Fabulous fungi!

Selectively growing fungi for myco-remediation

Learning about adaptation, selection and biotechnology while doing something useful for the environment!

Russel Barsh, Madrona Murphy, Nathan Hodges, and Eleni Petrou
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There has been increasing interest in using "mushrooms" to help vascular plants remove pollution from rain runoff in roadside ditches, bioswales and "rain gardens". A mushroom is simply the fruiting body of some kinds of fungi. The real work takes place underground in mycelium, a web of very fine fibers that can form mats extending from a few square feet to hundreds of acres. Mycelia collect water and nutrients from water and soil and use them to produce proteins and more mycelia. Mycelia occasional send shoots to the surface of the soil—this includes mushrooms—to disperse spores and colonize new ground. Mycelia protect themselves from bacteria by making antibiotics, and sometimes also use chemical defenses to ward off other fungal species' mycelia.

Fungi have had a very long and important association with plants. Many species of fungi "eat" plants, either parasitizing living ones or breaking down dead ones. In fact, fungi are essential for recycling nutrients in terrestrial ecosystems. They are everywhere in soils, in leaf litter and decaying wood. In addition, many fungal species have evolved symbiotic "mycorrhizal" relationships with plants, exchanging micronutrients and water collected by the mycelium for sugars produced by the plant through photosynthesis. The so-called mycorrhizal species of fungi are especially important in forests, where layers of different mycelia feed different species of trees. This is one reason why logging, fire and soil disturbance can make it hard to re-grow a biologically diverse and resilient forest.

Some plants have become completely dependent on fungi instead and cannot live without them. Some, like the Coralroot orchids (Corallorhiza), no longer photosynthesize and let their fungal partners do all the work. Likewise, mycorrhizal fungi associated with trees cannot be grown without their plant partners; truffles are a well-known example.

Combining plants with fungi should increase the ability of a bio-remedial garden to hold water, filter metals out of the water, and metabolize hydrocarbons. But it is not as simple as planting a garden and sprinkling fungal spores over it! The fungi must already be well adapted to living with the soils and plant species involved. And they must have a demonstrated capacity to filter, accumulate or degrade contaminants—capabilities we can enhance through careful selection over multiple generations.

Our lesson plans introduce students to identifying, collecting, and growing local fungi; selecting fungal strains for enhanced bio-remedial properties; and confirming these properties using standard methods of analytical chemistry. We use automotive motor oil to test fungal strains' ability to degrade simple aliphatic hydrocarbons. Alternatively, you may use copper (from Cupric Sulfate) to test fungal strains' ability to sequester metals. A more advanced (and costly) option is to evaluate fungal degradation of PAHs (polycyclic aromatic hydrocarbons) using Phenanthrene as a standard with an immunoassay.

LESSON PLANS

1. Collect and identify local fungi

Purpose:

Acquire a source stock of a fungal species that can be cultured in the classroom, and evaluated for its remedial properties.

Principles:

For fungi to grow well, they must already be adapted to local soils, weather, and plant species. Moreover, fungi that grow on woody plants are the most likely to have useful biochemical capabilities!

Learning Objectives:

- Learn when and where to find the fruiting bodies of local fungi.
- Learn basic life history of local fungi, and how to identify them.
- Learn to use scientific (binomial) names and taxonomic keys.

Materials:

A reliable, thorough guide to your region's native fungi[*]

Magnifiers and garden trowels

Activities:

After a week or two of fall rainfall (but before any significant snowfall), visit a relatively undisturbed woodland where there are decaying tree trunks or stumps as well as healthy mature trees. Examine logs and stumps for wood polypores or "shelf fungi," Oyster mushrooms (*Pleurotus spp*), Chicken of the Woods, and Turkey Tails. Search the ground around larger trees for flushes or "fairy rings" of mycorrhizal mushrooms such as *Russula*, *Lactarius*, *Suillus* and *Amanita*, and gently trowel the earth around them to look for the buried cotton-candy-like mycelium and its connections with the tree roots. Make a collection of 10 conspicuous "tree eaters" and mycorrhizal fungi you discover—a single fresh specimen of each will do—and bring them back to the classroom. Note: fungi will remain viable for several days if simply left in a brown paper bag. Brush off excess dirt, but do not wash, bruise, or refrigerate.

In the classroom, lay specimens out on a table, and try to identify them using both published photographs and a key. Some keys will require spore prints (leave the fruiting body on a sheet of blue paper for 2-3 days with its gills or pores facing down) or cutting a cross-section of the fruiting body with a sharp knife.

Use Google Scholar to find out whether any of the wood-eating species you have chosen have already been tested for accumulating or metabolizing hydrocarbon pollution. If not, you can break fresh ground scientifically, by studying a species about which we know little or nothing!

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[*] We recommend: *Mushrooms of Northeastern North America* (Bessette, Fisher, Bessette); *Mushrooms of the Southeastern United States* (Bessette, Roody, Bessette, Dunaway), and *Mushrooms Demystified* (Arora) for the Western States.

2. Culture fungi in the classroom

Purpose:

Prepare identical "daughter" colonies of a fungal species that you would like to evaluate so that you can maintain the original "wild type" strain, while trying to develop an "improved" strain through selection.

Principles:

Small samples of tissue from freshly collected fungi can be maintained *in vitro* as long as they have adequate supplies of nutrients (simple sugars) and are protected from bacteria and molds that compete for the nutrients. Fungal cultures will grow faster, fruit and reproduce ("spawn") if given additional nutrients including starch (a complex sugar or polysaccharide) and protein.

Learning Objectives:

- Learn to make sterile growth media and keep them sterile
- Learn to inoculate growth media with fungi successfully
- Learn to recognize fungal, bacterial, yeast and mold colonies

Materials:

Autoclave (or home pressure cooker)

Electronic scale (accurate to 10 milligrams or less)

1-liter glass jar with snug nylon or metal closure

Glass Petri dishes (12 pairs, sterilized), or pre-sterilized plastic ones

Ingredients for Malt Extract Agar (maltose yeast extract, agar, water)

Alcohol lamp, scalpels and/or forceps

Alcohol-resistant laboratory marking pen

Disposable laboratory gloves (Nitrile or Latex)

Parafilm™ or masking tape, and aluminum foil

Magnifiers (10X to 20X)

Activities:

Prepare and autoclave 1 liter of Malt Extract Agar.[*] If you are using glass Petri dishes (or "plates") instead of pre-sterilized disposable plastic Petri dishes from a sealed package, be sure to sterilize them too before filling them with medium.

[*] Combine 20 grams agar, 20 grams maltose or barley sugar, 2 grams yeast extract or nutritional yeast, and (optional) 1 gram of peptone, and add tap water to 1 liter. Autoclave medium in glass jar for 15 minutes at 121°C or process in a household pressure cooker for 30 minutes at 20 pounds. Cool until jar can be handled comfortably before pouring medium into Petri dishes.

When the agar has cooled enough to just handle comfortably, open one plate at a time, and holding the lid by the outside rim to prevent contamination, slowly pour enough into the dish to completely cover the bottom (a layer about 2mm thick), replace the lid before pouring the next dish (dishes may be stacked before pouring). Let the medium cool completely before moving.

Choose a single clean, unblemished specimen of a wood-eating fungus from Lesson 1 to serve as the "wild type" parent. Wipe the outside of the fungus clean with a paper towel and carefully split it in half down the middle of the stem and up through the cap. Be careful not to touch the newly exposed flesh. Flame your scalpel or forceps by dipping briefly in ethanol and passing through the alcohol lamp flame (do not hold the flaming scalpel over your container of ethanol!), without touching anything with the blade, let the scalpel cool briefly (30 seconds) and use the blade to slice a small cube of flesh from just above the gills or pores of your fungus, being sure to only cut from the freshly exposed, clean flesh.

Using the scalpel carefully transfer the cube of fungus to the center of the media on a cooled Malt Extract Agar plate (being sure that nothing else touches the surface of the media and holding the lid by the outside rim to prevent contamination). Use Parafilm™ (or masking tape) to seal the edges of the plate and label it with the species of fungus, the date and the student or team name.

Inoculate at least plate for each student or team of students, flaming the scalpel between inoculations and switching to the other half of the fungus if needed to find clean flesh. Reserve two plates as controls. Keep one of them sealed. If anything grows on it, your plates and/or growth medium were contaminated. Open the other one and leave it open in the classroom for 10 minutes before closing it again. It will grow colonies of any bacteria or other microbes that are floating in the air of the classroom. Use these controls to check whether students have successfully maintained sterile conditions.

Incubate the wrapped plates at room temperature or 20 ^{o}C out of direct sunlight until the fungus colonizes the plates.

Check plates after a week. A fine web of mycelium should be visible around each of the transplanted cubes of fungus; and there should be no round colored colonies of other microbes. Have students draw the contents of each plate, including the controls, in color, and use magnifiers to try to identify each colony as fungal, bacterial, or other, from its shape, color and small features such as mycelial threads or hyphae. There are excellent on-line resources to get started.[*]

Wait one more week before using the first set of plates to grow a new set of plates using the same sterile techniques, as described further in the next lesson plan.

[*] http://www.sciencebuddies.org/science-fair-projects/project_ideas/MicroBio_Interpreting_Plates.shtml

3. Select a fungal strain for remedial power

Purpose:

Challenge the chosen fungal strain to adapt to, and tolerate high concentrations of a contaminant, potentially "learning" to sequester, hyper-accumulate, or degrade the contaminant, through a process of progressive exposure and selection.

Principles:

Like bacteria, fungi multiply rapidly, and they can evolve new strains in response to changing environmental conditions that enable them to survive. Sometimes, in fact, they "discover" through mutations how to metabolize a new substance they encounter, changing it from a threat into a resource. Progressively increasing the concentration of the new substance continues the process of adaptive evolution to its biochemical limits.

Learning Objectives:

1. Learn to stimulate fungal adaptation by dosing growth media
2. Learn to identify and select fungal strains that tolerate contaminants
3. Learn to use area coverage as a measure of fungal reproduction rates

Materials:

Fungal colonies grown for Lesson 2

Autoclave (or home pressure cooker)

Electronic scale (accurate to 10 milligrams or less)

1-liter glass jar and 250 mL jar with snug nylon or metal closures

Glass Petri dishes (12 pairs, sterilized), or pre-sterilized plastic ones

Ingredients for Malt Extract Agar (maltose yeast extract, agar, water)

Alcohol lamp, scalpels and/or forceps

Parafilm™ or masking tape, and aluminum foil

Disposable laboratory gloves (Nitrile or Latex)

Alcohol-resistant laboratory marking pen

Graph paper and low-power magnifiers

Motor oil, Cupric Sulfate, or Phenanthrene[*]

Activities:

Prepare another set of 12 sterile Petri dishes ("plates") with Malt Extract Agar, this time with one modification: After mixing up the growth medium in a 1-liter jar, pour about 200 mL into a second, smaller jar, close it, and set it aside. Then into the l-liter jar

[*] Phenanthrene is a relatively low toxicity PAH; nonetheless, be sure to wear goggles and gloves, avoid any spills or inhalation, and dispose of containers that have held Phenanthrene solutions or dosed agar.

add the contaminant "challenge" (1 gram Cupric Sulfate, 20 mL motor oil, or 0.1 gram of Phenanthrene), cap securely, and swirl—do not shake—the contents to mix thoroughly. Autoclave both jars of medium, one dosed and the other not dosed.

After autoclaving, pour ten plates of dosed medium, being careful to swirl the jar gently between pours to keep the added contaminants evenly mixed. This is especially important for motor oils, and for lipophilic compounds such as pesticides, anti-microbial agents or antibiotics.

Fill the remaining two plates from the jar that was not dosed. They will serve as untreated controls that preserve the original "wild type" fungus strain.

Have students examine the plates they prepared for Lesson 2, and choose up to six that have conspicuous growth of fresh mycelium, and lack colonies of bacteria, yeast or mold. Each of these healthy strains will be used to inoculate two or more fresh plates (at random) including the controls. For example, if only four of the plates from Lesson 2 are growing healthy, uncontaminated fungi, each of them will be used to inoculate three fresh plates out of the 12 (including controls) that you have poured for Lesson 3.

To inoculate a fresh plate from one of the original plates, use sterile technique to cut about a square centimeter out of the agar with fresh fungal growth and as quickly as possible—to avoid contamination from the air in the classroom—transfer this tiny square to the surface of a fresh plate. Close, seal, and mark each new plate as in Lesson 2, and incubate as before in a dark quiet space at room temperature or about 20^{o}C for a week.

After a week, examine the 12 plates for evidence of fungal growth. Tape a round of graph paper to the bottom of each Petri dish, hold the dish up to a strong light (without opening it) and estimate the area of each fungal colony by counting the number of graph paper squares that it covers. Make a chart to compare colonies by size. Set aside the two dosed plates with the largest fungal colonies, and the healthiest not-dosed plate. Dispose of all of the other plates and their contents.[*]

Prepare another batch of Petri dishes, 8 with dosed Malt Extract Agar, and 4 with medium that has not been dosed. Inoculate the 8 fresh dosed plates from the two largest fungal colonies found on the first batch of dosed plates. Inoculate the 4 untreated plates from the healthiest fungal colony found on the first batch of not-dosed control plates.

Close, seal, mark, and incubate for one to two weeks. Examine the plates, using a round of graph paper to measure the size of each fungal colony. Select two of the healthy dosed colonies, and the healthiest control colony, for testing in Lesson 4. To qualify as healthy, the fungus should have colonized at least half the plate, and there should be no round shiny bacterial or yeast colonies, or fuzzy black or green mold colonies.

[*] Dispose of inoculated plates, and whatever is growing on them, by autoclaving them before placing them, sealed and tied in a plastic bag, into ordinary school solid waste. Do not re-use plastic plates. Glass ones can be re-used only after autoclaving them with their contents, scraping out the contents into a plastic bag for solid waste disposal, washing and re-autoclaving. Always wear gloves when handling inoculated plates.

4. Test fungi for remedial effectiveness

Purpose:

Produce a source stock of the selected contaminant-tolerant fungal strain that can be evaluated for its remedial properties *in vitro* in the classroom, and compare its remedial activity with the original wild- Bio-remedial type strain.

Principles:

Fungal strains that have adapted to living with a particular contaminant are likely to have evolved biochemical means of sequestering or degrading the contaminant that were absent in the original wild type. Remedial activity can be evaluated by measuring the "clearance rate" of contaminants in fungal cultures: how quickly a given dose of contaminant disappears from the growth medium.

Learning Objectives:

1. Learn to design a simple experiment with treatment and controls
2. Learn to use a spectrophotometer, a basic tool of analytical chemistry
3. Learn one or more methods of analytical quantitative chemistry

Materials:

Fungal colonies grown for Lesson 3

Autoclave (or home pressure cooker)

100 mL and 500 mL glass graduated cylinders

12 clean wide-mouth one-cup glass jelly jars

1 liter of dry rye grain berries

Alcohol lamp, scalpels and/or forceps

Disposable laboratory gloves (Nitrile or Latex)

Alcohol-resistant laboratory marking pen

Motor oil, *or* 1% Cupric Sulfate solution, *or* 0.1% Phenanthrene solution[*]

Reagents for determining concentrations of toxics (*see* **Laboratory Manual**)

Activities:

We hypothesized: the fungal strains which survived dosing with contaminants are not only better able to tolerate those contaminants than the wild-type fungi, but may also be able to sequester or degrade those contaminants. To test this hypothesis, we must first grow larger colonies of the tolerant fungal strains, and the wild type, for comparison. For this purpose, we will switch from agar plates to grain as the growth medium.

[*] For 1% Cupric Sulfate, measure 0.5 gram dry Cupric Sulfate into 500 mL distilled water; it will dissolve readily. For 0.1% Phenanthrene, measure 50 mg Phenanthrene into a mixture of 10 mL of laboratory grade Methanol and 10 mL distilled water. Once the Phenanthrene has fully dissolved, top up the mixture to 500 mL with distilled water and swirl to combine.

Measure 75 mL of dry rye berries into each jar; then add 100 mL of tap water to each jar. This should leave at least an inch between the top of the liquid and the rims of the jars. Add the contaminant you have selected for study to each jar: 1 mL of motor oil; or 1 mL of 1% Cupric Sulfate (equivalent to 1 mg $CuSO_4$ or 0.4 mg Copper in each jar); or 1 mL of 0.1% Phenanthrene (equivalent to 0.1 mg Phenanthrene for each jar). Cap jars securely, and shake the contents to mix them thoroughly. Cover jars loosely, and let the rye berries soak overnight.

The next day, autoclave jars for 30 minutes at 121°C, or process in a household pressure cooker for one hour at 20 pounds. Be sure that lids are loosened before placing jars in the autoclave or pressure cooker, to allow steam to escape freely. After processing the jars, let them cool completely before tightening their lids to preserve sterility.

After jars have cooled completely, inoculate them with fungi grown for Lesson 3. Make six Treatment jars and six Controls from the dosed and not-dosed plates chosen at the conclusion of Lesson 3 as follows:

- Three jars from one of the dosed plates (Novel Strain A)
- Three jars from the other dosed plate (Novel Strain B)
- Three jars from the not-dosed plate (Wild-type Control)
- Three jars with no fungi (Negative Control) – just open and close them

Before inoculating a jar of rye, shake it and loosen its lid. Unwrap the Petri dishes chosen from Lesson 3. Flame your scalpel by dipping it briefly in ethanol and passing it through the flame of the alcohol lamp. Give it a few seconds to cool. Then, holding the lid of the petri dish by the outside rim to prevent contamination, use the sterilized scalpel to cut a centimeter-square wedge of agar that has fungal hyphae growing on it. Use the scalpel to lift the wedge out of the Petri dish, close the dish, and transfer the wedge to the jar of rye, being careful to avoid contamination, and ensuring that nothing comes in contact with the inside of the lid of the jar. Close the jar tightly and flame your scalpel.

Shake all the jars to bring the agar in contact with the rye berries. Incubate jars at 20°C or at room temperature. You should see fine white threads of mycelium after one or two weeks. Allow mycelia to grow for another two weeks before measuring contaminant concentrations by the methods set out in the **Laboratory Manual**.

5. Scale up your propagation program

Purpose:

Grow sufficient quantities of fungal species that can be cultured in the classroom, and evaluated for its remedial properties.

Principles:

For fungi to grow well, they must already be adapted to local soils, weather, and plant species. Moreover, fungi that grow on woody plants are the most likely to have useful biochemical capabilities!

Learning Objectives:

4. Learn when and where to find the fruiting bodies of local fungi.
5. Learn basic life history of local fungi, and how to identify them.
6. Learn to use scientific (binomial) names and taxonomic keys.

Materials:

Fungal colonies chosen as a result of Lesson 4

Autoclave (or home pressure cooker)

Electronic scale (in tenths of grams, to 20 grams or more)

10 glass one-quart canning jars with secure metal or nylon lids

10 quarts of dry rye grain berries

Alcohol lamp, scalpels and/or forceps

Parafilm™ or masking tape, and aluminum foil

Disposable laboratory gloves (Nitrile or Latex)

Alcohol-resistant laboratory marking pen

Activities:

If Lesson 4 confirms that your fungal selection program has been successful, you should now move from the laboratory to the field and inoculate a bioswale or rain garden with your bio-remedial fungal strain! The first step is to grow a sufficient quantity of the oil-eating (or PAH-degrading, or copper sequestering) fungal strain to inoculate at least a cubic yard of appropriate bioswale fill such as hardwood chips. You can do this by using the same basic technique you have already used to grow mycelia on rye berries. Prepare more quart jars than you think you will need, because some may become contaminated or simply not grow vigorously.

Measure 400 mL of dry rye berries into each jar; then add 500 mL of tap water to each jar. This should leave at least an inch between the top of the liquid and the rims of the jars. Cover jars loosely, and let the rye berries soak overnight.

The next day, autoclave jars for 30 minutes at 121°C, or process in a household pressure cooker for one hour at 20 pounds. Be sure that lids are loosened before placing jars in the autoclave or pressure cooker, to allow steam to escape freely. After processing the jars, let them cool completely before tightening their lids to preserve sterility.

After jars have cooled completely, inoculate them with a strain of fungi grown for Lesson 3* and tested successfully for its bio-remedial properties in Lesson 4.

Before inoculating each jar of rye, shake it and loosen its lid. Flame your scalpel by dipping it briefly in ethanol and passing it through the flame of the alcohol lamp. Let it cool briefly. Hold the lid of the petri dish by the outside rim to prevent contamination, and use the sterilized scalpel to cut a centimeter-square wedge of agar with fungal hyphae growing on it. Use the scalpel to lift the wedge out of the Petri dish, close the dish, and transfer the wedge to the jar of rye, being careful to avoid contamination, and ensuring that nothing comes in contact with the inside of the lid of the jar. Close the jar tightly and flame your scalpel again before inoculating the next jar.

Shake all the jars to bring the agar in contact with the rye berries. Incubate jars at 20°C or at room temperature. You should see fine white threads of mycelium after one or two weeks, and you can let them grow for another month before using them to "spawn" a bed of moist hardwood chips or other medium in a bioswale or "rain garden".

Reserve at least one jar of healthy mycelium to preserve the contaminant-adapted strain, and grow more of it for future projects. Hint: acquired characteristics may be lost, if they no longer confer a selective advantage! Consider "challenging" your fungal strain periodically by adding contaminants to growth media.

* If kept sealed in a cool dark place, the fungal cultures you grew for Lesson 3 can survive for a month of longer without additional nutrients.

A. Technical note on motor oil composition

Motor oils mainly consist of long aliphatic (straight chain) hydrocarbons, roughly 20 to 50 carbon atoms in length, which are easily metabolized by many fungi and bacteria similarly to the way humans and other mammals metabolize sugars. Motor oil products contain as much as 5-10% other compounds, however, which include deliberate additives intended to enhance performance, as well as byproducts of the original formation of the oil and its manufacture.

The "base stock" of motor oil products is distilled from petroleum, or synthesized from other hydrocarbon sources such as vegetable oil. Petroleum is formed from organic residues of ancient swamps and lagoons, and usually contains metals, as well as complex aromatic (carbon ring) hydrocarbons created by the same underground heat and pressure required to convert plant biomass into aliphatic oils. As a result, petroleum may contain a small amount of carcinogenic compounds such as PAHs. More PAHs are created when motor oils are heated or burn in an engine. Burning gasoline and motor oil also creates dioxins and furans, which can be carcinogenic.

Since motor oils are used to lubricate very hot moving metal parts, used motor oil contains a considerable amount of fine metal particles produced by engine wear. Much is iron, of course, since engines are largely composed of steel; but there can be just as much copper in used oil, and copper is very toxic to fish. Copper is soft and wears down more quickly than iron, so more of the copper in an engine ends up in lubricating oils.

A floating film of fresh or used motor oil may therefore contain a wide variety of dissolved, and relatively persistent toxic hydrocarbons as well as metals such as lead and cadmium. The base stock itself functions as a solvent and facilitates the dispersal of toxic metals and compounds dissolved in it. When fungi metabolize the base stock, they may accumulate metals and dissolved toxic hydrocarbons as well—filtering out, but generally not degrading them.

Additives can improve performance of motor oils, but also increase the toxic load. Most products contain detergents such as Magnesium Sulfonates, for example, and Zinc Dialkylithiophosphate and other Zinc compounds are added to most motor oils to reduce friction and engine wear. Zinc is very toxic to aquatic organisms. Anti-oxidants added to motor oils to prolong their useful life include phenols that become PAHs when burned.

Detergents and surfactants added to motor oils increase their mobility if they are spilled or leak into the environment. At high concentrations, most are also toxic to small aquatic organisms at the base of the food web.

Silicones are also routinely added to motor oils as dispersers, emulsifiers, and de-foamants. Silicones are polymers that contain a carbon-silicon skeleton with oxygen and hydrogen. They are non-reactive and heat resistant, persistent, but probably non-toxic in the environment, breaking down slowly into carbon dioxide, water, and mineral silicates (many minerals including the clay minerals are silicates). Silicones may not pose much of a toxic threat in the environment, but they may slow the metabolism of oils by fungi.

2. Determination of motor oil (base stock)

Motor oil is hydrophobic; it does not dissolve in water. Whatever oil remains in each jar is likely to be adsorbed to the surfaces of the rye berries, mycelia, and the glass jar itself. Motor oil will dissolve in weakly non-polar organic solvent such as an alcohol, however; hence we can recover any remaining oil from our jars simply by flushing them with alcohol. Some traces of vegetable oils from the rye berries may also be dissolved in the alcohol, but they can be "salted out" (precipitated with Sodium Chloride).

Since it is difficult, if not impractical, to separate the mycelium from the grain for separate chemical analysis, you will test extracts of the entire contents of each jar (growth medium together with fungi). If degradation has taken place, the total oil recovered from jars containing mycelia should be significantly less than the oil recovered from Controls. Without further analysis (which would require liquid or gas chromatography), we cannot determine what compounds the missing oil has been turned into by fungal metabolism; nor can we be absolutely certain that all the missing oil was metabolized by fungi, and not by unintended, invisible colonies of bacteria or yeasts.

Materials

Spectrophotometer or colorimeter set to a wavelength of 660 nm
Laboratory vortexer (student muscle power is an acceptable substitute)
Electronic laboratory scale (in tenths of a gram, to 20 grams or more)
Disposable plastic weigh boats for use with the scale
Twelve 3-mL (1-cm path length) plastic or glass spectrophotometer cuvettes
Twelve clean small glass beakers (between 20 and 50 mL are best)
50 mL graduated glass cylinder with a secure stopper
Disposable soft plastic transfer pipette (1-mL or 3-mL capacity)
Twelve 20-mL disposable plastic Luer-lock syringes
Twelve 0.45-micron PTFE Luer-lock syringe filters
1 liter of analytical grade Isopropyl alcohol (Isopropanol) [*]
2 liters of distilled water
100 mL of 30% NaCl solution (30 grams NaCl into 100 mL distilled water)
Motor Oil left over from Lessons 2 and 3, to serve as a standard

Extraction

Rinse each of the cuvettes and beakers with Isopropyl alcohol to remove any oils or soaps. Lean them on their sides on an absorbent paper mat or towel for at least an hour to evaporate any residual alcohol.

Pull each syringe apart, mount a fresh syringe filter on the barrel by screwing it in and then place the syringe-mounted barrel and plunger in a clean beaker. Also be sure to prepare the 30% NaCl solution in advance. Dissolved that much salt in water will require heating, and the solution should be back to room temperature before use.

[*] Isopropyl "rubbing alcohol" sold in pharmacies and grocery stores is often diluted and may also include additives that can interfere with test results. Purer, inexpensive Isopropyl alcohol is readily procured from educational science suppliers such as Carolina Biological and Ward's.

Open each jar of rye berries from Lesson 4 and carefully add 50 mL of Isopropyl alcohol. Immediately re-close each jar tightly, and shake vigorously for one minute. Let the contents of the jar settle for 10 minutes, then fill a filter-mounted 20-mL syringe with supernatant liquid. Insert the plunger into the syringe barrel, and firmly but slowly press down, expressing the filtered liquid into a glass beaker. Cover each filled beaker tightly with Parafilm™ or plastic wrap until you are ready to measure the dissolved oils.

It can also be useful to allow the contents of each beaker to dry, then scrape them out and weigh them. Fungal growth will metabolize some of the rye berries, but will also potentially utilize water, oxygen and carbon dioxide to manufacture more fungal tissue.

Measurement

Measure 10 mL of filtered Isopropanol extract into the 50-mL graduated cylinder. Add distilled water to 50 mL. Next use the soft plastic transfer pipette to add 1 mL of the salt solution. Cap the graduated cylinder securely, vortex or shake it vigorously for three minutes, and then let it rest for five minutes. Dissolved motor oil will produce turbidity: that is, cloudiness or a milky color.

To "read" the turbidity, pour some of the liquid from the graduated cylinder into a cuvette (nearly to the top) and measure Absorbance at 660 nm in the spectrophotometer.[*] Record the result. The greater the absorbance, the more oil was present.

Be sure to rinse the graduated cylinder and cuvettes with Isopropyl alcohol before re-using them for another measurement.

Calibration

To be able to interpret results more meaningfully, in absolute concentrations of oil rather than relative Absorbance, you need to prepare three standard solutions of the motor oil you used to dose the jars of rye berries in Lesson 4.

In preparing the Treatment and Control jars for Lesson 4 you used a dose of 1 mL of motor oil per jar. You extracted each jar's contents into 50 mL of Isopropanol. If no oil was lost or metabolized inside a jar, and the extraction of residual oil from the jar was 100% successful, the extract should contain 1 mL oil in 50 mL alcohol or a little less than 2 percent oil by volume. One standard solution should therefore be 2% of the same oil by volume since that is the maximum concentration of oil you can expect to recover from any of the jars of rye berries. Other standards should be less. We recommend using 0.5, 1.0, and 2.0% solutions of oil in Isopropyl alcohol as standards.

Measure out 10 mL of each of these three standard solutions, dilute into 50 mL of distilled water, salt out with 1 mL 30% NaCl, shake, rest, and measure Absorbance at 660 nm just as you have done with extracts from the jars of rye berries. Use the three results from the standards to plot a calibration curve (Absorbance versus Concentration of motor oil), and use the calibration curve to convert the measurements of Absorbance from your twelve Treatments and Controls into "equivalents to X% motor oil by volume".

[*] If you are using a dual-beam spectrophotometer, use a reference cuvette with10% Isopropyl alcohol in distilled water. If using a single-beam spectrophotometer, blank on a cuvette of 10% Isopropyl before shooting any samples.

3. Determination of copper

Unlike motor oil, which consists of large hydrocarbon molecules that can be split and re-assembled into different, more or less toxic structures by fungal and bacterial cells as a means of generating metabolic energy, copper is an element. It cannot "go away" or be degraded into something that is non-toxic. It can only be relocated, from water or soil into the tissues of a fungus, bacterium or plant: bio-filtration or accumulation. This could be useful so long as the organism accumulating copper remains alive. Copper sequestered by trees in their wood is removed from the environment for many years, while any copper that accumulates in fruit, seeds, or grasses may be returned to the food web within a year.

To determine whether your fungal strain accumulates or sequesters copper it will be necessary to test the fungus and the medium (rye berries) separately. This would be a much easier task if we were studying plants, and could simply shake soil off their roots! At least we can assume that no copper has escaped from the experimental jars, so that we can also assume that whatever copper is not sequestered in fungal tissue, is still adsorbed to the rye berries or the walls of the jars.

Copper ions tends to adsorb to surfaces, but can be mobilized by reducing the pH of the environment. On the other hand, copper incorporated into the tissue of fungi will need to be extricated somehow—for example by using a stronger acid. Most laboratory methods begin by incinerating plant tissues, leaving an ashy mixture of carbon and metals from which metals can be dissolved into strong acid. Alternately, enzymes can be used to break down cellulose and cell walls.

Materials

Spectrophotometer or colorimeter set to a wavelength of 450 nm
Hotplates, preferably with adjustable temperature controls
Electronic laboratory scale (in tenths of a gram, to 20 grams or more)
Disposable plastic weigh boats for use with the scale
Twelve 3-mL (1-cm path length) plastic or glass spectrophotometer cuvettes
Twelve clean small glass beakers (between 20 and 50 mL are best)
Twelve clean small ceramic crucibles
10 mL graduated glass cylinder
Adjustable Pipettor with 250-microliter or 1000-microliter tips
Twelve 20-mL disposable plastic Luer-lock syringes
Twelve 0.45-micron PTFE Luer-lock syringe filters
Diethyldithiocarbamate (DDC) copper reagent[*]
10M (37%) analytical grade Hydrochloric Acid
Sodium Bicarbonate (preferably analytical grade, not from the corner store)
Cupric Sulfate, also known as Copper (II) Sulfate
2 liters of distilled water
Paper plates and glass stir rods
Stainless steel spoon, spatula, and/or stainless steel scalpel

[*] Available from LaMotte, Hach, and other manufacturers of environmental testing supplies.

Extraction

Rinse the ceramic crucibles with Hydrochloric Acid, then with distilled water, and let them dry turned upside down on paper towels. It is important to use acid that has less than 0.0001% copper (=100 parts per million). Most acids contain minute metal residues from their manufacture, and these contaminants can produce spurious analytical results. Manufacturers generally provide assay data on labels.

Pull each syringe apart, mount a fresh syringe filter on the barrel by screwing it in and then place the syringe-mounted barrel and plunger in a clean beaker. Rinse all other containers in dilute Hydrochloric Acid (50 mL of 10M acid into 1 liter of distilled water), and leave them to dry overnight prior to use.

Using a stainless steel spoon or spatula, scrape the contents of each Treatment and Control jar onto a paper plate. Rinse the tool with distilled water between uses.

While the contents of each jar are still fresh and moist, use a scalpel to separate as much of the fungal mass as practicable from the rye berries. Focus on obtaining a sample of *pure fungus without any rye attached*, rather than trying to get every bit of fungus that can be seen. We will be testing for the concentration of copper in pure fungal tissue; the total amount of copper the fungus has sequestered can only be estimated because threads of mycelium too small to see will have spread throughout the rye.

Use the electonic scale and weigh boats to measure the mass of fungus recovered from each Treatment jar and record the results (as "live weight"). For the Controls, there should of course be no fungus at all, so instead, count out and weigh 10 rye berries. This will tell us something about how much copper is absorbed by the rye (=growth medium).

Gently heat fungal samples in crucibles on the hotplate until they shrink, darken, and begin to carbonize. Remove the crucibles and allow them to cool. Then add 1 mL of Hydrochloric Acid to each crucible and leave overnight. Mash and mix the sludge at the bottom of each crucible with a glass stir rod. Add 9 mL of distilled water, and continue to mix gently in the crucible with a stir rod. Pour the liquid into a filter-mounted syringe barrel, insert the plunger, and press down firmly but slowly to express the liquid into one of the glass beakers. Cover beakers tightly with Parafilm™ or plastic wrap until you are ready to measure the copper concentration of the contents.

Measurement

Neutralize the acid in the contents of each beaker by adding 0.2 grams of Sodium Bicarbonate.[*] Swirl the liquid, which may bubble, and allow to rest for a minute or two, until it is still. Use the adjustable Pipettor to add 0.25 mL of DDC. (Some suppliers sell DDC reagent in dropper bottle: 0.25 mL = 250 microliters = 20 drops.) Swirl once gently to mix, and immediately measure Absorbance at 450 nm on the spectrophotometer, using distilled water as a reference or blank. The greater the absorbance, the more copper was extracted from the fungus (or the rye, in the Controls).

[*] A quarter-teaspoon measuring spoon from the kitchen will also do for this purpose: faster than weighing and sufficiently precise.

The DDC method is accurate within the range of approximately 0.01 to 3.00 parts per million (ppm). This means that if we expect results greater than 3.00 ppm, we should dilute samples before testing them, to bring them within the reliable range of the method. Likewise, if we obtain Absorbance results greater than 2.5000, it is important to dilute the remaining portion of the sample to 50% and try again.*

Calibration

To be able to interpret results meaningfully, in absolute concentrations of copper rather than relative Absorbance, you need to prepare three standard solutions of the same Cupric Sulfate solution you used to dose the jars of rye berries in Lesson 4.

In preparing the Treatment and Control jars for Lesson 4 you added 1 mg Cupric Sulfate to each jar. Since copper is only about 41% of the mass of a molecule of Cupric Sulfate ($CuSO_4$), 1 mg Cupric Sulfate is only 0.4 mg (=400 micrograms) of ionic copper! You extracted the fungi from each jar into 1 mL of acid plus 9 mL of distilled water, so if the fungi sequestered all of the copper, the extract should contain 0.4 mg copper in 10 mL of liquid, which is equivalent to 40 mg copper in a liter, or a concentration of 40 parts per million. That's about 10 times the upper limit of the DDC test.

How much copper can we realistically expect to be sequestered by fungi over the course of a 3-4 week experiment? Mycelia would have to grow extensively in order just to reach all of the copper coated rye berries in each jar; once mycelia are in contact with copper, they may not be anywhere close to 100% efficient in transporting the copper into fungal tissues. Even 10% uptake of copper during this short experiment is a significant biofiltration effect, and 10% of 40 parts per million is 4 parts per million). This is still a lot of copper from both a toxicological and an analytical perspective. An additional 50% dilution of extracts prior to testing would be prudent.

For standards, prepare a stock solution of 1.0 gram Cupric Sulfate in 1.0 liter of distilled water—a 0.1% solution. Exactly 1.0 mL of this stock solution topped up to 100 mL with distilled water is a 0.00001 of $CuSO^4$ or 8 ppm of copper. Dilute this 8-ppm solution further to produce standards at 2 ppm, 1 ppm and 0.5 ppm.

Measure out 10 mL of each of these three standard solutions, add 250 microliters of DDC reagent to each of them, swirl gently, and measure Absorbance at 450 nm just as you have done with extracts from the jars of rye berries. Use the three results from the standards to plot a calibration curve (Absorbance versus Concentration of Copper), and use the calibration curve to convert the measurements of Absorbance from your twelve Treatments and Controls into "parts per million copper by weight".

You can back-calculate the concentrations of copper in acid extracts, to original concentrations in fungal tissues. If there is 1.5 ppm of copper in the 10-mL extract from 2.5 grams of fungal tissue, the concentration of copper in the fungus itself was 2.5/10 or 4 times 1.5 ppm = 6 ppm.

* Absorbance is a logarithmic function that is asymptotic with 4, that is, its functional maximum is 3.9999, and its greatest resolving power or precision is below 2.0000.

4. Determination of Persistent Biological Toxics by ELISA

We have looked at the fungal metabolism of simple aliphatic hydrocarbons in the base stock of motor oil, and fungal sequestration of an element, ionic copper. Can fungi also degrade or sequester toxic cyclic hydrocarbons in petroleum products such as PAHs, or the cyclic hydrocarbons sold as pesticides, or as pharmaceuticals?

Answering these questions requires sensitive analytical methods that can identify individual compounds accurately, and "see" down to a billion or trillion parts per million. For 50 years this meant mass spectrometry, which uses high voltage to break compounds into smaller ionized pieces and measures their mass. Under the same conditions, a given compound breaks down into the same sized pieces. Mass spectrometers are expensive to build and operate, however.

In the 1980s, a biochemical alternative was developed that uses specially prepared antibodies to bind to target analytes. Enzyme-linked Immunosorbent Assays (ELISA) are now widely used in clinical medicine and medical research, and have begun to find more applications in environmental monitoring. Some ELISA methods can detect as little as a single attogram (10^{-18} gram) of the target. ELISA methods vary in "specificity," however. Some methods can overstate the concentration of the target by cross-reacting with one or more similar chemical compounds. Cross-reactivity tables are included in manufacturers' specifications for ELISA antibody kits.

When using a spectrophotometer, concentration of the target analyte is ordinarily a function of Absorbance, but the reverse is true in ELISA! How does it work? ELISA uses a "competitive" reaction. Antibodies are bonded to magnetic nanoparticles or to the walls of depressions in a clear glass or plastic plate. The sample is added, and any target compounds that are present bind with the antibodies. Next is an "enzyme conjugate" that contains copies of the target compound coupled with an enzyme. The enzyme conjugate sticks to any unbound antibodies that remain on the magnetic particles or plate. Finally, any excess enzyme conjugate is washed off, and a reagent is added that reacts with the enzyme conjugate still stuck to the particles or plate, producing a yellow color that can be measured with a spectrophotometer at 450 nm. What the spectrophotometer reads is the concentration of bound enzyme conjugate, which is inversely proportional to the amount of target analyte that was in the original sample.

ELISA kits for environmental studies are available from Abraxis, Modern Water, BioSense, Oranoxis, and other companies. Kits with magnetic particle "formats" require purchase of a magnetic rack to separate the metal particles for washing ($400); the results can be read on a standard spectrophotometer.[*] Plates are more widely available, and made for a wider variety of analytes, but they can only be read by a "plate reader," a scanning spectrophotometer that few schools own. In either case you will also need an adjustable Pipettor to measure 200 to 500 microliter aliquots of reagents; a vortexer; and a supply of very pure HPLC-grade or MS-grade laboratory Methanol.

[*] Magnetic particle kits sufficient for 100 tests can cost $500 to $1800 depending on the target analyte. Ask companies about educational discounts, and discounts for kits that are close to their expiry date. Antibody kits have a nominal shelf life of 6-12 months, but are usually good for several months after that.

The following suggested exercise uses a magnetic particle format ELISA kit from Modern Water for PAHs. If the fungi grown in Lessons 2 and 3 are effective at degrading Phenanthrene, the PAH concentration of the contents of each Treatment jar should be less than the Control jars. We must be careful to extract the contents of jars fully. If we only succeed in rinsing Phenanthrene off the surfaces of rye berries or fungi, we will miss any Phenanthrene that was absorbed by rye and fungus tissues *without being degraded*. This would lead us to overestimate the bio-remedial efficiency of the fungus.

Materials

Spectrophotometer or colorimeter set to a wavelength of 450 nm
Magnetic rack for use for magnetic particle format ELISA
Modern Water RaPID Assay® PAH kit*
Adjustable Pipettor with 1000-microliter tips
Kitchen blender with glass receptacle an steel blades
Vortexer (this procedure is not appropriate for manual shaking)
Thirty-three 3-mL (1-cm path length) plastic spectrophotometer cuvettes
Twelve clean small glass vials with Nylon or Teflon caps (20 mL are best)
Twelve 20-mL disposable plastic Luer-lock syringes
Twelve 0.45-micron PTFE Luer-lock syringe filters
100 mL glass graduated cylinder for Methanol and distilled water

Extraction

PAHs dissolve only weakly in water but readily in methanol; a 50:50 solution of methanol and distilled water is ideal for extracting PAHs for environmental samples. To begin, prepare 1 liter of this diluted methanol solvent. Use this solvent to clean cuvettes and vials before and after each use, and for extracting Phenanthrene from rye and fungi.

Open the Treatment and Control jars and add 50 mL diluted methanol to each jar. Close jars securely and shake for two minutes. Allow contents to settle for five minutes. This should suffice to dissolve any Phenanthrene sticking to the surfaces of rye, fungi, or glass. Pour the contents of each jar in turn into the blender, add an additional 50 mL of diluted methanol, and run the blender for a minute at low speed: chop the rye and fungi into small bits but do not homogenize them. Pour the finely chopped contents back into the original jar, close it tightly, and let it settle overnight in a refrigerator. Rinse blender receptacle with diluted methanol after each use.

On the second day, remove jars and the ELISA kit from the refrigerator. Pull each syringe apart, mount a fresh syringe filter on the barrel by screwing it in and then place the syringe-mounted barrel and plunger beside a clean glass vial. Pour 10-20 mL of clear liquid from the top of each jar into a filter-mounted syringe barrel, insert the plunger, and firmly but slowly press the plunger to express clear filtrate into a glass vial. Cap the vials and dispose of the syringes and filters as solid waste. The remaining contents of jars can

* Kit includes test tubes and reagents. Keep it refrigerated, not frozen, at all times except the day it is used. Modern Water sells two PAH assay kits, one that is standardized to Phenanthrene that we recommend here, the other for carcinogenic or cPAHs standardized to BaP, a very toxic species that should not be handled by students. www.modernwater.com/assets/downloads/Factsheets/MW_Factsheet_Rapid%20Assay_PAH_highres.pdf

also be disposed of as solid waste; the jars themselves can be rinsed with methanol for re-use.

At the beginning of Lesson 4 you added 0.1 milligrams Phenanthrene to each jar, equivalent to 100 micrograms (100 μg). The extraction used 100 mL of diluted methanol, thus the extracts have a maximum possible concentration of 100μg in 100 mL or 1 μg/mL Phenanthrene, equivalent to 1 mg/L or one part per million. The upper limit of the ELISA assay method you will use is about 50 parts per billion (= 0.050 ppm). To be certain that the extracts are reliably measurable, they should be diluted at least 1:10 in 50% Methanol before carrying out the ELISA procedure!

Measurement

Insert 33 test tubes from the ELISA kit into the magnetic rack. Separate the rack, setting the magnetic base aside.

Set the Pipettor to 200 microliters. Beginning with the first test tube in the rack, add 200 microliters of the Diluent/Negative Control to tubes 1 and 2; 200 microliters of Standard 1 (0.1 parts per billion) to tubes 3 and 4; 200 microliters of Standard 2 (1.0 parts per billion) to tubes 5 and 6; 200 microliters of Standard 3 (5.0 parts per billion) to tubes 7 and 8; and 200 microliters of Positive Control (2.0 parts per billion) in tube 9. Change pipette tips whenever you change what you are measuring, to avoid cross-contamination.

Now add 200 microliters of the filtered extracts from Treatment and Control jars to pairs of test tubes, beginning at test tube 10: from the first jar to tubes 10 and 11, from the second to tubes 12 and 13, and so on. Change pipette tips each time that you change vials of methanolic extracts.

Using the pipettor, add 250 microliters of Enzyme Conjugate to each test tube. A single pipette tip can be used for this task.

Swirl the bottle of Magnetic Particles to mix them thoroughly (do not shake—it will cause foaming). Re-set your pipettor and add 500 microliters of particles solution to each tube. A single pipette tip can be used for this task. Swirl the bottle gently every two minutes so that magnetic particles do not settle out.

Vortex each tube for a few seconds, then incubate the test tubes for 20 minutes at room temperature.

Now re-combine the two parts of the magnetic rack. The magnets will draw all of the magnetic particles to the sides of the test tubes and hold them there as long as the rack remains together.

After 2 minutes, invert the rack (holding the two parts firmly together) over a sink to pour out the liquid contents. Blot the lips of the tubes on a paper towel.

Add 1 mL of Wash Solution to each test tube. Vortex each tube, wait 2 minutes, then pour out the contents and blot again.

Add 1 mL of Wash Solution to each test tube once again. Vortex each tube, wait 2 minutes, then pour out the contents and blot. Now separate the magnetic rack and keep it separated for the remainder of this procedure.

Use the pipettor to add 500 microliters of Color Solution to each test tube. Vortex all of the tubes, and incubate them for 20 minutes at room temperature. During this brief rest, turn on the spectrophotometer, set it to 450 nm, and blank it on a cuvette filled with Wash Solution. Meanwhile, the tubes will develop a blue color, inversely proportional to the concentration of Phenanthrene.

Use the pipettor to add 500 microliters of Stop Solution to each tube. The color of each tube will instantly change from blue to yellow. As quickly as possible, measure the Absorbance of the contents of each test tube by pouring it into a cuvette, and inserting the cuvette into the spectrophotometer. Write down the Absorbance for each tube.

Average the results from each matched pair of standards, controls and samples. If any measurement seems much too high or too low, you can also reject it as an outlier, and just use the paired measurement.

The mean Absorbance for the Negative Controls (test tubes 1 and 2) is called Bo. Now calculate the %B/Bo for each of the three Standards. If for example Standard 2 has a mean Absorbance of 0.8397 and the mean Absorbance of the Negative Control is 1.204 then the %B/Bo for 1.0 parts per billion Phenanthrene is 0.8397/1.204 x 100 or

Plot the %B/Bo for the 0.1, 1.0, and 5.0 parts per billion Standards. The X-axis of the graph is Concentration of Phenanthrene, and the Y-axis is %B/Bo at each of the three Standard concentrations. This is the calibration curve for interpreting the %B/Bo of each of the samples.

It is well worth using Excel to plot the calibration curve because it will instantly derive the best-fit equation, which you can then use to compute the Phenanthrene values for each of your samples. Usually the best fit will be a natural logarithmic function.

Remember that the Phenanthrene in each experimental jar was dissolved in 50 mL of diluted methanol. Since we added exactly the same amount of Phenanthrene to each jar at the beginning, and extracted what was left into the same amount of methanol—and our hypothesis relates to *relative* concentrations of PAHs in Treatment versus Control jars—it is unnecessary to back-calculate from the PAH concentrations measured in the extracts, to PAH concentrations in the biomass contained within each jar. However, if there were visible differences in the amount of fungal biomass amongst the Treatment jars, it would be interesting to compare them to differences in measurable bio-remediation!

If the %B/Bo of any of your samples suggests a Phenanthrene concentration over 10 parts per billion, you may want to run those samples again after diluting the remaining extracts to 25% with diluted methanol.

A simple example of data and calculations is attached: students used an ELISA kit to ascertain the mean body load of PAHs in tent caterpillars in an urban wetland, before it was turned into a runoff detention pond. They hypothesized that caterpillar body loads would increase as more contaminated water was diverted into the wetland, and this meant more PAHs would be transferred out of the wetland to insectivorous birds and bats. Note that one measurement of Standard 3 was anomalous and was “thrown out” of calculations as an outlier.

PAH loading of caterpillars: an example of ELISA calculations

PAHs by ELISA		Absorbance at 450 nm					
				Mean		ppb	%B/Bo
Calibration	0.0	0.6809	0.6942	0.6876		0	
	2.0	0.5880	0.6065	0.5972		2	86.9
	10.0	0.5163	0.4925	0.5044		10	73.4
	50.0	0.6575	0.3564	0.3569		50	51.9
Control	25.0	0.4009	calculates to 31 ppb				
Blank (MeOH)	0.0	0.7561	calculates to ND				

Ln(Concentration)= (%B/Bo - 95.77)/-10.873

	grams					Calculated	
Samples	**Biomass**	**Absorbance**		**Mean**	**%B/Bo**	**ppb**	**Adj Mean**
1	0.66	0.2711	0.3347	0.3029	44.1	116	176
2	0.77	0.2467	0.2253	0.2360	34.3	285	370
3	0.75	0.3148	0.2942	0.3045	44.3	114	152
4	0.78	0.2527	0.3027	0.2777	40.4	163	209
5	0.55	0.2870	0.2377	0.2624	38.2	199	362
6	0.51	0.3014	0.3001	0.3008	43.7	120	235

SD of all values 0.0338

Normalized to one gram of tissue

Mean concentration for all six larvae: 251