participation in week's activities. Decision made to import the CSL powder in order to standardise our experiment, as facilities to produce the CSL in Mauritius not existent. Due to delays in starting project between proposal submission and approval, and budgeting constraints, decision made to only take samples at 10cm and 20cm depth, as cleaning effort from authorities and volatility of hydrocarbons resulted in a high probability of 2cm depth samples being hydrocarbon free.

02/02/21	Purchase on credit of Corn Steep Powder from Chinese supplier after written assurance obtained from National Plant Protection Office that additional permit not required (Mrs Seebun, Principal Scientific Officer)
3/02/21	Purchase on credit and collection of Trichoderma Harzianum from BioEdge Ltd, at Tamarin
3/02/21	Meeting at SGS Ltd at Valentina, Phoenix Office, with Mr Daniel Julie and Miss Mungur, scheduled agreed, depending on approval from ministry. Also obtained suppliers details for PPE acquisition at favourable pricing
3/02/21	First site visit at low tides in South East Coast, suitability of ground for sampling, proximity to mangroves assessed, GPS coordinates sent to ministry official (Mrs Koonjul) for approval and stated aim for 19/02/2021
3/02/21	Informed by MRIC accounts section that funds could not be disbursed due to administrative reasons, causing delays to other planned purchases
4/02/21	Informed by Mrs Koonjul that 3 other alternative sites need to be chosen as per conditions of permit, schedule for next visit made for 5/02/21
5/02/21	Second site visit at low tides in South East Coast, GPS coordinates sent to Mrs Koonjul and informal approval obtained, still planning for 19/02/21 sediment collection
08/02/21	Visit to 3 PPE suppliers and quotations obtained for purchase
08/02/21	Delivery of imported CSL powder delayed as courier company unable to acknowledge official message from NPPO
15/02/21	Planned Purchase of sampling equipment completed (trowels, corer, funnel etc)
16/02/21	Decision to postpone 19/02/21 sediment collection due to lack of funds disbursement and unable to purchase PPE equipment essential to collection. New date of 05/03/21 decided, for low tides conditions during work hours specified by our collection permit
18/02/21	Informed by MRIC that disbursement cheque finally issued
19/02/21	Corn Steep liquor powder delivered
22/02/21	Attended mandatory MRIC dissemination event, discussion with other researchers doing related projects
22/02/21	Purchase order of 1st set of PPE equipment completed
04/03/21	Site visit to mark and pre-plan collection for the next day, collection of containers from SGS Ltd
05/03/21	Collection of sediments and delivery to SGS Ltd, processing for day zero testing, terms for first instalment agreed
08/03/21	Purchase of growth medium for fungi, delivered to SGS Ltd, with experimental phase due to start on 15th March due to upcoming public holidays

09/03/21	Sanitary curfew announced. SGS Ltd conferred and decision to postpone experimental phase until Covid19 and Work Access Permit situation resolved
12/03/21	Authorities announce that the SGS laboratory will be in a "red zone" and access will be severely restricted. Decision made to transfer all sediment samples to cold storage until further updates available.
19/03/21	First batch of test results processed and shared by SGS ltd. Results rechecked on our request, and shared with ministry of fisheries scientific department in view of discrepancy with their own results.
29/03/21	Zoom meeting with project team to revise experimental phase in view of day zero results as well as delay between collection and future experimentation. Protocol revised.
31/03/21	Authorities announce removal of SGS lab from Red zone. WAP to access lab awaited
15/04/21	Able to circulate to lab with WAP. SGS Lab informed in order to incubate fungi in preparation for experimental phase
22/04/21	Experimental phase started as per revised protocol. Revised day zero test to be done on 23rd April 2021.
4/5/2021 - 20/5/2021	Amended progress report submitted to MRIC. Processing until milestone 2 disbursement carried out
5/5/2021	PAH analysis of day 14 samples performed at SGS Labs, results only available after payment made
19/5/2021	PAH analysis of day 28 samples performed at SGS Labs, results only available after payment made
20/5/2021-30/6/2021	Purpose built greenhouse suppliers sourced, greenhouse ordered and installation initiated
1/6/2021–25/6/2021	Correspondence with Ministry of Fisheries for obtention of special permit to access beaches for seedling retrieval, in view of beach lockdown protocol.
3/6/2021	Results from SGS obtained and analysis
4/6/2021	Experimental design changed to reflect natural degradation of PAH in stored sediment, and preliminary microbiology set ups performed at SGS labs
4/6/2021	Purchase of Rhizotron containers
11/6/2021	Zoom meeting with research team to take stock and future planning, including experimental set up
14/6/2021 – 21/6/2021	Design of experimental setup with TsTh and CSL within the rhizotron
22/6/2021	Results from SGS obtained and analysis
29/6/2021	Experimental set up at SGS to find optimal dose of TsTh and CSL
29/6/2021	Purchase of lab and storage equipment
1/7/2021	Permit from Ministry of Fisheries for access to beaches obtained
6/7/2021 – 10/7/2021	Completion of outer nursery construction Placement of inner protective flooring
6/7/2021 – 13/7/2021	Optimising bioremediant combination with rhizotron set ups.

7/7/2021	Collection of suitable mangrove propagules, sorting, measurement and data collection, transfer to nursery facility
7/7/2021-22/7/2021	Results from SGS lab transmitted piecemeal
14/7/2021	Retrieval of sediment from SGS lab, set up of optimal bioremediant combination in controlled conditions
16/7/2021-21/7/2021	Sorting of propagule into experimental and controls after initial measurements, and placement in controlled conditions in nursery
21/7/2021 – 6/11/2021	Mangrove growth period, monitored by Mist and Spray Ltd
5/08/2021	Zoom meeting with team for results review and further publication plans
6/11/2021 – 30/11/2021	Retrieval of seedlings from rhizotrons, measurements, statistical analysis and report production
1/12/2021 – 31/07/2022	Report write up and drafts sent for journal publication, revision requested but not feasible in view of contaminated sediment no longer available

Documentation of work completed

1. Scope of Work document, for the attention of SGS Ltd, sent 19/02/2021	**Protocol Genius Dr /MRIC/ SGS:** **Roles:** Genius Dr Ltd - "GDL" – principal investigator: obtention and delivery of samples, addition of CSL and TsTh (to be provided by GDL), payment processing MRIC – funding body, disbursement of funds at relevant milestones SGS – provision of sampling containers, lab testing, storage of samples, incubation of TsTh **Field Protocol** 5 Sites – Bois des amourettes , BA Anse Jonchee, AJ Bambous virieux, BV Petit sable, PS 4 soeurs, QS **Sampling protocol:** 10cm and 20cm layers from each site, labelled at collection as BA1, BA2, AJ1, AJ2, BV1, BV2, PS1, PS2, QS1, QS2. Each site will be consider as a single overall location, with no testing for intra-site variation Trichoderma to be incubated for at least 24h, maximum 60hours in either potato dextrose agar or Sabouraud agar, all to be added to samples on day 10. **Testing scenarios** Phase 1 1. Detectable hydrocarbon in all 10 and 20cm sample >> proceed to 72 tests, except site M 2. Detectable hydrocarbon in only some 10cm samples or only 20cm samples >> exclude sites from further testing.(This is dependent on day zero sampling) and proceed for testing for remaining samples 3. No detectable hydrocarbon in any sample a. >> stop all testing, b. keep half of sediment for seedling growth c. >> introduce predetermined amount heavy oil in sediments in 4 experimental arms i. Proceed as per experimental schedule as above. ii. Total number of tests will 1+ 4 + 4

Test quantities
Scenario 1:

	Day 0	Day 28	Day 49	Total bottles
Each sample to be tested 200g		X 4 10cm X4 20cm 8 mini bottles	X4 10cm X4 20cm 8 mini bottles	18
Sites	ALL 5	All except site "M"	All except site "M"	72
Site M for phase 2 to be chosen based on hydrocarbon test (median content)		Day		
Total tests	**10**	**32**	**32**	**72**

Site "M" will be determined after first round of testing, and will be one of the five original sites. It will be excluded for testing until phase 2.
Nomenclature for subdivision of sample to be done at SGS: xxAAyBB, where xx is day of testing, AA is site of sample, y is depth of layer, and BB is branch of experiment
Example, for site BA:
2 tests on day zero, 00BA1NA, 00BA2NA
8 tests on day 28, 28BA1NA, 28BA2NA, 28BA1TS, 28BA2TS, 28BA1CS, 28BA2CS, 28BA1CT, 28BA2CT
8 tests on day 49, 49BA1NA, 49BA2NA, 49BA1TS, 49BA2TS, 49BA1CS, 49BA2CS, 49BA1CT, 49BA2CT
The above will be needed for 3 other sites, as site M will be excluded
NA = no addition (control)
TS = trichoderma
CS = corn steep
CT = Corn+Tricho
TS, CS and CT will be added on day 10 to their respective containers by GDL team. All are liquids

Phase 2:
As per scenarios In phase 1:

1. At day 28 (after results of day 21), 4 varying concentration of CSL + tsTh mixes in site M sediment, to be tested at day 21 and 42.ie 8 tests
2. At day 28 (after results of day 21), 4 varying concentration of CSL + tsTh mixes in sediment with median hydrocarbon content from the remaining contaminated sediment; ie 8 tests
3. At day 28, set up heavy oil + sediment + mixes as above

In summary:
Tests needed , minimum 35, maximum 80

2. Sampling questionnaire used

SOIL & SEDIMENT SAMPLE LOG SHEET

Page___ of___

Project Site Name: _____
Project No.: _____

[] Surface Soil
[] Subsurface Soil
[] Sediment
[] Other: _____
[] QA Sample Type: _____

Sample ID No.: _____
Sample Location: _____
Sampled By: _____
C.O.C. No.: _____

Type of Sample:
[] Low Concentration
[] High Concentration

GRAB SAMPLE DATA:

Date:	Depth Interval	Color	Description (Sand, Silt, Clay, Moisture, etc.)
Time:			
Method:			
Monitor Reading (ppm):			

COMPOSITE SAMPLE DATA:

Date:	Time	Depth Interval	Color	Description (Sand, Silt, Clay, Moisture, etc.)
Method:				
Monitor Readings (Range in ppm):				

SAMPLE COLLECTION INFORMATION:

Analysis	Container Requirements	Collected	Other

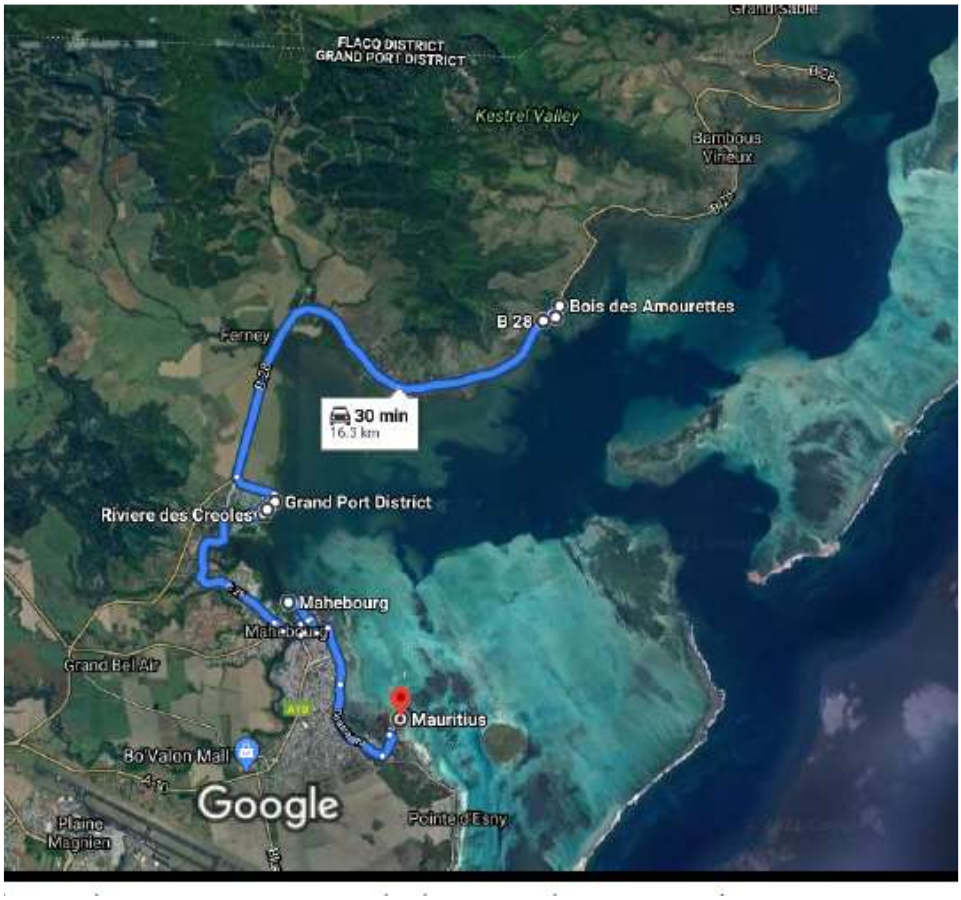
OBSERVATIONS / NOTES:

MAP:

Circle if Applicable:

MS/MSD	Duplicate ID No.:

Signature(s):

3. Site visit 03/02/21	 Proposed 5 sites: Location: Anse Jonchée/Bois des Amourettes Grand Port GPS coordinates: 20° 21' 51.19" S , 57° 44' 26.63" E GPS coordinates: 20° 21' 56.56" S , 57° 44' 24.53" E GPS Coordinates: 20° 21' 58.48" S , 57° 44' 18.70" E  Location: Morcellement Ferney, Grand Port GPS Coordinates: 20° 23' 21.58" S , 57° 42' 5.95" E GPS Coordinates: 20° 23' 25.31" S , 57° 42' 2.79" E GPS Coordinates: 20° 23' 27.31" S , 57° 42' 0.43" E

Location: Riviere des creoles, Grand Port

GPS Coordinates: 20° 23' 37.60" S , 57° 42' 2.92" E

GPS Coordinates: 20° 23' 38.86" S , 57° 42' 3.24" E

GPS Coordinates:20° 23' 40.73" S , 57° 42' 4.13" E

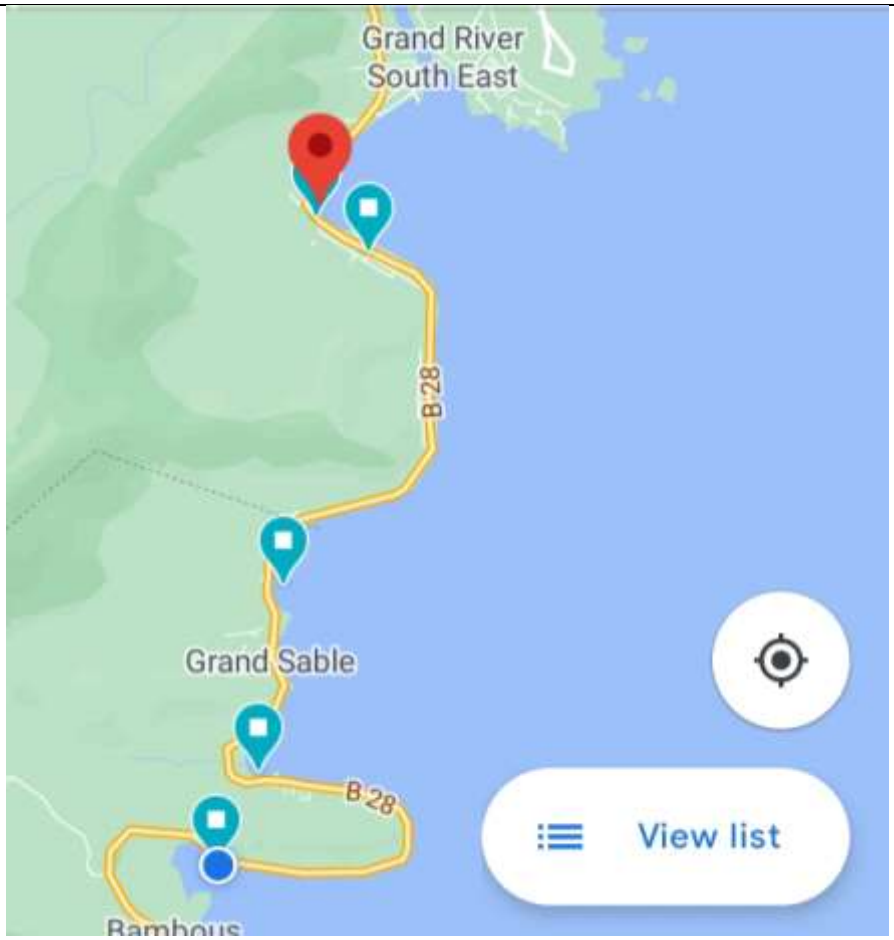
Location: Mahebourg, Grand Port

GPS Coordinates: 20° 24' 7.91" S , 57° 42' 12.96" E

GPS Coordinates: 20° 24' 9.51" S , 57° 42' 23.53" E

Location: Pointe Jerome, Grand Port

GPS Coordinates: 20° 25' 01.67" S , 57° 43' 08.10" E

Site visit 2 05/02/21	 Sites 1. (Northernmost site:) 4 Soeurs 20°29716 S, 57°77343 E  2. Petit Sable 20°33326 S, 57°76927 E

	 3. Bambous Virieux 20°33894 S, 57°7665170 E  4. Anse Jonchee - same location as on 3/02/21, not revisited 5. Bois des Amourettes (southernmost), same location as on 3/02/21, not revisited
04/03/21	Dress Rehearsal/Marking visit

Getting used to the terrain

Trying the tools out on the chosen sites

Measuring distances between sites

Determining suitable site

Removable Site marking

Close inspection

Containers for sediments

Pre-labelling of bottles

	 Getting used to Chemical Hazard PPE
05/03/21 Sample collection	 20x20cm square marked for collection

Trowel with 10cm markings for accurate depth

Corer with 10cm depth also used for sampling

Steel ruler for accurate depth measurement

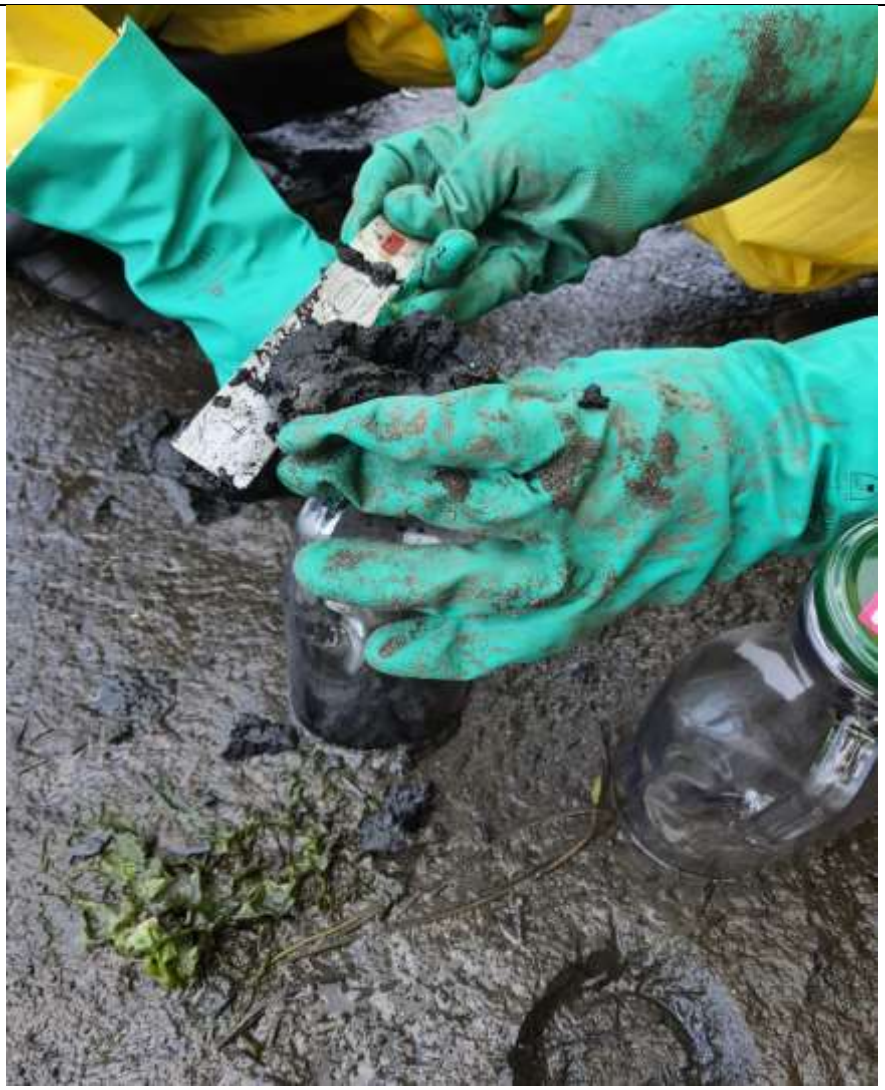
Site after sample taken and before being refilled with sediment

Working in teams of 2 to fill containers

Oil contaminated core sample

Before transfer into pre-labelled glass container

Working faster before tide traps us.



Macroscopically viscous oily sediment

Site 1 – Anse Jonchee

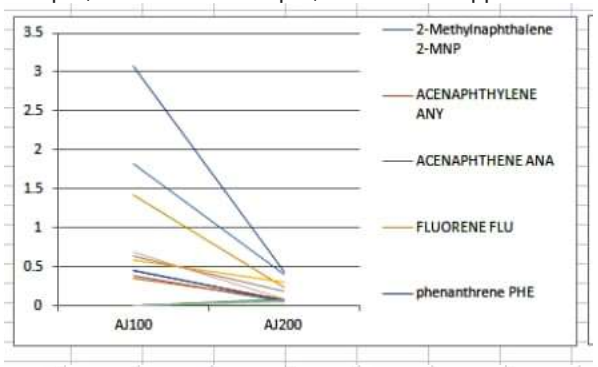
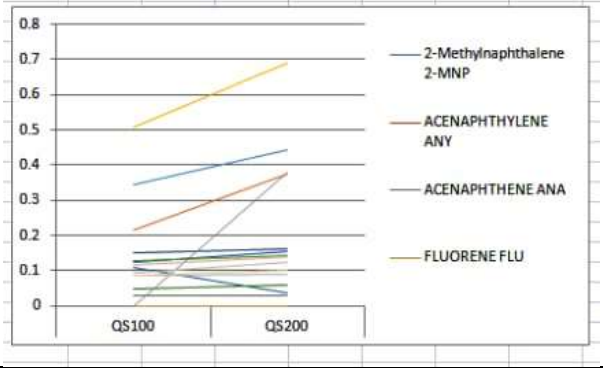
Site 2 – Bois des Amourettes

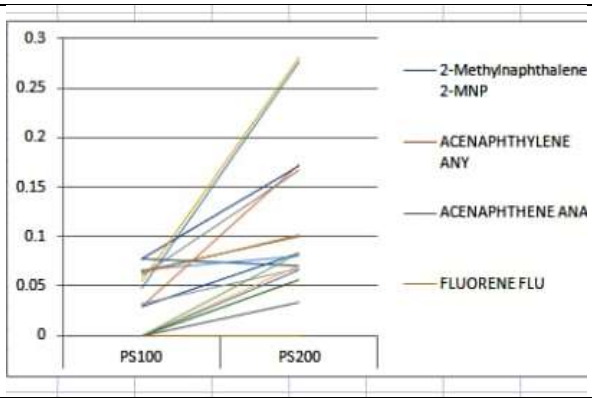
Site 3 – Bambous Virieux

Site 4 – Petit Sable

Site 5 – Quatre Soeurs

	 Sediment samples collected  Day zero samples divided and ready to be tested
08/03/2021 Microbiology protocol given to SGS	Microbiology protocol: **Fungal prep** Plate – Sabouraud agar, to be kept at 4 degrees in fridge prior to use. 3 grams Trichoderma dissolved by shaking in 5 ml distilled water at room temperature, all sterile containers (ie glass test tube). 5x 1ml drops per plate using glass pipette, added aseptically to avoid contamination, evenly distributed Incubation temperature: 30.1 degrees Celsius Incubation time: 72 -96 hours, in absence of UV light. Note that process should be planned for addition to experimental setup on the 15th of March. The process to be repeated for 16 plates total. The remaining unused plates to remain in fridge until Phase 2. The remaining Trichoderma should now be kept in fridge, with no light. **CSL prep** 1:1 mixture with sterile water, eg 10g CSL powder in 10ml water, mixed thoroughly. Powder to be kept in fridge in sealed container when not in use. **Experimental set up**

	Content from fungal culture to be retrieved using sterile metal corer and added to 25ml sterile water at room temperature per plate in conical flask and shaken at 150rpm for 2-3 minutes. 16 such flasks will be obtained, ie 400ml of fungal solution. Note: If quite uniform growth obtained from each plate, a single 400ml solution can be made up. If not, I will need to keep track of initial culture.
12/03/21 Storage protocol due to Lockdown	Correspondence to Mr Daniel Julie, Regional Business Manager, SGS ltd As things stand, our protocol for keeping the sediments at room temperature for an indeterminate length of time is no longer viable. Are you able to store our bottles at a lower temperature in the meantime? 4-10 degrees celsius range would be ideal and will slow things enough for us to still be able to use them.
19/3/21	Day zero sampling test results obtained. Hydrocarbons present in detectable amounts at Anse Jonchee, Petit Sable and Quatre Soeurs only, with none at Bambous Virieux and Bois Des Amourettes Tests performed: Gas Chromatography-Mass Spectrometry List of Molecules: • Benzo[a]pyrene • Dibenzo[a,h]anthracene • Indeno[1,2,3-c,d]pyrene • Pyrene • Benzo[b]fluoranthene • Benzo[k]fluoranthene • Benzo[g,h,i]perylene • Fluoranthene • Benz[a]anthracene • Chrysene • Benzo[j]fluoranthene • Benzo[e]pyrene • Cyclopenta(c,d)pyrene • Anthracene • Acenaphthene • Acenaphthylene • Fluorene • 2-Methylnaphthalene • Naphthalene • Phenanthrene Graphical representation of results: (AJ= Anse Jonchee, QS = Quatre Soeurs, PS = Petit Sable, 100 = 100mm depth, 200 = 200mm depth, units are 0.01ppm dL  



15/04/21 Revised protocol sent to SGS

Revised phase 1 protocol.

1. Mixing of sediment samples from each individual site
    a. Roughly 2.8kg/per depth/per site currently distributed in 3 bottles will be mixed together by me using my hand trowels.
    b. This should result in 4 large samples to be divided for the testing phase.
    c. The 4 large samples will be QS1NA, QS2NA, AJ1NA, AJ2NA
2. Due to the lockdown delay, we would like to test the above samples at the new day zero, ie 22nd April 2021
    a. Tests to be conducted on 00QS1NA, 00QS2NA, 00AJ1NA, 00AJ2NA (total = 4)
3. We will now be testing next batch at day 14 and day 28, ie 5th May 2021 and 19th May 2021
    a. Subdivisions will be as our initial protocol
    b. tests on 5th May 2021
    c. tests on 19th May 2021
4. On 22nd April, I will prepare and label 8 samples with no additive, 16 samples with a single additive, and 8 samples with 2 additives, with the respective dates
5. 480ml of CSL and 480ml of TsTh will be divided in the 24 experimental sample

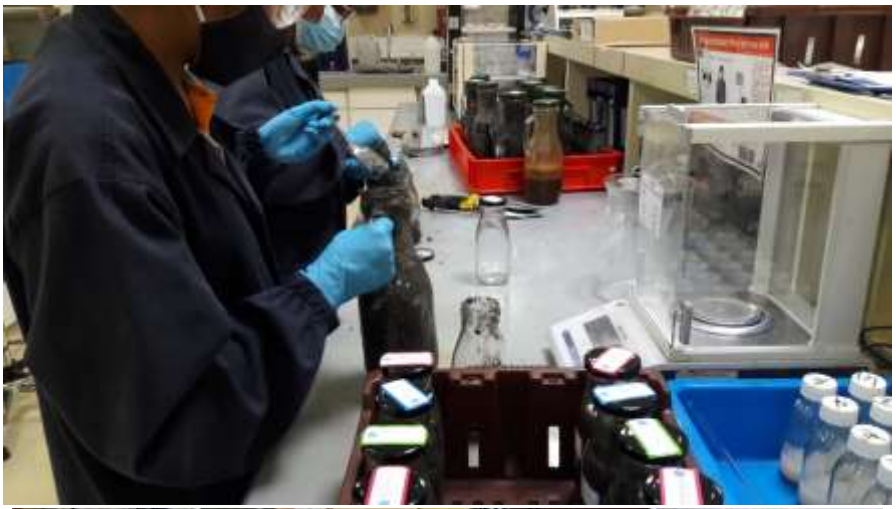
Additional tests:

1. Microbiology
    a. We had 4 spare fungal cultures today, and saw it as an opportunity for microbiological analysis.
    b. We would like a fungal colony concentration
        i. in a 1:1 CSL/TsTh mix at day 3 and day 7
        ii. in a 1:2 CSL/TsTh mix at day 3 and day 7
        iii. comparisons with control TsTh growth alone on day 3 and day 7
    c. We would like macroscopic and microscopic images of the above testing
    d. If we can obtain data for day 14 for as well, within the realm of possibility, I believe we will be having some novel scientific information

	2. Physico-chemical properties of the sediment a. By grain size analysis, we would like to get a distribution of the particle sizes (sand, silt, clay) as per the following protocol definitions: i. Silt: particle size 0.002-0.05 mm ii. Clay: particle size <0.002mm iii. Sand: very coarse, coarse, medium, fine, very fine, as per definition above iv. pH
22/04/2021 Phase 1 interventions	1. 240g CSL powder mixed with distilled water (240g) in a 1:1 ratio 

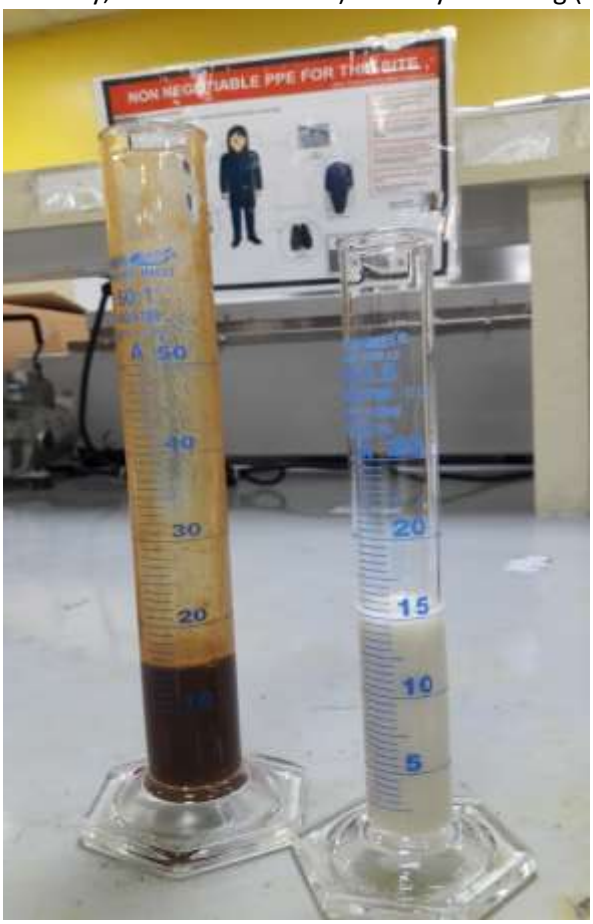
Shaken vigorously to obtain a homogeneous solution

Trichoderma solutions prepared as per protocol



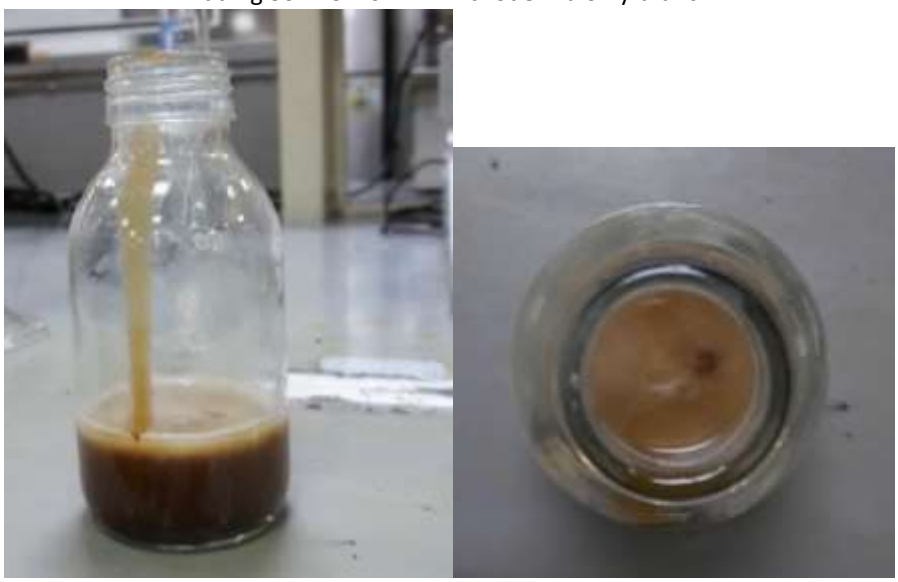
Composite mixtures made as per protocol

First batch of composites, labelled as per additive (none, Corn steep liquor only, trichoderma only, corn+Trichoderma) and day of testing (0, 14, 28)



CSL and TsTh added in 1:1 ratio in volume (15ml each)

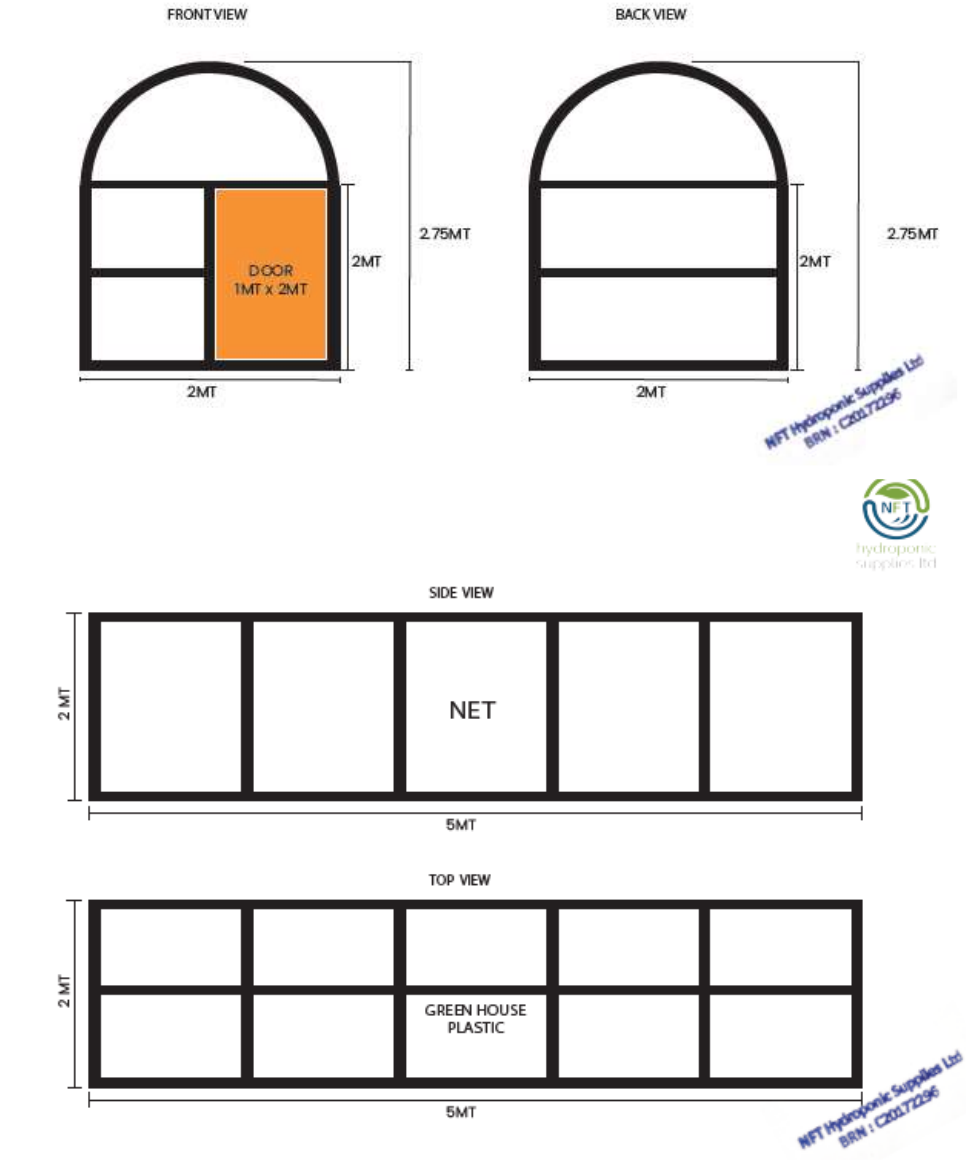
Adding 30ml of TsTh in Trichoderma only branch

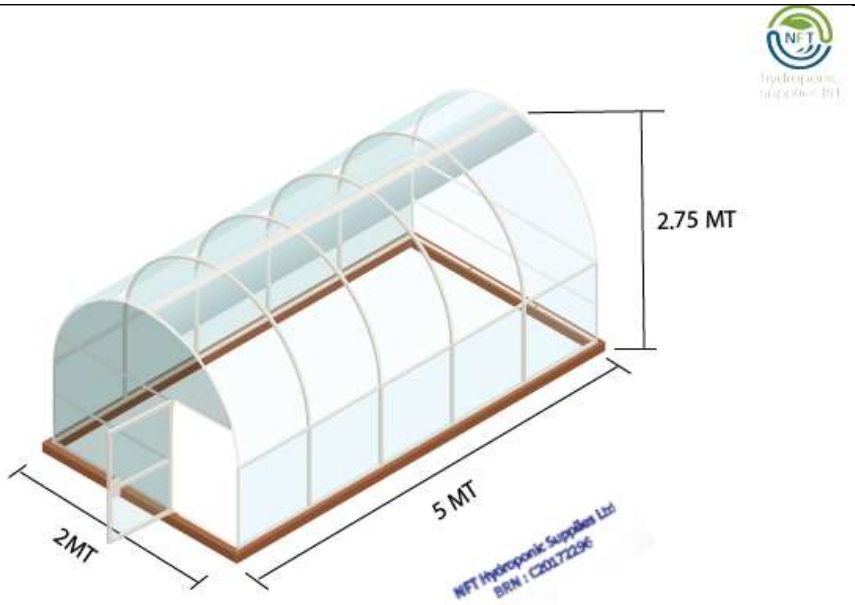


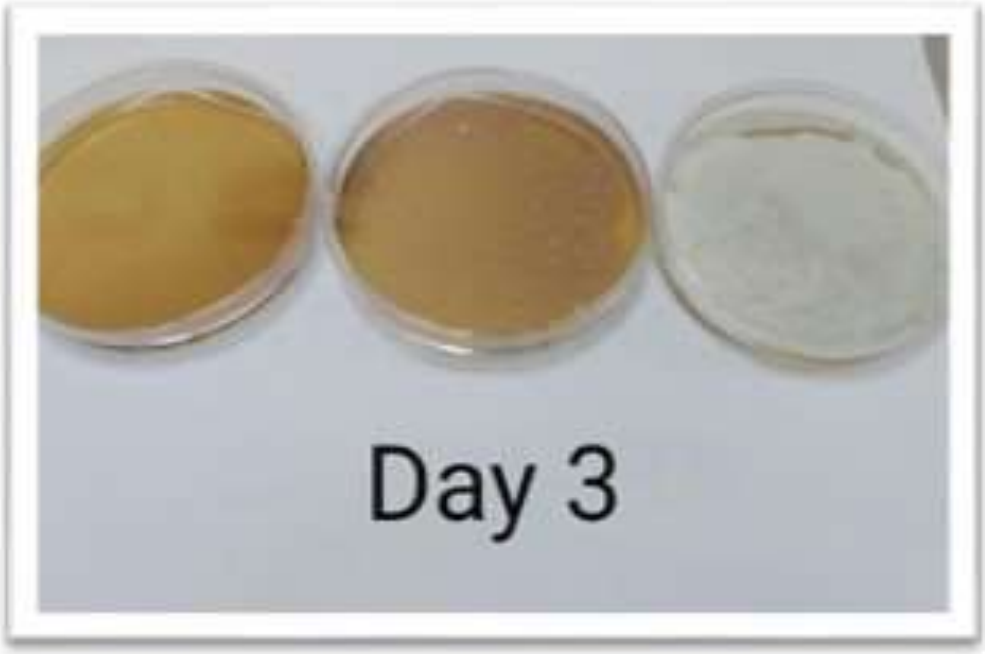
Physical appearance of 1st ever documented combination of CSL and TsTh
(side and top view) – A uniformly coloured mixture, with an emulsion of fungi at the surface.

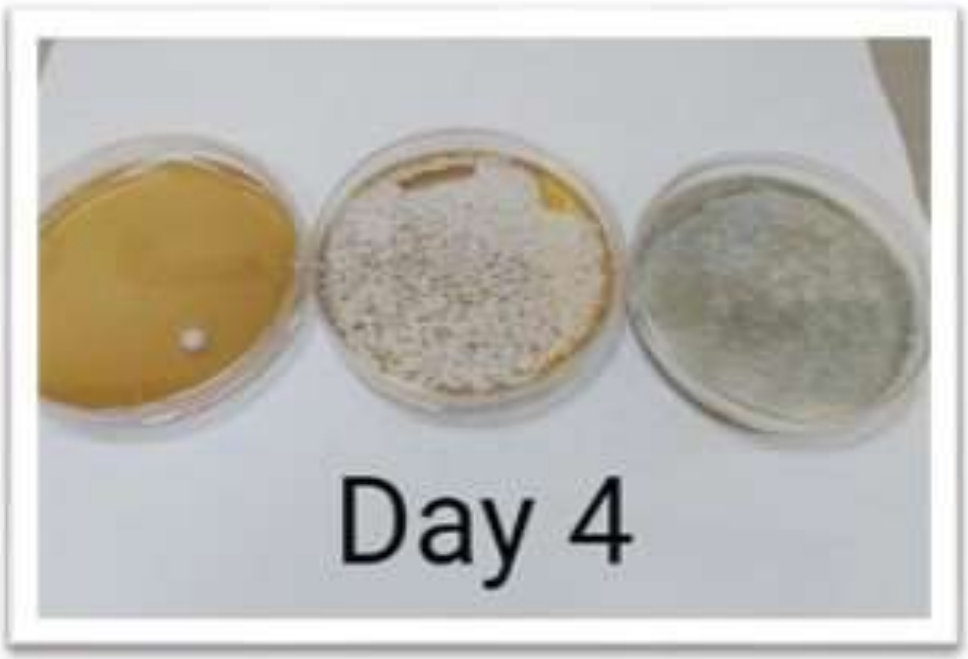
Completed experimental batch, to kept out of light at room temperature conditions. Additives added to the jar without any shaking or mixing. Each jar and its content weighed prior to account for potential dose related response.

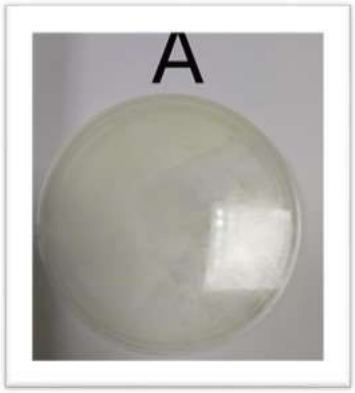
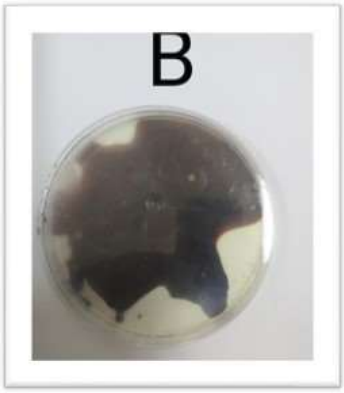
20/5/21	Plans for nursery

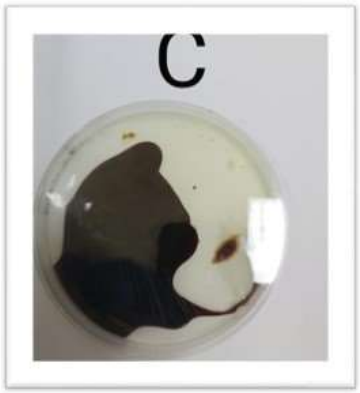

FRONT VIEW
BACK VIEW
DOOR
1MT x 2MT
2MT
2.75MT
2MT
2MT
2.75MT
2MT
SIDE VIEW
NET
2 MT
5MT
TOP VIEW
GREEN HOUSE
PLASTIC
2 MT
5MT

	
3/06/2021	Results from SGS for experimental set ups from 23/04/2021 on samples in storage since 12/03/2021, kept at 2-4 degrees Celsius Day 0 trace PAH detected in 1 sample only, none in the rest Day 14 (5/5/21): no PAH detected Day 28 (19/5/21): no PAH detected Microbiology tests Day 7  Day 14  Left plate: CSL:TsTh ratio is 1:2; middle plate ratio is 1:1; right plate is TsTh only

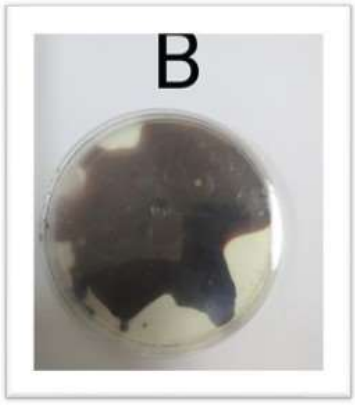

Day 3


Day 4

	 The above was suggestive of increased dilution of CSL would be less beneficial in combination with TsTh
4/6/2021	Experimental protocol, as per original scenario 3 where no hydrocarbons found in our sediment samples Addition of commercially available diesel in a petri dish of biological agents A. 30 ml TsTh + 30ml Diesel B. 20 ml TsTh + 20ml CSL + 20ml Diesel C. 23ml CSL + 23ml Diesel D. 30ml TsTh + 30ml CSL Dilution performed using sterile water, CSL and TsTh preparation as per protocol on 08/03/21  

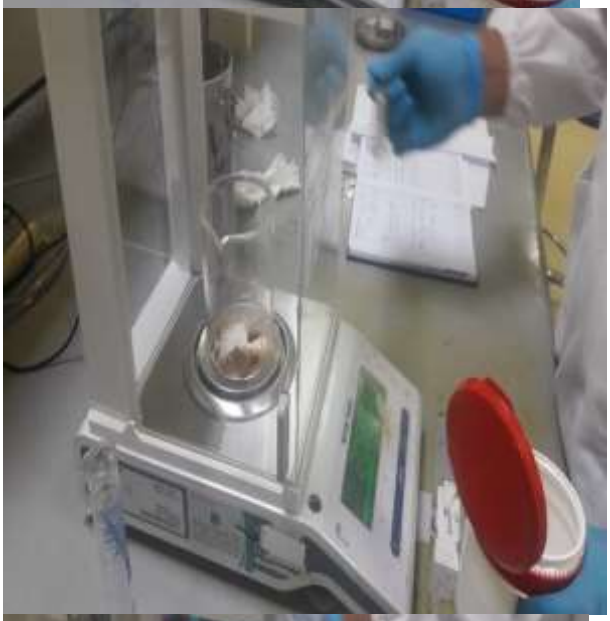
	 
6/6/21	Rhizotron trial set up to check for suitability of container  
11/06/21	Agenda and minutes for team meeting via Zoom: Agenda: 1. Progress so far: a. Tests march, oil on 3 sites (Anse Jonchee, Petit Sable, and Quatre Soeurs), 6/05/2021 b. Tests April 22, oil in zero site (Anse Jonchee, Quatre Soeurs), despite low temperature storage c. Evidence of some macroscopic growth in prepared sediment/corzianum bottles in sediments i. Pictures unfortunately corrupted d. Petri Dish – CSL + TsTh continue growing, better growth in 1:1 ratio (liquid 30:30ml) than in 1:2 ratio. Note that with CSL, TsTh has typical greenish colour appearance, while TsTh alone has whitish appearance only, and stops growing. e. We have done an experimental set up adding tsTh, TsTh/CSL, CSL to diesel in large petri dishes, to see how it behaves. The hydrophilic nature of the diesel means we have to rethink how to set this up 2. Technical issue and next phase

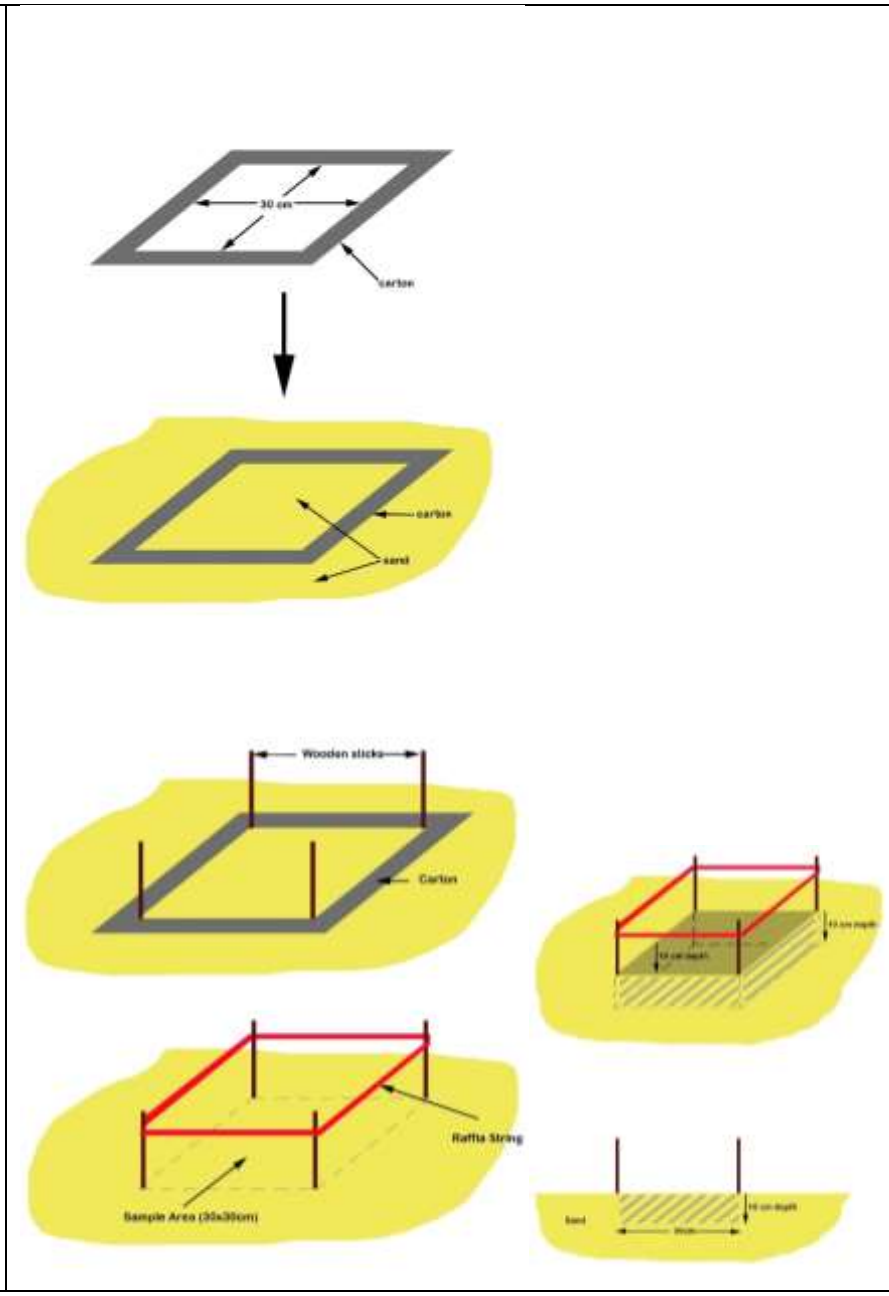
	a. What we are doing is different from several of the papers published and we referenced for this project i. All our additives are in powder form, and no clear guide on how to dilute it best ii. Adding the additives in liquid form poses the problem of mixing, so needs refining. iii. ?using droplets to add in a dish iv. Pre-fermenting the tsth v. ?mixing everything in dry form with sediment, then adding the liquid components for a more homogeneous solution? vi. Example – 200g sediment + 10g CSL powder + 1g TsTh powder + 10g distilled water + 1g Diesel, giving us a 0.43% Diesel (430ppm) contaminated soil, which we can test at day 7 and 14 with our remaining GC-MS allocation. We have 26 tests remaining 3. a. Ideal composition will be obtained from the above b. Greenhouse will be set up on 15th of June 2021 c. Permission to go on the beaches has already been sought, with a follow up call made d. Chosen areas – Poudre d'or, Grand gaube, bras D'eau, for propagule collection. With quarantine rules, we cannot do as for sediment and just go and collect on our chosen day e. We have cylindrical rhizotron set ups, amount of sediment to add is to be finetuned, we can reuse all our other sediment, considering there is no oil remaining, and we will be adding vastly significant amounts in our set up. f. We will monitor Ph,humiditiy. Salinity I need to clarify. (collect water sample from propagule collection site) 4. Write ups a. No update from Ministry re composition of their sediment analysis sadly b. I have uploaded some papers on pollution (heavy metals, dyes,) on google drive today c. Paper 1: our survey of the sediment, additionally we can do a visual analysis of the sediments, as sgs do not have the equipment to do more detailed analysis locally and would need to send samples abroad. We cannot directly link the oil findings to the wakashio, but I guess we can infer it d. Paper 2: on figuring out how to combine our bioremediants in an effective way e. Paper 3: on the practical exercise in the greenhouse 5. Tasks arising from above points: a. Photographs of greenhouse b. Dr Appadoo will send 1 page plan for papers
14/06/21	Nursery area cleared in readiness for delivery of nursery construction materials CSL + TsTh rhizotron trials continued in contained environment 
20/6/21	Construction materials delivered
22/6/21	Installation delayed by 48h as Crew tested positive for COVID Results from microbiology tests at SGS continue to show growth only in CSL+Tsth combination samples (B and D)

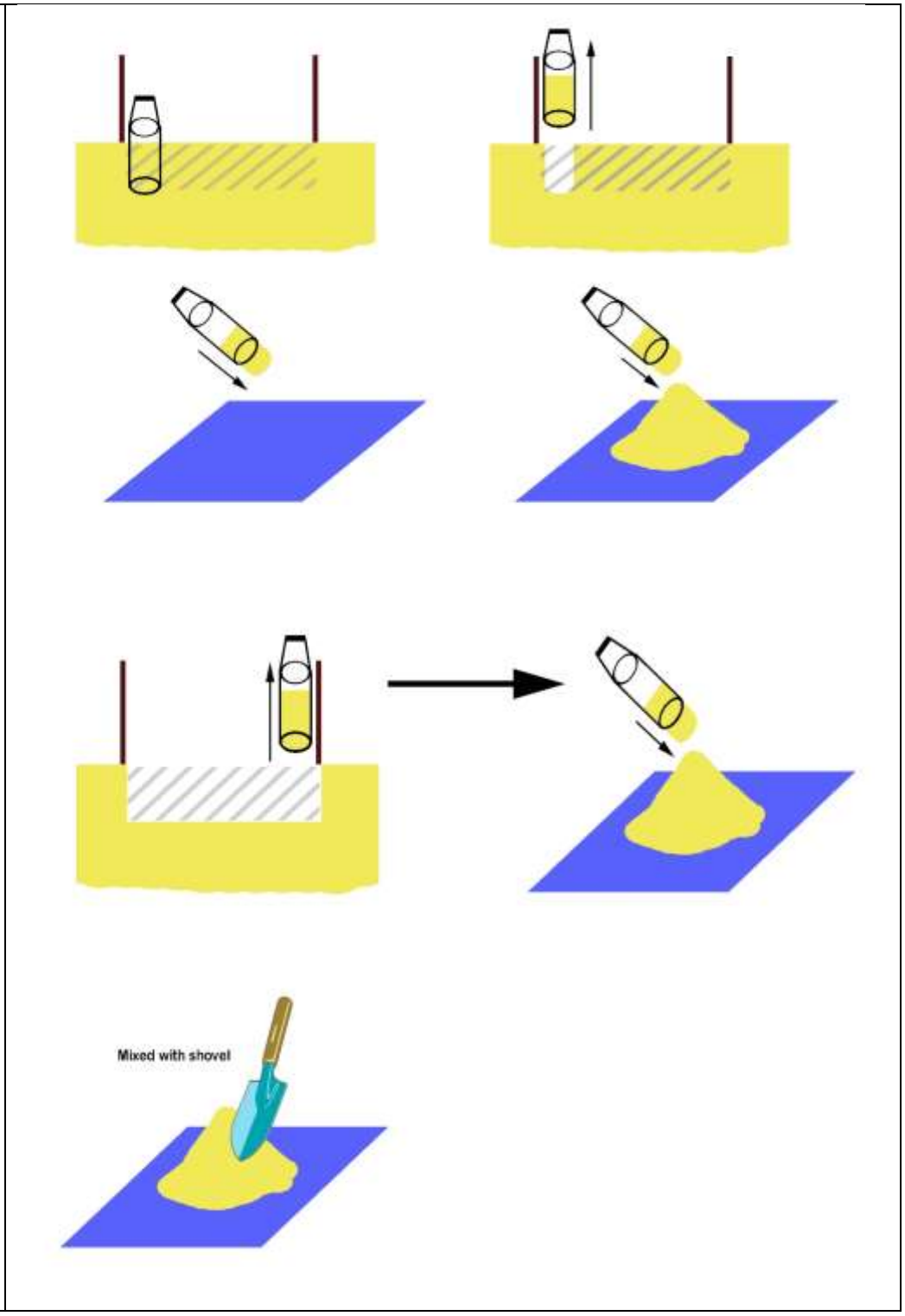
	 
24/6/21	Frame for greenhouse installed  
29/06/21	Protocol for final phase of laboratory tests **"29/06/2021 Plan** **1. Experimental set up** **a. Sediments from point aux sables retrieved and mixed together** **i. Expected total 2.7kg** **ii. Divided into 5 experimental branches , repeat x 2** **Branches Diesel amount** **1. 200g + 10g CSL + 4g TsTh + 10ml distilled H20 (total weight = 224g)** **2. 200g + 10g CSL + 6g TsTh + 10ml distilled H20 (226g)** **3. 200g + 10g CSL + 8g TsTh + 10ml distilled H20 (228g)** **4. 200g + 10g CSL + 10g TsTh + 10ml H20 (230g)** **5. 200g + 10g CSL + 2g TsTh + 10ml H20 (222g) 1g diesel** **For testing today: 200g sediment + 10ml H20 + 1g diesel** **Testing PAH** **Day 7 (6th July 2021)** **Day 14 (13th July 2021)** **2. Storage of above: dark cupboard, room temperature** **3. Add 10ml distilled H20 to day 14 samples at day 7 (6th July 2021) and mix, no testing to be done at that point** **4. Retrieve all sediments and store away."** At labs: Growth seen in all 4th June 2021 experimental petri dishes at varying degrees

Analysing Diesel + Tsth only using hand microscope . TsTh growth seen floating over the diesel

Circular growth of TsTH floating on the CSL+ diesel broth. Ample growth seen in left bottom corner, with no diesel added

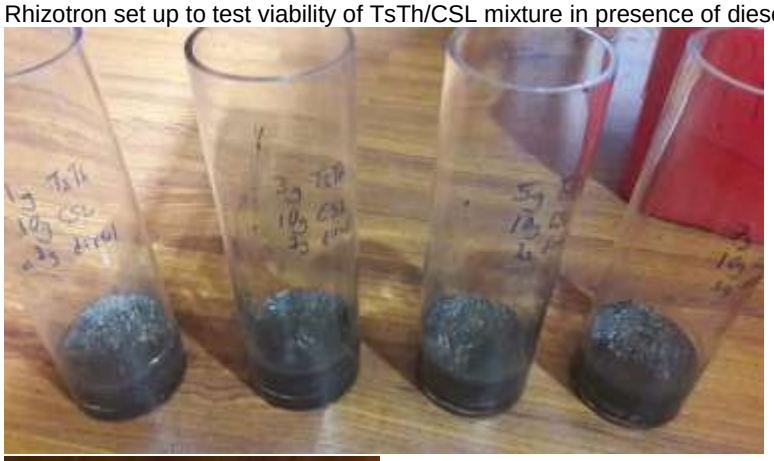
	Experimental set up as per the above protocol for PAH testing    Purchase and collection of laboratory equipment, and storage containers to retrieve sediments from SGS labs
30/06/21	Payment of fees to team members Preparation of documentation for dissemination


30 cm
carton
carton
sand
Wooden sticks
Carton
Raffia String
Sample Area (30x30cm)

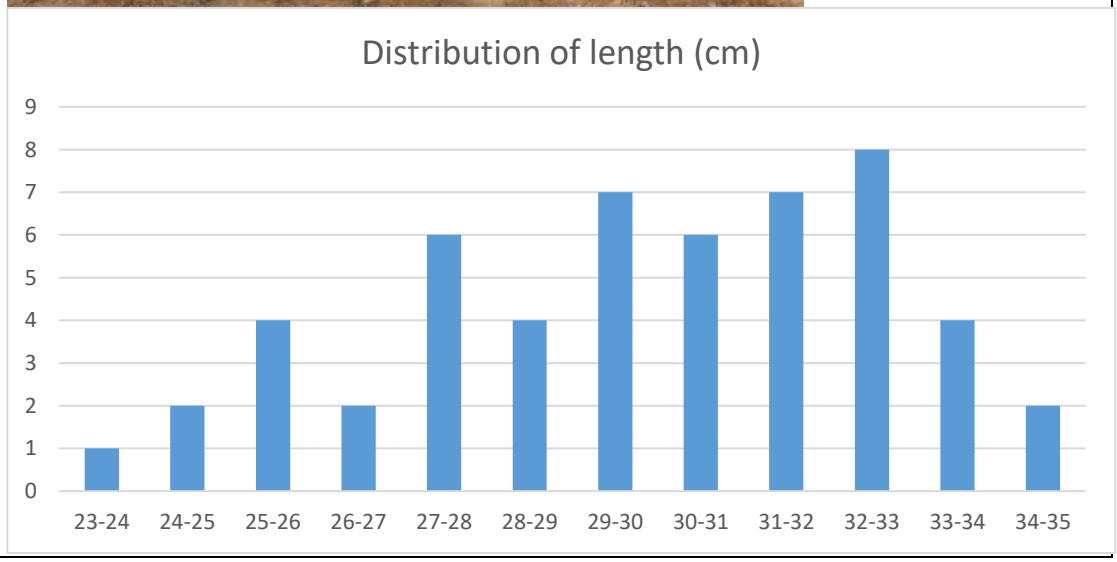

Mixed with shovel

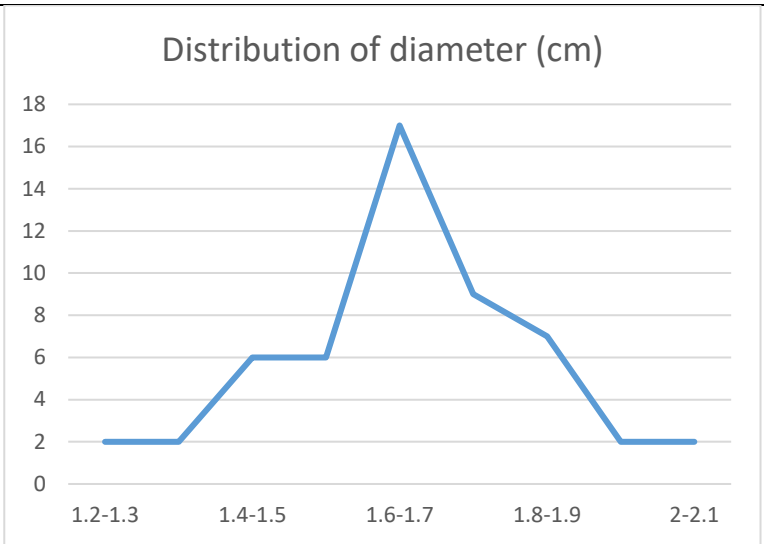
	

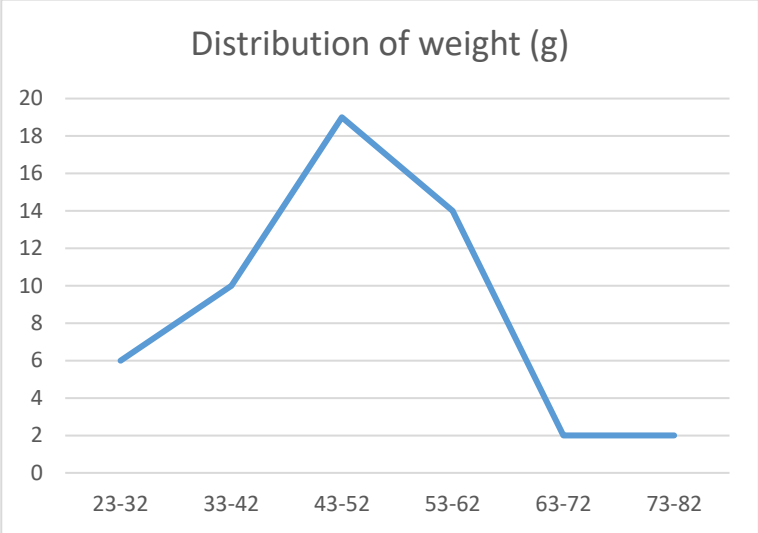
6/7/21	Nursery outer construction 

6/7/2021	Rhizotron set up to test viability of TsTh/CSL mixture in presence of diesel in sediment   

7/7/ 2021	 	Mangrove propagules collection from Grand Gaube Beach 20°01'34.8"S 57°40'57.3"E


Distribution of length (cm)
9
8
7
6
5
4
3
2
1
0
23-24
24-25
25-26
26-27
27-28
28-29
29-30
30-31
31-32
32-33
33-34
34-35





7/7/21 – 23/7/21	Results for final phase of laboratory tests on 29/6/21 **1. Experimental set up** **a. Sediments from point aux sables retrieved and mixed together** **i. Expected total 2.7kg** **ii. Divided into 5 experimental branches , repeat x 2** **Branches Diesel amount** **1. 200g + 10g CSL + 4g TsTh + 10ml distilled H20 (total weight = 224g)** **2. 200g + 10g CSL + 6g TsTh + 10ml distilled H20 (226g)** **3. 200g + 10g CSL + 8g TsTh + 10ml distilled H20 (228g)** **4. 200g + 10g CSL + 10g TsTh + 10ml H20 (230g)** **5. 200g + 10g CSL + 2g TsTh + 10ml H20 (222g) 1g diesel** **For testing today: 200g sediment + 10ml H20 + 1g diesel ("ONA" – results on 29/6/21 analysis)** **Testing PAH** **Day 7 (6th July 2021) (7CT2, 7CT4, 7CT6, 7CT8, 7CT10 – results on 6/7/21 analysis)** **Day 14 (13th July 2021) (14CT2, 14CT4, 14CT6, 14CT8, 14CT10 – results on 13/7 analysis)** **Day 21 (20th July 2021) – set up on 14th July 2021 (** **2. Storage of above: dark cupboard, room temperature** **3. Add 10ml distilled H20 to day 14 samples at day 7 (6th July 2021) and mix, no testing to be done at that point** **4. Retrieve all sediments and store away."**

Analysis performed on 29.06.2021

		ØNA
MASS		12.8039
VOLUME		10
DILUTION		5

		Results
Parameter	**Abbreviation**	**Concentration/PPM**
NAPHTHALENE	NAP	0.08401
2-Methylnaphthalene	2-MNP	0.279
ACENAPHTHYLENE	ANY	0.04016
ACENAPHTHENE	ANA	ND
FLUORENE	FLU	ND
phenanthrene	PHE	ND
ANTHRACENE	ANT	ND
fluoranthene	FLT	ND
pyrene	PYR	0.03904
Cyclopenta(c,d)pyrene	CPP	ND
benzo(a) anthracene	BaA	ND
chrysene	CHR	ND
benzo(b)fluoranthene-benzo(j)fluorenthene	BbF-BjF	ND
benzo(k)fluoranthene	BkF	ND
benzo(e)pyrene	BeP	ND
benzo(a)pyrene	BaP	ND
INDENO(1-2,3-cd)PYRENE	IPY	ND
DIBENZO(ah)ANTHRACENE	DBA	ND
BENZO(ghi)PERYLENE	BPE	ND

Note: ND: Not Detected

Analysis performed on 06.07.2021

		7CT2	7CT4	7CT6	7CT8	7CT10
MASS		12.2419	12.4046	12.7222	12.4349	12.0269
VOLUME		10	10	10	10	10
DILUTION						

		CONCENTRATION/PPM				
Parameter	**Abbreviation**					
NAPHTHALENE	NAP	0.04964	ND	0.09974	0.08957	ND
2-Methylnaphthalene	2-MNP	0.15405	0.07954	0.36088	0.33507	0.04127
ACENAPHTHYLENE	ANY	ND	ND	ND	ND	ND
ACENAPHTHENE	ANA	ND	ND	ND	ND	ND
FLUORENE	FLU	ND	ND	ND	ND	ND
Phenanthrene	PHE	ND	ND	ND	ND	ND
ANTHRACENE	ANT	ND	ND	ND	ND	ND
Fluoranthene	FLT	ND	ND	ND	ND	ND
Pyrene	PYR	0.03175	ND	0.13787	0.10267	ND
Cyclopenta(c,d)pyrene	CPP	ND	ND	ND	ND	ND
Benzo(a) anthracene	BaA	ND	ND	ND	ND	ND
Chrysene	CHR	ND	ND	ND	ND	ND
Benzo(b)fluoranthene-benzo(j)fluorenthene	BbF-BjF	ND	ND	ND	ND	ND
Benzo(k)fluoranthene	BkF	ND	ND	ND	ND	ND
Benzo(e)pyrene	BeP	ND	ND	ND	ND	ND
Benzo(a)pyrene	BaP	ND	ND	ND	ND	ND
INDENO(1-2,3-cd)PYRENE	IPY	ND	ND	ND	ND	ND
DIBENZO(ah)ANTHRACENE	DBA	ND	ND	ND	ND	ND
BENZO(ghi)PERYLENE	BPE	ND	ND	ND	ND	ND

Analysis performed on 13.07.2021

		14CT2	14CT4	14CT6	14CT8	14CT10
MASS		12.8742	12.2981	12.4104	12.4092	12.4351
VOLUME		10	10	10	10	10
DILUTION		5	1	1	1	1

		CONCENTRATION/PPM				
Parameter	**Abbreviation**					
NAPHTHALENE	NAP	ND	ND	ND	ND	ND
2-Methylnaphthalene	2-MNP	ND	ND	ND	ND	ND
ACENAPHTHYLENE	ANY	ND	ND	ND	ND	ND
ACENAPHTHENE	ANA	ND	ND	ND	ND	ND
FLUORENE	FLU	ND	ND	ND	ND	ND
Phenanthrene	PHE	ND	ND	ND	ND	ND
ANTHRACENE	ANT	ND	ND	ND	ND	ND
Fluoranthene	FLT	ND	ND	ND	ND	ND
Pyrene	PYR	ND	ND	ND	ND	ND
Cyclopenta(c,d)pyrene	CPP	ND	ND	ND	ND	ND
Benzo(a) anthracene	BaA	ND	ND	ND	ND	ND
Chrysene	CHR	ND	ND	ND	ND	ND
Benzo(b)fluoranthene-benzo(j)fluorenthene	BbF-BjF	ND	ND	ND	ND	ND
Benzo(k)fluoranthene	BkF	ND	ND	ND	ND	ND
Benzo(e)pyrene	BeP	ND	ND	ND	ND	ND
Benzo(a)pyrene	BaP	ND	ND	ND	ND	ND
INDENO(1-2,3-cd)PYRENE	IPY	ND	ND	ND	ND	ND
DIBENZO(ah)ANTHRACENE	DBA	ND	ND	ND	ND	ND
BENZO(ghi)PERYLENE	BPE	ND	ND	ND	ND	ND

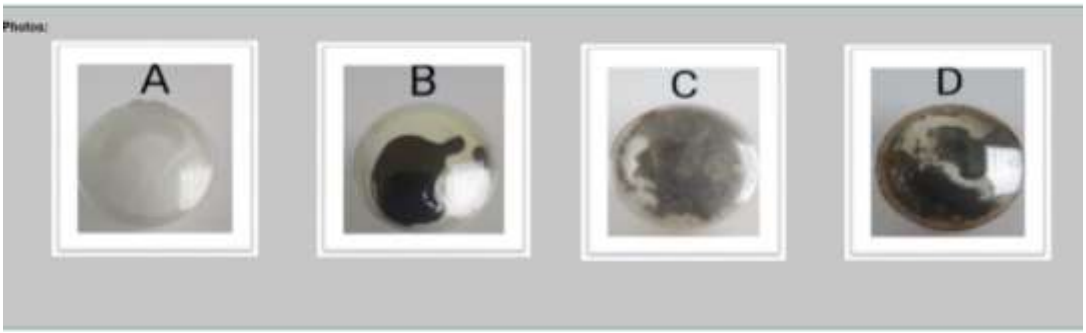
Analysis performed on 20.07.2021

		21CT2	21CT4	21CT6	21CT8	21CT10
MASS		12.003	12.007	12.0868	12.0121	12.0494
VOLUME		10	10	10	10	10

		CONCENTRATION/PPM				
Parameter	**Abbreviation**					
NAPHTHALENE	NAP	ND	ND	ND	ND	ND
2-Methylnaphthalene	2-MNP	ND	ND	ND	ND	ND
ACENAPHTHYLENE	ANY	ND	ND	ND	ND	ND
ACENAPHTHENE	ANA	ND	ND	ND	ND	ND
FLUORENE	FLU	ND	ND	ND	ND	ND
Phenanthrene	PHE	ND	ND	ND	ND	ND
ANTHRACENE	ANT	ND	ND	ND	ND	ND
Fluoranthene	FLT	ND	ND	ND	ND	ND
Pyrene	PYR	ND	ND	ND	ND	ND
Cyclopenta(c,d)pyrene	CPP	ND	ND	ND	ND	ND
Benzo(a) anthracene	BaA	ND	ND	ND	ND	ND
Chrysene	CHR	ND	ND	ND	ND	ND
Benzo(b)fluoranthene-benzo(j)fluorenthene	BbF-BjF	ND	ND	ND	ND	ND
Benzo(k)fluoranthene	BkF	ND	ND	ND	ND	ND
Benzo(e)pyrene	BeP	ND	ND	ND	ND	ND
Benzo(a)pyrene	BaP	ND	ND	ND	ND	ND
INDENO(1-2,3-cd)PYRENE	IPY	ND	ND	ND	ND	ND
DIBENZO(ah)ANTHRACENE	DBA	ND	ND	ND	ND	ND
BENZO(ghi)PERYLENE	BPE	ND	ND	ND	ND	ND

Testing viability of bioremediant on diesel used in laboratory conditions, kept at room temperature

A 30 ml Tsh + 30 ml Diesel	B 20 ml Ts Th + 20 ml CSL + 20 ml Diesel	C 23 CSL + 23 Diesel	D 30 ml Ts Th + 30 m CSL



Experimental protocol, as per original scenario 3 where no hydrocarbons found in our sediment samples
Addition of commercially available diesel in a petri dish of biological agents

A. 30 ml TsTh + 30ml Diesel
B. 20 ml TsTh + 20ml CSL + 20ml Diesel
C. 23ml CSL + 23ml Diesel
D. 30ml TsTh + 30ml CSL

14/7/21

Rhizotron set up for optimal dosage to use after 7 days

7g TsTh/CSL top and side view

5g TsTh/CSL top and side view

There was no visible growth in the 3g and 1g rhizotrons after 1 week.

21/7/21

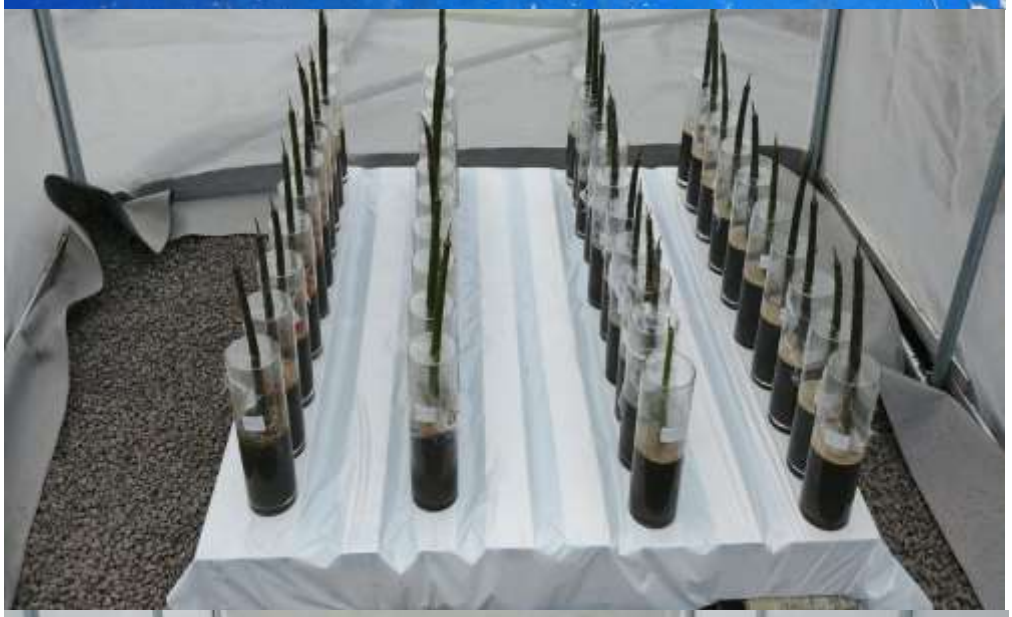
experimental branches (10 rhizotrons per branch) :

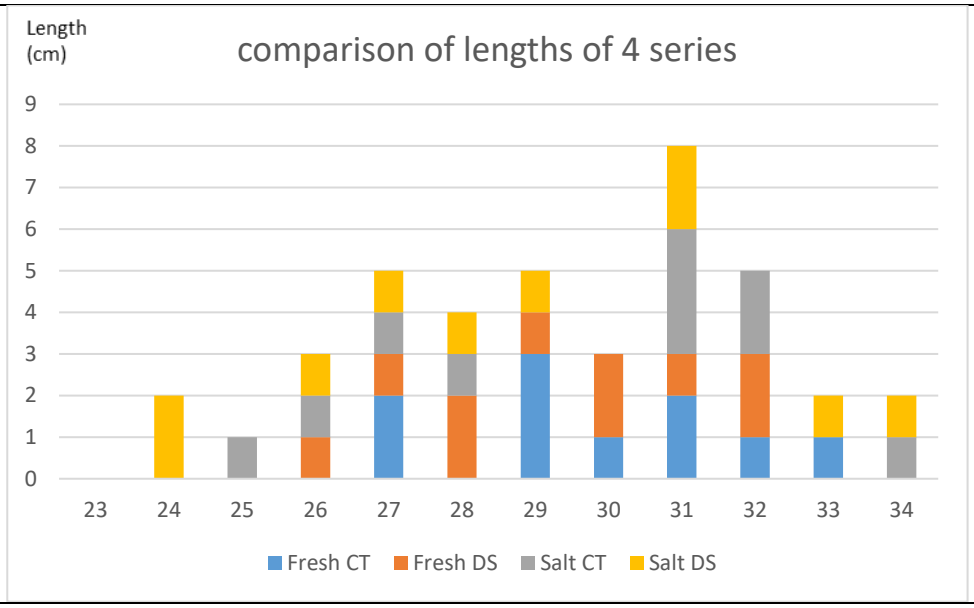
Nomenclature: contents:

	Trichoderma (g)	CSL (g)	Water (mL)	Diesel (g)	Salt (g)
RhCTSa	150	150	1500	15	30
RhCTFr	150	150	1500	15	0
RhDsSa	0	0	1500	15	30
RhDsFr	0	0	1500	15	0

procedure:

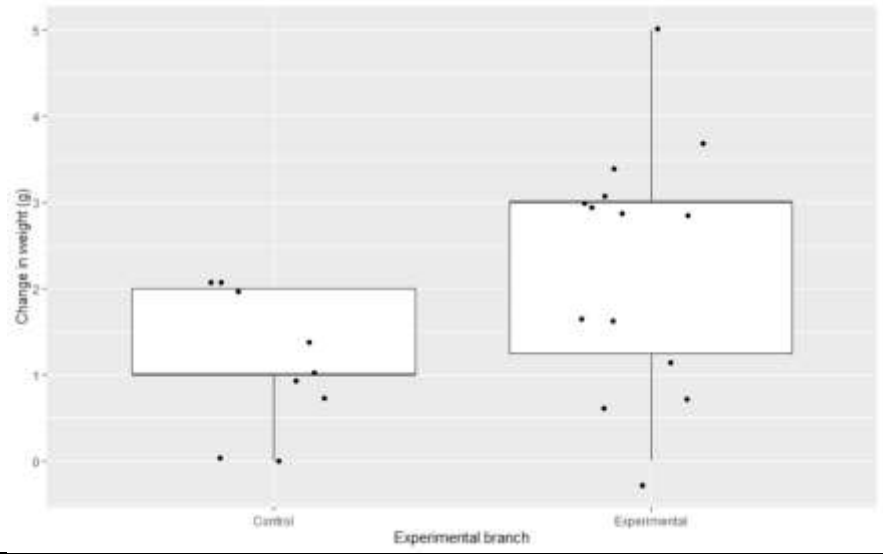
Step 1	Mix CSL & Diesel required
step 2	Divide into equal parts for each experimental branch
Step 3	Add each mix to CT labelled rhizotron
step 4	Add trichoderma required
step 5	Add water +/- salt
step 6	Cling film wrap and cut hole
step 7	Measure mangrove details
step 8	Insert mangrove root 5cm in sediment

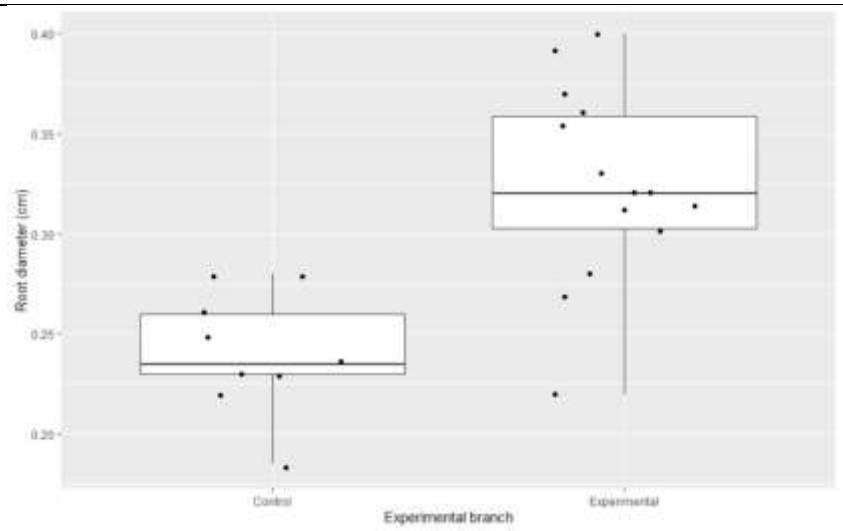
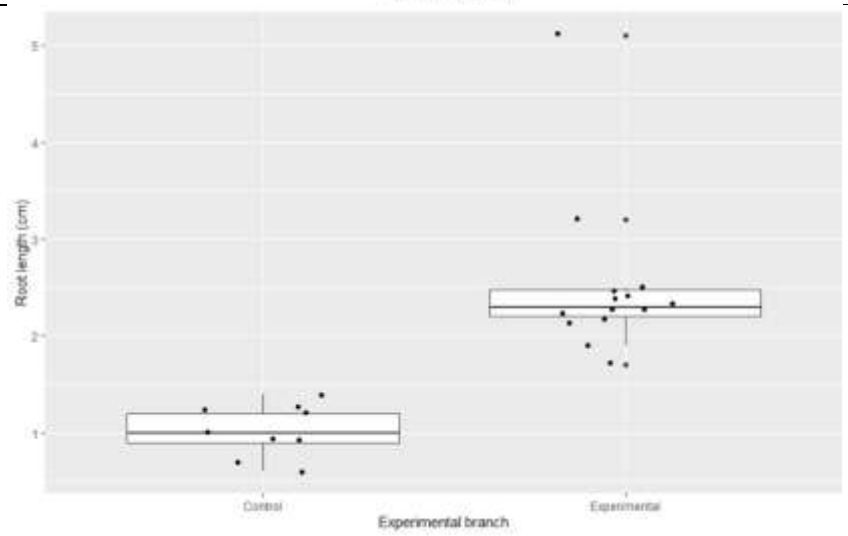
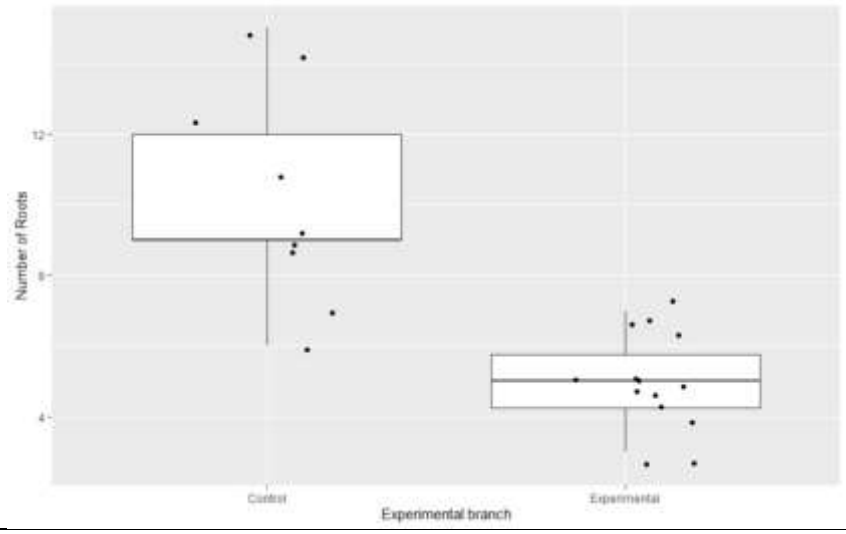


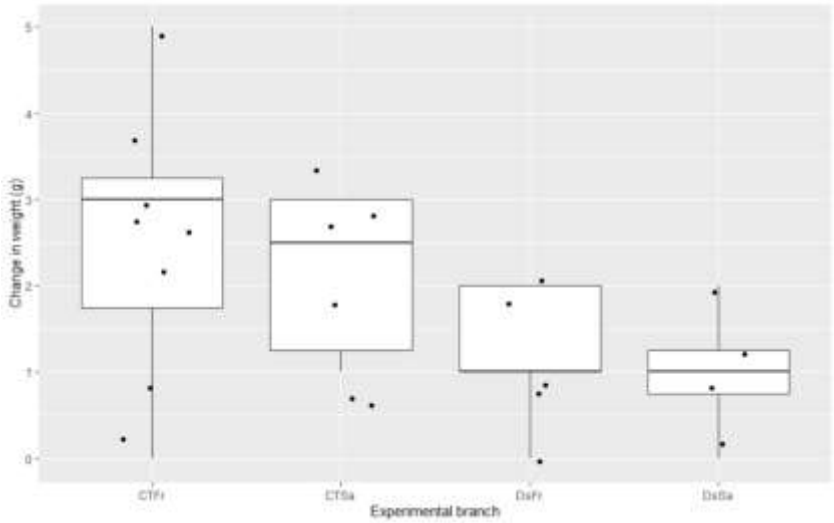
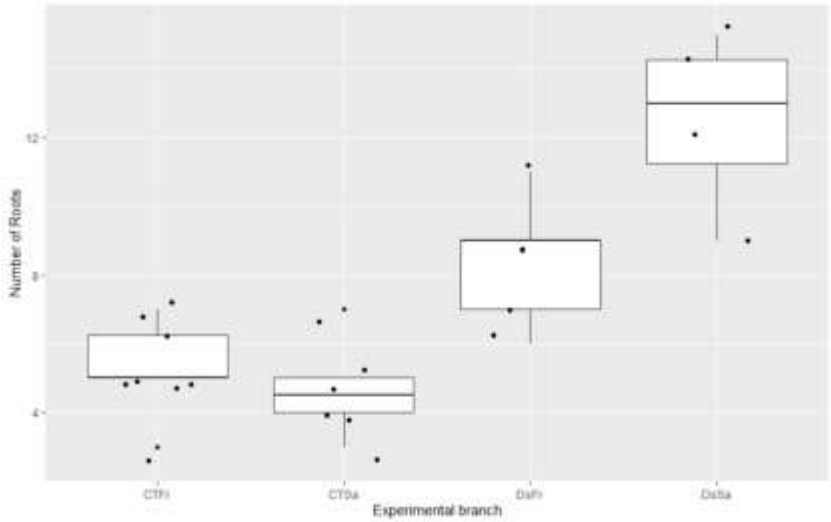
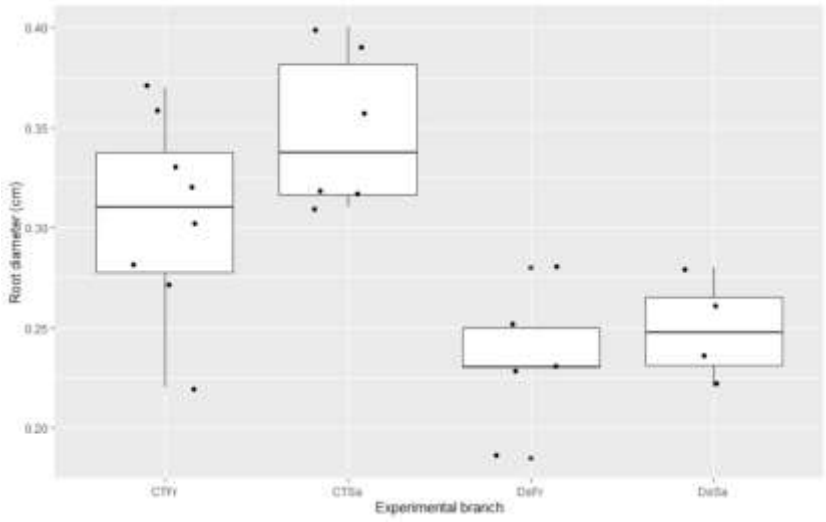


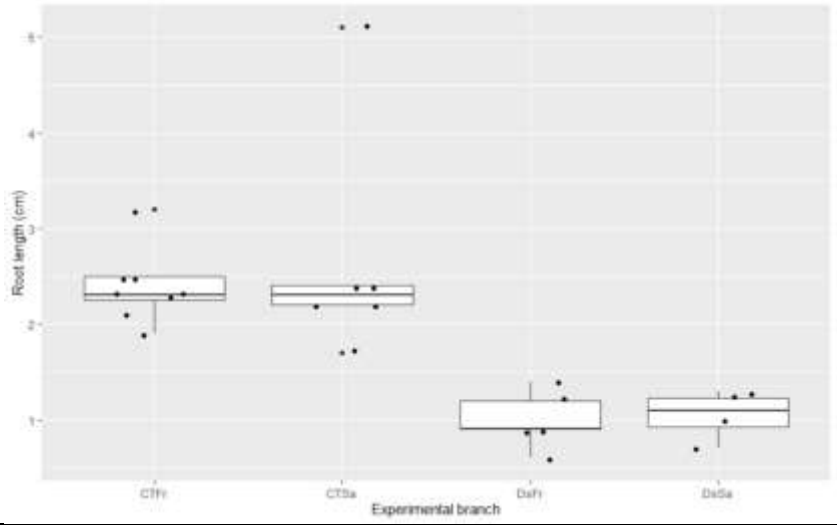
7/11/2021

Removal of propagules from Rhizotrons and measurements

		
16/11/2021	Statistical analysis of measurements – using R Script Note: 17 propagules did not survive; control = no additive, experimental = TsTh+ CSL Initial data was proven to be normally distributed, hence results are a true reflection of experimental differences	
		Average change in weight (g) is superior in experimental branch (p=0.0148, statistically significant)

		Average root diameter (cm) was superior in experimental branch (p = 0.0001, statistical significance reached)
		Average root length (cm) was superior in experimental branch (p<0.05, statistically significant)
		Average number of roots was higher in control branch, indicative of poor health (p<0.05, statistically significant)

	  	Further details with additional breakdown of fresh and salty water. Due to the propagule deaths, the end number was too small to carry out ANOVA analysis.



R Script code

```
# boxplot Rhizotron number of roots
rn <- ggplot(Mangrove, aes(Rhizotron,Number.of.roots)) +
  geom_boxplot()
brn <- rn + geom_jitter(shape=16, position=position_jitter(0.2))
print(brn + labs(y="Number of Roots", x = "Experimental branch"))

# boxplot Rhizotron root length
rl <- ggplot(Mangrove, aes(Rhizotron,Root.length..cm.)) +
  geom_boxplot()
brl <- rl + geom_jitter(shape=16, position=position_jitter(0.2))
print(brl + labs(y="Root length (cm)", x = "Experimental branch"))

# boxplot Rhizotron root diameter
rd <- ggplot(Mangrove, aes(Rhizotron,Root.diameter..cm.)) +
  geom_boxplot()
brd <- rd + geom_jitter(shape=16, position=position_jitter(0.2))
print(brd + labs(y="Root diameter (cm)", x = "Experimental branch"))

# boxplot Rhizotron change in weight
pw <- ggplot(Mangrove, aes(Rhizotron,Change.in.weight..g.)) +
  geom_boxplot()
bpw <- pw + geom_jitter(shape=16, position=position_jitter(0.2))
print(bpw + labs(y="Change in weight (g)", x = "Experimental branch"))


# boxplot setup number of roots
srn <- ggplot(Mangrove, aes(Setup,Number.of.roots)) +
  geom_boxplot()
sern <- rn + geom_jitter(shape=16, position=position_jitter(0.2))
print(sern + labs(y="Number of Roots", x = "Experimental branch"))

# boxplot setup root length
srl <- ggplot(Mangrove, aes(Setup,Root.length..cm.)) +
  geom_boxplot()
serl <- srl + geom_jitter(shape=16, position=position_jitter(0.2))
print(serl + labs(y="Root length (cm)", x = "Experimental branch"))

# boxplot setup root diameter
srd <- ggplot(Mangrove, aes(Setup,Root.diameter..cm.)) +
  geom_boxplot()
serd <- srd + geom_jitter(shape=16, position=position_jitter(0.2))
print(serd + labs(y="Root diameter (cm)", x = "Experimental branch"))

# boxplot setup change in weight
sw <- ggplot(Mangrove, aes(Setup,Change.in.weight..g.)) +
  geom_boxplot()
sew <- sw + geom_jitter(shape=16, position=position_jitter(0.2))
print(sew + labs(y="Change in weight (g)", x = "Experimental branch"))
```

Analysis appendix attached to submission

Key Deliverables	Status
15 sediment samples from polluted areas	Achieved 05/03/2021

Usable TsTh	Purchased 3/2/21, incubated 19/4/21 and used 22/4/21
Usable CSL	Purchased 2/2/21, delivered 19/2/21, used 22/4/21
Analysis of day zero samples (pre-lockdown)	Processed 5/3/21, results obtained 19/3/21
Analysis of new day zero samples (post-red zone)	Processed 23/4/21, results on 3/6/2021
Nursery facilities set up for mangrove	Purchased 20/5/21, partially built at 30/6/21, final phase pending
Obtain mangrove seedlings	Permissions sought from ministry on 4/6/21 in view of sanitary curfew situation, awaited
Analysis of phase 2 results and ascertainment of optimal dose	Established that CSL and TsTh are compatible bioremediants (29/6/21), PAH results set up and awaited in milestone 3
Protocol for nursery trial with mangrove seedlings	Rhizotron sourced and purchased, protocol finalised at 11/06 team meeting
Mangrove propagules collection	Started and completed on 7/7
Experimental set up and data collection	Started 14/7 and completed 7/11
Analysis of final phase 2 results	Confirmation that the bioremediant combination is effective against Diesel, phase completed
Analysis of phase 3 results	Completed 16/11 and included in report

4. PROBLEMS ENCOUNTERED

A brief account of the problems encountered to achieve the set objectives as well as for any delay and proposed remedial measures.

- First set date for collection postponed due to delays in disbursement in funds from MRIC and inability to purchase protective equipment in timely manner, despite daily phone calls to the MRIC office. As the field work was entirely dependent on low tides and the restricted hours imposed by the terms of our government issued permit, we could only reschedule for 2 weeks later to include the opportunity for a final pre-collection site visit.
    - Remedial measures proposed: use of speedy electronic bank transfer facilities instead of cheques for disbursement of funds to reduce delays in signing and subsequent need to obtain cheque and clearing of cheque from bank
- Courier companies caused delays in delivering imported raw material despite receiving written confirmation from the relevant authorities that no permit was required.
    - Suggest MRIC put this courier company (FedeX) on a blacklist and share information on the incompetence of this company when other researchers need to import permit-free raw materials.
- Covid-19 pandemic disrupted our schedule by a whole month, as the laboratory was in the Red Zone and there were delays in obtaining a WAP from the authorities. As

MRIC officers were working from home, it was very difficult to obtain additional assistance in speeding up the process.

- As a team, we went back to other additional scientific literature available to look for a revised protocol to be able to shorten the turnaround time without compromising results, and found a suitable alternative protocol.

- Access to shores was restricted, delaying collection of mangrove propagules as it stands. Additional time was requested.

- The Covid-19 pandemic disruption to our schedule by a whole month meant that the collected samples were compromised, with no testable PAH remaining in our samples. Delays in payment also meant that we might have unnecessarily tested samples
    - For milestone 2 we negotiated with the SGS lab to release the results even before payment from the funders, as the funds were guaranteed to be available, and the lab agreed to this condition.

Our initial target date was end of October, beyond which the research team could not fully commit to the project going forward. Our request for additional stipend without altering the final budget to ensure the time commitment was rejected by the project management committee. As such, further delays were foreseeable and inevitable.

5. FINANCIAL REPORT

MRIC funds disbursed: Rs112000 on 19th Feb 2021, Rs446,546 on 25th May 2021, : Rs186,340 on 22nd September 2021, and R75,176 on 3rd May 2022, totalling Rs820,062

Company loan from Genius Dr Ltd for running costs: Rs11858.25

Expenditures:

1. Laboratory fees for SGS Ltd: storage plus testing – Rs 80000 + Rs203,544 +Rs 98624 = Rs382168
2. Laboratory fees for Mist and Spray Ltd - Rs48275 + Rs1725 = Rs 50000
3. Purchase of consumables = Rs11862.25 + Rs1651 = Rs13513.25
4. Purchase of equipment = Rs19087 + Rs 3087 + Rs2700 = Rs24874
5. Nursery cost = Rs28112.35
6. Travelling = Rs 11684 +Rs7894 + Rs600 = Rs20178
7. Staff salary – Rs 210000
    a. Project leader – Rs75000
    b. Project collaborator – Rs 75000
    c. Project assistant – Rs60000

Total expenditures = Rs728, 845.60 + Rs11858.25 = Rs740703.85