Experiment 1: Fungal Growth Observation in Vactor Waste

Purpose: To observe the growth of 8 species of fungi in the presence of "vactor waste" (storm drain sludge) from the City of Spokane's decant facility. Analytical testing of components of Vactor Waste and media, including PCBs, will be done before and after fungal growth.

Overview: Each strain of fungi will be acclimated to digest the components of vactor waste by introducing them to a small amount of sterilized vactor waste in malt agar, on petri dishes (2 generations). Each generation (growth on one petri dish) will take roughly 1-2 weeks, depending on the fungal strain, and the strain's reaction to the VW. Then these 'acclimated' fungal cultures will be transfered to jars of sterilized rye grain and incubated for 3-5 weeks, then to pasteurized alder sawdust and incubated for 5-8 weeks. After sawdust jars are colonized we will use this 'sawdust spawn' to create various ratios of raw vactor waste, pasteurized sawdust, and sawdust spawn. We will incubate the ratio jars and visually observe fungal growth over a minimum of 8 weeks before sending samples to be analyzed for PCB degradation.

Approach/ reasoning: Each selected species shows promising traits for PCB breakdown. We aim to learn if they will survive and grow in the presence of vactor waste, which contains a multitude of substances/ pollutants including PCBs. Research suggests that fungi can potentially be 'trained' to digest certain substrates if introduced to them in small amounts at an early growth stage. *(G.F. Leatham, T.J. Griffin "Adapting liquid spawn Lentinus edodes to oak wood" Applied Microbiology and Biotechnology nov. 1984) (Growing Gourmet and Medicinal Mushrooms, Paul Stamets, pg 142)* This can trigger the production of enzymes that are effective at digesting the specific food source. Introducing a small amount of sterilized vactor waste to the cultured fungi in petri dishes at the beginning could give them a better chance of survival when mixed with large amounts of vactor waste later in the experiment. Observing mycelial growth in jars of various ratios of vactor waste and sawdust will be very informative to our long term goal of digesting PCBs in vactor waste.

Task 1: Collect Vactor waste (Heidi, Aimee, Adrianne, Jeff)

-rinse cement mixer with water
- use clean garden shovel to scoop vactor waste into cement mixer (rented from A to Z Rentals)
-run cement mixer to homogenize thoroughly
-use shovel to break up clods, mix as well as possible
- load homogenized VW into six 2.5-gallon glass jars by hand with nitrile gloves, and 2 quart canning jars.
- transport all jars to City of Spokane lab, (Riverside facility on Aubrey White Parkway) put large jars in fridge, autoclave 2 quart jars for 2 hours.

Task 2: train fungi to sterilized vactor waste

Step A: Make VW spiked petri dishes

1. Make VW spiked agar mixture in small batches (Taylors recommendation)
   Use the following recipe in each glass juice bottle, measuring and mixing dry ingredients first, then adding water:
   - 250mL water
   - 5 g malt extract
   - 5 g agar agar
   - 0.5g nutritional yeast
   - 1.05g dry VW (pulverize with mortar and pestle as fine as possible)
2. Cover each bottle mouth and scalpel with tin foil and load into pressure cooker
   sterilize bottles and scalpel in pressure cooker at 15 psi for 45 mins (following the All American Pressure Cooker/Canner Manual: [HYPERLINK "http://fantes.net/manuals/all-american-pressure-cooker-manual.pdf"])
3. pour agar mixture into petri dishes using sterile technique (described here: [HYPERLINK "http://vlab.amrita.edu/?sub=3&brch=73&sim=212&cnt=1"]) in laminar flow, swirling bottle in between each pour to suspend VW particles into media. Stack when cool and cover stack with original sleeve. Leave in laminar flow to set overnight. (24 VW spiked petri dishes for G1 (generation 1))

Step B: Make Nutrified Malt Agar petri dishes

1. follow steps in "Step 1" excluding the addition of the dry VW. Make 24 "nutag" dishes per generation.

   Nutrified malt agar recipe:
   - 1 Liter H_2O
   - 20 g agar agar

20 g malt
2 g nutritional yeast

Step C: Transfer fungi to "nut-ag" and "VW" dishes (G1)

1. in laminar flow/ sterile technique, transfer each fungal species to 3 'nut-agar' (nutrified agar), and to 3 'VW' petri dishes with stainless scalpel.
    a. On each dish carefully label the following: "species & strain", "nut-ag" OR "VW", "G1" (generation 1), "date". Number each dish in species group (6 petris total per species: nutag i, ii, iii, vw i, ii, iii)
    b. Transfer cutting (this is "G1 Day 0")
    c. tape dish shut, stack, cover stack of 10 with original sleeve.
2. Place all transferred dishes in incubation room at 75 degrees F. (48 total for G1)

Step D: Observe growth on G1 "VW" and "nut-ag" dishes

1. Record the following on days 4, 7, ~~and 11~~
    a. On each petri dish lid, outline the edges of the transferred cutting with fine tip sharpie. Using the center of cutting as the starting point, draw X and Y axis (at a 90 degree angle) to the edges of dish. Mark edge of mycelial growth at each axis (4 directions) with sharpie, measure in millimeters and record in scientific journal. (see growth chart)
    b. take photo with ruler in frame, for visual documentation.
    c. note visual observations in scientific journal (texture, density of mycelium, thin or thick, etc)
2. prepare to transfer before mycelium reaches edge of dish (taking a cutting of the actively growing outer edge of mycelium is ideal; growth slows after it reaches the edge of its food source)

Step E: Transfer fungi to 2nd "nut-ag" and "VW" dishes (G2)

1. For each species: choose 1 "nutag" dish and 1 "VW" dish to transfer that meets the following criteria:
    a. no contamination (no visible mold, bacteria, etc)
    b. fastest growth in category (ex: TVblack nutag i, ii, or iii)
    c. preferably, mycelium has not reached edge of dish
2. Transfer this 'VW' culture to 3 new 'VW' petri dishes in laminar flow using sterile technique. Transfer this 'nutag' culture to 3 new 'nutag' dishes in laminar flow using sterile technique.
    a. On each dish carefully label the following: "species & strain", "nut-ag" (i, ii, iii) OR "VW" (i, ii, iii), "G2" (generation 2), "date"
    b. Transfer cutting using sterile technique (this is "G2 Day 0")
    c. Tape dish shut, stack, cover stack with original sleeve.
3. Place all transferred dishes in incubation room at 75 degrees F. (48 total for G2)

Step F: Observe growth on G2 "VW" and "nut-ag" dishes

1. Record the following on days 2, 4, and 6 (amended based on speed of growth G1)
    a. On each petri dish lid, outline the edges of the transferred cutting with fine tip sharpie. Using the center of cutting as the starting point, draw X and Y axis (at 90 degree angle) to the edges of dish. Mark outer edge of mycelial growth along each axis (4 directions), measure in millimeters and record in scientific journal
    b. take photo with ruler in frame, for visual documentation
    c. note any visual observations in scientific journal (texture, density of mycelium, thin or thick, etc)
2. prepare to transfer before mycelium reaches edge of dish

'Training' to vactor waste treatment groups:

Species:	**Group A:** **Agar+Fungus**	**Group B:** **Agar+ Vactor waste+ Fungus**	**Species totals:**
Coprinus comatus h.crk			**6**
Grifola frondosa			**6**
Lentinula edodes			**6**
P. ostreatus brat			**6**
P. ostreatus columbinus			**6**
Pleurotus(sp?) CA creek			**6**
Stropharia r-annulata			**6**
Trametes versicolor			**6**
Group Totals:	**24 per gen**	**24 per generation**	
48 petri dishes per generation, 96 total for 2 generations			

Task 3: Grow Acclimated cultures on grain; "Grain Spawn"

Step A: hydrate grain (June 27th)

1. in large pot, submerge 16.5 cups organic rye grain in water, soak overnight (2 jars x 8 species= 16 jars. 1 cup dry grain per jar. Plus 0.5 cups for sample jar)

Step B: sterilize grain (June 28th)

2. following day, bring pot with grains to a boil and stir for 5 mins
3. strain hot grains through colander
4. load 2 cups hot grain into each quart jar (it expands when hydrated) and a bit into the half pint jar. Close each jar with filter patch lid (drilled hole covered in two layers of cloth athletic tape)
5. cover lids loosely with square of aluminum foil, load into pressure cooker
6. Sterilize the 17 grain jars (+ scalpel wrapped in foil) for 60-75 mins at 15 PSI in pressure cooker.
7. Turn off heat, allow to cool overnight in pressure cooker.

Step C: transfer cultures from VW petris to grain (Wed, June 29th)

1. The following day, open pressure cooker inside lab room. (Set aside the one small jar for analytical testing.)
2. For each species, select one "VW" dish that meets the following criteria:
    a. fastest growth (use growth measurements from Day 2, 4, & 6 to determine)
    b. no contamination (no visible mold, bacteria, etc)
3. Using sterile technique, transfer the selected 'acclimated' culture of each species with stainless scalpel from petri dish to 2 sterilized quart grain jars (16 total) in laminar flow, labeling carefully: "species", "date", "VW i/ ii/or iii". (This is Day 0.)

Step D: Incubate/ monitor

1. incubate at 75 degrees F. in incubation room, allowing colonization
2. Record the following on days 6, 12, 19, 26, 33 (1x per week) in chart*
    a. Quantify approximate percentage of visible mycelial growth on the following areas of jar:
        i. Upper surface of grain
        ii. Grain against vertical sides of jars
        iii. Grain against base of jar
    b. Quantify density of mycelium:
        i. Low- Can barely see the threads of mycelium, can see grains unobstructed
        ii. Moderate- bright white visible mycelium, threads not as well defined/ thicker mass of white, grains partially obstructed from being covered my mycelium, but can still make out individual grains
        iii. High- grain texture may be visible, but covered completely in white mycelium. Threads not individually visible, but continuous white mycelia 'mat'.
    c. take photo, for visual documentation

d. note visual observations in scientific journal (texture, density of mycelium, thin or thick, etc)
e. Note signs/ no signs of contamination

*grain jar growth measurement chart

	Jar #1					Jar #2				
	% growth on grain surface					% growth on grain surface				
Species	Upper	vertical	base	density (i, ii, iii)	notes	Upper	vertical	base	density (i, ii, iii)	notes
Pleurotus djamor										
Grifola frondosa										
Lentinula edodes										
Pleurotus ostreatus brat										
Pleurotus ostreatus columbinus										
Pleurotus wild CA creek										
Stropharia rugosoannulata										
Trametes versicolor black										

Task 4: Grow Acclimated cultures on pasteurized alder sawdust; "Sawdust Spawn"

Step A: sieve alder sawdust and retain material between sieves #4 (4.76mm) & #16 (1.18mm). (Need **9.5 gallons** total sawdust for experiment.)

Step B: Make filter lids for all 25 jars
Drill hole into the center of each autoclavable plastic jar lid, and cover hole with two layers of cloth athletic tape, placing the weave in opposite directions. This creates an effective filter allowing respiration but keeping contaminants/ competing organisms out.

Step C: hydrate 60 cups (3.75 gallons) sieved alder sawdust (7-25)
Make 3 jars x 8 species= 24 jars

- Need 2 jars (5 cups total) per species for ratios; make 3 jars (7.5 cups total) per species in case one gets contaminated.
- 2.5 cups hydrated sawdust x 24 jars= ***60 cups total hydrated sawdust***

(60 cups= 3.75 gallons)

a. Gradually add water to retained sieved sawdust while mixing with paint stir stick in clean Rubbermaid bin.
   i. Since the sawdust's initial moisture content depends on source/ ambient temp & humidity, hydration formulas/ recipes are not useful. Cultivators always achieve proper moisture content of a fungal growth substrate by feel (McCoy 2016, pg 255). Sawdust should readily clump and hold its form when squeezed in the hands but no water should drip out or be left standing on hands or in containers. Moisture is absorbed slowly: add water to sawdust in small amounts, mix thoroughly, and allow to sit for 20+ minutes before checking and adding more water if needed. (Avoid over-hydrating which can cause standing water/ mycelial suffocation/ anaerobic activity.)

Step D: Sterilize sawdust jars. (7-26)

1. Measure out and weigh 2.5 cups hydrated sawdust, record weight. (170g). Weigh out and load this amount (170g) into each quart jar (25 total, 24 for species + 1 for calculating moisture content). Close with filter lids. Cover lids in tin foil.
2. Sterilize in pressure cooker for 2 hours at 15 PSI, allow to cool overnight. (25 jars will require two rounds of pressure cooking, as only 19 fit inside.)
3. Calculate moisture content of extra jar, and record.
   a. Formula: **Moisture content (%)= (A-B)/(B-tare weight) x 100**
      i. A=mass of wet sample + weight of container
      ii. B=mass of dry sample + weight of container
      iii. Tare weight= weight of container

Step E: Transfer grain spawn to sawdust (7-27)

1. Select one grain spawn jar to transfer: TAKE PHOTOS OF EACH
   a. Fastest growth (determined by measurements in grain jar growth charts)
   b. Highest density of mycelium (determined by measurements in grain jar growth chart)
   c. No contamination (visual signs)
2. Transfer selected jar: Distribute grains evenly between 3 jars of sterilized sawdust in front of laminar flow hood, labeling carefully with species, date, VW trained. Close with filter lid, shake jar to distribute grains into sawdust as evenly as possible.
   a. incubate inoculated sawdust jars at 75 degrees F,
   b. once per week, make the following observations:
      a. take photo(s) for visual documentation

b. note visual observations in scientific journal using growth measurement chart

3. Allow to fully colonize (duration depending on strain)

Task 5: Vactor Waste Ratios

Step A: sieve alder sawdust and retain material between sieves #4 (4.76mm) & #16 (1.18mm) until retained 5.5 gallons.

1. calculate dry weight for sawdust in ratios
2. calculate dry weight for VW

Step B: hydrate 5.5 gallons sieved alder sawdust (following method in Task 4, Step C)

Step C: Sterilize sawdust jars.

1. For each species, load below measurements of hydrated sawdust into each quart jar (5 jars per species, 40 jars total). Label carefully with Jar#. Close with filter lids. Cover lids in tin foil to prevent addition of moisture.

WEIGHTS for each species	Jar #1	Jar #2	Jar #3	Jar #4	Jar #5
Hydrated sterilized sawdust	50g	75g	87.5g	94g	100g

2. Load 50g hydrated sawdust into a total of three jars. These will be the hydrated sawdust + VW control triplicate.
3. Load 19 jars and a stainless spoon into pressure cooker. sterilize for 2 hours at 15 PSI. allow to cool completely before removal. (40 jars require three rounds of pressure cooking, as only 19 jars fit inside.)

Step D: Create ratios

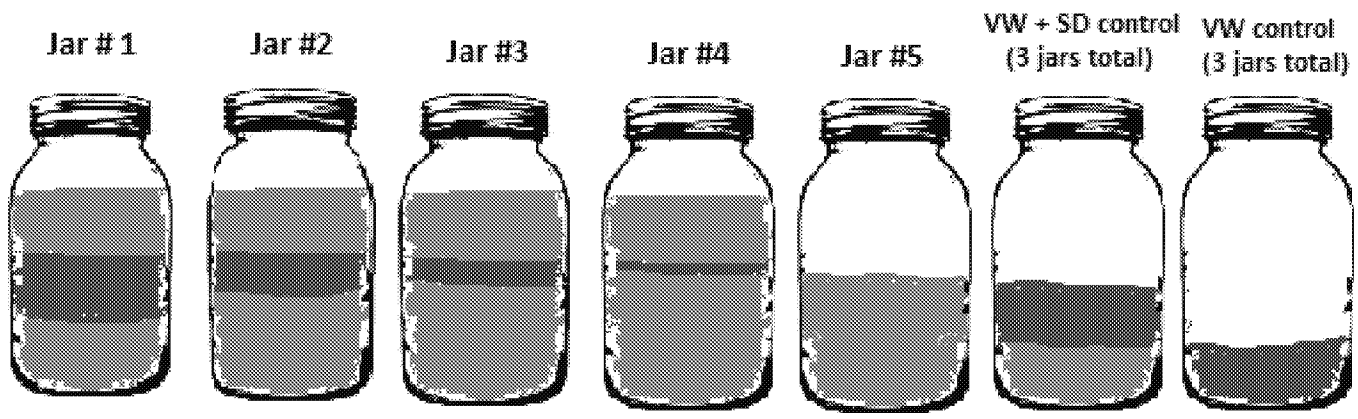
1. Create ratios for each species when the mycelium of that species has fully colonized the sawdust jars, at a density of iii (as defined in the growth measurement chart). Mix ratios soon after (within several days) full colonization is reached, as mycelium will start to lose vigor if left too long without more fresh food to consume. Be sure to create ratios with each species at this same maturity of growth (although it will be on different dates for each species), in order to replicate the scenario as accurately as possible.
2. Measure the following weights of VW into sterilized sawdust ratio jars. Add corresponding weights of Myceliated Sawdust (sawdust spawn from previous task), labeling with species and date. (Jar # is already labeled.) Close each jar with filter lid, and vigorously shake jar 20 times.

note: will be converted to dry weights

	WEIGHTS	Jar #1	Jar #2	Jar #3	Jar #4	Jar #5	Jar #6	Total for species:
For each species of fungus:	Hydrated sterilized sawdust	50g	75g	87.5g	94g	100g	50g	456.5g
	Vactor Waste (raw)	100g	50g	25g	12.5g	NONE	100g	287.5g
	Myceliated Sawdust	50g	50g	50g	50g	50g	NONE	250g

Controls without mycelium:	VW+SD control	100g VW + 50g HSD	100g VW + 50g HSD	100g VW + 50g HSD
	VW control	100g VW	100g VW	100g VW

Each of the eight species of fungi has a group of ratio jars, which are Jars#1-#5. Then there are two separate groups of controls, each containing a total of three jars. The first control contains a mixture of Vactor Waste and Hydrated Sterilized Sawdust, and the second control contains Vactor Waste only.



3. Incubate all ratio jars at 75 degrees F on wire rack in incubation room

4. Once per week:
    a. take photo(s) of each jar for visual documentation
    b. note visual observations in chart and insert into scientific journal (percentage of growth, density of mycelium, etc)

Allow to fully colonize (between 8 and 20 weeks).

Task 6: Sampling

Step A: Select 3 species to sample, and 2 jars per species based on the following criteria:

1. At least 50% mycelium colonization
2. At least some areas of "iii" density
3. Favor higher ratios of VW when possible within species

Step B: Take Samples

1. Gather supplies:
    a. Large Stainless steel bowl
    b. 4 oz jars (one per sample= 10 jars)
    c. Acetone (for rinsing)
    d. Stir spoon
    e. Latex gloves

Under fume hood*(?)*:

2. Rinse bowl and spoon with acetone and let dry
3. Open jar to be sampled, use spoon to loosen contents, pour entire contents into stainless bowl.
4. Use spoon to break up material as much as possible, and stir thoroughly to homogenize
5. Use spoon to fill 4oz jar completely with homogenized material.
6. Tightly close lid of 4oz jar, and label clearly with "species, ratio jar #, date" *(does pacific Rim have different/ specific labeling requirements?)*
7. Take careful notes in lab notebook: which jars were chosen, sampled, procedures, etc (species, ratio jar #, date ratio was mixed)
8. Freeze 4oz jars to be sent to pacific rim.
9. Send to Pacific Rim Laboratory for PCB testing/fingerprinting (EPA 1668)
10. Freeze all remaining samples for potential testing in the future. (@ TLC lab)