Drought experiment protocol – vers. 4.0

The Bromeliad Working Group.

PURPOSE

At each field site:

(1) To establish the relationship between precipitation features (total amount and temporal aggregation) and bromeliad community structure and functioning. Are there thresholds? Do different components of the food web respond differently? Do different functions respond differently?

(2) To compare the limits of bromeliad resilience (in both community structure and function) to altered precipitation features to (a) current inter-annual variability in these features and (b) future, projected inter-annual variability in these features. Will climate change going increase the frequency (probability) of exceeding resilience limits?

Between field sites:

(3) To determine whether there is broad congruence in the relationships between precipitation features and bromeliad structure and functioning, or whether the relationships are context-dependence. If there is context-dependence, can we explain some of this dependence in terms of either biogeographical difference in the species pool or bioclimatic differences in ambient precipitaion features?

(4) To determine whether differences between fieldsites in the probability of exceeding resilience limits due to climate change is primarily due to differences in the underlying precipitation-ecosystem functions, or regional differences in climate change scenarios.

In addition to these aims of the combined multi-site analysis, individual researchers are encouraged to add additional treatments to answer site-specific questions. This will allow each researcher to publish a first author publication centered on their own question. for example, Ignacio Barberis plans to examine how phenotypic plasticity of the bromeliads affects responses, Diane Srivastava plans to build a model of precipitation dynamics in bromeliads over the year to determine the frequency of drying directly. Obviously it is best if we minimize overlap between researchers on these site-specific questions.

LOCATION AND TIME OF EXPERIMENT

Ideally the experiment would be conducted in the same habitat in the same season in each field site. However, this is not possible. If you have the option of forested versus open sites, there is a preference for forested sites as the majority of field sites are forested. In terms of time of year, aim for when there is a high abundance of invertebrates to maximize potential impacts.

TREATMENTS

Each bromeliad will represent a different precipitation treatment. The precipitation treatments differ in two ways: the mean amount of rainfall per day (mu) and the frequency of large versus small rainfall events (k, the dispersion parameter). All treatments are expressed relative to the average current mu and k, therefore "mu0.1k0.5" means that the mean rainfall of this treatment is 1/10 of the normal rainfall, and that the rainfall is half as dispersed among days as normal.

The chart below describes the 30 precipitation treatments in terms of mu and k, where mu and k are to be calculated from past precipitation records as described shortly. IMPORTANT: Let us all label our treatments the same way, following this chart, to avoid later confusion. These treatment labels have been integrated into the R script described shortly.

	Mu*0.1	Mu*0.2	Mu*0.4	Mu*0.6	Mu*0.8	Mu	Mu*1.5	Mu*2	Mu*2.5	Mu*3
K*0.5	Mu0.1k0.5	Mu0.2k0.5	Mu0.4k0.5	Mu0.6k0.5	Mu0.8k0.5	Mu1k0.5	Mu1.5k0.5	Mu2k0.5	Mu2.5k0.5	Mu3k0.5
K	Mu0.1k1	Mu0.2k1	Mu0.4k1	Mu0.6k1	Mu0.8k1	Mu1k1	Mu1.5k1	Mu2k1	Mu2.5k1	Mu3k1
K*2	Mu0.1k2	Mu0.2k2	Mu0.4k2	Mu0.6k2	Mu0.8k2	Mu1k2	Mu1.5k2	Mu2k2	Mu2.5k2	Mu3k2

SELECTION OF BROMELIADS

Thirty bromeliads should be selected for the experiment. Aim for the most common size in your study area, so that the results are representative. We cannot attempt to standardise bromeliad size amongst study areas, given the large range in bromeliad sizes amongst field sites.

Bromeliads selected within a field site should be as similar in size as possible! Bromeliad size has effects on drying rates, water depth and species interactions, and so can confound our treatments (using bromeliad size as a statistical covariate in analysis can only partially help here given the complexity of these size related effects).

The most biologically relevant metric of bromeliad size is maximum volume ("capacity"), as it typically has the strongest correlations with invertebrate abundance and richness. Maximum volume can be roughly estimated in the field from a few physical measurements (number of leaves, longest leaf or plant diameter) using previously established allometric relationships.

Example: In Costa Rica we will mark 70-80 bromeliads, and record number of leaves and leaf width of each plant. We will then estimate maximum volume of each plant using equations previously developed for this field site (leaf width is measured at base of leaf):

Guzmania spp. *maximum volume=exp(-0.756521+0.078921*number of leaves+0.606163*leafwidth in cm)*

Vriesea spp.*maximum volume=exp(1.7695+0.034311*number of leaves+0.379343*leafwidth in cm)*

We will then chose 30 bromeliads that have similar volume, with an aim for volumes > 100 ml that contain the top predator, an odonate.

PRECIPITATION MANIPULATION

In general terms, these are the steps:

1. Obtain daily precipitation records for your fieldsite (or nearby), for a number of recent years, for the 60 day period (exact dates) that you want to run the precipitation manipulation (*e.g. in Costa Rica we are running the manipulation for Oct 9-Dec 7, so we obtained precipitation records for this 60 day period for the last five years. Note that the 60 days includes Oct 9 and Dec 7th. Data does not exist for earlier years*). When calculating 60 days, note that you include the final date (there are some date duration calculators on the internet that help avoid making mistakes here). Please note that the total experimental is longer than the 60 days – it is actually 68 days long, and likely requires a 2 week set-up period and a 1 week take down period.

2. Email Andrew MacDonald (macdonald@zoology.ubc.ca) a csv file of the daily precipitation in mm for this 60 days period for all the years you have (one column per year). We are happy to send you the R code so you can understand exactly what it does, but requires more computer power than the average laptop – we are running it on the UBC server- this is why it makes sense to have Andrew run this script for all groups. The R code fits the negative binomial model for each year, and estimates the average of two parameters: the mean (mu) and the dispersion parameter (k). Note that some texts (e.g. Crawley) more accurately refer to k as the clumping parameter. These two parameters describe your ambient or baseline scenario.

What the R script does (please see Andrew's pdf file for more exact descriptions):

- **The R script uses the ambient mu and k to first calculate all 30 experimental combinations of mu and k**. For each of these 30 combinations, the script then calculates the exact frequency of days with each precipitation level . Note that we explicitly do not use a random function to do this - we are not trying to "simulate" the distribution - because this just adds noise to the frequency estimates. Instead we have worked out how to convert a probability distribution with an infinitely long positive tail into a discrete frequency distribution.

- Next the R script shuffles the temporal order of rainfall in the ambient treatment until **we optimize the resemblance to the temporal autocorrelation structure of our real precipitation records**. Specifically, we use a method developed for the short census length of populations (60 days is also "short" in the world of temporal statistics) where the SD of the response variable for a certain number of consecutive days is plotted against the number of consecutive days ("census window"). We then minimize the deviation from actual and simulated relationships between SD and census length.

- **The R script rearranges the rainfall order** in each on the 29 non-ambient treatments so that the rank order of rainfall events is the same (e.g. the largest rainfall for a treatment occurs on the same day in all treatments, as does the smallest rainfall). This minimises the potential for random variation between treatments in the order of rainfall events to lead to high noise in ecosystem response. It also ensures that the temporal order of rainfall in all treatments is as natural as possible given the constraints of the selected mu and k parameters.

- **The R script also divides the 30 treatments into 3 blocks of 10.** The script randomly selects ten treatments to start on day 1, another ten on day 2, another ten on day 3. This jittering of start dates will allow enough time to sample invertebrates at end of experiment (10 bromeliads per day). Furthermore, the script checks with an ANOVA after randomly partitioning your 30 treatments into 3 blocks that there is no trend for mu or k to differ between the 3 blocks! A different randomization sequence is used for each fieldsite.

- **The R script adds days to the beginning and the end of the 60 day rainfall period.** All bromeliads begin with the median amount of rainfall of the ambient treatment, and the day after some pre-treatment samples are taken ("sample") before bromeliads begin to experience different rainfall scenarios. At the end of the rainfall manipulations we add on a day of rainfall equal to the median of that treatment. Why? We realize that the very last rainfall day may have substantial influence on results like water chemistry concentrations, so we wanted to ensure that the experiment ended with each bromeliad receiving a representative rainfall amount. To be clear, there will be 30 different median values in this experiment, one per bromeliad. The next day, is another "sample" day for water chemistry and micro-organisms, and then two days later is "insect" day when the bromeliad is harvested and the insects (etc.) quantified.

3. Once at your fieldsite, convert mm of water from Andrew's watering schedule to ml of water. You require two other bits of information for this: (1) A "combined correction factor", and (2) Mean catchment area (one value for all bromeliads). These are explained in the next section.

We will use these values to convert the depth of precipitation needed on any given day for any given precipitation scenario into volume as follows:

Volume of water to add to bromeliad = Mean surface area * Combined correction factor* Depth required for scenario

> *Example: In Costa Rica, we knew that the average catchment area of our bromeliads, from Image J analysis was 1622 cm^2, and the rainfall for our first day was scheduled to be 0.65 cm, and the combined correction factor was 0.34, so we concluded that 1622*0.65*0.34 = 357 ml of water needed to be added on the first day.*

Why are we not using the exact surface area for each bromeliad? There is enough variation in the experiment without adding further noise. Measurements of surface area have a lot of noise associated with them (e.g. the relationship between bromeliad surface area and effective catchment area is fairly inexact) and this error would be propagated into estimates of volume for each bromeliad. A better method is to minimize estimation error by taking the average of many estimates, and simply put bromeliad size in the analyses as a covariate to account for residual differences between bromeliads. In Costa Rica we carefully trimmed the bottom few leaves off bromeliads that were slightly larger than our desired volume until the desired capacity was reached.

> *A caution: as described shortly, there is a 3 day pre-rainfall schedule period, and a 3 day post-rainfall period, plus the blocks are staggered so the actual length of the experiment is 68 days, that is, longer than the 60 day rainfall schedule.*

FIELD SET-UP AND MONITORING OF EXPERIMENT

<u>Before the start of the experiment:</u>

1. **As much prior to the start of the experiment as possible, plant small bushes or saplings in pots, and water daily with 15N-nitrate salts**. A shrub < 1m tall should receive daily 50 ml of a labelled ammonium sulfate solution made by dissolving 5 g of Ammonium $^{15}N_2$ sulfate 10 atom % in 1 L of water for 30 days. Of course, if your plants require more total water, add unfertilized water as needed to keep the plants happy. Choose a species ("detritus species #1") whose leaves are similar to the tree leaves that enter bromeliads in your study area, and which you have determined will be consumed by detritivores. Net the bushes in order to catch any leaves that senesce and fall off. Obtain a small sample of each of :

    - Bush/sapling leaves (> 2g dry wt) **prior to 15N** fertilization to determine natural isotope signature in the detrital species. The detrital leaves should be dried at about 50 degrees C and kept in a dry envelope for isotopic analysis. (n=3). Humid fieldsites may need to keep dried material with packets of silica gel in an airtight bag.

    - Bush/sapling fertilized leaves **immediately prior to adding them to the experiment**. The detrital leaves should be dried and kept in a dry envelope for isotopic analysis. (n=3)

- Select three bromeliads not used in the experiment. If you label the central 3 leaves of the bromeliad as "leaf 1", "leaf 2", "leaf 3", please cut and collect leaf 2 and 3 to know background level (n=3). The leaves should be gently washed with distilled water to remove any epiphyll growth, dried with a clean paper towel, and dried at about 50 degrees C. Keep in a dry envelope for isotopic analysis.

> *Example: In Costa Rica we will be fertilizing 8 1 m tall saplings of a pioneer tree Conostegia xalatensis that we know the insects eat, and that we have successfully used for ^{15}N enrichment before. We have purchased 70 grams of labelled ammonium sulfate, which will make 14 L of solution, which will provide 35 days of fertilization. Approximate cost $800. We mailed the powder to our fieldsite in advance as prohibited on aircraft (fertilizers are used in bombs).*

2. **Determine which species of dead leaf ("detritus species #2) is favoured by bromeliad detritivores.** This does not need to be the same species that you are using for the ^{15}N fertilization, but of course can be! This can be done in several ways. First, you may already have experience studying detrital decomposition in your system, and have determined which detrital species are preferred. Or you could use observation. Finally, you could do a short (one week) test by placing a few common detritivores in tubes with a specific leaf species, repeated for other candidate leaf species, and determining which leaf species is preferred. Use this species for leaf packs (otherwise may get no detrital processing, as has happened to DS lab before!).

3. **Determine the catchment area of your bromeliads using image analysis.** To do this, place the bromeliad to be photographed in a container so that it stands upright with its leaves naturally positioned. Place next to it a ruler or other item of known length. If your bromeliad species has leaves that bend, such that water only falling on one side of the inflection point would drain towards the center, you will need to mark this point on the leaf so that you only include the appropriate part of the leaf in the total "catchment" area of the bromeliad. We have found that short pieces of white string are the fastest way to mark this on each leaf. Take the photograph looking straight down on the bromeliad and ruler, by standing on a chair. Use Image J to calculate the surface area of the bromeliad from the photograph (Appendix 1).

4. **Combined correction factor. This requires rainfall, so ensure that you leave enough time to do this before your experiment is scheduled to start.** Simply multiplying mm of rain by catchment area of the bromeliad overestimates the volume collected by the bromeliad. This is because precipitation is measured in an open area, but often bromeliads are under trees – and the canopy intercepts some of the rain. In addition, not all of the rain that hits the bromeliad leaf runs down the leaf – some is simply deflected off the plant by the force of impact. In Costa Rica we calculated that the canopy reduces rainfall by 30%, and that the deflection effect reduces rain entry into the bromeliad leaf wells by a further 33%. In other words, rain entering bromeliad = 0.34*rainfall in schedule. It would be better to calculate this value yourself, rather

than use ours, at it will be quite site specific. Here is how we calculated the above numbers with a team of 4 people. We waited until it was raining. One person had two buckets with a known opening area, which they placed in the open. One person had 10 pots of known opening area which they placed over a few square meters in the forest. Two people had a bromeliad each, whose "catchment area" had been previously measured by photography. All of the above was protected from the rain (inverted) until a whistle blew. Then all containers and bromeliads were allowed to catch rainfall, until a bromeliad was close to overflowing, at which point the whistle blew again. The 10 cups were placed in a bucket and covered immediately, the bromeliads covered by umbrellas, and open bucket also covered, and all were brought to the station to have the volume of collected rain measured. We repeated this several times and took the average of the following values (surprisingly consistent, save one outlier).

Forest mm rain fell = volume of rain in 10 forest cups/ total surface area of 10 cups

Open mm rain = volume of rain in open buckets/total surface area of buckets

Actual bromeliad catchment = bromeliad vol rain captured/forest mm rain

Canopy effect = Forest mm/Open mm

Deflection effect = actual catchment area/Image J catchment area

Combined correction factor = canopy effect * deflection effect

5. **Equalise abundances of the major invertebrate groups, and detritus amounts, between bromeliads.** For each bromeliad, wash out its contents as completely as humanly possible, and capture them in a bucket. Sieve the contents of the bucket, and sort the sieved contents into detritus and each of the main invertebrate families (DS uses sieves of size 850 um and 150 um to do this, but use what you have). Sorting to species will probably take more time than we have, and the aim is to only have the community structure approximately equal – oviposition during the experiment will add noise to initial compositions so little point in having them identical (can't estimate species-specific mortality in an open system), hence the recommendation to sort only to family. Keep the invertebrates in pots with lids, in water with a bit of detritus. Avoid overcrowding, especially for tipulids (high viral load, keep singly if possible). Tabanids prefer damp paper towel, predators should be fed every day. Keep detritus damp, do not cover. The aim is to sort coarsely to be able to complete this step in <5 days (to minimize invertebrate death). When you have sorted all 30 bromeliads this way, divide the total amount of detritus by 30, and the total invertebrate numbers in each family by 30.

Example: in Costa Rica we have budgeted 3 days for 7 people to complete this task. Each day we collected 10 bromeliads from the forest, took their photo for Image J, and washed out contents using a hose while holding each plant upside down over a giant funnel. We estimated average abundances of each family to put in each bromeliad, and will initially put 50% of this amount in, as mortality of insects while in captivity may reduce the amount that we have by the time the third block is assembled. Any insects remaining after 50% added were divided between bromeliads and supplemented by additional individuals obtained by pipetting further bromeliads. If we did not have enough of a particular family (e.g. 27 odonate individuals but 30 bromeliads) we also used supplemental organisms from pipetting.

6. **When the bromeliad is empty, measure maximum water volume.** Pour a known amount of water into a bromeliad until it overflows, capture and measure the overflow. The volume of water in the bromeliad is water added minus water overflowed.

7. **Reassemble bromeliads with identical quantities of detritus and invertebrate composition (at the family level).**

8. **Construct rain shelters over each bromeliad, in the field.** Rain shelters will be made of wire and transparent plastic sheet (Appendix 2). Bromeliads should either be rooted in the ground or hanging from trees, as appropriate to your field site. Hanging bromeliads should be secured so that they cannot swing in strong winds and spill water.

9. **Add ibuttons.** ibuttons must be programmed prior to using; program each ibutton to record every hour for the duration of the experiment. Wrap ibuttons tightly in parafilm. Add at least one ibutton (ideally two) to the central tank in each bromeliad (ibutton failure rate = 5%, so we will likely be missing data from one or more bromeliads if you only use one). Use another three to measure air temperature during the experiment – place each under a small box with holes cut for air passage, with the ibutton about 20 cm aboveground. Total ibuttons required = 63.

10. **Make 60 leaf packs using detritus species #2 (APPENDIX 5).** I suggest that you dry and weigh two - three leaves first, then place them in water to make them flexible, and then tie together the three leaves with the finest (thinnest) possible monofilament (fishing line) sewn along teh middle vein to create a leaf pack. If you attempt to sew together the leaves when they are dry the leaves will fragment.

11. **Start the watering schedule.** Remember that ten randomly selected treatments will start on day 1, another ten on day 2, another ten on day 3 (see step 7 of Precipitation Manipulation Schedule. This will allow enough time to sample invertebrates at end of experiment (10 bromeliads per day).

12. **Fill each bromeliad, on the date as indicated in the watering schedule, with non-chlorinated water.** The very first day of rainfall in each bromeliad, in the schedule, is the median amount of “rain” that the ambient treatment will receive This starts all bromeliads off the same, so we can have a pre-treatment period for bacteria and protists.

<u>At the very first “sample” day on the schedule, one day after the first addition of water:</u>

1. **Take turbidity, O2 andpH readings with handheld meters.** It is good practice to take measurements from at least two leaf wells, avoiding the central well or dead leaf wells. The oxygen meter reading usually takes a while to stabilize, so expect to spend a few minutes per bromeliad obtaining these reading. Only after this step is finished, should you go onto the next step that disturbs the water.

2. **Sample water from each bromeliad for protists, bacteria and chlorophyll-a (fluorometer method only).** You will mix up the water in a leaf well as follows: use a small pipet to suck up as much as possible of the volume of water in the leaf well, quickly to shoot the water back out of the tip so as to homogenise the contents of the leaf well, repeat another 2 times, and end by removing a few ml of water that you will put in a container. Repeat for another 9 or so leaves in the bromeliad, until you have about 20ml of water total that is a good representation of the water in the bromeliad. Avoid the central tank and dead outer leaves if possible, as they often have unusual water chemistry.

3. **For the protist sample**, stir the water collected in the above step, then remove 1ml and place in a 1.5 ml micro-centrifuge tube. Add enough drops of Lugol's solution that the sample is deep brown. If the samples become pale over time, more Lugol's should be added. We recommend that you also physically scratch the bromeliad ID on each micro-centrifuge tube, as if one tube opens the Lugols will dissolve all ink written on all tubes.

4. **For the chlorophyll-a sample (fluorometer method only)**, stir the water collected two steps above, and fill a cuvette with the water sample (approx. 2-3 ml). Place in a fluorometer.Wait exactly 2 minutes for the sample to settle and obtain your reading. If your fluorometer is equipped with a turbidity channel (420 nm), also obtain that reading now. If you do not have a fluorometer, you likely will not be able to measure chlorophyll-a at the beginning of the experiment (the filter method requires removal of up to 50 ml of water, which may be too great a perturbation for your bromeliads depending on their size– this will need to be judged on a site-by-site basis).

5. **For the bacterial sample,** stir the water collected three steps above, then remove 9 ml and place in a 10 ml cryovial (a vial suitable for -70degrees C). Add enough formaldehyde-Borax solution to obtain a final formaldehyde concentration in the sample of 4% (usually 1 ml: APPENDIX). If you don't have enough water for this, you can do epifluorescence on less volume (as little as 3 ml), but it is risky – there will not be any ability to repeat measurements. Bacterial abundance will be assessed by epi-fluorescence microscopy in the lab.

6. **Place two leaf packs in each bromeliad.** Leaf packs should be placed in each of two (marked) middle tanks of the bromeliad, avoid the central tank or dead bromeliad leaves.

<u>Precipitation manipulation</u>

1. Add water daily to each bromeliad according to bromeliad's treatment schedule. Water must not be chlorinated or contain insects. The amount of water to add is average bromeliad catchment area x canopy correction factor x precipitation of that day in that treatment. Water to be divided amongst leaves (roughly, by eye) and must be added slowly to avoid washing out insects more than a normal rainfall would. When more than a liter of water is to be added, we recommend dividing the total amount into a morning and evening addition. A small watering can with a long thin spout is very useful.

During the experiment:

1. **Measure water depth in central tank and two (marked) outer leaves in each bromeliad, every day if possible (at least every two days).** To be clear, record the identity of the leaf and bromeliad next to the depth measurement on each day, so that we can use leaf identity as a random factor in the analysis.

2. **Record daily precipitation and max/min temperature for your fieldsite.**

3. **Observe and record spiders and any other terrestrial predators.** This can be done as a visual survey every week, where the abundance of obvious (visible without disturbing bromeliad) spiders are recorded, and any other predator (though non-spider predators are usually impossible to see). Coarse taxonomy is fine here (e.g. record spiders as web building, hunting or jumping). We are expecting a shift from aquatic to terrestrial biota with our treatments, and this is one way to quantify this.

4. **Add 15N enriched leaves (detritus species #2) at midpoint of the experiment.** Enriched leaves should be dried and weighed prior to addition. Add as many leaves as possible, as long as each bromeliad has an equal mass of enriched leaves added. As a rough guide, aim to add 10-25% of the detrital amount already in the bromeliad. Ideally enriched senescent leaves that have fallen in the net would be used, but you will likely not have enough and need to supplement these with enriched green leaves too. Gustavo Romero has had success using green leaves from the enriched Pitanga plants, dried and weighed prior to addition.

At the end of the experiment:

"Sample" day

1. The day after median rainfall is marked "sample" on your watering schedule. This means that it is the day to sample for CO_2 and methane, turbidity, oxygen, pH, protists, bacteria and (optionally as mentioned below) chlorophyll-a.

2. ***CO_2 and methane*. Collect at dawn (just before the sun rises)before disturbing bromeliad with any further sampling.** If this is not possible, it can also be collected at dusk (just as the sun sets) the preceeding day. Water is collected in a glass syringe with no air bubbles, and injected into a vacuum tube ensuring no headspace. If water samples are immediately put in a cooler, it is possible to fly with the samples and have them analysed in an infrared gas analyser, although there is some risk: the vacuum conditions mean that the glass vials can break if the water gets too cold (expansion). Samples should be analysed within 72 hours of collection. Trisha Atwood is willing to collect samples, and fly them back to UBC for analysis (estimated cost: $2000 per field site because of flight, analyser, and stipend costs). These water samples should be done by someone with some training – pretty tricky to do correctly. 3ml water sample for each of CO_2 and methane, ideally with repeats.

3. **After the bromeliads have been undisturbed for at least two hours, take O2 and pH readings with handheld meters.** It is good practice to take measurements from at least two leaf wells, avoiding the central well or dead leaf wells. Only after this step is finished, should you go onto the next step that disturbs the water.

4. **Sample water from each bromeliad for protists, bacteria, turbidity** and (optionally) chlorophyll-a . You will mix up the water in a leaf well as follows: use a small pipet to suck up as much as possible of the volume of water in the leaf well, quickly to shoot the water back out of the tip so as to homogenise the contents of the leaf well, repeat another 2 times, and end by removing a few ml of water that you will put in a container. Repeat for another 9 or so leaves in the bromeliad, until you have about 20ml of water total that is a good representation of the water in the bromeliad. Avoid the central tank and dead outer leaves if possible, as they often have unusual water chemistry.

5. **For the protist sample**, stir thewater collected in the above step, then remove 1ml with a micropipet and place in a 1.5 ml micro-centrifuge tube. Add enough drops of Lugol's solution that the sample is deep brown. If the samples become pale over time, more Lugol's should be added. We recommend that you also physically scratch the bromeliad ID on each micro-centrifuge tube, as if one tube opens the Lugol's will dissolve all ink written on all tubes.

6. **For the bacterial sample,** stir the water collected three steps above, then remove 9 ml and place in a 10 ml cryovial (a vial suitable for -70degrees C) or split between two 5 ml cryovials. Add enough formaldehyde-Borax solution to obtain a final formaldehyde concentration in the sample of 4% (usually 1 ml: APPENDIX). Again, you could measure bacteria on a lower volume of sample (say 3 ml), but it is risky – no ability to redo a slide. Bacterial abundance will be assessed by epi-fluorescence microscopy in the lab.

7. **Optional step. You may elect to measure chlorophyll-a with a fluorometer at this point, as well as on the "insect " sample day.** The advantage to doing this is (a) you then have fallback data in case there is an issue with the "insect"day chlorophyll-a data, and (b) you have chlorophyll-a data at the end of the experiment that is collected in the same manner as the initital chlorophyll-a data. **For the chlorophyll-a sample (fluorometer method only)**, stir the water collected in the step above, and fill a cuvette with the water sample (approx. 2-3 ml). Place in a fluorometer. Wait exactly 2 minutes for the sample to settle and obtain your reading. **If your fluorometer is equipped with a turbidity channel (420 nm), also obtain that reading now.**

Example: In Costa Rica, the CO_2 and bacterial samples will be carried in a cool box (on towels over ice in a cooler, not directly on ice) to a refrigerator in town as soon as they are collected, and then flown back to UBC in a cool box. The bacterial samples will be flash frozen with liquid nitrogen upon arrival, and then kept at -70 C until they can be quantified with epi-fluorescence microscopy. The CO_2 samples will be analysed at the Gas Analyzer facility.

“Insect” day

1. **In your watering schedule, two days after the “sample” day is another day marked “insect”.** This is the final harvest of the experiment, when you will collect bromeliad tissue, measure the water volume, obtain whole-plant chlorophyll-a measurements, retrieve ibuttons and leaf packs, and –most importantly – census the macroinvertebrate community.

2. **Before you do anything else, cut two bromeliad leaves (inner leaf 2 and 3, avoid very centre one) above the water line and place in a paper envelope to be dried at 50 to 60 degrees C.** We need enough tissue to measure nitrogen, so if the leaves are unusually small you would need to collect more. This tissue sample should be clean of debris and – especially – epiphyll species like bryophytes , and dried at 50-60 degrees C in its paper envelope. Please be careful not to dry at higher temperatures. Samples will be analysed for N15 and %N at either UC Davis or Cornell, and Angelica Gonzalez (agonzale@zoology.ubc.ca) can provide instructions on sample preparation in the lab.

3. **Next, remove the leaf pack.** This will be transported back to the lab in a plastic bag, any associated invertebrates and algae removed with a paintbrush, and the remaining detritus will be dried at 60 degrees C and weighed.

4. **Homogenize the water within leaf wells by sucking in and out with a pipet, and remove (with large mouth pipet to avoid harming invertebrates) and measure the entire amount of water contained in each bromeliad and place in a bucket**. **DO NOT ADD ANY WATER TO THE BROMELIAD AT THIS POINT.** This allows us to convert our protist and bacterial density measurements to per bromeliad amounts. Add any detritus still stuck in the bromeliad that can be pulled out with forceps. Stir, let exactly 2 minutes elapse, and obtain either 5 ml (fluorometer method for chlorophyll-a) or a 50 ml (filter method for chlorophyll-a) water sample for chlorophyll (if 50 ml not possible, you could use less if the water is green enough). If you are doing the filter method, please be sure to remove all insects from the water sample before filtering it! If you are using the fluorometer method, please remember that you will need to calibrate the fluorometer (the numbers on the fluorometer are only relative to a standard), which requires measuring various samples with known chlorophyll-a concentration. For example, you could use the filter method to estimate chlorophyll-a in a serial dilution of bromeliad water or pond water, and take fluorometer readings on the same samples. This standardisation needs to be done in the field, as fluorometers are very sensitive to any travel back to the lab. Exact protocol in Appendix.

5. **Remove ibuttons at some point in the procedure: IMPORTANT** - make sure that your ibuttons can be linked to the bromeliad treatment, for example by placing each in a labelled envelope, recording ibutton ID, etc.

8. **You have two options for obtaining insects from bromeliads.** The bromeliads could be sampled either by dissection (maximum efficiency, but destructive) or by washing (results in bias, and efficiency will vary with bromeliad architecture). You can now add any water needed to aid in this process. **Dissection method:** Return bucket and bromeliad to field station. Immediately sort the water in the bucket for macroinvertebrates (the aim is to mimimize the time predators have access to prey in the bucket), then carefully dissect the bromeliad leaf by leaf, washing all detritus off the dissected leaves into a bucket with a hose. Sieving the contents of the bucket through a stack of 850 and 100 um sieves seems to speed up sorting times by clearing the water (keep the material that passes through the 100 um sieve for FPOM, see note below. No insects are small enough to be in this); here the material on the 850 sieve is resuspended and sorted in one white tray, and the material on the 150 um sieve is resuspended in water and sorted in another white tray. **Pipet method.** The trick here is to continually rinse out the bromeliad with strong water pressure (e.g. held upside down over a large tub with a hose) until nothing more comes out. Vinicius suggests a final rinse with a water-vinegar mixture to get insects to "let go". It is useful to figure out what proportion of insects are missed by pipet method at your study site if you ever have the opportunity to use the pipet and dissection methods on the same bromeliads (any bromeliads, not necessarily the ones in this study).

9. All aquatic invertebrates found will be identified to **species or morphospecies**, and it is **important that either body length or size class noted**. The latter size measurements will be combined with pre-determined allometric relationships that convert size measures to biomass for each taxa/taxonomic group. If you do not have good allometric equations for your field site, you could dry some of the insects collected in this experiment to create a set (there are also general ones posted in the BWG DropBox, see Diane).

10. **FPOM dry mass**. Once insects have been removed from detritus, sieve all detritus through a 100 um sieve if you haven't yet done so, collect water and sediment that passes through this sieve, and filter using standard Whatman filter paper in funnels. Dry the filters + FPOM at 50- 60 degees C until dry. The filter paper should be previously dried and weighed (or else you'll be trying to scrape FPOM off filter paper to weight it – almost impossible)!

If you find that filtering the FPOM is taking forever, then here is a fast way to subsample (from Edd Hammill). Use Whatham GFC 25mm filters and a 50 ml syringe (as for chlorophyll). Sieve all sorted bromeliad water through a 100 um sieve. Add cleanwater until total volume (stuff from plant AND water) = 6000ml. Stir thoroughly and take a sample of this water in a cup (about 300 ml will be plenty). Dry the GFC filters, and weigh. Stir the subsample and fill the 50 ml syringe with it. Then, using the syringe, push water from stirred subsample through the filter until absolutely clogged, record the volume pushed through. Dry the filter + sample and re-weigh. Calculate the mass added by the FPOM. Divide the FPOM mass by the volume pushed through (this gives mass in 1ml of water). Multiply the mass per ml by 6000, this is the total weight of FPOM in the plant.

ANALYSIS

The overall analysis will be a three factor regression (site x precipitation mean x precipitation aggregation, all fixed effects) with the following response variables:

Invertebrates: total biomass, abundance, richness, predator:prey biomass

Microfauna: total abundance of each of ciliates, rotifers, amoeba

Bacterial density

Chlorophyll

Ecosystem functions: Detrital processing, Nitrogen uptake by bromeliad, CO_2 and methane (at sites where facilities exist)

Environmental conditions: Days without water, mean hydroperiod (consecutive dry days), temperature variation, mean temperature, mean water depth.

Covariates: bromeliad size.

We will look for thresholds by using non-linear functions in glm or gls, and possibly by using gam models.

Multivariate analyses (e.g. ADONIS, PERMANOVA) are needed for composition. We expect minimal overlap in invertebrate species between sites, so a better approach here is analysis at the family level and the functional group level. Ciliate species data may need to be analysed similarly, or else composition effects can be determined at the site level, and the responses qualitatively compared between sites.

APPENDICES

APPENDIX 1. How to use Image J to calculate the surface area of the bromeliad from the photograph

APPENDIX 2. Construction of rain shelters from wire and transparent plastic sheet.

APPENDIX 3: Leaf packs

APPENDIX4. Microbial Formalin solution

APPENDIX 5. Chlorophyll estimation.

Appendix 1: Bromeliad Catchment Area Using Image J

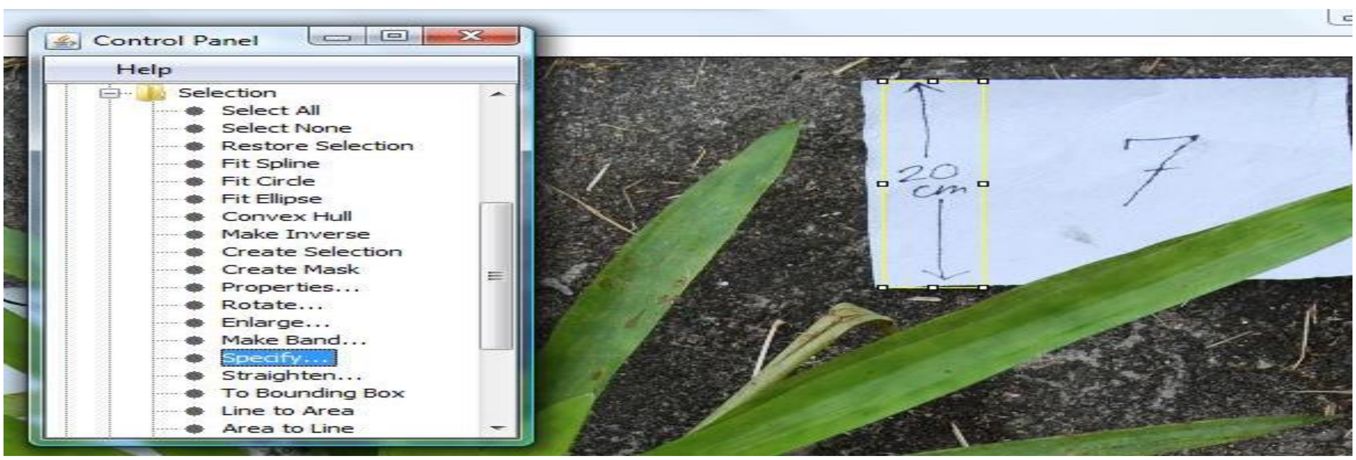
Here is an example of a bromeliad where string has been used to mark the point where water on the leaf drains towards the center of the bromeliad (i.e. limit of catchment area):

Below, we use the example of a bromeliad with rigid upright leaves, where the catchment area is the entire leaf surface, so we did not need to use strings.

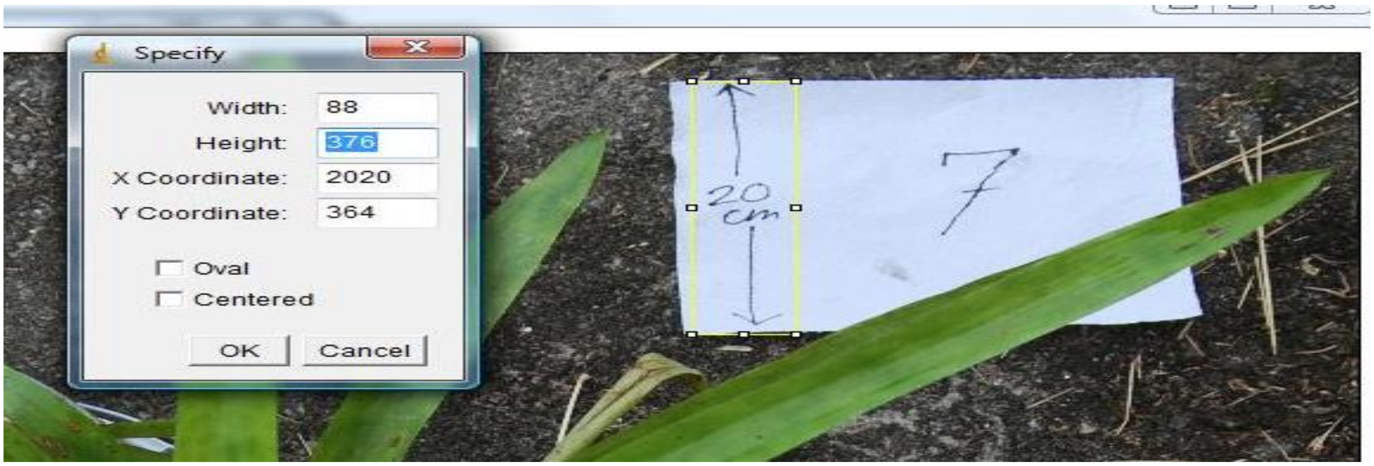
Open an image using the free software: Image J (http://rsbweb.nih.gov/ij/download.html).

1. Using an image that has a size reference, zoom in and select a box the height or width of the reference measurement using the rectangular drawing tool. Right click the box and select the **Control Panel**.

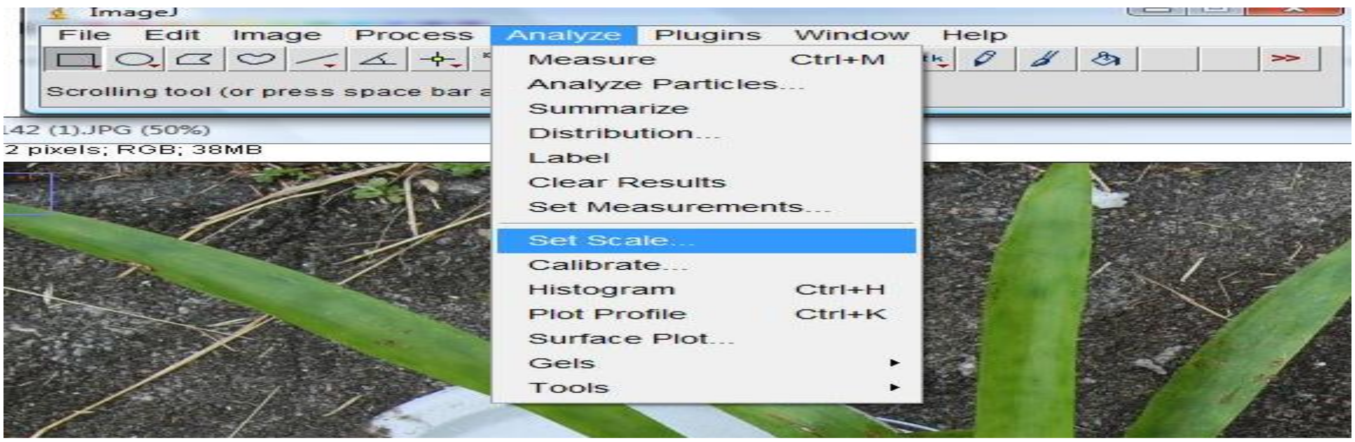
2. In the **Selection** folder, choose **Specify**.



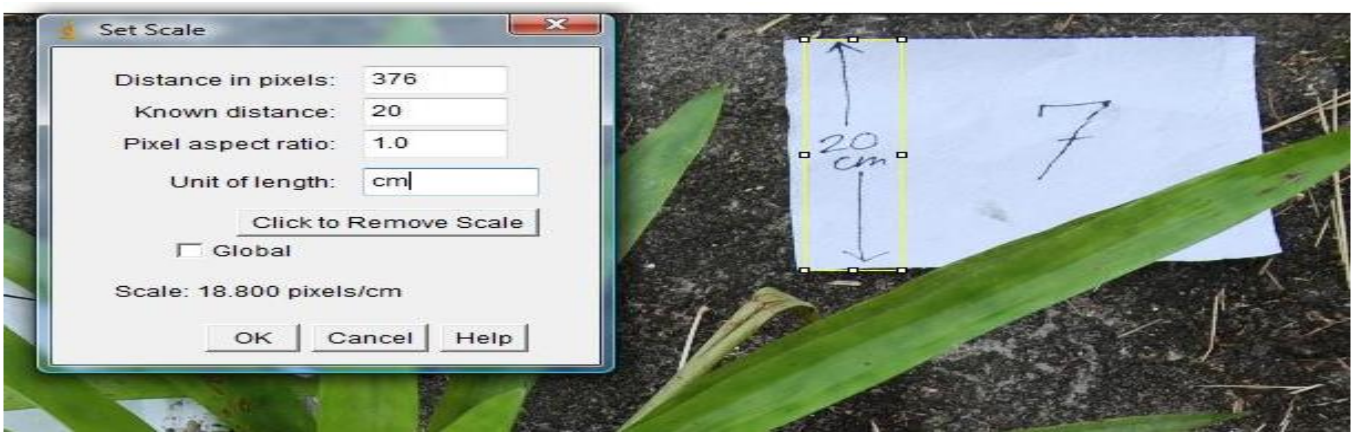
3. Choose the measurement specified based on the reference measurement in the image.



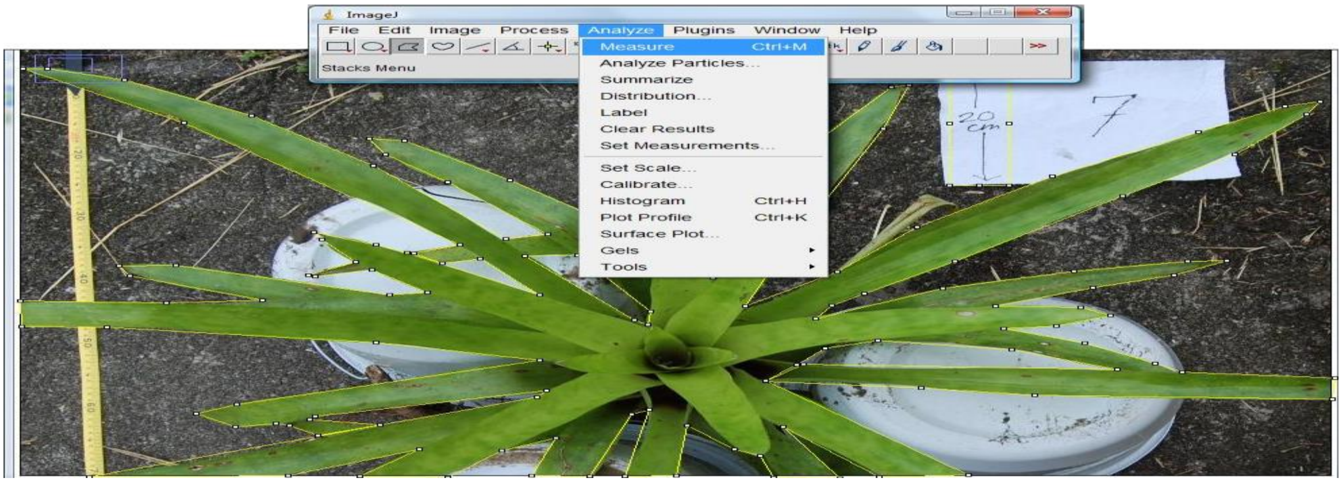
4. In the **Analyze** menu, select **Set Scale**.



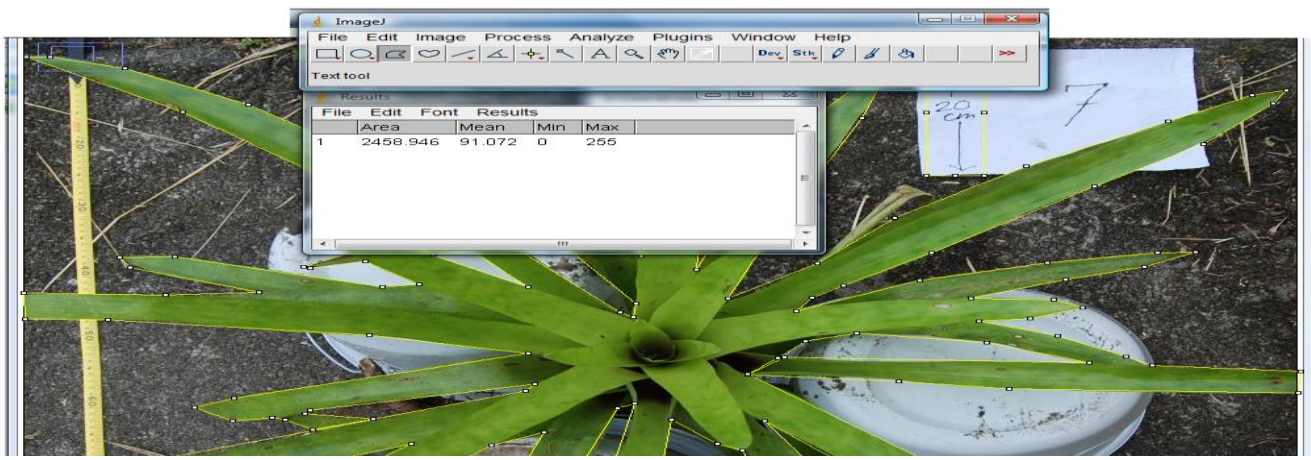
5. Enter the pixel distance from the Specify menu and the known distance from the image as well as the units used.



6. Use the freehand drawing tool or polygon drawing tool to select the entire perimeter of the bromeliad, go to the **Analyze** menu and select **Measure**. (If you look carefully at image below, you will see that the bromeliad has been outlined in yellow).



7. This will give a result for the area of the selected shape.



Appendix 4: Formalin-Borax solution (modified from A. Pires)

Reagents:

Formalin supersaturated with borax: Add Borax (sodium tetraborate) to formalin (formaldehyde 36-38%) until it precipitates, using a magnetic stirrer. Borax is the trademark for a cleaning compound made of sodium tetraborate sold in supermarkets, etc. Diluted formaldehyde is called formalin. You will need to add the Borax over one or two days until no further Borax can be dissolved. A bit of undissolved Borax will remain on the bottom of the flask. Transfer to a leakproof bottle (either amber glass or brown Nalgene). Although you technically only need 60 ml of this solution, we recommend making 200 ml.

Bacteria preservation

Water samples should be fixed with formaldehyde solution (Formalin) supersaturated with Borax. Avoid the solid Borax crystals at the bottom of the bottom (e.g. pre-fill your sample tubes at the field station before going to bromeliads). Add 1 ml of this formalin-Borax solution in 9 ml of sample. The final concentration of formaldehyde in your sample will therefore be about 3-4%. Store in the refrigerator until preparing the slides. If longer than a few days, flash freezing in liquid nitrogen is recommended.

Bacterial density

Bacterial density can be determined by epi-fluorescence microscopy (requires about 3-4 ml per slide) or a flow cytometer (requires less than 1 ml). Flow cytometry relies on the bacterial cells not being deformed, so is best when the samples can be flash frozen right away.

Bacterial abundance

Total water volume x bacterial density = bacterial abundance

Appendix 5: chlorophyll –a concentration (modified from V. Farjalla)

There are two different methodologies to perform the chlorophyll-a analysis. The first one is based on direct spectrofluorimetric analysis of the water sample and the other is based on indirect (filtered) spectrophotometric analyses of the water samples. Ideally, do both, but otherwise choose one. Remember that the water for these analyses is homogenized I the leaf well by the pipet prior to collecting.

Direct analysis by spectrofluorometry.

1. First, you must have a portable spectrofluorometer. We have been using one from Turner Designs (http://www.turnerdesigns.com/t2/instruments/aquafluor.html) with good results.
2. The measurement is done directly by using a 1-cm cuvette filled with unfiltered water sample.
3. The obtained value should be converted to chlorophyll-a concentration in the lab by performing a calibration curve with a known chlorophyll-a standard, either obtained from Turner or prepared in the lab (e.g. an algae culture or lettuce leaves). This calibration curve will require a different type of fluorometer, a lab spectrofluorometer that measures chlorophyll on acid-digested samples not living samples. Note that the next method also requires a spectrofluorometer. Details on this calibration step available from Angelica Gonzalez (agonzale@zoology.ubc.ca)

Analysis by spectrophotometry.

1. First you must filter the water samples. You can use a portable filter device composed by a 50-ml syringe, a re-usable 25-mm filter-cap, 25mm 1.2 um cellulose filters (GF-C – Whatman or similar) and vials to capture the water after it has been filtered (for water color analysis). I have shown this technique in the Bromeliad Workshop.
2. Filter as much water as you can before the filter becomes clogged. Put the clogged filter safely aside, replace with a new filter, and continue filtering the water. TAKE NOTE OF THE TOTAL VOLUME OF FILTERED WATER USED FOR BOTH FILTERS (i.e. sum the amount of water filtered for each filter). To be clear, you should therefore have two filters for each bromeliad.
3. Keep both filters (for chlorophyll-a analysis) and filtered water (for water color).
4. The filters should be kept in the dark and frozen (or, at least, refrigerated) as much as you can.
5. The best way to save the filters is to fold the filter in the half (with the filtered part in the interior), cover them with aluminum foil and put both replicates in Eppendorff® tubes.
6. The filtered water should be kept in the dark and refrigerated as much as you can. Freeze if you will not be analyzing within 2 or 3 days.
7. Upon return to your lab, you will need to digest these samples with acid, and measure with a spectrofluorometer. Details on this step available from Angelica Gonzalez (agonzale@zoology.ubc.ca)