**Contaminant Bioassay Protocol**

**Day 1: Set-up**

Part 1: Prep contaminant dilutions

1. The 750% treatment receives 0.5 ml from the already prepared stock (“high stock,” 0.5 for each contaminant for 1 ml total).
2. The 10% treatment will require a new stock (“low stock”):
   1. Mix 200 µl of the already prepared stock in 14.8 ml of acetone.
   2. The 10% treatments will receive 0.5 mL of this new stock (for each contaminant, 1 ml added total).

Part 2: Fill bottles with sample water

1. Each bottle should get a 3x DI water rinse then 3x sample rinse.
   1. Be relatively conservative with the sample rinse, just in case (though there should be plenty of water).
2. Fill each bottle with sample water to just below the top line near the cap (~250 ml).

Part 3: Add nutrients

1. Each bottle marked with red (indicating that they receive nutrients) should receive 0.5 mL of each nutrient stock (NaNO3 and KH2PO4), for a total addition of 1 mL.
   1. This should be 10 bottles total.

Part 4: Add contaminants

1. Bottles marked low should receive 0.5 mL of the freshly prepared 10% stock for the appropriate contaminants listed on the bottle.
2. Bottles marked high should receive 0.5 mL of the original, previously prepared stock.
   1. Work with one contaminant at a time and start with the lower concentration, then the higher concentration (to try and keep using the same pipette tip)

Part 5: Add acetone for solvent control

1. Add 1 mL of acetone to the bottles marked solvent control and solvent + nutrients.
2. Add 0.5 mL of acetone to the one contaminant only bottles (diclofenac for the 07/15-18/2025 bioassay) to bring the total addition of acetone to 1 mL (to match the combined treatments and the solvent control).

Part 5: Transfer to plates

1. Make sure each bottle is well mixed (gentle inversions and/or swirling).
2. Pipette 5 mL into a glass test tube.
3. Measure chlorophyll RFU.
4. Transfer from the test tube into a well in the appropriate row of the appropriate plate.
   1. This should all occur four times per bottle (four replicates).

Part 6: Place in Incubator

1. Put all plates and bottles into 25°C incubator in random order.

Part 7: PAM

1. Use water from the large sampling jug to measure a “Time Zero” Fv/Fm three times and a light curve once.

**Day 2: Rearranging and 24 h measurements**

1. Once in the late morning (~10:00) give the bottles and plates a light swirl/shake and rearrange in the incubator.
2. At 24h from the start of the experiment (about the time when all bottles were filled and contaminants were added and you started to measure Chl *a* on Day 1), measure Chl *a* fluorescence again:
   1. Pipette 5 ml from the well into a glass test tube.
   2. Measure fluorescence and record RFU.
   3. Pour the sample back into the well it came from.
   4. Repeat for all samples.
   5. Place plates back in the incubator randomly and gently invert and rearrange bottles.
3. Once in the late afternoon/evening, give the bottles and plates a light swirl/shake and rearrange in the incubator.

**Day 3: Rearranging and 48h measurements**

1. Once in the late morning (~10:00) give the bottles and plates a light swirl/shake and rearrange in the incubator.
2. At 48h from the start of the experiment (about the time when all bottles were filled and contaminants were added and you started to measure Chl *a* on Day 1), measure Chl *a* fluorescence again:
   1. Pipette 5 ml from the well into a glass test tube.
   2. Measure fluorescence and record RFU.
   3. Pour the sample back into the well it came from.
   4. Repeat for all samples.
   5. Place plates back in the incubator randomly and gently invert and rearrange bottles.
3. Once in the late afternoon/evening, give the bottles and plates a light swirl/shake and rearrange in the incubator.

**Day 4: Terminating the bioassay**

1. Once in the late morning (~10:00) give the bottles and plates a light swirl/shake and rearrange in the incubator.
2. At 72h from the start of the experiment (about the time when all bottles were filled and contaminants were added and you started to measure Chl *a* on Day 1), measure Chl *a* fluorescence again:
   1. Pipette 5 ml from the well into a glass test tube.
   2. Measure fluorescence and record RFU.
   3. Combine the four replicates for each treatment into a 50 ml falcon tube, this is called the composite and will be used for PAM data. This needs to be kept in the dark for at least 30 minutes before it can be used, so place the tubes in a dark box or something else dark.
3. Use the composite for each treatment to measure Fv/Fm three times and a light curve once.
   1. Make sure that you get the full regression when you run the light curve, otherwise do it again:

A screenshot of a data table

AI-generated content may be incorrect.

1. Use the bottles for filtering for HPLC:
   1. Filter two replicates per treatment, 100 ml each.
2. Create a voucher sample for each treatment using water from the bottle and preserving with Lugol’s.
3. Empty any leftover water and use 10% HCl to clean all materials.

**2025-07-15 Diclofenac Bioassay Treatment Groups**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Treatment | Solvent? | Nutrients? | Diclofenac | Carbamazepine | PFOS | 6ppd-q |
| Control | - | - | - | - | - | - |
| Solvent Control | 1 ml acetone | - | - | - | - | - |
| Nutrient Control | - | 0.5 ml each stock | - | - | - | - |
| Solvent + Nutrient | 1 ml acetone | 0.5 ml each stock | - | - | - | - |
| D Low | 0.5 ml acetone | - | 0.5 ml low stock | - | - | - |
| D High | 0.5 ml acetone | - | 0.5 ml high stock | - | - | - |
| D+C Low | - | - | 0.5 ml low stock | 0.5 ml low stock | - | - |
| D+C High | - | - | 0.5 ml high stock | 0.5 ml high stock | - | - |
| D+P Low | - | - | 0.5 ml low stock | - | 0.5 ml low stock | - |
| D+P High | - | - | 0.5 ml high stock | - | 0.5 ml high stock | - |
| D+Q Low | - | - | 0.5 ml low stock | - | - | 0.5 ml low stock |
| D+Q High | - | - | 0.5 ml high stock | - | - | 0.5 ml high stock |
| D Low + Nut | 0.5 ml acetone | 0.5 ml each stock | 0.5 ml low stock | - | - | - |
| D High + Nut | 0.5 ml acetone | 0.5 ml each stock | 0.5 ml high stock | - | - | - |
| D+C Low + Nut | - | 0.5 ml each stock | 0.5 ml low stock | 0.5 ml low stock | - | - |
| D+C High + Nut | - | 0.5 ml each stock | 0.5 ml high stock | 0.5 ml high stock | - | - |
| D+P Low + Nut | - | 0.5 ml each stock | 0.5 ml low stock | - | 0.5 ml low stock | - |
| D+P High + Nut | - | 0.5 ml each stock | 0.5 ml high stock | - | 0.5 ml high stock | - |
| D+Q Low + Nut | - | 0.5 ml each stock | 0.5 ml low stock | - | - | 0.5 ml low stock |
| D+Q High + Nut | - | 0.5 ml each stock | 0.5 ml high stock | - | - | 0.5 ml high stock |

**PAM protocol:**

1. Turn on the laptop and open WinControl (password: 1542)
2. Press the on button on the grey box (opening win control should do this, but if it doesn’t, press the button)
3. In the settings tab, reset to default (see the reset button in the top left). Make sure the measuring light intensity is set to 8 and gain (*not pm gain*) is set to 5.
4. Fill one cuvette with milli q water and one with sample water (wipe down outside of both).
5. Place the sample cuvette in the holder (this is the rightmost part of the equipment and has a circular lid). Replace the lid.
6. Let the sample dark acclimate again for a few seconds and watch the F (on the laptop or on the grey box). In win control under the settings tab, adjust pm-gain until F is ~800.
7. Remove the sample and place in a dark spot (could use the drawer, just be careful). Replace it with the milli q water cuvette.
8. On the box, press the up arrow until the little screen says auto zero (should just be once), then press set. When F is ~0 replace the milli q water with the sample. Double check that F is still around 800 (this doesn’t need to be exact, but if it’s way off try adjusting pm-gain and autozeroing again.)
9. In the win control program, go to report and make sure fv/fm is selected.
10. Go back to the light curve tab and hit start lc (bottom right of graph). You should hear two beeps, around ~30 seconds apart. After the second beep, hit stop lc.
    1. Make sure the SAT chart looks okay.
11. Take the sample cuvette out and replace it with more of the same sample. Then repeat step 10. Do this twice (for three total measurements).
12. Take the sample cuvette out and replace it with more of the same sample for a full light curve. Repeat step 10, but don’t stop the light curve. Let it run through, this should take about 5 minutes. Double check that the regression is calculated.
13. Save the data: save data > there should be a folder with Schlenker\_cont or something along those lines, save the file as the date collected and the sample id (ex: 2024-08-21\_C+D\_low)
14. Repeat for all samples (steps 4-11). Between samples rinse the cuvette 3x with mili q water and once with sample. Be conservative with the sample rinse.

Notes:

* I like to record what the pm-gain is set to for each sample.
* I also record the number of each run and the Fv/Fm value in my notebook (this is so there’s no confusion later in case files get saved weird, see “No.” and “Fv/Fm” in the Report tab).