Sampling protocol for WHONDRS Diel Cycling Study

A video protocol will be available at: https://www.youtube.com/channel/UC8d9IFF3qMRkJlo2SlWAz3Q

Note: We prefer that you ship your samples **immediately** after finishing the 48 hour sampling. You should keep the samples in a cooler with wet or blue ice during transit. See the end of the protocol for more shipping information.

In addition to the sampling supplies provided by WHONDRS, you will need the following:

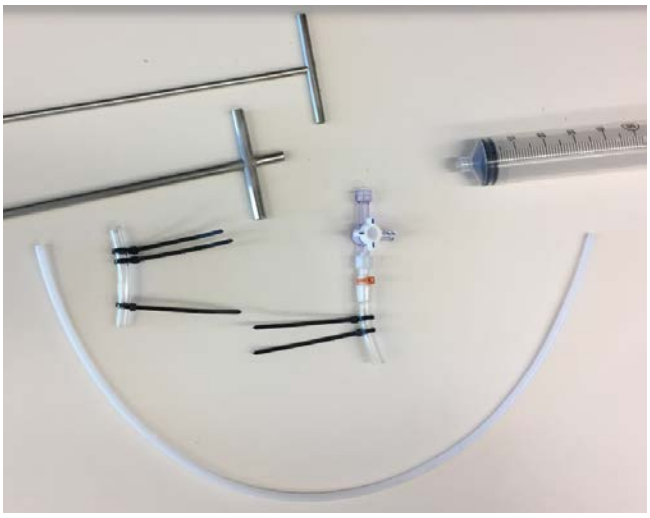
1) Cooler(s) with wet ice or blue ice for keeping samples cold in the field
2) Celsius thermometer for taking water temperature
3) A method for collecting latitude and longitude in decimal degrees in the field (a smart phone is sufficient)
4) A method for taking pictures of the field site (a smart phone is sufficient)
5) A refrigerator to keep samples cool if there is a shipping delay
6) Access to a FedEx office for shipping samples back overnight
7) Access to stream gauge data (**that can be made public**) for at least the 3 months prior to collecting the samples. Data must be recorded at a minimum frequency of every hour.
8) A mallet to aid in pushpoint installation if needed
9) Measuring tape with metric units
10) Container to collect used needles
11) Optional: tripod
12) Scissors, pen/pencils, permanent marker
13) Electrical/vinyl tape
14) A plan and supplies to ensure safe working conditions for the sampling crew over the 48-hour collection period

The sampling kit should contain the following items:

1) Hard copy data sheet and protocol
2) 60 mL Syringes (should be new and sealed) for water sampling, plus extras (40 total)
3) 5 mL syringes and needles to inject RNALater into the used sterivex filters, plus extras (45 total)
4) Nitrile gloves to minimize contamination, plus extras (1 box total)
5) Sterivex filters with needles attached, plus extras (40 total) (keep extras sealed unless needed)
6) Sets of vials for each of the 17 sampling time point plus extras. Each set has 4 for surface water ("SW") and 4 for pushpoints ("PP"). Each with a unique label. **Do not unscrew the cap from any vial**. Sample will be collected by piercing the septum with the needle attached to the filter.
   For each sampling time point and type (e.g. 3 hr surface water)
   - One vial will be clear. Fill this vial first.
   - Three vials will be amber. The **amber vials each contain 10 microliters of 85% phosphoric acid** to aid in sample preservation. Fill these vials after the clear vial to minimize acid transfer to the clear vial.
7) pH strips and color matching chart
8) Three 12" long, ¼" diameter pushpoint samplers made up of an external rod with a screened interval and an internal rod for stability during installation, plus an extra. **Always ensure the internal rod is fully inserted before attempting to install pushpoint or adjust pushpoint depth. If the internal rod is not fully inserted, the pushpoint will bend or break.**
9) Extra mesh pushpoint socks - The pushpoints will arrive with a mesh sock over the end with the screened interval. This is to help prevent clogging. If you begin installing a pushpoint and then

have to pull it back out for reinstallation, the sock will likely be damaged. You can remove it and attach one of the extras.

10) Two spools of Teflon tubing. You will need to cut the tubing into four lengths of Teflon tubing – three for pushpoint sampling and one for surface water sampling. We will also send short lengths of more flexible tygon tubing (4, plus extras) and also short lengths of tygon tubing already attached to 3-way valves (4, plus extras). Each length of thin teflon tubing will have a short length of flexible tygon tubing at each end. One end will be connected to the external pushpoint rod following pushpoint installation (or open to surface water for the surface water tube) and one end will run up bank and be connected to a 3-way valve for sampling. You will have to cut the Teflon tubing to your needed length and then attach the short lengths of tygon tubing to each end using zip ties. The protocol video and photos will help visualize the needed arrangement. For the surface water sample tube, you will have to attach a mesh sock just like the ones attached to the ends of the pushpoint rods so that it is covering one end of the tube to help reduce particulate matter. Use a zip tie to secure the mesh sock to the tubing If not all the teflon tubing is needed, please send it back with your other unused supplies.
11) Ten empty sand bags to weight the tubing down
12) Zip ties, two sizes (Unless you are outside of the US. If this is the case, we cannot ship zip



Unassembled: Pushpoint (internal and external rod), short tygon tubing, short tygon tubing with 3-way valve, example teflon tubing (much longer in reality), and 60mL syringe.

Assembled set-up for pushpoint sampling.

Close up of Teflon-to-tygon-to-3-way valve sampling port. Note that the Teflon tubing is pushed all the way to the luer lock connector. The tygon tubing is secured to the Teflon tubing with zip ties.

Close up of Teflon-to-tygon-to-pushpoint connections. Note that the Teflon tubing is pushed all the way to the pushpoint opening. The tygon tubing is secured to the Teflon tubing and to the pushpoint with zip ties.

ties. Please provide your own. We recommend very small ones and a larger size.)
13) Optional: One HOBO data logger for dissolved oxygen (DO) and one for specific conductance (SpC) already programmed and logging
14) Foil to keep the sampling ports protected from contamination when not in use
15) Luer lock caps to keep the RNALater in the used sterivex filters (early versions of the sample kit have one-way valves instead of luer lock caps. Close the valves to use them like caps).
16) Several 40mL clear glass vials filled with RNALater to preserve the sterivex filters
17) Whirlpak bags for the used, RNALater-filled, and capped sterivex filters, plus extras (40 total)
18) Several pieces of flagging tape to tie onto pushpoints for easier visualization

After confirming that you have all of the needed gear, choose the sampling location and collect field metadata as follows:

1) Choose your location to be as close as possible to a stream gauge. Also try to sample in a location with natural sediments as opposed to locations with artificial substrates.
2) Confirm there is downstream flow at the sampling location (e.g., drop in a leaf and make sure it moves downstream)
3) Identify the location to install your pushpoints. The water elevation at the site should be as low as possible in the context of anticipated flows over the 48-hour period. Attempt to install the pushpoints ~1m from the shoreline or wherever they will be inundated for the entire 48-hour period.
4) Install the first pushpoint by clearing away large gravel, placing the sock-covered tip of the pushpoint at the desired entry point, and manually pushing down on the entire pushpoint device. **Always ensure the internal rod is fully inserted before attempting to install pushpoint or adjust pushpoint depth. If the internal rod is not fully inserted, the pushpoint will bend or break.** If the pushpoint needs some additional force, lightly encourage entry using a rubber mallet. Push or pound on the pushpoint until almost the entire length is in the subsurface. **Do not use excessive force. If the pushpoint will not go as deep as desired, try a different location**. Using excessive force will bend or break the pushpoint.
5) Repeat the same process ~ 2 meters downstream from the first pushpoint and again ~2 meters upstream from the first pushpoint. **The pushpoints should be oriented parallel to flow**.
    - Naming convention:
        - PP1 = pushpoint most upstream
        - PP2= pushpoint middle
        - PP3= pushpoint most downstream
6) After the pushpoints are installed, carefully pull out the internal rod from each pushpoint, taking care to apply downward pressure to the external rod so that the entire device is not pulled out.
7) For each pushpoint, measure the distance from the streambed to the top of the external rod of the pushpoint. Record.
8) Measure the distance between PP1 and PP2 and between PP2 and PP3. Record.
9) Record latitude and longitude in **decimal degrees** for the middle pushpoint (PP2). For this, feel free to use a GPS or a smart phone app such as 'My GPS Coordinates' or Google maps
10) If you have HOBO loggers with your supplies, anchor the two HOBO data loggers to the middle pushpoint using zip ties. Record the time they enter the water.

IMPORTANT NOTE ABOUT SAMPLE CLEANLINESS:

The water samples will be analyzed for high-resolution carbon characterization. It is very easy to contaminate these samples. **Please be very careful not to touch the inlet or outlet of the filters, syringes, valves, and needles.** We have provided extra filters, syringes, needles, and valves. If you accidentally contaminate something, please replace it with the extra supplies. **Please be very careful not to touch the vial septa.**

When you are sampling one of the sampling ports, three sampling ports will not be in a state of use. **When sampling ports are not in use, please wrap them in several layers of clean foil** that is provided in your supplies. This will reduce the contamination from other carbon sources. Do not put the sampling port on the ground unless it is well wrapped in foil.

After installing the pushpoints, choose the monitoring location and collect field metadata as follows:

1) Tubing will run up bank from the push points to a monitoring location. Choose a monitoring location that will remain safe and dry throughout the 48 hour time period.
2) Take one length of tubing provided and attach one end to PP1 until the flexible tubing is over the PP opening and the smaller diameter white tubing is as close as possible to the PP opening. Secure in place using a zip tie to form a tight seal around the PP. Temporarily secure the other end of the tubing at your monitoring station and **wrap in foil** before placing on the ground. Do the same for PP2 and PP3. Use a permanent marker to write the port name directly on the tubing ("PP1", "PP2", "PP3")
3) The fourth length of tubing will be used for surface water sampling. Surface water sampling will only be conducted at the PP2 location. Use a zip tie to secure one end of the tubing to PP2 (middle pushpoint) at the elbow created by the pushpoint body and the two small metal handles extending from the sides. Ensure that the tubing is secured tightly but is not being compressed to the point of closure. The tubing will have a mesh filter sock on the end. Ensure that the tube opening is directed to the water column, not along the streambed. This will minimize sediment clogging. Temporarily secure the other end of the tubing at your monitoring station and **wrap in foil** before placing on the ground.
4) Fill the empty sand bags using sediment, gravel, or other items. Using the sand bags, weight the tubing from the pushpoints up to the monitoring station so that the lines are secure.
5) Take the following photos with a smart phone or camera (note these will be uploaded along with metadata). For the last three pictures (b, c, d), lay out a **measuring tape to 30cm** on the shoreline as a reference for scale and make sure it can be seen in the photos. The first figure (looking across the river) doesn't need to contain the measuring tape.
    a. Looking across the river to give a sense of how broad the river is
    b. Looking upstream, showing the river surface, shoreline sediments, and vegetation
    c. Looking downstream, showing the river surface, shoreline sediments, and vegetation
    d. Looking straight down onto a location with representative shoreline sediments
6) Choose a location to take a field photo at every time point. The photo will be taken from one stable location (near your monitoring site) that you will use for all time points and will therefore be safe and accessible at all time points. The photo should have the same angle and zoom every time. It is recommended to use a tripod that will be left in place for the entire 48-hour period. The other 4 pictures will only be taken one time, during site set-up
7) Fill out additional metadata on the hard copy data sheet prior to sampling (i.e., date, time zone, name, institution).

At each time point, collect samples as follows:

1) At the beginning of each time point, record the time on the data sheet and then take a photo of the written time point number (i.e., 0, 3, 6, 9, etc.) on your hard copy data sheet (zoom in on the printed number). This will help to keep track of the field photo you will take at every time point. Then, from your tripod or pre-chosen location, take a photo looking towards the pushpoints. At the end of the sampling period, you should have a total of 17 photographs of the same exact location through time. Please use flash or another method of illumination at night.
2) If accessible, measure the water depth at PP2 (middle pushpoint) and record it on the datasheet in cm. If the location is not accessible, do not record a value on the datasheet.
3) Measure the temperature of the water at **50% of the water column depth** as close to PP2 as reasonably possible and record it on the datasheet in degrees C.
4) Remove the pH strip from its protective plastic box, making sure to keep the paper dry.
5) Dip the strip into the river water, wait 10 seconds after wetting the pH strip, and compare the color to the color chart provided with the pH strip. Record the pH value on the metadata sheet.

Sample the surface water (vials name starts with "SW")

1) Put on the provided nitrile gloves (do your best if they don't fit well)
2) Take out one clear vial and three amber vials. Record their sample IDs on the hard copy data sheet.
3) Unwrap the surface water sampling port from its protective foil. Keep it loosely in the foil so that it can be pulled out.
4) Open the syringe package and remove the syringe. Please **don't touch the outlet of the syringe and do not fully remove the plunger from the syringe body**. This is important to avoid contamination.
5) Pull out the sampling port from the foil. Attach the syringe to one side of the 3-way valve for the surface water sampling tube. **Do not touch the outlet of the syringe or the inlet of the 3-way valve**.
6) Fill the syringe with water from surface water sampling tube. Expel the syringe contents onto the ground and repeat this two more times. If the syringe fills with air, rotate the 3-way valve to hold a vacuum on the tubing while expelling the air from the syringe. Repeat as needed to flush out the air and then a total of three syringe volumes of water.
7) After flushing the syringe/tubing, fill the syringe again; this is the sample water for the first replicate.
8) Open the filter package that has a needle attached to the filter.
9) Screw the filter/needle assembly onto the open side of the 3-way valve. Remove the plastic cover that is protecting the needle. Please **don't touch the outlet of the 3-way valve, the inlet of the filter, or any part of the needle**. This is important to avoid contamination. Also, retain the plastic needle cover. Note that it is okay to touch the cylindrical filter housing.
10) Rotate the 3-way valve to push a few mL of water through the filter/needle assembly. This water is not collected.
11) Beginning with the **clear** glass vial, remove the flip top cap on the glass vial. Please **don't touch the septum** that is exposed after removing the flip top cap. This is important to minimize contamination. **Do not unscrew the top of the vial**. Only remove the white disc that rests on top of the cap and conceals the septum.
12) Pierce the septum with the needle and fill the vial approximately half way full. Please **don't fill more than half way**. If the vial is more than half full, it will likely burst when frozen.
13) After injecting sample water into the vial, withdraw the needle from the septum.

14) Place the vial containing the sample into the cooler with wet or blue ice. Note there is no need to record any information on the sample label. There is no need to replace the flip top cap that previously covered the septum.
15) Taking care not to touch the needle on the other side of the 3-way valve, fill the syringe with water from the same tube. Repeat the above sampling procedure for the three amber vials. These are the second, third, and fourth surface water replicates. Note that **one filter and one needle should be used to collect all four surface water replicates**. The additional filters and needles sent with the kit should only be used if there is an issue with the first filter or needle. If an extra filter or needle is needed, please do not touch the inlet or outlet of the filter, and please don't touch the inlet of the needle or any part of the metal needle (it is okay to touch the plastic needle cover). If the extra filter or needle are needed, please record the reason on the paper metadata sheet. **If the extra needles or filters are not needed, they should be shipped unopened when the samples are shipped.**
16) After filling the amber vials, shake each one gently to incorporate acid into the sample. Then place into cooler.
17) After collecting the four water samples, save the used filter:
    A. To do this, first detach the 60mL syringe from the 3-way valve, expel any remaining water onto the ground, and fill the syringe with air. Then attach it to the 3-way valve again, and push the air through the filter. The goal is to expel as much water from the filter as possible. Repeat 2 or 3 times if needed.
    B. Discard the needle.
    C. Take one of the small luer lock caps provided in a bag and attach it to the open end of the filter. Note that the filter has a "male" side and a "female" side and therefore there are two types of caps provided. Place sampling port on foil.
    D. From the provided supplies, take out a clear glass vial filled with RNALater. This is the preservative for the filter. Also take out a 5mL syringe and a new needle.
    E. Connect the new needle to the 5mL syringe, flip off the cap on the RNALater to expose the septum, and invert the vial so the septum is facing the ground.
    F. Insert the new needle into the septum and fill the 5mL syringe with RNALater. The filter holds 3mL, so you can fill the syringe a little less than full.
    G. Withdraw the needle from the septum and place the RNALater somewhere protected. Cover the top with clean foil. You will use the RNALater at each time point.
    H. Discard the needle.
    I. Take the used filter off of the 3-way valve, taking care not to touch the inlet of the filter or the outlet of the valve. Place the sampling port back on the foil.
    J. Attach the 5mL syringe to the filter and rotate so that the syringe is facing down and the filter is below it. Push the plunger to slowly fill the filter with RNALater. Fill until you feel some resistant.
    K. Detach the syringe and put into a waste container. Cap the filter using one of the small luer lock caps provided.
    L. Put the capped syringe into a small whirlpak bag, tie up to seal, and place in the cooler. Please ensure no water is entering the whirlpak bag. When you have access to a freezer and refrigerator -- **DO NOT FREEZE the filters**. Only refrigerate them. Freezing the filters will cause them to burst,
18) Verify all sample IDs are recorded on hard copy data sheet.
19) Discard the used 60mL and 5mL syringes. Discard the used needles.
20) Make sure the sampling port is well wrapped in foil.

Sample the pushpoints (vials name starts with "PP")

21) Take out one clear vial and three amber vials. Record their sample IDs on the hard copy data sheet.
22) Unwrap one pushpoint sampling port from its protective foil. Keep it loosely in the foil so that it can be pulled out.
23) Open a new filter package that has a needle attached to the filter but leave the needle/filter assembly in the package
24) Open a new syringe package and remove the syringe. Please **don't touch the outlet of the syringe and do not fully remove the plunger from the syringe body**. This is important to avoid contamination. You will use this one syringe and one filter for all of the pushpoints.
25) Pull out the sampling port from the foil. Attach the syringe to one side of the 3-way valve for the pushpoint sampling tube. **Do not touch the outlet of the syringe or the inlet of the 3-way valve**.
26) Fill the syringe with water from one of the pushpoint sampling tubes **very slowly**. The preferred rate is one syringe full per minute (~1 mL/sec). Expel the syringe contents onto the ground and repeat this two more times. If the syringe fills with air, rotate the 3-way valve to hold a vacuum on the tubing while expelling the air from the syringe. Repeat as needed to flush out two syringe volumes of water.
27) On the last round of flushing the syringe, place a pH strip in the outflow from the syringe. Wait 10 seconds after wetting the pH strip and then compare the color to the color chart provided with the pH strip. Record the pH value on the metadata sheet. If you need to put the sampling port down, place it on the foil.
28) After flushing the syringe/tubing, fill the syringe again; this is the sample water for the first replicate.
29) Screw the filter/needle assembly onto the open side of the 3-way valve (The same filter will be used for all PP sites but a new needle is needed at each PP site). Remove the plastic cover that is protecting the needle. Please **don't touch the outlet of the 3-way valve, the inlet of the filter, or any part of the needle**. This is important to avoid contamination. Also, retain the plastic needle cover. Note that it is okay to touch the cylindrical filter housing.
30) Rotate the 3-way valve to push a few mL of water through the filter/needle assembly. This water is not collected.
31) Beginning with the **clear** glass vial, remove the flip top cap on the glass vial. Please **don't touch the septum** that is exposed after removing the flip top cap. This is important to minimize contamination. **Do not unscrew the top of the vial**. Only remove the white disc that rests on top of the cap and conceals the septum.
32) Pierce the septum with the needle and **fill the vial approximately 15% full**. **The clear vial will become a composite sample from all of the pushpoints.** The total volume after contributing from all the pushpoints will be just under 50% full.
33) After injecting sample water into the vial, withdraw the needle from the septum.
34) Place the vial containing the sample into the cooler with wet or blue ice, but ensure that the septum remains clean and the sample is still easily accessible. You will be adding additional water to this vial from the other pushpoints until the vial is 50% full.
35) Taking care not to touch the needle on the other side of the 3-way valve, fill the syringe with water from the same tube.
36) Repeat the above sampling procedure for one amber vial, but use the same syringe and same filter as the first pushpoint. **Fill the vial approximately 50% full**. Please **don't fill more than half way**. If the vial is more than half full, it will likely burst when frozen.
37) After injecting sample water into the vial, withdraw the needle from the septum.
38) Discard the needle.

39) Shake the amber vial gently to incorporate acid into the sample. Place into cooler. Record the time.
40) Disconnect the syringe from the sampling tube and place the sampling port on foil. Push out any remaining water from the syringe, and attach it to the next pushpoint sampling tube. Disconnect the filter from the prior sampling port and attach to the next pushpoint sampling tube. Attached a new needle. Note that **one filter and one syringe should be used to collect all pushpoint samples but a different needle should be used for each one**. If an extra filter or needle is needed, please do not touch the inlet or outlet of the filter, and please don't touch the inlet of the needle or any part of the metal needle (it is okay to touch the plastic needle cover). If the extra filter or needle are needed, please record the reason on the paper metadata sheet.
41) Wrap the prior sampling port in foil.
42) Repeat steps 21 to 40 on the new pushpoint tube. Ensure that you **use the same clear vial you used for the first pushpoint tube (and the same syringe and filter)**. After the second pushpoint tube, this vial should be 30% full.
43) After completing the second pushpoint tube, repeat steps 21 to 40 on the last pushpoint. Ensure that you **use the same clear vial you used for the first two pushpoint tubes (and the same syringe and filter)**. After the last pushpoint tube, this vial should be 45% full and is complete. Put in cooler.
44) After collecting the four water samples, save the used filter using the same method as the surface water sampling (step #17).
45) Verify all sample IDs are recorded.
46) Discard the used 60mL and 5mL syringes. Discard the used needles.
47) Make sure the sampling port is well wrapped in foil.
48) Repeat the entire sampling procedure every 3 hours for a total of 48 hours. Take care to fill out the metadata sheet as you go.

Prior to leaving the field site:

1) Please confirm that all fields on the paper metadata sheet are filled out prior to leaving the field, and take a picture of the data sheet. This picture will be uploaded with the metadata
2) Remove and empty sand bags. The empty sand bags do not need to be returned.
3) Measure the length of Teflon tubing used for each sampling run and record.
4) Pull pushpoints out of the ground and **rinse very well**. Replace internal rod of each one. If it feels gritty when you slide the internal rod back in, pull it out and rinse the pushpoint again. If the external rod is slightly bent, carefully bend back in place as you push the internal rod deeper. Pushpoints will be shipped back with the samples.

After samples are collected, transport them to a refrigerator, keeping them on wet or blue ice during transit, or preferably, transport them directly to a FedEx location where they can be shipped immediately.

Metadata:

1) After the samples are stored in the refrigerator or shipped, please email WHONDRS@pnnl.gov for a copy of the digital metadata form.
2) Please enter as much information as possible. The form indicates what information is required and what is optional.
3) The form also provides instructions on how to submit the field photos and the stream gauge data. For the photos, please follow the naming convention described in the metadata form (e.g.

LastName-2018-Across; LastName-2018-Up; LastName-2018-Down; LastName-2018-sed; LastName-2018-Data; LastName-2018-Hour0 (repeat last photo for all hours).

4) For the stream gauge data, it is critical to follow the instructions carefully to ensure consistent data format and consistent units across sampled locations. If the closest stream gauge provides downloadable real-time data, it is acceptable to provide the url and the PNNL WHONDRS team will download and format the data.

Shipping:

Note: We prefer that you ship your samples **immediately** after finishing the 48 hour sampling. You should keep the samples in a cooler with wet or blue ice during transit. Because we are not freezing the samples, the sooner they can be shipped, the better. If you finish your sampling at the end of the week (Thursday/Friday), please store the samples in a refrigerator and ship on Monday/Tuesday. Please contact WHONDRS@pnnl.gov **before** you do the sampling if you do not already have a return shipping label. See the end of the protocol for more shipping information.

1) Keep all samples refrigerated or in a cooler with wet/blue ice until shipping. Samples should be shipped immediately after sampling unless sampling finishes on a Thursday or Friday. If that is the case, refrigerate the samples and ship on Monday.
2) The sample vials should all be packed back into the two cardboard boxes they came in, with each vial in an individual slot. Put these boxes into the original box shipped to you. Add any remaining supplies, pushpoints, and the filter samples. Ship this box via overnight FedEx.

WHONDRS will provide you with a return shipping label for box. You should not have to pay for shipping.

Abridged sampling protocol

1) Record time
2) Take photo of data sheet time point and then pushpoints from single photography location
3) Measure water depth
4) Measure temperature
5) Measure pH of river.
6) Sample surface water
    - Flush tubing
    - Fill clear vial half full, put in cooler
    - Fill three amber vials half full, shake gently, and put in cooler
    - Expel air from filter, cap filter outlet, fill with RNALater, cap filter inlet, put in whirlpak.
    - Discard used syringes and needles
7) Sample pushpoints
    - PP1 - Flush tubing
    - Measure pH.
    - Fill clear vial 15% full, put in cooler but keep septum clean and keep vial accessible
    - Fill one amber vial half full, shake gently, and put in cooler
    - PP2 – Flush tubing (same syringe, same filter, different needle)
    - Measure pH.
    - Take the same clear vial as before and add water until 30% full, put in cooler, keep septum clean, keep vial accessible
    - Fill one amber vial half full, shake gently, and put in cooler
    - PP3 – Flush tubing (same syringe, same filter, different needle)
    - Measure pH.
    - Take the same clear vial as before and add water until 45% full, put in cooler
    - Fill one amber vial half full, shake gently, and put in cooler.
    - Expel air from filter, cap filter outlet, fill with RNALater, cap filter inlet, put in whirlpak.
    - Discard used syringes and needles
8) Repeat every 3 hours