**Airbrushing Protocol**

1. Grab ice.
2. Grab 4 nubbins from the -80C freezer and thaw at room temperature or on ice.
3. Label (sample ID, initials, and date) four 50ml conical tubes for tissue slurry.
4. Once thawed, put on safety gear including gloves and goggles. Put samples that you aren’t yet airbrushing on ice.
5. Sterilize shears with 70% EtOH and snip a 1-2cm piece to use for airbrushing. Put whatever fragment you’re not using back in the original Whirlpak bag (or other sample container) and put on ice. Once you’re done airbrushing, put these samples in the “Done” box in the -80 freezer.
6. Grab your first sample, a WhirlPak or Ziploc bag, and fill airbrush with filtered seawater (FSW).
7. Spray off all tissue into the bag.
8. Seal and cut corner off the bottom of the bag.
9. Pour tissue slurry into 50ml conical and place on ice.
10. Use super glue to attach skeleton to a petri dish and label the dish with the sample ID, initials, and the date. Place skeleton in chemical hood to dry.
11. When done with all samples, thoroughly clean the airbrush with DI and EtOH.
    1. Fill and run with one round of DI water, one round of EtOH, and one final round of DI water. Wipe down the body of the airbrush with DI.
12. IF you have time to do tissue homogenization, proceed to that protocol. If you don’t have time, you can temporarily store tissue slurries on ice and then transfer them into the -80 freezer once you are done with all of your samples for the day.

**Tissue Homogenization**

1. Go to the -80C freezer and grab N=4 tissue slurries from the appropriate bin.
2. Ensure the lids are on tightly on all 15ml conicals and thaw at room temp.
3. Once thawed, homogenize tissue on high for the following times (Remember: the bottom hole on the homogenizer must always be submerged while the top hole should *never* be submerged):

<5ml slurry: 2 minutes

5-10ml slurry: 3 minutes

10-30ml slurry: 4 minutes

>30ml slurry: 5 minutes

1. Clean the tissue homogenizer between each sample. Fill a 50ml conical about halfway with DI water (you can get this from the sink) and homogenize for about 20-30 seconds.
2. Once all samples are homogenized, place conical tubes in centrifuge (make sure it’s balanced!) and spin at 2000rpm for 3 mins.
3. While samples are in the centrifuge, label one 1.5ml tube for each sample. Make sure to include the sample name, date and your initials.
4. Remove samples from centrifuge (be careful not to disturb pellet).
5. Remove and discard the supernatant for each sample.
6. Resuspend to 15ml using FSW.
7. Add 600ul of Z-fix to each conical tube.
8. Vortex each sample briefly.
9. Aliquot 1000ul into the respective 1.5ml tubes.
10. Store all samples at 0 degrees C.
11. Add date of homogenization to the symbiont counting datasheet.

**Symbiont Counting**

1. Remove 1.5 mL tubes (samples) from 0℃ freezer that you will count today
   1. Don’t remove more than 4 at a time
   2. Let samples thaw to a liquid (in closed drawer)
2. Turn on microscope and gather counter, hemocytometer, and coverslips
   1. Counter, hemocytometer, and coverslips are in the top drawer to the left of the microscope
3. Clean hemocytometer and coverslips with Milli-Q water and dry with Kimwipes
4. Vortex all samples until well mixed (10-30 sec)
5. Add 20 uL of 1:1 Lugol’s iodine to each sample
   1. Solution is in Isotemp freezer (50 mL conical tube with orange lid covered in foil)
   2. Keep Lugol’s iodine solutions **OUT OF THE LIGHT**
   3. Once the solution is added to the samples, keep the samples **OUT OF THE LIGHT**
   4. Use a 20-200 uL micropipette and tips
6. Vortex 1 sample so orange color is evenly distributed in liquid
7. Take a 10 uL subsample of your sample
   1. Take subsample with tip halfway down into the 1.5 mL tube
   2. Use 2 - 20 uL micropipette and tips
8. Pipette subsample into hemocytometer
   1. First, place coverslip on hemocytometer with coverslip edge along the outside edge of one of the mirrored parts
   2. Orient pipette parallel to hemocytometer and insert tip into indentation into the mirrored part (tip should be underneath coverslip)
   3. Eject the subsample on the hemocytometer
9. Count the symbionts
   1. Place hemocytometer on the microscope (use blue 40x lens)
   2. Make sure lines and symbionts are in focus
   3. Use counter to count the number of symbionts (similarly sized green/brown dots) in each large square (25 total)
   4. Count symbionts that are within the three line outline
   5. A large square is outlined with three white lines on each side
   6. There are 16 smaller squares (single white outlined) in each large square
10. After you count the symbionts in each large square, write the number on the counter in the spreadsheet.
11. **RESET COUNTER TO 0**
12. Repeat steps 9b-11 two more times
13. When done with all three subsamples, calculate average and record in both the raw data spreadsheet and the final symbiont count spreadsheet.
14. Repeat steps 6-13 for each sample
    1. Remember to clean hemocytometer and coverslip with DI water/Kimwipes between each sample
15. When finished, place all counted samples (1.5 mL tubes) into the box labeled “Ana Counted” in the 0℃ freezer
    1. **Keep the lid on this box at all times** (samples cannot be exposed to light)
16. Clean up workstation
    1. Clean hemocytometer and coverslips with Milli-Q water/Kimwipes
    2. Turn off microscope (keep plugged in)