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Experiment 5
ESM 219L
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Biodegradation of Toluene and Effects of Nutrient Addition and Bioaugmentation

Introduction

Petroleum hydrocarbon (PHCs), specified contaminants compounded in fuel, pollution is a common form of environmental pollution (Puntas, 2017). The leading cause of PHC pollution is caused by leaking underground fuel tanks (LUFTs). Although not classified as a hazardous material by nature, LUFTs require immediate attention from state and federal agencies due to the negative environmental impact that can incur if left unmediated. PHCs negative effects include contamination of soil resulting in contaminated ground water or reduced utility of land for agriculture or other development purposes (CDC, 1999). For this reason, PHCs have been broken down by individual contaminants and classified as constituents of gasoline/fuel in order to further research contamination remediation as well as establish government protocols and regulations. Toluene, an identified contaminant within gasoline, is known to have biodegradable characteristics when introduced to microbial communities in soil and water. Biodegradation of Toluene is limited by volume and time, wherein high concentrations at the source of the spill can be toxic to microbial organisms. Limitations of biodegradation of Toluene at the source of the spill further constrain biodegradation rates due to low-oxygen levels. High Oxygen concentrations are incumbent for microbial aerobic biodegradation, as the Oxygen operates as terminal electron acceptors (TEA). Furthermore, Toluene is classified as a volatile organic compound (VOC), which diffuses rapidly in the presence of air allowing for shorter lag time between spill and biodegradation.

Method

For this experiment, the biodegradation of Toluene was measured over time through Carbon mineralization. Toluene biodegradation will be observed through the inoculation of a toluene degrading bacterium (bioaugmentation), addition of nutrients, and toluene concentrations.

Toluene biodegradation will be studied as a function of several treatments: inoculation with a toluene degrading bacterium (bioaugmentation), addition of nutrients, and two toluene concentrations. Toluene treatments were monitored over time

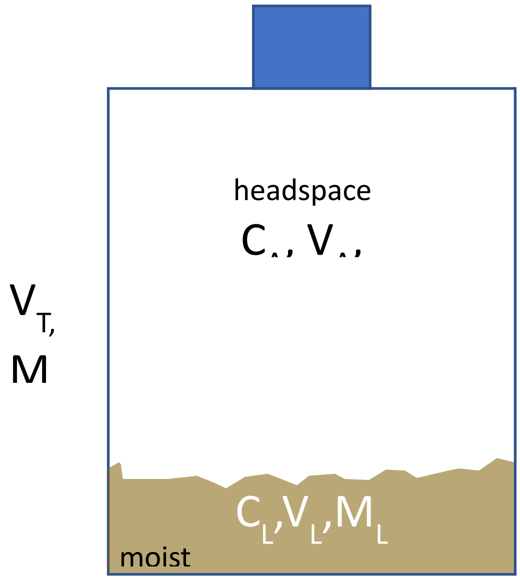
Toluene concentration in the test bottle headspace will be monitored over time to assess the effect of treatments on the process. Sterile controls will be from sterilized (autoclaved: 121°C,1 hr; repeat 3 times with a few days in between) soil. Below, is a breakdown of preparation steps:

1. In each treatment bottle, weigh 10 g (ODE) of soil. NOTE: the soil is assumed to have a 20% moisture content (g water / g ODE soil), and so12 grams of moist soil was weighed into the bottle.

2. In each sterile control, weigh 10 g of (ODE) sterile soil: NOTE: use aseptic technique so that soil is not contaminated in the process.

3. The inoculum solution was prepared by suspending a generous loop (flame-sterilized) of *P. putida* into 10 mL of sterile ½ 21C nutrient solution in a 15 mL blue capped tube. The bottle was capped and vortexed to mix pellet completely.

4. 5 mL of nutrients, water or *P. putida* suspension was added per treatment specifications (see table below). All bottles were placed on a roller to mix completely.



5. Using the gas tight syringes, the toluene was then transferred through the Mininert caps into the bottles to result in aqueous phase concentrations of 100 mg/L (Low/8.7 µL) or 200 mg/L (High/17 µL). Note: When toluene is added to a closed bottle with soil and soil water, the toluene will distribute between the gas phase and the aqueous phase, reaching equilibrium. We determined how much toluene to add to have a desired aqueous phase concentration, by calculating the distribution of toluene mass at equilibrium. This is called performing a mass balance. Review **appendix 1** for mass balance calculations.

6. 15 minutes on a roller was allowed for complete equilibration. The bottles were then incubated at room temperature (25 C) in the dark. It is important to note that simulating a nocturnal environment assists in limiting photodegradation of the Toluene to ensure unadulterated results of biodegradation from the various treatments (Marques, 2016).

7. At 15 minutes (t=0) and at intervals thereafter, the toluene concentration in each bottle was measured in the headspace using a gas chromatograph.

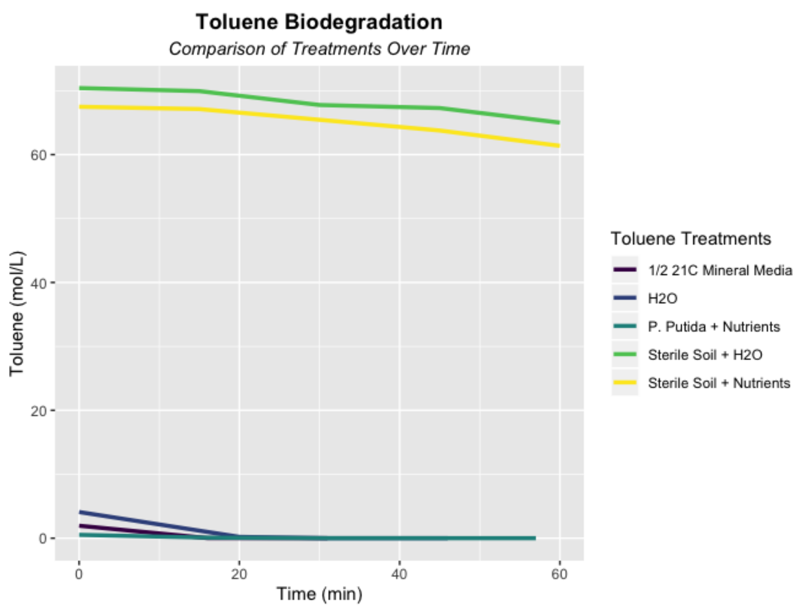
Table: Treatments in Experiment 5					
Treatment ID	Water	Nutrients	P. putida	High Toluene	Low Toluene
Soil 1	x			x	
Soil 2	x				x
Soil 3		x		x	
Soil 4		x			x
Soil 5			x	x	
Soil 6			x		x
Sterile Soil 7	x			x	
Sterile Soil 8	x				x
Sterile Soil 9		x		x	
Sterile Soil 10		x			x

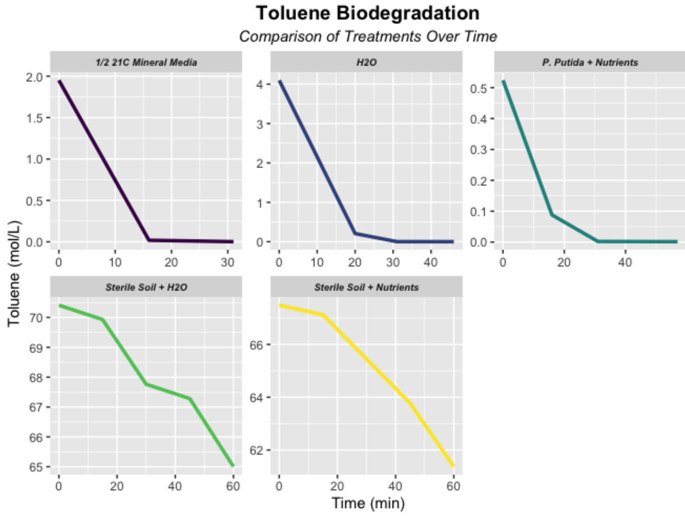
Results

The results were taken only for the high peak area and ran through the calculations below. To see the final calculations of all treatments see **appendix 2**.

$$M_{toluene} = \frac{Peak\ Area}{9653} * \frac{Density\ of\ Toluene}{Molecular\ Weight\ of\ Toluene} * 1000\ mL/L$$

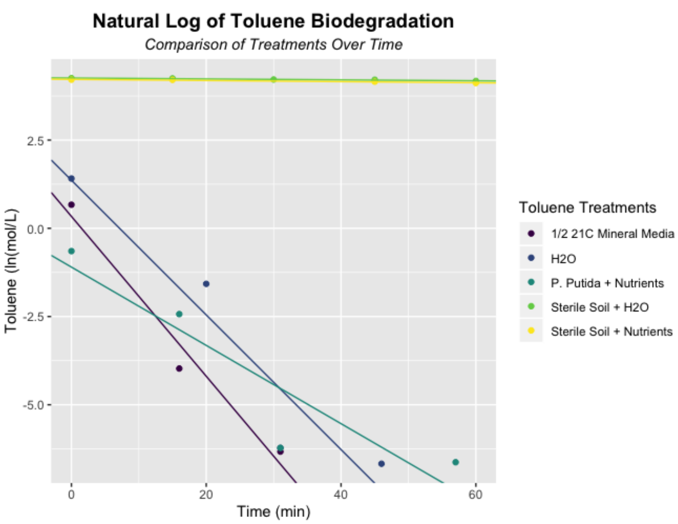
The Toluene biodegradation within the sterile soil control treatments held an extended lag time and inequivocally slow biodegradation rates. When comparing the controls to the healthy soil, there is a stark contrast in the biodegradaton rates. Furthermore, treatments with the healthy soil had infinitesminal lag times. There is minor differences in each of the healthy soil treatments depicted. For further scrutiny, a magnified view of the Toluene Treatment Levels is presented in the next section displayed below.





An in-depth review of each Toluene Treatment infers differences between the controls where the added nutrients held a greater biodegradation of 5 mol/L of Toluene and remained consistent in it's rate of biodegradation in comparison with the control water treatment. The P. Putida + Nutrients Treatment had the highest rate of biodegradation of Toluene which appears to have occurred immediately upon Toluene exposure to the treatment. The ½ 21C Mineral Media had the second highest biodegradation rate, followed by the water treatment. It appears all three of the heatlhy soils + bioaugmentation had minimal, if any, lag time in the biodegradation process.

<u>Discussion</u>



Due to the curvature kinetics of the treatments represented in the graphs depicted above, it is inferred that the reactions are following the integrated rate law of 1st order. For a zero-order rate to occur, a successive half-life must be present wherein the half-life decreases by a factor of two. Zero order rate would also mean the rate of biodegradation is independent of the concentration of the Toluene. Therefore, it is unlikely that the treatment rates are zero-order due to concentration levels of Toluene being significant to microbial biodegradation. It is possible to presume that the kinetic rate is 1st order due to the nature of the curved slope remaining relatively constant (Vallance, 2017). To prove this hypothesis, we use the integrated rate law for First Order kinetics which is applied in the graph to the left. The integrated rate law formula is as follows:

$$\ln(Concentration\ of\ Toluene) = -kt + \ln(Initial\ concentration\ of\ Toluene)$$

The purpose of the sterile controls was to emphasize differentiation in Toluene biodegradation when bioaugmentation and/or healthy soil is present. As noted in the results, Toluene biodegradation is severely limited under sterile conditions regardless if H_2O or nutrient additives are introduced. The sterile controls assist in the evaluation of biodegradation in healthy soil conditions and variant degrees of bioaugmentation.

Aforementioned in the results section, there is a substantial difference in the biodegradation of Toluene between sterile soils and purportedly healthy soils. Further evaluation of the healthy soil treatments distinguished bioaugmentation as increasing microbial biodegradation. The p. putida treatment was determined to have the highest rate of Toluene biodegradation. However, both the nutrient and H_2O treatment were competitive in rates of biodegradation to the p. putida treatment. What can be inferred from this experiment is that there is significant value in healthy soil and its promotion of biodegradation. Although the experiment results are useful in theoretical principles, the experiment did not account for seasonal fluctuations of temperature, Ph levels in the soil, nor diurnal environments that affect Toluene biodegradation.

Appendix 1. Calculations of Mass of Toluene Needed

Calculations of the amount of liquid phase (neat) toluene to add to bottles for establishing desired concentrations in soil water.

Assume conditions are standard pressure, and temperature is 25 °C.

Toluene is a volatile organic compound (VOC). Its formula is C_7H_8 and its Molecular Weight is 92 g/mole. Its water solubility is 518 mg/L (25 °C).

When added to a closed system (our bottle, in this case), toluene will equilibrate between the gas phase (headspace and soil gas-filled pore spaces) and soil water (aqueous phase). We use Henry's Law to relate the gas and aqueous phase concentrations, so that we can predict the distribution of toluene and determine the amount of total toluene to be added in order to achieve a desired concentration in soil water.

See Sander (2015) for a compilation of H_L values for toluene. These are for 298 °K or 25 °C. Values can be expressed as dimensionless. In this case, for toluene, the gas/water value is assumed to be 0.29.

When toluene is added to a closed bottle with soil and soil water, the toluene will distribute between the gas phase and the aqueous phase, reaching equilibrium. If we want to determine how much toluene to add such that we have the desired aqueous phase concentrations, then we must calculate the distribution of toluene mass at equilibrium. This is called performing a mass balance.

First inventory what will be in the bottles: 10 g soil (ODE), 5 mL of solution (water, bacterial suspension, or nutrients). Assume that the soil is 20% moisture (gravimetric, so g water / g ODE soil). This means that the moist soil needed is 12 g, and that there are 2 g or 2 mL of water associated with the soil. The total moisture in the bottle is thus 7 mL (sum of the soil water and the aqueous phase amendment for each of the treatments).

We have a total bottle volume of 250 mL, but some volume—besides being occupied by water—is also occupied by the soil. If we assume that the soil solid phase density is 1.4 g / mL, then the volume occupied by the soil is 7.1 mL. Thus, the gas phase in the bottle (not occupied by water or by soil solids) is: 250 mL – 7.1 mL – 7 mL = approximately 236 mL.

Now, to calculate the mass of toluene to be added to reach a concentration of 200 mg/L in the aqueous phase, first calculate the mass in the aqueous phase: 200 mg/L * 0.007 L = 1.4 mg.

Now, this mass in the liquid is in equilibrium with the mass in the air in the bottle. This means that the total mass in the bottle is the sum of the mass in the liquid plus the mass in the air:

$M_T = M_A + M_L$ and M_L is related to M_A by H_L such that $(M_A/V_A) / (M_L/V_L) = H_L = C_A/C_L$
Or, rearranging and expressing the mass balance:
$M_T = C_AV_A + C_LV_L = (H_L*C_L)V_A + C_LV_L = C_L(H_LV_A + V_L)$

Now, we can substitute values and calculate:
M_T = 200 mg/L (0.29*0.236L + 0.007L) = 15 mg = (15/1000) g = 0.015 g

Now, assume that the density of toluene is 0.86 g/mL.

Therefore, the volume of liquid toluene to be added to the closed bottle is:
(0.015 g / (0.86 g/mL)) = 0.017 mL = (0.017 mL) * (1000 µL / mL) = 17 µL

Performing the same overall calculation for **100 mg/L** final concentration in the aqueous phase of the soil yields half that, or 8.7 µL.

Appendix 2. *Calculations of Peak Area to mol/L*

Treatment	Time	Peak Area	Toluene (mL)	Toluene (g)	Toluene (mol), per ml	Toluene (mol/L)	ln(Toluene (mol/L))
Toluene + H2O	0	4204.6508	0.4355797	0.37760404	0.00409816	4.098155394	1.410536969
Toluene + H2O	20	212.5594	0.02202004	0.01908917	0.00020718	0.207175695	-1.574188078
Toluene + H2O	31	2.0312	0.00021042	0.00018242	1.98E-06	0.001979754	-6.224782684
Toluene + H2O	46	1.2988	0.00013455	0.00011664	1.27E-06	0.001265904	-6.671968788
Toluene + 1/2 21C mineral media (nutrients)	0	2001.5316	0.20734814	0.1797501	0.00195084	1.950836802	0.66825841
Toluene + 1/2 21C mineral media (nutrients)	16	19.2944	0.0019988	0.00173276	1.88E-05	0.018805711	-3.973594679
Toluene + 1/2 21C mineral media (nutrients)	31	1.8308	0.00018966	0.00016442	1.78E-06	0.001784429	-6.328656803
Toluene + p. putida + nutrients	0	537.5188	0.05568412	0.04827256	0.00052391	0.523904522	-0.646445821
Toluene + p. putida + nutrients	16	90.2054	0.00934481	0.00810101	8.79E-05	0.087920677	-2.431320269
Toluene + p. putida + nutrients	31	2.0328	0.00021059	0.00018256	1.98E-06	0.001981313	-6.223995523
Toluene + p. putida + nutrients	57	1.3562	0.0001405	0.0001218	1.32E-06	0.00132185	-6.628723008
Toluene + sterile soil + H2O	0	72240.0876	7.4836929	6.48761338	0.07041039	70.41039047	4.254340844
Toluene + sterile soil + H2O	15	71748.8224	7.43280041	6.44349468	0.06993157	69.93156804	4.247517164
Toluene + sterile soil + H2O	30	69524.7116	7.20239424	6.24375557	0.06776379	67.76378952	4.216027974
Toluene + sterile soil + H2O	45	69030.206	7.15116606	6.19934586	0.06728181	67.28180876	4.208889899
Toluene + sterile soil + H2O	60	66693.6252	6.90910859	5.98950624	0.06500441	65.00440889	4.174455097
Toluene + sterile soil + nutrients	0	69239.8092	7.17287985	6.21816954	0.0674861	67.48610313	4.211921697
Toluene + sterile soil + nutrients	15	68871.2864	7.13470283	6.18507388	0.06712691	67.12691428	4.206585071
Toluene + sterile soil + nutrients	45	65428.5735	6.77805589	5.87589665	0.0637714	63.77139842	4.155304789
Toluene + sterile soil + nutrients	60	62966.6188	6.52301034	5.65479766	0.0613718	61.37180012	4.116950448

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